THE ROLE OF PHOSPHOLIPASE C IN SIGNAL TRANSDUCTION DURING DROUGHT AND COLD STRESSES IN WHEAT (*Triticum aestivum* L.)

NESREEN SABRY[1], F. M. ABDEL-TAWAB[2] AND P. GULICK[3]

1. *Banha University, Banha, Egypt*
2. *Ain-Shams University, Cairo, Egypt*
3. *Concordia University, Montreal, Canada*

Environmental stresses are one of the major challenges to crop production. Molecular biologists have investigated these problems through the study of the genes whose expression could be changed in response to stress with the hypothesis that these genes may contribute to tolerance to these stresses.

Zhu (2002) stated that salt and drought stress signal transduction consists of ionic and osmotic homeostasis signaling pathways, detoxification response pathways, and growth regulation pathways. Some phospholipids systems are activated by osmotic stress, generating a diverse array of messenger molecules, some of them function upstream of the osmotic stress-activated protein kinases. Also, ABA biosynthesis is regulated by osmotic stress. Both ABA dependent and independent osmotic stress signaling start with the modification of the expressed transcription factors, leading to the expression of early response transcriptional activators, which then activate downstream, stress tolerance effect or genes.

Many cold responsive genes have been identified and characterized since the 1980's. More recently DNA microarrays analysis using a 24 K GeneChips representing about 24000 Arabidopsis genes have been used to characterize changes in gene expression in response to cold treatment (Zhu *et al.,* 2007). One study identified 655 genes cold upregulated and 284 as cold downregulated genes after chilling treatment (Lee *et al*., 2005). Many of the early cold responsive genes encode transcription factors that probably activate the genes that are induced after longer periods of exposure to the cold. Only one transcription factor was down regulated during early cold stress, suggesting that cold responses in plants are started by transcriptional activation and not by repression of genes. To investigate the interaction of proteins that may have signalling or regulatory function, protein-protein interaction was studied with yeast 2 hybrid analysis (Tardif *et al.,* 2007).

Phospholipases are sets of enzymes which have the ability to hydrolize phospholipids. They have

different forms in plants, including phosphlipases C, D and A. Phospho-lipase C and D have important roles in signal transduction. The general structure of phospholipids consists of two fatty acyl chains esterified to a glycerol backbone at the sn-1 and sn-2 positions, and a phosphate at sn-3 position to which a variable head group (R) is attached. Phosphatidylglycerol plays a specific role in photosynthesis (Sakurai *et al.,* 2007) by helping in the organization and function of thylakoid membranes in plant cells. The first cell change during stress injury is an alteration in the structure and function of cell membranes. In many plants, alterations in lipids, particularly in phospholipids and sterols were very clear due to water stress (Liljenberg, 1992) or salt treatment (Kuiper, 1984). These alterations in the membrane seem to control the fluidity and the surrounding proteins, which influences membrane functions such as bilayer permeability carrier-mediated transport and the activity of membrane bound enzymes including ATPase activity (Surjus and Durand, 1996).

In plants, G-proteins are involved in the regulation of ion channel and abscisic acid signalling, modulation of cell proliferation, and in many other processes such as seed germination, shoot and root growth, and stomatal regulation. Phospholipase C (PLC) has been shown to function as an intercellular effector molecule in plants for the α-subunit of pea heterotrimeric G-proteins whose activity is regulated by salinity stress (Misra *et al.,* 2007). Gα protein interacts with pea phospholipase-C (PLC) at the calcium-binding domain (C2) leading to increasing GTPase activity, thus the signal transduction can be reduced by PLC association with the Gα-protein (Tuteja, 2007).

The main objective of this study was to characterize the expression profiles of three full length cDNA clones from two phosphogyceride specific phospholipase C's wheat genes under drought and cold stresses.

MATERIALS AND METHODS

Triticum aestivum (L) genetic stocks

Hexaploid Norstar *T. aestivum* cultivar was used in this study and was obtained from Agriculture and Agri-Food Canada, Plant Gene Resources of Canada (PGRC), Saskatoon Research Centre.

cDNA Clones

A cDNA clone for phosphatidylglycerol phospholipase Cs, Ta-PG-PLC 2-1 corresponding to GenBank EST accession gi:39569057 was obtained from the Genome Canada Functional Genomics of Abiotic Stress (FGAS) program (Houde *et al.,* 2006) and Ta-PG-PLC2-2 corresponding to GenBank EST Accession gi:22028920 was obtained from Arizona Genomics Institute (AGI). The clones were sequenced at the Genome Quebec Innovation Centre, McGill University. Additional partial cDNA clone of *Ta-PG-PLC2-3* corresponding to GenBank EST accession gi:20331802 was assembled from EST sequences. Sequences were

compared to gene sequences from rice obtained from GenBank using BLASTn and BLASTx.

