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Expression of retrotransposon-like sequences in Scots pine (*Pinus sylvestris*) in response to heat stress

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Abstract

Mobile genetic elements, or transposable elements, are ubiquitous components of plant genomes, and in pine these sequences have been estimated to comprise up to 70 % of the genome. Retrotransposon activity can be a major factor in genome instability, rearrangements and therefore also plasticity of the genome and adaptation to changing environmental conditions. Plant retrotransposons have been shown to be activated under stress conditions. In our study we demonstrate transcriptional activation of many non-specific retrotransposon-like sequences in Scots pine (*Pinus sylvestris*) after exposure to heat stress. Active retrotransposons have not been described in gymnosperms, one of the most ancient plant groups. Our results suggest the existence of several groups of active retrotransposons in the Scots pine genome, which share different levels of similarity with known retro-elements from other plant species. Further studies of retrotransposon activation in pine are needed to increase understanding of their possible function and evolution.

Key words: expression, genome rearrangement, heat stress, primer binding site, retrotransposons, Scots pine.
Abbreviations: bp, base pairs; cDNA, complementary DNA; ESTs, expressed sequence tags; LINE, long interspersed repetitive elements; LTR, long terminal repeats; PBS, primer binding site; SINE, short interspersed repetitive elements; TE, transposable elements.

Introduction

The study of impact of environmental change on species and ecosystems is a vital area of research. This includes the study of adaptation of organisms to unfavourable environmental conditions and survival mechanisms. In species with long generation times, such as conifers, phenotypic plasticity plays an important role in adaptation and survival. The rates of recombination and mutation events in genomes increase in stress conditions. Recently it has been proposed that the non-coding portion of genomes is functionally important, as it shapes chromosome structure, is involved in genome rearrangements, and therefore might define genome structure stability or instability in stress conditions (Madlung, Comai 2004). Transcriptional activation of retrotransposons has been observed in several well studied plant species under various abiotic and biotic stress conditions. For example, tobacco *Tnt1* and *Tto1* are activated by protoplast isolation, cell culture, wounding, methyl jasmonate, $CuCl_2$, salicylic acid, and pathogen attack (Hirochika 1993; Mhiri et al. 1997; Moreau-Mhiri et al. 1996; Takeda et al. 1998). Some retrotransposons are active also during different developmental stages, for example maize Prem-2 element in early microspores (Turcich et al. 1996) or in actively proliferating tissues. Cis-acting sequences were located in the U3 region of active retrotransposons that is similar to some stress response gene promoters (Kumar, Bennetzen 1999). For example, structural properties and the effect of stress conditions on transcriptional regulation have been well investigated for the *Tnt1* retrotransposon from Solanaceae (Grandbastien et al. 2005).

In many plant genomes, repetitive sequences, including retrotransposons, are the major structural part of the genome and range from 15% in *Arabidopsis thaliana* to 90% in Liliacea (Sabot, Schulman 2006). Transposable elements (TEs) are characterized by a high level of diversity and form up to tens of thousands in different plant families, which complicates their classification (Wicker et al. 2007). Class I transposable elements or retrotransposons use an intermediate RNA step to transpose into a new location in the genome, and they are most abundant and widespread elements found in plants. Depending on their structural properties, TEs are divided into superfamilies and families. Long terminal repeat (LTR) retrotransposons contain 100 bp to 5 kbp direct repeats in their termini and one or several open reading frames for the genes gag, int and pol. LTR containing elements have been divided into two superfamilies, *Copia* and *Gypsy*. In LTR retrotransposons, a segment located downstream of a 5' long terminal repeat constitutes the primer binding site (PBS) complementary to the 3' terminal nucleotides of a tRNA molecule. This site is very conservative among different TE families and therefore could be used in genome analyses of species with

poor sequence information or for isolation of novel LTR retrotransposons (Kalendar et al. 2010). Non-LTR elements include LINE (long interspersed repetitive elements) and SINE (short interspersed repetitive elements) type elements. LINEs have gag and pol genes, but lack the int gene. SINEs are non-autonomous elements containing intronless pseudogenes. The chromosomal location of TEs in the genome is dependent on the element type and host organism. Large-scale sequencing studies of various species have shown that retrotransposons tend to form clusters, but dispersed elements have also been found (Kumar, Bennetzen 1999).

