**Random amplified polymorphic DNA markers detected
in a segregating hybrid population of
Solanum chacoense × *S. phureja****

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ABSTRACT

Reproducible random amplified polymorphic DNA (RAPD) patterns were obtained in two potato species (*Solanum chacoense* and *S. phureja*), one of their F₁'s, and its derived F₂ population, by adjusting temperature profiles of the amplification process and template DNA concentrations (1 ng/μl reaction vol.). Although the number of amplified products and detectable differences between the two species increased with increasing GC content of the primer, 50% or 60% GC is recommended for maximizing scorable RAPDs in the F₂ population. Using 82 primers, 589 RAPDs were detected between the parents, and 70% of them (409 RAPDs) in the F₁ clone. The number of RAPDs reliably scored for their segregations in the F₂ population was significantly lowered because of complicated RAPD patterns. Consequently, 22% of the RAPDs detected in the parents (129 RAPDs), an average of 1.57 RAPDs per primer, were obtained as genetic markers, which can be used for the construction of a genetic map for this particular population.

1. INTRODUCTION

Diploid potatoes and their related wild species are mostly self-incompatible, and maintain a high level of heterozygosity. Self-compatible plants within self-incompatible species sometimes occur (Cipar et al., 1964). One of those found in *Solanum chacoense* was selfed over generations and became near-inbred lines (Hanneman, 1985). A cultivated diploid species, *S. phureja*, is in the same genomic category with *S. chacoense*, resulting in reasonably good crossability between them and normal chromosome pairing behavior at meiosis in the F₁ (Matsubayashi, 1983). In order to conduct a detailed genetic analysis on the gene action of self-compatibility, one of near-inbred lines was crossed with *S. phureja*

* Reference to a specific brand or firm name does not constitute endorsement by the US Department of Agriculture over others of similar nature not mentioned.

and an F_2 population was obtained, in which self-compatibility is segregating. A DNA marker-based linkage map is required for mapping a gene inducing self-compatibility.

Restriction fragment length polymorphisms (RFLPs) are well established genetic markers in the potato. Two independent and their combined RFLP maps have already been constructed (Bonierbale et al., 1988; Gebhardt et al., 1989b, 1991; Tanksley et al., 1992); however, RFLP assay is laborious and lengthy.

Amplification of genomic DNA by polymerase chain reaction (PCR) with single short oligonucleotides of arbitrary sequence could provide polymorphic DNA bands called random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990). As most RAPDs are simply detected as the presence versus absence of DNA segments, RAPDs are mostly dominant genetic markers inherited in a Mendelian fashion (Williams et al., 1990). Quiros et al. (1993) demonstrated disomic and tetrasomic inheritance of RAPDs in diploid and tetraploid potatoes. Because of their versatility, the RAPD assay has a wide application in plant genetics. It has been used for varietal identification in various crop plants including rice (Fukuoka et al., 1992), adzuki bean (Kaga et al., 1993), *Brassica* species (Hu and Quiros, 1991; Demeke et al., 1992), soybean (Williams et al., 1990), cocoa (Wilde et al. 1992), and potato (Mori et al., 1993; Demeke et al., 1993).

The advantages of RAPDs are simplicity, rapidity and the need of only minute amounts of DNA. The RAPD markers can facilitate construction of genetic maps and mapping genes (Rafalski et al., 1991). Reiter et al. (1992) identified 225 RAPD marker loci in *Arabidopsis thaliana* and constructed a genetic map in a four month period. RAPD markers tightly linked to specific disease resistance were identified in a short period of time by using near-isogenic lines or bulked segregants in a segregating population. Such RAPD markers, for example, have been identified for *Pseudomonas* resistance (Martin et al., 1991) and nematode resistance (Klein-Lankhorst et al., 1991) in tomato, and downy mildew resistance in lettuce (Michelmore et al., 1991; Paran et al., 1991).

We report here RAPD markers detected in the *S. chacoense* $\times$ *S. phureja* population, and discuss especially on reproducibility, primer design, inheritance of marker bands and detection frequency of scorable RAPDs.

