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Restriction fragment length polymorphism (RFLP) analyses of plants produced by in vitro anther culture of *Solanum chacoense* Bitt

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Summary. In this study, a novel approach was used to characterize the genetic architecture of plants produced by in vitro anther culture of two lines of self-incompatible *Solanum chacoense* Bitt. ($2n = 2x = 24$). We used cytological observations to determine the ploidy level of the regenerated plants and scanned genomic DNA of the anther donor plants to identify heterozygous sequences. Restriction fragment length polymorphism (RFLP) analyses permitted the visualization of DNA variations. Several heterozygous DNA markers were found within single anther donor plants. Completely homozygous lines could be easily identified. Somatically derived plants could be separated from diploid plants produced from 2n (unreduced) microspores. Our results demonstrate first division restitution (FDR) as the mechanism operating during the production of 2n microspores in one of our *S. chacoense* line. Potential applications of RFLP analyses for genetic mapping, identification of lethal alleles and quantitative trait loci (QTL) with haploid or homozygous diploid plants and determination of gene-centromere distance with diploid plants derived from 2n microspores will be discussed.

Key words: RFLP markers – Anther culture – *Solanum* sp. – FDR – Self-incompatibility

Introduction

It is known from reports on in vitro anther culture of several plant species that haploids are not exclusively produced and that plants with other ploidy levels are also regenerated (Bajaj and Sopory 1986). In *S. chacoense,* a tuber-bearing diploid potato, in vitro anther culture often produces diploid plants (Cappadocia et al. 1984). The precise determination of the genetic architecture of these plants is important, whether for using homozygous diploids in potato breeding programs or for genetic analyses of the gametophytic self-incompatibility system of this species (Bajaj and Sopory 1986; Cappadocia et al. 1986). Diploid plants produced from anther culture will be fully homozygous only if originating from reduced microspores where nuclear fusion or spontaneous chromosomes doubling occurred during the first stage of culture or during the first pollen mitosis (Sunderland 1974). By contrast, diploid plants originating from somatic tissues of the anther will be genetically identical to the anther donor plant. Some diploids would be partially heterozygous if they originated from 2n (unreduced) microspores. In such cases, FDR (first division restitution) or SDR (second division restitution) mechanisms will generate lines homozygous for some genetic markers and heterozygous for others (Veilleux 1985). Similar results will also be obtained if the line is an aneuploid (monosomic/deletion). This complicates the identification of truly homozygous lines when the number of informative genetic markers available in the anther donor plant is limited. The availability of many polymorphic markers within a single anther donor plant would overcome these difficulties and provide an accurate method to determine the genetic make-up of diploid plants produced by anther culture.

Molecular biology techniques now offer the opportunity to rapidly identify large numbers of heterozygous genetic markers within a single plant. In this approach, cloned DNA sequences are used to probe specific regions of a plant genome for the presence of variations at the DNA sequence level. These variations are detected by

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restriction endonucleases and are revealed by separating DNA fragments according to their sizes by electrophoresis. Polymorphism is seen as variations in the length of genomic DNA fragment(s) homologous to the radioactively labelled cloned DNA sequence. Such variations in fragment length between individuals have been termed restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980). Methods and applications of RFLP analyses for plant improvement have been discussed previously (Landry and Michelmore 1987). In this paper, we demonstrate the possibility for a novel application of RFLP analysis, namely to distinguish true homozygous diploids from somatically derived plants and diploids arising from 2n microspores during anther culture of *S. chacoense*. Parallel cytological and morphological observations were used to complement the data obtained from RFLP analyses.

Materials and methods

Plant resources

Two diploid lines of *S. chacoense* (2n = 2x = 24), obtained from the Potato Introduction Station of Sturgeon Bay, Wisconsin, were used as anther donors for in vitro culture. Line PI230582 is strictly self-incompatible and line PI458314 displays erratic pseudo-compatibility. These two lines differ also in the process by which plants are produced from anther culture. Plants regenerated from anthers of PI458314 arise only through embryogenesis, while plants produced from PI230582 go through a callus phase before regeneration. The methods for in vitro anther culture have been described previously (Cappadocia et al. 1984, for PI458314; Cappadocia and Ahmim 1988, for PI230582). Cytological analyses permitted the identification of the ploidy level of all the plant material used in this study. A sample of six haploid, six diploid and one tetraploid plants regenerated from PI230582 and six haploid and six diploid plants regenerated from PI458314 were analyzed. Four plants produced from selfing of PI458314 were also included in our analyses.

