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En/Spm-LIKE TRANSPOSONS IN POACEAE SPECIES: TRANSPOSASE SEQUENCE VARIABILITY AND CHROMOSOMAL DISTRIBUTION

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Abstract: Belonging to Class II of transposable elements, *En/Spm* transposons are widespread in a variety of distantly related plant species. Here, we report on the sequence conservation of the transposase region from sequence analyses of *En/Spm*-like transposons from Poaceae species, namely *Zingeria biebersteiniana*, *Zingeria trichopoda*, *Triticum monococcum*, *Triticum urartu*, *Hordeum spontaneum*, and *Aegilops speltoides*. The transposase region of *En/Spm*-like transposons was cloned, sequenced, and compared with equivalent regions of *Oryza* and *Arabidopsis* from the gene bank database. Southern blot analysis indicated that the *En/Spm* transposon was present in low (*Hordeum spontaneum*, *Triticum monococcum*, *Triticum urartu*) through medium (*Zingeria bieberstiana*, *Zingeria trichopoda*) to relatively high (*Aegilops speltoides*) copy numbers in Poaceae species. A cytogenetic analysis of the chromosomal distribution of *En/Spm* transposons revealed the concurence of the chromosomal localization of the *En/Spm* clusters with mobile clusters of rDNA. An analysis of *En/Spm*-like transposase amino acid sequences was carried out to investigate sequence divergence between 5 genera – *Triticum*, *Aegilops*, *Zingeria*, *Oryza* and *Arabidopsis*. A distance matrix was generated; apparently, *En/Spm*-like transposase sequences shared the highest sequence homology intra-generically and, as expected, these sequences were significantly diverged from those of

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Abbreviations used: TE – transposable elements; TIR – terminal inverted repeats; TPase – transposase

O. sativa and *A. thaliana*. A sequence comparison of *En/Spm*-like transposase coding regions defined that the intra-genomic complex of *En/Spm*-like transposons could be viewed as relatively independent, vertically transmitted, and permanently active systems inside higher plant genomes.
The sequence data from this article was deposited in the EMBL/GenBank Data Libraries under the accession nos. AY707995 - AY707996 - AY707997 - AY707998 - AY707999 - AY708000 - AY708001 - AY708002 - AY708003 - AY708004 - AY708005 - AY708005 - AY265312.

Key words: Transposase, *En/Spm*, Poaceae, Sequence, Evolution, *In situ* hybridization

INTRODUCTION

The most visible peculiarity of plant genomes, particularly the large genomes of cereals, is the high percentage of transposable elements (TE; up to 80% of a given cereal's nuclear genome) [1]. From the investigation of the *Drosophila* genomes, it is known that TE can give rise to significant genomic changes by creating mutations, altering gene expression, conducting telomeric function, and promoting chromosomal aberrations [2-4]. TE was divided into two classes. Class I (RNA) elements transpose via an RNA intermediate [5, 6]. Class II (DNA) elements transpose via a DNA intermediate and usually have short terminal inverted repeats (TIR). Class II TE is classified to several super families [7]. Members of the *En/Spm* family, which are the main subjects of this study, are widespread among plants [8]. They share a common sequence (5'-CACTA-3') at their TIR termini and were found in a variety of distantly related species [9-13], but only a few elements were characterized at the molecular level [7, 8]. For *Arabidopsis*, where the system was shown to transpose with high efficiency, *En/Spm*-mutagenised populations were developed and successfully employed for gene tagging [14-18]. Greco *et al.* [19] demonstrated that the maize *En/Spm-I/dSpm* two-component system can transpose in rice. *En/Spm* transposons are known to be linked with genes and their expression; transposition is controlled by interacting autoregulatory and/or epigenetic mechanisms and is, therefore, relatively independent of their chromosomal localization [20]. Each *En/Spm* transposon possesses a gene encoding a TPase that has several conserved domains, and it is technically possible to locate the chromosomal positions of these elements by fluorescent *in situ* hybridization (FISH) of TPase fragments amplified from the genomic DNA of the species [8].
Here, we present our results from a sequence analysis of *En/Spm*-like TPase conserved regions and their chromosomal distribution in five Poaceae species. TPases were compared to similar sequences in *Oryza sativa* and *Arabidopsis thaliana* to shed light on the sequence conservation/divergence of *En/Spm*-like TPase conserved regions in the Poaceae family.

MATERIALS AND METHODS

Plant material and DNA isolation

Z. biebersteiniana, *Z. trichopoda*, *T. monoccoccum*, *T. urartu*, *Ae. speltoides*, and *H. spontaneum* (Tab. 1) were used for the preparations of chromosome spreads and PCR amplifications. The plants were grown in the greenhouse and genomic DNA was isolated from the leaves via DNA extraction, using the CTAB method of Kidwell and Osborn [21] for the amplification of the *En/Spm* transposon transposase-conserved regions.