2. MATERIALS AND METHODS

Plant materials and DNA isolation

Plant materials used were a self-compatible *S. chacoense* line (clone chc525-3, S₇), *S. phureja* (clone 1.22) and one of their F_1 clones (clone F₁-1) and 60 F_2 individuals derived by selfing the clone F₁-1. Total DNA was extracted from the fresh leaves by the method of Doyle and Doyle (1987). DNA concentration was determined by a mini-gel method in comparison with lambda DNA of known

concentration.

RAPD assay techniques

Standard amplification reactions were performed in volumes of 10 μ l consisting of 10 ng of genomic DNA, 0.2 μ M primer, 10 mM Tris-Cl buffer (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μ M each of dATP, dCTP, dGTP and dTTP (Takara), 0.2 unit *Taq* DNA polymerase (Ampli Taq^R , Perkin Elmer Cetus). Primers with an arbitrary sequence of 10 bases were commercially synthesized by TOYOBO (Osaka, Japan) or Akitsu Keisoku (Tokyo, Japan), or purchased from Operon Technologies (CA, USA). The reaction mixture was overlaid with a drop of mineral oil. Two thermo-cyclers were used: Thermal Sequencer (Iwaki Glass Co., Ltd., Tokyo, Japan), composed of three oil or water baths, reaction tubes mechanically transferred from one to the other, and BioOven (BioTherm Corporation, Arlington, VA, USA), a forced air circulating oven. The Thermal Sequencer was set for 45 cycles of 1 min at 94°C, 2 min at 36°C and 3 min at 72°C, followed by one cycle of 1 min at 72°C. The BioOven was set for 45 cycles of 30 sec at 95°C, 1 min at 36°C and 2 min at 73°C, followed by one cycle of 1 min at 73°C. The entire reaction mixture, mixed with loading dye, was taken from under the mineral oil and separated by electrophoresis on a 1.4% or 2% agarose gel (SeaKem R LE, FMC) or a 3% NuSieve 3:1 agarose R gel (FMC) in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA) containing ethidium bromide. The electrophoresed gel was observed and photographed over UV light.

Monitoring temperatures in PCR

Actual temperatures in the reaction mixtures were measured by a digital thermometer (Anritsu, model: HL-100) and a sensor (Anritsu, B0.5-K-J1M3-50). The tip of the sensor was dipped into the reaction mixture through a small hole in the center of the lid of a reaction tube.

3. RESULTS

Temperature profile at PCR

Random DNA segments were amplified from plant genomic DNA by *Taq* DNA polymerase through three steps of temperature changes; double-stranded DNA is denatured to single stranded DNA at over 90°C, annealed with primers at 36°C, and DNA extended by *Taq* DNA polymerase at 73°C. Two different thermo-cyclers with comparatively different transition rates between temperatures were tested at different annealing temperatures of 42, 36 and 30°C (Fig. 1). Quick changes from denaturing to annealing and/or from annealing to DNA extension, performed by Thermal Sequencer (A), yielded different banding patterns at different annealing temperatures. In contrast, slow changes, performed by BioOven (B), gave more or less identical patterns at different annealing tempera-

Fig. 1. Responses of PCR to different thermo-cyclers (A, Thermal Sequencer; B, BioOven) and annealing temperatures (1, 42°C; 2, 36°C; 3, 30°C). F₁-1 amplified with a primer 5'-TGGTCACTGA-3' (left panel) and *S. phureja* (clone 1.22) amplified with a primer 5'-TGGTGACTGA-3' (right panel) were separated on a 1.4% agarose gel. The right lane in each panel contains *Hind*III digested lambda DNA. Arrowed bands were sensitive to the temperatures (see text).

tures. The DNA segments clearly amplified either at 36 or 30°C by Thermal Sequencer (indicated by arrows in Fig. 1) were stably amplified by BioOven both at 36 and 30°C. The thermo-cycler having slow transition rates (BioOven) might have higher tolerance against temperature fluctuations. As small temperature variation across the set of reaction tubes is usually inevitable, BioOven was used in the following experiments.

