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Cre-*lox* site-specific recombination between *Arabidopsis* and tobacco chromosomes

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Summary

To create hybrid chromosomes, we tested the Cre-*lox* system to mediate recombination between *Arabidopsis thaliana* and *Nicotiana tabacum* chromosomes. Protoplasts of the two plants were fused to allow site-specific recombination to join a promoter from tobacco to a hygromycin resistance coding-region from *Arabidopsis*. The expected recombination junction was detected in hygromycin-resistant calli. Analysis of one hybrid suspension cell line revealed the presence of markers corresponding to the north arm of *Arabidopsis* chromosome III, but not markers from other chromosome arms. However, these markers were not detected in regenerated plants. With a second hybrid cell line we obtained a single hygromycin-resistant progeny from approximately 18 000 self-fertilized seeds of one regenerated plant. Molecular analysis of this hybrid indicated that a small portion of the north arm of *Arabidopsis* chromosome V is present in the tobacco genome. However, neither the recombination junction nor *Arabidopsis* DNA was detected in tissue from the plant grown without selection or in the subsequent generation. Thus interspecies transfer of a chromosome arm between plant cells is possible, but maintenance of the hybrid chromosome in a plant is unlikely. The feasibility of site-specific recombination between genomes of different species offers new possibilities for engineering hybrid chromosomes that may be maintained in cell culture.

Keywords: protoplast fusion, Cre-*lox* recombination, recombinase-mediated translocation, *Arabidopsis*, *Nicotiana*.

Introduction

Pioneering fusion experiments between rodent and human cells have set the precedence of using somatic cell hybridization to clone chromosomes (Migeon and Miller, 1968; Weiss and Green, 1967). After rodent and human cells are fused, human chromosomes are rapidly lost in the dividing hybrid cells. This produces rodent cell lines that harbor one or a few human chromosomes or chromosome segments. In contrast, the fusion of protoplasts from related plant species often results in hybrid plants that contain significant amounts of both parental genomes (Forsberg *et al.*, 1994; Gleba and Hoffmann, 1978; Piastuch and Bates, 1990; Wolters *et al.*, 1993). The fusion of plant protoplasts from phylogenetically distant genomes usually leads to hybrid plants with an asymmetric combination of the two genomes. Parts of one or both genomes are lost in a largely unpredictable manner (Hinnisdaels *et al.*, 1988). In rare instances, a fraction of 'donor genome' incorporates into the 'recipient genome' (Hinnisdaels *et al.*, 1991; Hinnisdaels *et al.*, 1992). As these events are random, it is impossible to predict the composition and location of donor DNA within the recipient genome.

In this study, we tested the feasibility of using site-specific recombination to move a large segment of *Arabidopsis thaliana* DNA to a specific location within a *Nicotiana tabacum* genome. Site-specific recombination systems can be used to rearrange transgenes and chromosomes in eukaryotic cells. Examples include the site-specific recombination between homologous chromosomes in *Drosophila* (Golic, 1991) and between non-homologous chromosomes in yeast (Matsuzaki *et al.*, 1990). Previously, we used the Cre-*lox* system to generate

chromosome translocations in tobacco (Qin *et al.*, 1994). Similar achievements have been shown in mouse embryonic stem cells (Smith *et al.*, 1995; Van Deursen *et al.*, 1995), and in *Arabidopsis* (Chou *et al.*, 1999). Collectively, these results demonstrate the ability of recombinase systems to translocate DNA between non-homologous chromosomes.

To advance beyond directing the recombination of chromosomes within a species, we tested the ability of site-specific recombination to mediate chromosome translocation between plants from different genera. Here we describe the site-specific recombination between tobacco and *Arabidopsis* chromosomes. The results of these experiments provide evidence of site-specific intergeneric chromosomal translocation, a technology that may lead to the controlled, stable generation of hybrid chromosomes.

Results

Experimental design

We adapted a strategy used to generate interchromosomal recombination in tobacco (Qin *et al.*, 1994). *Arabidopsis* was transformed with p*loxP-hpt* containing the wild-type 34 bp *lox* sequence that also serves as an untranslated leader sequence preceding *hpt*, the coding region of the hygromycin phosphotransferase gene. Due to the lack of a promoter, hygromycin resistance is not conferred. The tobacco lines harboring *35S-lox-cre* have been described previously (Albert *et al.*, 1995; Qin *et al.*, 1994). In these plants the *35S* RNA promoter of cauliflower mosaic virus (*35S*) promotes transcription of *cre*. When tobacco protoplasts harboring *35S-lox-cre* (nt-c lines) are fused with *Arabidopsis* protoplasts harboring *lox-hpt* (at-h lines), the hybrid will have both transgenic chromosomes. Cre-mediated recombination between the *lox* sites causes reciprocal translocation of the chromosome arms (Figure 1). This translocation should produce two new transgenes: (1) *35S-lox-hpt*, which confers hygromycin resistance; and (2) *lox-cre*, which abolishes *cre* expression due to lack of a promoter. This recombination event simultaneously produces a selectable hygromycin-resistant (Hyg^R) phenotype and terminates *cre* transcription.

