Genomic instability in *Solanum tuberosum* × *Solanum kurtzianum* interspecific hybrids

C.F. Marfil, R.W. Masuelli, J. Davison, and L. Comai

Abstract: The use of interspecific crosses in breeding is an important strategy in improving the genetic base of the modern cultivated potato, *Solanum tuberosum* L. Until now, it has normally been interspecific *Solanum* hybrids that have been morphologically and cytologically characterized. However, little is known about the genomic changes that may occur in the hybrid nucleus owing to the combination of genomes of different origin. We have observed novel AFLP bands in *Solanum tuberosum* × *Solanum kurtzianum* diploid hybrids; 40 novel fragments were detected out of 138 AFLP fragments analyzed. No cytological abnormalities were observed in the hybrids; however, we found DNA methylation changes that could be the cause of the observed genomic instabilities. Of 277 MSAP fragments analyzed, 14% showed methylation patterns that differed between the parental species and the hybrids. We also observed frequent methylation changes in the BC_1 progeny. Variation patterns among F_1 and BC_1 plants suggest that some methylation changes occurred at random. The changes observed may have implications for potato breeding as an additional source of variability.

Key words: DNA methylation, genome instability, interspecific hybrids, potato, *Solanum*.

Résumé : Le recours aux croisements interspécifiques constitue une importante stratégique en amélioration génétique pour accroître la diversité génétique chez la pomme de terre cultivée moderne, *Solanum tuberosum* L. Habituellement, les hybrides interspécifiques chez le genre *Solanum* ont été caractérisés sur les plans morphologique et cytologique. Cependant, nous en savons peu au sujet des changements génomiques qui pourraient se produire dans le noyau hybride en raison de la combinaison de génomes de diverses origines. Les auteurs ont observé de nouvelles bandes AFLP chez des hybrides diploïdes *Solanum tuberosum* × *Solanum kurtzianum*. Sur un total de 138 fragments AFLP, 40 produits nouveaux ont été détectés. Aucune anomalie cytologique a été observée chez les hybrides alors que des changements au niveau de la méthylation de l'ADN ont été observés et pourraient s'avérer la cause des instabilités génomiques observées. De 277 fragments MSAP, 14 % ont montré une méthylation différente entre les parents et les hybrides. Les auteurs ont aussi fréquemment observé des changements de méthylation au sein des progénitures BC_1. La variation observée au sein des plantes F_1 et BC_1 suggère qu'une partie des changements de méthylation seraient survenus au hasard. Les changements observés pourraient avoir des implications pour la sélection en constituant une source additionnelle de variabilité.

Mots clés : méthylation de l'ADN, instabilité du génome, hybrides interspécifiques, pomme de terre, *Solanum*.

[Traduit par la Rédaction]

Introduction

Wild *Solanum* species constitute an important reservoir of genetic diversity and resistance to biotic and abiotic stresses for potato improvement. Pre- and post-zygotic barriers hinder the incorporation of genes from diploid wild species into the cultivated potato *Solanum tuberosum* (tbr, $2n = 4x = 48$). These barriers can be overcome by ploidy manipulations: either haploidization of the tetraploid species or polyploidization via chromosome doubling of the diploid parent (asexual) or 2*n* gamete (sexual); in this way, desirable characters from the wild diploid species have been introduced into tetraploid potato cultivars (Ross 1986).

In plant breeding, the success of gene introgression via sexual hybridization depends on the phylogenetic relationships between species, opportunities for genetic recombination, and stability of the introgressed gene. Cytological analyses are usually performed to evaluate the meiotic process in experimental hybrids (Hermsen 1994). Species with close genetic affinity produce fertile hybrids with regular chromosome pairing, while the hybrids of those more distantly related species have meiotic irregularities and are sterile. In both cases, however, little is known about the genomic changes that may occur in the interspecific hybrids owing to the combination of genomes of different origin in the nucleus. Liu et al. (1999), for example, found DNA methylation changes in both random genomic DNA and specific genes, in introgression lines derived from wide crosses

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Table 1. Pollen stainability of the tbr × ktz diploid hybrids and results of crosses between the hybrids and ktz SCL 4550.

Female parent	Pollen stainability (%)	No. of pollinations	No. of berries	Berry set (%)	Average no. of seeds/berry
Hybrid 1	68	6	4	66.6	161
Hybrid 2	62	4	3	75	174
Hybrid 3	59	12	9	75	153
Hybrid 4	52	9	5	55.5	97

between cultivated rice *Oryza sativa* and the wild species *Zizania latifolia* (Griseb.). It is known that DNA methylation changes affect gene expression. For example, promoter methylation represses transcription, whereas its demethylation activates silent genes. Furthermore, genomic methylation changes are involved in most of the epigenetic phenomena observed in plants (Martienssen and Colot 2001). Thus, information on these phenomena should assist in understanding the outcome of hybridization.

We are working with interspecific hybrids between a tbr haploid ($2n = 2x = 24$) and the wild potato *Solanum kurtzianum* (ktz, $2n = 2x = 24$) with the objective of introducing desirable ktz genes in the cultivated potato. This wild species, which grows in northwest Argentina, is probably better adapted to dry environments than any other Argentinian wild potato. It is found in very dry sandy soils, on hillsides, and sometimes in the open, but often protected by bushes, shrubs, and trees (Hawkes and Hjerting 1969). It exhibits resistance to the potato cyst nematode, *Globodera rostochiensis* (Huijsman 1960), and the root knot nematode, *Meloidogyne incognita* (R.W. Masuelli, unpublished data). Both tbr and ktz belong to series Tuberosa (Hawkes 1990) and share the basic A genome (Matsubayashi 1991). Based on the assigned genome constitution, we did not anticipate meiotic irregularities in the hybrids and expected that introgression of ktz genes into the tbr genome would be feasible. Notwithstanding the cytological behavior, the DNA methylation status in the interspecific hybrids in comparison to the parents is not known. If methylation changes occur in the hybrids, the expression of genes could be epigenetically altered.

