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Short-term hybridisation activates Tnt1 and Tto1 Copia retrotransposons in wild tuber-bearing *Solanum* species

Running head: Hybridisation activates retrotransposons in wild potato

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ABSTRACT

Interspecific hybridisation in tuber-bearing species of *Solanum* is a common phenomenon and represents an important source of variability, crucial for adaptation and speciation of potato species. In this regard, the effects of interspecific hybridisation on retrotransposon families present in the genomes, and their consequent effects on generation of genetic variability in wild tuber-bearing *Solanum* species, are poorly characterised. The aim of this study was to analyse the activity of retrotransposons in inter- and intraspecific hybrids between *S. kurtzianum* and *S. microdontum,* obtained by controlled crosses, and the effects on morphological, genetic and epigenetic variability. For genetic and epigenetic analysis, S-SAP (sequence-specific amplification polymorphism) and TMD (transposon methylation display) techniques were used, respectively, with specific primers for Tnt1 and Tto1 retrotransposon families (Order LTR, Superfamily Copia). The results indicate that at morphological level, interspecific hybrid genotypes differ from their parental species, whereas derived intraspecific hybrids do not. In both cases, we observed significant reductions in pollen grain viability, and a negative correlation with Tnt1 mobility. Both retrotransposons, Tto1 and Tnt1, were mobilised in the genotypes analysed, with mobility ranging from 0 to 7.8%. Furthermore, at the epigenetic level, demethylation was detected in the vicinity of Tnt1 and Tto1 in the hybrids compared with the parental genotypes. These patterns were positively correlated with the activity of the retrotransposons. The results suggest a possible mechanism through which hybridisation events generate genetic variability in tuber-bearing species of *Solanum* through retrotranposon activation.

INTRODUCTION

It has long been recognised that hybridisation and introgression occur widely among natural plant populations (Anderson 1949), constituting the major forces in plant evolution by contributing to diversification and speciation (Mallet 2007; Rieseberg & Wills 2007). Although these mechanisms do not necessarily lead to immediate hybrid speciation, they are an important means for transfer and/or *de novo* origin of ecological adaptations. Thus, they play an important role in facilitating speciation in variable niches (Arnold 2004; Wang *et al*. 2009). In the case of tuber-bearing *Solanum* species, there is high morphological variability and a wide range of adaptation to different environmental conditions. This provides strong evidence of the role that homoploid hybridisation played as an evolutionary force in this group (Masuelli *et al.* 2009).

The effect of hybridisation on plant genomes was primarily thought as a 'genomic shock', caused by a response to stressful conditions, as first proposed by the maize geneticist Barbara McClintock (1984). This shock would lead to a genome-wide relaxation of gene expression, including transposon activation. Actually, it is known that this process is often accompanied with rapid and expansive genomic and epigenomic changes that include fragment gain and loss, through chromosome rearrangement or activation of transposable elements (Kashkush *et al*. 2003a; Kashkush & Khasdan 2007), extensive alteration of DNA cytosine methylation (Kraitshtein *et al.* 2010; Zhao *et al.* 2011), histone modifications (Marfil *et al.* 2009) and small RNA changes (Ha *et al*. 2009). These changes are considered a stabilising mechanism for the establishment of new species (Feldman & Levy 2005; Cara *et al*. 2013).

Newly synthesised or recent hybridisation events allow one to examine immediate responses to short-term changes (Feldman & Levy 2009) and to elucidate the impact of genome merging. In this sense, genome reorganisation associated with mobile elements can be quantified by comparing their organisation in parental and hybrid genotypes using transposon display strategies. To provide further evidence of transposon activation by hybridisation in tuber-bearing species of *Solanum*, we carried out an experimental design based on inter- and intraspecific hybrids, obtained by controlled crosses between genotypes

classified as *S. kurtzianum* and *S. microdontum.* The morphological and reproductive performance of parents and respective hybrids was analysed. Additionally, a comparative study of the genetic and epigenetic behaviour of Tnt1 and Tto1 – two retroelement inhabitants of *Solanum* spp. genomes – was performed using sequence-specific amplified polymorphism (S-SAP) and transposon methylation display (TMD). The first technique allows identification of new insertions in the hybrids that are absent in parental lines, whereas the second identifies changes in methylation status in the vicinity of retrotransposons.

Our data show moderate mobility of both retrotransposons responding in a genotype- and cross-dependent manner. We also observed correlations between the occurrence of new insertions in the hybrids with low pollen viability and hypomethylation in the proximity of these elements. These results suggest a possible mechanism by which hybridisation events generate genetic variability in tuber-bearing species of *Solanum* through retrotranposon activation.

MATERIAL AND METHODS

Plant material and experimental design

Parental genotypes of *S. kurtzianum* (ktz) and *S. microdontum* (mcd) were kindly provided by the Genebank Potato and Forage, National Institute of Agricultural Technology (INTA), Balcarce, Argentina (Table 1A). Intraspecific (ktz × ktz) and interspecific (ktz × mcd) synthetic hybrids (F_1) were obtained through controlled crosses (Table 1B, Fig. S1A). In both cases, a fixed pollen donor genotype was selected to perform the crosses (Ma and Ka for inter- and intraspecific crosses, respectively).

