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DYNAMICS OF mRNA OF GLYCINE-RICH RNA-BINDING PROTEIN DURING WOUNDING, COLD AND SALT STRESSES IN *NICOTIANA TABACUM*

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Abstract

Glycine-rich RNA-binding proteins (GRPs) are RNA-binding proteins that contain one or more RNA recognition motif (RRM) or consensus RNA-binding domains at their N-terminus and a glycine-rich domain at their C-terminus. Their function is not well characterized but GRPs are implicated in plant stress responses, owing to the fact that their mRNA level increases under these conditions. In order to gain an insight into the role at molecular level, the study was designed to observe the dynamics of *Nt*GRP1 during exposure to stresses i.e. wounding, cold and salt stress by real-time PCR. Expression was found to be modulated by wounding in five months old plants. For cold stress, plants were transferred to 4°C for 24 hours at different developmental stages, wherein expression of mRNA was found to be up-regulated. Variation in transcript level was also seen in response to treatment with various concentrations of NaCl. The detailed characterization of GRPs is expected to enhance our understanding about role of GRPs in plant stress metabolism.

Introduction

The glycine-rich proteins that lack an amino terminal signal peptide and contain one or more consensus sequence RNA-binding domain (CS-RBD) or RNA recognition motif (RRM) at their N-terminus and a highly glycine- rich region at their C-terminus are termed glycine-rich RNA-binding proteins (GRPs). GRPs are widely distributed in plants both in angiosperms and gymnosperms (Sachetto-Martins *et al*., 2000) and have been isolated also in various organisms ranging from yeast to man, for instance, in cyanobacteria (Sato, 1995), vertebrates such as mice (Nishiyama *et al.,* 1997) and human beings (Derry *et al.,* 1995). GRPs have been identified in plants including monocots such as *Zea mays* (Gendra *et al.,* 2004) and rice (Kim *et al.,* 2010) and also reported in dicots e.g. *Arabidopsis* (Kim *et al.,* 2010), tobacco (Naqvi *et al.,* 1998; Lee *et al.,* 2009) and *Brassica* (Kim *et al.,* 2012).

Post-transcriptional gene regulation is poorly understood in plants (Kidokoro *et al*., 2009). Although *Arabidopsis* genome encodes for more than 200 putative RBPs, only a few RBPs have been critically analyzed for their functions (Fedoroff, 2002). Members of GRPs family have been implicated in a variety of roles in RNA metabolism including transcriptional termination (Gott-lieb & Steitz, 1989), mRNA stability (Minvielle-Sebastia *et al.,* 1991), splicing (Scherly *et al.,* 1990) and translational initiation (Milburn *et al.,* 1990).

The functions of GRPs are largely unknown, but the affinity of the GRPs for variety of nucleic acids indicates their functional diversity. In ribohomopolymer-binding assays, GRP from maize, barley and *Arabidopsis* show higher affinity for poly r(U) and poly r(G) than to poly r(A) and poly r(C) (Kim *et al.,* 2005) suggesting that cellular RNA ligands possess more U and G residues. This feature shows their involvement in regulation of gene expression at post-transcriptional level as the RBPs.

Recently studies have been conducted to understand various aspects of plant stress physiology. Acka & Samsunlu (2012) examined salt tolerance with respect to changes in parameters such as proline, chlorophyll contents, nutrients accumulation and K/Na ratio. Root morphological traits were taken into consideration under drought stress by Xu *et al.,(* 2012) while another study was especially focused on the effect of growth regulators wherein salicylic acid was found to improve plant growth under drought (Ullah *et al.,* 2012).

GRP mRNA accumulation levels were studied by northern blotting and reported to be modified during plant stress responses such as drought, UV radiation, salinity, wounding, salt, flooding, viral infection, abscicic acid treatment (ABA), cold, dehydration and heavy metal exposure (Naqvi *et al.,* 1998; Lee *et al.,* 2009). Presently, real-time PCR is increasingly employed to study mRNA quantitatively. Furthermore, technique is highly reliable and now it has become more powerful and used to validate results after microarray experiments conducted for gene expression analysis (Wong & Medrano, 2005).