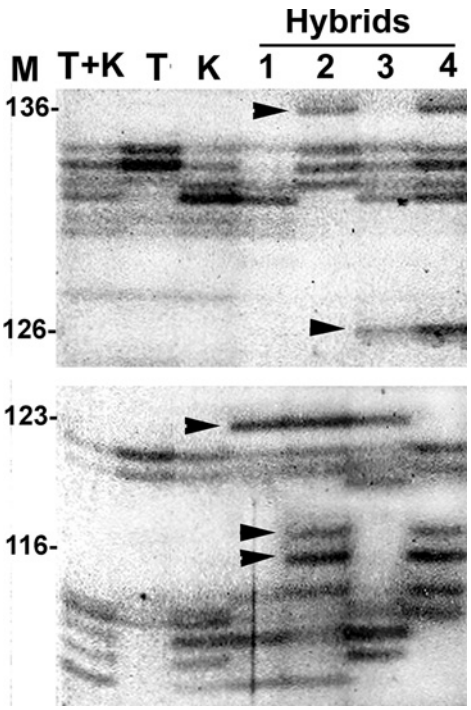
In this paper, we describe genomic and methylation alterations in interspecific tbr × ktz F_1 hybrids and BC_1 progenies of F_1 hybrid × ktz crosses, as revealed by molecular analysis of random sequences and genes of known function.

Materials and methods

Plant material

Four interspecific hybrids (H1 to H4) were obtained by embryo rescue (Nitsch and Nitsch 1969) from crosses between clone B91.1158–49 ($2n = 2x = 24$), a tbr haploid, and one plant of ktz accession SCL 4550 ($2n = 2x = 24$) that were provided, respectively, by the Laboratory of Genetics and the Potato Germplasm Bank, EEA Balcarce, INTA, Argentina. Intraspecific hybrids were obtained by crossing accessions SCL 4550 and SCL 4560 of *S. kurtzianum*, which were also provided by the Potato Germplasm Bank. The plants were grown in pots in a greenhouse, where pollinations were carried out after flower emasculation. Hybrids 1 and 4 were crossed as females with ktz to produce BC_1 plants.

Fig. 1. AFLP analysis of *Solanum kurtzianum* (K), *Solanum tuberosum* (T), and the hybrids (1–4). Representative AFLP profiles using the primer combinations M-CAG–E-AGG (upper panel) and M-CAG–E-AAG (lower panel); 1:1 mix of DNA from the parents. The band sizes on the left were deduced by comparison of the fragments with the 100 bp molecular marker (Promega). Arrowheads indicate new fragments present in the hybrids.



Cytological analysis

Chromosome counts were carried out on the root tips following the standard technique of pretreatment with 8-hydroxyquinoline (0.29 g/L) for 24 h, hydrolysis in 1 mol/L HCl for 10 min at 60 °C and staining with basic fuchsin (Coleman 1938).

Meiotic analyses were performed on the flower buds of the F_1 hybrids, fixed for 48 h in a solution of ethanol – acetic acid (*v*/*v*, 3:1) at room temperature, and stored in 70% ethanol at 4 °C until use. Anther squashes were stained on a glass slide with 1% acetocarmine solution. Pollen viability was estimated after staining.

AFLP analyses

DNA was extracted from leaves according to Dellaporta et al. (1983). After spectrophotometric measurement of DNA concentration (GeneQuant RNA/DNA Calculator, Pharmacia

Table 2. Sequence of novel AFLP fragments and database search.

AFLP fragment[a]	Length (bp)[b]	Primers[c] (*Mse*I–*Eco*RI)	Hybrids with novel fragments[d]	Homology product, nucleotide accession[e]	Nucleotides identified[f] (%)	BLAST *E* score
MM 15	123	CAT–AGG	H_1, H_2, H_3	Similar to *A. thaliana* far-red impaired response protein, AF262043.1	68	2.5
MM 17	118	CAT–AGG	H_2, H_4	Related to pathogen resistance genes from *S. tuberosum*, U60074.1	82	5.2×10^{-10}
MM 23	107	CAT–AGG	H_1, H_2, H_3, H_4	Similar to *A. thaliana* Dof zinc finger protein, TC177855	67	3
MM 47	199	CAG–AGG	H_2, H_4	Similar to dihydrolipoamide *S*-acetyltransferase in *Clostridium magnum*, BQ508691	74	1.1×10^{-5}

[a]Working designation.
[b]Length of the sequence read.
[c]AFLP nucleotide extension primers.
[d]Hybrids in which the novel fragments appear.
[e]Accession in TIGR gene indices.
[f]Nucleotide identity.

Biotech), DNA was diluted in 1× TE buffer to a concetration of 100 ng/μL for use in PCR analysis.

AFLP analysis of parental plants and the 4 F_1 hybrids was performed as described by Vos et al. (1995), using the AFLP™ Analysis system I AFLP Starter Primer Kit (Gibco BRL Life Technologies Inc., Gaithersburg, Md.). The amplification products were electrophoresed on 6% polyacrylamide gels and stained with silver.

Methylation-sensitive amplification polymorphism

Parental plants, the 4 F_1 hybrids and 6 BC_1 plants of hybrids 1 and 4 were analyzed using methylation-sensitive amplification polymorphism (MSAP). The protocol developed by Reyna-López et al. (1997) and adapted for rice by Xiong et al. (1999) was followed. This is an adaptation of the original AFLP protocol to incorporate the use of methylation-sensitive restriction enzymes. The isoschizomers *Hpa*II and *Msp*I were used as frequent cutters and *Eco*RI was used as the rare cutter. The adapters for *Eco*RI were the same as those used in the AFLP protocol. The adapters for *Hpa*II–*Msp*I digest fragments were designed according to Xiong et al. (1999).

