Characterization of *Stowaway* MITEs in pea (*Pisum sativum* L.) and identification of their potential master elements

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Abstract: We have investigated miniature inverted-repeat transposable elements (MITEs) of the *Stowaway* family and corresponding *Mariner*-like master elements that could potentially facilitate their mobilization in the genome of the garden pea (*Pisum sativum* L.). The population of pea *Stowaway* MITEs consists of 10^3 – 10^4 copies dispersed in the genome. Judging from a sequence analysis of 17 isolated *Stowaway* elements and their flanking genomic regions, the elements are relatively uniform in size and sequence and occur in the vicinity of genes as well as within repetitive sequences. Insertional polymorphism of several elements was detected among various *Pisum* accessions, suggesting they were still transpositionally active during diversification of these taxa. The identification of several *Mariner*-like elements (MLEs) harboring intact open reading frames, capable of encoding a transposase, further supports a recent mobilization of the *Stowaway* elements. Using transposase-coding sequences as a hybridization probe, we estimated that there are about 50 MLE sequences in the pea genome. Among the 5 elements sequenced, 3 distinct subfamilies showing mutual similarities within their transposase-coding regions, but otherwise diverged in sequence, were distinguished and designated as *Psmar-1* to *Psmar-3*. The terminal inverted repeats (TIRs) of these MLE subfamilies differed in their homology to the TIRs of *Stowaway* MITEs. The homology ranged from 9 bp in *Psmar-3* to 30 bp in *Psmar-1*, which corresponds to the complete *Stowaway* TIR sequence. Based on this feature, the *Psmar-1* elements are believed to be the most likely candidates for the master elements of the *Stowaway* MITEs in pea.

Key words: *Mariner*-like transposons, master elements, *Stowaway* MITEs, insertional polymorphism, *Pisum sativum*.

Résumé : Les auteurs ont étudié les transposons MITE (« miniature inverted-repeat transposable elements ») de la famille *Stowaway* et les éléments autonomes correspondants de type *Mariner*, lesquels pourraient potentiellement causer la mobilité de ces éléments chez le petit pois (*Pisum sativum* L.). Le génome du pois compte 10^3 – 10^4 copies dispersées de MITE *Stowaway*. L'analyse de la séquence de 17 éléments *Stowaway* et de l'ADN génomique qui les borde a révélé que ceux-ci sont relativement uniformes tant en matière de taille que de séquence et qu'ils sont situés à la fois à proximité de gènes et au sein de séquences répétées. Du polymorphisme insertionnel pour plusieurs éléments a été détecté parmi diverses accessions de *Pisum*, ce qui suggère qu'ils étaient encore mobiles lors de la différenciation de ces taxons. L'identification de plusieurs éléments de type *Mariner* (MLE, « *Mariner*-like elements ») ayant des cadres de lecture ouverts intacts, capables de coder pour une transposase, vient appuyer davantage l'hypothèse d'une récente mobilité de ces éléments *Stowaway*. En employant la séquence codant pour la transposase comme sonde, les auteurs estiment qu'il y aurait environ 50 séquences MLE au sein du génome du pois. Parmi les 5 éléments séquencés, 3 sous-familles distinctes montrant des similitudes au sein des régions codant pour la transposase, mais autrement assez divergentes, ont été observées et appelées *Psmar-1* à *Psmar-3*. Les répétitions terminales inversées (TIR) de ces sous-familles de MLE présentaient différents degrés d'homologie avec les TIR des MITE *Stowaway*. L'homologie variait entre 9 pb chez *Psmar-3* et 30 pb chez *Psmar-1*, ce qui, dans ce dernier cas, correspond à la séquence complète du TIR de *Stowaway*. Sur cette base, les éléments *Psmar-1* sont considérés comme étant les meilleurs candidats à titre d'éléments autonomes des MITE *Stowaway* chez le pois.

Mots clés : transposons de type *Mariner*, éléments autonomes, MITE *Stowaway*, polymorphisme insertionnel, *Pisum sativum*.

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Introduction

Nuclear genomes of the majority of higher plants are predominantly composed of numerous families of repetitive elements differing in their origin, genomic organization, and nucleotide sequences. Among these families, mobile elements (also called transposons) often represent the most abundant fraction (Kumar et al. 1997; SanMiguel and Bennetzen 1998; Neumann et al. 2001). This is mainly due to the accumulation of retrotransposons, elements that spread

