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Trans-activation of the maize transposable element, *Ds*, in *Brassica napus*

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Abstract A two-component transposable element system consisting of a stabilized *Activator* (*Ac*st) and a chimeric *Dissociation* (*Ds*) element has been introduced into the genome of *Brassica napus*. Analyses performed on F2 progeny derived from crosses between *Ac*st- and *Ds*-bearing parents confirm that *Ac* transposase catalyzes the somatic excision of the *Ds* element in both embryonic and non-embryonic tissues of this important crop species. The data further reveal that the vast majority of plants containing both *Ac*st and *Ds* exhibit *Ds* excision. However, the level of excision is low and germinal *Ds* excision events are not observed. We estimate that germinal excision of *Ds* occurs at a frequency of < 0.2%. RT-PCR analysis of the *Ac*st transcript in somatically active seedlings reveals that introns III and IV are highly misprocessed. The pattern of transcript processing is very similar to that observed in germinally inactive but somatically active Arabidopsis seedlings. We suggest that *Ds* excision activity in *B. napus* is highly dependent on the efficiency of *Ac*st transcript processing.

Keywords Activator · Dissociation · *Brassica napus* · Transposon · Transposase

Introduction

The genus *Brassica* is made up of several important cultivated species that include vegetable, oilseed and fodder crops. The economic importance of this genus has led to numerous molecular genetics research programs. Long-term goals of these programs include cloning genes of agronomic interest and their allelic variants. While T-DNA-based insertional systems can be used for gene isolation (Feldmann 1991; Krysan et al. 1996), they have limited utility in species such as *Brassica*. Generating the large number of independent T-DNA insertion events required to mutagenize a specific gene is prohibitive in plants that are difficult or labor intensive to transform. However, since transposons are mobile, a large number of independent insertion events can be generated from the progeny of a single, starting transformant.

In recent years a small number of endogenous transposons or transposon-like sequences have been identified in *Brassica* species (Pastuglia et al. 1997; Cui et al. 1999; Suzuki et al. 1999). To date, however, these elements have not been characterized in detail and there is no data suggesting that any are still mobile. For this reason, we have chosen to explore the utility of a stabilized version of the maize *Activator* (*Ac*st) and chimeric *Dissociation* (*Ds*) transposable elements for developing a two-component heterologous insertional system in *Brassica napus*.

Ac is an autonomous element that can mobilize itself as well as the non-autonomous *Ds* element. In 1986, Baker et al. were the first to report that *Ac* retained its activity in the heterologous host, tobacco. Since then, *Ac* has been introduced into numerous plant species including tomato, Arabidopsis, *Petunia*, lettuce (reviewed by Kunze et al. 1997) and wheat (Takumi et al. 1999). *Ac* encoded transposase was demonstrated to be functional in all these hosts since it could catalyze the excision of both *Ac* and *Ds* elements. In addition, reintegration of *Ac* and *Ds* elements into the genome after excision has been demonstrated in all tested species with the exception of wheat (Takumi et al. 1999). Germline transmission of the reintegrated elements has also been demonstrated in all well-characterized systems.

Among heterologous hosts, activity of the native *Ac* element is highly variable. In solanaceous crops like tomato and tobacco, *Ac* germinal excision rates are high (Belzile et al. 1989; Hehl and Baker 1990), while in plants like Arabidopsis and lettuce excision is low or barely detectable (Schmidt and Willmitzer 1989; Yang et al. 1993). Studies suggest that the low excision frequen-

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cies are the direct result of low levels of functional transposase as excision rates increase concomitantly with transposase levels. Modifications of the native *Ac* element have led successfully to higher excision levels. One of the most successful alterations involved replacing 5′ regulatory *Ac* sequences with strong heterologous promoters such as the CaMV 35S, Arabidopsis *rbcS* and T-DNA 2′ promoters (Ellis et al. 1992; Grevelding et al. 1992; Honma et al. 1993). Another successful modification involved expressing transposase from a cDNA copy, thus avoiding problems associated with misprocessing of the *Ac* transcript (Ellis et al. 1992; Grevelding et al. 1992; Yang et al. 1993; Martin et al. 1997).

As an important first step in establishing an *Ac*/*Ds* insertional system in *Brassica* species, we have introduced modified versions of the maize elements into *B. napus* and have characterized the activity of the system in this important crop plant. Among our findings is the very important observation that *Ac* can catalyze the excision of the *Ds* element in somatic tissue of *B. napus* plants. To our knowledge, this is the first report demonstrating *Ac* transposase-mediated *Ds* excision in *B. napus*.

Materials and methods

DNA constructs

Chimeric *Ac* and *Ds* T-DNA elements were constructed in the binary vector pRD400 (Datla et al. 1992); the NPTII gene serves as the T-DNA selectable marker (Fig. 1). The source of *Ac* transposase is an immobile or stable *Ac* element, Ac^{st} under the transcriptional regulation of the CaMV 35S promoter (pAB80 and pAB83) or the *rbcS* promoter (pAB81 and pAB84). These chimeric Ac^{st} elements are identical to those used and described in Honma et al. (1993). The screenable marker for the Ac^{st} T-DNA is the $35S_p$-*Lc*-$rbcS_t$ chimeric gene (Lloyd et al. 1992). The *Lc* cDNA in pAB80 and pAB81 is 2.2 kb in length, while the version in pAB83 and pAB84 contains an additional 200 bp of 5′ untranslated sequences (Lloyd et al. 1992). The complete T-DNA element is schematically represented in Fig. 1A.