Cytological and morphological observations

The ploidy level of each plant was determined by chromosome counts on root tip cells as described by Cappadocia et al. (1984). Pollen fertility and size were determined on mature samples by staining with 1% acetocarmine. Microsporogenesis was studied with paraffin section (10 μm) of anthers stained by the Schiff's reagent and mounted according to standard procedures (Berlyn and Miksche 1976). Observations on pollen tube growth in styles of pollinated flowers were done using fluorescence microscopy technique (Martin 1959). They were used to supplement the data on fruit and seed setting.

Source of probes

Polysomal poly(A)$^+$ mRNAs were isolated from *S. tuberosum* tubers as described previously (Marineau et al. 1987). Double-stranded cDNAs were prepared from these mRNAs according to the procedure of Gubler and Hoffman (1983), using oligo d(T) primers. The cDNAs were inserted into the PstI site of pBR322 by homopolymeric tailing, and used to transform *E. coli*. A cDNA library containing 1.4×10^4 clones was obtained and small-scale plasmid preparations were made from individual colonies (Rodriguez and Tait 1983). cDNA inserts larger than 500 bp were isolated by using a modification of the freeze-squeeze procedure (Tautz and Renz 1983) and used as probes on Southern blots of *S. chacoense* genomic DNA.

Isolation of S. chacoense genomic DNA

Genomic DNA of *S. chacoense* was isolated as described for lettuce, with the exception that the sucrose concentration was increased to 0.6 *M* in the extraction buffer and the concentration of β-mercaptoethanol was doubled in all the solutions (Landry et al. 1987). The nuclei pellets were also resuspended with a small brush to minimize the abrasive effect of starch during resuspension.

Southern blot analysis

DNA samples of each *S. chacoense* line were individually digested with each of the following restriction endonucleases: BamHI, EcoRI, EcoRV, HindIII. Digested DNAs (10 μg/lane) were electrophoresed in 0.8% TAE agarose gels and transferred onto Zetaprobe membranes (Bio-Rad) by capillarity (Southern 1975). Four duplicate Southern blot sets were made to simultaneously test four cDNA probes. DNA inserts from cDNA clones were labeled with ^{32}P by nick-translation (Rigby et al. 1977). Hybridizations were performed as previously described (Landry et al. 1987). Filters were washed in 2 × SSC, 1% SDS at room temperature for 10–20 min, then twice in 0.1 × SSC, 1% SDS and 0.1% sodium pyrophosphate in a 4-l beaker placed on a microprocessor-controlled hot plate stirrer (Fisher), with the temperature probe set at 63 °C, stirring at 325 rpm for 30 min. Filters were then exposed to XAR-5 X-ray film (Kodak) for two days at −80 °C using two Lightning Plus intensifier screens (DuPont). After exposure, probes were stripped from the membrane by washing in 0.1 × SSC, 0.1% SDS at 85 °C for 30 min. The membranes were rehybridized up to ten times.

Analyses of polymorphisms

Heterozygous cDNA probes were identified on the X-Ray film as differences in banding patterns between individuals regenerated from anther culture. Allelic forms of each polymorphic cDNA probe were determined from the banding patterns of haploid plants and a number was assigned to each different allele detected with a given probe-enzyme combination. Since we studied lines derived from two plants (PI458314 and PI230582), a maximum of four different alleles could be observed for each locus detected with a given combination of probe-enzyme. When a single cDNA probe detected several loci, individual loci were designated by the same name but distinguished by a lower case letter (e.g. *sb17a, sb17b* and *sb17c*). When more than one enzyme displayed polymorphism for the same locus, only one enzyme × probe combination was reported.