The study was carried out for over 2 years (for a diagrammatic scheme, see Fig. S1B). In the first year, 15 F_1 botanical seeds from each cross were sown in Petri dishes containing water-agar (0.8%) for 15 days. All the seedlings obtained were transplanted into humidity chambers in pots with transparent covers and containing sterilised coconut fibre as a substrate to prevent drying and favour plant establishment. All the established plants were transplanted to individual cylindrical pots (8 × 10 cm) containing sterile soil for plant

development and tuberisation. Parental plants were always grown from tubers in a sterilised soil substrate. Plants were watered once a week with water, and nutrients were supplemented monthly by fertigation with NPK. During all these stages, plants were grown in chambers with a 16h /8 h photoperiod at 24 °C/19 °C (day/night) and 60/80±5% relative humidity. In total, we worked with five parental genotypes and 25 surviving genotypes (Table 1). When plants developed sufficient leaf biomass, samples of young leaves were taken for molecular analysis.

In the second year, morphological analysis was completed (Fig. S1C). For this purpose, all 30 genotypes were grown from tubers obtained in the previous year during spring (October 2012) in pots of 10-cm diameter with sterile soil. Each genotype was planted in triplicate (n=3), giving a total of 90 experimental units. The experiment was conducted using a completely randomised design under uniform conditions in an insect-proof greenhouse in Luján de Cuyo, Mendoza, Argentina.

Morphological and reproductive analyses

Morphological evaluation of the genotypes was performed when the plants were at the flowering stage. Thirty morphological characters, previously described in Raimondi *et al.* (2005), were measured per genotype (n=3 plants), in the tenth true leaf from the base of each plant, the immediate stem internode below the tenth leaf, three flowers from the first inflorescence to appear, and all the tubers produced per genotype.

Two flowers were removed from each plant (six flowers per genotype) to indirectly estimate pollen viability. Briefly, pollen from each flower was sprinkled on a slide and stained with 2 %acetocarmine. In a light microscope and 400x magnification, two fields per flower were digitalised and photographed. Total viable (stained) and nonviable (not stained) pollen grains were counted per field using ImagePro software, and the percentage of viable pollen grains recorded.

Retrotransposon families

Two families of retrotransposon (Order LTR, Superfamily Ty1-copia), Tnt1 (Grandbastien *et al.* 1989) and Tto 1 (Hirochika 1993), were selected for this study according to their mobility under stress conditions in *Nicotiana tabacum.* Digestion profiles of both retrotransposons with the restriction enzymes used in this work are available in Figs S2 and S3, respectively.

Sequence-specific amplification polymorphism

The technique for S-SAP (Van der Broech *et al.* 1998; Syed & Flavell 2007) is a modification of the AFLP technique (Vos *et al.* 1995), adapted to detect polymorphisms associated with the mobility of retrotransposons. Unlike the original technique that employs a specific primer designed according to the known sequence to be amplified, fluorescent-labelled primers specific to the two families of retrotransposon mentioned above were used.

The DNA extraction from each genotype was performed using the CTAB protocol (Doyle & Doyle 1987). DNA digestion was carried out in duplicate with 300 ng DNA, 4 U EcoRI (New England Biolabs, Ipswich, MA, USA), 2 µl 10× buffer and 100 ng μl^{-1} BSA in a final volume of 20 µl. The samples were incubated at 37 °C for 3 h. The quality of the digestion reactions was monitored with electrophoresis in 1% agarose gel (w/v) at a constant 90 V for 40 min. The following steps were performed independently on each digestion (n=2).

The ligation reaction was performed with 10 µl of the digested DNA, 1.25 µl 20 mM EcoRI adapter (5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3'), 2 µl T4 DNA ligase buffer 10× and 0.75 U T4 ligase (Promega, Madison, WI, USA) in a final volume of 20 µl. Adapters were ligated by incubating the samples at 20 °C for 3 h.

Pre-selective amplification was performed using specific primers for the adapter sequence EcoRI+00 (5'-AGACTGCGTACCAATTC-3'). Each PCR reaction was performed with 2 µl of ligation product, 0.2 µl of each 50 ng μl^{-1} primer, 0.4 µl 5 mM

dNTPs, buffer 1× DNA polymerase and 1 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil) in a final volume of 20 µl. The amplification conditions were: 20 cycles at 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The amplification products were monitored using 5 µl of the reaction for electrophoresis on 1% agarose gel, as described above.

Selective amplification was carried out with the primer specific to the EcoRI adapter sequence '+00' (5'- GACGATGAGTCCTGAGTAG-3') and primers specific for each family of retrotransposon, labelled with different fluorophores, Tnt1 – OL16 (TAM* 5'-TTCCCACCTCACTACAATATCGC-3') and Tto1 – 6-FAM* (5'-CACTCCCCTGTTAGGAAACATTC-3'). The PCR reaction was prepared using 2 µl of 1:3 dilution of the pre-amplification product, 0.2 µl primer EcoRI+00 50 ng μl^{-1}, 0.2 µl primer-labelled specific retrotransposon 50 ng μl^{-1}; 0.8 µl 5 mM dNTPs, buffer 1× DNA polymerase and 1 U Taq DNA polymerase (Invitrogen) in a final volume of 20 µl. The amplification programme used was: 14 cycles at 94 °C for 30 s, 55 °C for 30 s (decreasing 0.7 °C per cycle) and 72 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 30 s and 72 °C for 2 min.