All primers designed for the *Eco*RI fragments had the same core and enzyme-specific sequence (5′-GACTGCGTACCAATTC-3′); the following combinations of 3 selective nucleotides were added to the basic sequence: ACA, AGA, ACC, AAA, and AAC. The *Eco*RI primers were used in combination with two *Hpa*II–*Msp*I primers bearing 4 selective nucleotides (in italics): 5′-CATGAGTCCTGCTCGG*TCAA*-3′ and 5′-CATGAGTCCTGCTCGG*TCCA*-3.

Genomic DNA (1 μg) was digested with 20 U *Eco*RI (New England Biolabs, Ipswich, Mass.) in a final volume of 40 μL of the appropriate buffer for 3 h at 37 °C. For the second digestion, 20 U *Hpa*II (Gibco BRL) or *Msp*I (New England Biolabs) were used. The digested fragments were ligated to the adapters in a buffer containing 0.5 mmol/L of DTT, 1 mmol/L ATP, and 20 U T_4 DNA ligase (New England Biolabs) and incubated at 37 °C for 2 h. Preamplification was performed using 1 μL of the ligation products and 0.2 μmol/L of the *Eco*RI and *Hpa*II–*Msp*I primers, without the selective nucleotides, in a final volume of 50 μL containing 1× PCR buffer, 0.1 mmol/L dNTP and 1 U *Taq* polymerase. The PCRs were performed with the following protocol: 30 s at 72 °C, 3 min at 94 °C, and 30 cycles consisting of 1 min at 94 °C, 1 min at 56 °C, and 2 min at 72 °C, with a final extension step of 5 min at 72 °C.

The preamplification products were diluted 1:10. One microlitre was end labeled with [γ^{32}P]ATP and used in the selective amplification reaction with the *Eco*RI and *Hpa*II–*Msp*I primers in a final volume of 20 μL. The other components were the same as the preamplification reactions. The PCR protocol was the same as in the AFLP protocol (Vos et al. 1995).

Methylation analysis of the *patatin* gene

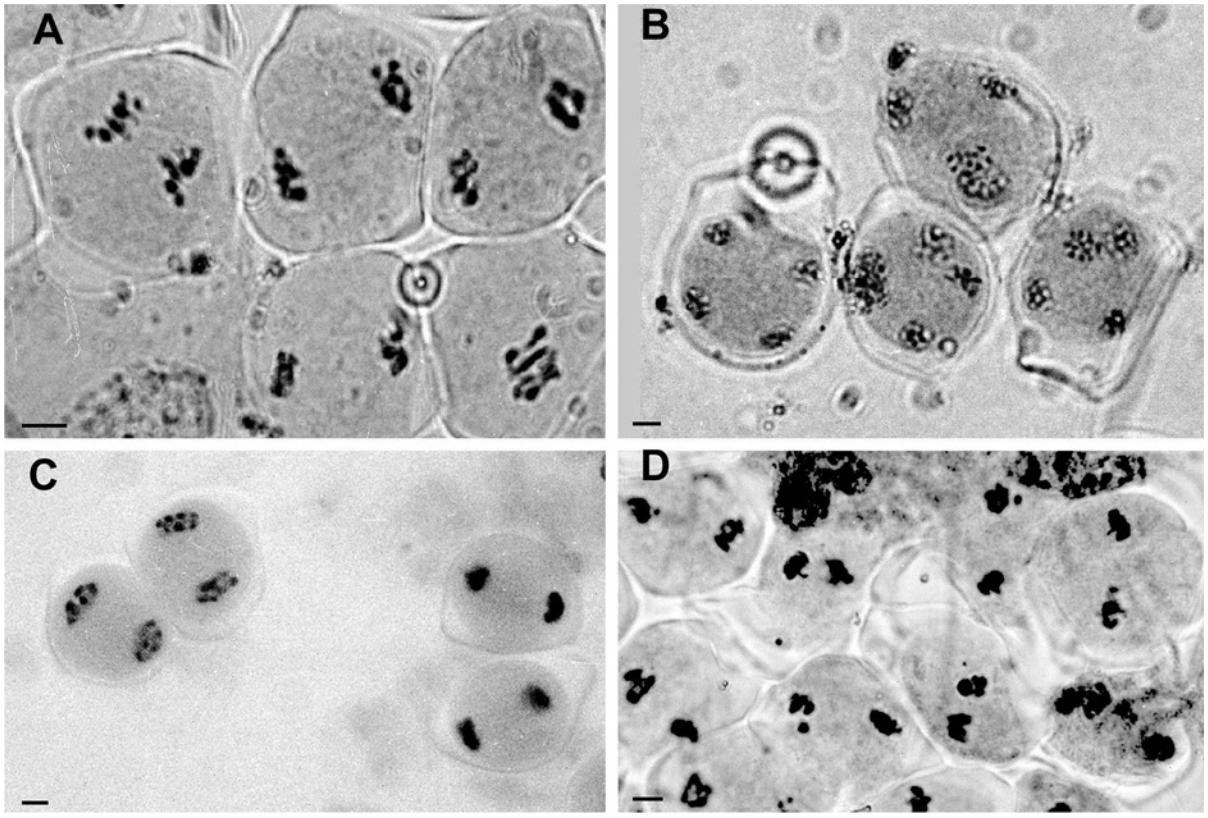
The sequence of the expressed sequence tag (EST) TC13721 from the TIGR potato (*Solanum tuberosum*) gene index (StGI) was used to design specific primers to amplify the gene for the potato tuber protein Patatin. This sequence contains the restriction site CCGG, which is recognized by isoschizomers *Hpa*II and *Msp*I. Genomic DNA from the parental plants and the 4 F_1 hybrids was digested with the isoschizomers and then used for PCR amplification with the primers 5′-GCTGCTGCTGTGGAATAACA-3′ (Pat 1) and 5′-TCATTCCGGCTACCATTCTC-3′ (Pat 2). The thermocycler (PTC-100, MJ Research, Waltham, Mass.) was programmed for an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of 92 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min, with a final extension step of 5 min at 72 °C. The PCR products were resolved by electrophoresis on a 1.2% agarose gel.