Unique pattern of gene expression suggests that glycine-rich RNA-binding proteins represent a class of RNA-binding proteins involved in general molecular responses mediated by post-transcriptional regulatory mechanisms. However understanding of their functions is severely limited due to the lack of clear functional data. Present study was aimed at analyzing the expression of mRNA of *Nt*GRP1 during exposure to different stresses i.e., wounding, cold and salt stress.

Materials and Methods

Seeds of *Nicotiana tabacum* cv samsun were obtained from National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad. Seeds were surface sterilized with 5 % Clorox and were cultured on half strength MS media (Murashige & Skoog, 1962) containing 30 g/L of sucrose, vitamins and 2 g/L of phytagel. Plants were kept at 25°C in growth room with a 16-h light/8-h dark photoperiod. Sampling was done at definite intervals following the stress treatment and were immediately kept frozen in liquid nitrogen and stored at -80ºC. Three months old plants were selected for salt stress treatment. Plants were treated with 0, 50 and 100 mM salt solution. Leaf, stem and roots were harvested from stressed and non-stressed plants at 0, 6 and 24 hours, post-treatment. Leaves of five months old plants were scraped with pins causing small holes to achieve visible wounding. Sample was collected from control and

wounded plants at specific time interval such as 0, 3, 6, 12, 24 hours. Low temperature treatment was given to 20, 30 and 40 days post-germination to *in vitro* grown plants. Treatment was administered by shifting the plants to 4°C in a 16-hrs light/8-hrs dark cycle and samples were harvested at 0, 6, 12 and 24 hours.

Two sets of primers were designed: one specific for *Nt*GRP1 i.e., *Nt*GRP1-F: 5'- AGT TGA ATA CAG GTG CTT C -3' and *Nt*GRP1-R: 5'- TTG ACG GTG ATG TTA CGA -3' and the second one for internal control i.e., Actin-F: 5'- GGA ATC CAC GAG ACT ACA -3' and Actin-R: 5'- TGA GGG AAG CCA AGA TAG -3'.

RNA was isolated using SV Total RNA Isolation System (Promega Cat.# Z3100) according to the instructions of the manufacture. The extracted RNAs were stored at -80°C until used. Total RNA was used directly for cDNA synthesis by using Reverse Transcription System (Promega Cat.# A3500). The synthesized cDNA was stored at -20°C prior to use. For the expression analysis of *Nt*GRP1 in *Nicotiana tabacum* under different stress treatments such as wounding, cold and salt stress, Real-time PCR was performed. Maxima SYBR Green qPCR Master Mix (2X) kit (Fermentas Life Sciences Cat.# K0221) was used. Reaction volume was 12 µl containing 1 µl of cDNA (1:10 dilution), 6 µl Maxima SYBR Green qPCR Master Mix (Maxima Hot Start Taq DNA polymerase, Maxima SYBR Green qPCR Buffer, SYBR Green I, ROX Passive Reference Dye, dUTP), 1 µl 25 pM primer mix (Forward & Reverse), and 4 µl nuclease free water. Line-Gene K Fluorescence Quantitative PCR Detection System (BIOER) was used for relative quantification. Thermal profile for real-time PCR included pre-amplification denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing at 62ºC for 30 seconds and extension at 72ºC for 30 seconds. Data was obtained by real time PCR Line-Gene K Fluorescence Quantitative PCR Detection System and was analyzed by $2^{-\Delta\Delta CT}$ method.

Results and Discussion

The *Nt*GRP1 mRNA expression was monitored in different stress treatments i.e., wounding, cold and salt stress by Real Time PCR. On exposure wounding, no change in mRNA expression was observed after 6 hours post-treatment. At 12 hours, 2.5 fold increase in mRNA level was observed (Fig. 1). However, the expression was back to normal after 24 hours post-wounding. Bove *et al.,* (2008) also reported significant induction in mRNA level of a glycine-rich RNA-binding protein (UBA2) by wounding in a hormone independent manner in *Arabidopsis.* Although wounding increased *Nt*GRP1expression however, it was not much pronounced.