The *Ds* element (pAB62) is comprised of 5′ and 3′ ends derived from the native *Ac* element; these ends (250 bp on the 5′ end and 456 bp on the 3′ end) flank a $35S_p$-*BAR*-NOS_t chimeric gene and plasmid rescue sequences (Fig. 1B). The chimeric *BAR* gene serves as the *Ds* selectable marker (De Block et al. 1989). Plasmid rescue sequences are derived from 2.57 kbp of pACYC184 DNA sequences (Rose 1988) and contain the p15A bacterial origin of replication and a gene that confers resistance to chloramphenicol when expressed in bacteria. The 1′-*ALS* chimeric gene serves as the *Ds* excision marker. The *Ds* element is cloned within the synthetic 5′ untranslated leader of the Arabidopsis mutant *ALS* gene (Olszewski et al. 1988) thereby preventing *ALS* expression (Fig. 1B). Upon excision of the *Ds* element, the mutant *ALS* gene is expressed under the control of the 1′ promoter and thereby confers resistance to the herbicide chlorsulfuron.

The *Ds* element was removed from pAB62 by *Sal*I digestion to create the *Ds* excision control vector, pAB61 (Fig. 1C). Detailed description on the assembly of the Ac^{st}, *Ds* and *Ds* excision control constructs will be provided upon request.

The Ac^{st}, *Ds* and *Ds* excision control binary plasmids, described above, were mobilized into the *Agrobacterium tumefaciens* strain, GV3101 (pMP90) (Koncz and Schell 1986) by tri-parental mating with the helper strain, MM294 (pRK2013) (Ditta et al. 1980) to create the corresponding *Agrobacterium* strains.

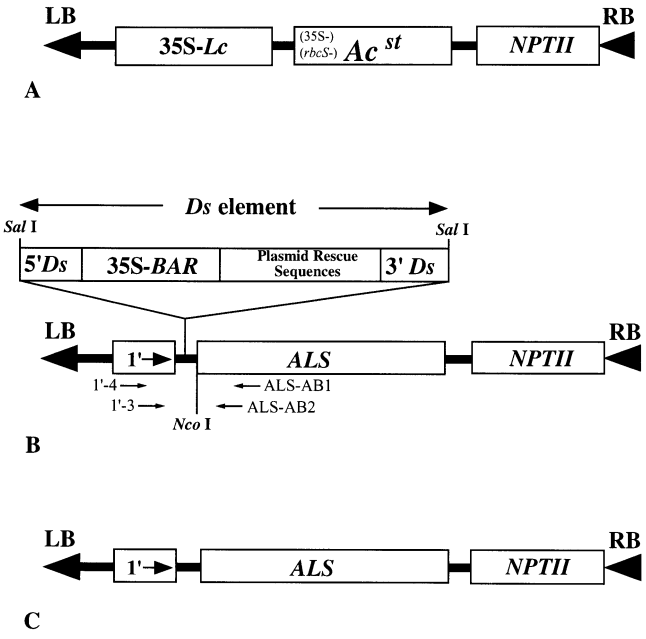

Fig. 1A–C Schematic diagrams of the T-DNA elements introduced into *B. napus* **A** Ac^{st} T-DNA in pAB80, pAB81, pAB83 and pAB84, **B** *Ds* T-DNA in pAB62, and **C** *Ds* excision control T-DNA in pAB61. *LB*: left-border, *RB*: right-border, *arrowheads* indicate the direction of the R and L borders. *35S*: 35S promoter, *rbcS*: *rbcS* promoter, *1′*: 1′ promoter. *Lc*: expression of *Lc* results in a highly visible accumulation of anthocyanins and overproduction of trichomes. *NPTII*: neomycin phosphotransferase II gene confers resistance to kanamycin. *ALS*: Arabidopsis mutant acetolactate synthase gene confers resistance to chlorsulfuron. *BAR*: phosphinothricin-N-acetyl-transferase gene confers resistance to phosphinothricin. *Small arrows* indicate the approximate positions and orientation of DNA primers. *Primer names* as indicated. Figure not drawn to scale

Transformation and selection of transgenic *B. napus* plants

Cotyledons of *B. napus*, cultivar Westar, were transformed using the *Agrobacterium* strains harbouring the Ac^{st}, *Ds* and *Ds* excision control binary plasmids. Transformation was done according to the method of Moloney et al., (1989) with modifications (http://molomac1.bio.ucalgary.ca/LabProtocols/Brassica_Protocol.html). Rooted primary transformants (T0) were transferred to soil and placed in a growth chamber (85% relative humidity, temperature 20 °C, 16-h daylength at a photon flux density of 200 μmol m^{-2} s^{-1}) and allowed to set T1 seeds. T1 seeds were harvested and subsequent generations were derived through selfing under the same growth conditions or under standard greenhouse conditions. Names were assigned to T0 plants and their subsequent lines based on the T-DNA they carried. Names assigned to the Ac^{st} lines also reflected the numerical order in which they were recovered from tissue-culture. The number of independent T-DNA lines used in this study are 13 35S-Ac^{st}, 14 *rbcS*-Ac^{st}, six *Ds* and one *Ds* excision control line. Only those Ac^{st} lines that transactivate *Ds* are listed in Table 1.