DNA gel blot analysis

Genomic DNA was digested with *Hpa*II and *Bam*HI (Promega, Madison, Wis.) unless specified otherwise. Fifteen micrograms of the digested DNA was separated by electrophoresis on a 0.8% agarose gel and transferred onto a Hybond N+ membrane by the alkaline method specified by the supplier (Amersham Pharmacia, Little Chalfont, UK).

The probe used was a 1600 bp PCR product corresponding to the *patatin* gene, which is specific to the ktz genome. The biotin-labeled probe was synthesized by PCR from the

Fig. 2. Meiotic observations of the hybrids. (A) Meiocytes at telophase I and metaphase I (lower right) and (B) at telophase II of hybrid 2. (C) Cells at telophase I in hybrid 3 and (D) at metaphase – anaphase II in hybrid 4. Bars = 5 μm.



cloned ktz-specific *patatin* fragment using a biotinylated Pat 1 primer. The membranes were hybridized overnight at 68 °C in 7% SDS, 0.5 mol/L Na_2HPO_4 (pH 7.2), and 1 mmol/L EDTA (pH 8). Washes were as follows: twice with 2× SSC and 0.1% SDS for 10 min at room temperature, once for 30 min with 1× SSC and 0.1% SDS at 66 °C, and once for 30 min with 0.2× SSC and 0.1% SDS at 66 °C. Signals were detected using the BrightStar™ BioDetect™ kit for nonisotopic detection of a biotinylated DNA probe (Ambion Inc., Austin, Tex.). Blots were placed in a protective plastic sheet and exposed to X-ray film for 24 h.

Isolation and sequencing of AFLP and MSAP fragments

Novel AFLP fragments present in the hybrids, but absent in the parents, and MSAP fragments differentially amplified from the two digests between the parents and the hybrids were eluted from the polyacrylamide gel by rehydrating the gel in boiling water for 5 min. The isolated DNA was re-amplified with the appropriate primers and ligated into a pGEM-T Easy Vector kit (Promega). Similarity analysis of the DNA sequences obtained was performed using the BLAST program searching the TIGR potato (*Solanum tuberosum*) database.

Results

Fertility of hybrids and genome analysis

The hybrids obtained were diploid. Pollen stainability was taken as a measure of male fertility. The hybrids varied between 52% and 68% in terms of pollen stainability. The tbr haploid clone B91.1158-49 did not produce stainable pollen and was used as the female parent in crosses; the male parent, ktz accession SCL 4550, showed a pollen stainability of 98%. In the backcrosses to ktz, all of the hybrid plants used as females produced berries with seeds; the average number of seeds/berry ranged from 97 to 174 (Table 1).

Table 3. Cytological analysis of 3 tbr × ktz diploid hybrids.

	Anaphase – Telophase I		Tetrad stage	
Hybrids	No. of cells	Cells with laggard (%)	No. of cells	Microcells/ tetrad (%)
2	40	7.5	60	0
3	64	12.5	100	0
4	25	10	100	0

The hybrid nature of the plants obtained from tbr × ktz crosses was confirmed by AFLP analysis. The four hybrids had a combination of AFLP fragments from both parents. However, novel fragments, not present in the parents, were observed in the hybrids (Fig. 1). As both parental species are highly heterozygous, we focused the analysis on new fragments, since fragments absent in the F_1 hybrids could be the result of segregation. Also, fragments that were not present in the mixed parental DNA were not scored. Using 4 different primer combinations, 138 AFLP fragments were obtained. Of these, the number of new fragments was 14 and 18 for hybrids 1 and 2 and was 8 for hybrids 3 and 4 (Fig. 1). Four AFLP bands present in the hybrids but absent in the parents were eluted from the gel, re-amplified, cloned,

Fig. 3. MSAP fragments detected in *Solanum tuberosum* (T), *Solanum kurtzianum* (K), and the hybrids (H1, H2, H3, and H4); the primer combination used was HM-TCCA–E-AAC (A) and HM-TCCA–E-ACA (B). H and M, MSAP profile of DNA digested with *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I, respectively. Arrows indicate segregation of AFLPs fragments. Arrowheads indicate differential MSAP products resulting from full methylation (FM) or demethylation (D).

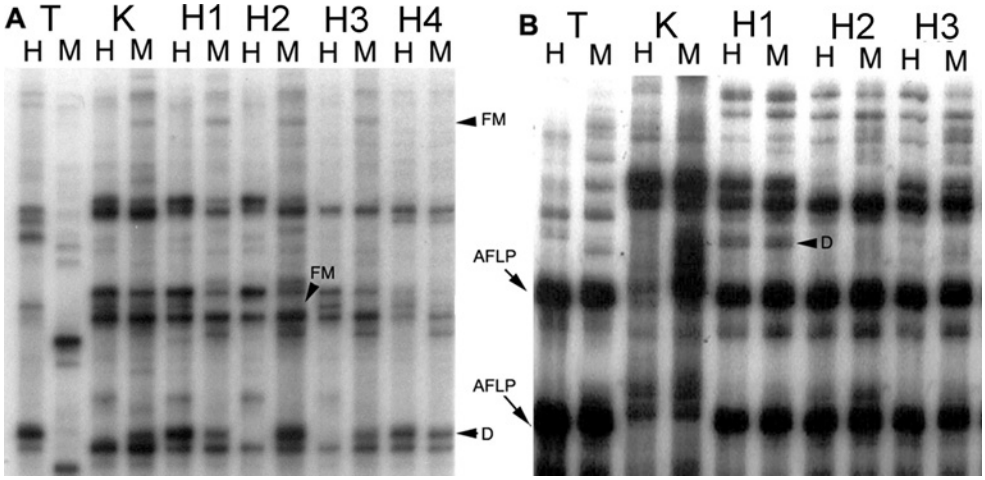


Table 4. Total number of fragments and number of methylation changes in 4 tbr × ktz hybrids detected by MSAP.

