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Tissue-culture enhanced transposition of the maize transposable element *Dissociation* in *Brassica oleracea* var. '*Italica*'

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Abstract To investigate the potential of heterologous transposons as a gene tagging system in broccoli (*Brassica oleracea* var. *Italica*), we have introduced a *Ds*-based two-element transposon system. *Ds* has been cloned into a 35S-SPT excision-marker system, with transposition being driven by an independent 35S-transposase gene construct (*Tpase*). In three successive selfed generations of plants there was no evidence of germinal-excision events. To overcome this apparent inability to produce *B. oleracea* plants with germinal excisions, we performed a novel tissue-culture technique to select for fully green shoots from seed with somatic-excision events. The results showed a very high efficiency of regeneration of fully green plants (up to 65%) and molecular analysis indicated that the plants genetically were like plants that contain a germinal-excision event. Further molecular analysis of these plants showed that 69% exhibited reinsertion of *Ds* back into the plant genome. Sequencing of donor-site footprints after *Ds* excision, revealed that there is an indication of more-severe deletions and rearrangements when higher concentrations of streptomycin are used in the tissue-culture selection process. Adapted versions of this regeneration technique have a high potential for providing germinal excision-like events in heterologous plants species which show low transposon activity. Alternatively, there is the potential to increase the proportion of 'germinal' plants in earlier generations of more-active plant species.

Keywords *Brassica oleracea* · Callus · Dissociation (*Ds*) · Streptomycin · Tissue culture · Transposition

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Introduction

The well-characterised maize transposable elements *Activator* (*Ac*) and *Dissociation* (*Ds*) have been shown to transpose in several plant species; for example, tobacco (Baker et al. 1988), tomato (Yoder et al. 1988), *Arabidopsis thaliana* (Van Sluys et al. 1987), potato (Knapp et al. 1988), *Petunia* (Haring et al. 1989), rice (Izawa et al. 1991), soybean (Zhou and Atherly 1988) and the legume *Lotus japonicus* (Thykjaer et al. 1995). The *Ds* element does not contain a functional transposase gene. Therefore, it cannot induce its own transpostion (non-autonomous). A *Ds* element transposes only under the influence of transposase produced by an *Ac* element (autonomous) within the same plant. A two-element system using the *Ds* element mobilised by the presence of an *Ac*-element derivative, which is itself unable to transpose (*TPase*), has been found to be effective in transposon-tagging experiments in *A. thaliana* (Bancroft et al. 1993; Long et al. 1993; Altmann et al. 1995; Springer et al. 1995), tomato (Jones et al. 1994; Van Der Biezen et al. 1996) and tobacco (Witham et al. 1994).

The *Ac*/*Ds* system of transposons has been used as an insertional mutagen, where the element inactivates the function of any gene that it inserts into. The two-element system has the advantage over the *Ac* element for transposon-tagging strategies because the resulting transposon-induced mutations can be stabilised by segregating away the transposase source (*TPase*) to prevent further excision events and thus possibly restoring the mutated gene's function. Once stabilised, the *Ds* element can be used as a tag to identify the flanking sequences of the mutated gene. Once tagged the wild-type gene can be cloned and isolated. In addition, a stabilised transposon is also important to overcome the possibility that, even after transposon excision away from a mutated gene, the gene function would not be restored as a consequence of a footprint left following transposon activity. This would fatally effect tagging experiments, as the mutated gene would not be flanking the transposon.

In this study we have introduced a two-element transposon system into *Brassica oleracea* spp. *italica* (broccoli). We anticipated that the behaviour of the system would be similar to that found in previous studies of heterologous plant species, but observed very low activity of the *Ac*/*Ds* system in broccoli.

A crucial requirement for the use of the *Ac*/*Ds* transposon system for gene isolation is to obtain germinal-excision events or plants with transposon excision in all cells (Carroll et al. 1995). The non-cell autonomous phenotype of the streptomycin-excision marker system in *Brassica* cotyledons has made the accurate determination of excision frequencies difficult. In fact, observation of fully green seedlings during screening on streptomycin (Jones et al. 1989), which is a strong indication of germinal excision in other autonomous plant species, is not a reliable indicator of germinal-excision events in broccoli. Transposon activity can be stimulated by tissue-culture selection (Peschke et al. 1987) and we have developed a novel tissue-culture approach to regenerate fully streptomycin-resistant plants from calli that are derived from non-fully resistant somatic cells. We have subsequently assessed the behaviour of the *Ds* elements within these regenerated plants with a view to the eventual isolation of genes by insertional mutagenesis in this economically important *Brassica* species.

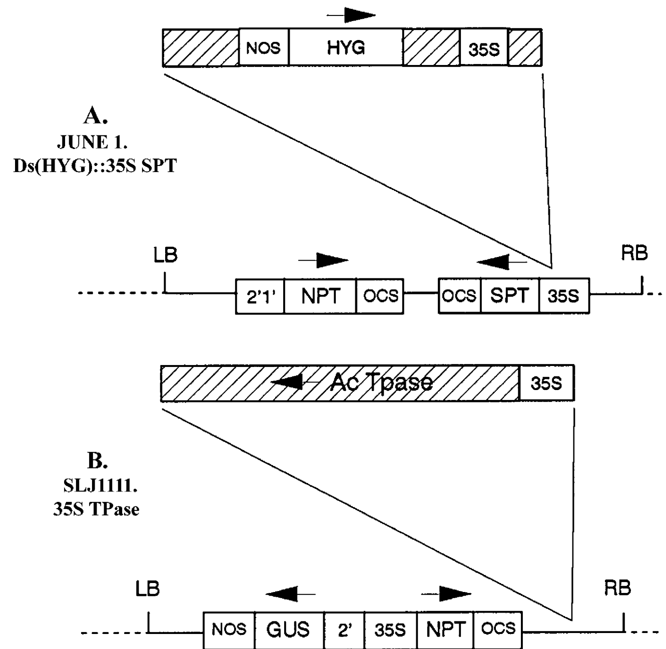

Fig. 1 Diagrammatic representation of the binary constructs used in this study. *A* June 1 (*Ds*(hyg)::35S SPT). The *hatched sections* represent *Ac* sequences in the constructs. *B* SLJ1111 (35S*Tr*), a stabilised *Ac* element used to stimulate transposition of the *Ds* element

Materials and methods

Plasmids

The two binary plasmid constructs used in this study are shown in Fig. 1. The *Ds* construct, termed *Ds* (HYG)::35S–SPT (Fig. 1A), was constructed by first introducing the Cauliflower mosaic virus promoter 35S (CaMV 35S) from a *Xho*I and *Cla*I cleavage from vector pKU33c into the *Activator* sequence of plasmid pJJ4361 cut with the same restriction endonucleases. This promoter is located at the 5′ end of the element and expresses outwards. Following transposition, this may result in the constitutive over-expression of a plant gene adjacent to the reinserted transposable element. The resulting construction was cleaved with *Xho*I and the HYG (hygromycin B. Phosphotransferase) coding region driven by the nopaline synthase (Nos) promoter, which was cloned into this from a *Xho*I/*Sal*I-cut from plasmid pCL19H. This completed *Ds* element was transferred into the T-DNA of the binary vector pCLO111 within the untranslated leader of the SPT (streptomycin phosphotransferase) gene, which is used as an excision marker to monitor the transposition of the *Ds* element by selection on media containing streptomycin (Jones et al. 1989). G. Coupland and C. Dean (JIC UK.) supplied plasmids pKU33c, pCL19H and pCLO111; J. Jones (JIC UK.) supplied plasmid pJJ4361.

The transposase source needed for the transposition of *Ds* in *trans* was provided by the plasmid SLJ1111 (Fig. 1B). This construct consists of a stabilised *Ac* element driven by the CaMV 35S promoter (35S-*TPase*.) which has been found to give elevated excision frequencies in tobacco and *Arabidopsis* (Scofield et al. 1992; Swinburne et al. 1992). The construction of this plasmid has previously been described by Scofield et al. (1992).

Plant transformation

The binary plasmids *Ds* (HYG)::35S–SPT and 35S-*TPase* were introduced into *B. oleracea* subspecies *italica* (Italian green sprouting 577) cotyledon petioles or hypocotyl segments by *Agrobacterium*-mediated transfer with the methods of De Block et al. (1989) and Moloney et al. (1989), using *Agrobacterium tumefaciens* strains C58C1 or LBA 4404.

Streptomycin screening and rescue

Progeny of the *Ds* (HYG)::35S–SPT and 35S-*TPase* crosses were screened on streptomycin to detect evidence of transposition events (Jones et al. 1989). Seeds were surface-sterilised, first by washing in 70% ethanol for 2 min, followed by a 20-min soak in 5% sodium hypochlorite, then washed 3-times with sterile distilled water for 10 min each. Seeds were sown onto basal MS (Murashige and Skoog) media (Imperial Labs) with 0.8% phytoagar (Gibco BRL), inositol 100 mg/l, thiamine 10 mg/l, pyridoxine 1 mg/l, nicotinic acid 1 mg/l, 3% sucrose and 250 μg/ml of streptomycin sulphate (Sigma). The results of sensitivity to streptomycin were scored after 1–2 weeks incubation in a culture room at 23 °C, and a 16-h photoperiod at an intensity of 70–80 $\mu Em^{-2} S^{-1}$.

When whole seedlings were taken from streptomycin selection and placed onto non-selective media there was poor root development. To overcome this, the seedlings were removed from the selective medium and cut halfway down the hypocotyl, then placed on MS medium as described above but without the streptomycin antibiotic, and with the addition of 0.1 mg/l of NAA (α naphthalene-acetic acid) to promote root growth. After rooting from the hypocotyl the seedlings were transferred to peat pots (Jiffy 7) to stimulate further rooting, and later moved into larger pots in the glasshouse.

Callus and shoot regeneration on streptomycin media

To overcome the apparent inability for *B. oleracea* plants to produce germinal excision events we have developed a selection

method to regenerate fully streptomycin-resistant plants from somatic cells in which there had been a transposition event.

A line displaying high somatic-excision activity (F_3 805–2, 66% of seedlings displaying excision) was chosen as the seed stock for selection. Seeds were surface-sterilised, as described previously, and plated at a density of ten seeds per Petri dish (sterilin) containing basal MS, 3% sucrose and vitamins as described above. Seeds were allowed to germinate for 5–7 days. The cotyledon or hypocotyl explants were excised and plated onto MS medium with the addition of BAP (benzyl aminopurine Sigma) at a concentration of 3.75 mg/l, and streptomycin sulphate at 25, 50, 100, 200 or 300 μg/ml. Samples of cotyledons were co-cultivated on MS medium without BAP or streptomycin for 3 days prior to antibiotic selection.

Explants were subcultured onto the same medium-recipe every 2–3 weeks. After 4–6 weeks of selection, depending on the concentration of selection used, distinct green sectors first appeared within the callus. Soon after this, shoots began to appear and grew rapidly. When fully green shoots were about 1–2 cm in length they were excised from the callus and transferred to 100-ml plastic jars (Richardson) containing the MS medium with streptomycin but no BAP. The green shoots were cultivated on this medium for 2–3 weeks and transferred to MS selection medium also containing NAA to promote root growth. Shoots were subcultured every 2 weeks and any white leaves or shoots were cut out so that only green shoots were allowed to grow. Plantlets with root initiation were transferred to Jiffy 7 peat pots in Magenta jars (Sigma). These were allowed to grow until roots protruded from the peat pots, and the plants were re-potted into a glasshouse (Fig. 2ii shows examples of the various stages of plant regeneration and development).

Polymerase chain reaction (PCR)

DNA template preparation from leaf or cotyledon tissue was performed using the method of Edwards et al. (1991). Ten microliters of the resultant 125–150 μl of the template preparation was employed for each 50-μl PCR reaction containing 1 × PCR Buffer (Boehringer), 250 μm of each nucleotide, 175 ng of each oligonucleotide primer and 2.5 Units of Boehringer *Taq* DNA Polymerase. The oligonucleotides used were:

(1) Detection of excision events, SPTP1: 5′-TATCCAGCTCGA-GTGGGTGAG-3′, and RBP1: 5′-CCATCGTAGGTGAAGG-TG GAAAATTAAT-3′ (Dean et al. 1992).
(2) Detection of non-excision events, SPTP1: as before, and DSN1: 5′-TACTCCCTGHCATGGCTGGCATTAACAG-3′.
(3) Reinsertion of *Ds* back into the genome. HYGP1: 5′-GCT-GGGGCGTCGGTTTCCACTATC-3′, and HYGP2: 5′-CGCA-TAACAGCGGTCATTGACTGGAGC-3′.

The relative position of these oligonucleotides and the expected fragment size produced is indicated in Fig. 3.

The thermal cycler programme parameters were 4 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and $1\frac{1}{2}$ min at 72 °C, followed by an autoextension of 30 s at 72 °C for each cycle. The programme finished with 10 min at 72 °C before holding at 4 °C.

Plant genomic DNA isolation and Southern-hybridisation analysis

Plant genomic DNA was extracted as described by the procedure of Sharp et al. (1988), except that the Kirby mix, containing tri-iso propylnaphthalenesulphonate (1%), 4-Aminosalycilate (6%), phenol (5%) and 50 mm of Tris-HCL pH 8.4 (Covey and Hull 1981), was used for the first-extraction step of the milled freeze-dried leaf material. The genomic DNA was quantified flourometrically following the method of Labarca and Paigen (1980). Ten micrograms of genomic DNA were digested to completion using various restriction endonucleases and run out by electrophoresis on 0.8% TAE agarose gels and blotted onto Hybond N + membranes (Amersham).

