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Water Deficit Induction of *Copia* and *Gypsy* Genomic Retrotransposons

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ABSTRACT

LTR-retrotransposons and other mobile DNA elements respond directly or indirectly to a wide variety of stresses by increasing or decreasing their copy numbers. This effect is specific for different retrotransposons and stresses. A potential osmotically-stressed action has been ascribed to sorbitol, but *in vivo* evidence of this action and its genomic impact remains elusive. However, sorbitol is still widely used to mimic the effects of drought and water deficit in plants. In the present investigation, the effect of sorbitol treatments was compared, in both *Copia* and *Gypsy* groups, of genomic retrotransposons with drought in barley. Transcriptional analysis showed that sorbitol exerted a strong influence upon *Copia* elements group after 4, 21 and 32 hours of sorbitol treatment. Transcription of the *BARE-1* retrotransposon family from the *Copia* group was actively induced *in vivo* by sorbitol treatment. When *BARE-1*-specific primers were used to amplify universal *Copia* cDNA products, this revealed unique and strong DNA bands at the same time points of *Copia* elements group which it belong. However, immunobloting of *BARE-1 GAG* protein-specific antibody showed no specific increase after treatments at the same time intervals. These results suggest that sorbitol has the capacity to increase the transcription activity of *Copia* elements, especially *BARE-1* retrotransposon.

Keywords: barley, drought impact, mobile element

INTRODUCTION

Retrotransposons are largely quiescent during development but are activated by stresses. Many stresses have been reported to cause an epigenetic activation of mobile elements, with or without long terminal repeats (LTRs), dispersed throughout the genome (Mansour 2007; Salazar *et al.* 2007). Various biotic and abiotic stresses are shown to increase the expression of various transcriptionally active LTR retrotransposons including chilling, infection, mechanical damage, *in vitro* regeneration, hybridization and generation of double haploids (Hirochika 1995; Grandbastien *et al*. 2005). For instance, it has been reported that rice retrotransposons are involved in mutations induced by tissue culture (Hirochika *et al.* 1996). In addition, exposure to cell-wall hydrolases was shown to activate specific expression of tobacco retrotransposons (Pouteau *et al.* 1991). In mammalian cells, it was reported that human endogenous retrovirus (HERV) elements are transactivated by viral infections in different cell lines (Nellaker *et al.* 2006).

Based on structural differences, there are two major families of LTR retrotransposons, *Gypsy* and *Copia*. Both families differ in the order of their encoded proteins, both are ubiquitous in plants and activated by stress (Flavell 1992; Voytas 1992; Suoniemi *et al*. 1998). Briefly, the two groups can be distinguished by the replacement of integrase (IN), which in *Copia*-like elements precedes the RT and ribonuclease H (RH) but in *Gypsy-like* elements follows them (**Fig. 1**). Earlier studies suggested that retrotransposons present in low-copy number are normally silent but are strongly activated by different kinds of biotic and a biotic stresses (Mansour 2007), whereas the abundant families are constitutively active, but at low levels (Vicient *et al*. 1999). However, the replication nature of retrotransposons combined with large size of the elements (5 to 10 kbp), indicates that active retrotransposon families have the potential to be major contributors to variation in genome size (Vicient *et al*. 2001). Therefore, it was important in this study to evaluate the stress activation of both families, *Gypsy* and *Copia*, with specific primers using different molecular techniques such as RT-PCR, cDNA and western blotting. We expected to see different pattern of activation in different families due to their structural differences (Mansour 2007).

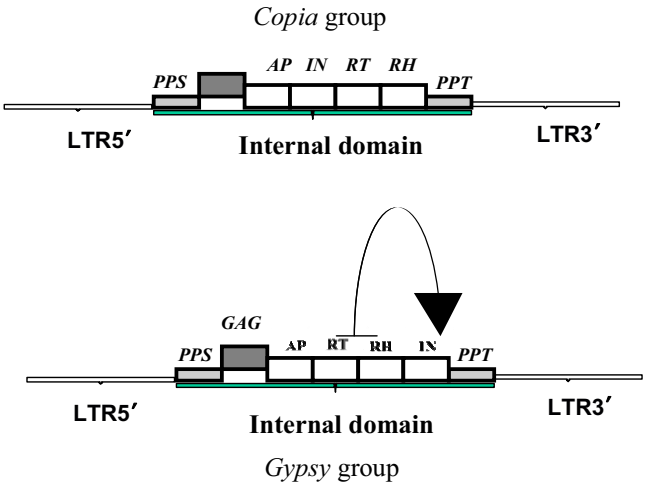

Fig. 1 Proposed structure of two major classes of retrotransposons. Both classes are flanked by long terminal repeats (LTRs). The primer binding site (PBS) and polypurine tract (PPT) are required for replication by reverse transcriptase (RT) in most elements. The protein coding region is usually separated into two domains by a frame shift between *GAG*, the capsid protein and aspartic domain, AP. The two groups can be distinguished by the replacement of integrase (IN), which in *Copia*-like elements precedes the RT and ribonuclease H (RH) but in *Gypsy*-like elements follows them.