Oligonucleotides (HM–E)[a]	No. of bands	No. of methylation changes			
		Hybrid 1	Hybrid 2	Hybrid 3	Hybrid 4
TCCA–AAC	66	7	8	6	6
TCAA–AGA	32	3	1	3	2
TCCA–ACC	37	1	1	2	3
TCAA–ACA	63	8	7	9	8
TCCA–AAA	79	6	7	3	7
Total	277	25 (9.0%)	24 (8.7%)	23 (8.3%)	26 (9.4%)

[a]Selective primers for *Hpa*II and *Msp*I and for *Eco*RI enzymes, respectively.

and sequenced. In all cases, satisfactory sequences were obtained and were compared with the TIGR database using the BLASTN program. Two had similarity to orthologous genes of the *Arabidopsis* genome and one had similarity to EST BQ508691, reported in StGI as having homology to a bacterial gene. The other had 82% similarity to a resistance gene characterized in potato (Table 2).

Cytological analysis

The novel fragments observed could be the result of either chromosome rearrangements involving the gain, loss, or relocation of chromosome segments in the hybrids, or epigenetic alteration. To exclude the possibility that the novel fragments observed could have originated from a DNA rearrangement such as sister chromatid exchange or interchromosomal recombination during mitosis, we examined the chromosome behavior at microsporogenesis. The meiotic analysis of the hybrids showed regular bivalent pairing at metaphase I (Fig. 2; Table 3). One or two lagging chromosome at anaphase I and anaphase II were observed. However, neither chromosome bridged, which denotes inversions or other chromosome mutations, nor did either form microcells at tetrad stages (Fig. 2). This analysis showed that meiotic abnormalities were not evident in the hybrids, as was expected from the genome constitution of the parents.

Methylation changes in the hybrids and BC_1 progeny

To test if epigenetic modifications occur in the hybrids, we analyzed the methylation status of the hybrids in comparison with the parental plant. The methylation pattern at the 5′-CCGG sites was analyzed using the isoschizomer methylation-sensitive enzymes *Hpa*II and *Msp*I in both random genomic DNA, using the MSAP technique, and in genes of known function. *Hpa*II is sensitive to full methylation (both strands methylated) of either cytosine, but cleaves the hemimethylated external cytosine; *Msp*I is sensitive only to methylation of the external cytosine (McClelland et al. 1994; Roberts and Macelis 2001).

For MSAP analysis, 5 pairs of primers were used and a total of 277 fragments were amplified. Forty fragments differentially amplified from the two digests between the parents and the hybrids (Fig. 3; Table 4). Of these, 6 fragments had an invariable pattern among the 4 hybrids analyzed, whereas 34 showed a variable pattern. The following three types of changes were observed: (*i*) full methylation of both cytosine residues at the recognition site results in neither *Msp*I nor *Hpa*II cleavage; (*ii*) full methylation of the internal cytosine results in *Msp*I cleavage, but not *Hpa*II cleavage; (*iii*) hemimethylation of the external cytosine results in cleavage by *Hpa*II, but not *Msp*I. The percentage of methylation changes among the hybrids varied between 8.3% and

Fig. 4. Methylation analysis of the *patatin* PCR products. Left panel, undigested PCR products. Central panel, *Hpa*II digestion of template DNA prior to amplification. Right panel, *Msp*I digestion of template DNA prior amplification. M, 100 bp molecular marker. T, *Solanum tuberosum*; K, *Solanum kurtzianum*; 1–4, F_1 hybrids.

