Restriction Fragment Length Polymorphism Analysis of Anther-culture-derived *Solanum chacoense*

Ram K. Birhman, Sylvain R. Rivard, and Mario Cappadocia

Institut de Recherche en Biologie Végétale, University de Montréal, 4101 Sherbrooke est, Montréal H1X 2B2, Canada

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Abstract. Using restriction fragment length polymorphism (RFLP) analysis, the genetic architecture of some anther-culture-derived *S. chacoense* Bitt. plants was studied, and their origins were elucidated. Our RFLP analyses showed that 1) several plants, even of different ploidy but otherwise genetically identical (clones), can be regenerated from callus originating from a single microspore and, conversely, that 2) some plants regenerated from single callus can have different genetic constitutions and, therefore, must have originated from two different microspore. These findings imply that previous anther culture efficiency estimates might have to be reconsidered.

Haploids (monohaploids and dihaploids) and homozygous doubled-haploids are important tools for studying potato genetics and improving potatoes (Cappadocia, 1990; Cappadocia et al., 1986; Sopory and Bajaj, 1987; Thach et al., 1993). Haploidization is usually obtained through either parthenogenesis or anther culture. The parthenogenetic method is time consuming and, therefore, is used rarely for obtaining monohaploids (Sopory and Bajaj, 1987); accordingly, anther culture is used widely to obtain haploids (Meyer et al., 1993).

Sometimes, researchers have assumed that each regenerated plant, whether monohaploid or not, originates from a single microspore; therefore, the number of individual plants somehow corresponds to the number of different genotypes regenerated. This assumption may be valid when gametic embryogenesis takes place, but recent work on maize (*Zea mays* L.) anther culture indicates that multiple embryo-like structures with identical genetic constitution can originate from a single microspore (Wan and Widholm, 1992). In systems regenerating via organogenesis preceded by a callus phase, even more caution is required because multiple plants can arise from a single microspore (D'Amato, 1985). Conversely, more than one cell in the callus can give rise to a shoot, which could result in genetic mosaic and chimera formation (Marcotrigiano, 1990).

Analysis with proper genetic markers as well as isozyme use and other biochemical markers can help define, in a first approximation, the regenerant's genetic architecture. For each plant's satisfactory characterization, however, using many such markers is required, and these markers may not be available in some cases. Restriction fragment length polymorphism (RFLP) analysis is an excellent technique for unequivocal plant genotype characterization and precise origin determination (Bentolila et al., 1992; Meyer et al., 1993; Rivard et al., 1989). In our study, we used RFLP to characterize several anther-culture-derived *Solanum chacoense* plants, a tuber-bearing diploid wild species regarded as promising for cultivated potato genetic improvement (Hawkes and Hjerking, 1969).

Materials and Methods

Plant material. We analyzed two self-incompatible diploid ($2n = 2x = 24$) *S. chacoense* parental lines (PI 230582 and PI 458314); some of their progeny from anther culture; an F₁ hybrid of PI 230582 and PI 458314 named B₁; and 21 anther-culture-derived plants from B₁. Details on the anther culture procedures have been reported by Veronneau et al. (1992). When two or more plants were regenerated from a single anther, they apparently came from one callus and regeneration occurred by shoot organogenesis. Each plant's ploidy level was determined by chromosome counts in root-tip cells. Isolated from *Solanum tuberosum* L. cv. Kennebec (Marineau et al., 1987), cDNA clones were used as probes on Southern blots of genomic DNA extracted from leaves harvested at once from mature plants grown in a greenhouse. These leaves were frozen and stored at -80°C until used.

Isolation of genomic DNA. Genomic DNA was isolated by the method described by Rivard et al. (1989) with the exception that Na₂-EDTA was increased to 70 mM and that 1% β -mercaptoethanol was replaced with 20 mM sodium metabisulphite in the extraction buffer.

The nuclei pellets were resuspended with a small brush to minimize the starch's abrasive effect during resuspension. In addition, all DNA samples were purified by cesium chloride-ethidium bromide preparative ultracentrifugation [300,000 $\times$ g for 48 h in a Ti 70.1 rotor, (Beckman, Palo Alto, Calif.)] to ensure consistent and complete DNA digestion when treated with restriction endonucleases.