Various probes were used in this study (Fig. 1) which were prepared by the standard methods of Sambrook et al. (1989). Probe DNA was isolated from agarose gels (low melt point) using the Geneclean II kit (Bio101, La Jolla Calif/Anachem) and labelled using the random primer method (Feinberg and Vogelstein 1984).

Sequencing

Sequencing of the footprints observed following the PCR reactions using primers SPTP1 and RBP1 was performed with the fMOL sequencing kit (Promega). The PCR product was run out on 1.5% TAE agarose electrophoresis gels and the variable footprints were isolated from the gels using the Geneclean II kit. The protocol employed for sequencing followed the steps for using End-labelled primer, where 100 ng of SPTP1 primer was labelled with $\gamma^{33}P$ ATP and the extension/termination reactions were made up using 50 ng of template DNA (PCR products). The cycle sequencing conditions were 95 °C for 2 min, then 30 cycles of 95 °C for 30 s and 70 °C for 30 s. The sequencing products were run out on 6% denaturing acrylamide/bisacrylamide gels.

Results

Screening of plants to assess transposon activity

Independent lines of *Ds*(HYG)::35S SPT and 35S TPase plants were crossed. The progeny were selfed for up to three generations. F2 and F3 seed was assessed for transposon activity by screening on streptomycin. Results showed that the streptomycin excision marker-system does give bleached and green regions, although there is not the distinct sectoring that was observed in tobacco and *Arabidopsis* (Dean et al. 1992; Scofield et al. 1992). The observed sectoring in *B. oleracea* is consistent with that found in tomato using the spectinomycin (SPEC) excision marker-system (Jones et al. 1993) which gave diffuse sectoring on the cotyledons at concentrations of the antibiotic above the de-toxification level of the cells. This non-cell autonomous nature of streptomycin selection in brassicas makes accurate determination of transposition frequencies difficult to assess. Especially for seedlings exhibiting low levels of excision, where green sectoring is so diffuse, it is difficult to distinguish it from bleached areas. Overall the excision marker is reliable enough to detect and select for excision events; however, molecular screening by PCR would give more-reliable determination of excision freqencies. PCR and Southern-blot analysis was performed on selected seedlings and, although evidence of somatic transposition was seen, no germinal transposition was evident even in three generations of selfed seed.

A further problem encountered was self-incompatibility. To self-pollinate plants, which is critical for use in gene-isolation procedures, involves bud pollination (emasculating unopened flower buds and pollinating the exposed stigmas with pollen from the same plant). However, one plant ($F_2$805-2), after rescuing from streptomycin selection, was found to have a degree of self-compatibility. The excision frequency for the self-compatible lines $F_2$805 and $F_3$805 were both found to show around

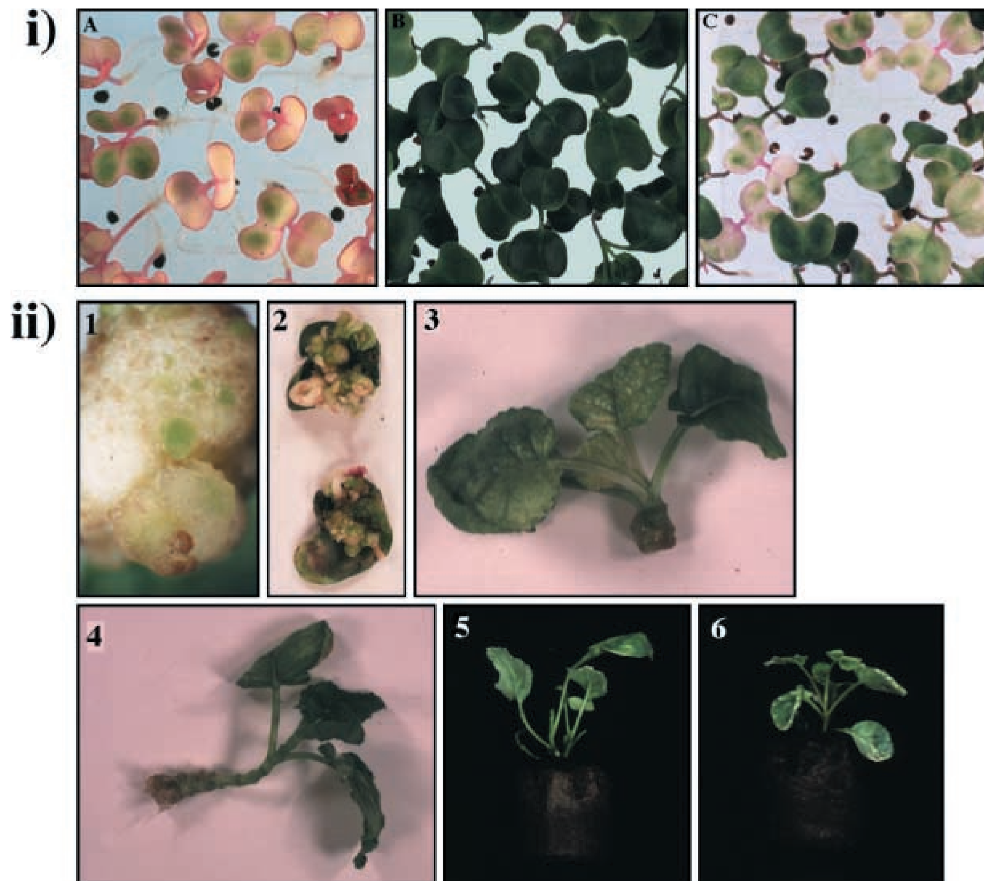

Fig. 2 **i**) Seedlings screened on streptomycin (250 μg/ml) at 2-weeks selection. *A* The F_3 805-2 seed stock used for the regeneration experiments. *B* Progeny from a 'germinal' regenerated plant showing fully resistant seedlings. *C* Progeny from another 'germinal' regenerated plant showing some inactivation of the streptomycin resistance gene. **ii**) Various stages of the streptomycin-selection regeneration technique. *1* Callus at 4 weeks, showing distinct green sectors. *2* Extensive *shoot* growth at 7–9 weeks. *3* Green plantlets transferred to 100-ml Richardson containers at 9–11 weeks. *4* A root-initiated fully green plantlet at 10–12 weeks. *5* An example of a 'germinal' plant transferred to sterile peat pots (Jiffy 7) at 12–14 weeks. *6* An example of a 'somatic' regenerated plant at 12–14 weeks. Note the bleaching patterns on the leaves

66% somatic excision activity. This was determined by the number of screened seedlings with observable green sectoring and by PCR analysis. (Fig. 2i, photograph A shows the streptomycin screen for $F_3$805). The transgene construct copy number of the $F_1$805 line was found to contain two loci of the *Ds* construct and one locus of the TPase plasmid. The genotypes for the F_2 and F_3 generations were one-locus each of the two constructs (Southern data not shown). The F_3 line was chosen for the callus shoot regeneration experiment because: (1) a large number of seeds were available; (2) it was partly self-compatible; and (3) it had a high somatic-excision frequency.