Results

Cytological and morphological observations

Chromosome counts on root tip cells permitted a rapid determination of the ploidy level of each plant analyzed in this study (Tables 1 and 2). Haploids could be clearly differentiated from diploids, as they exhibited reduced size and lower vigor. The tetraploid individual (B13), by contrast, was extremely vigorous, exceeding all the plants

Table 1. RFLP, morphological and cytological data of plants derived from in vitro anther culture of *S. chacoense* line PI 230582

Locus	Enzyme	PI 230582	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13
sb1	HindIII	1,2[a]	1	1	1,2	1,2	1,2	1,2	1	1	2	1	1	1	1,2
sb2	EcoRI	1,2	1	1	1,2	2	1,2	1,2	2	2	1	2	2	2	1,2
sb3	EcoRI	1,2	1	2	1,2	1,2	1,2	1,2	1	1	2	1	1	2	1,2
sb4	EcoRI	1,2	1	2	1,2	1,2	1,2	1,2	1	2	2	1	1	2	1,2
sb5	EcoRV	1,2	1	2	1,2	1,2	1,2	1,2	2	2	1	1	1	2	1,2
sb6	EcoRI	1,2	1	2	1,2	2	1,2	1,2	1	1	1	1	1	2	1,2
sb7	EcoRV	1,2	1	1	1,2	2	1,2	1,2	1	2	2	1	1	2	1,2
sb8	HindII	1,2	1	1	1,2	1,2	1,2	1,2	2	2	1	2	2	1	1,2
sb9	EcoRI	1,2	2	2	1,2	2	1,2	1,2	1	1	2	1	1	2	1,2
sb10	EcoRI	1,2	1	2	1,2	1	1,2	1,2	1	1	2	2	2	1	1,2
sb11	EcoRI	1,2	2	1	1,2	1,2	1,2	1,2	2	2	1	2	2	1	1,2
sb12	EcoRI	1,2	1	1	1,2	1,2	1,2	1,2	1	1	2	1	1	1	1,2
sb13	EcoRI	1,2	2	2	1	1,2	1,2	2	1	2	1	1	1	2	1,2
sb14	HindIII	1,2	1	1	1,2	1,2	1,2	1,2	2	2	1	2	2	1	1,2
sb15	EcoRI	1,2	1	2	1,2	1	1,2	2	1	1	2	1	1	2	1,2
sb16	EcoRV	1,2	2	1	1,2	1,2	1,2	1,2	2	2	1	2	2	1	1,2
sb17a	EcoRV	1,2	2	1	1,2	1,2	1,2	1,2	2	2	2	2	2	1	1,2
sb17b	EcoRI	1,2	1	1	1,2	1,2	1,2	1,2	2	1	1	1	1	1	1,2
sb17c	EcoRI	1,2	1	2	1,2	1,2	1,2	1,2	2	2	1	1	1	2	1,2
sb18	EcoRV	1,2	2	1	1	2	1,2	2	2	1	1	1	1	2	1,2
sb19	EcoRV	1,2	2	1	1,2	1	1,2	1,2	1	1	2	2	2	1	1,2
sb20	EcoRI	1,2	1	2	1,2	1	1,2	2	1	1	2	1	1	2	1,2
sb21	EcoRI	1,2	1	1	1,2	1,2	1,2	1,2	2	1	1	1	1	1	1,2
sb22	EcoRI	1,2	1	2	1,2	1,2	1,2	1,2	1	1	2	1	1	2	1,2
sb23	EcoRI	1,2	2	1	2	1	1,2	1	2	2	1	2	2	1	1,2
No. chromosomes		24	24	24	24	24	24	24	12	12	12	12	12	12	48
Ploidy level		2×	2×	2×	2×	2×	2×	2×	1×	1×	1×	1×	1×	1×	4×
Flower formation		+	–	–	+	+	+	+	+	–	+	+	+	–	+
Pollen formation		+	–	–	+	+	+	+	–	–	–	–	–	–	+
Pollen fertility		>95%	–	–	>95%	>95%	>95%	60%	–	–	–	–	–	–	>95%
Fruit setting		+	–	–	+	–	+	+	–	–	–	–	–	–	+

[a] Numbers indicate plant genotypes: 1 – homozygous for allele 1; 2 – homozygous for allele 2; 1,2 – heterozygous for allele 1 and 2

in several morphological traits such as height, leaf and flower sizes, and leaf thickness. Aneuploids were not found in our plant sample.

Flower, pollen production, pollen fertility and fruit setting were also determined (Tables 1 and 2). Line PI230582 produced variable amount of 2n pollen (up to 7%) while PI458314 only sporadically did so. In the former line, parallel spindle fiber formations during meiotic divisions were observed (Fig. 1). This indicates FDR mechanism operating during the formation of 2n microspores (Veilleux 1985).

