

Detection of LTR Retrotransposons Reactivation induced by *in vitro* Environmental Stresses in Barley (*Hordeum vulgare*) via RT-qPCR

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Abstract: The effects of four environmental stresses of heat, drought, wounding and cell culture, on the transcriptional reactivation of seven long terminal repeat retrotransposons (LTRs) of barley (*Hordeum vulgare*) genome, were investigated. These LTRs included two *Copia*-type (*Bare* and *Maximus*); and five *Gypsy*-type (*Erika*, *Jeli*, *Sabrina*, *Sukkula1* and *Sukkula3*) LTRs. RT-qPCR analyses revealed that *Erika1* LTR was highly reactivated under heat, drought, and wounding with 28.1, 9.9 and 9.4 fold increments, respectively. *Sabrina* LTR was 6.2 fold reactivated under cell culture. *Bare1* LTR was reactivated by drought (4.1 fold) and cell culture (3.4 fold). Transcription activity of *Jeli* LTR was increased by 3.4 fold under cell culture. *Sukkula1*, *Sukkula3* and *Maximus* LTRs were slightly reactivated under drought, wounding and cell culture. These changes in the reactivation pattern of LTRs provide fingerprints for tracking the molecular changes occurred in barley genome upon exposure to environmental stresses, which might result in gain or loss of yield. Such sensitive LTR expression profiles underline one of the important role of LTR genetics in agriculture.

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List of Abbreviations: LARD-LTRs (large retrotransposons derivatives), LTRs (long terminal repeat retrotransposons), RT-qPCR (reverse transcription–quantitative polymerase chain reaction), TREP (triticeae repeat sequence database server).

1. Introduction

Transposable elements (TEs) are major genetic elements of the eukaryotic genomes (Jurka *et al.*, 2007; Mansour, 2007). In plant, TEs comprise about 15% (*Arabidopsis thaliana*), 50-80% (most grass genomes), or more (some *Liliaceae* species) of the nuclear genome (Sabot and Schulman, 2006). In human, TEs comprise nearly half (42%) of the nuclear genome (IHGSC, 2001).

Next to DNA transposons, retrotransposons (RTs) are the main groups of TEs. The latter move ('or jump') by copy-and-paste way of life cycles through molecular steps of transcription, reverse transcription and integration of the cDNA copies back into host genome (Alisch *et al.*, 2005; Grandbastien *et al.*, 2005; Brady *et al.*, 2007; Saito *et al.*, 2008; Geuking *et al.*, 2009). LTR retrotransposons (long terminal repeats or LTRs) are the most abundant class of RTs in plant (Wicker *et al.*, 2005). On average, LTRs comprise 60% of the genomes of maize, wheat and barley (Vicent *et al.*, 1999; Myers *et al.*, 2001; Wicker *et al.*, 2001). LTRs are the main source of insertional inactivation mutagenesis (Zedek *et al.*, 2010), which results in

'genomic shock' (McClintock, 1984), polyploidy (Vitte and Panaud, 2005), genome remodeling (Wicker *et al.*, 2005), changes in gene expression (Servant *et al.*, 2008), and genome size enlargement. The latter can occur within one plant generation (Bennetzen, 2002), and might be reversible resulting in genome fluctuation by expansion and contraction (Shirasu *et al.*, 2000; Bennetzen, 2002).

Experiments confirmed the elevated transcriptional activities (reactivation) of LTRs induced by exogenous environmental stresses of chilling, salt, light, infections, nitrate limitations, mechanical damage, and also by *in vitro* regeneration. These activities result in the development of doubled haploids and hybridization (Grandbastien *et al.*, 2005; Nellaker *et al.*, 2006; Stribinskis and Ramos, 2006; Salazar *et al.*, 2007; Sharma *et al.*, 2008; Maumus *et al.*, 2009; Woodrow *et al.*, 2010). As a result of plant defense mechanisms, LTRs can be silenced epigenetically by hypermethylation (Kumar and Bennetzen, 1999).

In the present study, the reactivation of seven barley LTRs; two *Copia*-type (*Bare1* and *Maximus*) and five *Gypsy*-type (*Erika*, *Jeli*, *Sabrina*, *Sukkula1*

and *Sukkula3*) induced by three abiotic stresses (heat, drought and wounding) and cell culture was successfully detected via RT-qPCR. Utilization of these retrotransposons can provide an indirect estimation of transcriptional patterns of these repetitive elements and play a major role in improving the annotation of genomic sequences used to search EST databases.

2. Materials and Methods

Plant materials

Barley (*Hordeum vulgare*; $2n=2x=14$ chromosomes; ~ 5500 MB genome size) seeds of the cultivar Giza 2000 were germinated and grown in the greenhouse under controlled growth conditions.

Environmental stresses

For mild heat stress, five-leaf-stage seedlings were grown for 3 days under high temperature (37°C) as recommended for cereals (Yildiz and Terzi, 2008). For drought stress, seedlings were grown for 27 days with minimum watering to keep them at the wilting stage. Eight replicated pots were used in which two seeds were planted in each plastic pot of 5130 cm³ containing 4000 g of dry soil in the greenhouse. Mass water content (kg/kg) corresponding to soil matric potentials of ≈ -20 and -500 kPa were determined from the retention curve to simulate well-watered and severe water deficit treatments, respectively. It was experimentally determined that addition of 500 and 100 ml of water every other day was required to maintain the desired soil matric potentials for plants up to 8–10 weeks. Soil water status thus presumably became progressively slightly ‘drier’ (lower matric potential) than the target levels as plants grew further and used more soil water. For wounding stress, seedlings were cut into pieces. For cell culture condition, cell suspension culture was applied for 27 days in liquid, aseptic nutritive media according to Mansour *et al.* (2008) following Bittsánszky *et al.* (2006). Statistical analyses for different experiments were performed following the procedure outlined by Gomez and Gomez (1984).

RNA extraction and synthesis of first-strand cDNA

Barley leaves (0.1 g) were used to isolate total RNA with Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. In 200 μ l, 1x DNase I buffer (10 mM Tris-HCl, pH 7.5; 2.5 mM MgCl₂, 0.1 mM CaCl₂, 20 U DNase I [Fermentas International Inc., Burlington, Canada], 5 mM DTT, 100 U RiboLock™ Ribonuclease Inhibitor) and the RNA samples (20 μ l each) were applied and incubated at 37°C for 60 min. Total RNA, in 1x TE,

was incubated at 70°C for 5 min and chilled on ice. The reaction mixture (50 μ l), composed of 1x reaction buffer for reverse transcription (50 mM Tris-HCl, pH 8.3, at 25°C), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, 10 mg of total RNA, 50 U (RiboLocka) ribonuclease inhibitor, 5 μ M of random primers, 1 mM dNTPs, was incubated at 5°C for 10 min and chilled on ice. Then, 1000 U RevertAida, M-MuLV reverse transcriptase (Fermentas International Inc., Burlington, Canada) were added. Then, the reaction mixture was incubated at 4°C for 60 min. Finally, 150 μ l TE was added and solution was stored in -20°C until use (Mansour *et al.*, 2008).

RT-qPCR

The QIAGEN OneStep RT-PCR kit was used according to Gyulai *et al.* (2005) and Bittsánszky *et al.* (2006). The PCR reactions mixture (25 μ l) contained 3 μ l cDNA, 1x PCR buffer (10 mM Tris-HCl, pH 8.8 at 25°C), 2.5 mM MgCl₂, 50 mM KCl, 0.1 % Triton X-100, 300 nM of each primer pair (Table 1), 0.2 mM dNTPs and 1 U DNase II DNA polymerase. PCR (PTC-225 DNA Engine Tetrad cycler, MJ Research, USA) cycles consisted of: 95°C for 2 min; 30 cycles of 95°C/15 sec, 52°C, 56°C or 60°C for 1 min; and 72°C for 2 min, with a final extension step at 72°C for 10 min. For gel electrophoresis (80V/3 h), samples (10 μ l) were mixed in 2x loading buffer and loaded to 1.7% agarose gel (1x STBE). Bands were detected by ethidium bromide staining. The expression levels of LTRs were determined by densitometer program of GelAnalyzer (<http://www.GelAnalyzer.com/>). Constitutively expressed α -tubulin gene was used as a control according to Suprunova *et al.* (2007).

