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Identification and potential use of a molecular marker for rust resistance in common bean*

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Summary. The Up_2 gene of common bean (*Phaseolus vulgaris* L.) is an important source of dominant genetic resistance to the bean rust pathogen [*Uromyces appendiculatus* (Pers. ex Pers.) Unger var 'appendiculatus' [syn *U. phaseoli* (Reben) Wint.]. Up_2 in combination with other rust resistance genes may be used to obtain potentially stable genetic resistance. It is difficult, however, to combine rust resistance genes effective against a single race due to epistatic interactions that frequently occur between them. A strategy that employed bulked DNA samples formed separately from the DNA of three BC_6F_2 individuals with Up_2 and three without Up_2 as contrasting near-isogenic lines (NILs) was used to identify random amplified polymorphic DNA fragments (RAPDs) tightly linked to the Up_2 locus. Only 1 of 931 fragments amplified by 167 10-mer primers of arbitrary sequence in the polymerase chain reaction (PCR) was polymorphic. The RAPD marker ($OA14_{1100}$) amplified by the 5′-TCTGTGCTGG-3′ primer was repeatable and its presence and absence easy to score. No recombination was observed between $OA14_{1100}$ and the dominant Up_2 allele within a segregating BC_6F_2 population of 84 individuals. This result suggests that $OA14_{1100}$ and Up_2 are tightly linked. Andean and Mesoamerican bean germ plasm, with and without the Up_2 allele, were assayed for the presence of $OA14_{1100}$. Apparently, the marker is of Andean origin because all Andean lines, with or without the Up_2 allele, contained the marker, and the marker was absent in all Mesoamerican germ plasm except the lines to which *Up*-2 had been purposely transferred. These results suggest that $OA14_{1100}$ will be most useful for pyramiding Up_2 with other rust resistance genes into germ plasm of Mesoamerican origin where the marker does not traditionally exist. The use of bulked DNA samples may have concentrated resources toward the identification of RAPDs that were tightly linked to the target locus. Marker-based selection may provide an alternative to the time-consuming testcrosses required to pyramid bean rust resistance genes that exhibit epistasis.

Key words: Polymerase chain reaction – Random amplified polymorphic DNA (RAPD) – *Phaseolus vulgaris* – *Uromyces appendiculatus* – Gene pyramiding

Introduction

High levels of pathogenic specialization (Stavely et al. 1989) for the bean rust pathogen, *Uromyces appendiculatus*, present a challenge to plant breeders working to develop stable genetic resistance. Marker-based selection may provide breeders with a means to facilitate stabilization of genetic resistance through gene pyramiding. Indirect selection as an efficient and practical means to develop resistance to bean rust will depend upon markers that are easy to detect and tightly linked to the resistance factors. The chance of identifying morphological or isozyme markers tightly linked to a specific trait such as the *Aps-1* isozyme locus for the *Mi* nematode resistance gene in tomato (Medina-Filho 1980) is slight because of the limited number of these marker types. Conversely, a better

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chance exists for identifying an RFLP (restriction fragment length polymorphism) marker tightly linked to a specific trait as Young et al. (1988) did for the *Tm-2a* virus resistance gene in tomato because RFLP markers are more numerous. However, probing Southern blots of restriction enzyme-digested genomic DNA for polymorphisms represents a laborious undertaking in our laboratory.

Recently, a novel technique that rapidly generates and screens random DNA segments for polymorphisms between different genotypes was developed simultaneously by Welsh and McClelland (1990) and Williams et al. (1990). The technique is based on amplification of random DNA segments in the polymerase chain reaction (PCR) using a single primer of arbitrary sequence. Martin et al. (1991) used near-isogenic lines (NILs) in tomato, and Michelmore et al. (1991) developed a DNA bulked segregant analysis strategy in lettuce to screen randomly amplified DNA fragments for polymorphisms from introgressed segments of DNA that contained specific disease resistance genes. The polymorphic DNA fragments, termed random amplified polymorphic DNA (RAPDs) (Williams et al. 1990), were detected as unique bands in agarose gels for one NIL or DNA bulk but not for the contrasting line or bulked phenotype. Putative linkages of RAPDs to the target loci were established with segregating populations.