Plant RFLP analyses

Thirty-nine cDNA probes were tested on a total of 56 DNA-enzyme combinations (14 plant DNA samples × 4 restriction endonucleases) for line PI230582 and on 68 DNA-enzyme combinations (17 plant DNA samples × 4 restriction endonucleases) for line PI458314. Twenty-three cDNA probes (56%) detected 25 polymorphic loci in PI230582 (Table 1). Similarly, 17 cDNA probes (44%) detected 18 polymorphic loci in PI458314 (Table 2).

Examples of the type of RFLPs detected in PI230582 and PI458314 are shown in Fig. 2. Completely homozygous plants displayed only one or the other but never both allelic forms of any DNA marker. Somatically derived diploid plants displayed both allelic forms of all the DNA markers. Partially heterozygous plants were also found; they displayed both alleles of most DNA markers but, in some plants, only one or the other allele was present.

RFLP data of haploid plants (B7–B12) derived from PI230582 were consistent with the cytological observations; only one or the other allele of each RFLP marker was present (Table 1). The two alleles were not found at the same time in any of these haploid plants. Haploid plants B10 and B11 displayed the same RFLP patterns for all the 25 polymorphic loci. The frequency of such a random event is 2.98×10^{-8} (0.5^{25}). Therefore, B10 and B11 are clones derived from the same microspore. Diploids B1 and B2 are completely homozygous plants, since no heterozygosity was detected with any of the 25 loci tested. RFLP patterns of diploid plant B5 were identical to its progenitor PI230582, suggesting that this

Table 2. RFLP, morphological and cytological data of plants derived from in vitro anther culture of *S. chacoense* line PI 458314

Locus	Enzyme	PI 458314	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	S1	S2	S3	S4
sb1	HindIII	1,2[a]	1	2	2	1	2	2	1	2	2	2	2	2	1,2	1,2	1	1
sb3	EcoRV	1,2	1	1	1	1,2	1	1	1	1	1	1	1	1	1,2	1	1,2	1,2
sb4a	EcoRI	2,3	3	3	3	2,3	3	3	3	3	3	3	3	3	3	3	2,3	1,2
sb4b	HindIII	1,2	2	1	2	1,2	2	1	2	2	2	2	2	2	2	2	1,2	1,2
sb5	EcoRI	1,2	1	2	1	1,2	2	2	1	2	1	2	2	1	1,2	1,2	1,2	1,2
sb7	EcoRI	1,2	1	1	2	1,2	2	2	2	1	2	2	2	2	1,2	2	1,2	2
sb8	BamHI	3,4[b]	3	3	3	3,4	3	4	3	3	3	3	4	3	3,4	3,4	3,4	2,4
sb11	EcoRI	3,4	3	3	3	3,4	3	3	3	3	3	3	3	3	3,4	4	3,4	1,4
sb12	EcoRI	3,4	3	4	4	3,4	3	4	3	3	4	3	3	3	3,4	3,4	3	NS
sb13	BamHI	1,2	1	2	1	1,2	2	1	1	1	1	1	1	2	1	1	1,2	2
sb14	HindIII	3,4	3	3	3	3,4	3	4	3	3	3	3	4	3	3,4	3,4	3,4	4
sb16	EcoRV	1,3	1	1	1	1,3	1	1	1	1	1	1	1	1	1	1	1,3	1,3
sb17	EcoRV	3,4	3	4	4	3,4	4	3	4	4	4	4	3	4	3,4	4	3,4	4
sb18	EcoRV	3,4	4	3	4	3,4	4	3	3	4	3	3	3	4	3,4	3,4	3,4	1,4
sb21	EcoRI	1,2	1	1	1	1,2	1	1	1	1	1	1	1	1	1,2	1,2	1,2	2,2
sb23	EcoRI	1,3	1	3	3	1,3	1	3	3	1	3	3	1	1	1,3	1,3	1,3	1,3
sb24	EcoRI	1,2	1	2	2	1,2	2	2	1	1	1	1	2	1	1,2	1	1,2	1,2
sb25	BamHI	1,2	1	1	2	1,2	2	2	2	1	2	2	2	1	1	2	2	1,2
No. chromosomes		24	24	24	24	24	24	24	12	12	12	12	12	12	24	24	24	24
Ploidy level		2×	2×	2×	2×	2×	2×	2×	1×	1×	1×	1×	1×	1×	2×	2×	2×	2×
Flower formation		+	+	–	–	+	–	+	–	–	–	–	+	–	ND	ND	ND	ND
Pollen formation		+	–	–	–	+	–	+	–	–	–	–	–	–	ND	ND	ND	ND
Pollen fertility		>95%	–	–	–	>95%	–	<50	–	–	–	–	–	–	ND	ND	ND	ND
Fruit setting		+	–	–	–	+	–	–	–	–	–	–	–	–	ND	ND	ND	ND

