ACTIVATION OF *Bare-1* RETROTRANSPOSONS IN BARLEY UNDER SORBITOL STRESS

A. MANSOUR[1], M. J. JääSKELäINEN[2], W. CHANG[2] AND A. H. SCHULMAN[2]

1. *Genetics Department, Faculty of Agriculture, Zagazig University, Egypt*
2. *MTT/BI Plant Genomics Laboratory, Institute of Biotechnology, University of Helsinki, PO. Box 56, Viikinkaari 9, FIN-00014 Helsinki, Finland*

Many stresses have been reported to cause an epigenetic activation of mobile elements, with or without LTR, dispersed throughout the genome (Mansour, 2007; Salazar *et al*., 2007). For instance, various biotic and abiotic stresses are shown to increase expression of various transcriptionally active LTR retrotransposons including chilling, infection, mechanical damage, invitro regeneration, hybridization and generation of doubled haploids (Hirochika, 1995; Grandbastien *et al*., 2005). In plants, it has been reported that retrotransposons of rice involved in mutations induced by tissue culture (Hirochika *et al*., 1996). Also, exposure to cell-wall hydrolases activates specific expression of tobacco retrotransposon (Pouteau *et al.*, 1991). In human cells, it was reported that human endogenous retrovirus (HERV) elements is transactivated by viral infections. This transactivation was shown in the different celllines (Nellaker *et al*., 2006).

The retrotransposon *Bare-1*family of cultivated barley (*Hordeum vulgare* L.) comprises more than 1.6 x 10^4 genomic copies (Vicicient *et al.,* 2001) dispersed on all chromosomes (Suoniemi *et al.,* 1996). The *Bare-1* retrotransposon was reported to respond to sharp microclimatic divergence specially drought (Kalendar *et al.,* 2000). While abscisic acid (ABA) is known as the primary hormone mediating plant responses to stresses specially drought (Wu *et al.,* 1997), ABA has been also reported as inducing signals for the retrotransposon *Bare-1* from barley (Suoniemi *et al.,* 1996). With this regard, sorbitol was widely applied *in vitro* for mimicking the effect of drought. Both sorbitol and drought are seemed to have mutual effect on each other. For Instance, drought treatment has shown to affect sorbitol and ABA level as shown in rosaceae fruit trees (Kanayama *et al*., 2007). The genomic impact of sorbitol treatment on barley has not described yet. In this study, the missing connection between sorbitol treatment on barley plants and the activation of retroelements especially *Bare-1* was investigated in this report.

MATERIALS AND METHODS

Sorbitol treatment

Barley (Bomi) seeds were germinated in the darkness for 2-3 days, and then seedling moved to vermicular and continued to grow for 14 days. The young leaves were cut and floated on 1 M sorbitol for 4, 21 and 32 hours. Leaves from different seedlings, after sorbitol stress, were frozen in liquid nitrogen and stored at –80^{o}C for RNA extraction.

RNA extraction

Mature barley leaves (Hordeum vulgare) were used to isolate total RNA after applying different stresses using Trizol method (Invitrogen, Carlsbad, CA, USA) following the manufacture instruction.

RT -PCR reaction

The QIAGEN OneStep RT-PCR Kit was used. The RT-PCR was performed following the manufacturer instruction and condition.

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 (Fermentas), 5 mM DTT, 100 U RiboLock™ ribonuclease inhibitor and RNA ≈ 20ng. Then the mixture was incubated for 60 min at 37°C.

RNA was purified with chloroform and precipitatied with (3V 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 mins 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 RiboLocka ribonuclease inhibitor, 5 µM random primers and 1 mM dNTPs. The mix was incubated at 50°C for 10 mins and chilled on ice. Then, 1000 units of RevertAida, M-MuLV Reverse Transcriptase (Fermentas), were added. The reaction mixture was incubated at 4°C for 60 minutes. Finally, 150 µl TE was add and stored in -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 primers, 0.2 mM dNTP Mix. and 1U DNaseII DNA Polymerase. Amplification was performed using PCR machine PTC-225 (Mj Research, USA). The PCR reaction parameters consisted of: 95°C, 2 min; 30 cycles of 95°C for 15sec, 52 or 56 or 60°C for 60sec and 72°C for 2 min;

a final extension at 72°C for 10 min.10µl of the samples were mixed in 2X loading buffer, loaded in 1.7% agarose gel. Gel electrophoresis condition was 80V for 3h; the bands was separated with 1xSTBE and detected by ethidium bromide staining.