When 20, 30 and 40 days plants were compared for the variation in transcript level, *Nt*GRP1 gene showed transcriptional variation in response to low temperature (Fig. 2). The mRNA expression of the post treatment stayed the same in 20 days and 30 days old plants whereas it was up-regulated in 40 days old plants (Fig. 2). In 40 days old plant, mRNA level showed 12 fold increase after 6 hours of cold treatment after which the mRNA level decreased but was still 8 fold higher after 12 hours. Such increase in GRP transcript may be a cause or effect of the hardiness to cold. The induction in the transcript level is in accord to the fact that mRNA of glycine-rich RNA-binding proteins were observed to be induced in response to low temperature in various plant species including *Arabidopsis* and rice (Kim *et al.,* 2010, Shinwari *et al*., 1998). Cold stress leads to the differential expression of a number of genes (Guy, 1990). Induction during cold stress is mainly attributed to the fact that GRPs are reported to mediate cold acclimation process and hence contributes to freezing tolerance in plants as observed in the case of GRP7 that reportedly functioned as an RNA chaperone during cold adaptation process and successfully complimented the cold sensitivity of *E.coli* lacking cold shock proteins (Kwak *et al.,* 2011). Therefore, it can be inferred that *Nt*GRP1 is developmentally regulated in 40 days old plants and may have some role in responses against cold stress.

The expression of *Nt*GRP1 was slightly altered in leaf exposed to salt stress (Fig. 3). No change in expression was observed in 0 mM and 50 mM salt treated plants. Slight increase in transcript level was seen after 6 hours of treatment with 100 mM salt that continued to 2 fold after 24 hours post-treatment. Previously in a similar study conducted on *Arabidopsis* it was found that the expression patterns of GRPs varied from gene to gene following exposure to salt stress wherein GRP1 was up-regulated whereas the transcript level of GRPs 5, 6 and 8 did not alter significantly. *At*RZ1 was down-regulated marginally in response to salt stress (Kim *et al.,* 2005). GRP 3, 4, 6, 7 transcript decreased markedly (Kwak *et al.,* 2005). This behavior of *Nt*GRP1 indicates its involvement in salt stress but the underlying mechanism is not known.

In stem of plants exposed to salt stress, the steady state levels of *Nt*GRP1 slightly altered in response to salt treatment (Fig. 4). After 6 hours of 100 mM salt treatment, 2 fold induction in the mRNA level was observed followed by the down-regulation that remained continuous till 24 hours. In roots, *Nt*GRP1 expression level was very low so fluorescence was not detectable in real time PCR analysis. Although, several reports entail the broad spectrum expression of GRPs. Lin *et al.,* (2005) observed a GRP transcript in roots at different developmental stages but not in leaves and ripe fruit. In another study, *At*GRP4 transcript was observed in highly proliferating organs such as root tips and flowers whereas *At*GRP8 was identified in two weeks old plants, where its expression was high across all plant tissues which nevertheless get suppressed in later stages of development (Schöning *et al.,* 2008).

In conclusion, mRNA expression of *Nt*GRP1 showed variation during different stress treatments wherein slight increase in transcript was observed during wounding and salt stress exposure however, up-regulation was well pronounced after low temperature treatment of tobacco plants indicating some relationship of GRP in cold stress. Although, structure and function of GRPs is not well characterized yet these are implicated in plant stress responses and developmental processes. In most cases, GRPs have been found to make important part of cold adaptation process which strengthens the hypothesis that their synthesis is part of the plant's defense mechanism. The detail characterization and functional analysis of GRPs is expected to enhance the understanding of plant biology.

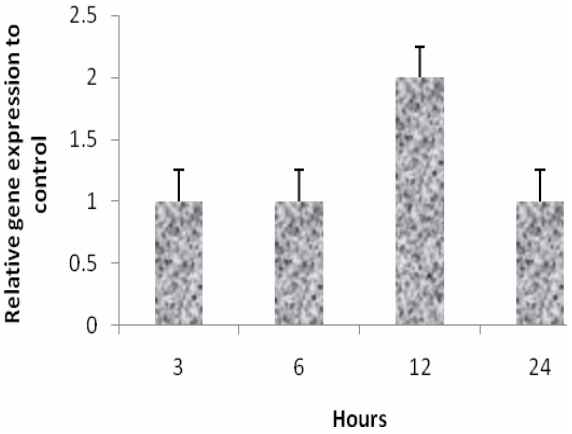

Fig. 1. Relative quantification of *Nt*GRP1 in wounded leaves by Real-time PCR.
Five months old plants were selected for wounding. The relative values (C_T) obtained by real-time PCR analysis were normalized by the C_T values of actin and were plotted against the time on which control and treated samples were harvested. Vertical bars indicate standard error. Expression in 0 hour control was 1 according to $2^{-\Delta\Delta CT}$ method.