through the genome via a copy-and-paste mechanism involving RNA intermediate (Bennetzen 2000). The other class of mobile elements, called DNA transposons, move within genomes using an excision–reintegration (cut-and-paste) mechanism and are therefore only moderately abundant. DNA transposons are characterized by the presence of terminal inverted repeats (TIRs) at their ends and, in the case of autonomous elements, by encoding a transposase, which facilitates their mobilization (reviewed in Galun 2003). Structurally distinct mobile elements that share some features with DNA transposons but are small and lack any coding capacity are called miniature inverted-repeat transposable elements (MITEs). First discovered in maize (Bureau and Wessler 1992), a number of MITEs distinguished by their nucleotide sequences were later identified in both monocot and dicot plants and also in animals (Feschotte et al. 2002a; Feschotte et al. 2002b). In contrast with other DNA transposons, MITEs occur in high copy numbers in plant genomes, reaching up to 10^4 copies per haploid genome (Bureau and Wessler 1992; Mao et al. 2000; Zhang et al. 2000). Recent progress in sequencing and computer analysis resulted in the classification of the majority of MITEs as nonautonomous deletion derivatives of longer DNA transposons belonging to either of the 2 main superfamilies, *Tc1/Mariner* and *PIF/Harbinger* (Feschotte et al. 2002b). This classification is based on sequence similarities of the TIRs and target site duplications (TSDs) of MITEs and respective DNA transposons, which reflect their mobilization by the same transposases encoded by the full-length DNA transposons (also called “master elements”).

Stowaway elements (Bureau and Wessler 1994) rank among the most abundant plant MITEs in terms of both their distribution in various plants taxa and their copy numbers in individual plant species (Bureau and Wessler 1994; Mao et al. 2000; Feschotte et al. 2002b; Feschotte et al. 2003). In the genome, *Stowaway* elements occur in close association with genes (Mao et al. 2000), which makes them ideal for designing molecular markers that target coding sequences. *Stowaway* elements are defined by having TIRs that include a 10-bp consensus sequence (5'-CTCCCTCCRT-3') and by generating TSD of the dinucleotide TA (Bureau and Wessler 1994; Feschotte et al. 2003). These features led to their association with the *Tc1/Mariner* superfamily of transposons (Feschotte et al. 2002b), more specifically with the group of *Mariner*-like elements (MLEs) (Feschotte et al. 2003). Although PCR-based detection of conserved domains coding for *Mariner*-like transposases revealed their presence in a wide range of plants (Feschotte and Wessler 2002), the full-length *Mariner*-like transposons that could act as master elements for *Stowaway* MITEs have been isolated from only soybean and rice (Jarvik and Lark 1998; Feschotte et al. 2003). Similarly, a comprehensive characterization of *Stowaway* elements is available for only a small number of extensively sequenced plant genomes, whereas information from other species is absent or very limited.

As part of our investigation of repetitive sequences present in the pea genome (*Pisum sativum* L.) we aimed to characterize the population of *Stowaway* MITEs. Owing to relatively limited amounts of sequence data from this species, only 3 copies of *Stowaway* have previously been identified using computer analysis of pea DNA sequences (Bureau

and Wessler 1994; Macas et al. 2003). Thus, to investigate the sequence diversity of these elements, we cloned and sequenced additional copies and characterized the abundance and insertional polymorphisms of *Stowaway* MITEs in selected taxa of pea. Moreover, we isolated and sequenced several full-length or partial *Mariner*-like elements, the potential “masters” of *Stowaway* MITEs. We analyzed these elements with respect to their structure, coding capacity, and eventual association with the *Stowaway* MITEs.

Materials and methods

Plant material and genomic DNA isolation

Seeds of plant species used in this study were obtained from the Plant Breeding Station at Borsov, Czech Republic (*Pisum sativum* L. ‘Carrera’), and the Western Regional Plant Introduction Station, Pullman, Washington, USA (*Pisum sativum* subsp. *sativum* (accession number PI116056), *Pisum sativum* subsp. *asiaticum* Govor. (W615036), *Pisum sativum* var. *arvense* (L.) Poir. (PI268480), *Pisum fulvum* Sm. (PI560063), *Pisum sativum* subsp. *elatius* (Steven ex M. Bieb.) Meikle (W615043), *Pisum sativum* var. *elatius* (Steven ex M. Bieb.) Meikle (W616220), *Pisum sativum* subsp. *abyssinicum* (A. Braun) Govor. (PI358610), *Pisum sativum* subsp. *transcaucasicum* Govor. (W612704), and *Pisum sativum* var. *pumilio* Meikle (PI560068)). Total genomic DNA was extracted from leaves pooled from 4–5 plants as described by Dellaporta et al. (1983). All DNA concentration measurements were done using the PicoGreen dye (Molecular Probes, Eugene, Oregon, USA) according to the manufacturer’s recommendations.

