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Identification of Differentially Expressed Genes Induced by Poly Ethylene Glycol (PEG) in Maize (*Zea mays L.)* for Callus Regeneration.

Manal M. Abdel-Rahman1, Asmaa M. El-Faramawy2, Ibrahim E. