PLASTID GENE TRANSFER IN HIGHER PLANTS

Homeologous Plastid DNA Transformation in Tobacco is Mediated by Multiple Recombination Events

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INTRODUCTION

The integration of cloned plastid DNA into the plastid genome occurs exclusively through site-specific homologous recombination in genera as diverse as *Chlamydomonas* (BOYNTON *et al.* 1992) and *Nicotiana* (MALIGA 1993), and excludes the foreign vector DNA. This is in striking contrast with the integration of cloned DNA into the nuclear genome which occurs randomly, and routinely involves the co-integration of vector DNA (OHL *et al.* 1994; ROCHAIX 1995). A characteristic feature of the plastid genome is its high recombinogenic activity, best demonstrated in the inverted repeat (IR) region as a continuous flip-flop recombination, and copy correction by gene conversion (GOULDING *et al.* 1996 and references therein). A growing body of evidence indicates that a RecA-mediated recombination system exists in chloroplasts (CERUTTI *et al.* 1992, 1993, 1995; CERUTTI and JAGENDORF 1993). It is probable that this mechanism, which maintains the uniformity of the plastid DNA population, also drives the homologous integration events (MEDGYESY *et al.* 1985; FEJES *et al.* 1990).

Plastid transformation is fast becoming a routine tool for exploring the contribution of plastid genes and their regulatory regions in the processes of photosynthesis and other plastid-related biosynthetic activities. There are, hovewer, very limited data on the mechanism and limits of DNA integration associated with plastid transformation. The apparent reason for this is that the usual strategy for introducing non-homologous DNA into the plastid genome of the two model species (*C. reinhardtii* and *N. tabacum*) involves the use of completely homologous flanking regions which ensure efficient integration through double exchange events (BOYNTON and GILLHAM 1993; MALIGA *et al.* 1994).

In *Chlamydomonas* an almost complete replacement of a continuous region of the plastid genome by the corresponding DNA fragment from the donor plasmid is the most common integration event, and, remarkably, a partial donor/recipient homology (plastid transformation in *C. reinhardtii* by *C. smithii* plastid DNA, and *vice versa*) decreased the integration frequency by 10-100 times (NEWMAN *et al.* 1990). A similarly striking dependence of the efficiency of integration on the extent of homology between the donor and target regions was observed in the case of site-specific nuclear transformation in yeast (SMOLIK-UTLAUT and PETES 1983; NEGRITTO *et al.* 1997) and mammalian cells (DENG and CAPECCHI 1992; TE RIELE *et al.* 1992), and in bacteriophage-plasmid recombinations in *Escherichia coli* (WATT *et al.* 1985; SHEN and HUANG 1986). The direct role of the mismatch repair system in limiting homeologous recombination has been demonstrated in such diverse genetic systems as nuclear transformation in mammalian cells (DE WIND *et al.* 1995), meiotic and mitotic chromosomal recombination in yeast (CAMBERS *et al.* 1996; DATTA *et al.* 1996), conjugational and transductional recombination between bacterial species (RAYSSIGUIER *et al.* 1989), and bacteriophage-plasmid recombination in *E. coli* (SHEN and HUANG 1989). There are only scant data on homeologous transformation in higher-plant plastids. The combination of *N. tabacum* and *N. plumbaginifolia* for plastid transformation has proved unsuitable for the analysis of the effect of partial homology due to the high nucleotide sequence identity in the region usually investigated by plastid transformation (O'NEILL *et al.* 1993). In the case of an intraspecific tobacco plastid transformation, where several RFLPs were introduced into the donor plastid DNA fragment (STAUB and MALIGA 1992), the RFLP analysis of two transformants indicated the incorporation of all or nearly all of the uninterrupted transforming DNA.

Nicotiana and *Solanum* are two genera belonging to different subfamilies in the family Solanaceae (D'ARCY 1979). There are therefore sufficient nucleotide sequence differences between the plastid genomes to facilitate the molecular analysis of recombination events (MEDGYESY 1994; HORVÁTH 1995). Here we demonstrate a highly efficient integration of cloned *Solanum* plastid DNA into the *Nicotiana* plastid genome mediated by multiple recombination events.

MATERIALS AND METHODS

Characterization of the pSSH1 plastid transformation plasmid: The location and cloning into pUC19 of the 7.8 kb *Hin*dIII plastid DNA fragment isolated from the *S. nigrum* StSp1 mutant was described previously (KAVANAGH *et al.* 1994). The complete nucleotide sequence of the 7,819 bp insert of pSSH1 was determined (EMBL Accession No). The corresponding plastid DNA region in tobacco extends from nucleotides 98,531 to 106,198 and from nucleotides 136,424 to 144,091 in IR_B and IR_A, respectively (SHINOZAKI *et al.* 1986; GenBank Accession No Z00044). The nucleotide numbering in the *16SrDNA* gene takes into consideration a correction (O'NEILL *et al.* 1993) of the published sequence.

Production of plastid transformant plants: *Nicotiana tabacum* L. cv. Petit Havana was maintained as shoot cultures on agar-solidified MS medium in the light (50 μE m^{-2} s^{-1}, 16 h day, 25°). PEG-mediated plastid transformation was performed as described (O'NEILL *et al.* 1993). Leaf protoplasts were isolated, DNA-treated protoplasts were cultured in liquid medium, then the colonies were further grown for the selection in antibiotic-containing solid medium, and plants were regenerated from the resistant calli as described previously (MEDGYESY 1994). The selective medium contained either 1,000 mg l^{-1} spectinomycin dihydrochloride or both spectinomycin dihydrochloride and streptomycin sulfate at 500 mg l^{-1} each. The same antibiotic (combination) was used during shoot regeneration as for the selection of the individual lines. Seedlings and leaf calli were tested for their resistance to the appropriate antibiotics as described by CSÉPLŐ (1994). In the resistance tests the two antibiotics were employed separately at 1,000 mg l^{-1} each.

Molecular analysis of plastid transformant plants: Chloroplasts were isolated from aseptically grown plants according to BOOKJANS *et al.* (1984). Lysis of chloroplasts and the purification of DNA followed standard protocols (SAMBROOK *et al.* 1989). Restriction enzyme digestions were performed according to the instructions of the suppliers. DNA fragments were separated by horizontal agarose-slab gel electrophoresis, and visualized by ethidium bromide staining following standard protocols. Non-radioactive DNA dot blot hybridization was performed using the Boehringer DIG DNA Labeling and Detection Kit. Undigested plastid DNA (1 μg) was denatured and bond to positively charged Amersham Hybond-N+ nylon membrane according to standard protocols, and probed by a mixture of *Dra*I-*Sma*I double- and *Apa*LI-*Ava*II-*Pvu*II triple-digested pUC19 vector DNA. The 7.8 kb *Hin*dIII plastid DNA fragment was cloned from several transformants as described in KAVANAGH *et al.* (1994). The sequence of oligonucleotide primers that were used for PCR and nucleotide sequence analysis of plastid transformants together with their position in IR_A within the *N. tabacum* plastid genome are as follows:

LRP1: [136,250-136,283]
5'-TCGTCTCTGGGTGCCTAGGTATCCACCGTAAGCC-3'
INS100: [136,757-136,738] 5'-GGACCGACCATAGAACCCTG-3'
SPC1: [138,552-138,576] 5'-GTATGGCTGACCGGCGATTACTAGC-3'
INS10-21: [140,674-140,694] 5'-GAAAGAGGTTGACCTCCTTGC-3'
NT1: [140,772-140,788] 5'-CAACAATTCATCAGACT-3'
SN1: [140,774-140,784] 5'-ACAGTTCATCACGGAAG-3'
SSH: [140,981-141,002] 5'-CTCGGAGACCCACCAAAGTACG-3'
CP1: [141,612-141,596] 5'-GGACCAATTTAGTCACG-3'
STR1: [141,729-141,753] 5'-TCTGTAGAGTGGCAGTAAGGGTGAC-3'
INS5TT10: [143,046-143,067] 5'-GAATGGCAGAGGCAAATAGAGC-3'
LRP2: [144,131-144,098]
5'-ACGTCAGGAGTCCATTGATGAGAAGGGGCTGGGG-3'

The oligonucleotide primers LRP1 and INS100 were used for the PCR detection of the 103 bp long *Solanum*-specific insert (Figure 2C), from total DNA of control and plastid transformants. Since both primer sequences flank the site of insertion, a diagnostic fragment of either 508 bp (*N. tabacum*) or 611 bp (*S. nigrum*) will be amplified. The primer SN1 is specific for the 10 bp long *S. nigrum* insertion (Figure 2B), while primer NT1 is specific for the wild-type nucleotide sequence at the same location. Primer SN1, when used in combination with primer CP1 (based on nucleotide sequence that is identical in both *N. tabacum* and *S. nigrum*), will amplify a product if the DNA template contains the insertion. Primer NT1, when used in combination with CP1, will only amplify a product if the DNA template lacks the insert. The oligonucleotide primers LRP1 and LRP2 were used to amplify, from total DNA or plastid DNA of the transformants, the entire plastid genome region corresponding to the the 7.8 kb *Hin*dIII fragment present in pSSH1. The priming sites for both LRP1 and LRP2 are located adjacent, but external to the 7.8 kb *Hin*dIII fragment on the plastid genome. Consequently, the primers cannot amplify sequences from within pSSH1 plasmid DNA. Efficient amplification of the 7.8 kb *Hin*dIII fragment was achieved using approximately 200 ng total DNA (or 20 ng plastid DNA) in a 50 μl reaction using the Boehringer ExpandTM Long Template PCR System and the following cycle parameters: 94°, 20 s; 63°, 30 s; 68°, 7 min; 35 cycles. DNA sequencing reactions were performed using either double-stranded plasmid DNA or long range PCR-amplified DNA templates, and Perkin-Elmer Applied Biosystems fluorescent dye-terminator or dye primer sequencing kits. Sequencing reactions were analysed on an Applied Biosystems Model 370 automatic sequencer.

RESULTS

Nucleotide sequence differences between *S. nigrum* and *N. tabacum* in the region inserted into the pSSH1 plastid transformation plasmid: The pSSH1 plasmid (Figure 1) contains the *16SrDNA* and the *rps*12(3') genes containing mutations conferring spectinomycin and streptomycin resistance, respectively, as a 7.8 kb *Hin*dIII fragment cloned from a *S. nigrum* plastid mutant (McCabe *et al.* 1989; Kavanagh *et al.* 1994). The spectinomycin resistance mutation is a G to A transition (as in strand A of IR_B) at nucleotide 1,139 of the *16SrDNA* gene. This nucleotide change causes the loss of an *Aat*II cut site. The streptomycin resistance mutation is an A to C transversion at the first nucleotide position in codon 88 (at position 85 of exon 2) of *rps12*(3'), converting a lysine to a glutamine. This nucleotide change creates a new *Mse*I cut site. At position 127 of the same exon of *rps12*(3') there is a neutral, species-specific, nucleotide difference (C in *S. nigrum* instead of T in *N. tabacum* at the first nucleotide position in codon 74). Complete nucleotide sequence analysis of the 7.8 kb cloned region identified additional species differences. The most characteristic *S. nigrum* feature is the presence of several, 5-100 bp insertions/deletions in comparison with the *N. tabacum* nucleotide sequence. The species-specific nucleotide sequence differences used in our analysis of the plastid transformant lines are summarized in Figure 2. These non-homologous sites are flanked on either side by regions sharing complete homology. The distinction between wild-type and transformed *Nicotiana* plastid DNA was facilitated by characteristic differences in the *Bam*HI cleavage site map (cf Figures 1 and 3C). These RFLPs involved a shortening of fragment No 5 by a *Solanum*-specific cut site, and a lengthening of fragment No 13 (formerly No 11 [SHINOZAKI *et al.* 1986]) due to *Solanum*-specific insertions (cf Figures 2 and 3C).

Efficient genetic transformation of the *Nicotiana* plastid genome by *Solanum* plastid DNA: Following PEG-mediated direct transformation of protoplasts using the pSSH1 plasmid, putative plastid transformant colonies were selected on the basis of their green color in a medium containing either spectinomycin or both spectinomycin and streptomycin. Tests for the presence of the antibiotic resistance markers were performed at three stages: the primary calli, the regenerated plants, and their progeny. Each line exhibited stable retention of the initial resistance phenotype, and proved to be completely homoplasmic (Table 1). The frequency of double-resistant calli (about 1 in 10^4 viable colonies, or in 10^5 protoplasts treated), considered as plastid transformants, was very similar after either initial single or double resistance selection (Table 1). A single spectinomycin-resistant spontaneous mutant (not included in Table 1) was identified by its streptomycin sensitivity and the presence of the diagnostic, *Nicotiana*-specific, *Aat*II cut site.

All but one of the 11 double-resistant *Nicotiana* plants characterized contained at least one of the unselected *Bam*HI markers diagnostic for *S. nigrum* (for representative RFLP patterns see Figure 3A). Apart from the *Solanum*-specific RFLPs, no other deviation from the expected plastid DNA fragment patterns was observed in the transformants. This indicated the site-specific integration of the targeting plastid DNA in each case, and a lack of integration of the pUC19 vector DNA. The latter conclusion was verified by a complete lack of dot blot hybridization of the vector DNA to plastid DNA of each of the transformants (data not shown). The polymorphic DNA regions (revealed as RFLPs between the *Solanum* and the *Nicotiana* plastid DNA) were shown to be homozygous in all but one of the transformants demonstrating complete intraorganellar plastid DNA segregation after transformation. This conclusion was corroborated by PCR analysis of *Solanum*- and *Nicotiana*-specific regions in each of the transformants (data not shown). In one of the primary regenerants a single locus, the peripheral *Solanum*-specific *Bam*HI marker, proved to be mixed, ie, both the *Nicotiana*- and the *Solanum*-specific *Bam*HI fragment No 5 were observed in the RFLP pattern (data not shown). The *Solanum*-specific fragment gradually faded over successive rounds of vegetative propagation and was absent in the sexual progeny of this plant (12 plants investigated, data not shown). A diagnostic test for the presence of the *Solanum* spectinomycin resistance site was based on a concomitant loss of a *Nicotiana*-specific *Aat*II cut site which resulted in distinctive changes in the

*Pst*I-*Aat*II restriction fragment pattern diagnostic for IR_A and IR_B (Figure 3C). The RFLP patterns demonstrated the identity of the copies of the invertedly repeated region with respect to the spectinomycin resistance site in all of the plastid transformant plants analysed (for representative RFLP patterns see Figure 3B).

Efficient co-integration of homologous and heterologous regions of the *S. nigrum* plastid DNA into the *N. tabacum* plastid genome: A preliminary compilation of the *Bam*HI RFLPs (for representative RFLP patterns see Figure 3A) in the 11 double-resistant, pSSH1-mediated plastid transformants indicated that the typical event was a full-length integration of the targeting plastid DNA. This was found in eight of the transformants while in two transformants only the peripheral *Solanum*-specific *Bam*HI marker was missing. In a single transformant, despite using so few markers, multiple recombination events were detected. In this particular transformant both the peripheral and the internal (located between the two resistance markers) *Solanum*-specific *Bam*HI markers were missing. These observations were corroborated by another experiment (HORVÁTH *et al.* 1997; E. M. HORVÁTH, S. O. PETER, T. JOET, D. RUMEAU, G. V. HORVÁTH, C. SCHÄFER, G. PELTIER and P. MEDGYESY, manuscript in preparation) in which the plasmid pSSH1M was used as the donor DNA, and spectinomycin alone was used to select for plastid transformants. In this latter experiment the pSSH1 plasmid was modified (pSSH1M) to include a *Sma*I cut site (Figure 4) near to that end of the

*Hin*dIII plastid DNA insert which possessed no RFLP in the original construct. A *Solanum*-specific *Xho*I site was also included in the analysis of the pSSH1M-mediated plastid transformants. This *Solanum*-specific *Xho*I site is the result of a single nucleotide difference (ctcGag instead of ctcCag) at position 1,411 (in *N. tabacum*) of the intergenic region between *rps*12(3') and *trn*V (data not shown). The use of these additional markers confirmed the previously observed dominance of uninterrupted integration and a bias against the integration of peripheral donor markers (Figure 4). However, in one third of the 18 transformants investigated in this experiment, integrations via quadruple recombination events were also revealed (Figure 4). The frequency of recombination events between the individual markers, calculated as a percentage of the transformants recombined in a particular interval, was in the range of 10-50% for internal regions. Furthermore, 50 and 60% of the transformants possessed the peripheral donor marker located 113 bp (*Sma*I) and 231 bp (*Bam*HI) from the cloning site, respectively (Figure 4), demonstrating that the peripheral recombination events occured very close to the vector-insert junction in these particular transformants. It was notable that in spite of the multiple exchange events the integration of the *Xho*I site (marking a single nucleotide difference), the internal *Bam*HI site (marking 10-21 bp insertions) and the peripheral *Bam*HI site (marking the 103 bp insertion) of the donor DNA (cf Figures 2 and 3) occured similarly (in 70, 70 and 60% of the transformants, respectively). The remarkably frequent recombination events via a 231 bp homologous peripheral region located between the 103 bp *Solanum*-specific insertion and the pUC19 vector, and the occurence of multiple recombination events in several transformants, prompted a more detailed analysis of heterologous sites across the region.

Nucleotide sequence analysis of all of the initial 11 pSSH1-transformed plants was performed, using cloned plastid DNA or long-range PCR products covering the region of insertion, in order to confirm the presence or absence of the heterologous sites and the resistance mutations. In most of the transformants showing full-length integration on the basis of the genetic and RFLP analyses, multiple recombination events were revealed (Figure 5). These integrations involved mainly quadruple but also hexuple or octuple recombination events. On the basis of the 14 distinct nucleotide sequence markers scored only one of the 11 transformants proved to be homogeneously *Solanum* type, showing uninterrupted full-length integration. Within the limitations of the analysis due to the relatively low number of transformants, and the complete homology of large regions of the donor and target plastid DNA, no polarity in marker recombination/incorporation was detected along the targeted region. Nucleotide sequence analysis did not reveal any examples of illegitimate recombination, inaccurate exchange or spontaneous mutation in the DNA regions investigated. Natural plastid DNA polymorphism as a potential source of the differences was excluded by the nucleotide sequence analysis of five independently isolated clones of the 7.8 kb *Hin*dIII plastid DNA fragment obtained from the original *S. nigrum* StSp1 mutant. Where a recombination site consisted of only a single nucleotide difference, a similar approach was used to exclude PCR-generated errors. The selection conditions had little or no influence on the distribution of the transformants among the different recombination classes (Figure 5). For example, of the two plants in which the neighbouring *Solanum*-specific sites are missing on both sides of the streptomycin resistance site, one was regenerated after selection on spectinomycin alone, and the other after double antibiotic selection. Recombination events were revealed in between the resistance markers in four out of six double-selected, and two out of five spectinomycin-selected, transformants. The frequencies of co-integration between the spectinomycin resistance and the different types of unselected *Solanum* markers, calculated as a percentage of transformants possessing both markers, were comparable: 45-90% for single nucleotide differences, 45-90% for 5-21 bp long insertions/deletions, and 75% for the 103 bp long insertion.

Marked position effects resulted in a non-random distribution of the recombination events on the targeted plastid DNA region: The recombination events were restricted in several cases to short (41-141 bp) homologous regions (cf Figures 2 and 5). No recombination was detected in the three locations where the *Solanum*-specific sites are separated by 1-22 nucleotides. The recombination frequency in the individual internal sections, calculated as a percentage of the transformants recombined in the particular interval, was in a similar range when for example the five 41-290 bp (10-45%) and the five 415-2,140 bp (10-55%) internal sections were compared. The recombination frequency in the entire 6.8 kb region between the peripheral *Solanum* markers (35%), or between one of the peripheral *Solanum* markers and the spectinomycin resistance marker (30% in both cases) was also in this range. This suggests no linkage between the markers separated by more than 40 bp. The distribution of the recombination events, however, was not random. In 90% of the transformants the peripheral recombination events were observed in one of the two short, 762 and 231 bp, peripheral regions, and in 50% of the cases they were found in both (Figure 5). These data were corroborated by similar results (70 and 40%) obtained by RFLP analysis in the other tobacco plastid transformation experiment (Figure 4) or in *N. plumbaginifolia* (15 transformants investigated, data not shown). A dependence of the recombination frequency on the length and the location of the particular intervals determined by the *Solanum*-specific sites, was further addressed in both the pSSH1- and the pSSH1M-mediated plastid transformation experiments (Figure 6). The observed frequency of recombination (calculated as percent of the intervals recombined in the region) was compared with the expected frequency (calculated to be proportional with the length of the individual intervals in the region). The recombination frequency of the individual sections calculated in this way also showed a hot spot in the shortest peripheral sections between the vector DNA and the first marker. The observed frequency in the 762, 231 and 113 bp peripheral sections was two, seven and 10 times higher than the expected frequency, respectively. The frequent recombination events at the short homologous regions adjacent to the heterologous vector DNA resulted in a very frequent co-integration of (at least one of) the peripheral donor markers. Integration events, however, never expanded into the vector DNA demonstrating that several kb of completely non-homologous DNA was an efficient barrier to recombination. The recombination frequencies were relatively low in the intervals adjacent to the selected spectinomycin resistance site, although this was pronounced only in the pSSH1 experiment. The lack of a conclusively strong effect of the selection was also indicated by the recombination frequencies in the intervals adjacent to the streptomycin resistance site, which were very similar in the double-selected (13 and 6%) and the spectinomycin-selected (14 and 4%) transformants of the pSSH1 experiment. All these position effects were independent of the length of the non-homologous sites scored indicating that the plastid DNA heterologies, at least up to a hundred nucleotides, did not hamper their own integration.

DISCUSSION

The present work demonstrates efficient transformation of the *Nicotiana tabacum* plastid genome by plastid DNA of *Solanum nigrum* which belongs to a separate subfamily in the Solanaceae. The phylogenetic difference between these species is sufficient to prevent the production of green cybrids with new nucleus-plastid combinations (THANH *et al.* 1988). The previously reported production of a normal green plastid DNA recombinant plant between tobacco and potato through protoplast fusion (THANH and MEDGYESY 1989), although predicting the feasibility of direct plastid gene transfer in such a taxonomically disctinct combination, left open the question of efficiency. In the present work the frequency of PEG-mediated plastid transformation using the incompletely homologous (homeologous) 7.8 kb segment of *Solanum* plastid DNA was approximately 10 transformants per 10^6 protoplasts treated, using either *N. tabacum* or *N. plumbaginifolia* (data not shown) as recipient in several independent experiments. This figure is similar to that observed in other experiments, in which either a 3-4 kb segment of completely homologous plastid DNA (GOLDS *et al.* 1993; O'NEILL *et al.* 1993) or a foreign gene flanked by 1-4 kb of completely homologous plastid DNA was used (KOOP *et al.* 1996). In a recent independent experiment (B. UIJTEWAAL, personal communication) similar plastid transformation frequencies were obtained in tobacco irrespective of whether completely homologous (pTB116; O'NEILL *et al.*

1993) or homeologous (pSSH1; this thesis) transforming plasmid was used (both plasmids confer streptomycin and spectinomycin resistance and target the same IR region). Therefore, in contrast to interspecific plastid DNA transformation in *Chlamydomonas*, in *Nicotiana* no reduction in the transformation frequency was observed using partially homologous donor DNA. This observation may reflect fundamental differences in the mechanism of integration in the plastid genome of the two organisms. In *Chlamydomonas* plastid transformants the integration of the donor plastid DNA occurred exclusively via one double-exchange-event (NEWMAN *et al.* 1990), even if a recombination hot spot was located in the recipient DNA region (NEWMAN *et al.* 1992). Consequently, complete or partial, but uninterrupted, DNA integration was always observed, even if 6-7 kb donor fragments labelled with six or seven RFLP/genetic markers were used (NEWMAN *et al.* 1990). The *Nicotiana* plastid transformants reported here, however, exhibited multiple internal exchange events in 33-73% of the transformants (depending on the number of markers scored), resulting in a mosaic-type integration of the donor DNA. It was notable that, in spite of the multiple recombination events, a larger extent of integrative recombination was also observed. A peripheral diagnostic marker located either 762, 231 or 113 bp from the vector-insert junction was co-integrated in 73, 61-73 and 50% of the *Nicotiana* transformants, respectively. In *Chlamydomonas*, however, a decrease in the distance between the vector DNA and the first donor marker from 1.35 kb to 150 bp resulted in a decrease in the ratio of transformants possessing that marker from 34-61% to 2-9% (NEWMAN *et al.* 1992). In our experiments, therefore, the mechanism resulting in multiple recombination events was believed to facilitate both the frequency and the efficacy of the co-integration of homologous and heterologous plastid DNA regions of the targeting DNA.

Recombination between homeologous nucleotide sequences is influenced by the extent and degree of DNA homology which is monitored by the recombination/repair enzymes. A central component of the system that promotes homologous recombination in bacteria is the RecA protein, and its functional homologs in eukaryotes (KOWALCZYKOWSKI and EGGLESTON 1994; CAMERINI-OTERO and HSIEH 1995). RecA protein is broadly responsible for facilitating recombination steps involving DNA pairing, strand transfer and branch migration. The presence and activity of a RecA homolog has also been observed in higher plant plastids (CERUTTI *et al.* 1992, 1993; CERUTTI and JAGENDORF 1993). Furthermore, the expression of the wild type and a dominant negative mutant of the *E. coli recA* gene located on the *Chlamydomonas* plastid genome substantially facilitated and diminished plastid DNA recombination, respectively (CERUTTI *et al.* 1995). Data presented in this thesis are fully compatible with a RecA-mediated recombination mechanism. The observed local recombination frequencies near the vector-insert junction were up to 10 times higher than would be predicted. This phenomenon was most probably due to the stimulation of homologous recombination adjacent to regions of extensive heterology. An enhanced neighbouring recombination frequency as a result of non-homology has been reported in crosses between bacteriophages (LIEB *et al.* 1984). It was concluded that large heterologous blocks in the recombining molecules can present a barrier to branch migration of Holliday structures which, consequently, would lead to an increased probability of resolution by cleavage in the adjacent region (LIEB *et al.* 1984). A central element of current recombination models is random walking of the branch point of a Holliday intermediate along the homologous region, which is then resolved by either integration before or dissociation at the end of the homology (ALANI *et al.* 1994; FUJITANI *et al.* 1995). It is notable however that the RecA-mediated DNA strand-exchange reaction *in vitro* readily bypasses short internal regions of heterology up to approximately 100 base pairs (MOREL *et al.* 1994). The lack of internal non-homologous regions longer than 100 bp which might have posed a barrier to branch migration, and in contrast the blocking effect of the extensive non-homologous vector DNA, is the most plausible explanation of the high frequency of integration of both the internal and peripheral donor plastid DNA markers observed in our experiments. In this context the mechanism promoting multiple recombination events is likely to involve random resolution of branch migration along the entire targeted region. Unfortunately the inherent difficulty in detecting each type of segregant after plastid recombinations in higher plants mitigates against a detailed investigation of the recombination mechanism (occurence and ratio of gene conversions and cross-overs). The above considerations, however, have taken into account only the effects of the RecA-mediated recombination/repair system.

The most potent inhibitor of recombination between moderately diverged nucleotide sequences is the mismatch repair system (MODRICH and LAHUE 1996). Mispaired bases in heteroduplex regions formed in the recombination reaction trigger long-patch mismatch repair leading to dissociation of the heteroduplex and abortion of the recombination reaction. *In vitro* studies demonstrated that mismatch repair proteins can directly inhibit the branch-migration stage of RecA-catalyzed strand transfer when mismatched base pairs occur within the hetroduplex (WORTH *et al.* 1994). Even 1% nucleotide sequence difference causes an order of magnitude reduction in the frequency of DNA recombination in various genetic systems (PETIT *et al.* 1991; TE RIELE *et al.* 1992; DATTA *et al.* 1997; ZAHRT and MALOY 1997; ELLIOT *et al.* 1998). A similar observation was reported in the case of interspecific plastid transformation in *Chlamydomonas* (NEWMAN *et al.* 1990), although the involvement of a recombination hot spot can conceal the effect (NEWMAN *et al.* 1992). The most direct proof of the role of the mismatch repair system is that mutations impairing defined mismatch repair genes increase the frequency of homeologous recombination by one-two orders of magnitude (see INTRODUCTION). Remarkably, homeologous recombination in different organisms typically results in the incorporation of continuous tracts (MATIC *et al.* 1994; SWEETSER *et al.* 1994; PORTER *et al.* 1996; DOONER and

MARTÍNEZ-FÉREZ 1997; YANG and WALDMAN 1997; ELLIOT *et al.* 1998). Our results revealed recombination frequencies and efficiencies typical of those found in homologous plastid DNA transformation despite the 2% nucleotide sequence divergence between *Nicotiana* and *Solanum* plastid DNA in the transformed region. Furthermore, we have demonstrated a primarily discontinuous homeologous integration (multiple recombination events involving closely located markers). All these data suggest that the mismatch repair system might be naturally suppressed in higher-plant plastids.

If further investigations support this hypothesis the phenomenon can be related to the lack of plastid DNA recombination in sexual crosses in higher plants. Meiotic recombination between chromosomes is a fundamental feature of the sexual cycle (ROEDER 1990; RADMAN and WAGNER 1993), and homologous recombination is an inherent part of bacterial gene transfer processes (CLARK and LOW 1988). Recombination occurs between parental plastid DNAs in *Chlamydomonas* (BOYNTON *et al.* 1992), even in interspecific crosses (LEMIEUX *et al.* 1981), because plastid fusion and the occasional mixing of parental plastid DNAs is a natural part of mating (CAVALIER-SMITH 1970; KUROIWA *et al.* 1982). In interspecific *Chlamydomonas* plastid recombinants the frequency and distribution of recombination events are determined by the plastid genome region and the species combination (LEMIEUX and LEE 1987; LEMIEUX *et al.* 1990; NEWMAN *et al.* 1992). In most higher plants, hovewer, the particular mechanisms of plastid transmission during the sexual cycle appear to prevent the mixing of parental plastids

(MOGENSEN 1996). Even in those species where plastids are inherited biparentally, recombination between the parental plastid genomes cannot be demonstrated, suggesting that plastid fusion itself is precluded in higher plants (HAGEMANN 1992). Whatever the evolutionary significance of the asexuality of plastids in higher plants (BIRKY 1995), we hypothesize that it may have resulted in the deterioration of a mismatch repair system that was no longer required to defend the plastid genome against the potentially destabilizing consequences of homeologous recombination.

In the present work the primary plastid transformant shoots regenerated following antibiotic selection proved to be homoplasmic. The regeneration of homoplasmic shoots in a single step occurs routinely when selection is based on antibiotic insensitivity mutations located in plastid genes (MEDGYESY 1990, 1994; DIX and KAVANAGH 1995). In the case of a multicellular, multiplastidic organism in which each plastid contains a multicopy genome, homoplasmy is achieved by a complex segregation process. This initially involves copy correction of the changes introduced into the inverted repeat region, and segregation of the plastid DNA copies during multiplication of the individual plastids. Under selective conditions the individual resistant plastids have a selective advantage during partitioning at the subsequent cell divisions, and gradually come to dominate the intracellular plastid population in the daughter cells. A sufficiently strong selection pressure results in early and complete segregation of plastids to the pure transformed type in the cell colonies prior to shoot regeneration. In our experiments a single plant was regenerated from an individual cell line, *i.e.*, from a protoplast-derived primary resistant callus. Therefore the individual plants investigated represent independent primary transformation events.

There is an increasing recognition that the plastid genome is a particularly attractive target for the introduction of genes carrying agriculturally valuable traits in various crop species (DIX and KAVANAGH 1995), a prospect exploited till now only in tobacco (MCBRIDE *et al.* 1995; DANIELL *et al.* 1998). This poses the question as to whether it will be necessary to develope species specific plastid transformation vectors for each particular crop species in order to ensure efficient and site specific integration of transgenes. The gene order and the coding sequences in the ribosomal RNA gene operon of the plastid genome is highly conserved in higher plants (DELP and KÖSSEL 1991). The data presented in this thesis demonstrate that the nucleotide sequence divergence in the targeted region (at least up to 2%) does not hamper the integration of the donor plastid DNA. These considerations indicate that plastid transformation vectors directed to this region are not required to be species specific. Further investigations are necessary however to determine the limits of nucleotide sequence heterogeneity allowing efficient integration. The use of the *Solanum* plastid rRNA gene-based plasmid pSSH1M in tobacco proved to be efficient for the targeted inactivation of a photosynthetic gene linked at a 5 kb distance to the marker gene (HORVÁTH *et al.* 1997; E. M. HORVÁTH, S.

O. PETER, T. JOET, D. RUMEAU, G. V. HORVÁTH, C. SCHÄFER, G. PELTIER and P. MEDGYESY, manuscript in preparation).

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ABSTRACT

Efficient plastid transformation has been achieved in *Nicotiana tabacum* using cloned plastid DNA of *Solanum nigrum* carrying mutations conferring spectinomycin and streptomycin resistance. The use of the incompletely homologous (homeologous) *Solanum* plastid DNA as donor resulted in a *Nicotiana* plastid transformation frequency comparable with that of other experiments where completely homologous plastid DNA was introduced. Physical mapping and nucleotide sequence analysis of the targeted plastid DNA region in the transformants demonstrated efficient site-specific integration of the 7.8 kb *Solanum* plastid DNA, and the exclusion of the vector DNA. The integration of the cloned *Solanum* plastid DNA into the *Nicotiana* plastid genome involved multiple recombination events as revealed by the presence of discontinuous tracts of *Solanum*-specific sequences which were interspersed between *Nicotiana*-specific markers. Marked position effects resulted in very frequent co-integration of the non-selected peripheral donor markers located adjacent to the vector DNA. Data presented here on the efficiency and features of homeologous plastid DNA recombination are consistent with the existence of an active RecA-mediated, but a diminished mismatch, recombination/repair system in higher-plant plastids.

ÖSSZEFOGLALÓ

A színtestek genetikai transzformációja magasabb rendű növényekben egyre inkább rutineljárássá válik, mégis nagyon kevés adat van a transzformáció során lejátszódó DNS-beépülés sajátosságairól. A szakirodalomban eddig megjelent plasztisztranszformációs kísérletekben a bevitt és a transzformált plasztisz-DNS-ek teljes homológiát mutattak, és a beépülés helyspecifikusan, homológ rekombinációval történt. Ez akkor is igaz volt, amikor idegen gént vittek be, ui. ezekben az esetekben homológ határoló szakaszokat használtak, tehát a tényleges rekombináció teljesen homológ szakaszok között történt. Kísérletünkben azt vizsgáltuk, hogy magasabb rendű növényekben részlegesen homológ (homeológ) plasztisz-DNS használata hogyan befolyásolja a DNS-integrációt. A pSSH1 jelű plasztisztranszformációs vektor egy *Solanum nigrum* (fekete csucsor) plasztiszmutánsból származó, 7,8 kbp hosszúságú plasztisz-DNS-szakaszt hordoz. Ezen DNS-szakasz nukleotidsorrendje, a szelektálható szpektinomicin- és sztreptomicinrezisztenciát okozó mutációk mellett, összesen 2%-nyi fajspecifikus eltérést mutat a *Nicotiana tabacum* (közönséges dohány) megfelelő plasztisz-DNS-szakaszáéval összehasonlítva. Ezt a részlegesen homológ plasztisztranszformációs vektort dohány-hártyássejtekbe (protoplasztokba) vittük be polietilénglikolos kezeléssel. A feltételezett plasztisztranszformáns sejtkolóniákat szpektinomicinrezisztenciájuk alapján izoláltuk, majd a regenerált növényeket sztreptomicinrezisztenciájuk és a plasztisz-DNS-ük restrikciós-fragmentmintázata alapján azonosítottuk. Meglepő módon a homeológ *Solanum*-plasztisz-DNS-sel történő transzformáció gyakorisága (átlagosan egy plasztisztranszformáns 10^4 sejtkolónia között) nagyon hasonló volt a homológ plasztisztranszformációs kísérletekben kapott gyakoriságokhoz. Tizenegy transzformáns növény plasztisz-DNS-ét nukleotidszekvencia-analízissel vizsgáltuk. Összesen 14, a donor DNS-re jellemző molekuláris marker (deléció, inszerció, pontmutáció) integrációját vizsgáltuk meg mindegyik plasztisztranszformánsban. Meglepő módon azt mutattuk ki, hogy a transzformánsok 70%-ában a donor DNS helyspecifikus beépülése számos belső rekombinációs esemény közvetítésével játszódott le, vagyis a DNS-integráció mozaikszerűen történt. Más genetikai rendszerekben a DNS-szakaszok nukleotidsorrendjének 1%-os eltérése a transzformáció, ill. rekombináció hatékonyságát 10-100-szorosan csökkenti, és a homeológ DNS-szakaszok leggyakrabban egységes darabként épülnek be. A homeológ rekombináció gátlását ezen esetekben egy speciális hibajavító rekombinációs rendszer (*mismatch repair system*) működése okozza. Ezen eredmények fényében a magasabb rendű növények kloroplasztiszaiban a DNS-rekombináció általunk kimutatott módja egyedülállónak tűnik, melynek egyik lehetséges magyarázata az, hogy a citoplazmás organellumokra jellemző anyai öröklődés miatt a növényi kloroplasztiszoknak nincs szexuális életük. Mivel a plasztiszgenom integritását nem veszélyezteti eltérő nukleotidsorrendű DNS-ekkel való rekombináció, feltehetően az ezt kivédendő speciális rendszer az evolúció során leépült. Ennek következtében a mesterségesen bevitt homeológ DNS beépülése hatékonyan, kizárólag a RecA-típusú homológ rekombinációs rendszer hatása alatt történik.

TABLES AND FIGURES

TABLE 1. Frequency and resistance phenotype of plastid transformants regenerated from N. tabacum protoplasts PEG-treated with the pSSH1 plasmid

Total number of				Resistance phenotypes in					
				callus[b]		leaf [c]		seedling[d]	
protoplasts	colonies	Selection	Lines[a]	spe	str	spe	str	spe	str
5.0×10^5	7.7×10^4	spe	5	+	+	+	+	+	+
5.0×10^5	5.2×10^4	spe + str	6	+	+	+	+	+	+

[a] The single spontaneous mutant, homoplasmic in resistance (+) and sensitivity (-) to spectinomycin (spe) and streptomycin (str), respectively, is not shown.

[b] Eight callus pieces/line/antibiotics tested from each primary callus (cell line).

[c] Eight leaf pieces/line/antibiotics tested (one plant regenerated from each cell line).

[d] Approximately 500 seedlings/line/antibiotics tested, obtained from crosses of the primary regenerants with the wild type as the male parent.

FIGURE 1

Genetic map of the pSSH1 plastid transformation plasmid. The 7.8 kb *S. nigrum* plastid DNA insert was cloned into the *Hin*dIII cut site of the pUC19 vector (thick line). The plastid genes (filled bands) shown inside and outside the circle are transcribed anti-clockwise and clockwise, respectively. The relative position and the direction of transcription of the ampicillin resistance gene in pUC19 is shown by a long arrow. Asterisks mark intron containing genes. *Bam*HI restriction sites of the insert are shown. Sites of the streptomycin (str^r) and the spectinomycin (spe^r) resistance mutations are shown by short arrows.

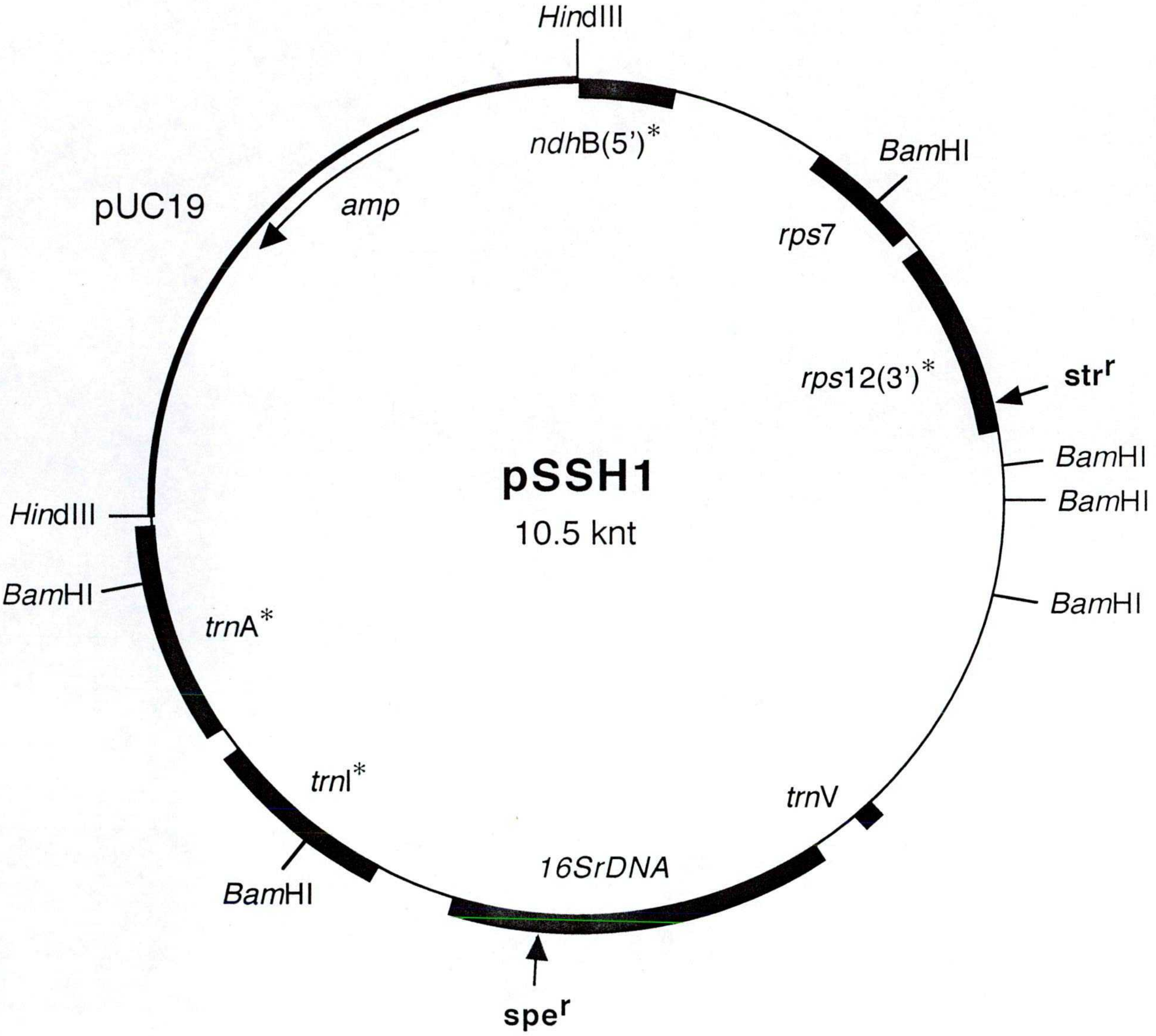

*Hin*dIII
*ndh*B(5')*
*Bam*HI
pUC19
amp
*rps*7
*rps*12(3')*
str^r
pSSH1
10.5 knt
*Bam*HI
*Bam*HI
*Hin*dIII
*Bam*HI
*Bam*HI
*trn*A*
*trn*I*
*trn*V
*Bam*HI
16SrDNA
spe^r

FIGURE 2

Alignment of nucleotide sequence differences between *N. tabacum* and *S. nigrum* used as physical markers of plastid DNA transformation. Portions of the full *N. tabacum* (NT) nucleotide sequence are shown, together with the differences detected in *S. nigrum* (SN), as they appear in IR_B (5' to 3' direction, strand A), (A) from position 365 of the intergenic region between *ndh*B and *rps*7, (B) from positions 445 and 941 (in *N. tabacum*) of the intergenic region between *rps*12(3') and *trn*V, and (C) from position 510 of the intron region in the *trn*A gene. Selected restriction enzyme cut sites characteristic of the particular region are also shown. Identical (.) and missing (-) nucleotides are marked.

A *ndh*B - *rps*7 spacer

```
          BspHI
NT  (5') TCATGAATTAGTAACTGCTTCCAATTTCCAAAAAAAA--TCCCAATTGTG
SN  (5') .....................................AA.A.........

NT       TCGAACTTTCCATTTTTGGAATAGTTACGGAATCTCCATGAATAGGATCA
SN       ..................................................

NT       AACCTTATTCCATGGTATTTACATGAGGTTCCTCTTTAAGAAAGTCCCCG
SN       ..................................................

NT       AGAGGCTTAGTTGATCCATGATTTATGTTTCATC-----TTTCCTTTTCG (3')
SN       ..................................TTTCG........... (3')
```

B *rps*12(3') - *trn*V spacer

```
NT  (5') AGTCCCATAGGTTTGATCCTGTAGAATTTGACCCATTTTCTCATTGAACG
SN  (5') ................................T.................

NT       AAAGGTACGAAATAAATCAGATTGATAAAAGTACCATGTGAAATCTTCGG
SN       ..................................................
                         BsaBI
NT       TTTTTCCCCTTCCTCGATCCCTATCCCATAGGTTAGGTACAGTGTTTGAA
SN       .......................A---------.................

NT       TCAATAGAGAACCTTTTCTTCTGTATGAATCGATATTATTCCATTCCAAA
SN       ..................................................
                                             BamHI
NT       TCCTTCCCGATACCTCCCAAGGAAAATCTCGAATTTGGATCCCAAATTGA (3')
SN       ..............................T................... (3')


NT  (5') CTATTATATTAGATTAGTATTAGTT---------------------AGTG
SN  (5') .........................TATATTAGATTAGTATTAGTT....
                                                  BanI
NT       ATCCCGACTTAGTGAGTC----------TGATGAATTGTTGGCACCAGTC (3')
SN       ..................CTTTCTTCCG.......C.............. (3')
```

C *trn*A intron

```
NT  (5') CGGAGAAGGGCAATGACTCATTCTTA------------------------
SN  (5') ..........................AAACCAGCGTTCTTAAGACCAAAG

NT       --------------------------------------------------
SN       AGTCGGGCGGAAGGGGGGGAAAGCCCTCCGTTCCTGGTTCTCCTGTAGTT
          BamHI
NT       -----------------------------GTTAGAATGGGATTCCAACTC (3')
SN       GGATCCTCCGGAAGCCACAAGAATCCTTA..................... (3')
```

FIGURE 3

*Bam*HI and *Pst*I-*Aat*II restriction fragment patterns and partial physical maps of the *N. tabacum* plastid transformants. (A) The gel electrophoretic separation of *Bam*HI-digested plastid DNA of wild-type *N. tabacum* (lane 1) and several plastid transformants (lanes 2-4) reveals different combinations of the *Solanum*-specific cut sites. Only fragments larger than 2.6 kb are shown. The transformant-specific fragments are marked by arrowheads. On the left a *Hin*dIII digest of lambda DNA is also shown. (B) The fragment patterns obtained with *Pst*I-*Aat*II double-digested plastid DNA in wild-type *N. tabacum* (lane 1) and several plastid transformants (lanes 2-4) demonstrate the identity of the copies of the invertedly repeated region in the plastid transformants for the spectinomycin resistance site. Only fragments larger than 5 kb are shown. The transformant-specific fragments are marked by arrowheads. On the left a *Hin*dIII digest of lambda DNA is also shown. (C) *Bam*HI and *Pst*I-*Aat*II cleavage site maps covering a part of the inverted repeat (filled bars) and the entire small single copy region, as they appear in the wild type (WT) and a *N. tabacum* plastid transformant (TR) possessing the full-length *S. nigrum* insert (the 7.8 kb area of integration is labeled by bordered lines). All the *Bam*HI, *Pst*I (P) and *Aat*II (A) cut sites of the region displayed are marked (short vertical lines). Numbers refer to the corresponding digestion fragments ordered by their size.