We anticipated that the resulting hybrid would eliminate either the tobacco or the *Arabidopsis* genome. Culture conditions were set to favor maintaining the tobacco genome because of two considerations: (1) plant regeneration from protoplasts is easier with tobacco than with *Arabidopsis*; and (2) as *N. tabacum* is an amphidiploid, an aneuploid state would be expected to have a less deleterious effect on phenotype or fertility (Papp *et al.*, 1996). An aneuploid state is anticipated, as the transfer of an *Arabidopsis* chromosome arm to a tobacco centromere

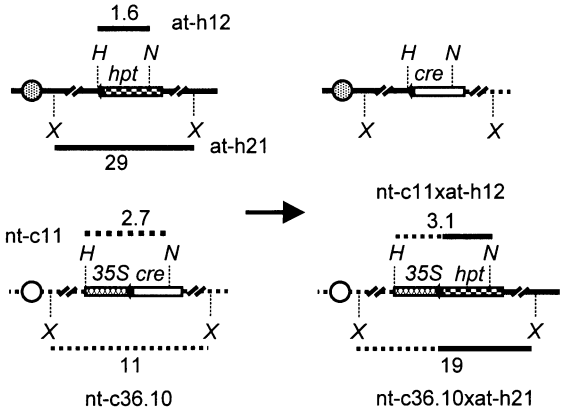

Figure 1. Experimental design for reciprocal chromosome arm translocation.
Parental transgenic chromosomes of *Arabidopsis* and tobacco (upper and lower left, respectively) and chromosomes after protoplast fusion and Cre-*lox*-mediated reciprocal translocation (right). Depicted are centromeres (circles); chromosomal DNA (lines); transgenes (boxes); and *lox*-sites (arrowheads). Shaded circles and solid lines represent *Arabidopsis*, open circles and dotted lines represent tobacco. As a reference for Figure 2 the expected fragment sizes, in kb, resulting from cleavage with *Nsi*I (*N*) + *Hin*dIII (*H*), and observed fragments sizes, in kb, resulting from cleavage with *Xho*I (*X*) are shown.

is concomitant with the transfer of a tobacco chromosome arm to an *Arabidopsis* centromere. If only the recipient-type centromere is maintained, a chromosome arm from the recipient genome would be lost in the exchange.

Two conditions were assumed to be necessary for maintaining a hybrid chromosome. (1) The *lox* sites must be in the same orientation with respect to the centromere, permitting formation of two unicentric chromosomes. If the *lox*-sites are in the opposite orientation, a dicentric and an acentric chromosome will arise and will be unstable. (2) The *35S-lox-hpt* transgene must transcribe towards the telomere. This permits the formation of a hybrid chromosome that contains a tobacco centromere. If the *35S-lox-hpt* transgene was transcribed towards the centromere, the hybrid chromosome would harbor an *Arabidopsis* centromere. The functionality of an *Arabidopsis* centromere in tobacco is not known. As there is a 50% chance that a *35S-lox-cre* transgene is oriented towards the telomere, five different tobacco lines were used for the fusion experiments. This gives a 97% chance of at least one being in the correct orientation.

Recombination products

Protoplasts of six *Arabidopsis* (at-h) and five tobacco (nt-c) lines were fused in 28 of 30 possible pairwise combinations. For most combinations, several independent fusions were made. Hyg^R calli were produced from 10 of the 28 combinations (Table 1). A *35S-lox-hpt* junction, indicative of Cre-mediated recombination, was detected by

PCR from these 10 fusion combinations. However, the PCR detection rate was low among the tested calli. The PCR junction was found in approximately 20% of the Hyg^R calli tested. It is possible that some of the observed Hyg^R calli may be false positives. It is also possible that the low detection rate is related to a limitation of the assay on calli that were quite small, grew very slowly, and were probably chimeric for the recombination event.

After 4 months some of the Hyg^R calli developed green leaflets; however shoot development was rare. Shoots that formed roots on hygromycin-containing medium were recovered from 57 calli: five from the fusion of nt-c36.4 and at-h12 (nt-c36.4 × at-h12); 18 from nt-c11 × at-h12; two from nt-c5 × at-h16; and 32 from nt-c36.10 × at-h21 (Table 1). The Hyg^R phenotype was not stable in these shoots. Detection of the *35S-lox-hpt* PCR product decreased in leaves further away from the base of the shoot (Figure 2a). When these plantlets reached approximately 10 cm in height the *35S-lox-hpt* PCR product was no longer detected, and the leaves did not reshoot in the presence of hygromycin. Hyg^R shoots did not form in control experiments using only nt-c protoplasts.

Molecular analysis of a cell line

As plants failed to maintain the Hyg^R trait, we attempted to propagate Hyg^R calli as suspension cultures. Hyg^R calli from each of the four fusions that gave rise to shoots were placed in hygromycin-containing liquid medium. All

Table 1. Hyg^R lines from fusion between *Arabidopsis* (at-h) and tobacco (nt-c) lines

	at-h12	at-h14	at-h16	at-h17	at-h19	at-h21
nt-c36.4	+,s(5)	–	+	–	–	+
nt-c36.10	+	–	–	–	–	+,s(32),p
nt-c3	–	–	–	–	–	–
nt-c5	–	–	+,s(2)	nd	nd	–
nt-c11	+,s(18),c	–	+	–	+	+

+, Hyg^R calluses recovered, PCR detection of *35S–hpt* junction; –, Hyg^R calluses not recovered; nd, no data for the fusion combination; s, shoot (number) regenerated from Hyg^R calli; c, cell suspension line maintained; p, one Hyg^R progeny from approximately 18 000 self-fertilized seeds.