Tab. 1. The accession numbers and sources of plant material

Accession	Origin	Source
Zingeria biebersteiniana	Russia	BIN RAN*
Zingeria trichopoda	Russia	BIN RAN
Triticum monoccoccum (PI 428154, G-1391)	Turkey	USDA**
Triticum urartu (TR38540)	Turkey	AARI***
Aegilops speltoides	Israel	IE****
Hordeum spontaneum	Maalot, Israel	IE

*BIN RAN – Komarov Botanical Institute of the Russian Academy of Sciences, **USDA – United States Department of Agriculture, ***AARI – Aegean Agricultural Research Institute, Turkey, ****IE – Institute of Evolution, Haifa, Israel.

Amplification of TPase fragments from *En/Spm*-like elements

Degenerate oligonucleotide primers were used for PCR amplification from the genomic DNA of *En/Spm*-like transposon TPase-conserved regions. The primers and PCR conditions were as described in Staginnus *et al*. [8] for *T. monoccoccum*, *T. urartu*, *Ae. speltoides* and *H. spontaneum*. For *Z. biebersteiniana* and *Z. trichopoda*, *En/Spm* element-specific primers (based on the sequence of the ESas-2 clone) [22] were used for PCR amplifications.

The cloning and sequencing of amplified fragments

Amplified fragments were gel separated and purified using the Qiagen Purification Kit (USA). Purified PCR products were ligated into a pGEM-T Easy Vector (Promega, USA) and transformed into *E. coli* XL1blue competent cells. Recombinant clones were isolated using the Qiagen plasmid isolation kit (USA), screened for inserts by digestion with *Eco*RI, and sequenced. The sequences of *En/Spm*-like transposon TPase fragments described here were deposited in the GenBank database with the accession nos. AY708005, AY708006 (*Z. biebersteiniana*, clones ESzb-2, ESzb-7, respectively), AY708002, AY708003, AY708004 (*Z. trichopoda*, clones ESzt-6, ESzt-7, ESzt-8, respectively), AY707998, AY707999, AY708000, AY708001 (*T. monococcum*, clones EStm-2, EStm-3, EStm-4, EStm-5, respectively), AY707996, AY707997 (*T. urartu*, clones EStu-8, EStu-9, respectively), AY265312 (*Ae. speltoides*, clone ESas-3), and AY707995 (*H. spontaneum*, clone EShs-2).

Genome walking and DNA sequencing

The DNA Walking Speed Up Kit (Seegene, USA) was used to conduct genome walking via a PCR-based method on the *En/Spm*-like transposon TPase conserved region in *Z. biebersteiniana* in the 5' and 3' directions according to the user's manual. Primary and secondary genome walking amplifications were carried out with the combination of the "DNA Walking ACP" (DW-ACP) primers provided in the kit and gene-specific primers (based on the sequence of the ESzb-2 clone). From the new sequences, primers were designed with the assistance of the computer program "Primer Premier 5" (Premier Biosoft International, CA, USA), and used for walking on genomic DNA for sequencing in both directions (Tab. 2). The resulting secondary PCR products were gel separated, isolated, cloned into pGEM-T Easy Vector (Promega, USA) according to the standard manufacturer's protocol, and sequenced. The sequence of the *En/Spm*-like transposon TPase fragment described here was deposited in the GenBank database (Accession number: AY772017). The TPase open reading frame was assembled by removing introns using the FGENES 2.0 Program (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind).

Tab. 2. The primers used for extending the *En/Spm*-like TPase region on *Zingeria bieberstiana* genomic DNA.

Primer	Sequence (5' to 3')
Z1-sense	CAT TTG GCT GTT CAT CTA CC
Z2-sense	TCT ACC TGA TGA GGC ACT AC
Z3-sense	TAC CTG ATG AGG CAC TAC TT
Z1-antisense	TCC CGT GGT TTA CCT TCA TC
Z2-antisense	CCC GTG GTT TAC CTT CAT CTT
Z3-antisense	GTA TCT TTC GTC TTG CCT TC
Zbi3	GGC ACT ACT TAG AGG TCC AG
Zbi4	CTC CAG CCT TCT TTG ATG TG
Zbi5	AAG TGC TTC TCC AAT GTC CC
Zbi6	CGT CCT CGT CCA CAA CAT CA
Zbi7	ATG TCC CGG AAT CAG GTT TG

Database searches and sequence comparisons

The sequences obtained in this study were analyzed for similarity to known sequences using the BLAST package provided at the National Centre for Biotechnology Information (NCBI) [23], and compared to each other with the ALIGN program (http://vega.igh.cnrs.fr/bin/align-guess.cgi). Several selected TPase nucleotide sequences obtained from PCR amplifications were conceptually translated into amino acid sequences and aligned with each other and with the known homologous sequences of *Oryza sativa* (NP 920256.1) and

Arabidopsis thaliana (BAB09502.1) with CLUSTALW. Multiple sequence alignments used to generate the phylogenetic tree with maximum parsimony were performed with the CLUSTALW server, available at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/) with default parameters. Multiple sequence alignments were used to generate a distance matrix between selected sequences in the FASTA format with WebPhylip (http://biocore.unl.edu/WEBPHYLIP/).