Scots pine (*Pinus sylvestris*) belongs to the gymnosperms (Pinophyta), which are one of the most ancient plant groups. In total, 70 to 75% of the Scots pine genome comprises of repetitive sequences (Kole et al. 2007). At present, active mobile genetic elements have not been identified in conifers. The genome of Scots pine has not been studied in relation to retrotransposon activity and adaptation to environmental conditions or response to stress conditions. To date, most studies have focused on the activation of individual, previously described retrotransposons or using conserved domains of one TE family in response to different stress conditions (Hirochika 1993; Lucas et al. 1995; Grandbastien et al. 2005; Lin et al. 2006; Ramallo et al. 2008; Woodrow et al. 2010). Therefore the aim of our study was to observe and compare the transcriptional activation of different types of retrotransposon elements in the Scots pine genome in response to heat-stress conditions.

Materials and methods

Two year old Scots pine (*Pinus sylvestris* L.) ramets were provided by the Latvian State Forestry department „Seeds and Plants" Jaunkalsnava, Latvia. Prior to the experiment, ramets were stored in the greenhouse. DNA was isolated from pine needles using a TRIS-based method. Genotyping with pine SSR (Simple Sequence Repeat) markers PTTX3107, PTTX4001 and PTTX4011 (Soranzo et al. 1998) with known diversity index in Latvian pine population (DI = 0.85 to 0.87) was performed in order to confirm the clonal identity of the ramets.

Heat stress treatment was performed under controlled conditions in a refrigerated precision cabinet with light, temperature and humidity control HOTCOLD-GL (Selecta, Spain). Temperature was gradually increased to 40 °C over eight hours and was maintained for 16 h. Photoperiod was set as follows: 10 h light and 14 h dark. Needles from two ramets exposed to heat stress and one non-stressed control ramet growing under normal conditions were collected and immediately frozen in liquid nitrogen.

RNA from pine needles was isolated using a CTAB-based method (Chang et al. 1993) followed by several purifications with the Turbo DNA-free kit (Ambion) to ensure complete removal of DNA. Purification was continued until amplification of the extracted RNA using a standard PCR protocol with genomic primers showed no PCR products on ethidium bromide stained agarose gels. Stress initiation was confirmed by detection of expression of genes whose expression has been shown to respond to a wide range of stress conditions and whose sequence information was available for *P. sylvestris* (Fig. 1): *LEA* (late embryogenesis abundant protein) F-TCCGCAGAGGTTACAGACATCG; R- 5'CTATTTGCGC TCAGGAGTCGAA-3; *P5CS2* (delta-1-pyrroline-5-carboxylate synthase 2) F-GATCCCAAGAGGTCAGCA, R-GAATCCTGCTTGTGCTTATTCC, *AbaH* (abscisic acid and water-stress induced protein) F-AGGACAACGT TAATTCTGGCTC, R-AATCGGCCTTATAACCAGTG TCG, and one housekeeping gene *GapC1* (glyceraldehyde-3-phosphate dehydrogenase) F-ACGGTTTTGGTCGA ATTG, R-CCCACGAGCTCGATATCAT. Primers were designed using the FastPCR program v. 6.1. (Kalendar et al. 2009).

Reverse transcription was performed with random hexamer primers using 1 µg of RNA with the TaqMan reverse transcription Kit (Applied Biosystems). This reaction was diluted with DEPC-treated water 1:2 (v/v) and 2 µL of complementary DNA (cDNA) was used in the subsequent reactions. PCR reactions with stress sensitive control markers were optimized depending on the primer used. Typical reaction was performed in a 20 µL reaction mixture containing cDNA, 1x Taq buffer (Fermentas), 1.5 to 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 µM of each primer, and 0.7 U Taq polymerase (Fermentas). Cycling conditions consisted of a denaturation step at 94 °C for 3 min, followed by 38 cycles at 94 °C for 30 s, 55 to 62 °C for 30 s, 72 °C for 40 s; and a final elongation step at 72 °C for 10 min. Amplification products were visualized after electrophoresis at 94 V for 2 h in 1xTAE buffer in a 1.5% agarose gel stained with ethidium bromide. Inter PBS (iPBS) reaction and product visualization was performed as described (Kalendar et al. 2010). The following 15 PBS primers were used: 2001, 2009, 2010, 2076, 2080, 2081, 2083, 2095, 2220, 2239, 2271, 2380, 2097, 2384, 2242.

Differentially expressed fragments amplified from stressed tree samples, but not from control tree samples, were excised and purified using the Gel Extraction Kit (Qiagene) following the manufacturer's protocol. Fragments were re-amplified with a touch-down PCR program from 55 to 47 °C with a final elongation step of 30 minutes. PCR reaction mixture contained 30 ng purified PCR product, 1x Dream Taq buffer (Fermentas), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1 µM PBS primer, and 0.8 U Dream Taq polymerase (Fermentas). Re-amplified fragments were examined by electrophoresis, purified with Sephadex-G50 and ligated into the plasmid pTZ57R/T using the InsTAclone PCR Cloning Kit (Fermentas), following the manufacturers protocol. Competent *E. coli* cells were prepared using the Inoue Method (Inoue et al. 1990) and transformed with

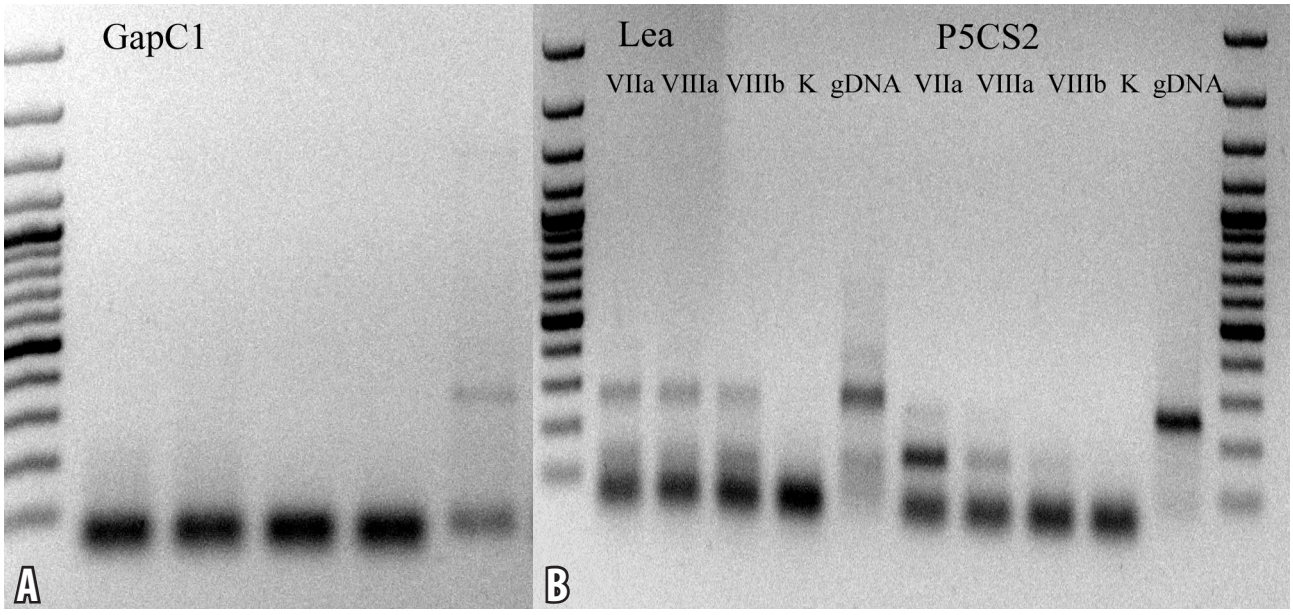

Fig. 1. A - Control primer from housekeeping gene GapC1 with the expected 71 bp PCR product. B - Amplification of stress response related genes LEA (280 bp) and P5CS2 (190 bp) from experimental samples. In the first and last lanes is size marker GeneRuler DNA Ladder Mix (Fermentas). In panels A and B - VIIa , VIIIa and VIIIb are cDNA samples from stressed trees, K is a cDNA sample from control tree, gDNS is amplification with genomic DNA sample from the same ramet.

plasmids using the heat shock procedure described by the same authors. Plasmids were isolated using the alkaline lysis method. Sequencing was performed with M13 primers and a BigDye® Terminator v3.1 Cycle sequencing Kit (Applied Biosystems) and analysed using an ABI Prism 3130x-Avant Genetic Analyzer (Applied Biosystems). Sequences were processed with SequencingAnalysis v.5 software (Applied Biosystems). Multiple Sequence Alignment and similarity dendrograms were generated using CLUSTALW v. 1.83 (http://www.genome.jp/tools/clustalw/). Sequence analyses and mobile element identification was performed as proposed by Wicker et al. (2007). Searches were conducted in the NCBI data base (http://www.ncbi.nlm.nih.gov/BLAST/); GrainGenes Triticeae Repeat Sequence Database (http://wheat.pw.usda.gov/ITMI/Repeats/blastrepeats3.html) and Gypsy Database 2.0 (http://gydb.org/index.php/Blast) (Llorens et al. 2011).

Results

Amplification with stress response gene markers to confirm transcriptional changes due to stress initiation were initially performed. One housekeeping gene marker was used as a control for cDNA synthesis using a sample from the control (Fig. 1A). LEA and P5CS2 gene PCR primers produced amplification fragments in stressed plants and showed no amplification in the control plant (Fig. 1B). For iPBS amplification initially 15 primers were tested; of which only 10 primers amplified PCR fragments from Scots pine genomic DNA samples and only five amplified differentially expressed PCR fragments using cDNA from experimental samples (Fig. 2). Amplification products from both high temperature stressed trees were similar with rare exceptions. Only fragments of one size that were amplified in both stressed plant samples were excised for further analysis. For each excised fragment, ten transformed colonies were taken for further analyses. Isolated plasmid insertions were analyzed and fragments with different sizes were sequenced and compared. If all plasmid inserts were of one size, five randomly chosen plasmids were sequenced using the M13 forward primer. Products with differing sequences were sequenced with the M13 reverse primer and full length sequences were obtained.

Sequences derived from one excised fragment were compared and divided into groups with greater than 80% similarity within each group. Multiple sequence alignments were performed (Fig. 3). One representative sequence from each group was screened against sequence databases. From a total number of 22 unique fragments, 15 fragments showed similarity with various transposable elements (TEs) annotated in the databases. A sequence was considered to have a match in the database when the total score was greater than 50 and sequence identity was more than 40%. Five fragments had weak similarity with TEs in the databases and three of them were similar to unclassified elements (Table 1).

Seven fragments showed strong similarity to sequences in the databases, with more than 80% identities in blastn database searches, in which nucleotide residues were compared. Three of them were similar to the *Gypsy* TE repeat region and showed no similarity with TE polyprotein domains. All these TEs were from *Triticeae Erika, Sumaya;*

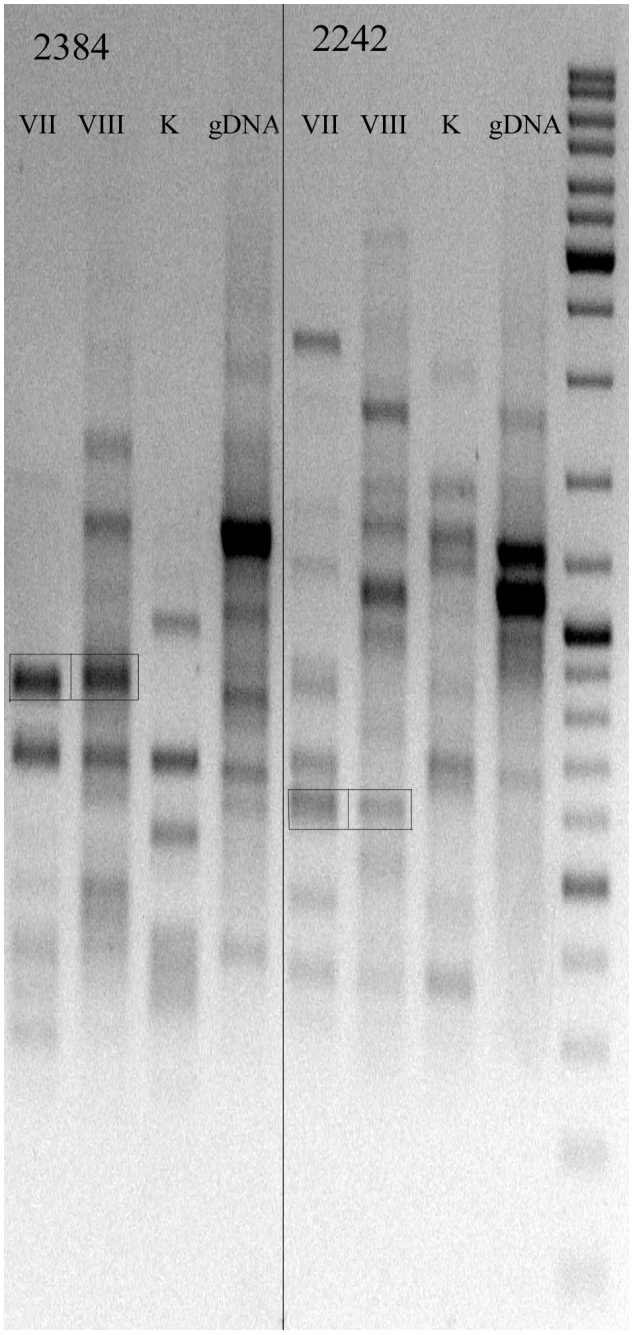

Fig. 2. Inter PBS amplification in experimental samples using primers 2384 and 2242. Lanes VII and VIII show fragments amplified from cDNA samples from heat stressed trees, lane K is the cDNA sample from the control tree, lane gDNS shows amplification with genomic DNA of the same ramet, and the last lane is size marker GeneRuler DNA Ladder Mix (Fermentas). Excised fragments are indicated.

Wham and *Laura* (Table 2). Four fragments also contained protein coding regions. The DNA sequence of fragment 9.2 was similar to the *Gypsy* TE *Ifis* from *Triticum turgidum*, and the translated protein sequence was similar to the *Carmilla* polyprotein. One fragment was similar to the LINE non-LTR TE genomic sequence *Persephone* from barley, as well as the polyprotein *Karin* from barley. Another fragment amplified from both stressed plants was similar to ORF2 of DNA transposon *Conan* from Triticeae. A second group of fragments showed similarity only in blastx searches of the databases, which indicates that they belong to a known superfamily of TEs, but could not be classified to a known TE family. Two fragments had a strong similarity to *Copia*-like LTR Retrotransposon integrase domains (Table 2). Four fragments had a weaker similarity to *Gypsy*-like LTR TEs. The remaining four fragments showed no similarity to any sequences in the databases and three fragments were only similar to unannotated expressed sequence tags (ESTs). Six fragments that showed similarity with TEs were also similar to EST sequences that were derived from various studies of stress-induced transcripts in several *Pinus* species.

Discussion

Differentially expressed fragments were studied under heat stress conditions. Universal PBS primers were used for evaluation of polymorphism in the transcriptome of genetically identical pine ramets subjected to heat stress. The PBS primers amplify the regions between PBS, and when they are applied to genomic DNA it is possible to obtain many amplification products, as TEs are found often in clusters within the genome. The iPBS amplification from cDNA can be explained by results from recent studies in higher eukaryotes, which showed that non-coding expressed RNA is complex, and that intergenic and intronic sequences are extensively transcribed (Mattick, Makunin 2006). The amount and quality of RNA molecules extracted from samples and the efficiency of all subsequent steps in the experimental analysis may affect the amplification of a particular fragment (Fig. 2). Differing amplification could be a result of the non-specific nature of the PBS primers and competition between targets during PCR amplification (Kalendar et al. 2010). Identical treatment and stress conditions were applied to both experimental trees. However, it is possible that some slight differences before or during the heat stress treatment affected the transcription. When differentially expressed fragments of the same size were sequenced from the two stressed individuals, they all formed one group, with a minimum of 90 % sequence similarity. Sequence differences within the groups could be expected since the reverse transcriptase enzyme can introduce errors, and thus even within a single active element sequence variations may be found between expressed fragments. The presence of sequence variation may also indicate that the transcripts originate from different copies in the pine genome (Fig. 3). Transposable elements are characterized by a high level of diversity and form up to ten of thousands of different families in plants that complicates the classification of them (Wicker et al. 2007).