Southern hybridization. The genomic DNA from each plant (10 mg/sample) was individually digested overnight with restriction endonucleases EcoRI and EcoRV, respectively, using 3 units/mg DNA at 37°C. The restriction fragments were separated by size through electrophoresis in 0.8% tris-acetate-EDTA (TAE) agarose gels and transferred on nylon membranes (Hybond N+; Amersham Co., Arlington Heights, Ill.) by capillarity. DNA inserts from cDNA clones were labeled with ³²P using multiprimer DNA labeling system (Amersham Co.). Details on digestion, electrophoresis, labeling method, prehybridization, hybridization, posthybridization washes, and auto-radiography have been described by Rivard et al. (1989). Allelic forms of each polymorphic cDNA probe were determined from the banding patterns of previously characterized haploid and homozygous diploid reference plants, and a number was assigned to each different allele detected with a given probe-enzyme combination (Fig. 1). When a single cDNA probe detected more than one locus, individual loci were designated by the same name but distinguished by a lowercase letter (e.g., sk3a and sk3b; Table 1).

Results and Discussion

RFLP analyses. Of the 16 probes tested on the 17 DNA-enzyme combinations, 11 probes detected 12 polymorphic loci (Fig. 1, Table 1). Haploid plant RFLP data corroborated the cytological observations by showing only one or the other RFLP marker allele. In addition, the RFLP profiles revealed that all the anther-culture-derived plants analyzed were either haploids or homozygous diploids (Table 1). This result indicates that none of the plants examined originated from unreduced microspore. Our experiment also provided results concerning the absence of plants regenerated from somatic tissue; we confirmed previous observations that sporophytic origin of anther-culture-derived plants is rare in *S. chacoense* (Rivard et al., 1989). Similar results have also been reported for rice (*Oryza sativa* L.) (Guiderdoni et al., 1989) and are in contrast to the predominantly sporophytic origin observed in other plant species such as *Arabidopsis* (Keathley and Scholl, 1983).

Eight plants (Table 1, a-h) from one single callus on anther 1 displayed identical RFLP patterns for the 12 polymorphic loci. The theoretical frequency for such an event to take place randomly is $(0.5^{12})^8 (= 1.26 \times 10^{-28})$. Thus, these plants represent clones originating from the same microspore. Plants i and j from anther 2 were also clones. Clone origin has been analyzed on some plants derived from anther culture of line PI 230582, and as many

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as 13 clones have been found to originate from a single microspore (S.R.R., unpublished data). Each of the remaining plants studied had a unique genetic architecture, indicating that each originated from a different microspore, even in those cases where two plants were regenerated from what appeared to be a single callus (Table 1, plants k and l from anther 3, and m and n from anther 4). Because the RFLP analysis in both cases showed that only three of 12 markers were shared by each pair of plants, it seems unlikely that these plants might be somaclonal variants. An alternative hy-

pothesis—that two microcalli originating from different microspores could proliferate together within the anther and give rise to shoots with distinct genetic constitution—is supported by our RFLP data. Furthermore, microscopic observations of squashed anthers where induction has occurred typically showed several scattered microscopic structures developing inside a single anther. On rare occasions, however, two or even three separate structures appeared to have grown in close proximity (Fig. 2). These observations need to be documented by additional histological studies.

Anther-culture-derived plants produced from lines B₂ and PI 230582 undergo a callus phase before regeneration (Cappadocia, 1990; Veronneau et al., 1992). This characteristic provides a mechanism for producing more than one plant from a single microspore, and by consequence, the plants would have the same genetic makeup. Of the eight clones (Table 1, a-h) regenerated from anther 1 of line B₂, three plants (a-c) were haploid, and the remaining five (d-h) were diploid. This result might be explained by diploidization in various sectors of the haploid callus. Polyploidization's first stages in potato callus derived from leaf segments have been monitored by Pijnacker et al. (1989), who have found that the degree of polyploidization depends on the initial ploidy level of the genotype used and is species dependent. In the case of regenerants i and j, both plants were diploid clones. Although callus diploidization could have occurred, spontaneous diploidization also may have occurred in the young pollen grain just after the first mitotic division through the fusion of the haploid nuclei, or even before, through endomitosis or endoreduplication at the microspore stage. Similar mechanisms have, in fact, been reported by Meyer et al. (1993) to explain the production of homozygous regenerants, via gametic embryogenesis, in anther culture of several diploid potato genotypes.