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On the other hand, the retrotransposon *BARE-1* family (Manninen and Schulman 1993) of cultivated barley (*Hordeum vulgare* L.) comprises more than 1.6×10^4 genomic copies (Vicient *et al.* 1999, 2001) dispersed on all chromosomes (Suoniemi *et al.* 1996a). The *BARE-1* retrotransposon was reported to respond to sharp microclimatic divergence, especially drought (Kalendar *et al.* 2000). Abscisic acid (ABA) is known as the primary hormone mediating plant responses to stresses, especially drought (Wu *et al*. 1997). ABA has also been reported as an inducing signal for the retrotransposon *BARE-1* from barley (Suoniemi *et al*. 1996a). In this regard, sorbitol was widely applied *in vitro* for mimicking the effect of drought. Both sorbitol and drought seem to have a mutual effect on each other. For instance, drought treatment has shown to affect sorbitol and ABA levels in *Rosaceae* fruit trees (Kanayama *et al*. 2007). In barley leaves, osmotic stress by sorbitol treatment was shown to induces accumulation of *a*-linolenic acid (a-18: 3), jasmonates and octadecanoids such as 12-oxo-phytodienoic acid followed by expression of genes inducible by endogenous jasmonates (Kramell *et al*. 2000). In addition, it was reported that accumulation of oxylipins occurs upon sorbitol treatment (Harwood and Quinn 2000). However, the genomic impact of sorbitol treatment, as compared to drought effect, on barley has not been described yet. In this study, the missing connection between sorbitol treatment on barley plants and the activation of retroelements, especially *BARE-1*, was investigated.

MATERIALS AND METHODS

Sorbitol treatment

Barley (*Hordeum vulgare* cv. 'Bomi') seeds were germinated in the dark for 2-3 days. Seedlings were then planted in vermiculite and grown for 14 days, until the leaves reached full shape. Cultures were incubated on a rotary shaker at 130 rpm with a 16-h photoperiod at 130 mMol m^{-2} s^{-1}, 24°C and 70% relative humidity. The young leaves, primary leaf segments from 14-d-old seedlings, were cut and floated on 1 M sorbitol (Harwood and Quinn 2000; Karmell *et al*. 2000) for 4, 21 and 32 hrs. On the other hand, samples were taken from parallel groups, young leaves from plants grown normally in soil and from leaves cut and floated in water, at the same time points as a control treatment. Stressed and control leaves from different seedlings were frozen in liquid nitrogen and stored at –80°C for RNA extraction.

Drought treatment

'Bomi' barley seeds were used in this study and germinated in a mixture of vermiculite, beat moss, black soil (1: 1: 1). The germinated seedlings were grown in a greenhouse at 22°C, with a 12 h photoperiod. Watering of plants was stopped 14 days after germination to initiate the drought treatment. Samples from individual plants were collected at different time intervals, after 1 and 21 days of drought. A parallel group of plants were planted without any drought treatment and watered normally as a control treatment. Samples from stress and control plants were frozen in liquid nitrogen and stored at –80°C for RNA extraction.

RNA extraction

Mature barley leaves were used to isolate total RNA after applying different stresses using the Trizol method (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

RT-PCR reaction

The Qiagen One-Step RT-PCR kit (Helsinki, Finland) was used. RT-PCR was performed following the manufacturer's instructions and conditions.

DNase I treatment

The reaction was performed in 200 µl mix containing 1X DNase I buffer (10 mM Tris-HCl, pH 7.5 (25°C), 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$), 20 U DNase I, 5 mM DTT, 100 U RiboLock™ ribonuclease inhibitor (Fermentas, Helsinki, Finland) and ≈20 ng RNA. Then the mixture was incubated for 60 min at 37°C. RNA was purified with chloroform and precipitated with 3 vol. ethanol and 0.2 M NaCl. The purified RNA was dissolved in 1X TE, pH 7.0. The RNA was treated twice for efficient removal of DNA contamination.