The utility of a genetic marker as a selection tool or potential starting point from which to conduct a chromosome walk will increase as its distance from the target locus decreases. Martin et al. (1991) using NILs and Michelmore et al. (1991) using bulked segregant analysis detected linkages of 6.8 and 6.0 cM between RAPD markers and the *Pto* gene conditioning resistance to *Pseudomonas syringae* pv. *tomato* and the *DM5/8* gene conditioning resistance to downy mildew, respectively. An approach that combines both NIL and bulked segregant analysis strategies may also be useful for identifying RAPD markers tightly linked to target loci (Michelmore et al. 1991).

Phaseolin seed protein, isozymes, and genomic and mitochondrial RFLPs have provided evidence for two distinct gene pools, Andean and Mesoamerican, of common bean (Gepts 1990; Khairallah et al 1990; Nodari et al. 1992). The dominant Up_2 gene was identified originally in the Andean gene pool (Christ and Groth 1982) and is an important source of genetic resistance because it is effective against 14 of 33 reported North American races of *U. appendiculatus* (Stavely 1984; Stavely et al. 1989) and 9 of 16 more recently identified races (J. R. Stavely unpublished). The study presented here describes how bulked DNA samples formed separately from three resistant and three susceptible individuals from a BC_6F_2 population of common bean were used to identify a RAPD marker tightly linked to the Up_2 gene that conditions hypersensitive resistance to *U. appendiculatus*.

Materials and methods

Plant materials

The dominant Up_2 allele was transferred from the Andean gene pool to the Mesoamerican gene pool by means of traditional backcross breeding using the snap bean 'Early Gallatin' as donor parent and navy bean 'C-20' as recurrent parent (Stavely et al. 1991). A BC_6F_2 population from this initial work was utilized in this study. The BC_6F_2 population consisted of 84 individuals segregating 3:1 (65R:19S, $\chi^2 = 0.25$, $P > 0.50$) for the Up_2 allele and served as a source of individuals for forming DNA bulks and as a segregating population to confirm the presence and degree of putative linkages between RAPD markers and the Up_2 allele.

BC_6F_2 individuals were grown in the greenhouse or field, and at about 10% bloom approximately 5 g of fresh leaves were collected from the younger leaflets for DNA extraction. Selfed seed was harvested for subsequent progeny testing. Between 10 and 20 plants belonging to BC_6F_2-derived F_3 families were inoculated with race 50 of *U. appendiculatus* to determine the genotype of each BC_6F_2 individual. Inoculation and disease rating followed the procedures of Stavely et al. (1989). Rust race 50 was used to distinguish the different genotypes because in the presence of the Up_2 allele it causes a distinct hypersensitive reaction (predominantly grade 2+, Stavely 1984) and with the susceptible recurrent parent C-20, which lacks a dominant Up_2 allele, it develops large pustules (grade 5 and 6).

DNA isolation

The extraction of DNA was based on that of Saghai-Maroof et al. (1984) and was similar to that of Nodari et al. (1992). Fresh leaf tissue stored at −70 °C was lyophilized and ground using a Wiley Mill with a 30- to 40-mesh screen. Homogenized leaf tissue (0.5 g) was placed in a tube with 10 ml of DNA extraction buffer (2% CTAB, 1.4 *M* NaCl, 0.2 *M* EDTA, 0.1 *M* TRIS-HCl pH 8.0, 1.0% 2-mercaptoethanol). The tubes were incubated in a water bath at 60 °C for 1 h with occasional swirling. After incubation 15 ml of chloroform-isoamyl alcohol (24:1, v/v) was added to the tube and gently mixed to form an emulsion. The tube was centrifuged at 3000 *g* at 4 °C for 10 min. The aqueous phase was removed, and the DNA was precipitated by adding two-thirds volume isopropanol. The precipitated DNA was spooled onto a glass rod, washed in 10 ml of 76% ethanol containing 10 m*M* ammonium acetate, and resuspended in 2 ml of TE (10 m*M* TRIS-HCl pH 8.0, 1 m*M* EDTA). The suspension was centrifuged at 12000 g at 4 °C, and the resulting pellet eliminated. The DNA was reprecipitated by adding 2 volumes of 100% ethanol. Reprecipitated DNA was pelleted at 3000 *g* for 10 min at 4 °C, washed with 70% ethanol for 20 min, then resuspended in TE (10 m*M* TRIS-HCl pH 8.0, 0.1 m*M* EDTA).