Southern blot analysis

Samples of 12 μg of genomic DNA were digested with *Hae*III restriction enzyme and then electrophoresed in 1% agarose gel, blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech Inc.), and hybridized with *Z. biebersteiniana* (ESzb-2), *Z. trichopoda* (ESzt-6), *H. spontaneum* (EShs-2), *Ae. speltoides* (ESas-3), or *T. urartu* (EStu-8) probes. Prehybridization and hybridization were carried out at 65ºC in 2.5X SSPE (sodium phosphate monobasic), 1% SDS, 0.01% sodium pyrophosphate and 0.1% PAES (polyanetolensulfonic acid). Washes were performed at 65ºC with 0.5X SSC and 0.1% SDS in the final wash. Radiolabeling of the ESzt-6, ESzb-2, EShs-2, ESas-3, EStu-8 and EStm-4 probes was performed using Random Primer DNA Labeling Mix (Biological Industries, Beit Haemek LTD, Israel). The probe was purified over a sephadex G-50 probe quant micro-column (Amersham Pharmacia, Biotech). The filters were autoradiographed at room temperature for four days.

Chromosome spread preparation, probe labeling, *in situ* hybridization and detection

Seeds were germinated on moist filter paper at 24ºC in the dark. Seedling shoot meristems were transferred to ice water for 24-28 h to accumulate metaphases, and then fixed in 3:1 (v/v) 100% ethanol:acetic acid. Chromosome spreads were prepared according to the technique of Pijnacker and Ferwerda [24] with modifications [22]. The internal part of the transposase (TPase) region of the plant *En/Spm*-like transposons was used as a probe for *in situ* hybridization (FISH) [8, 22]. The FISH procedure was previously described in detail [22, 25]. The clones ESzb-2 and ESas-3 were labeled with Cy-3 (Amersham) according to a standard oligolabeling protocol. For *in situ* (FISH) localization of the 45S rDNA and 5S rDNA gene regions, we used the pTa71 [26] and As5SDNAE [22, 27] probes, respectively. pTa71 was labeled with digoxigenin-11-dUTP (Roche) and detected with monoclonal anti-digoxigenin clone DI-22 (Sigma) / Alexa 350 (Molecular Probes). As5SDNAE was labeled with biotin-16-dUTP (Roche) and detected with Alexa 488 (Molecular Probes). Centromeres were localized by hybridization with a PCR-generated probe, CCS-1 [28], labeled with digoxigenin-11-dUTP (Roche) and detected with a rhodamine-conjugated sheep anti-digoxigenin Fab fragment (Roche). Hybridization was carried out at 63ºC for 3 h.

Differential staining procedures
After *in situ* hybridization, the same slides were successively stained with GC-specific fluorochrome chromomycin A_3 (CMA_3), AT-specific fluorochrome Hoechst 33258 [29], and Giemsa stain [30].

RESULTS AND DISCUSSION

Characterization and sequence comparison of *En/Spm*-like TPase fragments
The TPase regions of the *En/Spm*-like elements from *Z. biebersteiniana* (571 bp – AY708005; 572 bp – AY708006), *Z. trichopoda* (572 bp – AY708002; 572 bp – AY708003; 572bp – AY708004), *T. monococcum* (653 bp – AY707998; 657bp – AY707999; 653bp – AZY708000; 652bp – AY708001), *T. urartu* (653bp – AY707996; 652bp – AY707997), *H. spontaneum* (695bp – AY707995) and *A. speltoides* (586bp – AY265312) were cloned, sequenced and directly deposited in the GenBank. Comparing the TPase fragments with each other showed that clones from *Z. biebersteiniana*, *Z. trichopoda*, *T. monococcum*, *T. urartu* and *Ae. speltoides* exhibit significant homology. The intra-genus homology is 95-97%; the homology between genera is 83-87%. Nucleic Acid BLAST analysis and comparison with the known sequences of various *En/Spm* elements from other plant species such as *Triticum aestivum*, *Cicer arietinum*, *Elais guineensis*, *Pisum sativum*, *Beta vulgaris*, *Lens culinaris*, *Allium sativum*, *Lycopersicon esculatum*, *Arabidopsis thaliana* and *Oryza sativa* show that the obtained sequences are most similar to the TPase region of the *TNP2*-like transposons. The clone from *H. spontaneum* (AY707995) shared only 48-57% sequence homology to all our clones and approximately the same homology (52%) to the TPase sequence of the TNP2-like transposon of *O. sativa* (NP 920256.1). This may suggest that this clone belongs to another type of *En/Spm*-like transposon, and thus it was excluded from further sequence analysis.