Cloning of *Stowaway* and *Mariner*-like elements

The “shotgun” genomic library was prepared from *P. sativum* ‘Carrera’ DNA subjected to sonication, mung bean nuclease treatment, and size fractionation on an agarose gel. The fragments of about 700–1000 bp were purified from the gel, treated with polynucleotide kinase in the presence of ATP, and cloned into *Sma*I-cut and dephosphorylated plasmid vector (pBluescript II SK+; Stratagene, La Jolla, Oregon, USA). The library was screened by colony hybridization with the probe prepared by PCR using primers TIR1 (5'-CCT CCG TTC CAT TTC AAG TGT C-3') and TIR2 (5'-TTA CTT CCT CTG TTT T-3') designed according to TIR sequences of 3 known pea *Stowaway* elements (*Stow-Ps1* – *Stow-Ps3*, Table 1) and *P. sativum* genomic DNA as a template. PCR was performed in 50 µL of reaction mix consisting of 1× PCR buffer, 1.5 mmol MgCl₂/L, 0.2 mmol dNTPs/L, 0.2 µmol primers/L, 2.5 U of *Taq* polymerase (Promega, Madison, Wisconsin, USA), and 20 ng of template DNA. The reaction involved 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. PCR was preceded by an initial denaturation (94 °C for 2 min) and followed by a final extension step (72 °C for 10 min). The reaction products were labeled and hybridized using the AlkPhos Direct™ hybridization and detection kit (Amersham Biosciences, Freiburg, Germany). Probe detection was performed using CDP-Star® substrate (Amersham Biosciences) and the signals were captured on X-ray film. In addition to the short-insert “shotgun” library, the pea genomic library cloned into a phage vector (Lambda DASH II, Stratagene)

Table 1. *Stowaway* elements identified in the pea genome. TSD, target site duplication.

Element name	Length (bp)	TSD (5'/3') ^a	Clone name ^b	Length (bp)	GenBank accession	Element position	Notes
<i>Stow-Ps1</i>	275	cA/Tt	PEALCTN	3881	L11745	1548–1274	Identified by Bureau and Wessler (1994) in 5' flanking region of lectin gene
<i>Stow-Ps2</i>	274	cc/TA	PEACAB80	1993	K02067	123–396	Identified by Bureau and Wessler (1994) in 5' flanking region of CAB binding protein gene
<i>Stow-Ps3</i>	275	TA/aA	CYP73A9-v1	4090	AF175275	1901–2175	Identified by Macas et al. (2003) in intron of trans-cinnamic acid hydroxylase gene
<i>Stow-Ps4</i>	270	TA/TA	c189	1576	AY833536	879–1148	—
<i>Stow-Ps5</i>		—/TA	c190	1341	AY833537	1–206 ^c	—
<i>Stow-Ps6</i>		—/TA	c229	904	AY833538	1–155 ^c	—
<i>Stow-Ps7</i>	270	TA/TA	c231	1000	AY833539	389–658	—
<i>Stow-Ps8</i>		—/TA	c232	524	AY833540	1–196 ^c	—
<i>Stow-Ps9</i>	275	TA/TA	c233	572	AY833541	296–570	—
<i>Stow-Ps10</i>		—/Unknown	c234	950	AY833542	1–266 ^c	Right TIR not identified owing to sequence rearrangements (duplication/inversion) in this region.
<i>Stow-Ps11</i>	274	TA/TA	c238	1555	AY833543	379–652	—
<i>Stow-Ps12</i>	274	TA/TA	c239	1566	AY833544	989–1262	—
<i>Stow-Ps13</i>	274	TA/TA	c242b	1626	AY833545	137–410	—
<i>Stow-Ps14</i>	274	TA/TA	c243	1250	AY833546	127–400	—
<i>Stow-Ps15</i>	274	TA/TA	c246	1501	AY833547	233–506	—
<i>Stow-Ps16</i>	273	TA/TA	c247	1268	AY833548	368–508 + 785–917	Contains insertion of <i>Stow-Ps17</i>
<i>Stow-Ps17</i>	274	TA/TA	c247	1268	AY833548	509–782	—

^aTwo nucleotides flanking 5' and 3' element ends, respectively, are shown. Sequences differing from the consensus TSD (TA/TA) are in lowercase.

^bIn the cases of previously reported sequences (*Stow-Ps1* to *Stow-Ps3*) GenBank loci names are shown.

^cElement truncated because of the cloning.

was screened with the same probe and positive clones were purified as described earlier (Macas et al. 2003).

A hybridization probe specific for *Mariner*-like elements was prepared by PCR amplification of 50 ng of pea genomic DNA using the primers MLE3A and MLE5A (Feschotte and Wessler 2002). The reaction was performed in 50 µL of 1× PCR buffer, 2.0 mmol MgCl₂/L, 0.2 mmol dNTPs/L, 0.2 µmol primers/L, 2.5 U of Platinum[®] *Taq* polymerase (Invitrogen, Carlsbad, California, USA), employing 35 cycles of the same temperature profile as described earlier, except for the annealing at 45 °C. The reaction products were cloned using TOPO TA Cloning[®] Kit (Invitrogen) and sequenced to verify amplification of the desired sequences. Equimolar amounts of the DNA from 3 of these clones (GenBank accessions AY833533–AY833535) were mixed and used for probe preparation and library screening as described earlier.