The actual temperatures in the reaction mixtures using BioOven were monitored by a digital thermometer. The temperatures varied tube by tube: over 90.0°C lasted from 50 to 80 sec (the highest from 91.7 to 93.1°C), below 37.0°C lasted from 70 to 140 sec (the lowest from 32.2°C to 36.1°C), and the range between 70.0 and 74.0°C lasted from 110 to 160 sec. It took an average of 12.2 min for one cycle. However, temperature variation to this extent gave consistent reproducible DNA banding patterns.

Genomic DNA concentration

Effects of different concentrations of template DNA in the PCR (0.01, 0.05, 0.1, 0.5, 1, 2 and 3 ng/μl) were examined (Fig. 2). Amplification succeeded in the broad range of template DNA concentrations. However, some of bands (arrowed) showed continuous increase or decrease in band intensities depending on template DNA concentrations. Therefore, an equal concentration of genomic DNA was crucial for comparisons between samples. In the following experi-

Fig. 2. Effects of different concentrations of template DNA in PCR. Amplifications were performed under different template DNA concentrations (0.01, 0.05, 0.1, 0.5, 1, 2 and 3 ng/ μ l, respectively from lanes 1 to 7) with primers (a) 5'-TGGTCACTGA-3', (b) 5'-TGGTCGCTGA-3', and (c) 5'-TGGGCACTGA-3'. The amplified products were separated on a 1.4% agarose gel. The left lane contains *Hind*III digested lambda DNA. phu=*S. phureja* (clone 1.22), chc=*S. chacoense* (chc525-3). Arrowed fragments are dependent on template DNA concentrations (see text).

ments, genomic DNA was adjusted to approximately 1 ng/ μ l PCR reaction.

Even the template DNA concentration of 0.01 ng/ μ l could amplify some DNA segments, but seemed less efficient. Higher template DNA concentrations over 2 ng/ μ l sometimes failed to amplify, resulting in unusual bands (see lane 7 of *S. phureja*, 2nd primer, in Fig. 2) or smeared DNA background tailing from the sample well (not shown). When such occurred in other plant species, diluting template DNA was generally helpful for successful amplification.

Primer design and the size of amplified DNA segments

Eighty-two 10-mer primers with different GC content (11 primers with 40%, 10 primers with 50%, 45 primers with 60% and 16 primers with 70% GC content) were tested for amplification with *S. chacoense* and *S. phureja* (Table 1).

GC content of the primer affected amplification. Five of 11 primers with 40% GC content did not generate any bands in either DNA sample. In the range from 40% to 70% GC, the average band number per primer increased with increasing GC content from 2.45 to 9.31 in *S. chacoense*, and from 3.27 to 8.63 in *S. phureja* (Table 1), though the band number slightly decreased in GC content from 50% to 60% in both samples.

Amplified DNA segments were classified by size into three categories; (1) over 2.0 kbp, (2) between 2.0 and 0.56 kbp and (3) smaller than 0.56 kbp, determined by comparisons with a 2.0 kbp fragment and a 0.56 kbp fragment of *Hind*III digested lambda DNA fragments. 13.9% of bands amplified from *S. chacoense* were in category (1), 74.8% in (2) and 11.4% in (3). In *S. phureja*, the size distribution was; 10.7% in (1), 73.4% in (2) and 15.9% in (3). Approximately

Table 1. The number of amplified DNA segments, and their averages per primer in parentheses, with primers of different GC content