Following selective amplification, the PCR products were prepared for capillary electrophoresis by mixing 1 µl of each sample with 8.7 µl Hi-Di formamide and 0.3 µl molecular weight standard ROX Genescan 500HD (Applied Biosystems, Carlsbad, CA, USA). The samples were denatured at 95 °C for 5 min and resolved with capillary electrophoresis (Applied Biosystems 3130 Genetic Analyser). The genetic matrix was constructed for each genotype from the electropherograms obtained using GeneMapper version 3.7 software (Applied Biosystems). Only reproducible fragments were scored as present (1) or absent (0) in each genotype, and then recorded. The alleles were analysed taking into account their size (200 to 600 bp) and repeatability (present in both technical replicates). To determine the presence of new alleles, each hybrid profile was compared with their respective parents, considering a new event of retrotransposition when fragments present in hybrids were absent in both parents.

Transposon methylation display

The transposon methylation display (TMD) technique allows systematic analysis of genome-wide DNA methylation status of 5'-CCGG sites flanking retrotransposons (Kashkush & Khasdan 2007). The procedure is similar to that used in S-SAP, the only difference being the enzymes used. In this case, the isoschizomeric enzyme pair *HpaII* and *MspI* was used, which recognises the same restriction site, 5'-CCGG, but has different sensitivities to cytosine methylation (Fig. S4). *HpaII* cuts if the external cytosine is hemimethylated or unmethylated, but does not cut if the internal cytosines are methylated. *MspI* cuts only if the internal cytosine is fully methylated or unmethylated. None of the enzymes cut when the sites are full methylated.

For this technique, two separate digestions of 300 ng DNA were conducted using 2 U enzyme *HpaII* on one side and two units *MspI* on the other. Each digestion was performed in conjunction with 2 U *EcoRI*. Each pair of digestions was performed in duplicate, and the quality of reactions was monitored with electrophoresis in 1% agarose gel (w/v) at constant 90 V for 40 min. The ollowing steps were performed independently on each digestion (n=2). The end digested by *HpaII* and *MspI* was ligated to 1.25 µl HpaII/MspI adapter 20 mM (5'-GATCATGAGTCCTGCT-3' and 5'-3-CGAGCAGGACTCATGA') and the end digested with *EcoRI* was ligated to 0.75 µl EcoRI adapter 20 mM (described above). Reactions of pre-amplification and amplification were performed similarly to the S-SAP protocol described above, with the only difference being that the primers employed were *HpaII/MspI* (5'-CATGAGTCCTGCTCGG-3') and EcoRI+00 (described before). For the selective amplification reaction, the primer *HpaII/MspI* was used in combination with the retrotransposon-specific family primers described above and fluorescence-labelled. The reaction cycling, capillary electrophoresis and binary matrix construction were conducted in the same manner as described for the S-SAP technique.

Alterations in inherited methylation patterns may be attributed to an increase (hypermethylation) or a reduction (hypomethylation) in the number of methylated cytosine in the 5'-CCGG sites. For analysis of specific-site methylation states in the F_1 with respect to progenitors, we used the rule of methylation heredity described in Table S1, where three types of inherited pattern, types A, B and C, were identified. The Mendelian inheritance pattern corresponds to type A; type B corresponds to specific loci changes due to hypermethylation; and type C to hypomethylation.

Statistical analysis

Statistical analysis was performed using the program InfoStat version 2012 (Di Rienzo *et al*. 2012). Morphological data were analysed using discriminant analysis of multivariate statistics. The correlation matrix was estimated using standardised data and calculating eigenvalues and projected data graphically.

The results are expressed as means of the replicates used with the corresponding SD. The number of biological replicates for each determination is detailed in each analysis. To determine whether there were significant differences between treatments ANOVA ($\alpha < 0.05$) was performed in univariate data and MANOVA ($\alpha < 0.05$) in multivariate data. In cases where ANOVA and MANOVA were significant, mean comparisons were conducted using the Duncan test ($\alpha < 0.05$) and Lawley-Hotelling test ($\alpha < 0.01$), respectively. For analysis of correlations between variables, Pearson correlation coefficient was used.

RESULTS

Morphological and reproductive analyses of hybrids and respective parents

For an overall view of the morphology, a discriminant analysis was carried out with 30 morphological characters evaluated. In this sense, the first two canonical variables, axis 1 (62.41%) and axis 2 (19.45%), explained 81.76% of the total variation of the traits (Fig. 1). Along axis 1, left panel grouped ktz×ktz intraspecific crosses with ktz parental genotypes, whereas the right panel grouped ktz×mcd interspecific crosses with mcd parental genotypes. However, axis 2 separated the mcd genotype from the remaininh genotypes. The MANOVA revealed that there were significant differences between genotypes at a

morphological level (P <0.001). The comparison of means according to Lawley-Hotelling allowed grouping of genotypes into four main groups. The Mcd parental genotype was differentiated morphologically from all other genotypes. Furthermore, the ktz parental genotypes and ktz×ktz hybrids were indistinguishable morphologically, forming a separate group. Finally, offspring of interspecific crosses ktz × mcd formed two distinct morphological groups. While Kb × Ma and Kc × Ma hybrids showed no morphological differences among themselves, Kd × Ma hybrids formed a separate group (Fig. 1).