Extending the *En/Spm*-like TPase region by genome walking in *Z. biebersteiniana*
Eleven *En/Spm* TPase specific primers (Tab. 2) were designed based on the sequence of the ESzb-2 (AY708005) clone, and used for PCR-based genome walking with the DNA Walking Speed Up Kit (Seegene, USA). We obtained a 1413-bp long clone (accession no: AY772017). Nucleic Acid BLAST analysis of this clone revealed significant homology (80-96%) to the TPase sequences of *O. sativa* (putative transposase, *Rim2-M86* transposase, putative *TNP*-like transposase), *Triticum aestivum* (*En/Spm*-like transposon) and *Lolium perenne* (CACTA-family transposon). After the removal of the predicted introns from the AY772017 clone, it was found to contain an intact open reading frame that has 198 amino acids, and revealed 38-46% homology to *O. sativa* (putative transposase, putative transposon protein and *TNP2*-like transposon protein), 30% to *A. thaliana* (*En/Spm*-like transposon protein), and 37-42% to *Antirrhinum*

majus (TNP-2) and to garden snapdragon transposable element-(*Tnp2*) at the predicted amino acid level.

Southern blot analysis

Southern hybridization was performed to gain insight into the genomic organization of *En/Spm*-like transposable elements in 5 species of the Poaceae family (Fig. 1). Hybridization revealed a range of fragments (6.5 kb-1300 bp), with *Hae*III digested DNA with a background smear. The number and strength of the hybridization signals indicate the presence of the *En/Spm* transposon in low (*Z. bieberstiana*, *Z. trichopoda*), moderate (*H. spontaneum*, *T. monococcum*, *T. urartu*), or relatively high copy numbers (*Ae. speltoides*) in Poaceae species. Kubis *et al.* [31] looked at the genomic organization of *EnSpm*-like transposons in oil palm (*Elaeis guineensis*) and reported that southern hybridization on a range of oil palm accessions generated strong signals, indicating their amplification, and the copy number of *EnSpm* transposons was detected as medium.

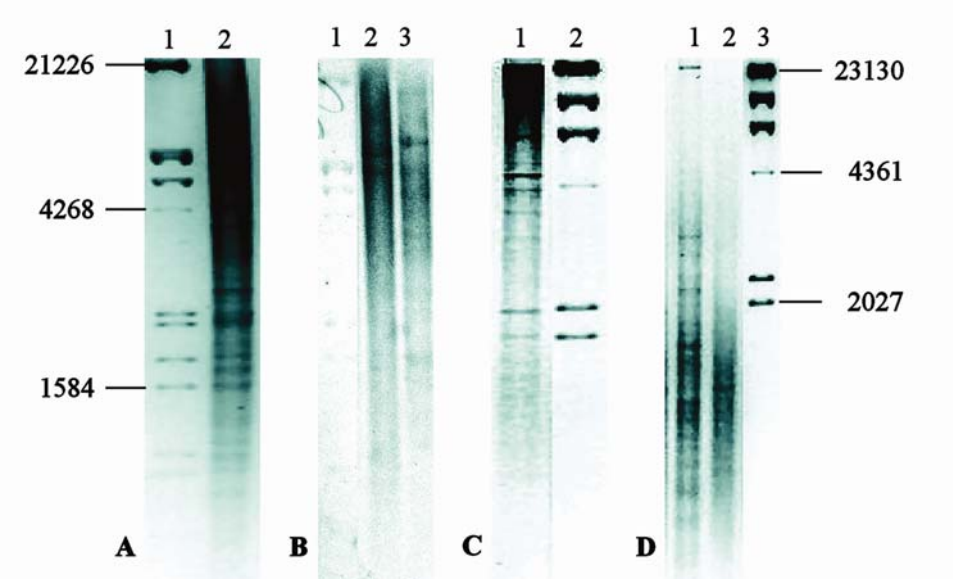


Fig. 1. Southern blot hybridization of the En/Spm transposons in the Poaceae family. Genomic DNAs were digested with *Hae*III. A - 1. Molecular size marker (lambda phage *Hind*III+*Eco*RI digest). 2. *Aegilops speltoides* hybridized with ESas-3 probe. B - 1. Molecular size marker (lambda phage *Hind*III+*Eco*RI digest). 2. *Zingeria trichopoda* hybridized with ESzt-6 probe 3. *Zingeria bieberstiana* hybridized with ESzb-2 probe. C - 1. *Hordeum spontaneum* hybridized with EShs-2 probe. 2. Molecular size marker (lambda phage *Hind*III digest). D - 1. *Triticum urartu* hybridized with EStu-8 probe 2. *Triticum monococcum* hybridized with EStm-4 probe. 3. Molecular size marker (lambda phage *Hind*III digest).