[a] Numbers indicate plant genotypes: 1 – homozygous for allele 1; 2 – homozygous for allele 2; 1,2 – heterozygous for allele 1 and 2
[b] Alleles numbered 3 and/or 4 are different from those seen in PI 230582 for the same probe/enzyme combination
ND – Not determined
NS – indicates a non-scorable genotype

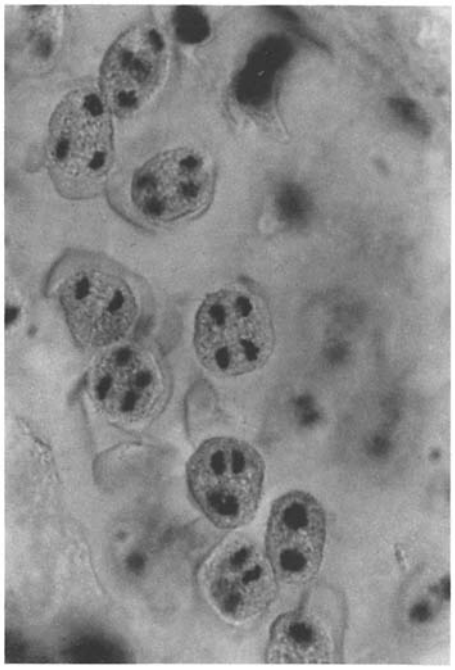

Fig. 1. Anaphase II of meiosis in pollen mother cells of *S. chacoense* line PI 230582 showing the parallel orientation of the spindle fibers in some cells

plant was regenerated from somatic tissues of the anther. Somatic origin was also concluded for plant B13, since all RFLP patterns were identical to PI230582 (Table 1). Chromosome counts indicated the tetraploid nature of plant B13. Therefore, plant B13 is most likely derived from somatic anther tissues where spontaneous chromosome doubling has occurred, probably during in vitro culture.

Diploid plants B3, B4 and B6 are partially heterozygous indicating either: (1) aneuploidy, (2) chimeras, (3) microdeletions, or (4) an origin from 2n microspores. Since chromosome counts consistently revealed 24 chromosomes, aneuploidy could not account for the partial heterozygosity of these plants. Chimeras would display variable hybridization intensities of the two alleles. Hybridization intensities of alleles in B3, B4 and B6 were not different, indicating equal amount of DNA for each allele (Fig. 2b, plants B3, B4, B6). Microdeletion could also be excluded since more than one random locus was homozygous in each plant; one would have to assume multiple deletions occurring in plants B3, B4 and B6. Diploid plants derived from 2n microspores would display partial heterozygosity similar to what was observed with plants B3, B4 and B6. Considering that production