Gene expression

Stresses

The wheat cultivar Norstar was tested under drought and cold (4C) conditions. (1) Seedlings were grown hydroponically with 1/2 Hogland solution for seven days and given an osmotic stress of 6% manitol for 0, 2, 4, 6, 12, 24 and 48 hours. (2) Plants grown in soil under green house conditions with 22ºC day and 20ºC night temperatures with supplemental light to achieve a light cycle of 16 hours light, and 8 hours of darkness. Plants were subsequently treated at 4ºC for 10 days. Leaf samples were taken at intervals 0, 2, 4, 6, 12, 24 and 48 hours during treatments.

Reverse transcription – polymerase chain reaction (RT-PCR)

RNA was extracted from plant tissue samples using QIAGEN RNeasy mini kit according to the manufacturer's protocol then mRNA were reverse transcribed to single strand cDNA using an oligo dT primer and the Invitrogen Super Script™ first- Strand synthesis system according to manufacturer's protocol. Gene specific primers were designed for each Ta-PG-PLC2 homeologe and the wheat actin gene using primer 3 software (Table 1). RT-PCR was conducted on samples for each of the three homeologs with the PCR temperature cycle: 94ºC/2 min (1 cycle), 94ºC/1 min, 58ºC/40 Sec, 72ºC/2 min (35 cycles); 72ºC/10 min (1 cycle) and 4ºC (hold) for Ta-PG-PLC2-2 and Ta-PG-PLC2-3. For Ta-PG-PLC2-1 the PCR temperature cycle was 94ºC/2 min (1 cycle), 94ºC/1 min, 70ºC/40 Sec, 72ºC/2 min (35 cycles); 72ºC/10 min (1 cycle) and 4ºC (hold).

RESULTS AND DISCUSSION

Wheat PLC gene sequence analysis

Two Ta-PG-PLC clones (Ta-PG-PLC2-1 (L5B008_C22) and Ta-PG-PLC2-2 (BQ804711.1)) were sequenced.

Gene expression studies by Reverse Transcriptase PCR

In response to osmotic treatment, which was 6% manitol treatment, Ta-PG-PLC2-1 showed downregulation of mRNA transcripts, followed by an upregulation while Ta-PG-PLC2-2 showed upregulation in response to 6 hrs of osmotic stress (6% manitol) followed by a significant downregulation at 48 hrs. Ta-PG-PLC2-3 showed a gradual upregulation in the level of expression under osmotic stress (Fig. 1 and Table 2).

In response to cold treatment, which was 4ºC, Ta-PG-PLC2-1 showed downregulation, then upregulation, then downregulation again by 48hrs of the mRNA transcripts while Ta-PG-PLC2-2 expression under cold stress showed downregulation by 12 hrs of cold followed by upregulation after 24 hrs then downregulation after 48hrs of the mRNA

transcripts. Ta-PG-PLC2-3 showed downregulation in mRNA levels after 4hrs of exposure to cold then gradual upregulation starting from 12hrs (Fig. 1 and Table 2).

These observations suggest that Ta-PG-PLC2 might be involved in the signal transduction pathways of drought and cold stresses but homeologous gene copies are regulated differently.

The differential expression of the PG-PLCs genes is due to the molecular mechanisms regulating responses of plant genes to water stress (Iskandar *et al.*, 2011) and there are many questions to be examined at the molecular level like the sensing mechanisms of water stress or osmotic stress, modulation of the stress signals to cellular signals, transduction of the cellular signals to the nucleus, transcriptional control of stress-inducible genes, and the function and cooperation of stress-inducible genes allowing water-stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 1997). Also, Analyses of stress-inducible gene expression have revealed the presence of multiple signal transduction pathways between the perception of water stress and gene expression. This explains the complex stress response observed after exposure of plants to drought. At least four different transcription factors have been suggested to function in the regulation of dehydration-inducible genes; two are ABA responsive and two are ABA independent. The transcriptional regulatory regions of the dehydration-induced genes have been analyzed to identify severa1 cis- and trans-acting elements that are involved in the water-stress response. An identified DRE cis element functions in the regulation of rapidly inducible genes in an ABA-independent manner. ABRE functions in the induction of genes after the accumulation of ABA during water stress. Severa1 genes for transcription factors are induced by water stress and ABA at transcriptional levels, which might be involved in the regulation of slowly induced stress-involved genes. In addition, many genes for factors involved in the signal transduction cascades, such as protein kinases and PLC, are regulated by water-stress signals (Shinozaki and Yamaguchi-Shinozaki, 1996; Mizoguchi *et al*., 1997). These signaling factors might be involved in the amplification of the stress signals and adaptation of plant cells to water-stress conditions (Shinozaki and Yamaguchi-Shinozaki, 1997).