Fluorescent *in situ* hybridization (FISH)

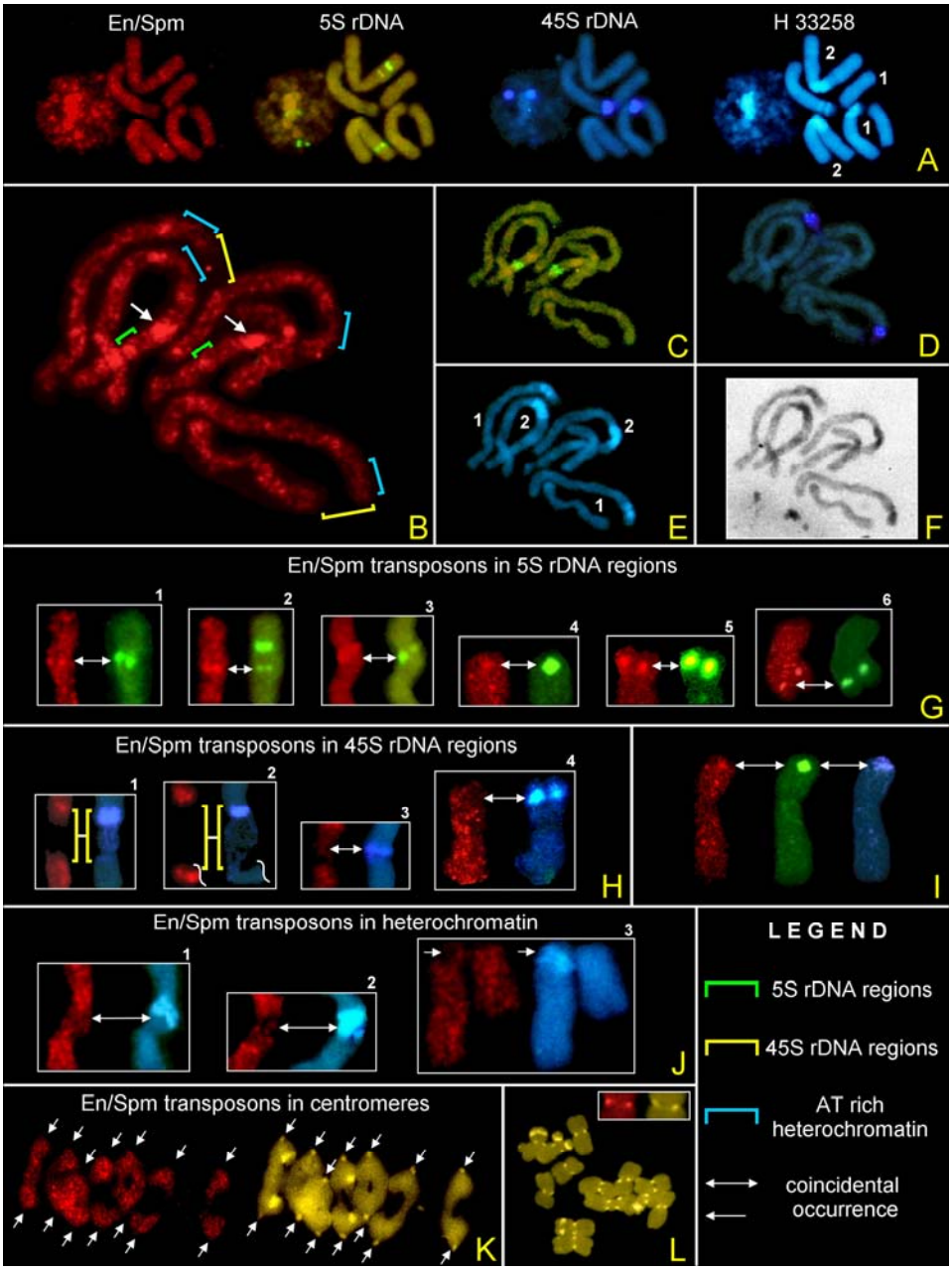
En/Spm transposons in 5S rDNA regions
Analysis of the chromosomal distribution of TPase fragments of *En/Spm* transposons in all the analyzed cereal species confirmed our previous data on the co-localization of *En/Spm* transposons and 5S rDNA regions [22]. Clusters of *En/Spm* transposons were mainly concurrent or adjacent to the regular 5S rDNA regions for *Z. biebersteiniana* (Fig. 2A-C, g: boxes 1, 3), *T. urartu* (not shown), *T. monococcum* (Fig. 2G: box 4), *Ae. speltoides* (Fig. 2G: box 5), and *H. spontaneum* (Fig. 2G: box 6). Additionally, mobile 5S rDNA sites also concurred with clusters of *En/Spm* transposons (Fig. 2 G: box 2).