The analysis of pollen viability in progenitors indicated that the genotype mcd had values of 75%, while ktz genotypes exhibited percentages close to 90%, with less than 5% variability between them (Fig. 2). However, pollen viability of the interspecific F_1 hybrids ranged between 67% and 90%, while in intraspecific crosses there were significant reductions with respect to their parents. In this sense, contrasts analysis between parents and their descendants revealed that only intraspecific F_1 hybrids exhibited a significant reductions in pollen viability (Kb × Ka hybrids *versus* Kb and Ka, $P < 0.0005$; Kc × Ka hybrids *versus* Kc and Ka, $P < 0.0001$), KcKa5, KcKa6 and KcKa7 being the most affected genotypes, with values of 55, 30 and 20%, respectively.

Activation analysis of LTR retrotransposons using S-SAP

The effect of hybridisation on the activation of retrotransposons was evaluated using the S-SAP technique. In total, 129 fragments were analysed for Tnt1 and 111 fragments for Tto1 (Table 2). Both families mobilised during hybridisation events, both at intra- and interspecific level (for comparison of new fragment sequence with Tnt1 and Tto1 5´end see Table S2). The analysis of genetic matrix obtained for both retrotransposons revealed that the Tnt1 family had two to ten new fragments in all crosses (1.5–7.7%) except for cross Kd × Ma, where mobility of Tnt1 was observed only in the KdMa2 genotype. The Tto1 family also exhibited mobility in both intra- and interspecific crosses, with one to seven new fragments (0.9–6.3%), with several genotypes without new fragments (KcKa1 and KbMa4), including all genotypes of cross Kd × Ma.

At crossing level, with the exception of genotypes from cross Kd × Ma, all hybridisation events showed increased mobility of the retrotransposons evaluated (Fig. 3). Tnt1 displayed the highest values of mobilisation in the cross Kc × Ma, followed by Kb × Ka, Kc × Ka and Kb × Ma, which did not show significant differences (Fig. 3A). Whereas in Tto1 maximum activity was observed in intraspecific crosses Kb × Ka and Kc × Ka, followed by interspecific Kb × Ma and Kc × Ma (Fig. 3B). Moreover, Pearson correlation analysis revealed a significant negative correlation between the number of novel fragments observed in Tnt1 and the percentage pollen viability (Table 3); a similar trend was observed for Tto1.

Analysis of methylation status of chimeric sequences (retrotransoposon/flanking sequence) with TMD

The global analysis of specific loci changes in methylation status in hybrids with respect to their parents (based on the matrix obtained from Table S1) can be viewed in Fig. S5. The most abundant pattern was A (parental inheritance), followed by C (hypomethylation) and then B (hypermethylation). In the case of genotypes from cross Kd × Ma, they exhibited less hypomethylated state changes with respect to the other hybrids.

Percentages of changes to hypomethylation states (pattern C) per cross revealed that similar trends were observed for both retrotransposons (Fig. 4). In this sense, hybrids from cross Kd × Ma exhibited the lowest values of hypomethylation states for both retrotransposons. Furthermore, with respect to Tto1, hybrids from interspecific crosses Kc × Ma and Kb × Ma presented less hypomethylation than intraspecific hybrids Kc × Ka and Kd × Ka, which had more changes to this state; Fig. 4B); while no difference was observed between these crosses in Tnt1 (Fig. 4A). The homology of the new fragments with 5-end of Tnt1 and Tto1 was corroborated by sequence analysis (Table S2).

The Pearson correlation analysis between S-SAP patterns and changes in methylation patterns in hybrids revealed that Tnt1 and Tto1 activity correlated with the hypomethylation of sites flanking retrotransposons (Table 4). However, we found a significant negative correlation only between Tnt1 activity and hypermethylation.

DISCUSSION

Genomic shock and reproductive biology

According to our results, morphological analysis of interspecific hybrids showed an intermediate phenotype between the parental species, while intraspecific hybrids resembled the phenotypic profile of the parental genotypes (Fig. 1). Interestingly, we observed significant reductions in pollen grain viability (from 20% to 90%; Fig. 2), retrotransposon activation (Table 2, Fig. 3) and changes in methylation patterns (Fig. 4) in hybrids obtained from intraspecific crosses. This association, in turn, was similar to that observed in the interspecific crosses Kb × Ma and Kc × Ma and opposite to hybrids of cross Kd × Ma, suggesting differences in genomic compatibility among parental species of Ktz.

These apparently inconsistent results have been observed previously in *Solanum* species. In this sense, the morphological analysis in accessions of *S. kurtzianum* revealed high morphological variability, attributed to its wide geographic distribution and overlap with populations of other species, such as *S. chacoense*, *S. maglia* and *S. spegazzinii* (Bedogni & Camadro 2009). There is evidence of hybridisation and introgression events attributed to incomplete isolation barriers between these species. Support for this statement is provided by the fact that some accessions of *S. kurtzianum* and S. *chacoense* were more similar to each other at morphological and genetic level than with accessions of the same species. Similar results were obtained following a thorough genetic analysis of germplasm accessions belonging to Petota section, which revealed that *S. kurztianun* does not constitute a distinct genetic unit, but rather is grouped together with species such as *S. maglia* and *S. spegazzinii* (Jacobs *et al.* 2011). Other genetic studies in natural populations of *S. kurtzianum* revealed that genetic diversity within populations is higher than between populations (Marfil & Masuelli 2013). These observations, in combination with our results, lead to the conclusion that there is an inconsistency between the morphological and genetic information determining the specific taxonomic range of potato tubers.