Kwon *et al.* (2009) reported that the imposition of dehydration to *Oryza sativa* L. caused significant loss of internal water and reduced the rate of water loss per minute per gram dry weight. This demonstrated that the dehydration imposition could cause osmotic stress due to water loss in tissue. Also, it reduced significantly relative water content and osmotic potentials over the time. The dehydration stress induced the mRNA expression of drought induced protein (Dip1), drought induced hydrophobic protein (DRR2) and mitogen activated protein kinase (MAPK). MAPKs were expressed before 20% water loss out of the initial water content. Dip1

and DRR2 were strongly expressed after 20-40 water loss out of the initial water content. Thus water loss rate and water status can be used to explore the genes related to simple response and/ or tolerance against internal water deficit.

During cold acclimation, different physiological and molecular changes that occur have been characterized by the transcriptional activation and repression of genes by low temperature. The reprogramming of gene expression results in the accumulation not only of protective proteins but also of metabolites (Zhu *et al.,* 2007). A set of transcription factors (CBF1-3) has been shown to play an important role in the regulation of gene expression (Pekka and Palva, 2003). CBF's control the production of proteins and metabolites that protect cellular structures and functions from the freezing and freeze-induced cellular dehydration. The complex signal network that is required for sensing and transduction of the low temperature and the interactions of the signal pathways involved have been explained. There is a strong relationship between metabolic status, post-transcriptional RNA processing, cold-responsive gene expression and chilling and freezing tolerance. There are a set of cold-regulated plant genes, which include those known as COR (cold regulated), KIN (cold-induced), LTI (low-temperature induced) or RD (responsive to dehydration) genes. C-repeat (CRT)-binding factors (CBFs), also known as dehydration-responsive-element-binding proteins (DREBs). Upstream transcription factors in the APETALA2 (AP2)/Ethylene response factor (ERF) gene family are known to bind to the promoter cis element and activate the expression of these cold-responsive genes (Thomashow, 1999). The CBF genes are induced by cold. Overexpression of CBFs in/ transgenic Arabidopsis results in the expression of downstream cold-inducible genes, even at warm temperatures, and in increased freezing tolerance (Zhu *et al.,* 2007). Several cis-elements in the CBF2 promoter are involved in the cold induction of CBF2. Inducer of CBF Expression1 (ICE1), abHLH (basic helix- loop-helix) protein, is an upstream transcription factor that binds to the CBF2 promoter and is required to activate its expression under cold stress. R2R3-type MYB transcription factor, AtMYB15, interacts with ICE1 and plays a negative role in CBF gene regulation under cold stress (Agarwal *et al.,* 2006). Furthermore, cold induction of three CBF genes is controlled by bHLHs (ICE1 and other related bHLHs) and MYB transcription factors. Some of these transcription factors cross-regulate each other. Similarly, CBF2 was known to regulate CBF1 and CBF3 negatively (Novillo *et al.,* 2004) and ZAT12 negatively regulates the expression of the CBF genes. However, the ZAT12 regulon includes fewer cold-responsive genes than the CBF regulon. Also, the expression of CBF genes activates the expression of several other cold-responsive transcription factors, such as the RAP2.1 and RAP2.6, which presumably control subregulons of the CBF regulon (Zhu *et al.,* 2007). These results support the role of CBFs in config-

uring the low temperature transcriptome in *Arabidopsis*.

A cDNA amplicon microarray for wheat which included many genes for signal transduction and regulatory genes were constructed to compare changes in gene expression in a highly cold-tolerant winter wheat cultivar and a less tolerant spring cultivar. During a time course of 14 days of cold acclimation 450 genes were observed to be significantly changed in their expression; 130 of these genes were annotated as genes involved in signaling or regulation. At all periods of cold acclimation, changes in transcript levels were seen in both cultivars. Ninety percent of all genes that had detectable increase in transcript levels and significant differential expression between the two cultivars over the course of seven days of acclimation showed significant changes in gene expression on the first day of acclimation. The expression differential between the two cultivars became more marked with time period of cold acclimation (Monroy *et al.*, 2007).