Synthesis of first-strand cDNA

Total RNA, in 1X TE, was incubated at 70°C for 5 min and chilled on ice. The reaction was performed in 50 µl containing 1X reaction buffer for reverse transcriptase (50 mM Tris-HCl, pH 8.3, 25°C), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 10 ng total RNA, 50 U RiboLock™ ribonuclease inhibitor, 5 µM random primers and 1 mM dNTPs. The mix was incubated at 50°C for 10 min and chilled on ice. Then, 1000 U of RevertAida, M- MuLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Fermentas, Finland) was added. The reaction mixture was incubated at 4°C for 60 min. Finally, 150 µl TE was added and stored at –20°C until use.

PCR reaction

The PCR reaction was performed in 25 µl reaction mixture containing 3 µl cDNA, 1X PCR buffer (10 mM Tris-HCl, pH 8.8 at 25°C), 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 300 nM for each primer, 0.2 mM dNTP mix and 1U DNaseII DNA Polymerase. Amplification was performed using a thermocycler (PTC-225, MJ Research, USA). The PCR reaction parameters consisted of: 95°C, 2 min; 30 cycles of 95°C for 15 sec, 52, 56 or 60°C (depending on TM of the used primers) for 60 sec and 72°C for 2 min; a final extension at 72°C for 10 min. 10 µl of the samples were mixed in 2X loading buffer, then loaded in a 1.7% agarose gel (SeaKem LE, FMC) and run for 80 V for 3 h; bands were separated with 1X STBE and detected by ethidium bromide (0.5 ng/µl) staining. The gel was photographed on a UV transilluminator (Pharmacia) by a Canon S5 digital camera with a UV filter adaptor. A negative control which contained all the necessary PCR components except template DNA was included in the PCR runs.

Specific primer sequences

Degenerated primers, specific for *Copia*-like elements (RT+ caratg gaygtnaarac and RT- catrtcrtcnacrta) (Hirochika and Hirochika 1993) and degenerated primers, specific for *Gypsy*-like elements (RT+ arcatrtcrtciacrta and RT- tayccihticcicgiathga) were used, as previously described by Flavell *et al.* (1992) and Muthukumar and Bennetzen (2004). The barley α-tubulin gene was amplified with specific primers 5′-AGTGTCCTGTCCACCCACTC-3′ and 5′-CCAAGGATCCACTTGATGCT-3′ (acc. no. U40042) was used as a constitutive control in all experiments with expected band size of 400 bp (Suprunova *et al*. 2007). The RT-PCR products were visualized by electrophoresis on a 2% agarose gel. For *BARE-1*, specific primers 5′-acgacacctccgcgttcagc-3′ (forward) and 5′-ccgaccacatgc ctccacggtttttcct-3′ (reverse) were designed from the consensus sequence of *BARE-1* LTR using FastPCR software (PrimerDigital Ltd.). The amplified bands size, as expected, was almost 450 bp.

GAG immunoblotting

Protein samples were prepared as described by Vicient *et al*. (2001). The protein was extracted from leaves of greenhouse plants in an eppendorf tube (1.5 ml) with plastic grinding sticks and sea sand. The equivalent of 20 µ*g* protein for each sample was separated by SDS-PAGE electrophoresis. Gel electrophoresis, blotting, and immunoreactions were carried out as described by Jääskeläinen *et al*. (1999). The antisera against full-length *BARE-1 GAG*, which recognizes 150, 95, and 32 kD proteins was used as described by Vicient *et al*. (2001).

Statistics and experimental design

To the extent possible, this experiment is designed to control the variables likely to affect responses to treatments. Nevertheless,

variables frequently exist, which can be measured and which affect the results of the treatment but cannot be readily controlled. In the present investigation, for comparisons among several treatments, three to five replicates of each were scored and data was statistically analyzed. All obtained data from the experiment was subjected to analysis of variance according to Snedecor and Cochran (1980) and the comparison of means was done using LSD test at $P = 0.05$ (Cochran 1977). All experiments were performed at least twice and representative data are shown.

RESULTS AND DISCUSSION

Sorbitol time course activation of *Copia* retrotransposons detected by RT-PCR

Sorbitol is widely applied *in vitro* for mimicking the effect of drought (Kanayama *et al*. 2007). It was proposed that sorbitol has an adaptive role of metabolism versus a maintenance role of sucrose metabolism under drought stress (Lo Bianco *et al*. 2000). In the present study, sorbitol (1 M) was applied to barley seedlings at different time intervals to investigate its effects on genomic retrotransposons. Specific *Copia* primers, as described in Hirochika and Hirochika (1993), were used to amplify the extracted RNA using RT-PCR. A substantial increase in the bands was noticed after 4 and 21 hrs of treatment. This increment declined again after 32 hrs of treatment (**Fig. 2**). Using specific *Gypsy* primers had no effect, however (data not shown). These results may have arisen because of the differences in structure between *Copia* and *Gypsy* families.