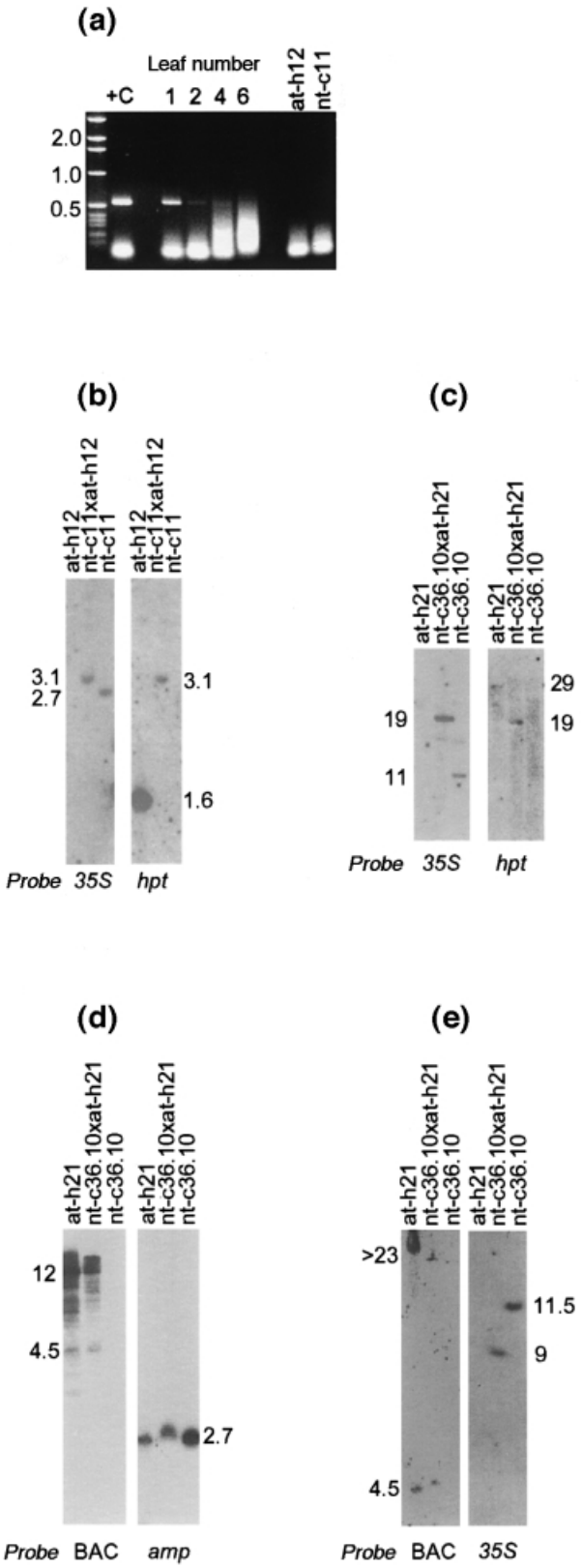

Figure 2. Molecular analysis of fusion products. Size markers are in kb.
(a) PCR analysis of leaves from a shoot regenerated from nt-c11 × at-h12. Leaf number 1 was at the bottom of the plant.
(b) Southern blot of DNA from nt-c11 × at-h12 suspension cell line treated with *Nsi*I and *Hin*dIII and probed with *35S* or *hpt*. Control lanes contain DNA from at-h12 and nt-c11.
(c) Southern blot of DNA from nt-c36.10 × at-h21 F_1 plant treated with *Xho*I and probed with *35S* or *hpt*. Control lanes contain DNA from at-h21 and nt-c36.10.
(d) Southern blot of DNA from nt-c36.10 × at-h21 F_1 plant treated with *Eco*R1 and probed with BAC (TAMU10O8) or *amp* DNA. Control lanes contain DNA from at-h21 and nt-c36.10.
(e) Southern blot of DNA from nt-c36.10 × at-h21 F_1 plant treated with *Eco*N1 and probed with BAC (TAMU10O8) or *35S* DNA. Control lanes contain DNA from at-h21 and nt-c36.10.

cultures grew slowly, and after several months of serial passages only one of the four lines survived. PCR analysis of this suspension line, from nt-c11 × at-h12, indicated that the expected 0.58 kb *35S-lox-hpt* junction was maintained. The parental junctions, *35S-lox-cre* and *lox-hpt*, and the second product junction, *lox-cre,* were not detected.

Genomic DNA from the nt-c11 × at-h12 suspension line was cleaved with both *Nsi*I and *Hin*dIII (Figures 1, 2b). A *35S* probe hybridized to a 3.1 kb band in nt-c11 × at-h12 and a 2.7 kb band in nt-c11, but not to at-h12 DNA. An *hpt* probe hybridized to a 3.1 kb band in nt-c11 × at-h12 and a 1.6 kb band in at-h12 DNA, but not to nt-c11 DNA. These results indicate that an intact *35S-lox-hpt* junction had formed in the nt-c11 × at-h12 suspension line.

When the DNA was cleaved with *Xho*I, however, Southern blots revealed a likely rearrangement of DNA outside the *35S-lox-hpt* fragment. *Xho*I is expected to cut outside the transgene (Figure 1). A *35S* probe detected a 16 kb band in nt-c11 DNA, and an *hpt* probe detected a 19 kb band in at-h12 DNA (data not shown). Based on the physical maps and reconstruction controls, we had expected to detect a 12 kb band in nt-c11 × at-h12 DNA with either the *35S* or *hpt* probes. However, a 5 kb band was found instead (data not shown). Although this is consistent with *35S* and *hpt* being on the same DNA fragment, the 5 kb fragment size is considerably shorter. We suspect that the recombination locus had suffered a deletion internal to the *Xho*I sites, or had acquired a new *Xho*I site near the *35S-lox-hpt* linkage.

Molecular analysis of F_1 *plants*

The behavior of the regenerated plantlets suggested that the hybrid chromosomes were not maintained. To investigate whether germinal cells might harbor and transmit the chimeric chromosome, flowers from 32 regenerated plants were self-fertilized or crossed to wild-type tobacco as the male or female donor. From the approximately 1.8 million F_1 seeds germinated in the presence of hygromycin, a single Hyg^R plant was recovered. This Hyg^R F_1 progeny came from approximately 18 000 self-fertilized seeds from a single nt-c36.10 × at-h21 plant. PCR analysis confirmed the presence of the expected 0.58 kb *35S-lox-hpt* junction. The parental junctions, the other product junction, and markers of the *Arabidopsis* chromosomes were not detected (data not shown).