Primer		<i>S. chacoense</i> *				<i>S. phureja</i> *				Between parents		Detected in F ₁ -1	Scored in F ₂
GC	No.	1	2	3	Total	1	2	3	Total	Common	Different		
40%	11	3	22	2	27	2	30	4	36	12	39	23	6
		(0.27)	(2.00)	(0.18)	(2.45)	(0.18)	(2.73)	(0.36)	(3.27)	(1.09)	(3.55)	(2.09)	(0.55)
50%	10	9	67	8	84	5	61	12	78	42	78	55	20
		(0.90)	(6.70)	(0.80)	(8.40)	(0.50)	(6.10)	(1.20)	(7.80)	(4.20)	(7.80)	(5.50)	(2.00)
60%	45	40	243	43	326	36	246	67	349	164	347	245	79
		(0.89)	(5.40)	(0.96)	(7.24)	(0.80)	(5.47)	(1.49)	(7.76)	(3.64)	(7.71)	(5.44)	(1.76)
70%	16	29	104	16	149	21	103	14	138	81	125	86	24
		(1.81)	(6.50)	(1.00)	(9.31)	(1.31)	(6.44)	(0.88)	(8.63)	(5.06)	(7.81)	(5.38)	(1.50)
Total	82	81	436	69	586	64	440	97	601	299	589	409	129
		(0.99)	(5.32)	(0.84)	(7.15)	(0.78)	(5.37)	(1.18)	(7.33)	(3.65)	(7.18)	(4.99)	(1.57)

* 1 = >2.0 kbp, 2 = 2.0–0.56 kbp, 3 = <0.56 kbp

three fourths of the amplified segments fell between 2.0 kbp and 0.56 kbp. The percentages of large fragments over 2 kbp tended to increase with increasing GC content of primers from 11.1% to 19.5% in *S. chacoense* and from 5.6% to 15.2% in *S. phureja*.

The effect of single-base substitutions of a primer on the banding pattern was examined. Each of 10 positions of the original sequence, 5'-TGGTCACTGA-3', was substituted for one of the other three bases (Fig. 3). Single-base changes

Fig. 3. Effects of single-base substitutions of primers on amplifications. *S. phureja* (clone 1.22) was amplified with primers, in which a single-base was substituted for one of the other three bases in each of 10 positions from the 5' end of the decamer primer (5'-TGGTCACTGA-3'). Sets of four lanes in order of A, T, C and G substitutions are given in parallel on a 2.0% agarose gel. The left lane contains *Hind*III digested lambda DNA.

Fig. 4. Genetic segregation of four RAPD markers (arrowed) detected by the primer 5'-GTCCCGACGA-3'. The parents P (*S. phureja* clone 1.22) and C (*S. chacoense* chc525-3), their F_1 (F_1 -1), and 60 F_2 's were separated on a 3.0% NuSieve 3:1 agarose gel. The left lane contains *Hind*III digested lambda DNA.

altered banding patterns, although common bands were often observed as well. Substitutions at the 1st and 2nd positions from the 5' end were less effective in generating new bands. Base changes from A or T to G or C increased the number of amplified products probably because of the increase of GC content of the primer. The ten primers tested in Fig. 3 had a GC content of 40%, in which five primers did not amplify any discrete DNA segments.

Different bands detected between S. chacoense and S. phureja

Eighty-two primers amplified a total of 586 bands in *S. chacoense* (an average of 7.15 bands per primer) and 601 bands in *S. phureja* (an average of 7.33 bands per primer) (Table 1). Among them, 299 bands, or an average of 3.65 bands per primer, were common in both samples. A total of 589 different bands, or RAPDs, were detected between *S. chacoense* and *S. phureja*. Although the number of amplified products increased with increasing GC content of the primers, the number of detectable RAPDs per primer was not significantly different between 50 and 70% GC content. An overall average was 7.18 RAPDs per primer between the two species.

Detection of RAPDs in F_1 and F_2

From 589 RAPDs identified between *S. chacoense* and *S. phureja*, 409 (69.4%) were detected in a single F_1 (F_1 -1, self-compatible) (Table 1).

Genetic segregations were investigated in 60 F_2 plants. For example, four bands (arrowed in Fig. 4), three from *S. phureja* (44a, 44b and 44d) and one from *S. chacoense* (44c), segregated (presence vs. absence) in the F_2 population.