Transgenic plants were grown aseptically on germination media (GM) as described in Babwah and Waddell (2000). GM was supplemented with 25 μg/ml of kanamycin (kan) (Sigma), 12.5 μg/ml of phosphinothricin (PPT) (a gift from AgrEvo, Saskatoon, SK) or 350 ng/ml of chlorsulfuron (Chem Service, West Chester, Pa.).

Molecular and genetic analyses of Ac^{st} and *Ds* transgenic lines

The number of *Ds* and Ac^{st} T-DNA loci harboured in each transgenic line was determined from genetic segregation and Southern

analyses. Genetic analysis was performed on selfed and F2 progeny (derived from *Ac*st × *Ds* crosses, see below) that segregated PPT^r and PPT^s phenotypes (*Ds* parents) or kan^r and kan^s and Lc$^+$ and Lc^o phenotypes (*Ac*st parents). Southern analysis of the right and left *Ds* and *Ac*st T-DNA borders was performed on genomic DNA isolated from the cross parents using right- and left-border specific probes as previously described (Babwah and Waddell 2000).

Acst×*Ds* crosses

Crosses were performed between *Ac*st and *Ds* transgenic plants. The choice of male and female parents was random and depended solely on the availability of flowers at the time when crosses were performed. Twenty nine crosses, representing 27 *Ac*st and six *Ds* lines, were performed. For those lines used in multiple crosses, the same plant was used as the parent in all crosses. Of the 29 crosses performed, only one was in reciprocal direction. One to seven F1 PPT^r, Lc$^+$ individuals from each cross was grown and selfed under standard greenhouse conditions to generate populations of F2 seeds. F1 and F2 progeny derived from a cross between a *Ds* line and an *Ac*st line represent a family. Eighty-six F2 populations representing 29 families were established and screened for *Ds* excision events.

Detection of *Ds* excision events

Two hundred seedlings per F2 population, as well as seeds from the *Ds* excision control line, *Ds* parent, *Ac*st parent and non-transformed Westar were initially plated on GM containing chlorsulfuron. After 2–3 weeks of selective growth, seedlings were examined for expression of the Lc phenotypes and sensitivity to chlorsulfuron. Detailed genetic and statistical analyses were performed on these seedlings. Subsequent to this, more F2 seeds were germinated on GM containing chlorsulfuron and these were examined for the chlorsulfuron resistant phenotype. In total, approximately 500 seeds were examined per F2 population. Germinal excision frequency was calculated as < 1 in 500 individuals, or < 0.2%.

A PCR assay for *Ds* excision was performed on the DNA isolated from seedlings grown on chlorsulfuron containing media. All 86 F2 populations were tested. Using a scaled-down version of the protocol by Dellaporta et al. (1983), DNA was extracted separately from the cotyledons (embryonic) and primary leaves (non-embryonic) of individual F2 seedlings. DNA was also extracted from the combined tissues (cotyledons, primary leaves, hypocotyls and roots) of pooled F2 seedlings after it was determined that *Ds* excises in both embryonic and non-embryonic tissues. For the control lines, DNA was extracted only from the combined tissues of pooled seedlings. A primary PCR was performed using approximately 500 ng of genomic DNA and the primers 1′-4 (5′-GTCACGCGCATTCCGTTCTTGCTG-3′) and ALS-AB1 (5′-GCTTCGACGAGGATATCAGCGCC-3′) (Fig. 1B). Subsequently, a nested PCR was performed using 1 µl of the primary PCR product and the primers 1′-3 (5′-GGTACACTTTTGACTAGCGAGGC-3′) and ALS-AB2 (5′-GGTTGATCTGGAGCGAATCGG-3′) (Fig. 1B). The reactions consisted of 100µl volumes and cycling conditions included an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 25 s and 72 °C for 45 s. PCR products were resolved on a 1.0% agarose gel. PCR products from five nested reactions representing five F2 families were cloned into the vector pCR 2.1 (Invitrogen). These products were sequenced and compared to the sequence data obtained from the *Ds* plasmid, pAB62, in order to confirm that excision had occurred.

RT-PCR analysis of *Ac*st transcripts

Total RNA was extracted according to Ausubel et al. (1994) from the leaves of two month old soil-grown Westar and somatically active F2 *Brassica* plants containing *Ac*st and *Ds* elements. Total RNA was also extracted from entire 2-week old Arabidopsis plants, ecotype Nossen (No-0), and F2 Arabidopsis plants containing *Ac*st and *Ds* elements grown in liquid culture (Windsor and Waddell 2000). These plants represented three groups, one that displays high germinal *Ds* excision activity, one that displays low germinal excision activity and one that displays only somatic activity. Poly A$^+$ mRNA was prepared from total RNA using the Oligotex mRNA kit (Qiagen). RT-PCR was performed using the Titan One Tube RT-PCR kit (Roche Molecular Biochemicals) and protocol suggested by the manufacturer. Analysis of introns I and II was performed using primers A-F (5′-GATGTCTACCACAAGCGCGCC-3′) and A-R (5′-CCACTCCTCGGCTTTAGGAC-3′) (see Fig. 5). Primers A-F and A-R are identical to primers ZZZ;aZZZ; by Martin et al. (1997). Analysis of introns III and IV was performed using primers INT3-F (5′-GTCTGCGTTCAGTGCTGGT-3′) and INT4-R (5′-CACTTGCTCACATCTGGATCA-3′) (Jarvis et al. 1997) (see Fig. 5). The PCR products were resolved on either an ethidium bromide stained 0.8% gel (for analysis of A-F/A-R products) or 2.0% gel (for analysis of INT3-F/INT4-R products).