Genomic DNA from the nt-c36.10 × at-h21 derived F_1 plant grown in the presence of hygromycin was examined by Southern hybridization. When cleaved with *Xho*I, which cuts outside the transgenes (Figure 1), the *35S* probe hybridized to a 11 kb band in nt-c36.10 DNA, and the *hpt* probe hybridized to an approximately 29 kb band in at-h21 DNA (Figure 2c). With nt-c36.10 × at-h21 DNA, both probes detected an approximately 19 kb *Xho*I band. This is consistent with *35S* and *hpt* being on the same DNA fragment. As demonstrated previously (Qin *et al.*, 1994), Southern analysis of nt-c36.10 revealed a faint second band that could be interpreted as a second but truncated copy of the transgene. This faint band was indeed found when hybridized to the *35S* probe, and its size remained unchanged in the nt-c36.10 × at-h21 lane.

As with the parent plant, the Hyg^R phenotype in the nt-c36.10 × at-h21-derived F_1 progeny was unstable. In the presence of hygromycin, chlorotic lesions appeared on the leaves (Figure 3). After 2 months without selection, the Hyg^R phenotype was lost and the recombination junction could no longer be detected. The Hyg^R phenotype was not found in approximately 50 000 F_2 seeds, and the recombination junction was not detected among 50 F_2 seedlings examined by PCR.

Map location of at-h12 and at-h21 transgenes

A 3.0 kb fragment of *Arabidopsis* DNA linked to *lox-hpt* in at-h12 was cloned by iPCR. Comparison of 450 bp of sequence from the rescued DNA failed to reveal a match to entries in GenBank. The 3.0 kb fragment hybridized to a 17 kb *Eco*NI band in *Arabidopsis* wild-type DNA, and to a 24 kb band in at-h12 DNA (data not shown). A 24 kb *Eco*NI band in at-h12 DNA was also detected by an *hpt* probe (data not shown). This indicates that the rescued DNA is linked to the transgene.

The DNA rescued from at-h12 did not hybridize with DNA from the CIC *Arabidopsis* YAC library. The at-h12 fragment hybridized to a *Sal*I RFLP in recombinant inbred lines. Mapmaker analysis of the RFLP segregation indicated that the DNA fragment rescued from at-h12 mapped to chromosome III between *GAPA* and *GL1* (41–45 cM). The centromere location on chromosome III is tentatively placed between *GL1* and *NIT1* (45–55 cM) (Copenhaver *et al.*, 1998). Thus the transgene in at-h12 is north of the centromere.

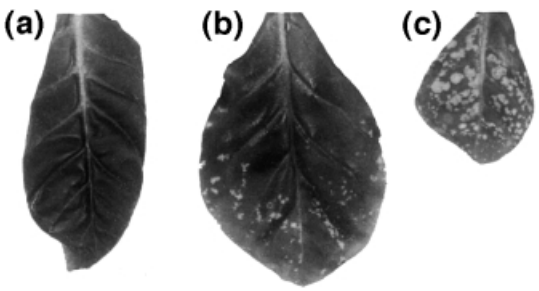

Figure 3. Lesions on leaves of the Hyg^R nt-c36.10 × at-h21 F_1 plant (approximately 6 cm high).
(a) Mid-leaf of plant grown without hygromycin; (b) mid- and (c) lower leaves of a plant (vegetative clone) grown with hygromycin.

In at-h21, a 2.0 kb fragment was isolated by iPCR, and approximately 350 bp were sequenced. The sequenced DNA failed to show a match to GenBank entries. The 2.0 kb fragment hybridized to a 15 kb *Eco*NI band in wild-type *Arabidopsis* DNA, and to a 19 kb *Eco*NI band in at-h21 DNA (data not shown). An *hpt* probe also hybridized to a 19 kb *Eco*NI band in at-h21 DNA (data not shown). This indicates that the DNA rescued from at-h21 is linked to the *lox-hpt* transgene. The rescued DNA hybridized to YAC clones CIC2B9 and CIC4D2 (data not shown), both located on chromosome V at approximately 0.5 cM (Schmidt *et al.*, 1997). These data indicate that the at-h21 transgene is at the northern tip of chromosome V.

Arabidopsis DNA in the nt-c11 × at-h12 cell line

The slow-growing nt-c11 × at-h12 suspension-cell line provided limited material for analysis. Therefore a PCR-based approach was used to monitor the extent of *Arabidopsis* DNA present in this cell line. In DNA isolated from callus a month after fusion, PCR products corresponding to chromosome locations scattered throughout the *Arabidopsis* genome were detected. As suspension cultures were established, a subset of these PCR products remained detectable.

Further analysis was conducted on a 20-month-old suspension culture. The chromosome III markers *GAPC*, C6 and *GAPA* could be amplified (Figure 4b), while the markers *ADH* of chromosome I, m429 of chromosome II, *BGL1* of chromosome III, *GA1* of chromosome IV, and *ASAI* of chromosome V were all undetectable (data not shown). The amplified markers reside north of the *lox-hpt* map position. A PCR product was not produced with primers specific for markers south of the *lox-hpt* map position (*ALS*, *AFC1* and *BGL1*). The simplest interpretation is that the DNA north of the *lox-hpt* transgene is selectively maintained in this cell line. However, YuP4G12RE, AtDMC1 and m255, all located on the north arm of chromosome III, were not found (Figure 4b). Therefore it appears that not all of the DNA north of the *lox-hpt* transgene was maintained. Other markers on the north arm of chromosome III were tested (myb4, S6, B4, g4711, AB13 and *GL1*). However, these markers were uninformative as the primers amplified similarly sized bands from tobacco DNA (data not shown). As the cell line was cultured over an extended period, it is possible that spontaneous chromosome rearrangements had occurred. A deletion of the telomere and of the DNA between C6 and *GAPA* would be consistent with the data.