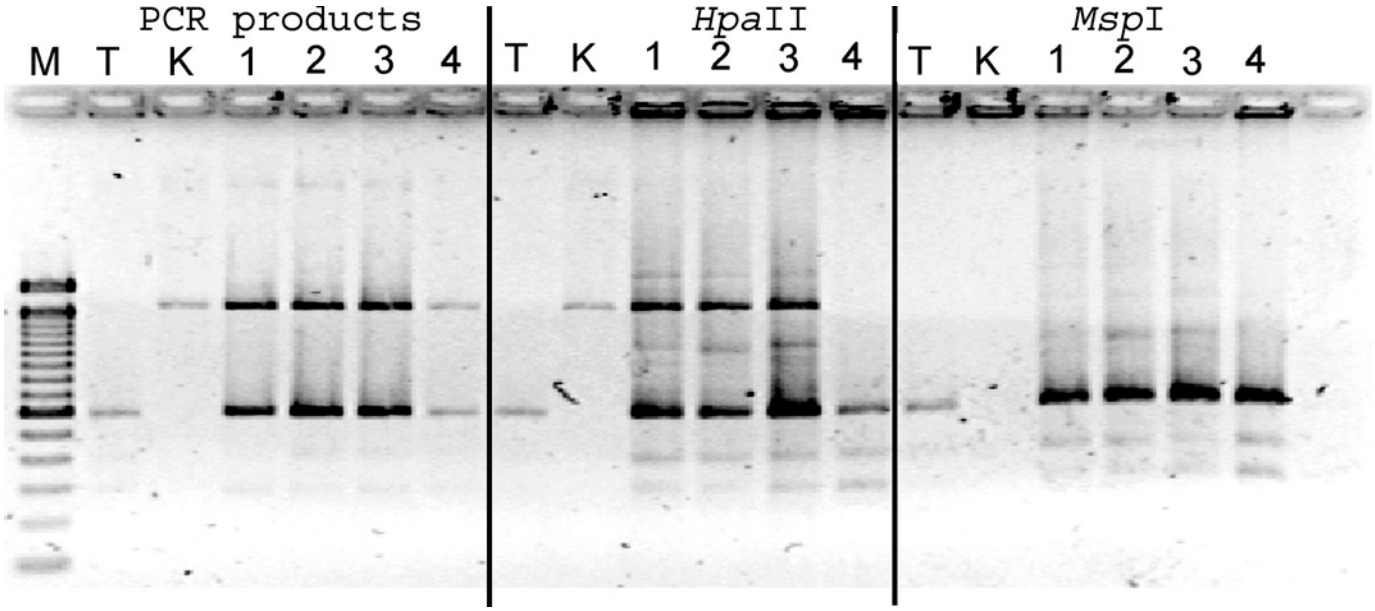
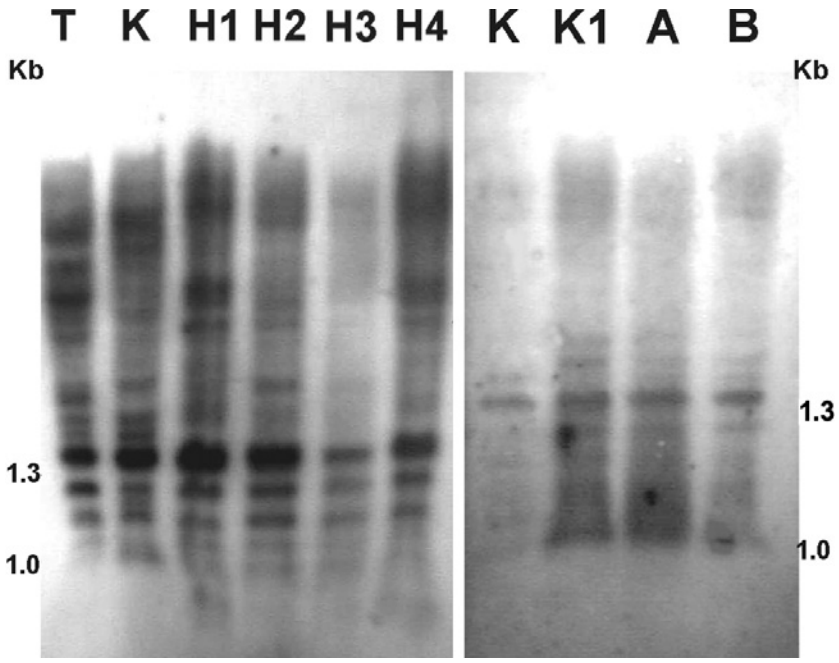
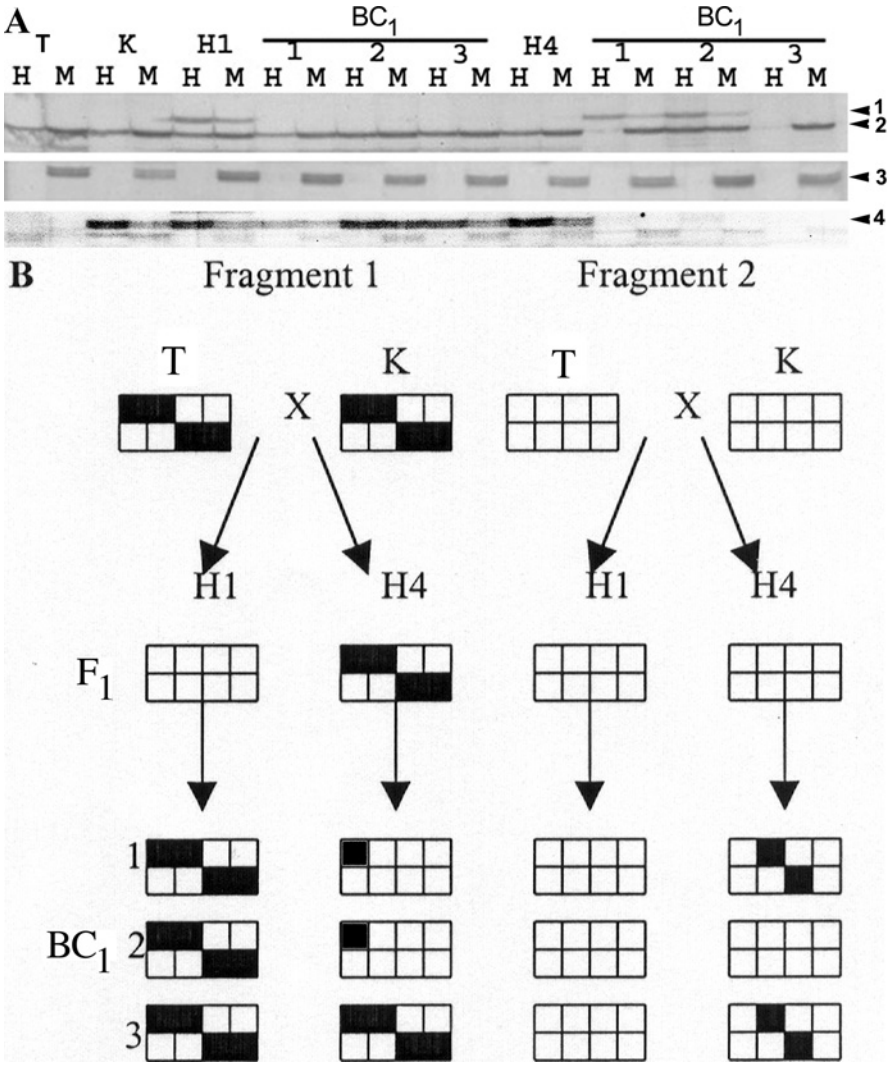


Fig. 5. Southern blot analysis of DNA methylation. DNA of plants of inter- (left panel) and intra-specific (right panel) crosses were digested with *Hpa*II and *Bam*HI and probed with the 1.6 kb PCR product of the *patatin* gene. T, *Solanum tuberosum*; K and K1, two accessions of *Solanum kurtzianum*; H1–H4, intraspecific hybrids; A and B, plants obtained from K × K1 intraspecifics crosses.