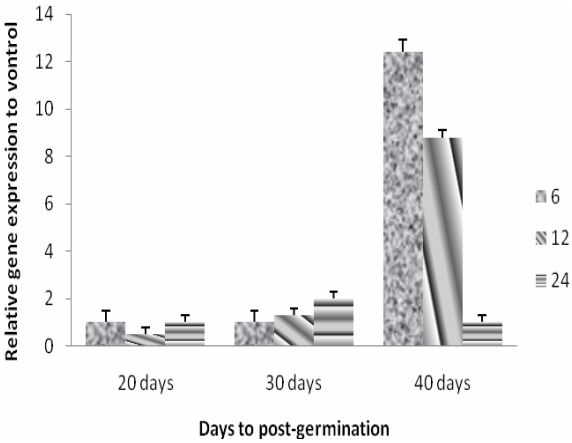

Fig. 2. Relative quantification of *Nt*GRP1 in plants exposed to cold stress by Real-time PCR.
Plants with different days of post-germination were selected for cold treatment. The relative values (C_T) obtained by real-time PCR analysis were normalized by the C_T values of actin and were plotted against time on which control and treated samples were harvested. Vertical bars indicate standard error. Expression in 0 hour control was 1 according to $2^{-\Delta\Delta CT}$ method.

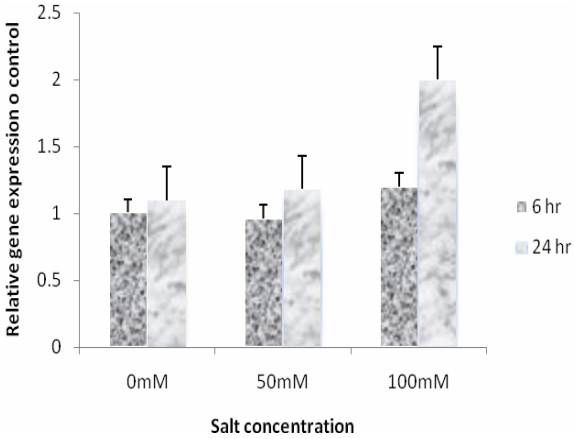

Fig. 3. Relative quantification of *Nt*GRP1 in leaf exposed to salt stress by Real-time PCR
Three months old plants were selected for salt stress. The relative values (C_T) obtained by real-time PCR analysis were normalized by the C_T values of actin and were plotted against the hours on which control and treated leaf samples were withdrawn. Vertical bars indicate standard error. Expression in 0 hour control was 1 according to $2^{-\Delta\Delta CT}$ method

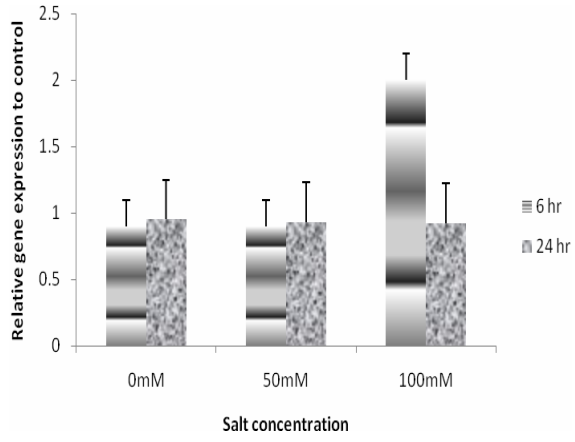

Fig. 4. Relative quantification of *NtGRP1* in stem exposed to salt stress by Real-time PCR
Three months old plants were selected for salt stress. The relative values (C_T) obtained by real-time PCR analysis were normalized by the C_T values of actin and were plotted against the hours on which control and treated stem samples were withdrawn. Vertical bars indicate standard error. Expression in 0 hour control was 1 according to $2^{-\Delta\Delta CT}$ method.

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