The PCR analysis was followed by DNA hybridization experiments. A *GAPC* probe hybridized to a single 3.8 kb *Eco*NI and a single 7.0 kb *Xho*I band in *Arabidopsis* DNA, but did not hybridize to tobacco DNA (data not shown). Therefore this probe represents a single- or low-copy *Arabidopsis* gene lacking extensive homology with tobacco DNA. In dot-blot hybridization, the *GAPC* probe hybridized to DNA from at-h12, at-h12 mixed with nt-c11, and nt-c11 × at-h12, but not to DNA from nt-c11 alone (Figure 4c). DNA loading was sufficient as tobacco DNA hybridized to all samples (Figure 4c). Similar results were obtained with C6 and with *GAPA* probes (data not shown). YuP4G12RE, AtDMC1 and m255 probes did not hybridize to nt-c11 × at-h12 DNA (data not shown). Many other *Arabidopsis* probes significantly cross-hybridized with tobacco DNA, and tobacco DNA readily hybridized to at-h12 DNA. This illustrates the general problem of DNA cross-hybridization between plant species, even those from different genera.

The conclusion is that a fragmented portion of the north arm of *Arabidopsis* chromosome III is linked to the tobacco

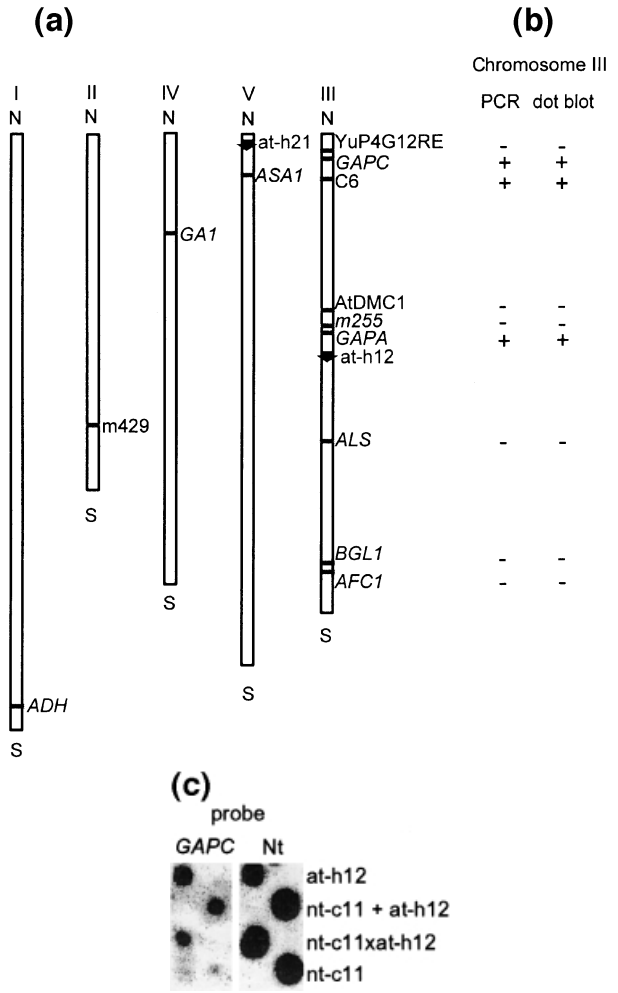

Figure 4. *Arabidopsis* chromosome III markers present in the nt-c11 × at-h12 suspension cell line.
(a) Chromosome locations of relevant markers and *lox-hpt* insertions, with deduced *lox* site orientations as shown.
(b) Detection (+) or non-detection (–) of specific chromosome markers by PCR and dot-blot analysis.
(c) Representative dot-blot analysis probed with *GAPC* or total tobacco DNA (Nt).

genome and is maintained in a tobacco cell line. This segment encompasses the DNA from 4.9 cM to at least the 11 cM marker and possibly to 29 cM.

Arabidopsis DNA in the nt-c36.10 × at-h21 F_1 plant

Arabidopsis DNA flanking the *lox-hpt* transgene in at-h21 hybridized to YAC clones CIC2B9 and CIC4D2 (data not shown). This indicates that the transgene lies 300–750 kb from the north end of chromosome V. The YAC clone yUP8A9 lies completely north of this region. yUP8A9 hybridized to the BAC clone TAMU10O8. Vector-free *Arabidopsis* DNA was isolated from TAMU10O8 after cleavage with *Not*I and fractionation in a CHEF gel. This DNA was used to probe *Eco*RI- or *Eco*NI-cleaved DNA. With *Eco*RI-digested DNA, the vector-free TAMU10O8 probe detected a number of bands in at-h21 and nt-c36.10 × at-h21 (Figure 2d). Most noticable are the common approximately 12 and 4.5 kb bands. Hybridization was not detected with nt-c36.10 DNA. This was not due to insufficient sample loading, as when the same blot was reprobed with a plasmid encoded ampicillin resistance gene fragment (*amp*), the expected 2.7 kb fragment was found in all DNA. With *Eco*NI-cleaved DNA, the vector-free TAMU10O8 probe detected approximately 4.5 and >23 kb bands in at-h21 and nt-c36.10 × at-h21, but not in nt-c36.10 (Figure 2e). A *35S* probe hybridized to an 11.5 kb band in the nt-c36.10 and a 9 kb band in the nt-c36.10 × at-h21 DNA. These results are consistent with the interpretation that *Arabidopsis* DNA corresponding to this 300 kb region is retained in the nt-c36.10 × at-h21 plant, at least when the plant is maintained in the presence of selection.