Moreover, the finding of significant reductions in seed germination (Table 1) and in the viability of pollen grains (Fig. 2) in some of the intraspecific hybrids originating from Kc × Ka and Kd × Ka crosses indicates the presence of some post-zygotic internal barrier (Camadro *et al*. 2004) between genotypes in *S. kurtzianum.* This result allows us to speculate that they correspond to hybrid entities. Similar results were obtained during the characterisation of nine accessions of *S. spegazzinii* from the Argentine northwest. These accessions were grouped based on their morphological phenotypes and show a marked variability in the viability of pollen grains (from 61 to 94%; Erazzú *et al.* 1999). Moreover, in a similar study, two populations tentatively classified as *S. chacoense* exhibited very low levels of viable pollen grains (one population with 5.7% and the other 32.7%). These percentages were lower than those from artificial hybrids *S. tuberosum* × *S. gourlayi,* and accessions presumed to be the interspecific hybrid *S. gourlayi* × *S. infundibuliforme* (Larrosa *et al.* 2012). In this work, reductions in viability were associated with meiotic abnormalities, indicating a potential genomic conflict at nuclear or nuclear–cytoplasmic level. These results support the hypothesis of the importance of hybridisation in natural populations.

Activation of retrotransposons and epigenetic changes associated with genomic shock

In the present study, we detected novel fragments of the retrotransposons Tnt1 and Tto1 in recently formed synthetic hybrids (Table 2). Previously, it was found that both families of retrotransposon are present in a quiescent state in healthy plants of Solanaceae genomes and are activated in response to different types of stress (Hirochika 1993; Grandbastien 1998; Grandbastien *et al.* 2005). Considering that all plants were grown in the same environmental conditions, it is possible that events of activation of these retrotransposons are due to the effect of genomic shock induced by hybridisation.

Both Tnt1 and Tto1 are among the best-characterised plant retroelements at a structural and functional level. The detailed structure and function of their respective LTR sequence analyses revealed complex transcriptional regulation (Takeda *et al.* 1998; Manetti *et al.* 2009), where the promoter and regulatory sequences involved in the expression of active copies concentrate in the U3´ region (Gransbastien *et al*. 2005; Petit *et al.* 2010).

This region is extremely variable within these families and, in the case of Tnt1 (and homologues in other *Solanum* sp.), exhibited similar motifs to those involved in the regulation of genes related to pathogen defence (Vernhettes *et al*. 1997; Grandbastien 1998; Melayah *et al*. 2001) and abiotic plant stress response (Salazar *et al*. 2007). Homologue copies of both retrotransposons have been identified in different *Solanum* genomes, such as *S. tuberosum* and *S. lycopersicon* in the case of Tto1 (Hirochika 1993), and different wild *Solanum* species, including those used in the present work in the case of Tnt1 (Manetti *et al.* 2007; Tam *et al.* 2007). The evaluation of polymorphic insertions of Tnt1 in different accessions of *S. lycopersicum* revealed that the majority are located in centromeric and pericentromeric regions of the tomato genome, being selectively neutral in most cases (Tam *et al.* 2007). However, insertional mutagenesis using Tnt1 in the *S. tuberosum* genome reveals that this retroelement preferentially inserted into genic regions in the potato genome (Duangpan *et al.* 2013). In the case of analysis of *de novo* insertion fragments analysed in this work, we identified only two gene insertions (polyphenol oxidase PPO, ID U22921 and fasciclin-like arabinogalactan protein, ID AK329135), corresponding to most of the genomic flanking 5-end to intergenic regions (data not shown).

In our work, moderate activity of Tnt1 and Tto1 was detected (0.9–7.8%) in response to hybridisation. In this regard, a study of the behaviour of Tnt1 through four generations of synthetic allopolyploid *Nicotiana sylvestris* × *Nicotiana tomentosiformis* revealed prolific activation of this retrotransposon only in the fourth generation (40%; Petit *et al.* 2010). The authors suggest that activation of Tnt1 would not be immediate, but it would require a meiotic event during which the homeologous genomes could interact (Petit *et al.* 2010). Comparisons of S-SAP profiles of the parental species *Spartina alterniflora* and *Spartina maritima* revealed few new insertions of retrotransposons (5%) in the F_1 hybrids, suggesting that a massive activation did not occur in the investigated families (Parisod *et al.* 2009). Other models of short-term hybridisation failed to demonstrate the mobility of the following mobile elements: ACC and Ac-III Wis-like (*A. thaliana* × *A. lyrata*; Beaulieu *et al.* 2009); Wis2-1A (*Aegilops sharonensis* × *Triticum monococcum*; Kashkush *et al*. 2003b); Athila-like and MITEs (*Brassica rapa* × *Brassica oleracea*; Parisod *et al*. 2009) and CACTA (*Triticum turgidum* × *Aegilops taushii*; Parisod *et al.*

2009). Furthermore, by comparing the activity of Tnt1 under abiotic (*in vitro* culture) and biotic stress conditions (protoplast isolation induced with fungal extracts), significant differential activation values of 2.7% and 24.4% were observed, respectively. Thus, experimental evidence suggests that the trigger for retrotransposon mobility is not a common phenomenon and is restricted to specific families of these elements in particular species, and mostly in young populations. Moreover, the response of particular retrotransposons depends on what kind of stress the genomic shock imposes, as well as the genomic background of the host.