Sequencing and computer analysis

Positive clones identified by colony or plaque-lift hybridizations were sequenced using the dideoxy chain termination method (Sanger et al. 1977) either directly (short-insert plasmid clones) or following subcloning of selected restriction fragments (phage clones). In the case of subclones longer than 700–800 bp, the sequencing templates were generated using GeneJumper[™] Primer Insertion Kit (Invitrogen). Sequence assembly and basic analysis were done with Staden Package software (Staden et al. 1996) and program tools implemented at the Biology WorkBench website (<http://workbench.sdsc.edu/>). *Stowaway* and *Mariner*-like elements and

their surrounding regions were then tested for homology to other plant sequences using BLAST (Altschul et al. 1997), FASTA (Pearson and Lipman 1988), and Dotter (Sonnhammer and Durbin 1995). Homology searches in new GenBank entries using BLAST and FASTA were periodically repeated until October 2004. Multiple sequence alignments were performed using ClustalW (Thompson et al. 1994) and similarities within the alignments were visualized employing Boxshade (created by K. Hofmann and M.D. Baron) and WebLogo (Schneider and Stephens 1990; Crooks et al. 2004) programs.

Characterization of abundance, genomic organization, and insertional polymorphism

To estimate the copy number of *Stowaway* elements in the pea genome, serial dilutions of genomic DNA were quantitatively dot-blotted on Hybond[™] N+ membrane (Amersham Biosciences) together with a cloned fragment containing the *Stow-Ps3* element as a hybridization standard. The quantity of spotted genomic DNA corresponding to 10³ to 10⁵ copies of the haploid genome (1C) was compared with 10⁶ to 5×10⁹ copies of the hybridization standard. Dot blots were hybridized with the probe that was prepared by PCR using TIR1 and TIR2 primers as described earlier. The probe was labeled and hybridized as described for library screening. The hybridization and washing temperatures were varied from 45 to 55 °C to achieve moderate or high stringency. Alternatively, estimation of copy numbers for both *Stowaway* and *Mariner*-like elements were done based on the number of positive phage clones observed after the screening with the

respective probes, taking into account the pea genome size (1C = 4337 Mbp; Baranyi and Greilhuber 1996) and average insert size in the library (15 kbp).

Southern blots of the pea genomic DNA were prepared by running 2 µg aliquots digested with various restriction endonucleases on 1% or 2% agarose gels and blotting to Hybond N+ membrane. The blots were hybridized using the AlkPhos Kit, as described earlier, using hybridization and a washing temperature of 50 °C.

Screening for insertional polymorphisms of *Stowaway* elements in various pea accessions was performed using 20 ng of genomic DNA as a template in 30 µL reactions composed of 1× PCR buffer, 0.2 mmol dNTPs/L, 1.5 mmol MgCl₂/L, 0.2 µmol primers/L, and 1.2 U of Platinum *Taq* polymerase. Primer pairs used for amplification of individual loci are given in Table 2. The PCR was performed for 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The reaction was preceded by an initial denaturation (94 °C for 2 min) and followed by a final extension step (72 °C for 10 min). The amplification products were resolved by ethidium bromide-stained 2% agarose gel electrophoresis.

Results

Isolation and characterization of *Stowaway* elements

To isolate additional copies of pea *Stowaway* elements, PCR primers were designed based on conserved terminal sequences of the 3 previously identified elements (*Stow-Ps1*, *Stow-Ps2*, and *Stow-Ps3*, Table 1). PCRs, using pea genomic DNA as a template, amplified fragments of expected length (about 250 bp, data not shown), which were used as a probe to screen pea genomic libraries cloned into plasmid or phage vectors. Among 24 500 plasmid and 20 700 phage clones subjected to screening, 8 and 94, respectively, gave positive hybridization signals. These numbers indicated that there are about 1 500 copies of *Stowaway* elements per haploid pea genome. This estimate was also confirmed using quantitative dot-blot hybridization of serial dilutions of genomic DNA, which produced signals equivalent to 1 000–10 000 element copies (supplementary material, Fig. S1²). Thirteen randomly picked positive clones were sequenced and analyzed, yielding a total of 14 element sequences (Table 1). Except for 4 elements truncated because of the cloning process, all MITEs were 270–274 bp in length and the majority of them were flanked by a duplication of their integration site (dinucleotide TA). One clone (c247, accession No. AY833548) contained 2 copies of elements, with 1 element nested into another, and both of them surrounded by TSDs. Multiple alignment of all newly identified and the 3 previously described pea *Stowaway* sequences revealed their relative homogeneity (78%–98% nucleotide similarity between full-length elements) and the presence of 30-bp TIRs (Figs. 1 and 4A). The terminal parts of TIRs included a 10-bp signature motif (5'-CTCCCTCCGT-3') typical for *Stowaway* elements. Southern hybridization of the *Stowaway* probe to pea genomic DNA digested with various restriction endonuc-

leases produced mainly a continuous smear indicative of dispersed genomic organization of these sequences (Fig. 2).