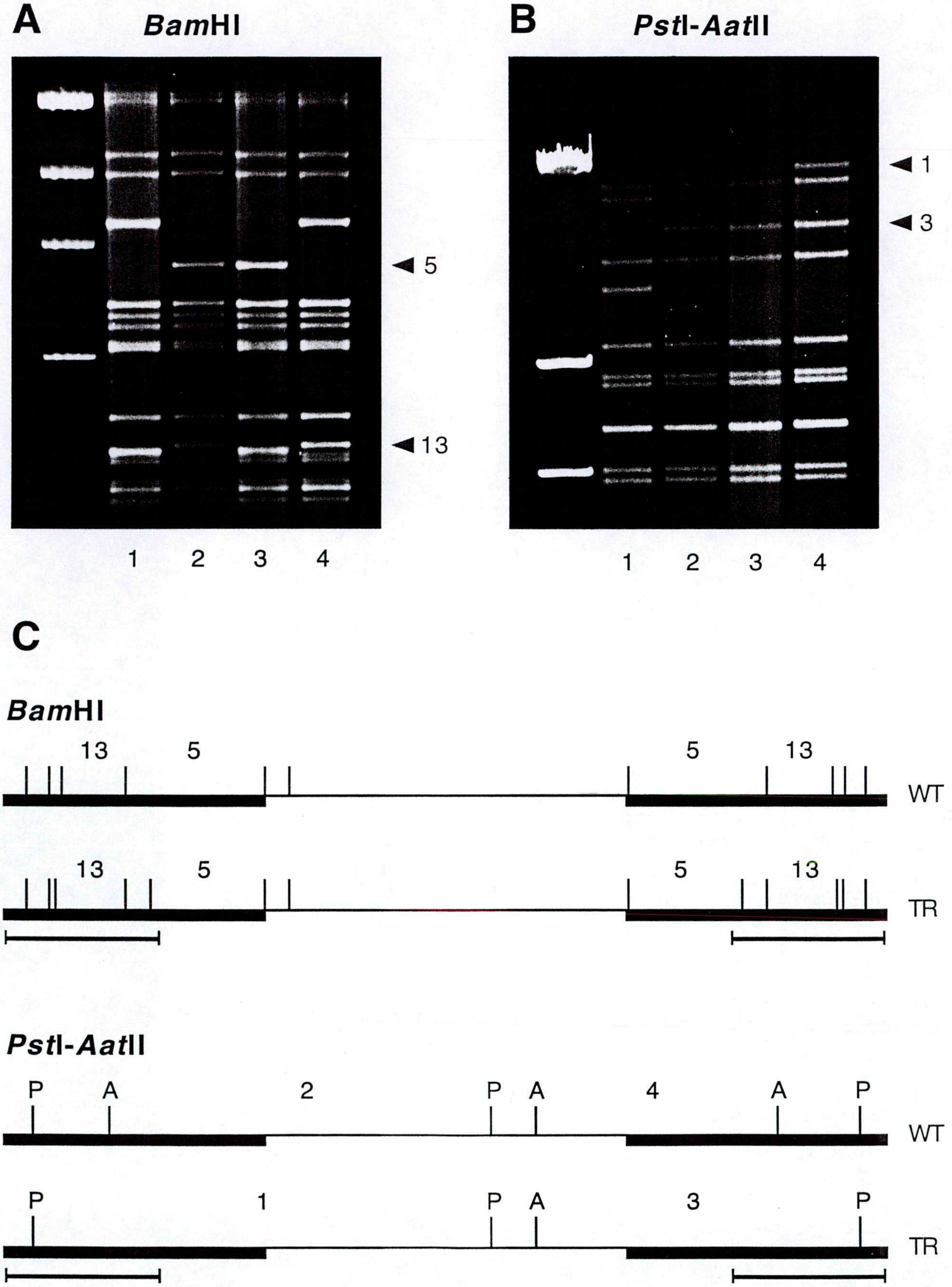

A
*Bam*HI
5
13
1 2 3 4
B
*Pst*I-*Aat*II
1
3
1 2 3 4
C
*Bam*HI
13 5 5 13
WT
13 5 5 13
TR
*Pst*I-*Aat*II
P A 2 P A 4 A P
WT
P 1 P A 3 P
TR

FIGURE 4

Schematic interpretation of the postulated recombination events following transformation of *N. tabacum* plastids with the pSSH1M plasmid. The 7.8 kb donor insert of the pSSH1M plasmid is shown at the top of the figure. Arrows mark the location of the *Solanum*-specific resistance and RFLP sites scored (a restriction endonuclease in brackets means the absence of a *Nicotiana*-specific cut site). Asterisks mark intron containing genes. Diagrams are presented for the origin of each class of plastid transformants. Each diagram is a single line with horizontal segments representing *Solanum*-type (upper, thick line) and *Nicotiana*-type (lower, dotted line) regions which are specified by a particular distribution of the donor genetic and physical markers (labeled by short vertical lines), and are separated by transverse segments (thin line) representing the regions of the postulated recombination events. The number of transformants belonging to a certain class is indicated beside each diagram. In this experiment only spectinomycin selection has been used.

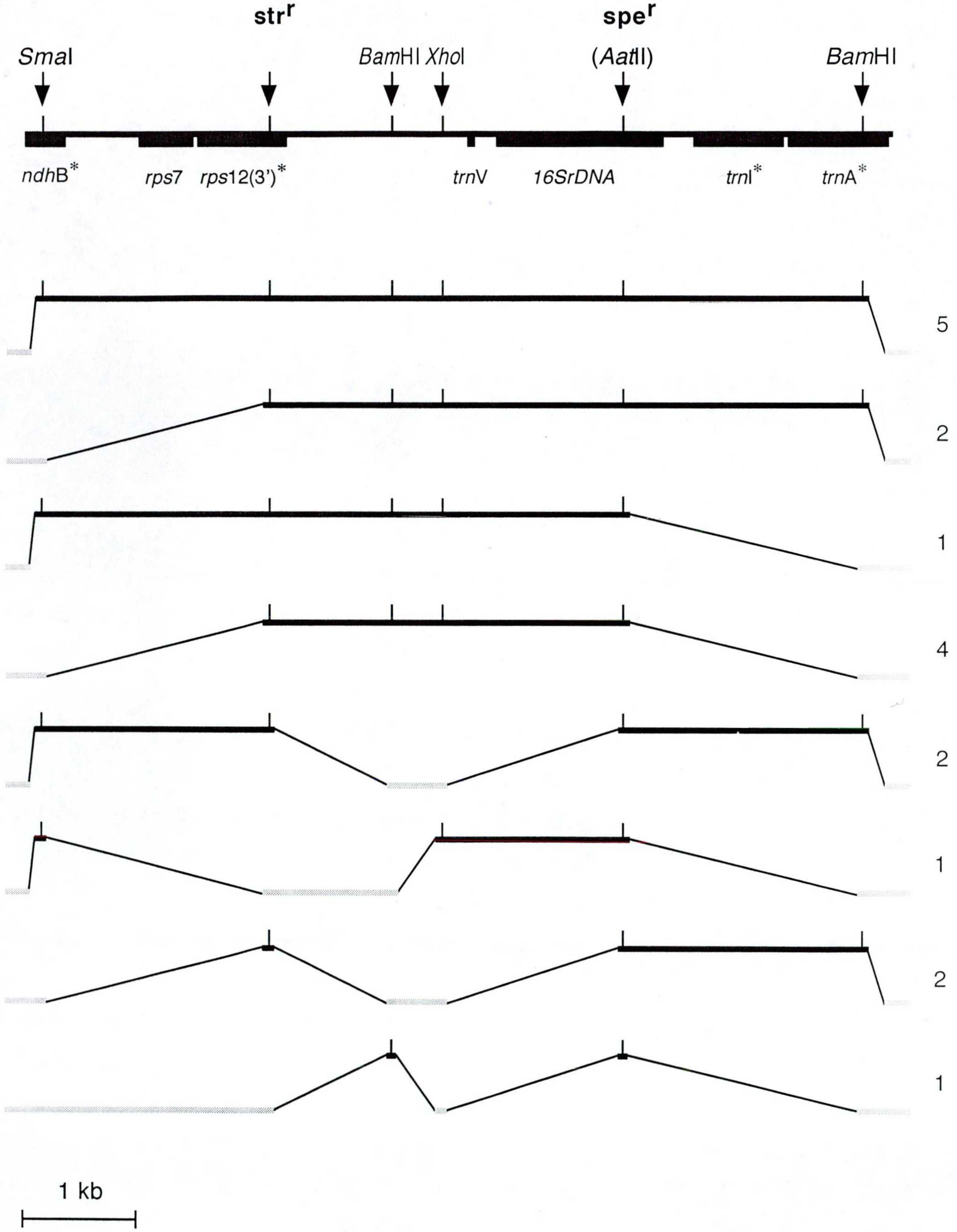

str^r
spe^r
SmaI
BamHI
XhoI
(AatII)
BamHI
ndhB*
rps7
rps12(3')*
trnV
16SrDNA
trnI*
trnA*
5
2
1
4
2
1
2
1
1 kb

FIGURE 5

Schematic interpretation of the distribution of postulated recombination events, on the basis of nucleotide sequence analysis of the *N. tabacum* plastid transformants, following transformation of *N. tabacum* plastids with the pSSH1 plasmid. The 7.8 kb donor insert of the pSSH1 plasmid is shown at the top of the figure. Arrows mark the location of the resistance mutations. Short vertical lines mark the regions of nucleotide sequence differences characteristic for the donor plastid DNA. The capital letters in brackets above them refer to the appropriate nucleotide sequence alignments shown in Figure 2. The resistance mutations (and the *Solanum*-specific nucleotide adjacent to the streptomycin resistance site) are described in the first section of the RESULTS. The *Xho*I site is described in the third section of the RESULTS. Asterisks mark intron containing genes. Diagrams are presented for the origin of each class of plastid transformants. Each diagram is a single line with horizontal segments representing *Solanum*-type (upper, thick line) and *Nicotiana*-type (lower, dotted line) regions which are specified by a particular distribution of the donor genetic and physical markers (labeled by short vertical lines), and are separated by transverse segments (thin line) representing the regions of the postulated recombination events. The number of transformants (spectinomycin-selected plus double-selected) belonging to a certain class is indicated beside each diagram.