The experimental evidence of regulatory mechanisms of retrotransposon activity indicates that they are mainly epigenetic in nature. The first indications of the role between loss of DNA methylation and activation of mobile elements were reported in maize, where methylation changes in the promoter of the Spm transposon induced its activation (Chandler & Walbot 1986; Fedoroff 2012). In this sense, it was reported that synthetic hybridisation might induce chromosomal instability associated with changes in epigenetic patterns (Comai 2000; Li *et al*. 2010). Our results revealed a positive correlation between retrotransposon mobility and hypomethylation in the vicinity of both retrotransposons in the synthetic hybrids analysed. In this respect, massive alterations in methylation patterns near retrotransposon sequences have been observed in *Cucumis* allopolyploid hybrids (Chen & Chen 2008) and in the first four generations of allohexaploid hybrids in wheat (Yaakov & Kashkush 2011). In the latter, changes in the methylation status (mostly toward hypomethylated states) were correlated with new Veju LTR retrotransposon insertions. However, on subsequent generations the authors observed a trend to hypermethylation states, screening the rapid response of epigenetic control mechanisms of the host genome (Kraitshtein *et al*. 2010). In addition, the study of several families of retrotransposon in different rice lines revealed that the environment of methylation in the vicinity of the sequences of these families tends to be highly methylated and shows that the degree of methylation patterns of a retrotransposon is characteristic of each family (Takata *et al*. 2007).

Some of the mechanisms underlying the silencing and inactivity of retrotransposons have been elucidated using transgenesis techniques, introducing active copies of Tnt1 and Tto1 retrotransposons and evaluating their behaviour in the genomic background of *A. thaliana* (Lucas *et al.* 1995; Hirochika *et al*. 2000). Unlike other plant species, the genome of *Arabidopsis* contains very few endogenous retrotransposons, and most families are present in low copy numbers, being mostly methylated and inactive (Feschotte *et al.* 2002). Following colonisation by Tnt1 and Tto1 in the genome of *Arabidopsis*, the analysis revealed an initial active transposition accompanied by hypermethylation silencing in the subsequent generations (Hirochika *et al*. 2000; Pérez-Hormaeche *et al*. 2008). Using the *Arabidopsis* mutant line *ddm1* (decreased in DNA methylation), these authors could associate Tnt1, Tto1 and another endogenous LTR retrotransposon activity with the non-methylated state of these sequences (Hirochika *et al*. 2000).

In contrast to what was observed in *Arabidopsis*, in the tobacco genome both Tnt1 and Tto1 display transcriptionally active copies with copy numbers of about 30 to 300, respectively (Hirochika 1993; Melayah *et al.* 2001). Why these active copies of retrotransposons escape gene silencing in tobacco still remains unanswered. One possible explanation is that silencing is not induced due to the large heterogeneity of sequences observed in the LTR region of both families (Casacuberta *et al*. 1995). Alternatively, it is possible that regulatory mechanisms of the activity of retrotransposons in the tobacco genome are not as efficient as in the *Arabidopsis* genome, which could be reflected in its genome size.

In regard to the genus *Solanum*, there is still no information linking the methylation status in the genome with activity of retrotransposons, this being the first report. However, previous results of our research team have shown that interspecific hybridisation events between wild species of *Solanum* section *Petota* induce structural genomic rearrangements (Ferrer MS, unpublished results). These changes have been associated with a general demethylation of the natural hybrids *S.* × *rechei* regarding the parental genomes (*S. kurtzianum* and *S. microdontum*; Cara *et al.* 2013) and morphological abnormalities at vegetative and floral level were observed (Marfil *et al*. 2009; Ferrer *et al*., unpublished

results). Together, these mechanisms might explain part of the large morphological diversity and adaptability observed in the tuber-bearing species of the genus *Solanum*.

CONCLUSION

In brief, the morphological analysis of synthetic hybrids of *Solanum* species revealed that crosses between plants classified as *S. kurtzianum* maintain morphological traits, whereas crosses between plants classified as different species (*S. kurtzianum* × *S. microdontum*) exhibited an intermediate phenotype. Despite this phenotypic behaviour, our results of pollen viability evidenced significant reductions in viability in all intra- and interspecific crosses Kb × Ma and Kc × Ma, whereas in the interspecific hybrids Kd × Ma this parameter was not reduced. These results suggest some degree of genomic incompatibility between parental lines classified as *S. kurtzianum* based on a morphological taxonomic concept. Besides, in all the evaluated genotypes activation of Tnt1 and Tto1 retrotransposons was observed in moderated ranges between 0.9 and 7.8%, except for genotypes of the interspecific cross KdMa, which exhibited mostly null mobility. In the case of Tnt1, there was a significant negative correlation between new insertions and reductions in pollen viability. On the other hand, epigenetic changes accompanied those results, where a trend to hypomethylated 5'CCGG islands in the vicinity of the retrotransposons analysed was observed, suggesting a possible mechanism for Tnt1 and Tto1 activation.

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Figure legends:

Figure 1. MANOVA results depicting the first and second canonical variables of the 30 morphological variables evaluated. Only the first two dimensions of this space are shown, because the first two canonical variables accounted for more than 81.76% of the variation among groups. Line spacing circles (--) group morphologically similar genotypes. The

circles (—) denote 95% confidence intervals of hybrids, and group means are represented by the point in the centre. MANOVA significant differences between groups are indicated with an asterisk ($P < 0.001$).
Symbols: Parental genotypes: (▲) Mc; (◇) Ka; (△) Kb; (⌓) Kc; (○) Kd. Intraspecific hybrids: (▪) KbKa; (◆) KcKa. Interspecific hybrids: (▽) KbMa; (●) KcMa; (□) KdMa.