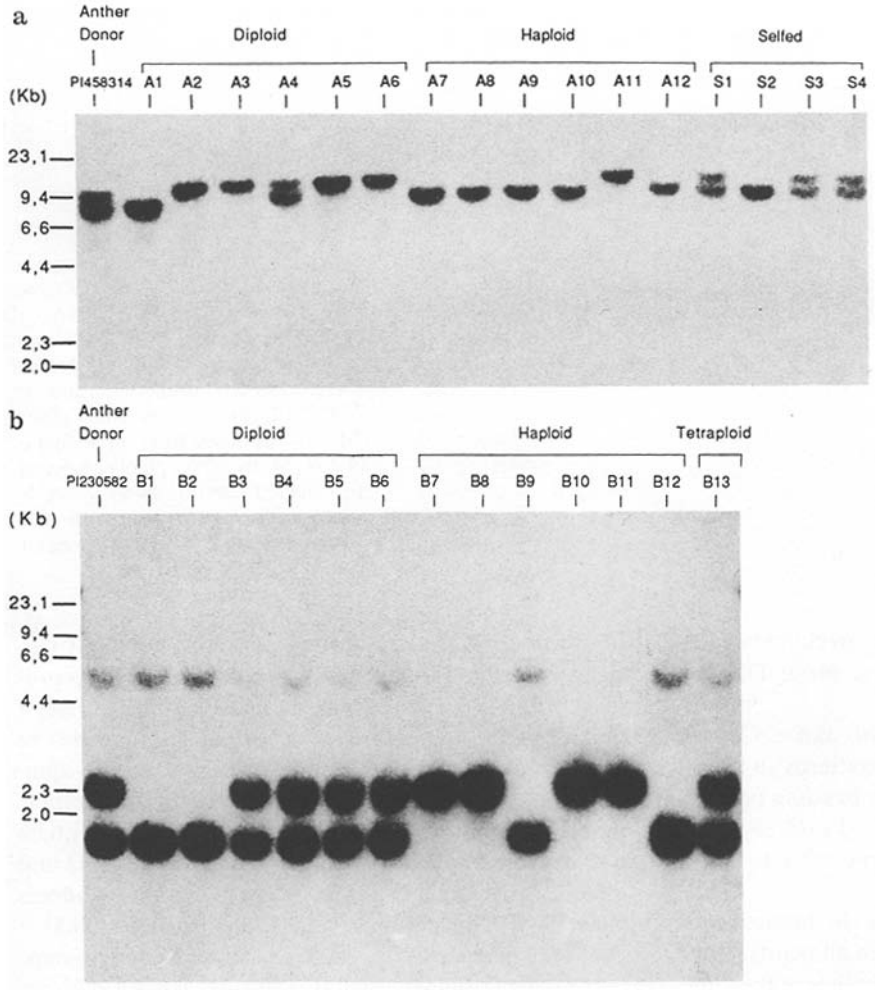

Fig. 2 a and b. Two patterns of RFLP segregation observed on Southern blots of plants derived from PI 230582 and PI 458314. The origin is at the top and DNA size markers are indicated in kilobases in the left margin. **a** RFLPs detected with cDNA clone *sb24* on EcoRI-digested genomic DNAs of PI 458314, six diploid (A1–A6) and six haploid (A7–A12) plants derived from in vitro anther culture of PI458314, as well as four plants (S1–S4) produced from the selfing of PI 458314. **b** RFLPs detected with cDNA clone *sb14* on HindIII-digested genomic DNAs of PI 230582, six diploid (B1–B6), six haploid (B7–B12) and one tetraploid (B13) plants derived from in vitro anther culture of PI 230582. In both **a** and **b**, allele 1 is the top band and allele 2 is the bottom band

of 2n pollen is relatively frequent with PI230582, that parallel spindles have been observed during meiosis (Fig. 1) and that most of the RFLP markers remained heterozygous (Table 1), plants B3, B4 and B6 are most likely derived from 2n microspores that were generated via FDR. If SDR was operating, most of the RFLP markers would have become homozygous (Veilleux 1985; Veilleux et al. 1985).

RFLP data on haploid plants (A7–A12) produced from PI458314 were also consistent with the cytological observations. Only one or the other allele of each RFLP marker was present (Table 2). Both alleles were never found in any of the haploid plants analyzed. Diploid plants A1, A2, A3, A5 and A6 displayed RFLP patterns typical of completely homozygous individuals; no heterozygous loci were observed. Diploid plant A4 is partially heterozygous. It was probably regenerated from a 2n microspore that was produced via FDR, since only one locus (*sb1*) became homozygous. In this case, a single microdeletion, undetectable by cytological observations, could not be excluded.

The pseudo-compatibility of line PI458314 allowed the production of selfed plants. We analyzed four putative selfed individuals, namely S1, S2, S3 and S4. Only alleles present in PI458314 were found in S1, S2 and S3 for the 39 cDNA probes analyzed; this indicates the selfed origin of these plants. However, new alleles not present in PI458314 were found in plants S4 at loci *sb4a, sb8, sb11,* and *sb18* (Table 2) and at five additional RFLP loci which were homozygous in PI458314 (data not shown). Therefore, plant S4 is likely to be the result of accidental outcrossing.