Analysis of adjacent genomic sequences

We characterized the insertion sites of pea *Stowaway* MITEs by investigating the sequences of genomic regions surrounding the elements. Similarity searches of these regions were performed against known plant sequences (Table 2). In many cases, no detectable homologies or homologies to only poorly annotated entries prevented unambiguous classification of the surrounding sequences. However, there were at least 2 elements (*Stow-Ps4* and *Stow-Ps10*) located within putative gene encoding regions. *Stow-Ps4* was inserted within the intron of a gene sequence with high similarity to translation elongation factor genes and expressed sequence tags (ESTs) from several plant species. The sequences adjacent to *Stow-Ps10* were classified as potentially coding based on their homology to ESTs and predicted exon sequences from *Lupinus* and *Medicago*. On the other hand, we found insertion sites of 2 other elements (*Stow-Ps6* and *Stow-Ps12*) were located within repetitive sequences of retroelement origin. There were also instances (e.g., *Stow-Ps8*, *Stow-Ps11*) of significant similarities to GenBank accessions annotated as gene sequences but limited to intergenic regions that were due to our analysis containing repetitive elements.

Insertional polymorphism

The presence of *Stowaway* elements in respective genomic loci was tested for a total of 9 accessions (subspecies or cultivars) of *P. sativum* and *P. fulvum*. Primer pairs were designed according to 7 genomic sequences carrying element insertions identified in this study and also according to the element *Stow-Ps3* described in our previous work (Table 2). Three tested loci produced single bands in all accessions (*Stow-Ps4*, *Stow-Ps7*, and *Stow-Ps15*, data not shown); the band sizes corresponded to the size expected if the *Stowaway* element was present in these regions. Amplification of *Stow-Ps11* insertion site yielded fragments corresponding to the locus lacking the element in all accessions; however, 3 accessions yielded additional fragments of the size indicating the presence of the element (supplementary material, Fig. S2²). A typical insertional polymorphism was observed in the case of *Stow-Ps3* where each accession produced a single band corresponding to the locus either with or without the *Stowaway* element (Fig. 3A). This was also verified by cloning and sequencing the PCR products shown in the lanes 1, 3, 4, 5, 9, and 10. In all cases where the element was absent, there was no footprint detectable in the sequence, suggesting the element had never inserted into these loci (supplementary material, Fig. S3²). An interesting and highly polymorphic amplification pattern was observed in the case of *Stow-Ps16* + *Stow-Ps17*, which included 3 fragment sizes corresponding to the presence of 2, 1, or no element (Fig. 3B).

²Supplementary data for this article are available on the Web site or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0R6, Canada. DUD 4016. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Table 2. Flanking genomic sequences and insertional polymorphism of *Stowaway* elements.

Element	Flanking sequences homology ^a	Description	Insertional polymorphism PCR primers (5' to > 3')	Expected product (bp)	Polymorphism
<i>Stow-Ps3</i>	Trans-cinnamic acid hydroxylase gene (AF175275)	Gene	TGCTCAAAAGAAGGGTGAGA TGTAGGGATAATTTAGGAATAAATGC	526	Yes (Fig. 3A)
<i>Stow-Ps4</i>	AC149268.5 (BN: 9×10^{-57}), BAD30880.1 (putative translation elongation factor EF-1 alpha (<i>Oryza sativa</i>), BX: 1×10^{-13})	Gene	AGGCATCATAGGCAGCAAAG AAGTTGGTCTAGCCATATGTACCC	560	No
<i>Stow-Ps5</i>	—	—	Not tested	—	—
<i>Stow-Ps6</i>	AP004971 (F: 9.4×10^{-27}), U11716 (B: 2×10^{-15})	Retro-element	Not tested	—	—
<i>Stow-Ps7</i>	—	—	CGAATTTTCATCGGTCTTTTG TGGATTCCAAAACCTCAAAACC	468	No
<i>Stow-Ps8</i>	AF138704 (<i>P. sativum</i> gibberellin c20-oxidase gene, F: 5.6×10^{-27})	—	Not tested	—	—
<i>Stow-Ps9</i>	—	—	Not tested	—	—
<i>Stow-Ps10</i>	CA784088 (<i>Glycine max</i> EST, F: 1.2×10^{-4}), CA409569 (<i>Lupinus albus</i> EST, F: 8.8×10^{-4}), AC133864.10 (<i>Medicago truncatula</i> clone mth2-18d12, F: 1.8×10^{-2})	Putative gene	Not tested	—	—
<i>Stow-Ps11</i>	AB095738 (<i>P. sativum</i> OPR1 gene for 12-oxophytodienoic acid 10, 11-reductase, F: 1.5×10^{-35})	—	TCCCTTTCAAACATAGTCACG ATCGGATTGGAGACTGAACC	804	Yes
<i>Stow-Ps12</i>	D10003 (<i>P. sativum</i> gene for phenylalanine ammonia-lyase, F: 3.2×10^{-46}), AAG51247 (putative copia-type polyprotein (<i>Arabidopsis thaliana</i>), BX: 6×10^{-23})	Retro-element	Not tested	—	—
<i>Stow-Ps13</i>	X68216 (<i>P. sativum</i> PS-IAA4/5 gene, F: 3.9×10^{-17})	—	TTAGCCCCTACATGGAGAATGG GGGTTGTTCATGGTACAATTC	738	Yes ^b
<i>Stow-Ps14</i>	—	—	TTGTGAAAACTATCCTGAAATGTG GAAACACCACAGTATTTGGAGATG	597	No products
<i>Stow-Ps15</i>	U11716 (<i>P. sativum</i> disease resistance response protein 206-d gene, F: 8.2×10^{-43})	—	TTCATTTCCTCTTTATGTTTATAAGG CACTAGGATATGGAACATGGAGAC	849	No
<i>Stow-Ps16</i> + <i>Stow-Ps17</i>	AC134049.20 (<i>Medicago truncatula</i> clone mth2-21j3, F: 9.2×10^{-29}), AJ417911 (<i>M. truncatula</i> gene for Narf-like protein, F: 9.2×10^{-13})	—	AAGGACATAATTGAAAAAGTAACAA TC CCATTGTTCTTCACAACTCC	846	Yes (Fig. 3B)