En/Spm transposons in 45S rDNA regions
We observed a significant reduction in the intensity of signals from *En/Spm* transposons in 45S rDNA regions (Fig. 2H) in all the analyzed species except those where the 5S and 45S rDNA clusters are adjacent in the distal part of chromosomes 1 and 5, i.e. *T. urartu* and *T. monococcum* (Fig. 2I).

En/Spm transposons in euchromatin
Besides an interspersed signal, strong distinct clusters of *En/Spm* transposons were observed in the euchromatin of somatic and meiotic chromosomes of all five investigated species. Furthermore, main clusters proximal to the 5S rDNA sites in the short arm of both homologues of chromosome 2 were observed in prometaphase chromosomes of *Z. biebersteiniana* (Fig. 2B). Also, large intercalary clusters of *En/Spm* transposons were detected in the long arm, and adjacent to AT-enriched and C-positive heterochromatic bands on the short arm of both homologues of chromosome 1 (Fig. 2B).

En/Spm transposons in heterochromatin
Z. biebersteiniana possesses large AT-enriched heterochromatic blocks in the pericentromeric regions of both chromosomes. It was previously shown that these blocks are enriched with TE of Class I, namely, with Ty1-*copia* like retrotransposons [32]. In distinction from Ty1-*copia* elements, a significant reduction in the intensity of signals from *En/Spm* transposons in heterochromatin regions was observed (Fig. 2J). No signal of *En/Spm* transposons was detected in the often decondensed Giemsa-positive and CMA_3-positive (not shown) heterochromatic bands adjacent to the 45S rDNA sites on chromosome 1 (Fig. 2B, D, E). We also observed a strong reduction in the signal in GC-rich pericentromeric heterochromatin and in the secondary constriction regions of *H. spontaneum* (Fig. 2K, L).
In summarizing our results, we may make several generalizations concerning the physical location of *En/Spm* transposons. TE of this type distributed unevenly on the chromosomes of the investigated cereals. The majority of the large *En/Spm* clusters are associated with 5S rDNA sites, whereas no association with 45S rDNA sites was detected (Fig. 2H), except for in two *Triticum* species where the

5S and 45S rDNA clusters are coincident (Fig 4I). Mobile 5S rDNA sites are also associated with *En/Spm* clusters (Fig 4G: box 2). As we reported previously [22, 27], the appearance of at least part of the mobile 5S rDNA sites may be connected with *En/Spm* transposon activity.



Another important peculiarity of *En/Spm* transposon chromosomal distribution is the strong reduction in the intensity of the FISH signal in all the AT- and GC-enriched heterochromatin in all the investigated species (Fig. 2B, E, F, J). Heterochromatin is a highly heterogenic, complex genomic fraction, and is composed to a great extent of different TE families [33-35]. Saunders and Houben [32] detected signal accumulation of the Ty1-*copia*-like sequence in the heterochromatin of *Z. biebersteiniana*, while our results show a significant decrease in the *En/Spm* transposon amounts in these regions, and, hence, these types of transposable elements demonstrate inverted chromosomal patterns.

Evolutionary divergence of TPase sequences

In order to understand cereal genomics, it is essential to evaluate the contribution of transposable elements, as they are the most abundant non-genic DNA in plants, providing a rich source for genome evolution, and are especially abundant in grass genomes [36, 37]. TE are highly polymorphic in terms of their insertion sites and internal structure [38]; thus, it is possible to utilize them as source to develop molecular markers [39]. Sequence analysis of *En/Spm*-like TPase amino acid sequences was carried out to investigate the sequence divergence