Figure 2. Individual values (%) of pollen viability in genotypes classified as species and in artificial hybrids. Dot = mean and bars are SD (n=6 flowers per genotype; two fields per flower).
Symbols: Parental genotypes: (▲) Mc; (◇) Ka; (△) Kb; (⌓) Kc; (○) Kd. Intraspecific hybrids: (▪) KbKa; (◆) KcKa. Interspecific hybrids: (▽) KbMa; (●) KcMa; (□) KdMa.

Figure 3. Average values of percentage of *de novo* S-SAP fragments originating from each hybridisation event. (A) Tnt1 and (B) Tto1. Results obtained from S-SAP matrix. The values in each histogram having different letters are significantly different from each other (Duncan, $P < 0.01$).

Figure 4. Mean percentage change to hypomethylation state in the vicinity of retroelemets originating from each hybridisation event. (A) Tnt1 and (B) Tto1. Results obtained from TMD matrix. The values in each histogram having different letters are significantly different from each other (Duncan, $P < 0.01$).
Author: please change 'Hipomethylation' to 'Hypomethylation' in this figure.

Supplementary Figures

Figure S1. Experimental design scheme. (A) Parental plants and crosses to obtain hybrid genotypes. (B) Year 1: Seed germination of F_1 seeds and tuber sprouting to obtain leaves for genetic analysis and tubers for clonal multiplication. (C) Year 2: Morphological analysis. For more information, see Material and Methods in manuscript.

Figure S2. Complete nucleotide sequence of Tnt1. Restriction sites with HpaII/MspI (CC^GG) are highlighted in yellow. Specific target sites for EcoRI (G^AATTC) are absent

in this retrotransposon. Primer sequence and direction of amplification are indicated with arrows.

Figure S3. Complete nucleotide sequence of Tto1. Sequences highlighted in yellow correspond to HpaII/MspI (CC^GG) restriction sites, while blue highlights are specific targets sites for EcoRI (G^AATTC). Primer sequences and direction of amplification are indicated with arrows.

Figure S4. Molecular basis of TMD. Isoschizomers *Hpa*II/*Msp*I recognise the same restriction 5′-CCGG site but have different sensitivity to methylation of the cytosines. *Hpa*II digests only if the external cytosine is hemimethylated (single-strand) or non-methylated 5′-CCGG site but does not digest if either of the cytosines is fully (double-strand) methylated; while *Msp*I does not, cutting only if the internal cytosine is fully (double-strand) methylated or specifically non-methylated 5′-CCGG site. Neither *Hpa*II nor *Msp*I digest when other combinations of methylation in 5′-CCGG occur, or if the 5′-CCGG sequence has been changed.

Figure S5. Inheritance of methylation patterns in regions flanking Tnt1 and Tto1 determined with TMD. This figure was constructed based on a matrix of changes in methylation status of hybrids with respect to their respective parents (Table S1). Each column represents a genotype, and lines correspond to independent loci. The colours indicate the change of methylation status, where the patterns: A (blue) indicate no changes from parents, B (red) corresponds to the pattern shifts to hypermethylated, and C (yellow) to hypomethylated states.

Table 1. Plant material used in morphological and molecular analysis.

A) Parental genotypes

	Source	Germplasm population code	Putative taxon	Genotype designation	Province/Locality	Latitude	Longitude	Altitude (m)	No. of plants
Natural populations	Potato and Forage Germplasm Bank, INTA Balcarce	4544	*S. kurtzianum*	4544.1 (Kc)	Mendoza/Las Heras	32°33´ S	68°58´ W	680	1
		4505	*S. kurtzianum*	4505.3 (Kd)	Mendoza/Las Heras	32°24´ S	68°52´ W	1185	1
		4552	*S. kurtzianum*	4552.3 (Kb) 4552.6 (Ka)	Mendoza/Las Heras	32°32´ S	69°02´ W	2330	2[A]
		4631	*S. microdontum*	4631.4 (Ma)	Catamarca/Andalgalá	27°19´ S	66°39´ W	2030	1

B) Intra- and interspecific crosses between *S. kurtzianum* and *S. microdontum*.

	Cross	♀ × ♂		Seed germination No. (%)[B]	Established seedlings[C]	Designation of surviving hybrids
Synthetic crosses	Interspecific	4552.3 × 4631.4	(Kb× Ma)	11 (75%)	6	KbMa1, KbMa2, KbMa3, KbMa4, KbMa5, KbMa6
		4544.1 × 4631.4	(Kc×Ma)	6 (41%)	3	KcMa1, KcMa2, KcMa3
		4505.3 × 4631.4	(Kd×Ma)	9 (58%)	6	KdMa1, KdMa2, KdMa3, KdMa4, KdMa5, KdMa6
	Intraspecific	4552.3 × 4552.6	(Kb×Ka)	6 (41%)	3	KbKa1, KbKa2, KbKa3
		4544.1 × 4552.6	(Kc×Ka)	9 (58%)	7	KcKa1, KcKa2, KcKa3, KcKa4, KcKa5, KcKa6, KcKa7

[A] Both genotypes are seeds obtained from the same fruit.
[B] 15 seeds from each cross were sown, and number and % seed germination after 2 weeks was recorded.
[C] Number of surviving plants from each cross.