In summation, Representatives of phospholipase C's gene family were characterized and fully sequence in bread wheat. *T. aestivum* L. and it's related species. They were involved in signal transduction of drought and cold stresses. However, the three homologs of PLC's investigated herein, exhibited different gene expression at the mRNA's level in response to both osmotic and cold stresses at different time points. Our results could have a favourable impact on the molecular breeding for osmo and cold-tolerance in bread wheat, being a strategic commodity for human feeding.

SUMMARY

Phospholipasase Cs (PLC) is the major cell membrane enzymes which have been recognized as important enzymes for their roles in regulation and signal transduction. Molecular biologists have studied the genes whose expression is changed in response to stress with the hypothesis that these genes may contribute to tolerance to these stresses. Specific primers to each homolog of Ta-PG-PLC2 were designed and changes in mRNA levels were observed in response to osmotic and cold stresses for three homologs of Ta-PG-PLC2. These changes suggested that Ta-PG-PLC2 is involved in the signal transduction pathways of environmental stresses and each homolog is expressed differently under the same stress.

REFERENCES

Agarwal, M., Y. Hao, A. Kapoor, C. H. Dong, H. Fujii, X. Zheng and J. K. Zhu (2006). A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J. Biol. Chem., 281: 37636-37645.

Benson, A. A. and B. Maruo (1958). Plant phospholipids I. Identification of the phosphatidyl glycerols. Biochim. Biophys. Acta, 27: 189-195

Houde, M., M. Belcaid, F. Ouellet, J. Danyluk, A. Monroy, Ani Dryanova, P. Gulick, Anne Bergeron, A. Laroche, M. Links, L. Macarthy, W. Crosby and F. Sarhan (2006). Wheat EST resources for functional genomics of abiotic stress. BMC Genomics, 7: 149-170.

Iskandar, H. M, R. E. Casu, A. T. Fletcher, S. Schmidt, J. Xu, D. J. Maclean, J. M. Manners and G. D. Bonnett (2011). Identification of drought-response genes and a study of their expression during sucrose accumulation and water deficit in sugarcane culms. BMC Plant Biol., 11: 12-25.

Kuiper, P. J. C. (1984). Functioning of plant cell membranes under saline conditions: membrane lipid composition and ATPases. In: Staples RC, Toenniessen DH, eds. Salinity tolerance in plants. New York: Wiley, p. 67-76.

Kwon, T. R., J. O. Lee, S. K. Lee and S. C. Park (2009). Water loss gene expression of Rice (*Oryza sativa* L.) plants under dehydration. In: (Ashraf A) eds. Salinity and Water Stress Springer.

Lee, B. H., D. A. Henderson and J. K. Zhu (2005). The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. Plant Cell, 17: 3155-3175

Liljenberg, C. S. (1992). The effects of water deficit stress on plant membrane lipids. Progress in Lipid Research, 31: 335-43.

Misra, S., Y. Wu, G. Venkataraman, S. Sopory and N. Tuteja (2007). *Plant J.* (Doi: 10.1111/j.1365-313X.2007.03169.x).

Mizoguchi, T., K. Ichimura and K. Shinozaki K (1997). Environmental stress response in plants: the role of mitogen-activated protein kinases. Trends Biotechnol., 15: 15-19.

Monroy, A. F., A. Dryanova, B. Malette, D. H. Oren, M. R. Farajalla, W. Liu, J. Danyluk, L. W. Ubayasena, k. Kane, G. L. Scoles, F. Sarhan and P. J. Gulick (2007). Regulatory gene candidates and gene expression analysis of cold acclimation winter and spring wheat. Plant Molecular Biology, 64: 409-423.

Novillo, F., J. M. Alonso, J. R. Ecker and J. Salinas (2004). CBF2/ DREB1C is a negative regulator of CBF1/ DREB1B and CBF3/ DREB1A expression and plays a central role in stress tolerance in Arabidopsis. Proc. Natl. Acad. Sci., USA, 101: 3985-3990.

Pekka, H. and E. T. Palva (2003). Signal transduction in plant cold acclimation. Plant Responses to Abiotic Stress. Springer Berlin/ Heidelberg, 4: 151-186.

Sakurai, I., N. Mizusawa, S. Ohashi, M. Kobayashi and H. Wada (2007). Effects of the Lack of Phosphatidylglycerol on the Donor Side of Photosystem II. Plant Physiol., 144: 1336-1346

Shinozaki, K. and Kazuko Yamaguchi-Shinozaki (1996). Molecular responses to drought and cold stress. Curr Opin Biotechnol., 7: 161-167.