Fig. 2. *In situ* hybridization and differential staining on chromosomes of *Z. biebersteiniana*, *H. spontaneum*, *T. monococcum*, and *Ae. speltoides*. A - Metaphase chromosome of *Z. biebersteiniana* after simultaneous *in situ* hybridization with TPase fragments of *En/Spm* transposons (detected as red), 5S rDNA (detected as green), 45S rDNA (detected as blue), and differential staining with Hoechst 33258 (blue). The main blocks of the *En/Spm* transposons are concurrent with 5S rDNA sites. B-F - Undercondensed prometaphase chromosome of *Z. biebersteiniana* after simultaneous *in situ* hybridization with TPase fragments of *En/Spm* transposons (B), 5S rDNA (C), 45S rDNA (D), differential staining with Hoechst 33258 (E), and Giemsa (F). The main clusters of the *En/Spm* transposons in the euchromatin are arrowed in B. G - the concurrence of *En/Spm* transposon clusters and 5S rDNA sites in: *Z. biebersteiniana*, short arm of chromosome 2 (boxes 1-3), *T. monococcum*, short arm of chromosome 1 (box 4), *Ae. speltoides*, short arm of chromosome 5 (box 5), *H. spontaneum*, long arm of chromosome 3 (box 6). H - decrease in the signal of the *En/Spm* transposons (detected as red) in 45S rDNA blocks (detected as blue) in prometaphase (boxes 1-2) and metaphase (box 3) chromosome 1 of *Z. biebersteiniana,* and in chromosome 6 of *Ae. speltoides* (box 4). I - chromosome 1 of *T. monococcum* after simultaneous *in situ* hybridization with TPase fragments of the *En/Spm* transposons (detected as red), 5S rDNA (detected as green), and 45S rDNA (detected as blue). J - decrease in the signal of the *En/Spm* transposons (detected as red) in AT-rich pericentromeric hetrochromatin (blue) in prometaphase (boxes 1-2), and metaphase (box 3) chromosome 1 of *Z. biebersteiniana*. K - meiotic chromosome (metaphase I) of *H. spontaneum* after *in situ* hybridization with TPase fragments of *En/Spm* transposons (detected as red) and differential staining with CMA_3 (yellow). Reduction of the *En/Spm* signal in GC-rich centromeric heterochromatin was observed (arrowed). L - somatic chromosomes of *H. spontaneum* stained with CMA_3 (yellow). GC-rich heterochromatic blocks were observed in the secondary constriction regions and in the centromeres. In the box: CCS-1 probe (detected as red) matched with GC-rich centromeric heterochromatic block (yellow).

between 5 genera – *Triticum*, *Aegilops*, *Zingeria*, *Oryza* and *Arabidopsis* (Tab. 3). The defective sequences (containing inserts or missense codons) were excluded from the analysis to minimize possible errors. Alignment of the *En/Spm* TPase fragments of *Z. biebersteiniana*, *Z. trichopoda*, *T. monococcum*, *T. urartu* and *Ae. speltoides*, and comparison with the homologous regions of *O. sativa* (NP 920256) and *A. thaliana* (BAB09502) indicates that there are 38 single, fully conserved residues that have the same amino acid in a certain position in all the species, 28 strongly conserved groups, and 11 weakly conserved groups (Fig. 3). A distance matrix and phylogenetic tree were generated; apparently the *En/Spm*-like TPase sequences shared the highest sequence homology intra-generically. As expected, these sequences had significantly diverged from the *O. sativa* and *A. thaliana En/Spm*-like TPase sequences with respective distance values of 0.35639 and 0.36398 (Fig. 4, Tab. 3). Thus, the intra-genomic complex of *En/Spm*-like transposons can be viewed as a relatively independent, vertically transmitted and permanently active system inside the plant genome. A good proof for this assumption would be if the divergence in TPase sequences could be used as a "molecular clock", i.e. if the

Tab. 3. The distance matrix of *En/Spm*-like TPase fragments (in length).

	1	2	3	4	5
1. *Triticum*	-	-	-	-	-
2. *Aegilops*	0.07699	-	-	-	-
3. *Zingeria*	0.08639	0.06626	-	-	-
4. *Oryza*	0.78914	0.73246	0.76885	-	-
5. *Arabidopsis*	1.05899	0.95841	0.98717	0.90478	-

calculated time of divergence of the tested species will correlate with known paleobotanical data. For this calculation, we assumed that monocot-dicot divergence occured approximately 200±40 million years ago (mya) [40]. If 200 mya is taken as a benchmark, and the genus *Triticum* as the youngest (as it shows the largest distance from the dicotyledonous *Arabidopsis*, Tab. 3), the recalculation of the time of divergence of the Poaceae species on the basis of their distance matrix is: *Oryza* (Orizeae*)* approximately 150 mya, *Zingeria* (Aveneae) – 16 mya, and *Aegilops* (Triticeae) – 14 mya. In general, this timing is in agreement with paleobotanical data. The earliest reliable angiosperm macrofossils are about 120 myr old, and are already clearly divisible into monocotyledonous and dicotyledonous [40]. It is also known that the hydrophilous Orizeae is one of the most ancient Poaceae, nowadays represented by only a few Palaeogene relicts [39]. The other fact that confirms our approximate calculation is that the fossil record of the mid-Miocene epoch (≈ 15 mya) indicates an increase in the amount of grass pollen, and a major diversification of the modern cereal lineages including Triticeae and Aveneae was proposed for this period [41].