^aAccession numbers of sequences displaying the best homologies are shown. The programs used to detect the homology (F, Fasta; BN, BlastN; BX, BlastX) and the score are given in parentheses.

^bNo amplification products appear in the majority of accessions.

Identification and characterization of putative master elements

Primers designed according to conserved regions of *Mari-ner*-like transposases (Feschotte and Wessler 2002) were used in PCR to test occurrence of corresponding sequences in the pea genome. The reaction resulted in amplification of fragments approximately 380 bp in length, appearing as a

single band on agarose gel electrophoresis. Cloning and sequencing of 3 randomly picked fragments (GenBank accessions AY833533–AY833535) revealed that their nucleotide sequences were highly similar (70%–87% mutual similarities) and displayed significant homologies to known plant MLE transposases. Moreover, each could be translated as a single open reading frame (no stop codons present) and

Fig. 1. Sequence logo showing conservation of nucleotides within pea *Stowaway* elements. The logo was created using the method of Schneider and Stephens (1990) as implemented on the WebLogo server (Crooks et al. 2004) and it is based on multiple sequence alignment of elements listed in Table 1. The alignment is available from the EMBL_Align database under accession number ALIGN_000801.

Fig. 2. Southern blot of pea genomic DNA digested with *Rsa*I (lane 1), *Mse*I (lane 2), *Dra*I (lane 3), *Eco*RI (lane 4), *Eco*RV (lane 5), *Bgl*II (lane 6), and *Xba*I (lane 7), and hybridized with the probe specific for *Stowaway* sequences. Restriction digests were resolved on 2% (lanes 1–3) or 1% (lanes 4–7) agarose gels. Restriction fragment lengths are indicated separately for lanes 1–3 (left) and 4–7 (right).

their deduced protein sequences confirmed their assignment as parts of the MLE coding sequences. Using the PCR-amplified fragments as a probe, the pea genomic libraries were screened to identify and sequence full-length elements. Among 150 000 phage clones screened, 29 gave positive hybridization signals, which corresponds to the presence of 53 MLE sequences per haploid pea genome. Five positive clones were subcloned and the corresponding elements were either partially or completely sequenced (Table 3). Two almost identical full-length *Mariner*-like elements were identified and because of their high similarity (99% over their entire length) were designated *Psmar*-1A and *Psmar*-1B. The

Fig. 3. Examples of insertional polymorphism of *Stowaway* elements. Primer pairs derived from sequences surrounding *Stow-Ps3* (A) and *Stow-Ps16* + *Stow-Ps17* (B) insertion sites were used to amplify corresponding loci from *P. sativum* ‘Carrera’ (lane 1), *P. sativum* subsp. *sativum* (lane 2), *P. sativum* subsp. *elatus* (lane 3), *P. sativum* var. *elatus* (lane 4), *P. sativum* var. *arvense* (lane 5), *P. sativum* var. *pumilio* (lane 6), *P. sativum* subsp. *abyssinicum* (lane 7), *P. sativum* subsp. *transcaucasicum* (lane 8), *P. sativum* subsp. *asiaticum* (lane 9), and *P. fulvum* (lane 10). Lane 11 contains a PCR with no template (negative control). The amplification products were resolved on ethidium bromide-stained 2% agarose gels.