LTR-specific primer sequences

The sequences of retrotransposon families were collected from the Triticeae repeat sequence database server (TREP) (<http://wheat.pw.usda.gov/ITMI/Repeats/>). The server provides sequence entries for the *Copia*-type LTRs of *Bare1* (70 entries) and *Maximus* (9 entries), and the *Gypsy*-type LTRs of *Erika* (9 entries), *Jeli* (14 entries), *Sabrina* (61 entries), *Sukkula1* and *Sukkula3* (7 entries). Downloaded TREP sequences were aligned for determining consensus sequences by MULTALIN© server (Combet *et al.*, 2000) and FastPCR© program (Kalendar *et al.*, 2009). For the analyses of inter specific sequence diversity and phylogeny, LTRs were analyzed *in silico* by BioeEdit (Hall, 1999) and MEGA4 (Tamura *et al.*, 2007) programs.

Table 1. Nucleotide sequences of primer pairs used for RT-qPCR analyses of seven retrotransposons (LTRs) as well as the constitutively expressed α -tubulin gene used as a control (Suprunova *et al.*, 2007). Amplified fragment sizes in base pairs (bp) are indicated.

LRTs	Primer sequences	LRTs	Primer sequences
<i>α-tubulin</i> (400 bp)	F: tccatgatggccaagtgtga R: ctcattgtaccgtgggatgctc	<i>Jeli</i> (380 bp)	F: accatgaccacataactacaacgcag R: cgtcttctggttaattcttgcctcag
<i>Bare1</i> (198 bp)	F: acgacacctccgcgttcag R: ccgaccacatgcctccacggttttctc	<i>Sabrina</i> (326 bp)	F: ttgggttcataccgtgcggtgac R: ggtgaacaattactgtcagca
<i>Maximus</i> (422 bp)	F: tgtgtttgtgagtgtacacagg R: acttggcgtggctatcgaacggctc	<i>Sukkula1</i> (294 bp)	F: Tctcagagttgaggtttccac R: gtcagacataacccccacgtgctc
<i>Erika1</i> (209 bp)	F: ttatgttcccggattgttgcgtc R: gaccaacactcagaggagcac	<i>Sukkula3</i> (333 bp)	F: acgaccaagatgctgctcttcc R: agacagatgatcccagcgac

3. Results and Discussion

In silico sequence analyses

During primer design, two samples of LTR retrotransposon families of available *Copia*-type (all *Maximus*) and *Gypsy*-type (all *Jeli*) were studied *in silico* based on full TREP data. Sequences showed distinctive DNA compositions with higher GC content in *Jeli* LTRs and higher AT content in *Maximus* LTRs with a single exception (*Maximus* TREP1711) (Figure 1). This obviously indicates independent origin of the two LTR families studied (Wicker *et al.*, 2007).

Phylogeny of *Hordeum* LTRs (Figure 2) showed high level of sequence diversity, however, with consensus stretches useful for primer design (Table 1). Sequence diversities also indicated that the active life cycles of LTRs, as new sequence combinations of LTRs, are always generated during their life cycles due to the lack of proof-reading activity of the RNA-dependent DNA polymerase (RdDpol), the reverse transcriptase (RT), which amplify the double-stranded cDNA copy from the transcribed single-stranded RNA transcriptomes of the LTRs (Wilhelm and Wilhelm, 2001).

RT-qPCR analyses of the reactivation of LTRs

Copia-type LTRs

The *Copia*-type *Bare1* LTR family was the first highly abundant ($1.66 \pm 0.6 \times 10^4$ copies) full-length retrotransposon described in barley (*Hordeum vulgare*), which accounts for approximately 3% of barley genome (Chang and Schulman, 2008). It actively transcribes, translates and assembles into virus-like particles. It is involved in genomic diversification within the genus. The *Bare1* was also found active in genome remodeling (Kalendar *et al.*, 2000; Shirasu *et al.*, 2000). Our results showed that *Bare1* LTR retrotransposon was reactivated by drought (4.1 fold increment) and cell culture (3.4 fold), but not by heat or wounding (Figure 3, Table 2). The *Maximus* LTRs of cereal genomes were found to be slightly reactivated in our study by heat wounding (1.5 fold increment) and cell culture (1.9 fold) (Figure 3, Table 2).