All fragments that were similar to known TEs in

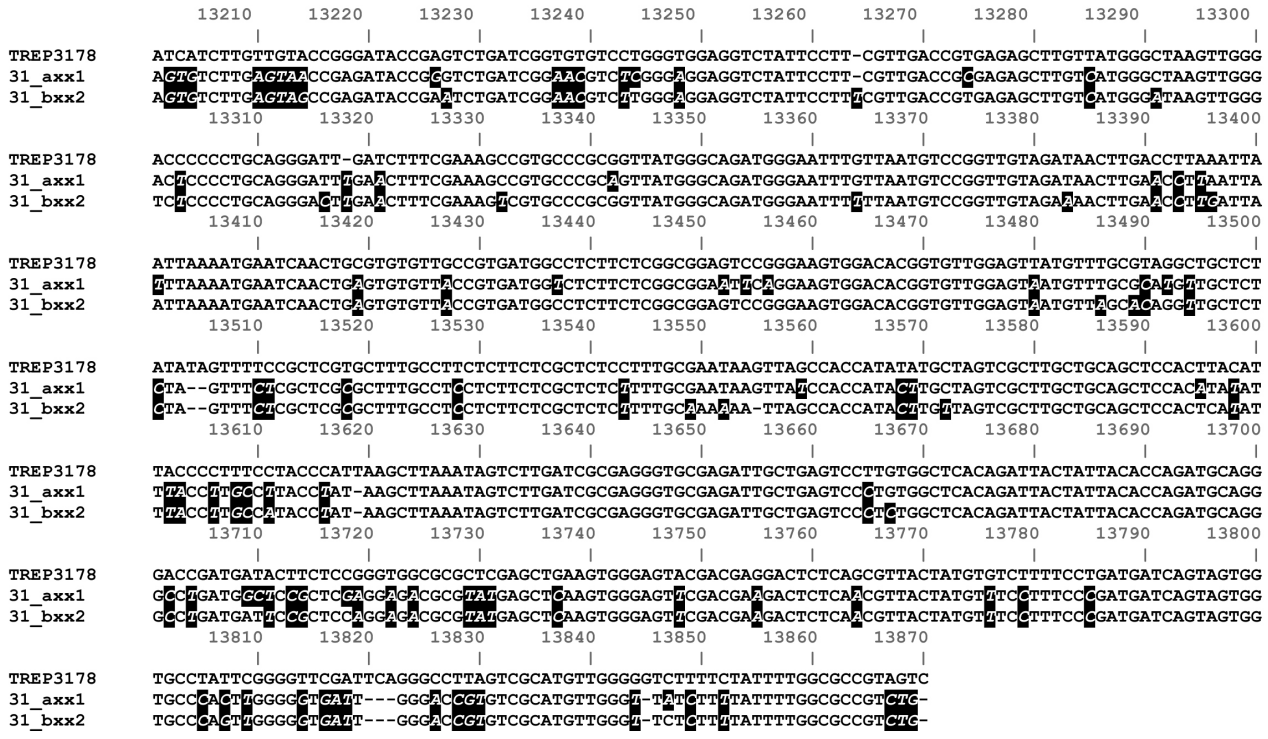

Fig. 3. Multiple sequence alignment of 3.1 group fragments with LTR *Gypsy*, *Erika* TE (data base number: TREP3178) nucleotide sequences.

Table 1. Results of similarity searches using the NCBI, TREP and GyDB databases

Fragment	LTR elements *Gypsy* superfamily	LTR elements *Copia* superfamily	Non-LTR elements LINE	DNA transposable elements CACTA	Unclassified elements	EST	No similarity	Blastn: Score / E-value / Positives	Blastx: Score / E-value / Identities / Positives
1.1							✓	–	–
1.2		✓						–	135 / 3e-34 / 41% / 61%
2.1	✓				✓	✓		–	55.1 / 1e-09 / 29% / 43%
2.2						✓		–	–
3.1	✓							670 / 0.0 / 88%	–
3.2	✓				✓	✓		–	54.7 / 2e-09 / 29% / 43%
3.3	✓							–	53.5 / 3e-09 / 28% / 47%
4				✓				620 / e-178 / 95%	274 / 2e-89 / 74% / 82%
5.1			✓					56 / 6e-08 / 86%	179 / 3e-47 / 67% / 77%
5.2							✓	–	–
5.3							✓	–	–
6.1							✓	–	–
6.2						✓		–	–
7.1						✓		–	–
7.2	✓					✓		–	–
7.3	✓							722 / 0.0 / 93%	–
8	✓				✓	✓		–	52.8 / 6e-09 / 31% /43%
9.1				✓				680 / 0.0/96%	295 / 4e-82 / 68% / 76%
9.2	✓					✓		50.1 /1e-06 / 83%	89 / 1e-20 / 52% / 64%
9.3		✓						–	–
10.1	✓					✓		686 / 0.0 / 90%	–
10.2		✓						–	84.7 / 8e-19 / 59%/73%

Table 2. Classification of analysed fragment sequences according to transposable elements from the TREP and GyDB databases