Specific primer sequences

Degenerated primers, Specific for *Copia*-like elements, (RT+ caratggaygtnaarac & RT- catrtcrtcnacrta) (Hirochika and Hirochika 1993) and degenerated Primer, Specific for *Gypsy*-like elements (RT+ arcatrtcrtciacrta &RT-tayccihticcicgiathga), as previously described by Fllavel et al, (1992), were used. Barley a-tubulin gene amplified with the specific primers 5-AGTGTCCTGTCCACCCACTC-3 and 5-CCAAGGATCCACTTGATGCT-3 (acc. no. U40042) was used as a constitutive control in all experiments (Suprunova *et al*, 2007). The RT-PCR products were visualized by electrophoresis on 2% agarose gel. For *Bare-1*, the specific primer F5`acgacacctccgcgttcagc-3` R and 5`ccgaccacatgcctccacggtttttcct-3` were designed from the consensus sequence of Bare-LTR.

GAG immunoblotting

The protein samples were prepared as described by Vicient *et al*., (2001). The protein was extracted from leaf eppendorf with plastic grinding sticks and sea sand. Equivalent of 20 µg 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 the *Bare-1* GAG full-length, which recognizes 150, 95, and 32 kD proteins was used as described (Vicient et al., 2001).

RESULTS AND DISCUSSION

Major classes of long terminal repeats (LTR) retrotransposon

Based on the structure differences, there are two major classes of long terminal repeats (LTR) retrotransposon, *Gypsy* and *Copia*. Both families are differing in the order of their encoded proteins, both are ubiquitous throughout the plants and stress activated (Flavell, 1992; Suoniemi *et al*., 1998) (Fig. 1). Earlier studies suggested that the retrotransposons present in low-copy number are normally silent but are strongly activated by stress, whereas the abundant families are constitutively active, but at low levels (Vicient *et al*., 1999). However, the replication nature of retrotransposon 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). Thus, it was important to evaluate the stress activation of both families 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 structure differences (Mansour, 2007).

Time course activation by sorbitol of Copia retrotransposon using 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 this study, sorbitol (1M) was applied on barley seedlings in different time intervals as described in material and methods section. Specific *Copia* primers (Hirochika and Hirochika, 1993) were used to amplify the extracted RNA using RT-PCR. Substantial increase in the bands was noticed after 4 hours and 25 hours of treatment. This increment was declined again after 32 hours of treatment (Fig. 2). On contrary, using specific *Gypsy* primers had no effect (data not shown). This could be explained by the difference in structure between *Copia* and *Gypsy* families.

Activation of Copia retrotransposon by sorbitol using cDNA

In this study, 1M sorbitol was applied on 14 day old young leaves of barley (Bomi) at different time intervals. The extracted RNA was used to generate cDNAs from the treated samples. Amplifying the cDNA samples using the same specific *Copia* primers resulted in the formation of two strong bands after 4 and 25 hours from sorbitol treatment confirming the previous results (Fig. 3). Thus, the activation pattern of *Copia* family and *Gypsy* were similar at different time intervals using both RT-PCR and cDNA. Beguiristain *et al*. (2001) have shown that three Tnt1 subfamilies were induced by stress, but their promoters have a different response to different stress-associated signaling molecules. This agrees with our 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

Amplification pattern of *Bare-1*-LTR specific primers, with the cDNA, resulted the same patterns of increment in the RT-PCR results after 4 and 25 hours of sorbitol treatment (Fig. 3). The activation pattern of *Copia* and *Bare-1*were similar at different time intervals, however, *Gypsy* showed no activation. This could be explained because *Bare-1* belongs to *Copia* family. These results shows different Stress-associated patterns of expression with different retrotransposon families which agrees with the results of (Beguiristain *et al*, 2001) and confirm the specific stress activation model of each elements with different stresses (Mansour, 2007).

Accumulation of capsid GAG antibodies after sorbitol treatment

The accumulation of *Bare-1* GAG (capsid) proteins was also detected on immunoblots with full-length *Bare-1* anti-GAG antibodies using western blotting technique (Vicient *et al*., 2001) (Fig. 5). Despite the activation of *Bare-1*on the

transcription level, no specific increase in *Bare-1*-GAG after sorbitol treatment was observed. This could be explained by the genome development mechanisms for controlling the TEs activity and their mutagenic potentiality known as post-transcriptional gene silencing (PTGS) (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 very effective silencing mechanism (Cheng *et al*., 2006). Although they are usually inactive, some retrotransposon 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 the role of retrotransposons in genome dynamics requires analysis of the regulation of the various steps of their life cycle.

Proposed model of LTR retrotrasposon activation by stress

The 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 (Okamoto and Hirochika, 2000), stress activation (Grandbastien, 1998; Kalendar *et al.*, 2000), translation (Jääskeläinen *et al*., 1999) and integration at specific loci (Hirochika *et al.*, 1996). Based on the above mentioned facts we tried to draw a hypothetical scheme of stress activation in plants (Fig 6). That scheme illustrates the role of different elements of retrotransposon structure in its activation (Mansour, 2007). It also 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 and 2005).