Results

Ds displays somatic excision in *B. napus*

To determine if the maize *Ac* encoded transposase can catalyze the excision of a *Ds* element in *B. napus*, crosses were performed between *Ac*st- and *Ds*-bearing plants (see Fig. 1A, B). From these crosses, 86 F2 populations representing 29 families were established and subsequently screened for *Ds* excision events on chlorsulfuron containing media. Seedlings bearing a *Ds* excision control (Fig. 1C) display clear chlorsulfuron resistance and produce long roots and hypocotyl, fully expanded cotyledons and well developed primary true leaves (Fig. 2). Similar chlorsulfuron-resistant seedlings were not observed among the F2 progeny, suggesting that there were no germinal excision events. On average, 500 seedlings per F2 family were screened. However, 50 F2 populations (Table 1) segregated individuals that were clearly more resistant than the sensitive *Ds* parent seedlings (Fig. 2). These chlorsulfuron semi-resistant seedlings were almost as tall as the fully resistant seedlings but had shorter roots, partially expanded cotyledons and small spindly shaped true leaves (Fig. 2). The chlorsulfuron semi-resistant phenotype suggests somatic activity of the *Ds* element rather than germinal excision events.

To verify that the semi-resistant phenotype does indeed reflect *Ds* excision activity, F2 plants were subjected to a PCR assay. DNA was isolated from the cotyledons or primary leaves of semi-resistant F2 individuals and used in a PCR with primers 1′-4 and ALS-AB1 (Fig. 1B). While a single 712-bp product was obtained from *Ds* excision control samples, no products were observed from DNA isolated from any of the F2 semi-resistant individuals (data not shown). However, a second nested reaction using primers 1′-3 and ALS-AB2 (Fig. 1B), yielded products of the expected size (645 bp) from both the positive excision control and the F2 semi-resistant individuals (Fig. 3A). *Nco*I digestion of these PCR products produced two fragments of the expected

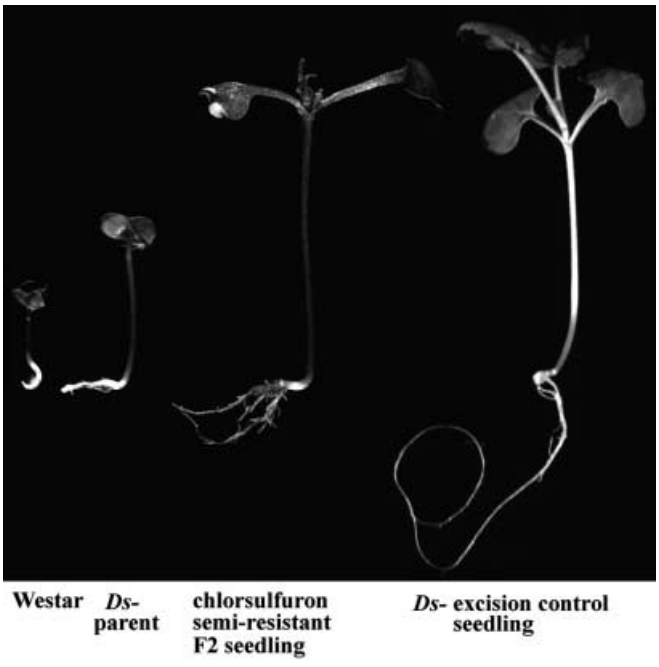

Fig. 2 Phenotypes of 3-week old *B. napus* seedlings germinated directly on GM containing 350 ng/ml of chlorsulfuron. Seedlings shown from left to right are: Westar, *Ds* parent (62-48), representative chlorsulfuron semi-resistant F2 [from cross 62-48 × 81-3B (family no. 14)] and chlorsulfuron resistant *Ds* excision control seedling (61-1)

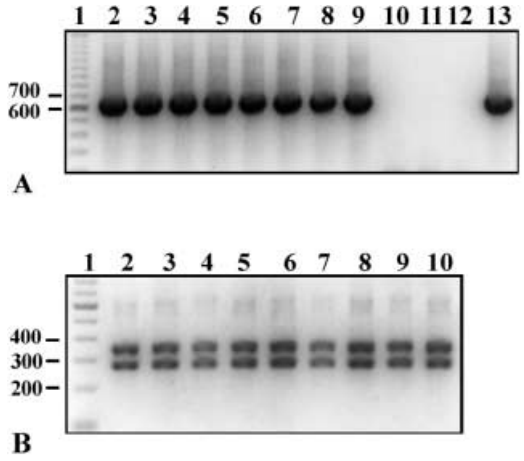

Fig. 3A, B PCR analysis of excision events in chlorsulfuron semi-resistant F2 seedlings. PCR assay (**A**) and *Nco*I restriction analysis (**B**) for detecting and confirming *Ds* excision respectively, among F2 and control Westar seedlings. **A, B** DNA molecular weight marker (*lane 1*); cotyledons (*lanes 2 to 5*) and primary true leaves (*lanes 6 to 9*) from four chlorsulfuron semi-resistant F2 seedlings derived from the cross 62-13 × 83-1B (family no. 2). **A** Westar seedling (*lane 10*); *Ds* parent seedling, 62-13 (*lane 11*); Ac^{st} parent seedling, 83-1B (*lane 12*) and *Ds* excision control seedling, 61-1 (*lane 13*). **B** *Ds* excision control seedling, 61-1 (*lane 10*)

size, 361 bp and 284 bp, confirming that the PCR amplified authentic *Ds* excision events (Figs. 1B and 3B). Similar results were obtained in PCRs performed on DNA isolated from chlorsulfuron semi-resistant individuals from all 50 F2 populations (data not shown). DNA isolated from chlorsulfuron sensitive F2 seedlings failed to produce products in either the primary or the nested reaction.