Plant transformation – callus shoot regeneration experiment

The objective was to regenerate plants from a tissue-culture system, similar to that used for transformation, to obtain plants made up entirely of streptomycin-resistant cells. Throughout this report we refer to these regenerant plants, that are completely resistant to streptomycin, as 'germinals'. Regenerant plants that were not fully streptomycin-resistant, where there are still *Ds* elements present within the donor site, are termed 'somatics'. To obtain these regenerant plants, we used MS-media plates containing either 25, 50, 100, 200 or 300 μg/ml of streptomycin sulphate and the explants were propagated on these media following the protocol outlined in the Materials and methods section. The results from the separate experiments are combined and summarised in Table 1. No plants were regenerated from 200 and 300 μg/ml of streptomycin; in the majority of these cases the callus blackened and died. Any shoots that did appear were completely white. Two further experiments were performed with SPT 100 μg/ml-selection using cotyledon explants. The regeneration efficiencies for the two experiments were very similar and gave 12 regenerants from a total of 150 explants tested (8% regeneration frequency). Regeneration efficiencies improved substantially with reduction in the concentration of antibiotic used in the selection process, as does the appearance of green shoots and the speed of shoot growth. The use of SPT 50 μg/ml-selection resulted in eight plants from 60 explants tested (13.3% regeneration rate), and SPT 25 μg/ml-selection resulted in 65 plants from 60 cotyledon explants (108%). Hypocotyl explants, like cotyledons, do regenerate into viable green plants, but the efficiency at specific concentrations of streptomycin was much lower. In addition, regenerants from hypocotyl explants took twice as long to form callus and regenerate shoots. Explants harbouring the *Ds* construct and no Tr, the Tr construct and no *Ds*, or no constructs at all, were also screened on all concentrations of streptomycin. In all the control explants there was no evidence of green

Table 1 Plants regenerated from two explant types on media containing various concentrations of streptomycin

Streptomycin concentration µg/ml	Explant type	Number of explants screened	Number of plants regenerated	Number of plants 'germinal'	Number of plants 'germinal' and *Ds* reinsertion
25	Cotyledon	60	65 (108%)[a]	42 (65%)[b]	29 (69%)[c]
25	Hypocotyl	60	4 (7%)	2 (50%)	2 (100%)
50	Cotyledon	60	8 (13%)	6 (75%)	6 (100%)
50	Hypocotyl	60	0 (0%)	0 (0%)	0 (0%)
100	Cotyledon	150	12 (8%)	7 (58%)	4 (57%)
100	Hypocotyl	50	1 (2%)	1 (100%)	0 (0%)
200	Cotyledon	100	0 (0%)	0 (0%)	0 (0%)
300	Cotyledon	100	0 (0%)	0 (0%)	0 (0%)

[a] Regeneration frequency (number of regenerated plants/number of explants cultured × 100), some explants regenerated more than one shoot (plant)
[b] Percentage of regenerated plants that were 'germinal'
[c] Percentage of 'germinals' in which *Ds* was reinserted back into the genome after excision

shoots or transposon movement, as determined by PCR screening.

Molecular analysis of transgenic callus-shoot regenerated plants

PCR analysis of regenerated plants

All regenerated plants were analysed by PCR to characterise the nature of transposition events. Three PCR oligonucleotide pairings were used, and characterisation was based on the combined results of all three reactions. Excision was confirmed by the presence of the 'empty donor site' fragment of 542 bp using primers SPTP1 and RBP1, and it was found that all the plants exhibited this fragment or a fragment of similar size (except the control explants). The presence of a 1,036-bp band following PCR using SPTP1 and *Ds*N1 shows that some tissues still harboured non-excised *Ds* elements. Reinsertion of the *Ds* element back into the plant genome was identified by the presence of a 375-bp fragment using primers HYGP1 and P2, but only for plants that have the 542-bp excision signal without the 1,036 bp fragment from PCR using SPTP1 and *Ds*N1. Figure 3 shows the PCR-analysis for selected plants. The PCR-reactions were performed separately, but are combined in the figure to show clearly the type of excision event that is exhibited by each respective plant.

PCR-analysis of SPT 100 µg/ml-selected regenerant plants determined that 58.3% of plants had the equivalent of a germinal-excision event (4.7% of total explants screened), of which 57% of these showed reinsertion of *Ds* back into genome (33% of the regenerated plants or 2.7% of the explants tested). SPT 50 µg/ml-selection resulted in 75% plants to be 'germinals' (10% of the explants screened), which all showed reinsertion of *Ds* back into the genome. Finally, for SPT 25 µg/ml, it was observed that 64.6% of the regenerated plants showed 'germinal'-excision events, and 69% of these had *Ds* reinsertion (44.6% of the regenerant plants).

SPT screening of 'germinal' plant progeny

Inheritance of excision events was determined by screening the progeny from selected plants on 250 µg/ml of streptomycin. 'Germinal' plants were expected to be fully green on streptomycin, but seven out of the nine families screened up to 60% of the seedlings and had a green variegation patterning (see Fig. 2i, photographs B and C) as observed for the somatic-excision events. DNA from these variegated seedlings was extracted and subjected to PCR. The presence of 'germinal' events was confirmed, as the variegated plants did not show a non-excision band (1,036 bp) from PCR using oliogonucleotides SPTP1 and *Ds*N1, which would be expected if these were 'somatic' events. It is thought that the streptomycin gene has been partially inactivated or silenced in these progeny.

Sequence analysis of variable footprints

In 14 out of the 90 regenerated plants, the empty donor-site fragment (PCR using oligonucleotides SPTP1 and RBP1) differed significantly in size (by observation on the electrophoresis gel) from the expected 542-bp fragment. A total of 22 footprints have been sequenced using the fMol sequencing kit (Promega). Figure 4 shows the electrophoresis gel for the 'footprint' PCR product bands that were sequenced. Cycle-sequencing using SPTP1 as the labelled primer, revealed sequence differences around the *Ds* fusion site within the untranslated leader of the streptomycin gene. Figure 4 also shows the sequence data for the variable region of the 22 sequenced PCR products.