PCR amplification

The PCR reactions were performed in 25 µl volumes of 10 m*M* TRIS-HCl pH 8.3, 10 m*M* KCl, 4 m*M* $MgCl_2$, and 200 u*M* each of dATP, dCTP, dGTP, and dTTP, containing 0.2 u*M* primer, 2 units of *Stoffel Fragment* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.), and 25 ng template DNA. Amplification was performed with a Perkin Elmer Cetus 480 thermal cycler for 47 cycles of 1 min at 94 °C, 1 min at 35 °C, followed

by 72 °C for 2 min extended by an additional second each cycle. Amplification products were resolved by electrophoresis in 1.4% agarose gels containing ethidium bromide (Williams et al. 1990).

General procedure

Two DNA bulks were formed as follows. One bulk consisted of equal amounts of DNA from three BC_6F_2 individuals that carried the Up_2 allele, and the other bulk was similarly formed from three BC_6F_2 individuals that were susceptible. The Up_2 genotypes for individuals comprising the resistant bulk were unknown because bulks were formed prior to F_3 progeny tests. It was determined later that the resistant bulk contained both homozygous and heterozygous individuals for the Up_2 allele.

The two DNA bulks, representing a contrasting NIL pair, were screened with 167 single 10-mer primers of arbitrary sequence (Operon Technology, Alameda, Calif.). Random amplified fragments observed from only the resistant bulk were classified in the entire segregating population. Linkages were calculated by maximum likelihood (Allard 1956). A RAPD found linked to the dominant Up_2 allele was screened across a sample of bean germ plasm with and without the Up_2 allele to examine utility as a genetic marker.

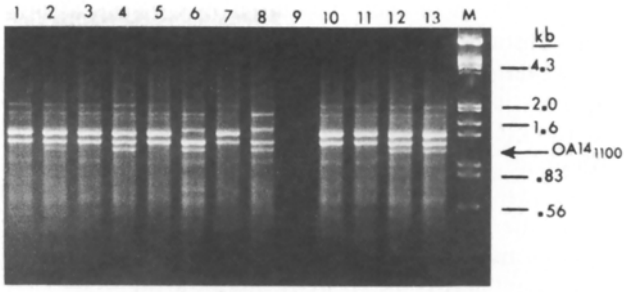

Fig. 1. DNA segments of BC_6F_2 individuals segregating for the Up_2 allele that were amplified using PCR with primer A14. *Lanes 1, 3,* and *5,* and *2, 4,* and *6* are the individuals that comprised the susceptible (up_2) and resistant (Up_2) bulks, respectively. *Lanes 7* and *8* contain the recurrent and donor parents, 'C-20' and 'Early Gallatin', respectively. *Lane 9* is a negative control that contained the A14 primer but no template DNA. *Lanes 10* and *11*, and *12* and *13* are repetitions of the susceptible and resistant bulks, respectively. *M* Molecular size marker

Results

Nine hundred and thirty-one discrete fragments of DNA, for an average of 5.6 DNA fragments amplified per primer, ranging in size from 0.1 to 2.0 kb, were amplified with the 167 arbitrary 10-mer primers. A 1.1-kb DNA fragment was amplified from the resistant bulked DNA sample and not from the susceptible bulked DNA sample with primer A14 (5′-TCTGTG CTGG-3′) (Fig. 1). This particular RAPD was designated $OA14_{1100}$ – 'O' for the source of the primer (Operon Technologies), A14 for the specific kit (A) and primer number (14), and $_{1100}$ for the size (bp) of the polymorphic fragment – using the same nomenclature as that of Michelmore et al. (1991) in describing RAPD loci in lettuce. No other RAPDs were observed.