Gypsy-type LTRs

The internal domain sequence of *Erika1* is 63% identical to the *Gypsy*-type *Bagy-1* retrotransposon of barley (NCBI Y14573) (Wicker *et al.*, 2007), and showed 69% identity to *Sukkula* LTRs with a poor match at the 5' end. A maize (*Zea mays*) *gypsy/Ty-3* LTR was detected recently in common millet (*Panicum miliaceum*) by AFLP analysis (Gyulai *et al.*, 2011). The transcription activity of *Erika1* LTR was elevated by stresses of heat (28.1 fold increment), drought (19.9 fold) and wounding (9.4 fold), however, the stress during cell culture showed no influence on the transcription activity (Figure 3, Table 2). The *Jeli* LTR, described first in hexaploid wheat (*Triticum aestivum*) genome, provided useful multiple genetic markers for common wheat (Melnikova *et al.*, 2011). Our results showed that *Jeli* LTRs in barley has no activation with most stresses studied, except with a slight reactivation during cell culture with 3.4 fold increment (Figure 3, Table 2). The *Sabrina* LTR, described as an active retrotransposons in common grasses (Todorovska, 2007), showed reactivation capacity by heat (1.4 fold) and during cell culture with 6.2 fold transcriptional increments (Figure 3, Table 2). *Sukkula* LTRs, described as Solo-LTR elements, are the most highly expressed elements in the cultivated barley (Shirasu *et al.*, 2000), however, they lack the coding sequences making it the main type of LARD-LTRs (large retrotransposons derivatives). Both *Sukkula1* and *Sukkula3* were transcriptionally slightly reactivated during cell culture (1.3 and 1.7 fold increment, respectively) and not by heat and wounding stresses. Nevertheless, *Sukkula3* was slightly reactivated by drought (1.6 fold increment) stress (Figure 3, Table 2).

Generally speaking, our results showed different retrotransposon responses to different environmental stresses (data not shown) in accordance with Beguiristain *et al.* (2001), Salazar *et al.* (2007) and Chang and Schulman (2008). *Tnt1A* element of tobacco was reactivated by wounding, biotic elicitors and pathogen attacks of fungal extracts (Melayah *et*

al., 2001). These reactivations seemed to be mainly caused by the *Tnr1A* promoter that has the potential to be activated by various biotic and abiotic stimuli (Grandbastien *et al.*, 2005; 2007). These stimuli were specifically repressed in tobacco when the LTR promoter was replaced in a heterologous position (Grandbastien, 1998).

The sequences of LTR retrotransposons contain environmental stress responsive elements (Niinemetts and Valladares, 2004). The *cis*-regulatory elements, similar to those of plant stress responsive genes, may be involved in binding active retrotransposons to similar defense-induced transcription factors (Casacuberta and Santiago, 2003; Dunn *et al.*, 2006). Also, there are numerous stress inductive gene promoters that share strong sequence similarities with LTRs (Casacuberta and Santiago, 2003; Dunn *et al.*, 2006). The *TLC1.1* retrotransposon was reactivated by multiple stress-related signaling molecules of salicylic acid (SA), abscisic acid (ABA), methyl jasmonate (MeJA), hydrogen peroxide (H₂O₂) and the synthetic auxin 2,4-D (Salazar *et al.*, 2007).

In conclusion, our results show that the studied LTR retrotransposon families responded to environ-

mental stresses with different rates of expression. RT-qPCR analyses revealed that *Erika1* LTR was highly reactivated under heat, drought, and wounding with 28.1, 9.9, and 9.4 fold increment, respectively. *Sabrina* LTR was reactivated under cell culture with 6.2 fold increment. *Bare1* LTR was reactivated by drought (4.1 fold) and under cell culture (3.4 fold). Transcription activity of *Jeli* LTR was increased by 3.4 fold under cell culture. *Sukkula1*, *Sukkula3* and *Maximus* LTRs were reactivated slightly under drought, wounding and cell culture. No explanation can be given for this phenomenon. Changes in the studied transcriptional activities of LTRs provide sensitive molecular fingerprints for tracking the molecular changes occurring in the barley genome exposed to environmental stresses (Koorneef *et al.*, 2004). This indicates that global warming might have an influence in the near future. These stress responsive LTR reactivations might result in gain or loss of yield in economically important crop plants like barley, which underline the important role of LTR genetics in agriculture.