Southern-blot analysis for confirmation of excision and reinsertion events

The streptomycin-selection plant regeneration method and PCR-reactions both monitor excision events and establish the occurrence of 'germinal' or 'somatic' transposition. They also assess the reinsertion of the element

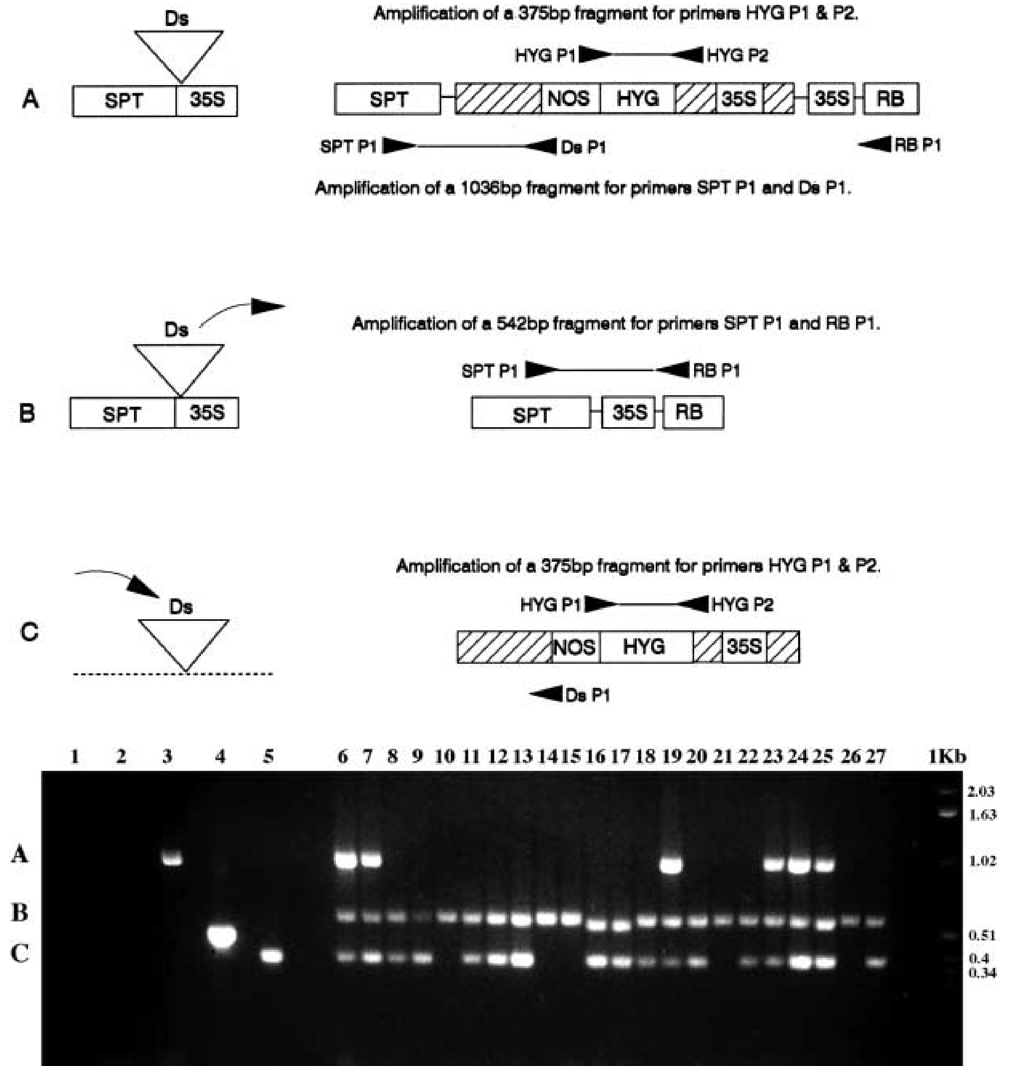

Fig. 3 PCR analysis of regenerated plants. **Top**. Schematic representation of the oligonucleotide primers used for each specific excision event. *A* Fragment sizes expected for non-excision of *Ds*. *B* Expected fragment size for excision events. *C* Expected fragment size for reinsertion of *Ds* (depending on previous excision events as in *B*.). **Bottom**. Gel electrophoresis of a PCR screen from regenerated plants selected on 25 µg/ml of streptomycin. *Lane 1* no DNA control. *Lane 2* non-transformed *B. oleracea* control. *Lane 3* control for non-excision of *Ds* using primer SPTP1 and **Ds**P1. *Lane 4* 35S::SPT positive control plasmid to show excision events using primers SPTP1 and RBP1. *Lane 5* Control for detection of the hygromycin gene using primers HYGP1 and 2. *Lanes 6–27* regenerated plant samples. Plant samples showing the presence of three fragments represent 'somatic'-excision events; the presence of excision and hygromycin fragments but no non-excision signal represents a 'germinal' event with reinsertion of *Ds*. Lack of the hygromycin fragments in 'germinal' plants show no reinsertion of **Ds** back into the plant genome

back into the genome in 'germinal' plants. However, these methods do not provide information on whether the excision events are independent, how many reinsertion events have occurred, or characterise reinsertion of *Ds* in the 'somatic' events. To address these questions, Southern-blot analysis was performed on selected regenerant plants.

Plant genomic DNA (10 µg) was digested to completion using either S*sp*1 or *Eco*R1 restriction endonucleases, following electrophoresis and blotting; the resulting filters were hybridised to specific probes as represented in Fig. 5 and 6. A S*sp*I-digest blot, probed with SPT II (Fig. 5), reveals the empty donor site as a 1.55-kb band (A). The variable footprints, as with the SPTP1 and RBP1 PCR reaction, are clearly defined. The non-excision allele is represented by a 2.45-kb fragment (B). The two larger fragments (C and D) are the terminator Ocs 3′ specific hybridisation bands (the probe used contained Ocs 3′ sequences), these correspond to Ocs terminator sequences in the *Ds* construct (D) and the transposase source (C), as visualised by the control-plant samples on the blot (lanes 2 and 3).

The *Eco*RI Southern hybridisation with the hygromycin probe (Fig. 6) indicates the presence of independent transposition events as well as confirming reinsertion of the *Ds* element back into the genome. The 1.25-kb fragment (band A) corresponds to internal *Eco*RI sites within the hygromycin gene and the *Ds* element. An approximately 11-kb fragment (band B) represents the *Eco*RI site within the hygromycin gene to the nearest *Eco*RI site within the plant genomic DNA adjacent to the left border, for the original T-DNA insertion event. This corresponds to the expected fragment size for an non-excised *Ds* element. The other fragment sizes represent integration sites of the *Ds* elements, the size depending on the nearest *Eco*RI site relative to the reinserted *Ds* element within the plant genomic DNA. All 'somatic' regenerant plants exhibit band B and all except 8–25 and 2–100 (lanes 6 and 16), also have *Ds* reinsertion. 'Germinal' excised plants 4–100 and 9–100 (lanes 18 and 22) are further examples where *Ds* has failed to reinsert. All the remaining plants show independent excision and reinsertion events except 43–25 and 45–25 (lanes 10 and 11), which show identical band patterns. These plants are