Table 1. Survey of common bean germ plasm, with (+) and without (−) the Up_2 allele for rust resistance, for presence (+) or absence (−) of the DNA marker $OA14_{1100}$

Andean gene pool	Up_2	$OA14_{1100}$	Mesoamerican gene pool	Up_2	$OA14_{1100}$
Snap			*Navy*		
Early Gallatin	+	+	C-20	−	−
BBL47	+	+	Bunsi	−	−
Atlantic	+	+	Mayflower	−	−
Eagle	+	+	Seafarer	−	−
Labrador	+	+	Fleetwood	−	−
Hystyle	+	+	BelMiDak-RR-1	+	+
Benton	+	+	BelMiDak-RR-2	+	+
Podsquad	+	+	BelMiDak-RR-3	−	−
Sprite	+	+	BelMiDak-RR-4	−	−
Rebel	−	+	BelMiDak-RR-5	−	−
Raider	−	+	BelMiDak-RR-6	−	−
Bountiful	−	+	4-5753	+	+
Provider	−	+	*Pinto*		
Slenderwhite	−	+	90T4042	−	−
Resisto	−	+	Sierra	−	−
Kidney			Olathe	−	−
Mecosta	−	+	UI-114	−	−
Sacramento	−	+	Aztec	−	−
Charlevoix	−	+	*Black*		
Chinook	−	+	Blackhawk	−	−
Idaho LRK	−	+	B90222	−	−
Mecosta	−	+	B90223	−	−

The DNA of each individual that contributed to the resistant bulk contained the $OA14_{1100}$ fragment. However, the intensity of the fragment within an agarose gel for one individual that was heterozygous at the Up_2 locus (lane 2; Fig. 1) was about half that observed for the other two individuals that were homozygous for the dominant Up_2 allele. This pattern of a difference in fragment intensity between heterozygous and homozygous resistant individuals was not consistent across the entire population; therefore, fragment intensity could not be used to determine the number of Up_2 alleles present at the locus. Likewise, Williams et al. (1990) stated that a RAPD marker would not differentiate between one or two copies of a particular allele at a locus.

No recombination was observed between $OA14_{1100}$ and the Up_2 gene in the segregating BC_6F_2 population of 84 individuals, suggesting that $OA14_{1100}$ and Up_2 were very tightly linked. A linkage estimate of 1.27 cM 1.23 (SE) between $OA14_{1100}$ and Up_2 would have been obtained if a single recombination event had occurred in this population. A tight linkage in the absence of recombination suppression due to an alien background would suggest that $OA14_{1100}$ may have utility as a genetic marker and as a starting point from which to initiate chromosome walks and jumps (Rommens et al. 1989) in an attempt to isolate the gene.

The $OA14_{1100}$ marker appears to be of Andean origin because of its presence in all Andean germ plasm and absence in all Mesoamerican germ plasm surveyed except our lines to which Up_2 had been purposely transferred (Table 1). The $OA14_{1100} - Up_2$ linkage remained intact in the navy bean germ plasm releases Belmidak-RR-1 and -2 (Stavely et al. 1991), which had acquired Up_2 from the parental BC_5 line 4-5753 that was developed with C-20 as the recurrent parent and 'Early Gallatin' as the donor parent (Table 1). This result shows that $OA14_{1100}$ can be used to monitor the introgression of the Up_2 allele into dry bean germ plasm of Mesoamerican origin. $OA14_{1100}$ will have less utility as a genetic marker within the Andean gene pool because it is ubiquitous within this gene pool.

Discussion

A genetic marker tightly linked to the Up_2 rust resistance gene was found using a strategy that combined NIL and bulked segregant analysis to screen PCR-amplified fragments for polymorphism. When contrasting NILs are used, the probability of identifying a DNA marker will depend, in part, upon the sequence divergence between the NILs for the introgressed region (Martin et al. 1991; Muehlbauer et al. 1991; Young et al. 1988). The 60% to 70% polymorphism observed between Andean and Mesoamerican gene pools with genomic probes (RFLPs) (Nodari et al. 1992) and arbitrary primers (RAPDs) (Miklas and Kelly 1992) suggested the potential for DNA sequence divergence between the introgressed segment containing the Up_2 allele of Andean origin and the comparable segment of the recurrent parent of Mesoamerican origin in our population.