To examine the *Ds* excision events in greater detail we sequenced the excision products from five individuals representing independent F2 families derived from both 35S-Ac^{st} and *rbcS*-Ac^{st} parents. As expected, the sequence data confirm that *Ds* excised in each of the F2 individuals (Fig. 4). The data also showed that excision was not precise and typical *Ds* footprints were generated at the site of excision (Fig. 4). Small deletions of up to three bp of 5′ and two bp of 3′ flanking *Ds* sequences were observed in all individuals. In addition, nucleotide substitutions occurred at, or very near, the point of *Ds* excision in two of the five individuals examined (Fig. 4).

These results indicate that functional *Ac* transposase is produced in transgenic *Brassica* plants and that chlorsulfuron semi-resistant F2 individuals represent individuals in which the *Ds* element is somatically active. Results also indicate that *Ds* excises in both embryonic (cotyledons) and non-embryonic (primary leaves) tissues (Fig. 3).

Genetic evidence for *Ds* somatic excision events in *B. napus*

To further confirm that the semi-resistant phenotype was due to somatic excision events, we investigated whether this phenotype co-segregated with the presence of both Ac^{st} and *Ds* elements in the progeny from various crosses. Table 1 compares the observed number of chlorsulfuron semi-resistant seedlings with the number of seedlings predicted to contain both Ac^{st} and *Ds*. The genetic analysis is based on the assumption that Ac^{st} and *Ds* loci segregate independently. In nine of the 16 F2 families there is good agreement between the observed and expected values in all or most F2 populations suggesting that the vast majority of plants containing both Ac^{st} and *Ds* exhibit chlorsulfuron semi-resistance as the result of *Ds* excision. In the remaining seven families there are significant differences between the observed and expected values in all F2 populations. However, we still observe that the majority of the progeny in these families express chlorsulfuron semi-resistance. In most of these populations the observed number of semi-resistant progeny most closely match the predicted value for the segregation of a single active Ac^{st} and *Ds* element (Table 1). This suggests that not all loci are active in these lines.

Excision of *Ds* is dependent on the activity of the Ac^{st} line

During our analysis, we observed that 13 F2 families produced populations with no evidence of somatic activity. This led us to examine what factors may have influ-

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                    SalI   11bp TIR      11bp TIR   SalI
1  gtggatccctGTCGACgCAGGGATGAAA//TTTCATCCCTAtGTCGACggtactcgataa
2  gtggatccctGTCGACc-------------------------GTCGACggtactcgataa
3  gtggatccctGTCG----------------------------GTCGACggtactcgataa
4  gtggatccctGTCGA---------------------------TCGACggtactcgataa
5  gtggatccctGTCGACc-------------------------GTTGACggtactcgataa
6  gtggatccctGTCGAC--------------------------GTCGACggtactcgataa
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Fig. 4 Sequence analysis of *Ds* excision footprints in chlorsulfuron semi-resistant F2 seedlings. DNA sequences of the 5′ and 3′ junctions of the *Ds* element in pAB62 (*1*), the corresponding region in the *Ds* excision products isolated from F2 *B. napus* individuals derived from the crosses 62-48 × 81-3B (family no. 14) (*2*), 62-27 × 81-7 (family no. 12) (*3*), 62-13 × 83-1B (family no. 2) (*4*), 62-48 × 80-6 (family no. 5) (*5*) and 80-10 × 62-13 (family no. 1) (*6*). TIR terminal inverted repeat. The 11-bp TIR sequences are indicated in *bold uppercase*; the *Sal*I restriction sites are indicated in *uppercase*; the T-DNA sequences flanking *Ds* are indicated in *lowercase*; missing nucleotides are indicated by *dashes* and base substitutions are *underlined*

enced *Ds* activity. The data in Table 1 clearly indicate that the 35S-*Ac*st and *rbcS*-*Ac*st elements are both capable of trans-activating the *Ds* element. Among the 16 F2 families displaying *Ds* excision activity, nine are 35S-*Ac*st and seven are *rbcS*-*Ac*st families. Of the 13 F2 families that lack detectable *Ds* activity, five are 35S-*Ac*st and eight are *rbcS*-*Ac*st families. Since both active and inactive F2 families were derived from the same *Ds* parents, but never from the same *Ac*st parents, we can conclude that the *Ac*st lines used for generating the F2 families is the largest variable in determining *Ds* excision activity.

The results were then analyzed to determine if the *Ac*st copy number affects *Ds* excision activity. It is clear from the data that excision occurred in the presence of one to multiple *Ac*st loci. It is also clear that some F2 plants containing a single element were as effective in promoting *Ds* excision as F2 plants containing multiple elements (compare activities in families 62-48 × 80-4 (family no. 4) and 62-48 × 80-6 (family no. 5) and families 62-48 × 84-2 (family 15) and 62-48 × 84-4 (family no. 16)).