SUMMARY

LTR-retrotransposons and other repetitive DNA elements are directly or indirectly responding to a wide variety of stresses by increasing or decreasing its copies. This effect is specific for different retrotransposons or stresses. The *Bare-1* retrotransposon members are actively transcribed *in vivo* in barley. *Bare-1* family was reported to respond to sharp microclimatic divergence specially drought. Sorbitol has been used widely to mimic the effects of drought. A potential osmotically-stressed action has been ascribed to sorbitol, but invivo evidence of this remains elusive. In the present work, the effect of sorbitol was compared in both *Copia* and *Gypsy* groups of retrotransposon using specific primers for both groups. One step RT-PCR analysis showed that sorbitol exerted a strong

influence upon *Copia* elements group after 4, 24 and 34 hours of sorbitol treatment. When *Bare-1* specific primers were used to amplify *Copia* cDNA products, it revealed unique strong DNA bands at the same time points. The immunobloting of *Bare-1* Gag protein specific antibody showed no specific increase after these treatments. Hence, sorbitol, has the capacity, in barley plant, to increase the transcriptional activity of *Copia* elements specially *Bare-1* retrotransposon.

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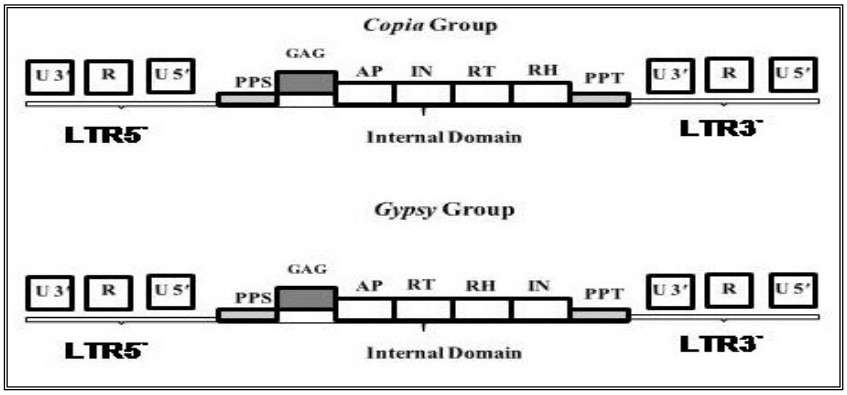

Fig. (1): Proposed structure of two major classes of retrotransposons. Both classes are bound by long terminal repeats (LTRs). The LTRs contain inverted repeat at their termini. The primer binding site (PBS) and polypurine tract (PPT) are present in most elements and are required for replication by reverse transcriptase (RT). 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 these units.

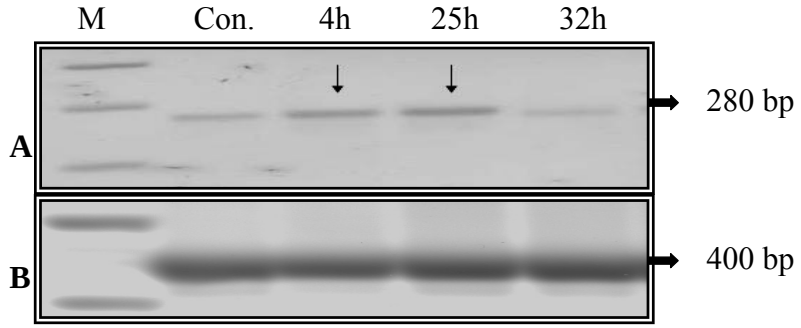

Fig. (2): RT-PCR amplification of *Coipa* like elements under Sorbitol stress using *Copia* universal primer. A) Substantial increase in the banding pattern was detected after 4hour and 25hours of sorbitol treatment. B) The amount of RNA was normalized using *α-tubilin* primers as a control.

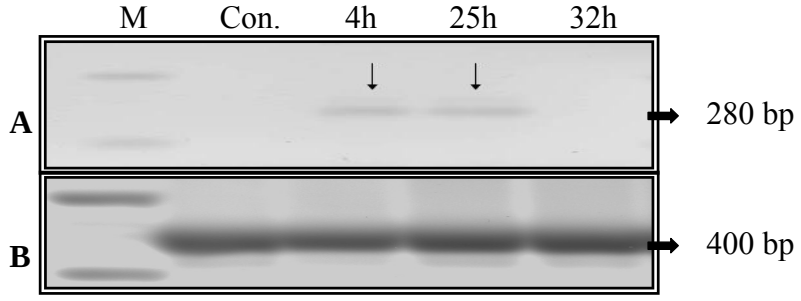

Fig. (3): Amplification of cDNA generated under sorbitol stress using *Copia* universal primer. A) Unique bands were formed after 4hours and 25 hours from of sorbitol treatment . B) The cDNA samples were the same in each lane and normalized using *α-tubilin* primers as a control.

