SEMI-QUANTITATIVE RT-PCR ANALYSIS FOR DEHYDRIN GENE EXPRESSION LEVELS IN WHEAT UNDER DROUGHT STRESS

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Drought is a worldwide problem, seriously influencing plant (crop) productivity. Wheat is a stable food for 35% of the world population. However, about 60% of world land area exists in arid and semi-arid zone. Rampino *et al.* (2006) reported that water deficit is a severe environmental stress and the major constraint on plant productivity with an adverse effect on plant growth. They noticed that *Triticum* and *Aegilops* seedlings differed in their response to drought stress at the physiological and molecular levels. Carding *et al.* (1992) reported that reverse transcription-polymerase chain reaction (RT-PCR) is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limited amounts. In most cases, when RNA analysis is required, a qualitative study is not sufficient to deliver a satisfactory answer (Marone *et al.*, 2000; Pierelli *et al.*, 2000). A common question is the quantification of specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions. Investigators have often been faced with the problem of detecting poorly expressed transcripts, as well as that of handling small amounts of unique samples.

A number of protocols and improved PCR techniques are now available, which were discussed by Marone *et al.* (1997) and Marone *et al.* (2001a). They reported that they may be not easily accessible to a standard laboratory and have pitfalls together with the advantages for which they were created. Although reproducibility is always an essential requirement, extreme accuracy may not be, in most studies a paramount requirement where the focus is not to measure minor changes or the exact number of molecules, but an increase or decrease by at least 1to 2-folds in expression levels. Hence, despite the greater accuracy of recently developed techniques, semi-quantitative methods are still widely used and appropriate for many purposes.

Marone *et al.* (2001b) described a semi quantitative RT-PCR protocol to extract RNA from as little as 10,000 cells and to measure the expression levels of several targets of mRNAs from each sample. They reported that as for all quantitative techniques, great care must be taken in all optimization steps: the

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necessary controls to ensure a rough quantitative (semi-quantitative) analysis. Lee *et al.* (2005) investigated the full-length (1.1 kb) cDNA, of *Oryza sativa* Dehydrin 1 gene (*OsDhn*1). The results indicated that the deduced protein is hydrophilic and belongs to the acidic dehydrin family, which includes wheat WCOR410 and Arabidopsis COR47. Expression of *OsDhn*1 was strongly induced by drought. Induction of *OsDhn*1 was also up-regulated in *UBI*: *CBF*1/*DREB*1b transgenic plants indicating that it is regulated by the *CBF*/*DREB* stress signaling pathway.

Modifications in the expression level of five dehydrin (*DHN*) genes were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (Rampino *et al*., 2006). Five cDNAs coding for different *DHN*s in wheat were identified and characterized. Four of these cDNAs were related to novel DHN sequences. The results obtained clearly indicated a relationship between the expression of these genes and tissue water content. In particular, in the resistant genotypes, the expression of DHN genes was initiated even though tissue hydration levels were still high, indicating the involvement of these proteins in water retention.

The aim of this investigation is to study the modifications in dehydrin gene(s) expression in response to drought stress as compared to control condition in wheat on RNA level using semi-quantitative RT- PCR and to validate the results using cDNA for northern blotting technique.

MATERIALS AND METHODS

Two wheat cultivars (Sahel1 and Giza160) were cultivated in a hydroponic experiment under control conditions and drought stress using 8 g/L polyethylene glycol for two days. Leaf samples of the two cultivars under investigation were collected from stressed plants and control to study the modifications in dehydrin gene(s) expression on mRNA level using semi quantitative RT-PCR technique.