Formation of more than one plant from the same microspore during anther culture has been previously reported in rice (Guiderdoni, 1991; Guiderdoni et al., 1989) and more recently in specific maize genotypes (Wan and Widholm, 1992). Our results show that this is

Fig. 1. RFLP patterns detected with cDNA clone sm17 on Southern blots of EcoRI-digested genomic DNAs. The origin is at the top and DNA size markers are indicated in kilobase pairs in the left margin. The parental genotypes (PI 230582 and PI 458314), reference plants carrying allele 1 (P26-haploid) or allele 2 (P51-haploid) were regenerated from PI 230582 anther culture; reference plants carrying allele 3 (H20-haploid) or allele 4 (H61-haploid) were regenerated from PI 458314 anther culture; the F₁ hybrid B₂, plants a-u (nine haploids and 12 diploids) were derived from B₂, which inherited alleles 1 and 3. Plants a-h, k, n, s, and t inherited allele 1, whereas plants i, j, l, o, q, r, and u inherited allele 3.

Table 1. RFLP and cytological data of plants derived from in vitro anther culture of *Solanum chacoense* F₁ hybrid B₂.

Genotype	Anther donor no.	Ploidy ^a	Loci											
			sb4	sb16	sm3	sm4	sm17	sm21	sk1	sk2	sk3a	sk3b	sk4	sk5
PI 230582		2x	1,2 ^b	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
PI 458314		2x	3,4	3,4	3,4	3,4	3,4	3,4	3,4	3,4	3,4	3,4	3,4	3,4
B ₂		2x	2,4	1,4	1,4	2,3	1,3	2,3	1,3	1,4	2,3	1,4	1,3	1,4
Anther-culture-derived plants														
a	1	1x	4	4	4	3	1	3	1	4	3	4	3	4
b	1	1x	4	4	4	3	1	3	1	4	3	4	3	4
c	1	1x	4	4	4	3	1	3	1	4	3	4	3	4
d	1	2x	4	4	4	3	1	3	1	4	3	4	3	4
e	1	2x	4	4	4	3	1	3	1	4	3	4	3	4
f	1	2x	4	4	4	3	1	3	1	4	3	4	3	4
g	1	2x	4	4	4	3	1	3	1	4	3	4	3	4
h	1	2x	4	4	4	3	1	3	1	4	3	4	3	4
i	2	2x	4	4	4	2	3	2	3	1	2	1	3	1
j	2	2x	4	4	4	2	3	2	3	1	2	1	3	1
k	3	1x	2	1	1	2	1	3	1	1	3	ND ^c	1	1
l	3	1x	4	4	4	2	3	3	3	4	2	ND	3	1
m	4	1x	2	4	4	3	ND	2	1	4	3	4	3	4
n	4	1x	2	1	1	2	1	2	1	1	2	1	1	1
o	5	1x	4	4	4	2	3	2	3	4	3	4	3	1
p	6	1x	2	1	4	2	ND	3	3	1	2	1	1	1
q	7	2x	4	4	4	2	3	3	3	4	2	1	3	1
r	8	2x	4	4	4	2	3	3	1	4	3	4	3	1
s	9	2x	2	1	1	2	1	3	1	1	2	1	1	1
t	10	2x	2	1	1	2	1	3	1	1	3	4	1	1
u	11	2x	4	4	4	3	3	2	1	4	2	1	3	1
			E ^d	V	E	E	E	E	V	E	E	V	V	V

^a1x = haploid (2n = 12); 2x = diploid (2n = 24).

^b1,2; 3,4; 1,3; 1,4; 2,3; 2,4 = heterozygous for the corresponding alleles. 1, 2, 3, 4 = homozygous for the corresponding allele.

^cND = Not determined.

^dE = EcoR I; V = EcoRV.

Fig. 2. Three microspore-derived microscopic structures, stained with lactoacetic orcein, developing inside a responsive anther of genotype B₁ after 2 weeks of culture.

also possible in *S. chacoense*. Furthermore, our results confirm previous observations that plants of different ploidy can be genetically identical, thus proving that they originated from the same microspore (Guiderdoni et al., 1989). Because plants that are indistinguishable by RFLP analyses may show different ploidy levels, the general idea that quantitative differences in ploidy levels imply qualitative differences in genetic content must be reconsidered. All these observations call for attention in breeding programs and in genetic studies (e.g., estimates of anther efficiency or segregation studies) where anther-culture-derived plants are used. These plants, however, can readily and unequivocally be characterized through RFLP analysis so that we may avoid discarding genetically different plants, simply because they apparently originate from a single callus, or, conversely, avoid working with several copies of the same genotype in crop breeding programs that use haploids.

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