The *Ac*st transcript is not fully processed in *B. napus*

Studies done in other systems suggest that excision frequency is dependent upon the efficiency of *Ac* transcript processing. We therefore utilized RT-PCR to analyze how efficiently the four introns of the *Ac* transposase transcript (Fig. 5A) are processed in F2 *Brassica* individuals. Our analysis was performed on somatically active F2 plants derived from crosses with the *Ds* parent, 62-48, and two different *Ac*st parents, 35S-*Ac*st parent 80-6 (family no. 5) and *rbcS*-*Ac*st parent 84-4 (family no. 16). *Ac* transcript-processing in these *Brassica* individu-

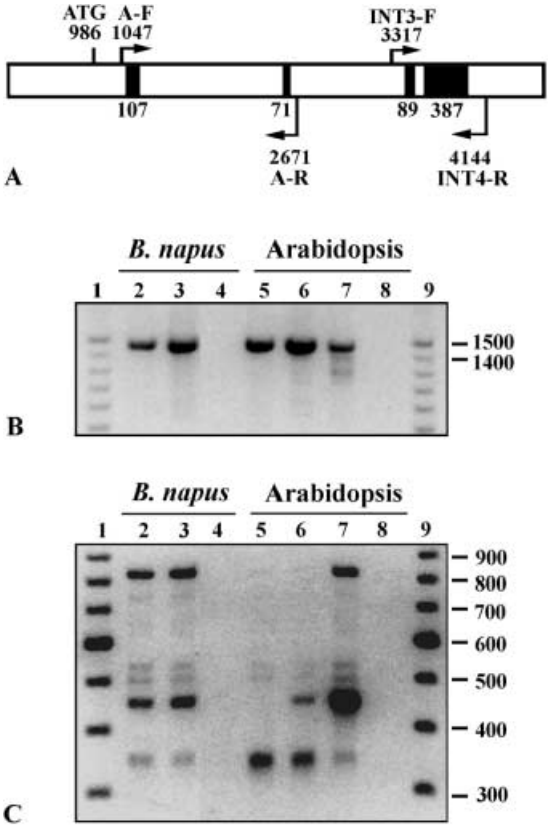

Fig. 5A–C RT-PCR analysis of the *Ac*st transcript in F2 *B. napus* and Arabidopsis plants. **A** Structure of the coding region of the *Ac*st transposase gene. *Small arrows* indicate the approximate position and orientation of DNA primers. Primer names as indicated. **B** Splicing of introns I and II (1,446-bp product corresponds to complete splicing of introns I and II). **C** Splicing of introns III and IV (351-bp product corresponds to complete splicing of introns III and IV; 440-bp product corresponds to splicing of intron IV only, 738-bp product corresponds to splicing of intron III only, and 827-bp product corresponds to no splicing of introns III and IV). **B and C** molecular-weight marker (*lanes 1 and 9*); somatically active *B. napus* F2 plants derived from crosses 62-48 × 80-6 (family no. 5) (*lane 2*) and 62-48 × 84-4 (family no. 16) (*lane 3*); Westar (*lane 4*); high germinal activity (55%) Arabidopsis F2 plants derived from population FA1608-9 (*lane 5*); low germinal activity (4%) Arabidopsis F2 plants derived from population FA1585-5 (*lane 6*); germinally inactive, somatically active Arabidopsis F2 plants derived from population FA1676-1 (*lane 7*); No-0 (*lane 8*)

Table 1 Genetic analysis of *Ds* and *Ac*st *B. napus* parents and their F2 families

Family no.	Crosses[a]		T-DNA loci[c]		Lc^{+}:Lc^{o} [d]	No. of chlorsulfuron semi-resistant seedlings		
	Ds parent	*Ac*st parent[b]	*Ds*	*Ac*st		Observed	Expected	
							All loci active[e]	1 *Ac*st and 1 *Ds* active[f]
1	62-13	80-10[a]	2	2	167:9	107	154.69	99.00*
			2	2	176:8	105	161.72	103.50*
			2	2	154:12	109	145.90	93.38
			2	2	147:12	123	139.75	89.44
2	62-13[a]	83-1B	2	≥3	162:0	87	149.50	91.13*
			2	≥3	175:0	89	161.50	98.44*
			2	≥3	175:0	75	161.50	98.44
			2	≥3	162:0	90	149.50	91.13*
3	62-48[a]	80-3	1	2	170:6	117	123.75*	
4	62-48[a]	80-4	1	1	123:39	85	91.13*	
			1	1	121:47	80	94.50*	
			1	1	132:43	82	98.44	
5	62-48[a]	80-6	1	≥3	187:0	132	138.06*	
			1	≥3	189:0	129	139.54*	
			1	≥3	172:0	131	126.98*	
			1	≥3	177:0	119	130.68*	
			1	≥3	181:0	121	133.63*	
6	62-48[a]	80-7	1	≥3	171:0	132	126.25*	
7	62-48	80-8[a]	1	≥3	163:0	102	120.34	91.69*
			1	≥3	172:0	97	126.98	96.75*
			1	≥3	177:0	99	130.68	99.56*
			1	≥3	169:0	101	124.77	95.06*
8	62-48	83-3A[a]	1	3	158:0	97	116.65	88.88*
			1	3	159:3	100	119.60	91.13*
9	62-59	83-1B[a]	2	≥3	142:0	83	131.04	79.88*
			2	≥3	159:0	87	146.73	89.44*
			2	≥3	147:0	79	135.66	82.69*
			2	≥3	151:0	85	139.35	84.94*
			2	≥3	155:0	73	143.04	87.19*
10	62-10	81-3C[a]	1	1	134:47	116	101.81*	
11	62-13[a]	84-5	2	≥3	182:0	167	167.96*	
			2	≥3	191:0	162	176.26	
			2	≥3	194:0	173	179.03*	
			2	≥3	187:0	187	172.57	
12	62-27[a]	81-7	2	2	163:10	116	152.05	97.31
			2	2	156:13	107	148.54	95.06*
			2	2	159:12	117	150.29	96.19
			2	2	172:9	117	159.08	101.81*
			2	2	171:12	119	160.84	102.94
			2	2	167:11	109	156.45	100.13*
			2	2	162:11	111	152.05	97.31*
13	62-48[a]	81-1A	1	1	131:51	88	102.38*	
14	62-48[a]	81-3B	1	1	137:57	131	109.13	
			1	1	138:52	127	106.88	
15	62-48[a]	84-2	1	1	137:36	85	97.31*	
			1	1	125:37	80	91.13*	
16	62-48[a]	84-4	1	≥3	186:0	107	137.32	
			1	≥3	189:0	127	139.54*	
			1	≥3	193:0	121	142.49	
			1	≥3	178:0	132	131.41*	