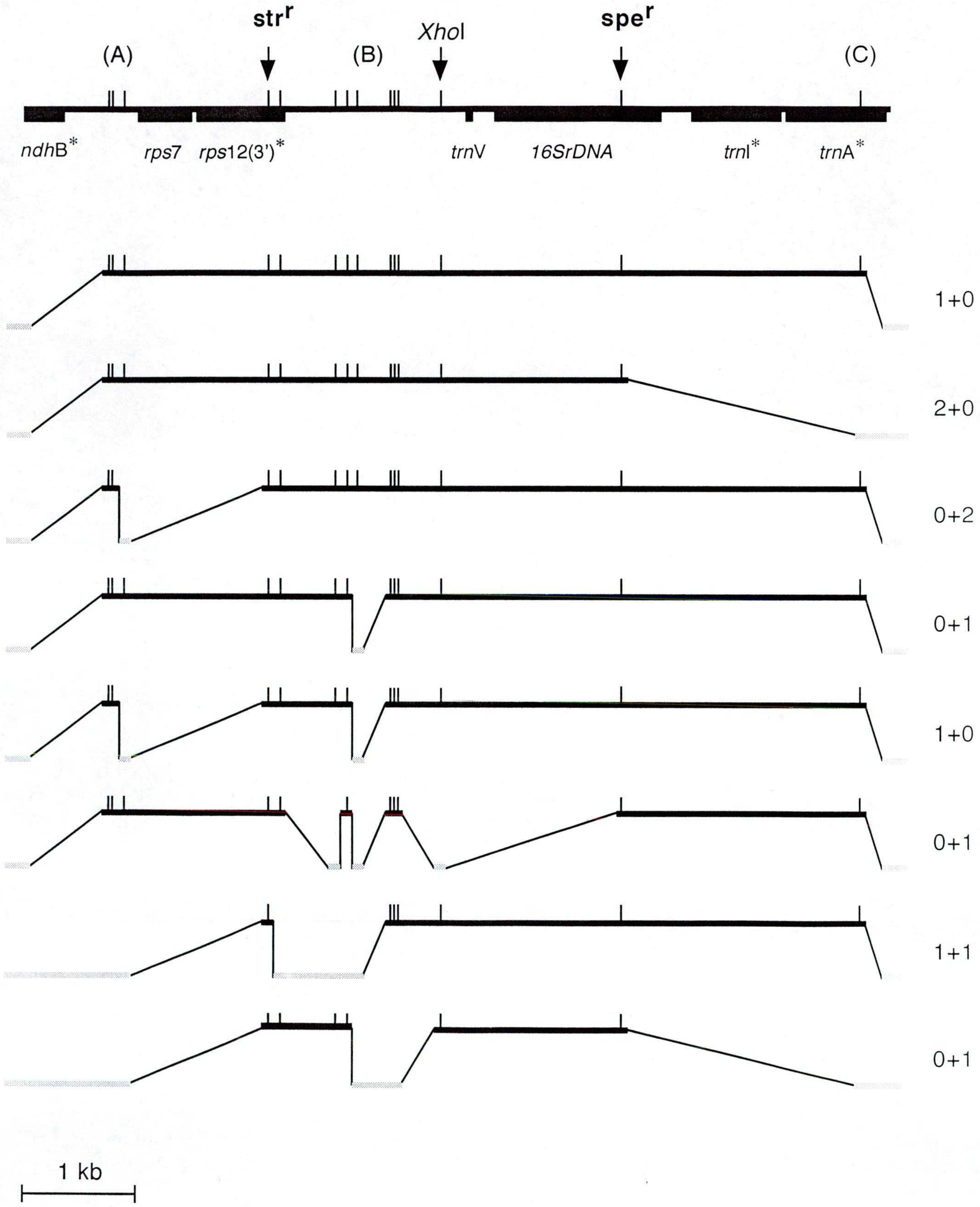
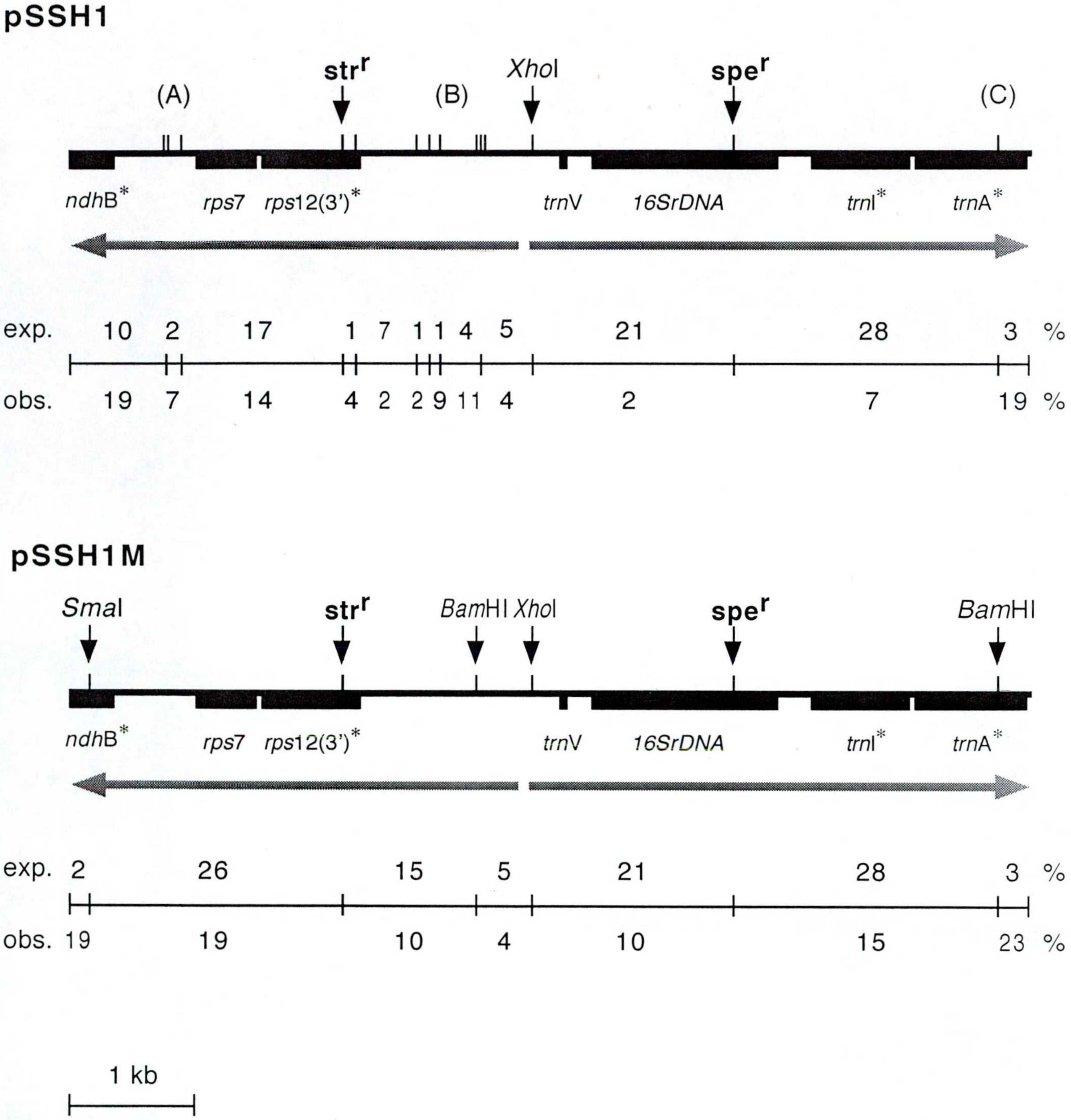

strʳ
speʳ
XhoI
(A)
(B)
(C)
ndhB*
rps7
rps12(3')*
trnV
16SrDNA
trnI*
trnA*
1+0
2+0
0+2
0+1
1+0
0+1
1+1
0+1
1 kb

FIGURE 6

Distribution of recombination frequencies in the individual sections of the targeted region in the *N. tabacum* plastid transformants. The 7.8 kb donor insert of the pSSH1 and the pSSH1M plasmids is shown at the top of the figures. Arrows mark the location of the *Solanum*-specific resistance and RFLP sites scored. Short vertical lines mark the regions of nucleotide sequence differences characteristic for the donor plastid DNA. The capital letters in brackets above them refer to the appropriate nucleotide sequence alignments showed in Figure 2. Asterisks mark intron containing genes. Dotted horizontal arrows mark the cotranscription units in the region (MATSUBAYASHI *et al.* 1987; HILDEBRAND *et al.* 1988; IRATNI *et al.* 1997). At the lower part of the figures the line is sectioned to show all intervals between the *Solanum*-specific sites. The minute intervals in which no recombination was detected are not shown. The expected and the observed recombination frequencies characteristic for the individual intervals are shown above and below the lines, respectively. The observed recombination frequency is expressed as the percent of the recombination events per interval. A 100% value represents the total number of recombined intervals of the entire region counted in all transformants. The expected recombination frequency is calculated as proportional with the length of the intervals considering 100% for the entire region between the cloning sites.


pSSH1
(A)
str^r
(B)
XhoI
spe^r
(C)
ndhB*
rps7
rps12(3')*
trnV
16SrDNA
trnI*
trnA*
exp. 10 2 17 1 7 1 1 4 5 21 28 3 %
obs. 19 7 14 4 2 2 9 11 4 2 7 19 %
pSSH1M
SmaI
str^r
BamHI
XhoI
spe^r
BamHI
ndhB*
rps7
rps12(3')*
trnV
16SrDNA
trnI*
trnA*
exp. 2 26 15 5 21 28 3 %
obs. 19 19 10 4 10 15 23 %
1 kb