Sorbitol activation of *Copia* retrotransposon investigated by cDNA amplification

In addition to RT-PCR technique, sorbitol transcriptional activation of *Copia* retrotransposon was also studied by using cDNA techniques. Briefly, 1 M of sorbitol was applied to 14-day-old young leaves of barley at different time intervals. The extracted RNA was used to generate cDNAs from treated samples. Amplifying the cDNA samples using the same specific *Copia* primers resulted in the formation of two strong bands after 4 and 21 hrs from sorbitol treatment,

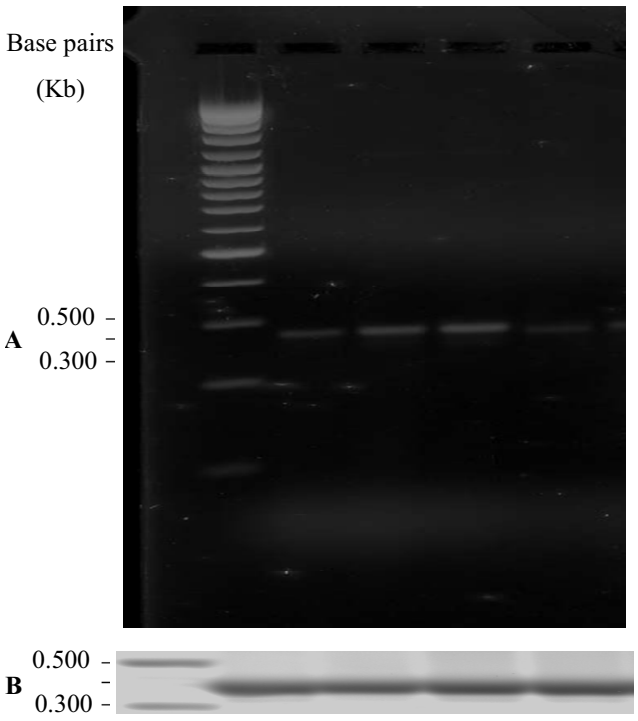

Fig. 2 RT-PCR amplification of *Copia*-like elements under sorbitol stress using *Copia* universal primer. (**A**) Substantial increase in the banding pattern was detected after 4 and 21 hrs of sorbitol treatment. (**B**) The amount of RNA was normalized using α-*tubulin* primers as a control.

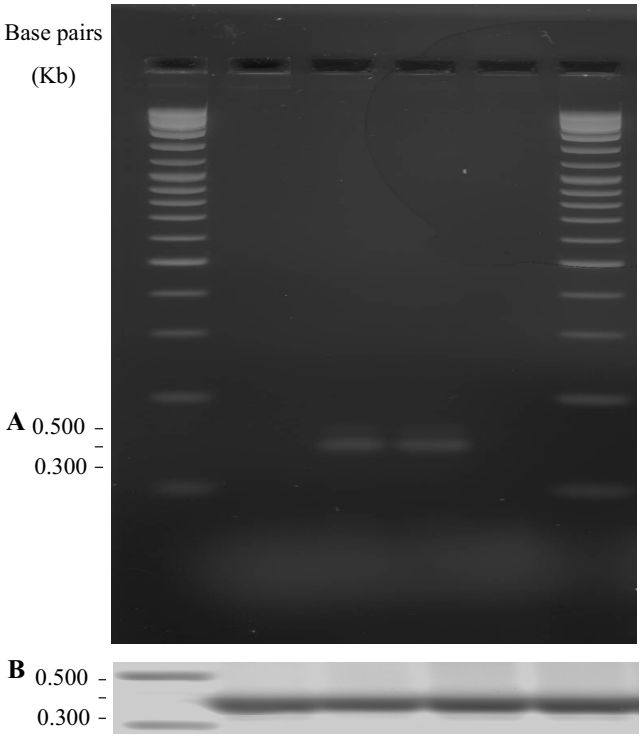

Fig. 3 Amplification of cDNA generated under sorbitol stress using *Copia* universal primer. (**A**) Unique bands were formed after 4 and 21 hrs of sorbitol treatment. (**B**) The cDNA samples were the same in each lane and normalized using *α-tubulin* primers as a control.

confirming the previous results (**Fig. 3**). Thus, the activation pattern of the *Copia* family was similar, at different time intervals, using both RT-PCR and cDNA. However, no activation was noted in *Gypsy* family lanes (results not shown). Beguiristain *et al*. (2001) showed that three Tnt1 subfamilies were induced by stress, but their promoters had a different response to different stress-associated signaling molecules. This agrees with the proposed hypothesis that different patterns of activation in different families are due to differences in their structure (Mansour 2007).