RNA extraction

RNA was extracted according to Ashoub *et al.* (2006), 0.2 g plant tissue was ground in liquid nitrogen, four volume of extraction solution (0.2M *MOPS*; pH 3-4) was added, equal volume of phenol/chloroform (1:1) saturated with 0.2 M *MOPS* was added. The mixture was incubated for 10 min at room temperature, and then centrifuged at 10000xg for 10 min at 4°C. The aqueous phase was collected with an equal volume of chloroform and re-centrifuged as described above. The aqueous phase was collected and 1/10 volume of 3M sodium acetate (pH 5.2) and 1 volume of absolute isopropanol was added. Samples were stored at -20°C for 2 hs. RNA was collected by centrifugation at 10000xg for 15 min. at 4°C. The supernatant was discarded and pellets were re-suspended in 100 µl 3 M sodium acetate, at pH 5.2. RNA was precipitated; pellets were washed with 100 µl of 70% ethanol and then precipi-

tated as above and re-suspended in 50 µl Nuclease free H_2O. RNA quality was examined on denature agarose gel electrophoresis.

RT-PCR

RNA was reverse transcribed in the presence of 5 mM MgCl, 1X PCR Buffer, 1 mM dNTPs, 25 U MuLV Reverse Transcriptase, 4 U RNA-guard Ribonuclease inhibitor and 2.5 µl of dehydrin reveres primer with the following sequence: (CTAGTGTCCAGTACATCCTCC) in a final reaction volume of 30 µl. Reactions were carried out at 42°C for 30 min, followed by a 10 min step at 94°C to denature the enzyme, and then by cooling to 4°C.

Standard PCR reaction for dehydrin

Equal amounts of the produced cDNA (2µg) of each sample were amplified using reaction mix are containing 1 unit Taq, 1x buffer, 1.5 mM MgCl, dNTPs and in the presence of the specific primers for dehydrin with the following sequences: {(F) ATGTCTCAGTATCAAAAC CAATATGTGC and (R) CTAGTG TCCAGTACATCCTCC}. Reactions were carried out in the following conditions, the first cycle for 5 minutes at 94°C, followed by 45 seconds at 94°C, 45 seconds at 55°C and 1 min at 72°C for 30 cycles. The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification, and that the two sets of primers used in each reaction did not compete with each other. Each set of reactions always included a no-sample negative control. We usually performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination. PCR products were resolved in 1.2 % agarose/TBE gel, visualized using ethedium bromide on UV transelluminator and photographed using gel documentation system. Furthermore, the concentration of the products was measured using spectrophotometer on the wave length 280/260 and was used as indicator for dehydrin activity in the different samples (semi-quantitative PCR).

cDNA northern blotting for dehydrin gene

Using the method of Jaakola *et al.* (2001), the cDNAs were run on a 1% agarose gel for 1 h at 100 V. The gel was stained for 30 min in 0.5 mM ethidium bromide. After two washes in both denaturation (0.5 M NaOH, 1.5 M NaCl) and neutralization (0.5 M Tris-HCl [pH 7.5], 3 M NaCl) buffers according to the standard Southern blotting procedure, the gel was transferred overnight to a positively charged nylon membrane (Roche) by capillary transfer using 20x SSC (standard saline-citrate). The membrane was baked at 120ºC for 30 min. A probe consisting of a 520 bp PCR amplified fragment of the dehydrin gene from wheat was labeled with digoxigenin-dUTP (Roche) according to the manufacturer's

instructions. Hybridization was performed overnight at 42°C in Ultrahyb hybridization solution (Ambion) in a probe concentration of 1 ng/mL. The membrane was washed twice in 2x SSC, 0.1% SDS for 5 min and twice in 0.1x SSC, 0.1% SDS for 15 min at 42°C. Nucleic acids were detected using the Dig high prime DNA labeling and Detection starter Kit I (cat. no. 1745832) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

When choosing between quantitative or semi-quantitative protocol to determine RNA expression levels by RT-PCR, the following parameters must be taken into consideration: 1) feasibility in the laboratory; 2) necessity to study different markers in the same sample; 3) availability of sample and 4) accuracy required for the specific application (i.e., need to measure the specific number of RNA molecules or rather rough variations in RNA levels). Relatively accurate methods have been developed, such as competitive PCR (MIMIC) or real-time PCR based on the use of fluorogenic probes. Quantification is rather accurate, but this technique requires a relatively large amount of cDNA and a large number of amplification reactions per sample. Moreover, it requires intensive initial work when different RNAs have to be analyzed. Another interesting, recently devised method, is the single-tube PCR which permits reverse transcription and PCR, this is a very convenient method when a single RNA marker has to be analyzed in a large number of samples. In addition, assaying several RNAs on the same sample.