Table 2. Expression levels of the seven LTR retrotransposons of barley (*Hordeum vulgare*) exposed to four stresses as compared to the constitutively expressed α -tubulin gene (control). Results of the RT-qPCR were calculated by GelAnalyzer densitometer program on gel photos (see Figure 3).

LTRs	Stress			
	Heat	Drought	Wounding	Cell culture
α -tubulin (control)	1.0	1.0	1.0	1.0
<i>Bare1</i>	1.0	4.1	1.0	3.4
<i>Maximus</i>	1.0	1.0	1.5	1.9
<i>Erika1</i>	58.1	19.9	9.4	1.0
<i>Jeli</i>	1.0	1.0	1.0	3.4
<i>Sabrina</i>	14.4	1.0	1.0	6.2
<i>Sukkula1</i>	1.0	1.0	1.0	1.3
<i>Sukkula3</i>	1.0	1.6	1.0	1.7

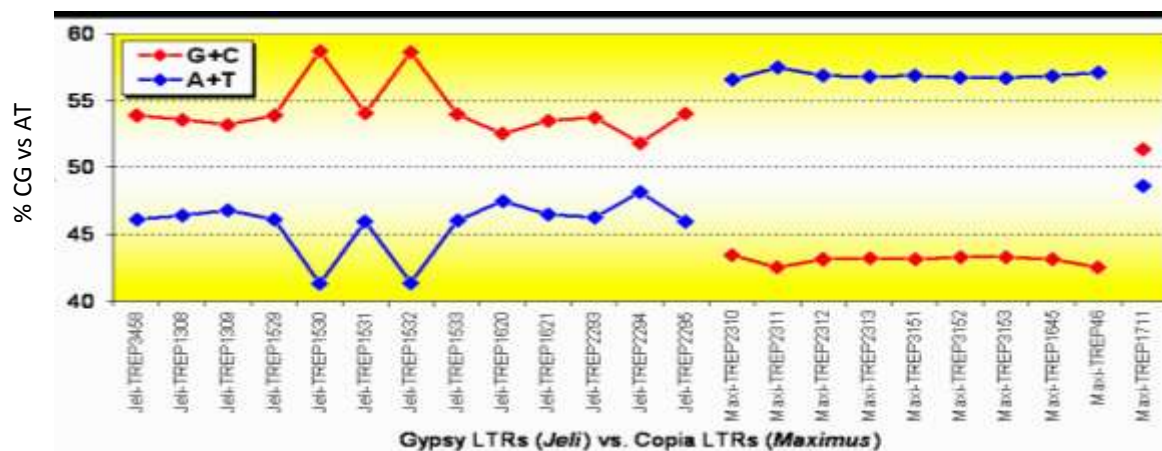


Figure 1. Differences between percentages of GC and AT nucleotide contents of *Copia*-type (all *Maximus* available) and *Gypsy*-type (all *Jeli* available) LTR retrotransposons. Full-length TREP sequences (indicated) were analyzed by BioEdit (Hall, 1999).