Activation of *BARE-1*- LTR retrotransposon by sorbitol treatment

The *BARE-1* retrotransposon belongs to the *Copia* family and is distributed throughout the barley genome (Waugh *et al.* 1997). The amplification pattern of *BARE-1* LTR-specific primers, with the cDNA, produced the same patterns of amplification in the RT-PCR results after 4 and 21 hrs of sorbitol treatment (**Fig. 4**). The activation pattern of *Copia* and *BARE-1* were similar at different time intervals.

Accumulation of *BARE-1 capsid protein (GAG*) after sorbitol treatment

Retrotransposons express their encoded proteins as a single polyprotein or as two (GAG and POL) separated by a frame shift (Jääskeläinen *et al.* 1999). It was demonstrated that GAG encoded by BARE-1 is translated and processed to the predicted mature size in dry and germinating embryos, leaves, and cell cultures of barley (Vicient *et al*. 2001). To detect the activity and impact of sorbitol at the translational level, the accumulation of *BARE-1 GAG* (capsid) proteins was detected on immunoblots with full-length *BARE-1* anti-*GAG* antibodies using western blotting (Vicient *et al*. 2001) (**Fig. 5**). Despite the activation of *BARE-1* at the transcriptional level, no specific increase in *BARE-1-GAG* after sorbitol treatment was observed. This could be explained by the genome development mechanisms for controlling transposable elements (TEs) activity and their mutagenic poten-

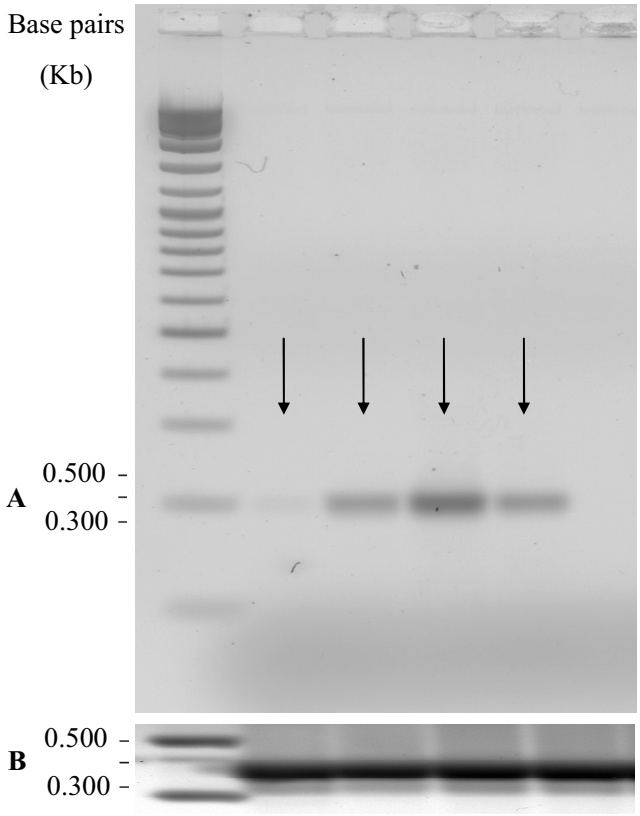

Fig. 4 Amplification *BARE-1*-LTR by designed primers for its conserved domain using generated cDNA based on universal *Copia* primers. (**A**) Substantial increase was noticed after 4, 21 and 32 h of sobitol treatment. (**B**) The cDNA samples were the same in each lane and normalized using *α-tubulin* primers as a control.

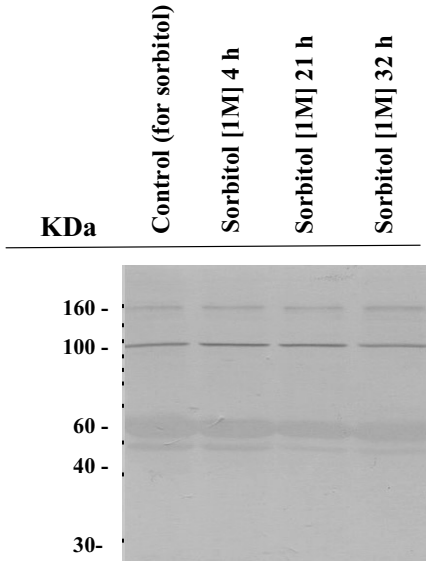

Fig. 5 Immuo-responses of leaf proteins separated by SDS-PAGE to anti-*GAG* antibodies. Immunoblot reacted with antibodies made to a full-length *BARE-1 GAG*, however, no substantial increase in the level of *BARE-1 GAG* was observed. The molecular weights are shown on the left axes.