Fragment	Order, Superfamily	Name of TE	Domain	Data base	Organism
1.2	LTR, *Copia*	*HORPIA2*	polyprotein	TREP, blastx	*Hordeum vulgare*
	LTR, *Copia*	*BARE1*	polyprotein	TREP, blastx	*Hordeum vulgare*
	LTR, *Copia*	*Oryco1-1*	INT	GyDB, cores	*Oryza sativa*
	LTR, *Copia*	*Tnt-1*	INT	GyDB, cores	*Nicotiana tabacum*
2.1/ 8/ 3.2	LTR, *Gypsy*	*Geneva*	GAG	TREP, blastx	*Hordeum vulgare*
	LTR, *Gypsy*	*Sabrina*	polyprotein	TREP, blastx	*Triticum turgidum*
3.1	LTR, *Gypsy*	*Erika*	genomic	TREP, blastn	*Triticeae*
3.3	LTR, *Gypsy*	*Sabrina*	polyprotein	TREP, blastx	*Triticum turgidum*
	LTR, *Gypsy*	*Diaspora*	GAG	GyDB, cores	*Glycine max*
4/ 9.1	DNS, TIR, CACTA	*Conan*	pol	TREP, blastn	*Triticeae*
5.1	LINE	*Persephone*	genomic	TREP, blastn	*Hordeum vulgare*
	LINE	*Karin*	polyprotein	TREP, blastx	*Hordeum vulgare*
7.3	LTR, *Gypsy*	*Wham*	genomic	TREP, blastn	*Triticeae*
9.2	LTR, *Gypsy*	*Ifis*	genomic	TREP, blastn	*Triticum turgidum*
	LTR, *Gypsy*	*Carmilla*	polyprotein	TREP, blastx	*Triticum aestivum*
10.1	LTR, *Gypsy*	*Laura*	genomic	TREP, blastn	*Triticeae*
10.2	LTR, *Copia*	*Maximus*	polyprotein	TREP, blastx	*Triticum aestivum*

databases could be classified to at least order (non-LTR, LINE) or superfamily (LTR *Gypsy*, LTR *Copia*) according to the classification proposed by Wicker et al. (2007). As pine belongs to the gymnosperms, which are evolutionary distinct from the majority of species present in the sequence databases, it was expected that higher similarity would be found at the amino acid level (using blastx to search databases). However, three fragments could be classified to particular TE families with high probability, as they showed high similarity at the nucleotide level (LTR, *Gypsy*, *Laura*; LTR, *Gypsy*, *Wham*; DNS, TIR CACTA, *Conan*; LTR, *Gypsy*, *Erica*). In this study several *Gypsy*-like elements were found and only two *Copia*-like elements. This might have resulted from the selection of the particular PBS primers used for analysis. Furthermore, given that there have been no published reports of retrotransposon activation in pine; the observed expression of different TE groups could not be compared to previous studies. For nine of the analysed sequences, similarities were found with EST sequences derived from cDNA libraries obtained in various studies of stress responses in pine. This confirms that the analysed sequences are consistently transcribed under various stress conditions. However, the existence of transcribed retrotransposon sequences in the RNA pool does not indicate proof of subsequent reinsertion into the genome, thus instigating genome rearrangement. Studies of correlation between increases in RNA level and increase in element copy number for some elements have suggested that particularly the regulation of TE transposition is controlled at the transcriptional level. However, some elements were transcribed, but no significant copy number increase was observed. Transcriptional activation is necessary in order to allow transposition, but also other steps are required and post-transcriptional regulation can be significant (Kumar, Bennetzen 1999). Future studies are necessary to obtain further data to elucidate this question.

One of the analysed sequences showed similarity to a DNA transposable element. These elements do not use RNA in transposition, but they can jump into LTR retrotransposons and be transcribed along with them. In a recent study of the wheat genome the *Conan* tandem repeat was found within the *Gypsy* LTR retrotransposon *Cereba* (Breen et al. 2010). This can explain the presence of these DNA transposon sequences in the transcribed RNA pool. Due to the clustering of retrotransposons within genomes, the identified sequences may be fragments or inactive elements within an active element. However, some of the analysed sequences were similar to several known active retroelement proteins (*BARE-1*, *Tnt-1*) which suggests that these retroelements possess transposable activity. These results indicate that further isolation of complete elements is needed to prove their transcriptional activation and to investigate activation of these elements in differing stress conditions.

Acknowledgements

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