Discussion

Intergenera chromosome recombination in cultured cells

The possibility of Cre-*lox*-mediated site-specific recombination between chromosomes of different organisms was tested with tobacco and *Arabidopsis*. We hypothesized that *Arabidopsis* chromosomes would eventually be eliminated, and that hybrid chromosomes with an *Arabidopsis* centromere would not be maintained. The centromeres of the yeast *Kluyveromyces lactis* do not function in the yeast *Saccharomyces cerevisiae*, and vice versa (Heus *et al.*, 1994). The experimental design therefore favored selection of a tobacco cell line with an *Arabidopsis* chromosome arm attached to a tobacco centromere. If the *lox*-site orientation is entirely random, only 25% of the recombination events are expected to yield a chromosome containing both the *35S-lox-hpt* marker and a tobacco centromere. Hyg^R calli were recovered in 33% of the fusion combinations. For every combination that yielded Hyg^R calli, we obtained PCR confirmation of the expected *35S-lox-hpt* junction, although on average this PCR junction was detected in only one of every five Hyg^R calli tested. This low PCR detection rate is probably due to the low sensitivity of the assay on limited material that is probably chimeric. The slightly higher 33% frequency is not significant; as it could be due to random chance, *lox* sites being unequally oriented, hybrid chromosomes arising from breakage of dicentric chromosomes, or maintenance of chromosomes with *Arabidopsis* centromeres long enough to confer hygromycin resistance to the calli.

The *lox-hpt* transgene in at-h12 was mapped to the middle of chromosome III near the centromere. Therefore Cre-mediated recombination is expected to transfer an arm of *Arabidopsis* chromosome III to a tobacco chromosome. Indeed, after the hybrid cell line had been in culture for more than a year, PCR and dot-blot hybridization detected three markers, all located on the north arm of chromosome III. Markers of other chromosome arms, which were initially detected, were not retained. The data are consistent with an interpretation that the hybrid cell line was initially composed of both genomes, and the *Arabidopsis* chromosomes were subsequently eliminated. It is puzzling that not all of the markers located at the northern section of chromosome III were found. A probable explanation is that the chromosome III arm has suffered deletions corresponding to the region of these markers. The cell line had been in continuous culture for over a year and chromosome rearrangements are known to occur in cultured cells (Wang and Marshall, 1996). It is possible that the tobacco line may be actively eliminating the non-tobacco DNA. This might have a variety of causes, including the possibility of undesirable effects of foreign gene expression and a non-optimal chromosome size. The molecular analysis of *Arabidopsis* DNA in the hybrid line was confined to the few PCR markers and probes empirically determined to lack cross-reaction with tobacco DNA. Due to this limitation, we cannot exclude the possibility that other regions of the genome are maintained in the hybrid line.

Instability of hybrid chromosomes in regenerated plants

Plantlets regenerated from four of the 10 protoplast combinations that yielded Hyg^R calli were similar. (1) They looked like tobacco; this is not surprising as culture conditions were set for tobacco regeneration. (2) The *35S-lox-hpt* junction was detected in newly emerging shoots, but not in subsequently formed leaves. This indicated that the hybrid chromosomes are not tolerated, and may be due to telomere differences or other unknown factors associated with somatic incompatibility. (3) 35 out of 36 plants did not transmit the Hyg^R trait to progeny. In the one plant that transmitted the Hyg^R trait, it was only found in a

single Hyg^R F_1 progeny out of approximately 18 000 seeds placed on selective medium. Thus a germ line cell maintaining the *35S-lox-hpt* junction long enough to transmit it to progeny is an extremely rare occurrence.

A notable feature about this Hyg^R F_1 progeny is that the *lox-hpt* transgene in the at-h21 donor line resides on the northern tip of chromosome V. Thus Cre-*lox*-mediated recombination should transfer only a small segment of *Arabidopsis* DNA to a tobacco chromosome. A BAC-derived DNA probe confirmed that a portion of the north arm of chromosome V was present in this hybrid plant. The fact that, at most, approximately 750 kb of *Arabidopsis* DNA is in the hybrid may have facilitated recovery of this plant. The recovery of highly asymmetric hybrids indicates that small amounts of foreign DNA are tolerated. Perhaps fewer foreign genes incompatible to organism development are expressed. This interpretation is consistent with the observation that the recombinant junction can still be amplified from second and third leaves of this hybrid plant, as long as hygromycin is present in the growth medium. Nonetheless, the development of chlorotic lesions in these leaves indicates development of sensitivity to hygromycin, and is consistent with the interpretation that the hybrid chromosome is being rejected.

Future prospects

These experiments demonstrate the ability of Cre-*lox*-mediated recombination to produce precise and predictable translocations between the chromosomes of different genera. The recombination reaction appears to occur efficiently, but hybrid chromosomes are not readily maintained in whole plants. This restriction appears to be less stringent with undifferentiated cells. However, the extent of DNA rearrangements that often result in cell lines is a serious concern, especially with cell lines that must be maintained through serial passages. If plant cell lines could be frozen, as is the case with rodent cell lines, this may minimize DNA rearrangements. The construction of hybrid chromosomes through site-specific recombination could result in novel uses in genome research. This technology could lead to plant or animal cell lines that harbor chromosome arms of other organisms, similarly to rodent cell lines that maintain human chromosome fragments (Migeon and Miller, 1968; Weiss and Green, 1967).