tial known as post-transcriptional gene silencing (PTGS) (Jensen *et al*. 1999; Vicient *et al*. 1999; Hirochika *et al*. 2000). PTGS, mediated by short interfering RNA (siRNA) and promoter inactivation by methylation (transcriptional gene silencing, TGS), is a very effective silencing mechanism (Vance and Vaucheret 2002; Cheng *et al*. 2006). Although they are usually inactive, some retrotransposons can escape silencing. In fact, transposable elements represent a threat to the integrity of their host genomes because of their mutagenic potential (Kidwell and Lisch 2000). Hence, an understanding of retrotransposons rule in genome dynamics requires analysis of the regulation of the various steps of their life cycle (**Fig. 6**).

Activation of *BARE-1* by dehydration stress

Although a large number of genes and proteins responding to stresses have been studied, most of the molecular components of the signaling transduction pathway involved in gene regulation and genomic impact under stress are still unidentified. Water-deficit stress is among the most frequently altered in gene expression (Hazen *et al.* 2005). In the current investigation we examined the effect of water-deficit stress on genomic *BARE-1* retrotransposon in barely after 1 and 21 days of drought by RT-PCR. The results show substantial increases in the *BARE-1* transcription level after 21 days of drought compared to the control (**Fig. 7**).

Accumulation of *BARE-1* (*GAG*) after drought treatment

Immunoblots with full-length *BARE-1* anti-*GAG* antibodies (Vicient *et al*. 2001) were able to detect accumulation of *BARE-1 GAG* (capsid) proteins after 21 days of water-deficit stress (**Fig. 8**). Thus, it could be concluded that the activation of *BARE-1* at the transcription level is associated with a specific increase in *BARE-1-GAG* capsid protein accumulation after water-deficit stress treatment.

Model of LTR retrotrasposon activation by stress

The life cycle of active retrotransposons are mainly composed of three major stages (transcription, translation and integration). They move by a cycle involving transcription, translation to generate the proteins needed for mobility, packaging into virus-like particles, reverse transcription to generate a cDNA, and integration of the cDNA back into the genome. For individual retroelements, evidence exists for transcription (Hirochika 1997; Vernhettes *et al*. 1997; Okamoto and Hirochika 2000; Morlaes *et al*. 2003; Sharma *et al*. 2008), stress activation (Wessler 1996; Grandbastien 1998; Kalendar *et al*. 2000; Nellaker *et al*. 2006; Stribinskis and Ramos 2006), translation (Hu *et al*. 1995; Jääskeläinen *et al*. 1999; Alisch *et al.* 2005; Saito *et al.* 2008), and integration at specific loci (Grandbastien *et al.* 1989; Hirochika *et al*. 1996; Brady *et al.* 2007; Geuking *et al.* 2009). Based on the above-mentioned references, the role of different elements of retrotransposon structure in its activation is hypothetically illustrated in **Fig. 9**.

The illustration shows that high variability in the nucleotide sequence as well as different *cis*-acting elements have been determined when promoter regions from different family members were compared. In that way, different retrotransposon families can respond differently to specific stress challenges (Grandbastien *et al.* 1998, 1989, 2005).

CONCLUSIONS

Sorbitol application is usually used to mimic the effects of drought and water deficit in plants (Kramell *et al*. 2000). The effect of sorbitol treatments was compared, in both *Copia* and *Gypsy* groups of genomic retrotransposons, with drought in barley (Flavell 1992; Voytas 1992). The cultivated barley 'Bomi' was used in this study for its relationship with its wild relatives (Pelger and Hoyer-Hansen 1989). The transcriptional activation of both groups was identified using RT-PCR and cDNA analysis. Results revealed that *Copia* elements are strongly affected by both stresses compared to the *Gypsy* group. This variation could have resulted from the structural differences of both groups (Suoniemi *et al*. 1998). In this regard, the result also showed that *BARE-1*, which belongs to the *Copia* group, was induced at the transcriptional and translational levels after 21 days of drought compared to the control. The results showed that different