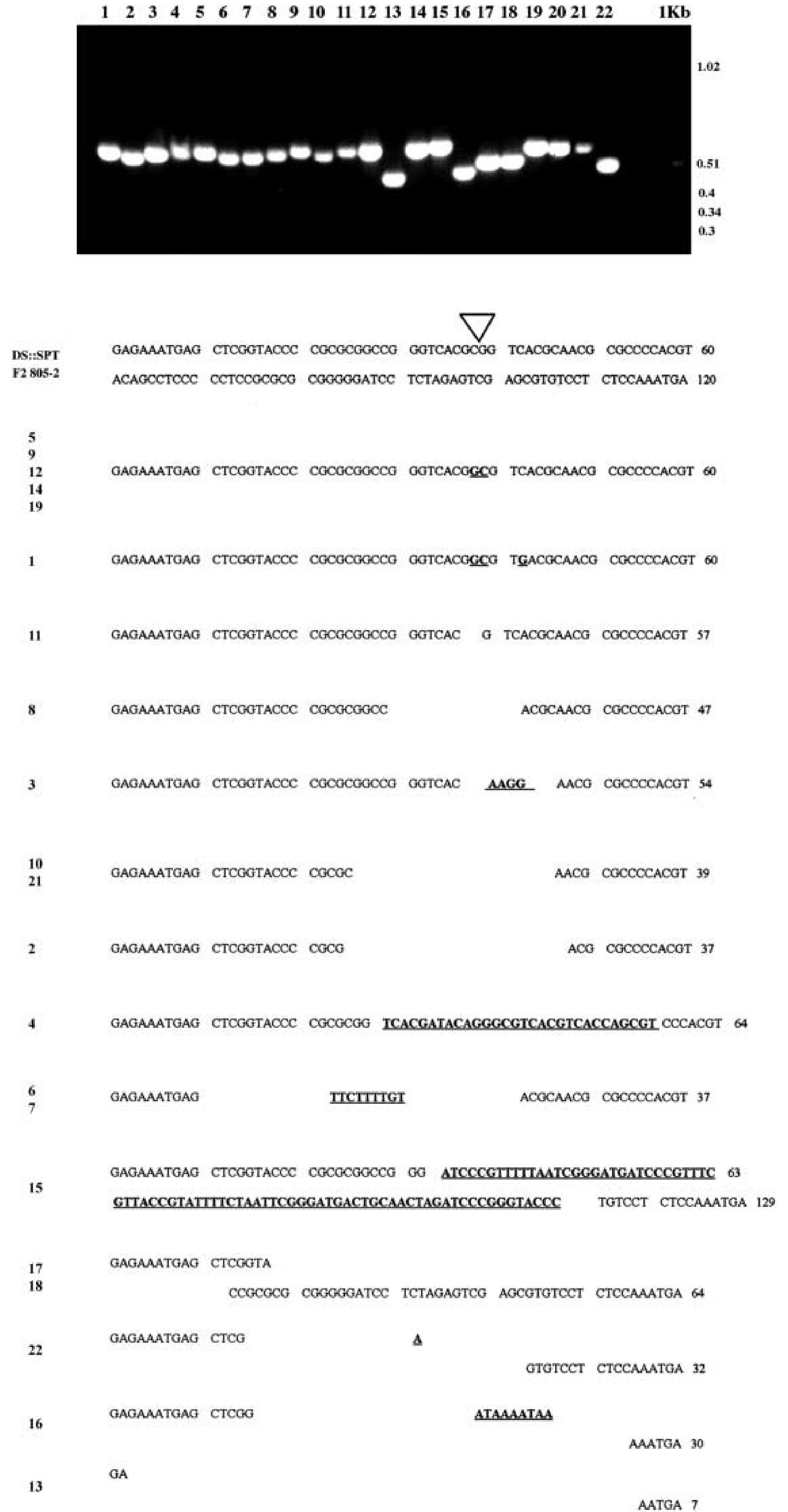

Fig. 4 Sequence analysis of the variable 'footprint' following excision of *Ds* from the donor site. **Top**. Gel electrophoresis of the variable footprints from a PCR reaction using SPTP1 and RBP1. *Lanes 1–9* SPT 25 μg/ml regenerated plants 5, 8, 14, 25, 29, 43, 45, 60 and 66. *Lanes 10 and 11* SPT 50 μg/ml regenerated plants 1 and 3. *Lanes 12–22* SPT 100 μg/ml plant samples 1, 2, 3, 4, 5, 6, 7, 8, 10, 11 and 12. **Bottom**. Sequence data of the PCR products ordered according to the level of 'severity' of the rearrangements and deletions. Sequence rearrangements are shown as *bold and underlined bases*, deletions are shown by *gaps* in the sequence data. Sequences from bases 60 to 120 not shown unless deletions or rearrangements occur here. The *numbering* of the plant samples in the sequence data represent the lane on the electrophoresis gel. The *triangle* on the F3 805-2 (Ds::SPT) sequence data shows where the *Ds* element has been inserted in the *Ds* construct