* Indicates non-significant difference $P > 0.02$

[a] Female parent

[b] The 35S-*Ac*st parents are designated as 80-X and 83-X; the *rbcS*-*Ac*st parents are designated as 81-X and 84-X

[c] All single locus lines contain a single T-DNA insertion

[d] Data based on plating 200 F2 seeds; the average germination frequency is 86.8%

[e] No. of plants predicted to contain *Ac*st and *Ds*, a locus number of 3 was used when the *Ac*st locus number was ≥3

[f] No. of plants predicted to contain one *Ac*st locus and one *Ds* locus

als was also compared to that in F2 Arabidopsis plants containing the identical 35S-*Ac*st and *Ds* elements. These Arabidopsis plants represent three F2 populations that were derived from crosses with one *Ds* parent and three independent 35S-*Ac*st parents conferring different levels of germinal excision activity. The Arabidopsis F2 population, FA1608-9, displays high germinal excision activity (55%), population FA1585-5 displays low germinal excision activity (4%) and population FA1676-1 has no detectable germinal activity (C. Chong, M. Supino and C. Waddell, unpublished data). Both of the germinally active lines have high levels of somatic activity while the germinally inactive line displays low somatic activity that, like the *Brassica* lines, requires a nested PCR to detect at the molecular level (data not shown).

Overall, our RT-PCR data suggest a direct correlation between the ability to process *Ac* transposase transcript and the frequency of somatic and germinal excision events. The processing defects are specifically observed with introns III and IV. RT-PCR analysis of introns I and II revealed the presence of a single PCR product, 1446 bp in size, among all Arabidopsis and *Brassica* plants (Fig. 5B), indicating that introns I and II are processed efficiently in both genera irrespective of transposition frequency. RT-PCR analysis of introns III and IV generated a number of different sized products, revealing that these introns are not always completely processed. Nonetheless, the fully spliced transcript (351-bp product) was observed among all Arabidopsis and *Brassica* plants (Fig. 5C). This fully spliced transcript is the primary product in the Arabidopsis high germinal excision activity line FA1608-9 (Fig. 5C, lane 5). All other populations generated additional PCR products indicative of incomplete transcript splicing. Arabidopsis plants from population FA1585-5, which displays low germinal excision activity, generated an additional 440 bp PCR product, the predicted size for splicing of intron IV only (Fig. 5C, lane 6). Population FA1676-1, which is germinally inactive but somatically active, generated this same 440-bp product as well as the full-length, unprocessed transcript (827-bp product) (Fig. 5C, lane 7). In addition to these two products, a very weak product is detected at the size predicted for splicing of intron III only (738 bp). This same profile of PCR products is seen in the two somatically active *Brassica* individuals (Fig. 5C, compare lanes 2 and 3 to lane 7). Two minor PCR products (approximately 500 and 525 bp) are also seen in all F2 plants (Fig. 5C). While the sizes of these products do not correspond to incomplete processing of introns III and IV, they may represent products arising from the use of alternative 5′ and 3′ splice sites within intron IV (Martin et al. 1997). Alternatively, they may represent non-specific products associated with the *Ac*st sequences present in all groups of plants.

Discussion

A stabilized version of the maize *Ac* element, *Ac*st, when introduced in *B. napus*, is capable of producing functional transposase and catalyzing the excision of a chimeric *Ds* element in somatic tissue. As is generally observed in other hosts, excision of the *Ds* element was imprecise and caused deletions and substitutions of the sequences at the excision site thereby creating characteristic *Ds* footprints. *Ac*st mediated excision of *Ds* in *B. napus* is therefore mechanistically similar to that reported in other species such as the native host, maize (Scott et al. 1996), as well as in heterologous hosts like tobacco, tomato and Arabidopsis (reviewed by Kunze et al. 1997). This result suggests that any host factors required for the excision step in transposition are also present in *Brassica*.