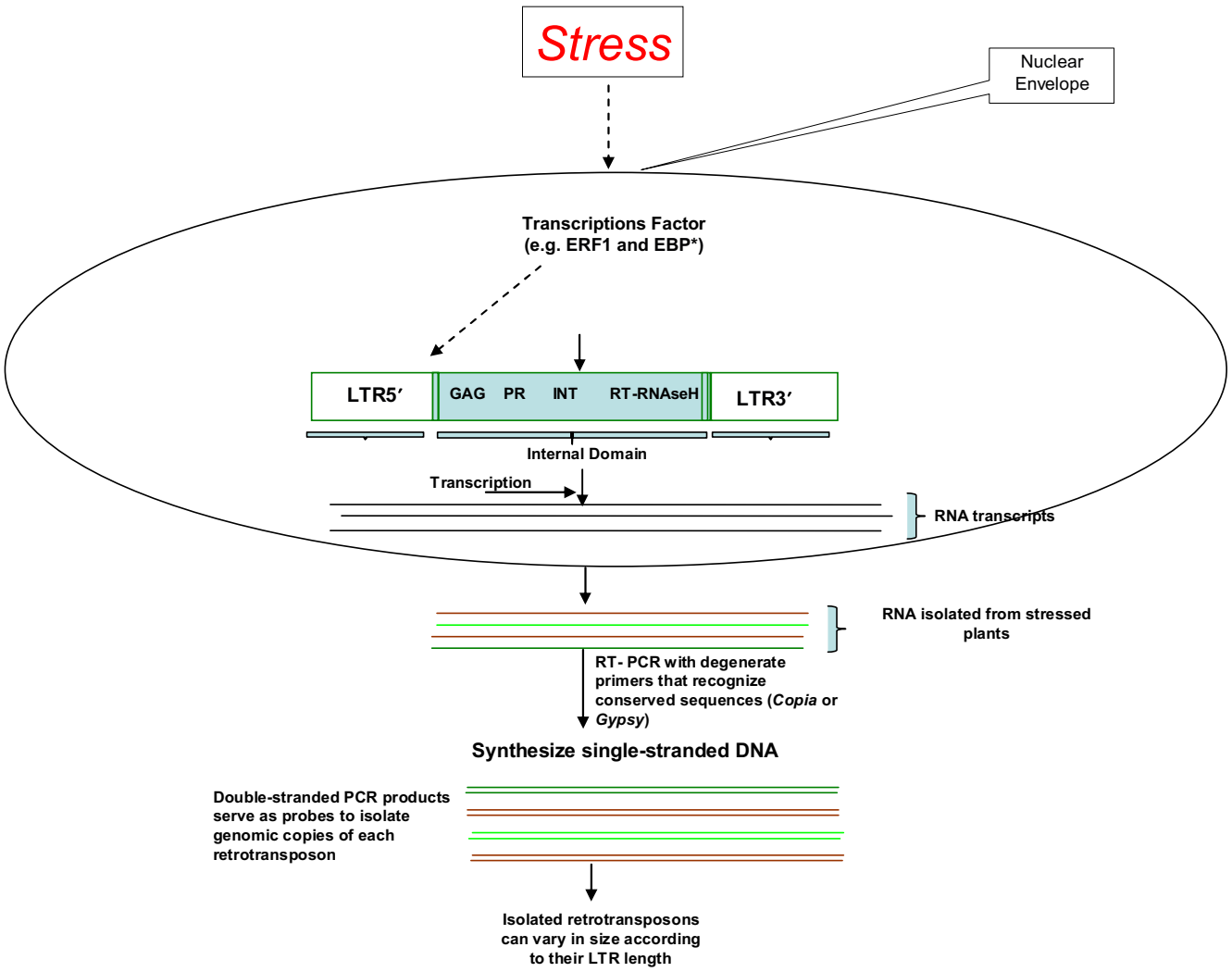

Fig. 6 Proposed schemes for studying stress activation of retrotransposon. RNA isolated from stressed plants or cultured cells is used to synthesize single-stranded DNA, which in turn is the substrate for amplification using the polymerase chain reaction with degenerate primers that recognize conserved sequences in all *Copia*-like reverse transcriptase (RT) domains. If other family is concerned, degenerate primers that recognize conserved sequences in that family should be used.

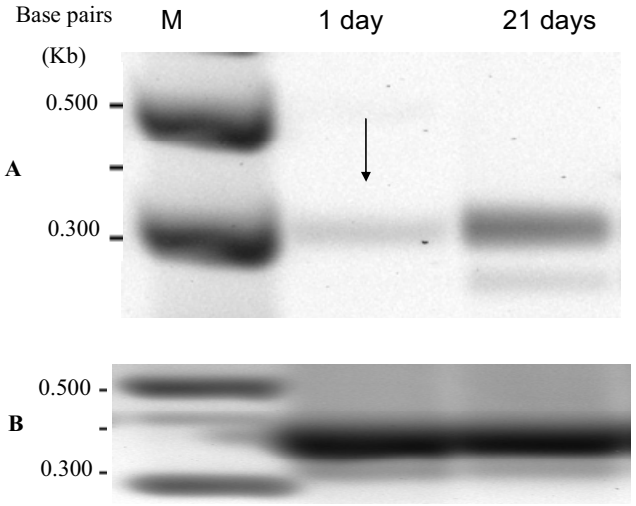

Fig. 7 The effect of water-deficit stress on genomic *BARE-1* retrotransposon in barely after 1 and 21 days of drought. By using RT-PCR, Substantial increases in *BARE-1* transcription were noticed after 21 days of drought comparing to the control.