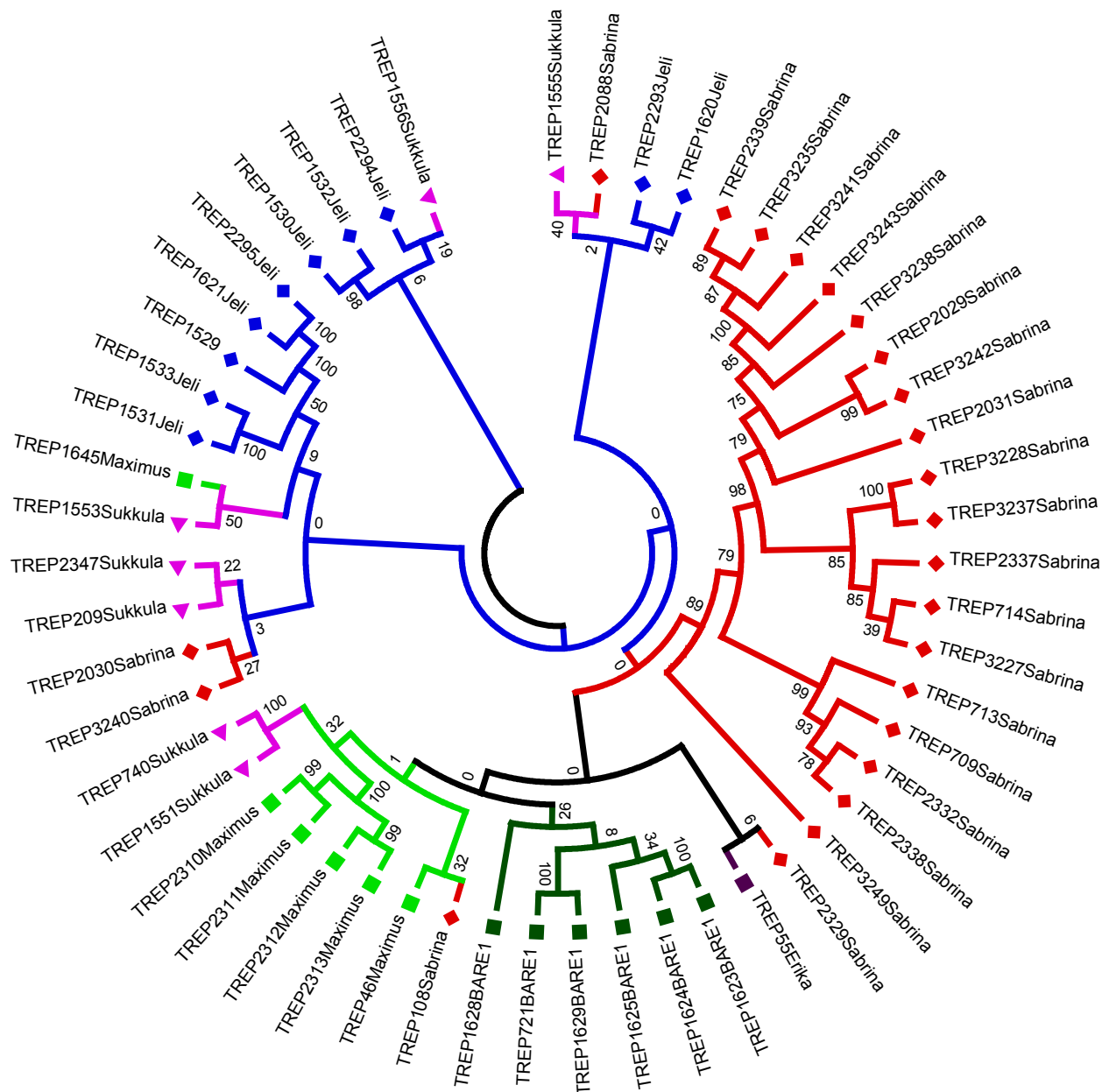


Figure 2. Bootstrap consensus dendrogram derived from the nucleotide sequences of LTR retrotransposons of *Hordeum* genome. Full-length TREP sequences of *Copia*-type (*Bare1* and *Maximus*) and *Gypsy*-type (*Erika*, *Jeli*, *Sabrina* and *Sukkula*) LTR retrotransposons were compared. Full-length TREP sequences of *Bare1* (six of the 65 LTRs available), *Maximus* (9 LTRs), *Erika* (1 LTR), *Jeli* (15 LTRs), *Sabrina* (23 LTRs) and *Sukkula* (7 LTRs) were aligned (BioEdit; Hall, 1999) and edited (MEGA4; Tamura *et al.*, 2007). LTRs of the same families are indicated with different colored symbols. Bootstrap supporting values from 1000 replicates are provided at node.