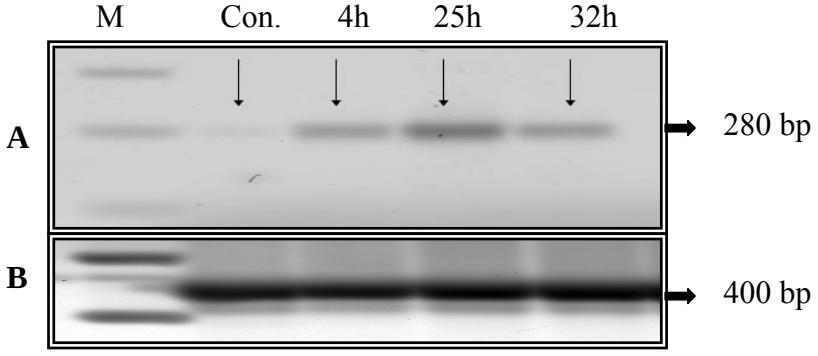

Fig. (4): Amplification *Bare-1*-LTR by designed primers for its conserved domain using generated cDNA based on universal *Copia* primers. A) Substantial increase was noticed after 4hours, 25 hours and 32 hours of sorbitol treatment. B) The cDNA samples were the same in each lane and normalized using *α-tubilin* primers as a control.

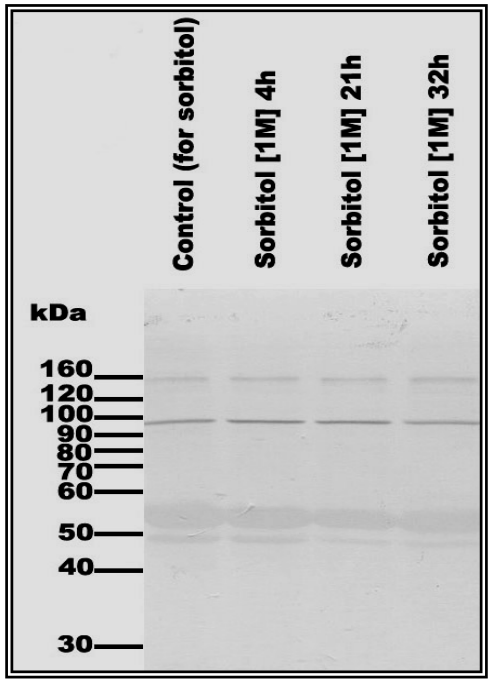

Fig. (5): Immuo-responses of leave proteins separated by SDS-PAGE to anti-GAG antibodies. Immunoblot reacted with antibodies made to a full-length *Bare-1* GAG. The molecular weights are shown on the left axes.

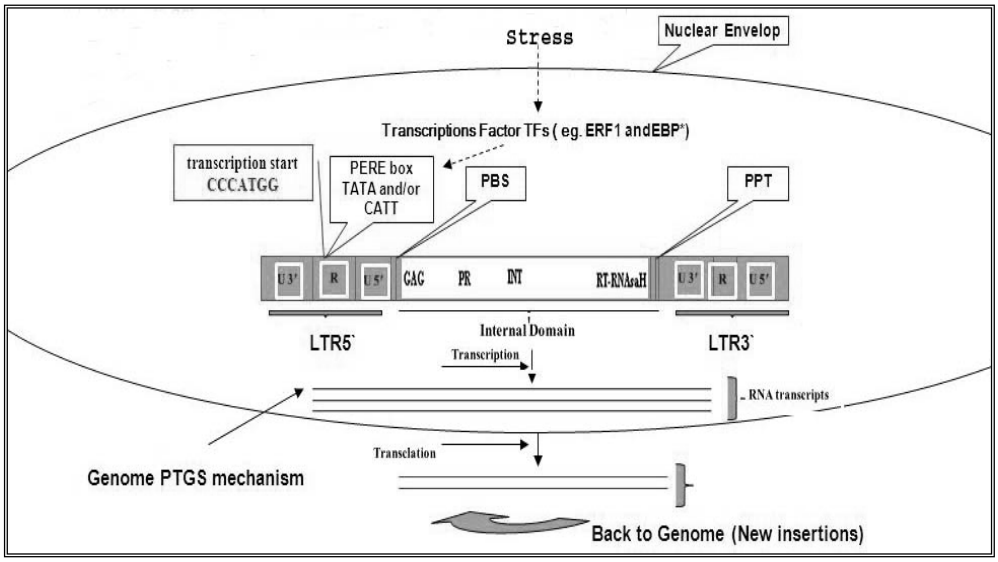

Fig. (6): Proposed scheme for stress activation of retrotransposon. Both the promoter region and the genome controlling mechanism can determine the activation of any element.