PCR data confirmed that while *Ds* excises in both embryonic and non-embryonic *B. napus* cells, the level of somatic excision events is low. This is based on the observation that *Ds* excision could only be detected in a nested PCR reaction; no evidence of transposition activity could be detected by Southern analysis (data not shown). To estimate the frequency of *Ds* excision in somatic tissues, the following was done. We performed a reconstruction experiment to simulate somatic *Ds* excision events occurring over a wide range of frequencies in *B. napus* tissues and determined the minimum number of excision events that could be detected by the nested PCR assay employed in this study. The reconstruction was created by preparing serial dilutions of DNA isolated from the *Ds* excision control into DNA isolated from non-transformed Westar. With these samples we subsequently determined that the nested PCR assay could detect as few as one excision event occurring in a population of one to two million cells. A similar dilution analysis was done with a single somatically active F2 population (family number 11). The results indicated that somatic activity was well above the detection limit at one excision event in 50,000 to 100,000 cells. This low frequency of *Ds* excision, possibly coupled with their spatial-temporal characteristics in somatic cells, was probably not ideal for being propagated into germinally inherited events. Plants form their gametes late in somatic development when shoot meristems convert into inflorescence or floral meristems. As a result, plants often pass somatic changes on to their progeny (Eisses et al. 1997). Based on our failure to identify germinal excision events, we estimate that germinal *Ds* excision occurs within a population of plants at a frequency of < 0.2% in this *Ac*st/*Ds* system.

Since *Brassica* and Arabidopsis are highly related genera, we believed that the *Ac*st/*Ds* system would have similar activity in the two heterologous hosts. For this reason we expressed transposase under the strong 35S and *rbcS* promoters; these transposase fusions are very effective in Arabidopsis (Honma et al. 1993). Surprisingly, these promoters are unable to promote high *Ds* excision frequencies in *Brassica*. To test the possibility that our *Ac* and *Ds* T-DNA constructs suffered deleterious changes, we introduced the identical T-DNA elements into Arabidopsis and tested all components for activity (data not shown). We found that the *Ac*st/*Ds* system was highly active and that *Ac*-encoded transposase generated

Ds germinal excision frequencies as high as 68% (C. Chong, M. Supino and C. Waddell, unpublished data). This observation suggests that the elements introduced into *B. napus* are fully functional.

Low excision frequencies can be the direct result of low levels of functional transposase. It is documented in several heterologous hosts that misprocessing of the *Ac* transcript is responsible for low levels of functional transposase (Grevelding et al. 1992; Finnegan et al. 1993; Takumi et al. 1999). Consequently, we determined how efficiently the *Ac* transcript was processed in *B. napus* cells and whether the level of processing might have had an effect on *Ds* excision events. RT-PCR analysis indicated that only a small proportion of the total Ac^{st} transcripts, expressed from both the 35S and *rbcS* promoters, was properly processed in *B. napus* F2 plants. The processing pattern was similar to that seen in somatically active but germinally inactive Arabidopsis F2 plants. In these plants, introns I and II are efficiently spliced, while introns III and IV are not. This pattern of transcript processing is generally similar to what has been reported for Arabidopsis *Ac* lines (Jarvis et al. 1997; Martin et al. 1997), except we observe less efficient splicing of intron III compared to intron IV. Our results lead us to conclude that the inability to process introns III and IV efficiently among *B. napus* and some Arabidopsis plants is a major cause of reduced *Ds* excision events and the lack of germinal activity.

Both correctly and incorrectly spliced transcripts have intact nuclear localization signals and DNA binding motifs (Boehm et al. 1995). Therefore, if partially spliced polyadenylated *Ac* mRNA is translated in *Brassica*, it could translocate to the nucleus and compete with the correctly made *Ac* transposase for binding sites in the *Ds* element (Martin et al. 1997). If this is occurring, *Ds* excision events would be significantly reduced and this can explain why germinal excision events are not recovered among our *Brassica* plants. This would suggest that high levels of transposition activity require that functional transposase be made over some threshold level.

It should be noted that while a germinally active *Ac*/*Ds* system in *B. napus* would greatly facilitate gene cloning endeavours, a somatically active system also has utility and can be used to perform somatic transposon-tagging (Hehl 1994). *Ds* elements can create visible somatic mutations by tagging a dominant allele of a given gene present in the heterozygous state. Since it is a somatic event, a single *Brassica* leaf can potentially carry a very large number of independent tagging events, representing multiple alleles. Many of these events can be recovered by regenerating fertile plants from the mutant tissue sectors and thereby reducing the number of plants that must be screened. This procedure has been used successfully to tag the semi-dominant *Sulfur* gene of tobacco (Fitzmaurice et al. 1999). Like tobacco, *B. napus* is an amphidiploid species that is easily regenerated into fertile plants using standard tissue culture practices and may therefore serve as an appropriate plant system for somatic transposon-tagging experiments. Furthermore, many of the genes that are of great interest in *Brassica* species are disease resistance genes (*R* genes) which are often single dominant genes, like the *N* gene of tobacco (Dinesh-Kumar et al. 1995).

In this pilot study, we demonstrate for the first time that the *Ac*-encoded transposase can catalyze the excision of a chimeric *Ds* element in the very important crop plant, *B. napus*. This system therefore has the potential to be used in tagging and isolating genes of interest. However, since we also show that *Ds* somatic excision frequency is low, further endeavours will first be required to increase this level of activity. Since our data strongly suggest that this low level of activity is in part due to the inefficient processing of the *Ac* transcript, it is worth exploring the utility of the *Ac* cDNA to supply transposase. Clearly, once transposition activity can be increased, *B. napus* seems very amenable to somatic-transposon tagging and germinal tagging experiments using an *Ac*/*Ds* system.

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