9.4% (Table 4). The MSAP technique assesses cytosine methylation throughout the genome and it is not known if the methylation status of known genes is altered in the hybrids. To test this possibility, we analyzed the methylation status at the 5′-CCGG site of the *patatin* gene. The PCR was set up for the amplification of 2 species-specific alleles, one of 500 bp from tbr and the other of 1600 bp from ktz. Digestion of DNA templates with *Hpa*II prior to amplification resulted in the disappearance of the 1600 bp allele only in H_4; the digestion with *Msp*I eliminated the 1600 bp alleles in ktz and the 4 hybrids (Fig. 4). These results showed that ktz and hybrids 1, 2, and 3 may have the internal cytosine methylated at the 5′-CCGG site and that H_4 was demethylated at this site. To further test this, the amplification products from the digested templates of both parents and the hybrids were pooled and digested with *Msp*I after PCR. The 1600 bp band was eliminated and the 500 bp band was present, confirming the internal methylation of the 5′-CCGG site in ktz and in hy-

Fig. 6. Segregation analysis of the methylation pattern of F_1 and BC_1 plants. (A) Portion of MSAP profile of an *Eco*RI–*Hpa*II (H) and *Eco*RI–*Msp*I (M) digest of DNA extracted from *Solanum tuberosum* (T), *Solanum kurtzianum* (K), the hybrids (H1 and H4), and three plants from the H1 × ktz and H4 × ktz backcrosses (BC_1). The methylation pattern of fragments 1 and 2 changed in the progeny and were considered unstable. Fragment 3 was scored as stable. Fragment 4 is an example of a novel methylation pattern in the BC_1 of hybrid 4 that was not scored as a methylation change because it could be interpreted as a segregating band. (B) Graphical interpretation of MSAP fragments 1 and 2 from A. The boxes represent the double-stranded recognition site (CCGG) of the *Hpa*II–*Msp*I isoeschizomer. Black boxes indicate methylated cytosine.



brids 1, 2, and 3, and the demethylation of hybrid 4 at this site. Also, this result showed that the 500 bp band does not have the 5′-CCGG restriction site. To confirm the decreased methylation in hybrid 4, we performed a gel-blot analysis of genomic DNA digested with *Bam*HI and *Hpa*II and probed with the *patatin* gene, present in high copy number (60 to 70) in the potato genome (Twell and Ooms 1998). In Fig. 5, the increased digestibility with *Hpa*II in hybrids 1 and 4 is presented, which can be visualized by a shift to lower-molecular-weight fragments, indicating hypomethylation of the 5′-CCGG sites in sequences of the *patatin* gene family. The *patatin* probe was also used to compare the methylation status among interspecific and intraspecific hybrids. The intraspecific hybrids did not show any hybridization signal below the 1 kb band (Fig. 5).

To know if the methylation changes in the hybrids were maintained or restructured in the offspring, we examined 74 MSAP fragments of the parental plants (tbr and ktz), 2 hybrids (H_1 and H_4), and 6 backcross plants from H_1 × ktz and H_4 × ktz crosses (Fig. 6). Differences between BC_1 plants and their parents were common. However, we discarded from the analysis the fragments that could be interpreted as polymorphisms inherited as heterozygous loci and segregating in the BC_1 progeny. The percentage of methylation changes among the backcross plants varied between 8.1% and 13.5% (Table 5). Of the 74 fragments scored, 40 (54%) maintained the parental methylation pattern and were classified as "stable fragments" and 34 (46%) showed variation in either the hybrids, the BC_1, or both, and were classified as "unstable fragments". Six unstable MSAP fragments were sequenced and all of them presented homology to coding sequences (Table 6). These results indicate that the methyl-

Table 5. Methylation changes in 6 (tbr × ktz) × ktz BC_1 plants.

		No. of methylation changes							
		H_1 × ktz			H_4 × ktz			Band classification[b]	
Oligonucleotides (HM–E)[a]	No. of bands	A	B	C	A	B	C	Stable	Unstable
TCAA–AAC	28	3	5	3	3	3	3	16	12
TCAA–AAA	46	7	4	6	3	7	4	24	22
Total	74	10 (13.5%)	9 (12.1%)	9 (12.1%)	6 (8.1%)	10 (13.5%)	7 (9.4%)	40 (54%)	34 (46%)

[a]Selective primers for *HpaII/MspI* and for *EcoRI* enzymes respectively.
[b]Stable, MSAP fragments that maintain the parental methylation pattern in the progeny; unstable, MSAP fragments susceptible to a change in their methylation status in F_1, BC_1, or both.

ation pattern in the BC_1 is subject to considerable changes and that several methylation-sensitive sites are reprogrammed in each generation.

Discussion

Genomic instabilities were observed in the interspecific tbr × ktz hybrids studied. The parental species used are closely related: both belong to the Tuberosa series and both have highly homologous genomes (Matsubayashi 1991). As expected, meiosis in the hybrids was normal, and both the pollen fertility and berry set were greater than 50%. However, we did find alterations in cytosine methylation. MSAP analysis displayed both increments and decrements in methylation and the methylation analysis of the ktz-specific *patatin* allele showed demethylation in hybrid 4. In addition, global demethylation of the multigene *patatin* family was observed in two interspecific hybrids compared with the parental genome. The MSAP changes were observed in all of the plants analyzed, suggesting that alteration of methylation status is a common phenomenon in these interspecific hybrids and, perhaps, in all hybrids of this type. However, the methylation pattern of MSAP and the *patatin* gene varied among the hybrids, indicating that at least some methylation changes were either random or depended on the presence of specific parental alleles. Also the DNA methylation changes were poorly inherited. This was demonstrated by the observation that the methylation pattern in 39% of the MSAP fragments in the BC_1 varied in comparison to the parents and that the methylation patterns were different among the 3 plants taken from the BC_1.