There were more amplified products obtained per primer in the study reported here than in the study Martin et al. (1991) conducted with tomato, possibly because they used a different DNA polymerase and some primers more than ten nucleotides in length. Miklas and Kelly (1992) obtained more amplified products with *Stoffel Fragment* than with *Amplitaq* DNA polymerase (Perkin Elmer Cetus) for an identical set of primers. Less than 35% of the products amplified by these two enzymes were in common.

It appears that a strategy that combines NIL and bulked segregant analysis may concentrate efforts and resources toward the identification of those RAPDs that are tightly linked to the target locus than either method used alone. As a result of bulking DNA samples, RAPDs associated with introgressed segments unlinked to Up_2 or associated with the introgressed segment containing the Up_2 locus but at a distance greater than 25 cM from the locus would, as a result of genetic recombination (Michelmore et al. 1991), likely appear as monomorphic bands due to their presence in both bulks. The probability of having no false positives will increase as the number of individuals comprising a bulk increases (Michelmore et al. 1991). Thus, bulked DNA samples formed with more than three individuals may be warranted for such studies in the future. Melchinger (1990) suggests screening two or more independently derived NILs from the same pair of recurrent and donor parents to decrease the occurrence of false positives.

Traditionally, breeding for resistance to bean rust is conducted on a regional basis with the incorporation of single gene resistances to the predominant races as a first step. Oligogenic resistance alone, however, may be easily overcome by the pathogenic variability that results from genetic recombination within the pathogen (Stavely et al. 1989). Therefore, breeding strategies that include multilines, non race-specific resistance factors such as the leaf pubescence avoidance mechanism (Steadman and Shaik 1988), and gene pyramiding (Coyne and Schuster 1975) have been recommended to stabilize resistance to the bean rust pathogen.

Pyramiding genes that confer resistance to all or most of the known North American rust races in combination with other genes effective against multiple races to deploy more than one gene for resistance to specific races has been accomplished for a range of dry bean genotypes (Stavely and Grafton 1989; Stavely et al. 1991). Pyramiding resistance to bean rust, how-

ever, is a difficult task because of the numerous progeny tests required for the different resistance genes. If a gene that conditions one kind of resistance to certain races of bean rust is epistatic to a gene that conditions another kind of resistance to the same races, the latter can not be detected without additional progeny tests (Stavely et al. 1989). In BelMiDak-Rust Resistant-1 and -2, resistance from PI 181996 is epistatic to that conditioned by Up_2. The use of rust races from other regions to identify the presence of specific resistance gene combinations could introduce new sources of pathogenic variability into native rust populations if great care is not taken. These problems and others (see review Melchinger 1990) associated with breeding for genetic resistance to plant diseases may be overcome using marker-based selection.

The utility of linked RAPDs as genetic markers will be dependent on the germ plasm used. For instance $OA14_{1100}$ will have greater utility as a genetic marker in beans of Mesoamerican origin where it traditionally does not exist. Conversely, $OA14_{1100}$ will have less utility within Andean germ plasm where it is ubiquitous, because progeny derived from Andean $(Up_2) \times$ Andean (up_2) hybridizations will not segregate for $OA14_{1100}$. The Cooperative MSU, USDA-ARS bean breeding program is currently using $OA14_{1100}$ to ascertain the presence of Up_2 in advanced navy bean (Mesoamerican) lines with resistance to all rust races from PI 181996 to supply a second resistance gene effective against 23 of 49 races. Normally, two additional generations to conduct a testcross and a subsequent progeny test would be required to determine the presence of Up_2 in this germ plasm. The application of marker-based selection for purposes of gene pyramiding to obtain adequate and stable levels of genetic resistance to this complex pathogen of common bean will increase further as genetic markers for other rust resistance genes are found.

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