Probe RFLP analyses

One cDNA clone detected two polymorphic loci, *sb4a* and *sb4b,* when probing DNA isolated from plants derived from PI458314, whereas only one polymorphic locus could be identified when it was used to probe DNA from plants derived from PI239582. One cDNA clone detected three polymorphic loci, *sb17a, sb17b* and *sb17c,* when probing DNA isolated from plants derived from

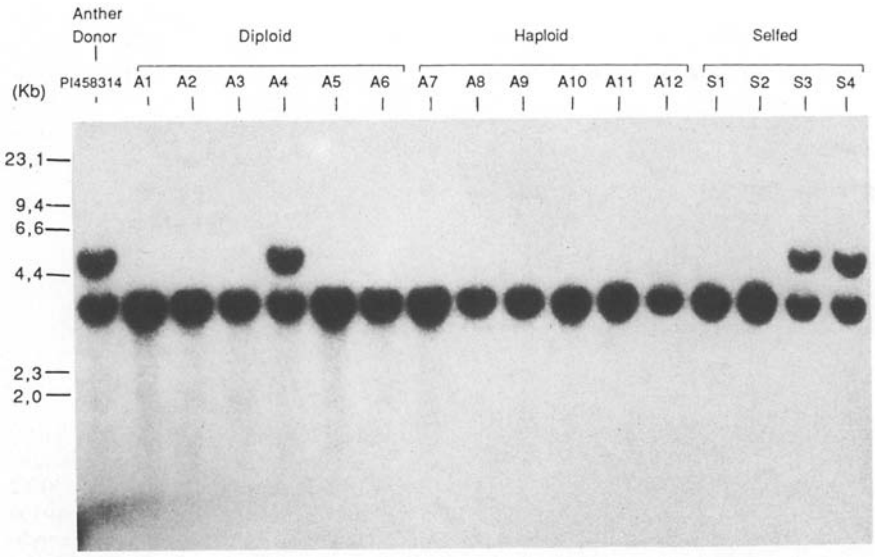

Fig. 3. Identification of a putative lethal allele with cDNA clone *sb16* on EcoRV-digested genomic DNAs of PI 458314, six diploid (A1–A6) and six haploid (A7–A12) plants derived from in vitro anther culture of PI 458314, as well as four plants (S1–S4) produced from the selfing of PI 458314. In all the plants analyzed, only allele 1 (bottom band) could be found in the homozygous state indicating lethality of allele 3 (top band)

PI230582, but only one polymorphic locus could be identified when the same clone was used to probe DNA of plants derived from PI458314.

Loci *sb11* and *sb16*, *sb15* and *sb20*, and *sb17b* and *sb21* displayed identical segregation patterns in plants derived from PI239582, although their banding patterns were different. Linkage between *sb11* and *sb16*, *sb15* and *sb20*, and *sb17b* and *sb21* might be responsible for these results.

Only one allele could be found in the homozygous state for loci *sb3*, *sb4a*, *sb11* and *sb16* in all plants issued from PI458314 (Table 2 and Fig. 3). Absence of the alternate homozygous genotype indicates possible lethality of the other allele or an allele at a closely linked locus. When comparing the same restriction enzyme × probe combinations on plants derived from PI230582, the putative lethal alleles of all four loci were not seen. Only the non-lethal allele and a different alternate allele were observed (Table 2). Conclusive evidence of the lethality of these alleles, however, will necessitate RFLP analyses of several other plants produced by in vitro anther culture.

Discussion

Traditionally, the identification of homozygous diploid plants produced from in vitro anther cultures has been done by monitoring one or few genetic markers. In *S. chacoense*, few genetic markers are characterized (Juned et al. 1988). Since few lines are amenable to anther culture, we are limited to the existing genetic variability within these lines. Isozyme analyses of several enzymatic systems were not informative in PI230582 and PI458314 (M. Cappadocia, unpublished results). The analyses of fertility characters such as flower and pollen production, pollen fertility and fruit setting were only partially indicative of the genetic architecture of plants produced from anthers of PI458314 and PI230582 (Tables 1 and 2). The high incidence of unreduced gametes as determined by variation in pollen size and parallel spindle formations during meiosis indicated that a multiple marker system would be necessary to clearly differentiate homozygous diploids, somatically derived diploids and diploids produced from 2n microspores (Cappadocia et al. 1984; Douches and Quiros 1988; Veilleux 1985).

In the present study, we demonstrated that haploids, homozygous diploids and somatically derived diploids produced from in vitro anther culture of *S. chacoense* could be easily separated with the combined use of cytological observations and RFLP analyses. Cytological observations were most useful in determining the ploidy level and in monitoring meiotic behavior. Completely homozygous genotypes were identified unambiguously by RFLP analyses, since they displayed one or the other but never both allelic forms of the DNA markers. Somatically derived diploid plants could also be clearly identified as they displayed both allelic forms of all the DNA markers.