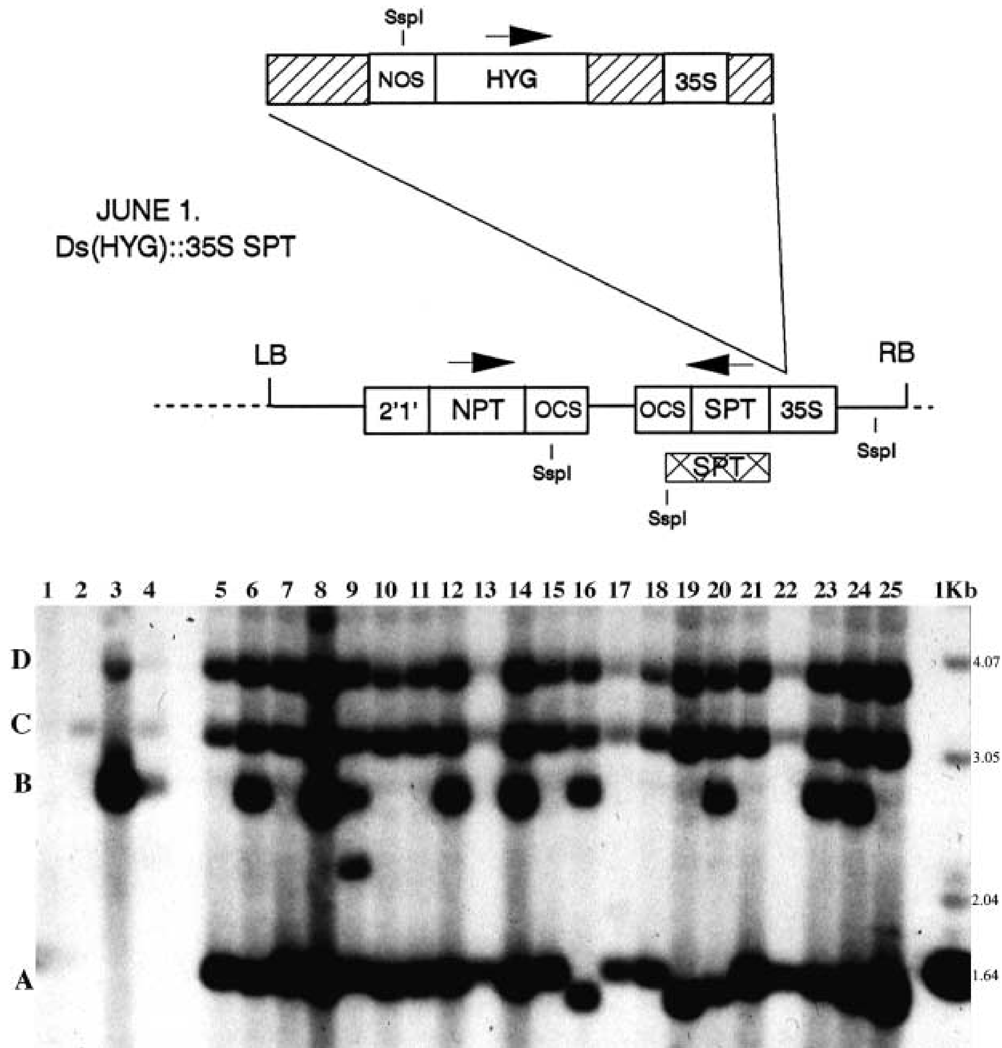

Fig. 5 **Top**. Schematic representation of the *Ds* construct showing the approximate position of the S*sp*I restriction sites. The *crosshatch box* represents the streptomycin and Ocs3′ terminator probe used in the Southern analysis. **Bottom**. S*sp*I Southern-blot hybridised with a 1-kb *Nco*I/*Ssp*I fragment from plasmid SLJ1491 (supplied by J. Jones), relating to the streptomycin phosphotransferase gene and partial Ocs3′ terminator sequences. *Fragment A* empty donor site after *Ds* excision (1.55 kb). *Fragment B* non-excision event (2.45 kb). *Fragments C and D* Ocs3′ specific hybridisation bands corresponding to Ocs terminator sequences in the 35STr construct (Fig. 1.) and additional sequences in the *Ds* construct (as indicated above). *Lanes 1* non-transformed *B. oleracea*, *2* T_0 35S*Tr* parent, *3* T_0 *Ds*(hyg)::35S spt parent, *4* F_3 805-2 parent plant. *Lanes 5–13* SPT 25 µg/ml regenerated plants 5, 8, 14, 25, 29, 43, 45, 60 and 66. *Lanes 14 and 15* SPT 50 µg/ml regenerated plants 1 and 3. *Lanes 16–25* SPT 100 µg/ml plant samples 2, 3, 4, 5, 7, 8, 9, 10, 11 and 12

from the same regenerated plant where the shoots became separated and grew as two individual plants. This occurrence was consistent with the sequence analysis of the variable footprints, which were found to be identical. Plants 4–25, 66–25 and 3–100 (lanes 7, 13 and 17) all exhibit ‘germinal’-type excision and only show a single reinsertion event. There is an indication of differences in intensity between fragments; for example, 29–25, 43–25, 45–25, 60–25, 1–50, 3–50, 7–100, 8–100, 10–100 and 11–100 (lanes 9, 10, 11, 12, 14, 19, 20, 21, 23 and 24). The lower intensity bands are thought to relate to quantity, where perhaps a reinsertion event occurred late in the development of the plant resulting in only a small part of the plant exhibiting this reinsertion event, or to further transposon movement from an earlier excision event. Either mechanism would be possible for ‘somatic’ plants. However for ‘germinal’ plants 43–25, 45–25, 3–50 and 8–100 further transposon movement is the most probable, as it is highly unlikely that the *Ds* element after excision in early development will stay in the cell for several cell-division cycles and then reinsert into the genome.

Discussion

The time-scale for the production of regenerated plants on 25 µg/ml of streptomycin is similar to that for normal T-DNA transformation in *B. oleracea* (3 to 4 months). However, there is a longer shoot-emergence period with increasing concentrations of the antibiotic. The efficiency of the regeneration technique, especially at 25 µg/ml of SPT in this transgenic line, resulted in numerous ‘germinal’ plants with single or multiple independent insertions of *Ds* using low numbers of cotyledon explants. It may be possible to combine a similar selection technique based on other plant lines or species with an optimal plant regeneration methodology to produce a higher proportion of ‘germinal’ plants and in earlier generations. This could be particularly valuable for species of plants that have low excision activity of *Ds*, or plant species that require a large amount of greenhouse space.

An interesting observation was the distinct green sectors found in the early stages of selection in the callus. This sectoring is consistent with cell-autonomous SPT selection in the cotyledons of tobacco (Jones et al. 1989)