Shinozaki, K. and Kazuko Yamaguchi-Shinozaki (1997). Gene expression and Signal Transduction in Water-Stress Response. Plant Phsiol., 115: 327-334

Soltis, D. E. and P. S. Soltis (1999). Polyploidy: recurrent formation and genome evolution. Trends in Ecology & Evolution, 14: 348-352.

Surjus, A. and M. Durand (1996). Lipid changes in soybean root membranes in response to salt treatment. J. Experimental Botany, 47: 17-23

Tardif, G., N. Kane, Hélène Adam, L. Labrie, G. Major, P. Gulick, F. Sarhan and J. F. Laliberté (2007). Interaction network of proteins associated with abiotic stress response and development in wheat. Plant Mol. Biol., 63: 703-718.

Thomashow, M. F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant. Physiol. Plant Mol. Biol., 50: 571-599

Tuteja, N. (2007). How Pea Phospholipase C Functions in Salinity Stress Tolerance. ISB News Report.

Zhu, J., C. H. Dong and J. K. Zhu (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current Opinion in Plant Biology, 10: 290-295

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. Annual Review of Plant Biology, 53: 247-273.

Table (1): Primers used for expression analysis by RT-PCR analysis.

Primer Name	Primer orienta-tion	Primer's Sequence	Melting Tempera-ture	G+C % ratio
Ta-PG-PLC2-1 (L5B008-C22)	Right	5`-TTGGACGGCGGCAACGGCGAC-3`	78.63	88.89%
Ta-PG-PLC2-1 (L5B008-C22)	Left	5`-CGGCGTCCGCGTCCCGGc-3`	78.02	71.43%
Ta-PG-PLC2-2(BQ804711.1)	Right	5`-CCAGTTTATCTATCTTTTTGGGACA-3`	59.80	36.00%
Ta-PG-PLC2-2(BQ804711.1)	Left	5`-GCGTCCATCTCGGACTTC-3`	59.30	61.11%
Ta-PG-PLC2-3(BQ169979)	Right	5`-CCCCAACGCATGTATCCT-3`	59.32	55.56%
Ta-PG-PLC2-3(BQ169979)	Left	5`-GCGACGATCTCGGACTTC-3`	59.46	61.11%
Control Wheat Actin	Right	5`-TTCATACAGCAGGCAAGCAC-3`	60.02	50.00%
Control Wheat Actin	Left	5`-CAACAACTGGGATGACATGG-3`	59.81	50.00%

Table (2): Summary of RT-PCR results of the three homologs under osmotic and cold stresses.

Homeologe	Hours	Osmotic stress	Cold
Ta-PG-PLC2-1	0		
	2		
	4		-
	6	-	+
	12	+	+
	24	+	+
	48	+	-
Ta-PG-PLC2-2	0		
	2		
	4		
	6	+	
	12	+	-
	24	+	+
	48	-	-
Ta-PG-PLC2-3	0		
	2	+	
	4	+	-
	6	+	
	12	+	+
	24	+	+
	48	+	+

(+) is upregulation, (-) is downregulation and (No) means no expression

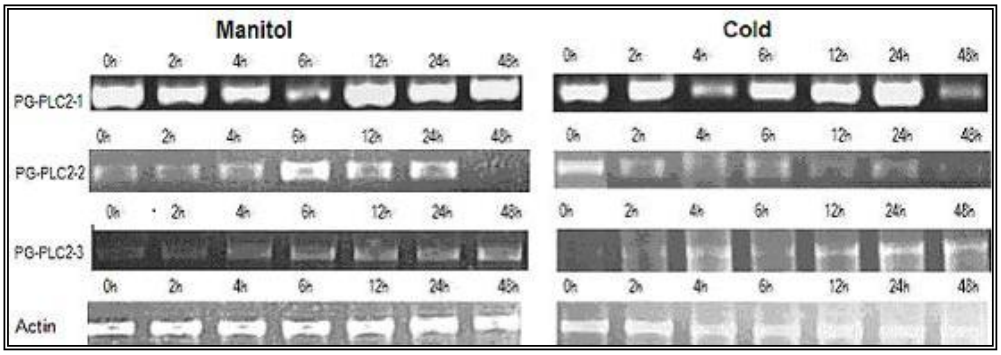

Fig. (1): RT-PCR for the three wheat Ta-PG-PLC2 homologs under two different stresses and the Actin used as a control.