elements *Psmar*-1A and *Psmar*-1B were 3204 and 3201 bp, respectively, and were inserted into unrelated genomic sequences. Their terminal inverted repeats included 31 nucleotides; in addition to this, the right TIRs included a 22-bp duplication. The terminal regions also displayed similarity to the TIRs of pea *Stowaway* MITEs, which spanned 30 bp (Fig. 4A). Both *Psmar*-1A and *Psmar*-1B elements harbor putative coding sequence of 1236 nucleotides uninterrupted by stop codons, thus potentially coding for proteins of 412 amino acids. These protein sequences were highly similar to *Mariner* transposases encoded by *Soymar1* and *Osmar1* ele-

Table 3. Characterization of clones containing *Mariner*-like sequences. ORF, open reading frame.

Element name	Length (bp)	ORF ^a	Clone name	Length (bp)	GenBank accession	Element position	Notes
<i>Psmar</i> -1A	3204	412 aa (872–2107)	c752_part	6339	AY833549	916–4119	Full-length element.
<i>Psmar</i> -1B	3201	412 aa (873–2108)	c756_part	5572	AY833550	1231–4431	Full-length element; 99% sequence similarity to <i>Psmar</i> -1A.
<i>Psmar</i> -2	Unknown	~ 412 aa ^b	c771_3end	4637	AY833551	(2771)–4451	Left TIR sequence not identified, right TIR ends at 4451. ORF contains mutations generating frameshifts and stop codons.
<i>Psmar</i> -3A	2205 (part.)	327 aa (1222–2399)	c775	2399	AY833552	195–2399	Partial sequence (3' truncated because of the cloning).
<i>Psmar</i> -3B	994 (part.)	—	c716	2588	AY833553	1595–2588	Partial sequence (3' truncated because of the cloning). The element sequence has 99% similarity to <i>Psmar</i> -3A except for an internal deletion of 1215 nucleotides at the position 2079.

^aOpen reading frame coding for putative transposase. The length of the deduced protein sequence and position of the ORF within the element (in parentheses) are given.

^bDeduced length after correction of frameshift and stop codon mutations.

ments (Jarvik and Lark 1998; Tarchini et al. 2000; Feschotte and Wessler 2002) as well as by previously unknown elements identified in the genome of *Medicago truncatula* (Fig. 4B).

In addition to *Psmar*-1A and *Psmar*-1B, three other *Mariner*-like elements were identified (Table 3). One of them, *Psmar*-2, probably represents a partially decayed element, which because of the accumulation of numerous mutations or sequence rearrangements lost its left TIR. However, the right TIR still could be identified, as well as the remnants of the coding sequence, which displayed similarity to other elements. Following corrections of 4 mutations (2 stop codons and 2 frameshifts), the protein sequence encoded by this region could be deduced and aligned with other MLE transposases (Fig. 4B).

The other 2 elements, *Psmar*-3A and *Psmar*-3B, displayed 99% mutual sequence similarity; but except for their coding regions, no homology to other elements was detected. Only the left TIR sequences were identified, as only partial sequences truncated at their right ends were obtained. Interestingly, these TIRs displayed much shorter homology to the pea *Stowaway* elements than *Psmar*-1; this homology was limited to the most terminal 9 bp (or 12 bp including 1 mismatch, Fig. 4A). As the sequence of *Psmar*-3B lacked 1215 bp because of internal deletion, only *Psmar*-3A was analyzed with respect to its coding capacity. An uninterrupted open reading frame was identified potentially coding for a protein with significant homology to those encoded by other *Psmar* elements (Fig. 4B).

Discussion

In the present study, we show that *Stowaway* MITEs are abundant in the pea genome, reaching 10^3 – 10^4 copies per haploid genome. Although the molecular mechanisms of MITE amplification remain to be explained, a comparably high abundance of some MITE families in various species suggests that these mechanisms are a common phenomenon (Feschotte et al. 2002a; Casacuberta and Santiago 2003). For

example, the *Stowaway* population in rice includes over 22 000 elements, which can be classified into a number of subfamilies based on their sequence similarity (Feschotte et al. 2003). In this respect, it is interesting that although the sample of the pea *Stowaway* sequences reported here is rather limited, the variability within the sample is surprisingly low. This observation cannot be fully explained by probe homology bias during the screening, as the hybridization stringency was only moderate to allow for identification of partially divergent targets. Moreover, the average mutual similarity of the 3 elements identified previously using a principally different approach (computer analysis of sequence database entries *Stow-Ps1*, *Stow-Ps2*, and *Stow-Ps3*) is very close to that of the whole set of the pea elements (84% versus 87%).