In this investigation, modification of dehydrin gene expression on RNA level in the plants of the two wheat cultivars under control and drought stress conditions was used by semi quantitative RT-PCR technique. cDNA produced from RNA extracted from stressed plants and control of the two wheat cultivars was used. The results in Figs (1 and 2) showed that a band with 480 bp was produced in the two cultivars under control and stress conditions but with different intensities. Higher intensity was observed in the stressed plants of cultivar G160 as compared with the control plants of the same cultivars indicating that dehydrin gene expression increased with high level as many folds under stress but the number of folds could not be determined from the gel. To determine how many folds increase in dehydrin gene expression under stress, the remained volumes of PCR products were measured using spectrophotometer on the wave length 280/260. The results indicated that 2.5 fold increase of dehydrin gene expression was observed in stressed plants as compared to control ones of this cultivar. On the other hand, in cultivar Sahel1, a band was obtained in the stressed plants as compared with the control plants but with lower intensity than that of cultivar G160. The results confirmed that the response for drought stress was greater in cultivar G160 than in cultivar Sahel1 regarding dehydrin gene expression. The spectrophotometeric

reading confirms the results of the gel where it indicated that the increase in dehydrin gene expression in the stressed plant of cultivar Sahel1 was only 0.297 folds of the control ones.

Dehydrin northern blotting

To confirm the results of semi quantitative RT-PCR, northern blotting was performed using dehydrin probe of the RT-PCR to detect the increasing in expression of the gene(s) conferring dehydrin in wheat using the same extracted RNA of the plants under investigation. The blotting results are shown in Fig. (3). The results indicated a greater response to drought stress in cultivar G160 than in cultivar Sahel1 as indicated by higher gene expression for the gene conferring dehydrin in the stressed plants as compared with the control ones. The results were in agreement with those obtained by semi quantitative RT-PCR. Dehydrin protein is known as an osmolyte which protects the plant cells from injures as it accumulates during drought stress, (Close, 1989; Ismael *et al*., 1999). The higher intensity of cDNA band in Giza 160 under stress could be due to over expression of dehydrin gene.

These findings were in agreement also with those of (Borovskii *et al*., 2002) who reported that drought stress led to higher accumulation of dehydrin-like protein (dlp) with 63 kDa in rye and wheat. Porat *et al*. (2004) reported that dehydrins are a family of plant proteins induced in response to environmental stresses such as water stress, and that citrus contains a small gene family encoding a unique class of dehydrins that identified by cDNA differential display analysis in a 'Navel' orange with 202-bp polymerase chain reaction (PCR) fragment.

Olave-Concha *et al*. (2004) reported that northern analysis showed two putative dehydrin transcripts of 1.0 kb accumulated only under exogenous ABA and 1.6 kb under osmotic and salt treatments, suggesting that *D. antarctica* would have ABA-dependent and independent pathways for regulation of dehydrin expression.

Rampino *et al*. (2006) observed a modification in the expression level of five dehydrin (DHN) genes in wheat by using reverse transcription-polymerase chain reaction (RT-PCR). Five cDNAs coding for different DHNs were identified and characterized. Four of these cDNAs were related to novel DHN sequences. The results obtained clearly indicate a relationship between the expression of these genes and tissue water content. In particular, in the resistant genotypes where the expression of DHN genes is initiated even though tissue hydration levels were still high, indicating also in wheat the involvement of these proteins in water retention.