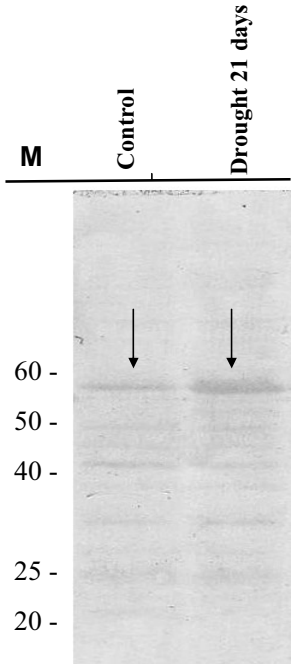

Fig. 8 Substantial increase in the level of *BARE-1 GAG* was observed after 21 days of water deficit.

kinds of stresses affect both groups differently. The results agree hypothetically with the theory that indicates that different patterns of activation, in different families, result from their structural differences (Mansour 2007).

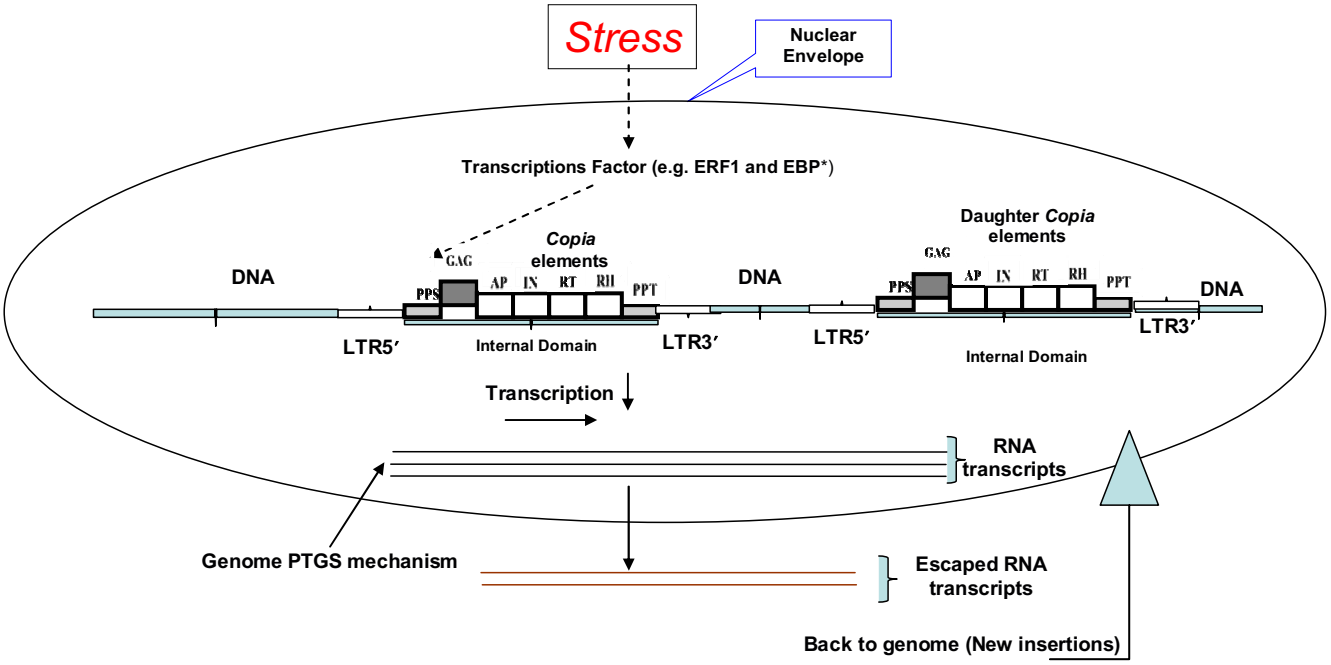

Fig. 9 Proposed models for stress activation and reinsertion of genomic retrotransposon. Stress activate specific transcription factor associated with it, which in turn, bind and activates the retroelement's transcription, translation and integration back to the genome.

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