Experimental procedures

Biological materials

The constructs p*35S-loxP-cre* and p*loxP-hpt* have been described (Albert *et al.*, 1995; Qin *et al.*, 1994). The relevant segment of each construct is shown in Figure 1 (additional details are available on request). The tobacco lines of Qin *et al.* (1994): 36.4, 36.10; and of Albert *et al.* (1995): nt35S-loxP-cre.3, nt35S-loxP-cre.5, are referred to in this paper as nt-c36.4, nt-c36.10, nt-c3 and nt-c5, respectively. The line nt-c11 is new. Each of the five tobacco lines has a single transgenic locus; and four of the lines harbor a single copy transgene. In nt-c36.10, DNA hybridization analysis revealed a faint second band that is interpreted as a second but truncated copy of the transgene (Qin *et al.*, 1994).

The *Arabidopsis* at-h plants are derived from *Agrobacterium*-mediated transformation (Valvekens *et al.*, 1988) of p*loxP-hpt* subcloned into pBIN19 (Bevan, 1984). Based on segregation of the kanamycin resistance marker among the progeny of the primary transformant, all six *Arabidopsis* lines harbor a single transgenic locus. Southern analysis revealed that at-h12, at-h16 and at-h21 harbor a single copy transgene, while at-h14, at-h17 and at-h19 contain two, three and four copies, respectively. The segregation pattern of at-h21 in a later generation was unusual. It behaved as though it had two kanamycin-resistant loci; the kanamycin-resistant: kanamycin-sensitive progeny from a self cross or from an outcross segregated 15 : 1, or 3 : 1, respectively. However, the Southern analysis based on *hpt* and *npt* probes of three different endonuclease cleavages (*Bcl*1, *Xho*1 or *Eco*N1) consistently indicated a single transgenic locus. This apparent anomaly can best be attributed to gene conversion or concerted gene evolution. The transgene is located near the telomere. Conversion of one of the four sister chromosomes derived from a homologue pair would yield this segregation pattern.

Protoplast fusion

Protoplasts were isolated from leaf tissue as described previously (Bancroft *et al.*, 1992; Damm and Willmitzer, 1988; Morgan and Ow, 1994). Fusion experiments were primarily conducted with polyethylene glycol (PEG). In some experiments, fluorescein diacetate-loaded albino callus *Arabidopsis* protoplasts were fused with tobacco mesophyll protoplasts. This allowed visualization of the fused cells as chloroplast-containing cells that fluoresce. Typically, 2×10^8 *Arabidopsis* protoplasts were fused with 2–4×10^8 tobacco protoplasts. For PEG-mediated fusion, PEG 3350 pH 5.8 was added to 25% total volume. The PEG-treated protoplasts were then diluted with W5 and embedded in 1% agarose (SeaPlaque). Calli were generated in K3A with 20 μg ml^{-1} hygromycin. Standard tobacco regeneration (Morgan and Ow, 1994) and cell culture (Smith, 1986) protocols were followed. Regeneration protocols specific for *Arabidopsis* were attempted (Damm and Willmitzer, 1988; Masson and Paszkowski, 1992), without success.

Mapping

Plasmid rescue and inverse PCR (iPCR) were used to retrieve the *Arabidopsis* DNA flanking the transgene. To confirm physical linkage of the rescued DNA to the transgene, rescued DNA was used as a probe in Southern analysis of DNA from wild-type and transgenic *Arabidopsis*. To map the transgene location, the rescued DNA was used as a probe in Southern analysis of an *Arabidopsis* CIC YAC library (Schmidt *et al.*, 1997). Map position was assigned if the hybridizing YAC clones were anchored to an *Arabidopsis* chromosome. Rescued DNAs that could not be mapped with the CIC YAC library were mapped through analysis of RFLP segregation in recombinant-inbred lines (Reiter *et al.*, 1992).

Molecular analysis

Standard conditions were used for PCR analysis and DNA hybridization. PCR detection of the parental and recombinant junctions was performed according to Albert *et al*. (1995) and Qin *et al*. (1994). Primers used for the *Arabidopsis* CAPS markers (http://genome-http://www.stanford.edu/Arabidopsis) have been described. CHEF gels were run according to recommendations by Bio-Rad (Hercules, CA, USA).

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References

Albert, H., Dale, E.C., Lee, E. and Ow, D.W. (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. *Plant J.* **7**, 649–659.

Bancroft, I., Westphal, L., Schmidt, R. and Dean, C. (1992) PFGE-resolved RFLP analysis and long range restriction mapping of the DNA of *Arabidopsis thaliana* using whole YAC clones as probes. *Nucl. Acids Res.* **20**, 6201–6207.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 8711–8720.

Chou, I.T., Kobayashi, J., Lee, E., Koshinsky, H., Medberry, S. and Ow, D. (1999) A collection of chromosome rearrangements in *Arabidopsis thaliana* generated through site specific recombination. In *Plant and Animal Genome VII Conference Abstracts.* San Diego, CA, 17–21 January 1999. http://www.intl-pag.org/pag/7/abstracts/pag7818.html

Copenhaver, G.P., Browne, W.E. and Preuss, D. (1998) Assaying genome-wide recombination and centromere functions with *Arabidopsis* tetrads. *Proc. Natl Acad. Sci. USA*, **95**, 247–252.

Damm, B. and Willmitzer, L. (1988) Regeneration of fertile plants from protoplasts of different *Arabidopsis thaliana* genotypes. *Mol. Gen. Genet*. **213**, 15–20.