SUMMARY

Semi quantitative RT-PCR protocol was optimized in our laboratory to extract mRNA from drought stressed and control plants of two wheat cultivars and

to measure the expression levels of several target mRNAs from each sample. In this investigation dehydrin gene expression was assessed. The detailed procedure for dehydrin expression levels under drought stress and control condition was described. The protocol was used as an internal control to normalize for sample to sample variations in total RNA amounts and for reaction efficiency. As for all quantitative techniques, great care must be taken in all optimization steps to ensure near quantitative (semi-quantitative) analyses. Also northern blotting for dehydrin was performed to confirm the results of semi quantitative RT-PCR in assessing the expression of gene(s) conferring dehydrin under drought stress and control condition. Generally, the results of the two techniques indicated that dehydrin gene expression increased under drought stress as compared with control plants particularly in cultivar G160.

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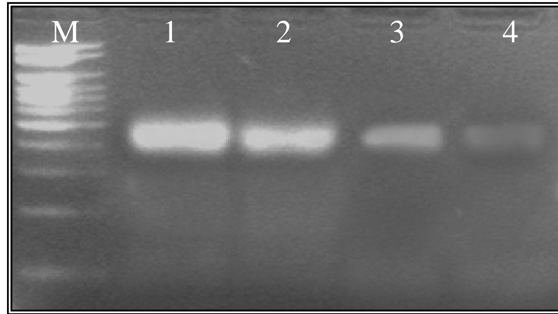

Fig. (1): RT-PCR products for dehydrin. Samples order from left to right are: DNA standard (M), 1- G160 drought stressed plants, 2- Sahel1 drought stressed plants, 3- Sahel1 control and 4- G160 control.

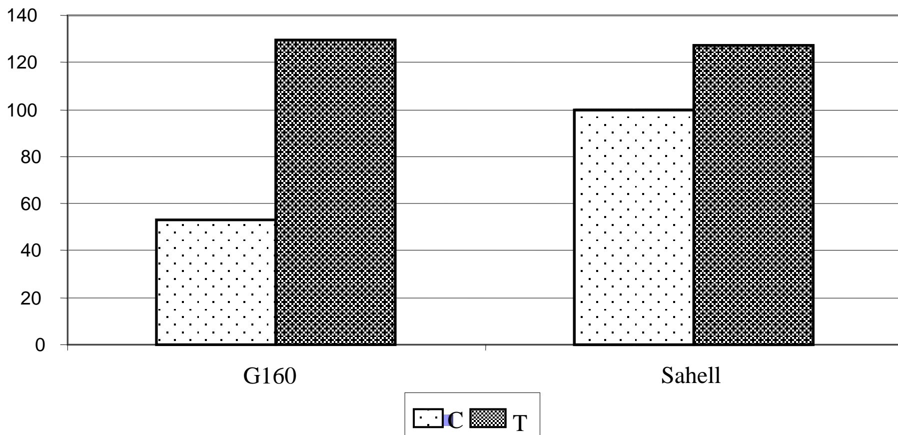

Fig. (2): Histogram reflecting the changing of spectrophotometric readings into µg/µl for the stressed (T) and control (C) plants of the two cultivars (G160 and Sahall) under investigation.

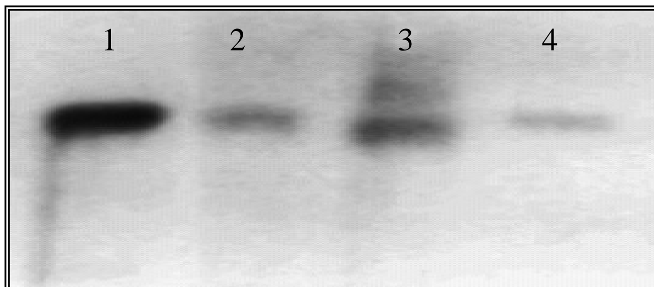

Fig. (3): cDNA-northern blotting for dehydrin. Samples order from left to right are: 1- G160 drought stressed plants, 2- Sahel1 drought stressed plants, 3- Sahel1 control and 4- G160 control.