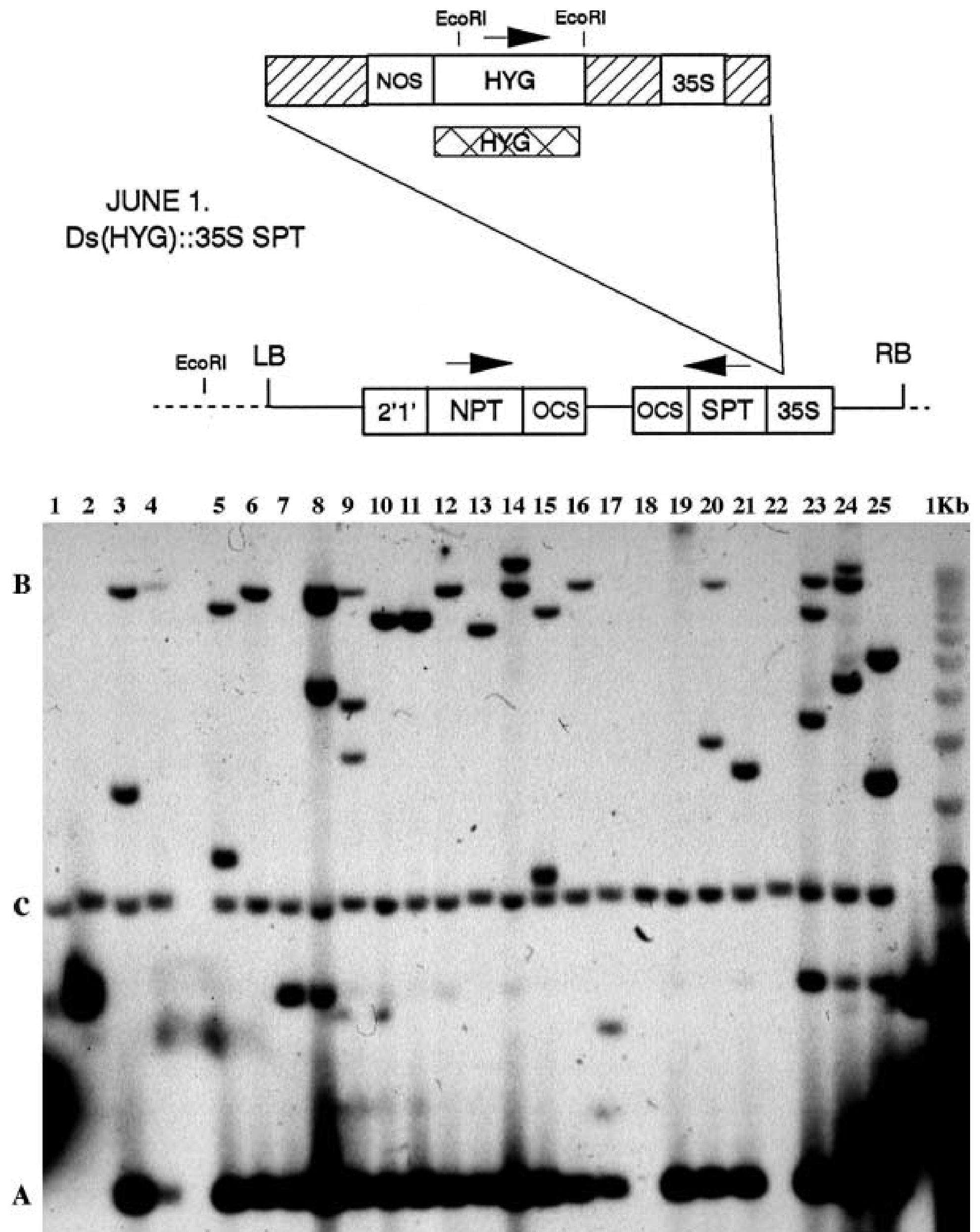

Fig. 6 Top. Schematic representation of the *Ds* construct showing the approximate position of the *Eco*RI restriction sites. The *crosshatch box* represents the hygromycin probe used in the Southern analysis. The*Eco*RI site on the *dashed line* adjacent to the left border sequences (*LB*) for the *Ds* construct represents the nearest *Eco*RI site to the T-DNA insertion site within the plant genomic DNA. **Bottom**. *Eco*RI Southern blot hybridised with a 1.46-kb *Sal*I/*Bgl*II fragment from plasmid pPCV720 (supplied by G. Coupland), corresponding to the reading frame of the hygromycin phosphotransferase gene. *band A* internal *Eco*RI sites in the *Ds* element (1.25 kb). *band B* non-excision of *Ds* (approximately 11 kb). *band C* unspecific hybridisation signal, thought to represent unrelated but homologous sequences in the brassica genome. This occurrence is very common with Southern-blot analysis in brassicas. *Lanes 1* non-transformed *B. oleracea*, *2* T_0 35S*Tr* parent, *3* T_0 *Ds*(hyg)::35S spt parent (two copies of *Ds* in the plant), *4* F_3 805-2 parent plant (one copy of *Ds*). *Lanes 5–13* SPT 25 µg/ml regenerated plants 5, 8, 14, 25, 29, 43, 45, 60 and 66. *Lanes 14 and 15* SPT 50 µg/ml regenerated plants 1 and 3. *Lanes 16–25* SPT 100 µg/ml plant samples 2, 3, 4, 5, 7, 8, 9, 10, 11 and 12

and *Arabidopsis* (Dean et al. 1992). This also highlights the possibility that SPT selection in the callus of *B.oleracea* is cell-autonomous allowing the selection method to function at low concentrations of the antibiotic. However, in broccoli when the shoots differentiate, the plantlets are thought to lose this cell-autonomous nature resulting in diffuse variegation patterns, as observed with seedling-screening on streptomycin.

In other heterologous plant species, for example tobacco (Marion-poll et al. 1993), tomato (Rommens et al. 1992, 1993), rice (Shimamoto et al. 1993) and *L. japonicus* (Thykjaer et al. 1995), all found that the empty donor-site sequences were very similar to those known in maize, with small deletions and GC inversion at the integration site. From our results we found 14 observable fragment-size differences for the empty donor site, following excision of *Ds* out of the 90 regenerated plants. The remaining plants all show the expected 542-bp band or a fragment of similar size. We sequenced several of these 'expected size' donor sites and found that these were similar to what was found in the other plant species mentioned above. However, in our work, comparing the results for a particular concentration of streptomycin, there seems to be a trend where the proportion of plants and the severity of the rearrangements and deletions increase with higher concentrations of antibiotic used in the selection. Considering SPT 100 µg/ml, 6 out of 12 individual plants (50%) show differences in footprint size and these are generally larger than for plants with lower antibiotic selection; these include 113-bp, 90-bp and 88-bp deletions, and a 81-bp rearrangement. The next largest deletion is 23 bp for 8–25

43/45–25 and a 30-bp rearrangement for 25–25. For SPT 50 μg/ml, one out of eight plants (12.5%) show an observable deletion greater than three, namely plant 1–50 with a 21-bp deletion. For SPT 25 μg/ml-selection, 5 of 68 (7.35%) plants that were produced from both cotyledon and hypocotyl explant experiments, show observable differences. The reason why we have more-severe alterations in the donor-site sequences is not clear; however, tissue-culture techiques often promote deletions and rearrangements, and mechanisms of somatic-excision events are not as well known as for germinals events. Overall the technique works best with 25 μg/ml SPT-selection, and these promote only a small proportion of severe alterations; moreover, as long as the rearrangement does not inactivate the SPT gene function when selecting, then this will not affect plant regeneration. In any case the efficiency of regeneration for this transgenic line at the 25 μg/ml SPT selection level was found to be the highest so there is little point in selecting with a higher concentration of streptomycin. This would increase the chances of rearrangement as well as decreasing regeneration efficiency.

The screening of 'germinal' plant progeny, which resulted in variegated patterning of the cotyledons, are thought to be due to silencing or partial inactivation of the SPT II gene. Considering that all the progeny harbour the same genomic transgenic locus, silencing is very likely. We have observed examples of such silencing of transgenes during other projects using various brassica lines in our laboratory (I. Senior, personal communication). Silencing of the SPT gene does add another complication to the reliable determination of excision events as this would have a detrimental effect on the selection process. It must be mentioned that transgenic silencing is quite common, would involve many factors and would vary between independent lines, so some account would need to be taken when performing experiments based on techniques outlined in this study.

The behaviour of the *Ds* elements with respect to reinsertion has not been fully investigated. Further work will be performed to examine if the system in *Brassica* is fundamentally the same as in other heterologous plant species. Is there duplication of sequences upon reinsertion into the genome, and also how far does the element move? Is it similar to other species, where the *Ac*/*Ds* system transposes predominantly to linked sites for germinal excision events? (Jones et al. 1990; Dooner et al. 1991; Bancroft and Dean 1993; Carroll et al. 1995; Van Der Biezen et al. 1996). In tomato it was found that transposable elements exhibiting somatic-transposition events have a tendency to move to unlinked sites (Osborne et al. 1991; Belzile and Yoder 1992; Healy et al. 1993). The antibiotic selection method used in our study was based on a somatic seed stock, so we need to consider whether this produces 'germinal' plants that have a tendency for *Ds* to move further than normally observed for germinal transposition events. This is of importance when devising transposon-tagging strategies.

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