The genomic changes observed could have resulted from several different processes, including intergenomic recombination, cytoplasmic–nuclear interaction, or DNA methylation among others. Cytological analysis showed that loss of chromosomes or chromosome segments is not the cause for these genomic changes. We cannot rule out the possibility of cytoplasmic–nuclear incompatibilities, owing to the fact that crosses were successful only when tbr was the female parent; the reciprocal crosses were not studied.

The changes in methylation could be the cause of the genomic instabilities observed. Remodeling of DNA methylation and phenotypic and transcriptional changes have been reported in synthetic *Arabidopsis* allotetraploids (Madlung et al. 2002). The appearance and disappearance of RFLP fragments were observed in interspecific and intergeneric hybrids in wheat (Liu et al. 1998*a*, 1998*b*). Also, Shaked et al. (2001) showed new methylation patterns using MSAP in interspecific wheat F_1 hybrids and allopolyploids. Cheng et al. (2002) detected new RAPD fragments in F_1 plants of *Brassica napus* × *Orychophragmus violaceus* intergeneric hybrids. In a recent paper, Ercolano et al. (2004) reported new AFLP fragments absent in both parental genotypes in 12 haploids obtained from tetraploid *S. tuberosum* through $4x \times 2x$ crosses with *Solanum phureja*. Their findings and our results are the only examples of the appearance of novel fragments in interspecific hybrids in potato. In both studies, the cytoplasm was from *S. tuberosum*; therefore, it is possible that the tbr plasmon induces genomic changes in the hybrids. According to McClintock (1984), hybridity and allopolyploidy are shocks that trigger a rapid and massive genomic response of the plant genome. The DNA gel blot analysis showed demethylation of the *patatin* sequences of the tbr × ktz interspecific hybrids on comparison with the ktz × ktz intraspecific hybrids.

The methylation changes observed in the interspecific potato hybrids and their progeny may have an implication for potato improvement in the introgression of genes from wild potato species via sexual hybridization. It is known that promoter methylation does repress transcription and that methylation patterns are inherited (Yoder and Bestor 1996). The activation of transposons and the methylation or demethylation of sequences in the hybrids could alter their expression, potentially silencing useful genes introgressed into the potato genome. One novel AFLP fragment sequenced here was highly similar to a characterized resistance gene (R gene) in potato. This change observed in two interspecific hybrids may indicate that inactivation of genes and partial suppression or modification of their expression may occur immediately after hybridization. On the other hand, minimal alterations in R gene sequence can either slightly alter taxonomic specificity or result in radical change in specificity. The first case results in the recognition of different pathogen strains or pathotypes; extreme examples of the second case include the single gene *Mi*, which gives resistance to a nematode and an aphid (de Ilarduya et al. 2001) and a single resistance-gene cluster in potato that confers resistance to both a virus and a nematode (van der Vossen et al. 2000).

In plants, genomic methylation is restricted mostly to transposons and other repeats (Colot and Rossignol 1999); transposons become activated when they lose methylation (Martienssen and Colot 2001). Further, it has been suggested that genes encoding transcription factors are more likely to

Table 6. Amplified patterns of unstable MSAP fragments and sequences similitude analysis.

Fragment/ length (bp)[a]	Amplification pattern[b]																Homology products, accession number[d]
				BC_1 H_1 × ktz					BC_1 H_4 × ktz								
	tbr[c]	ktz	H1	A	B	C	H4	A	B	C							
	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	
17-7/303	–	–	–	–	+	+	–	–	–	–	–	–	+	+	–	–	Similar to β-galactosidase II precursor in the tomato *Lycopersicon esculentum*, C98157
21-7/264	–	+	–	–	–	+	+	+	+	+	+	+	+	–	+	+	Homologue to signal recognition particle receptor protein, CK863079
38-6/177	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	Similar to cuticle protein, BQ509747
40-6/156	–	–	–	–	–	–	–	–	+	+	–	–	+	+	–	–	Unknown protein, CK717356
49-6/134	+	–	–	–	+	+	–	–	+	+	+	+	+	+	+	+	Similar to NAM-related protein (no apical meristem), TC97146
52-6/127	–	–	–	+	+	+	–	+	+	+	–	+	–	+	+	+	Similar to asparagine synthetase 2, TC102596

[a]Working designation and length of the sequence read.
[b]Amplified patterns of MSAP fragments in the different genotypes. H and M, amplifications using genomic DNA digested with *Hpa*II–*Eco*RI and *Msp*I–*Eco*RI respectively; +, presence; –, absence.
[c]tbr, *S. tuberosum*; ktz, *S. kurtzianum*; H_1 and H_4, interspecific hybrids between tbr and ktz; A, B, and C, plants of the BC_1 progeny.
[d]Accession in TIGR gene indices.

gain novel regulatory patterns through insertion of transposable elements in their promoter regions. Genes encoding transcription factors may also be more susceptible to epigenetic regulation (Martienssen 1998) and, thus, could become selectively unstable in interspecific hybrids. We have shown that methylation changes occur in a wide variety of genome sequences of the F_1 hybrids and BC_1 plants.

Our results showed that the *S. tuberosum* × *S. kurtzianum* interspecific hybrids present new AFLP fragments and changes in the methylation pattern. The mechanisms whereby a novel fragment occurs are unknown. However, it is possible that these newly generated bands might be derived form altered parental bands arising from epigenetic modifications such as cytosine methylation induced by interspecific hybridization. This reprogramming could possibly occur in the zygote of the interspecific hybrid, with the potential for many loci to be reprogrammed again in the next generation. It is possible that this process could continue until the hybrids are stabilized. The novel fragments and epigenetic modifications represent an additional source of variation for potato breeding and may have an implication in the origin and fitness of new species by interspecific hybridization.

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