An average of 2.00 RAPDs per primer was scored in F_2 using the 50% GC primers (Table 1). However, the primers with higher GC contents generated a larger number of amplified DNA segments, deriving complicated patterns in which the number of visually scorable RAPDs was actually decreased; 1.76 by 60%GC and 1.50 by 70% GC primers. Consequently, 129 of 409 RAPDs in F_1 were clearly scored in the F_2 population (1.57 RAPD markers per primer). The rest of them were unable to score because of complicated RAPD patterns.

4. DISCUSSION

PCR conditions and reproducibility

The RAPD assay entirely depends on PCR, which is, although a one-tube reaction, a complicated enzymatic process affected by various internal factors such as the concentrations of the template DNA, primer, deoxynucleotide, magnesium, *Taq* DNA polymerase concentration, an enzyme source, and their balance in a reaction mixture (Carlson et al., 1991; Devos and Gale, 1992). However, the same experimental conditions except for template DNA can be reproduced easily

in different laboratories.

External factors represented by different thermo-cyclers and their setting conditions are crucial for reproducible performance of RAPDs (Carlson et al., 1991). By comparing two thermo-cyclers in this study, it was confirmed that different thermal profiles altered PCR, resulting in different RAPD patterns. From our experience, most problems in experimental reproducibility were caused by temperature differences across the block or tube rack. As pointed out by Devos and Gale (1992), small temperature differences at the denaturing step influenced amplification, but those at the annealing temperature had negligible effects: when one reaction mixture was at 36°C and the other was at 32°C in BioOven, both presented similar RAPD patterns. Thus, it is important to monitor the actual temperature of each reaction mixture and set them within an acceptable temperature range.

Template DNA concentrations for obtaining reproducible RAPD patterns have been optimized in some plant species, which varies significantly; for example, 0.2–0.4 ng/μl for wheat (Devos and Gale, 1992), 8 ng/μl for Douglas-fir (Carlson et al., 1991), and 10 ng/μl for tomato (Klein-Lankhorst et al., 1991). This study proposed the concentration of 1 ng/μl for potatoes, although a broad range of template DNA concentrations (0.05–2 ng/μl) amplified successfully. With over 2 ng/μl of template DNA, smeared backgrounds were observed, which may have resulted from the annealing of the 3' end of various PCR products, extension and random termination after initial 10-mer primers were used up, as described by Devos and Gale (1992). In terms of reproducibility of RAPD patterns, adjusting genomic DNA concentration equally in each sample may be an initial step to obtain reproducible and comparable banding patterns.

Primer and RAPD pattern

The number of amplification products increased with increasing GC content of the primers. This tendency was previously reported in rice by Fukuoka et al. (1992), although Tulsieram et al. (1992) found no obvious association between GC content of primers and the number of detectable amplification products in conifers. Fritsch et al. (1993) confirmed the existence of a positive correlation between GC content of a primer and its ability and strength of amplification in flowering plants. These support that the higher stability of GC complementation (three hydrogen bonds) might yield a larger number of effective priming sites than AT complementation (two hydrogen bonds) (Fukuoka et al., 1992). Although a higher number of polymorphic bands was observed with primers of higher GC content, scorable bands were decreased, as discussed later. From a practical point of view for RAPD mapping, either 50% or 60% GC is suggested for efficient RAPD primers.

Approximately three fourths of the amplification products fell into the size range between 2.0 kbp and 0.56 kbp. Similar observations were made in rice

(Fukuoka et al., 1992) and cocoa (Wilde et al., 1992). The sizes of DNA segments amplified by *Taq* DNA polymerase are limited and those over 2 kbp are merely synthesized (Saiki et al., 1988). Based on the nuclear DNA content of the diploid *S. tuberosum* (1597–1862 Mbp) (Arumuganathan and Earle, 1991), the number of possible annealing sites which match perfectly with 10-mer primers with potato genomic DNA (around 1650 sites) (1597–1862 Mbp divided by 4^{10}) and the average distance between them (approximately 1050 kbp) can be calculated. The estimates indicate that shorter DNA segments are amplified less frequently. Seemingly, these two limiting factors for amplification could explain the observed size distribution in RAPD patterns.