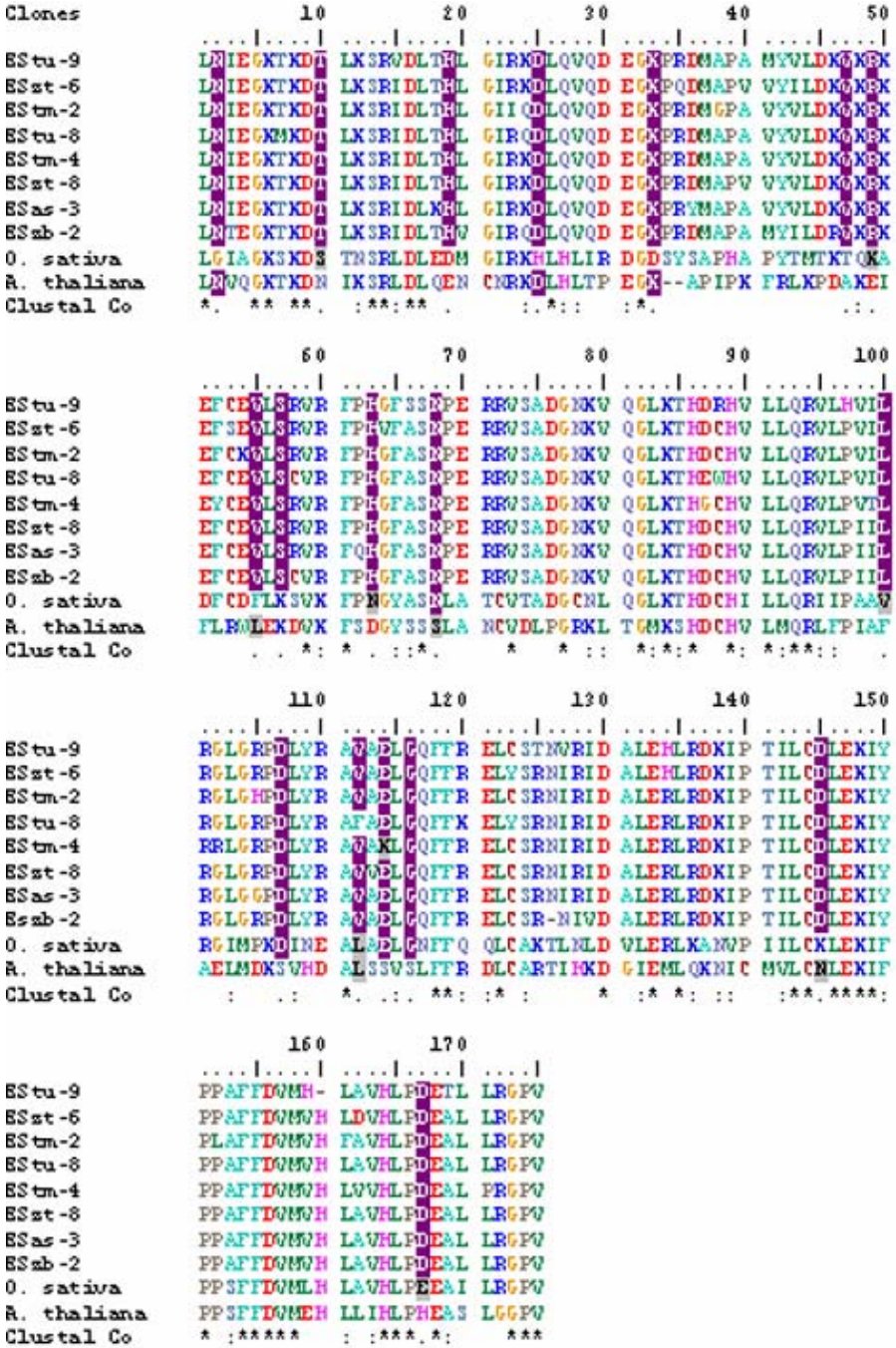


Fig. 3. Multiple alignment of the *En/Spm*-like TPase region. Sequences are named according to the species initial followed by the clone number. Conserved motifs and residues are shaded. (*) Single fully conserved residue, (:) strong group, (.) weak group.

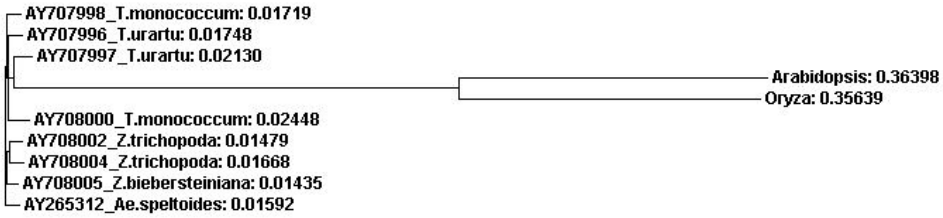


Fig. 4. The phylogenetic tree of the *En/Spm*-like TPase region.

Thus, the sequence comparison of the *En/Spm*-like transposase coding regions defined that the intra-genomic complex of the *En/Spm*-like transposons could be viewed as relatively independent, vertically transmitted, and permanently active systems inside higher plant genomes.

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