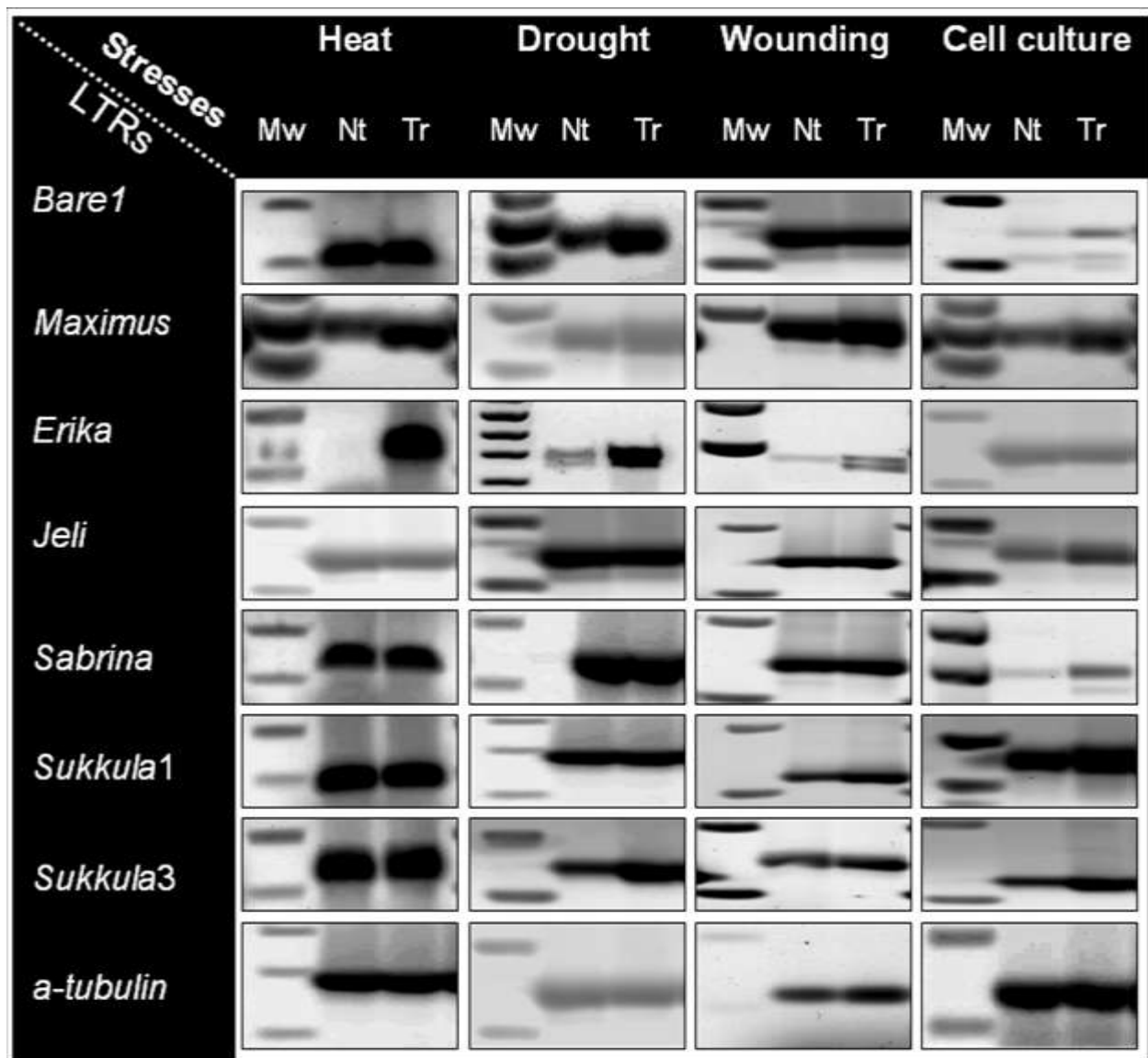


Figure 3. RT-qPCR analyses of the transcriptional reactivations of seven LTR retrotransposons of barley (*Hordeum vulgare*) genome. Two *Copia*-type (*Bare1* and *Maximus*); and five *Gypsy*-type (*Erika*, *Jeli*, *Sabrina*, *Sukkula1* and *Sukkula3*) LTR retrotransposons were investigated under stresses of heat, drought, wounding, and cell culture. For control, the level of the constitutively expressed α -*tubulin* gene was applied. Amplified fragment sizes are indicated in Table 1. *Mw* (molecular weight markers), *Nt* (Not treated), *Tr* (treated samples).

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