Pea *Stowaway* elements have all the features typical for this group of transposons. They are short, AT-rich (73% A + T), contain characteristic TIR sequences, and integrate into the dinucleotide TA (Bureau and Wessler 1994). Analysis of *Stowaway* elements present in the extensively sequenced rice genome revealed that they predominantly occur in the vicinity of potential gene sequences (Mao et al. 2000). This makes the *Stowaway* elements attractive for designing molecular markers based on insertional or inter-MITE polymorphisms (Casa et al. 2000; Chang et al. 2001) that could be targeted into gene-rich euchromatic regions. Our findings suggest that this approach is also feasible in pea, as at least part of the identified elements are located near potential gene sequences. Moreover, insertional polymorphism was already detected among various pea lines (subspecies and cultivars) for some of the elements (Fig. 3). On the other hand, some of the pea elements were found within repetitive (retroelement) sequences, which contrasts with the observation made about rice.

The successful identification of *Mariner*-like sequences in the pea genome is not surprising, as the presence of numerous subfamilies of MLE transposases was reported from a range of monocot and dicot species by using the PCR approach (Feschotte and Wessler 2002). In spite of these find-

Fig. 4. (A) Comparison of the TIR sequences of pea *Mariner*-like and *Stowaway* elements. Left (L) and reverse complements of right (R) TIRs are shown for 2 subfamilies of MLEs (*Psmar-1* and *Psmar-3*) and for the consensus sequence of *Stowaway* elements (*Stow-Ps*). In *Psmar-1*, the most terminal part of the right TIR is labeled R1, whereas the internally duplicated TIR region is labeled R2 (this sequence directly follows the R1 region). Identical nucleotides are indicated on black background. (B) Multiple alignment of predicted amino acid sequences of plant *Mariner*-like transposases. The sequences are derived from MLEs identified in pea (*Psmar*), soybean (*Soymar1*; Jarvik and Lark 1998), rice (*Osmar1*; Tarchini et al. 2000, Feschotte and Wessler 2002), and *Medicago truncatula* (Mt_AC137603; this is a newly identified element located at position 40833–44543 of the sequence AC137603.16). Position of the D.D39D motif is indicated with asterisks.

ings, up to now the only full-length elements have been reported for the soybean and rice genomes (Jarvik and Lark 1998; Tarchini et al. 2000; Feschotte and Wessler 2002). The assignment of the pea elements as *Mariner*-like transposons is strongly supported by their structure and the homology of their transposases, which are similar to those of *Soymar1* and *Osmar1* elements (Fig. 4B). In addition, the pea *Psmar* transposases include the D.D(39)D motif typical for plant MLEs (Feschotte and Wessler 2002).

The 5 sequenced pea elements represent 3 MLE subfamilies distinguished by their sequence similarity. We estimated that there are about 50 copies of MLEs; thus, the actual number of different subfamilies in the pea genome might be greater than the 3 reported here. A similar situation was reported for rice (Feschotte et al. 2003) and, based on the partial transposase sequences, also for other plant species (Feschotte and Wessler 2002). In addition, we made the

same observation when searching for potential MLEs in genomic sequences of a model legume plant *Medicago truncatula* in which several distinct subfamilies sharing sequence similarity only within their TIRs and transposase-coding regions were identified (J. Macas and P. Neumann, unpublished data). Contrary to a large proportion of the previously reported plant transposase-coding sequences, which were disrupted by mutations creating frameshifts or stop codons (Feschotte and Wessler 2002; Feschotte et al. 2003), only 1 of the 7 partial or full-length transposase sequences identified in pea was disrupted by mutations. This suggests that a large fraction of pea MLEs may be capable of producing functional transposase and thus mediating their own mobility as well as the nonautonomous *Stowaway* elements. It has been proposed that sequence identity of TIRs is required for cross-mobilization of nonautonomous MITEs by their master elements (Feschotte et al. 2003). Thus, the most

likely candidates for these master elements are the transposons of the *Psmar-1* subfamily, as their homology to *Stowaway* MITEs extends over the whole TIRs (Fig. 4A). In contrast, the similarity of *Psmar-3* TIRs to those of *Stowaway* and *Psmar-1* is limited to the most terminal region.

The present data obtained from pea as the representative of the legume family (Fabaceae) complement the findings reported for rice and other (mainly grass) species. It was shown that the pea genome contains a relatively homogeneous population of *Stowaway* MITEs in addition to several subfamilies of *Mariner*-like transposons that could potentially act as the master elements, providing functional transposase capable of mediating their mobility. This raises questions about the potentially ongoing transpositional activity of *Stowaway* elements in pea, which seems possible considering the presence of intact MLEs and the occurrence of insertional polymorphism of corresponding MITEs detected in different pea lines. Thus, the pea genome may represent a good model for investigating the biology and activity of *Mariner*-like and *Stowaway* elements in plants.

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