Partially heterozygous diploids were found during our RFLP analyses. Our results demonstrate the complexity of determining the genetic make-up of partially heterozygous plants and indicate the danger of conclusions drawn from a limited number of genetic markers. Somatic origin could erroneously be concluded when heterozygous markers are found in diploids produced from anther culture. With our plants, heterozygosity was rarely indicative of somatic origin even for a plant regeneration system that passed through a callus stage (PI230582). Only 1 out of 12 diploids produced by anther culture was derived from somatic tissues. Even with 25

polymorphic loci, we could not clearly draw the genetic architecture of heterozygous plant A4 which might have originated from a 2n microspore. A single microdeletion not visible by cytological observations or chromosomal rearrangements occurring early during in vitro culture cannot be ruled out, since only a single locus became homozygous in A4.

The reliable identification of homozygous diploids by RFLP analyses is now providing us with a precision tool to analyze the mechanisms involved in the generation of new alleles at the self-incompatibility locus in *S. chacoense* (Cappadocia et al. 1986). RFLP analyses also permitted the identification of an outcrossed plant from a small sample of plants originally thought to be selfed individuals. In studies of self-incompatibility, accidental outcrossing of a single plant could confuse the issue raised around the spontaneous generation of new self-incompatibility alleles (Nettancourt 1977). We are currently testing all the selfed individuals produced from PI458314 to identify other possible outcrossed individuals. To shorten the analysis process, polymorphic probes will be combined in a single hybridization reaction. We estimated that two sets of five polymorphic clones (ten loci) will be sufficient to accurately separate completely homozygous diploids from other diploids while one set composed of probes *sb8, sb11* und *sb18* will clearly separate selfed from outcrossed individuals.

An RFLP-based linkage map can be constructed in *S. chacoense*. However, the operation of its gametophytic self-incompatibility system would lead to disturbed segregation ratios for RFLP loci linked to the self-incompatibility loci as it has been shown in *Lycopersicon peruvianum* (Tanksley and Loaiza-Figueroa 1985). Theoretical considerations of the types of crosses, methods of linkage estimation, progeny sizes and controls needed for accurate analysis of disturbed segregation ratios caused by gametophytic self-incompatibility has been described by Leach (1988). A genetic analysis method, where sexual reproduction by pollination is avoided, would overcome the problem. Considering that PI458314 produces a high frequency of homozygous diploid plants, these could be used to construct a detailed RFLP linkage map of *S. chacoense,* since they represent a sample of the gametes produced by PI458314 not biased by self-incompatibility. Segregation ratios will not be altered by self-incompatibility because pollination will be bypassed. In addition, this approach will allow us to identify lethal alleles and alleles under negative selection. Such information could be useful to select genotypes suitable for anther culture in *S. chacoense*.

RFLP analyses permitted the discernment of diploid plants derived from 2n microspores from other diploids produced by anther culture and confirmed FDR as an operating mechanism during microsporogenesis. Taking into account the high proportion of plants regenerated from PI230582 that were derived from 2n microspores, we could calculate the distance between RFLP loci and centromeres by measuring the frequency of recombinant homozygotes for each RFLP locus. Sixty to 70 plants derived from 2n microspores would be sufficient to determine gene-centromere distances. RFLPs are expected to be detected frequently within line PI230582 since it is strictly self-incompatible and consequently highly heterozygous.

Finally, Cowen (1988) demonstrated that recombinant inbreds or homozygous diploids are ideal genetic materials to detect main effects linked to quantitative trait loci (QTL). The time necessary to produce recombinant inbred lines, however, is a major disadvantage. On the other hand, many homozygous diploid plants could be produced by in vitro anther culture of line PI458314. These lines can be propagated vegetatively by cuttings and tubers. Quantitative traits such as tuber size, plant height, fertility and response to anther culture could be scored in each homozygous diploid in replicated experiments during the process of constructing the RFLP linkage map. Recently developed computer-assisted analytical methods of QTLs will permit the mapping of such characters with a precision that approaches discrete Mendelian factors analysis (Paterson et al. 1988).

In conclusion, RFLP analyses of diploid plants regenerated from anther culture of *S. chacoense* revealed the diversity of their origins. Such analyses are also opening possibilities for new approaches to construct detailed linkage maps, calculate gene-centromere distances, identify lethal alleles and map QTL. Experiments along these lines are in progress.

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