Table 2. Total number and percentage of novel fragments of Tnt1 and Tto1 detected using S-SAP in each hybrid

A) Intraspecific crosses

Kb × Ka			Kc × Ka		
Hybrid	**N° new fragments (Tnt1/Tto1)**	**% new fragments (Tnt1/Tto1)**	**Hybrid**	**N° new fragments (Tnt1/Tto1)**	**% new fragments (Tnt1/Tto1)**
KbKa1	4 / 3	3.1 / 2.7	KcKa1	7 / 0	5.4 / 0.0
KbKa2	3 / 7	2.3 / 6.3	KcKa2	8 / 8	6.2 / 7.2
KbKa3	7 / 4	5.4 / 3.6	KcKa3	5 / 4	3.9 / 3.6
			KcKa4	4 / 6	3.1 / 5.4
			KcKa5	3 / 2	2.3 / 1.8
			KcKa6	10 / 5	7.7 / 4.5
			KcKa7	7 / 6	5.4 / 5.4

B) Interspecific crosses

Kb × Ma			Kc × Ma			Kd × Ma		
Hybrid	**N° new fragments (Tnt1/Tto1)**	**% new fragments (Tnt1/Tto1)**	**Hybrid**	**N° new fragments (Tnt1/Tto1)**	**% new fragments (Tnt1/Tto1)**	**Hybrid**	**N° new fragments (Tnt1/Tto1)**	**% new fragments (Tnt1/Tto1)**
KbMa1	3 / 1	2.3 / 0.9	KcMa1	10 / 1	7.8 / 0.9	KdMa1	0 / 0	0.0 / 0.0
KbMa2	2 / 5	1.5 / 4.5	KcMa2	10 / 4	7.8 / 3.6	KdMa2	2 /0	1.5 / 0.0
KbMa3	5 / 5	3.9 / 4.5	KcMa3	8 / 1	6.2 / 0.9	KdMa3	0 / 0	0.0 / 0.0

KbM a4	3 / 0	2.3 / 0.0	KdM a4	0 / 0	0.0 / 0.0
KbM a5	3 / 1	2.3 / 0.9	KdM a5	0 / 0	0.0 / 0.0
KbM a6	6 / 4	4.6 / 3.6	KdM a6	0 / 0	0.0 / 0.0

Total number of analysed fragments: 129 for Tnt1 and 111 for Tto1.

Table 3. Pearson correlation of Tnt1 and Tto1 activity and pollen viability. The values in parentheses correspond to significance level of Pearson statistic. * Indicates significant values with $P < 0.05$.

Activity detected with S-SAP	Pollen viability
Tnt1	-0.53* ($p_{0,05}$=0.0196)
Tto1	-0.35 ($p_{0,05}$= 0.1109)

Table 4. Pearson correlation analysis of Tnt1 and Tto1 activity detected using S-SAP with changes in methylation patterns analysed using TMD. The values in parentheses correspond to Pearson statistical significance. Significant values are indicated with (*) for $P < 0.01$ and (**) for $P < 0.05$.

Retrotransposon activity detected with SSAP	TMD methylation changes	
	Hypermethylation	Hypomethylation
Tnt1	-0.48* ($p_{0.05}$=0.036)	0.58** ($p_{0.05}$=0.008)
Tto1	-0.17 ($p_{0.01}$=0.436)	0.48* ($p_{0.05}$=0.016)

Cloning and sequencing of new fragments of S-SAP and TMD products.

To investigate the identity of new fragments identified using electropherograms analysis, selected S-SAP and TMD products from hybrids and their respective parents were denatured at 90 °C in 4 µl loading buffer, resolved with polyacrylamide gel (6%) electrophoresis at 85 V for 150 min and visualised with silver staining. New fragments in the hybrids were cut from the polyacrylamide gel and dissolved in 20 µl MiliQ water through incubation at 95 °C for 20 min. The extracted fragments were used as templates to perform PCR amplification to accumulate copies of targeted fragments. These products were separated with 1% agarose gel electrophoresis, and the target fragments extracted from agarose gels before ligation into plasmid vector pGEM-T Easy (Promega) and transformation into TOP10 chemical-competent *Escherichia coli* cells for overnight cultivation at 16 °C. Single clones, positive for inserts were selected for sequencing. The plasmid DNA of individual clones was obtained with the alkaline lysis procedure and digested with EcoRI enzyme. Digestion products were analysed with electrophoresis in 0.8% agarose gels to discard false positives.

Cloned products were sequenced using the M13 forward primer in an ABI PRISM 3100 DNA automated sequencer (Perkin Elmer). All nucleic acid sequences were screened for vector contamination using the Vector Screen program

(www.ncbi.nlm.nih.gov/VecScreen) and primers sequences were removed. The obtained nucleotide sequences were deposited in the DDJJ database (DNA Data Bank of Japan, www.ddbj.nig.ac.jp) under accession numbers AB981958 to AB981967. Homology search between the obtained sequences and their respective retrotransposons was conducted using the BLAST2 program of the National Center of Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Homology assignment criterion was based on maximum probability threshold per sequence and a minimum E-value of 10^{-10}.