Forsberg, J., Landgren, M. and Glimelius, K. (1994) Fertile somatic hybrids between *Brassica napus* and *Arabidopsis thaliana*. *Plant Sci.* **95**, 213–223.

Gleba, Y.Y. and Hoffmann, F. (1978) Hybrid cell lines *Arabidopsis thaliana* + *Brassica campestris*: no evidence for specific chromosome elimination. *Mol. Gen. Genet.* **165**, 257–264.

Golic, K.G. (1991) Site-specific recombination between homologous chromosomes in *Drosophila*. *Science*, **252**, 958–961.

Heus, J.J., Zonneveld, B.J.M., Steensma, H.Y. and van den Berg, J.A. (1994) Mutational analysis of centromeric DNA elements of *Kluyveromyces lactis* and their role in determining the species specificity of the highly homologous centromeres from *K. lactis* and *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **243**, 325–333.

Hinnisdaels, S., Negrutiu, I., Jacobs, M. and Sidorov, V. (1988) Plant somatic cell hybridizations: evaluations and prospectives. *Nat. Assoc. Plant Tissue Cult. Newsl.* **55**, 2–10.

Hinnisdaels, S., Bariller, L., Mouras, A., Sidorov, V., Del-Favero, J., Veuskens, J., Negrutiu, I. and Jacobs, M. (1991) Highly asymmetric intergeneric nuclear hybrids between *Nicotiana* and *Petunia*: evidence for recombinogenic and translocation events in somatic hybrid plants after 'gamma'-fusion. *Theor. Appl. Genet.* **82**, 609–614.

Hinnisdaels, S., Mouras, A., Salesses, G., Veuskens, J., Taylor, C., Gharti-Chhetri, G.B., Negrutiu, I. and Jacobs, M. (1992) Translocation events demonstrated by molecular, *in situ* hybridization and chromosome pairing analyses in highly asymmetric somatic hybrid plants. *Transgenic Res.* **1**, 170–176.

Masson, J. and Paszkowski, J. (1992) The culture response of *Arabidopsis thaliana* protoplasts is determined by the growth conditions of donor plants. *Plant J.* **2**, 829–833.

Matsuzaki, H., Nakajima, R., Nishiyhama, J., Araki, H. and Oshima, Y. (1990) Chromosome engineering in *Saccharomyces cerevisiae* by using a site-specific recombination system of a yeast plasmid. *J. Bacteriol.* **172**, 610–618.

Migeon, B.R. and Miller, C.S. (1968) Human–mouse somatic cell hybrids with single human chromosome (group E): link with thymidine kinase activity. *Science*, **162**, 1005–1006.

Morgan, M. and Ow, D. (1994) Polyethylene glycol-mediated transformation of tobacco leaf mesophyll protoplasts: an experiment in the study of Cre-*lox* recombination. In *Methods in Plant Molecular Biology, A Laboratory Manual* (Maliga, P., Klessig, D., Cashmore, A., Gruissem, W. and Varner, J., eds). Cold Spring Harbor: Cold Spring Harbor Press, pp. 1–17.

Papp, I., Iglesia, V.A., Moscone, E.A., Michalowski, S., Spiker, S., Park, Y.-D., Matzke, M.A. and Matzke, A.J.M. (1996) Structural instability of a transgene locus in tobacco is associated with aneuploidy. *Plant J.* **10**, 469–478.

Piastuch, W.C. and Bates, G.W. (1990) Chromosomal analysis of *Nicotiana* asymmetric somatic hybrids by dot blotting and *in situ* hybridization. *Mol. Gen. Genet.* **222**, 97–103.

Qin, M., Bayley, C., Stockton, T. and Ow, D.W. (1994) Cre recombinase-mediated site-specific recombination between plant chromosomes. *Proc. Natl Acad. Sci. USA*, **91**, 1706–1710.

Reiter, R.S., Williams, J.G.K., Feldmann, K.A., Rafalski, J.A., Tingey, S.V. and Scolnik, P.A. (1992) Global and local genome mapping in *A. thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. *Proc. Natl Acad. Sci. USA*, **89**, 1477–1481.

Schmidt, R.K., Love, K., West, J., Lenehan, Z. and Dean, C. (1997) Description of 31 YAC contigs spanning the majority of *A. thaliana* chromosome 5. *Plant J.* **11**, 563–572.

Smith, A.J., DeSousa, M.A., Kwabi-Addo, B., Heppell-Parton, A., Impey, H. and Rabbitts, P. (1995) A site-directed chromosomal translocation induced in embryonic stem cells by Cre-*loxP* recombination. *Nature Genet.* **9**, 376–385.

Smith, R.H. (1986) Establishment of calli and suspension cultures. *Meth. Enzymol.* **118**, 539–549.

Valvekens, D., van Montagu, M. and van Lijsebettens, M. (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl Acad. Sci. USA*, **85**, 5536–5540.

Van Deursen, J., Fornerod, M., van Rees, B. and Grosveld, G. (1995) Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. *Proc. Natl Acad. Sci. USA*, **92**, 7376–7380.

Wang, W.C. and Marshall, D. (1996) Genomic rearrangement in long-term shoot competent cell cultures of hexaploid wheat. *In Vitro Cell. Dev. Biol. Plant.* **32**, 18–25.

Weiss, M.C. and Green, H. (1967) Human–mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. *Proc. Natl Acad. Sci. USA*, **58**, 1104–1111.

Wolters, A.-M., Vergunst, A.C., van der Werff, F. and Koorneef, M. (1993) Analysis of nuclear and organellar DNA of somatic hybrid calli and plants between *Lycopersicon* spp. and *Nicotiana* spp. *Mol. Gen. Genet.* **241**, 707–718.