Amplification is carried out even when a primer was not perfectly matched with template DNA (Williams et al., 1990; Rafalski et al., 1991). This is supported by the results with single-base substituted primers which amplified similar fragments in addition to new fragments (Fig. 3).

RAPD detection frequency

Reiter et al. (1992) identified 392 RAPDs between two lines of *Arabidopsis thaliana* by screening 1200 primers; the RAPD detection frequency was 0.33 RAPDs per primer. An overall average of RAPD detection frequency between *S. chacoense* and *S. phureja* was 7.18 per primer, which exceeds significantly that of the above-mentioned *A. thaliana* and those between Japonica and Indica rice (2.9 per primer) (Fukuoka et al., 1992), two lines of *Zea mays* (1 per primer) (Williams et al., 1990), soybean *Glycine max* and *G. soja* (0.5 per primer) (Williams et al., 1990), two strains of *Neurospora crassa* (2.5 per primer) (Williams et al., 1990).

S. chacoense is a field weed in lowland pastures and has the white stellate corolla. In contrast, *S. phureja* is cultivated in wet mountain slopes and has the colored rotate corolla (Hawkes, 1990). Their distinctiveness is supported by chloroplast DNA analysis; *S. chacoense* has W type and *S. phureja* has S or A type chloroplast DNA (Hosaka and Hanneman, 1988). Bonierbale et al. (1990) positioned *S. chacoense* and *S. phureja* on different branches in a dendrogram constructed by RFLP markers. These are all in agreement with the high level of RAPD detection frequency, suggesting considerable differentiation between the two species.

*RAPD markers for the *S. chacoense* $\times$ *S. phureja* population*

The segregating bands in F_2 were either present or absent, and all were present in the F_1 plant, thus behaving like dominant markers. This agrees with previous reports on RAPD markers in potatoes (Quiros et al., 1993) and other species (Williams et al., 1990; Carlson et al., 1991; Hu and Quiros, 1991; Roy et al., 1992).

69.4% of RAPDs between the parents were identified in a single F_1 clone. A

parent homozygous at a given RAPD locus produces progenies all showing the RAPD band because of dominant nature. If it is heterozygous, 50% of progenies show the band. Thus, at least 30.6% of RAPD loci were obviously heterozygous in the parents, since those did not amplify the DNA segments in the F_1 clone. The other RAPD loci in the parents might be heterozygous, but were unable to be revealed in a single F_1 genotype. Most of the diploid potato species, including the two species used in this study, are outbreeding and maintain a high proportion of heterozygous loci as demonstrated at DNA level by RFLP markers (Gebhardt et al., 1989a) and at protein level as well (Hosaka and Hanneman, 1991).

RFLP markers used for map construction are usually single-copy DNA represented by very few bands on the Southern-blot (generally, one or two); thus, their segregations can be scored easily by visual observation. In contrast, the RAPD patterns averaged of 7.15 bands in *S. chacoense* and 7.33 bands in *S. phureja* and were crowded within about 2 cm distance on the gel, and in the F_2 , 4.99 bands segregated in addition to 3.65 monomorphic bands in a narrow area (Table 1). This complicated RAPD pattern hindered reliable scoring of RAPDs in segregation analysis. In this study, only 32% of 409 RAPDs which were expected to segregate in the F_2 population were reliably scored. A similar result was reported in *Arabidopsis thaliana*, in which 225 of 392 polymorphic DNA bands (57%) were scorable in a segregating population (Reiter et al., 1992).

In conclusion, 129 RAPDs were identified as useful genetic markers for the self-compatible *S. chacoense* $\times$ *S. phureja* population. A genetic linkage map is currently being constructed. These RAPD markers may make possible quick advances in constructing a genetic linkage map, filling a large gap and saturating the existing potato RFLP maps (Bonierbale et al., 1988; Gebhardt et al., 1989b, 1991; Tanksley et al., 1992).

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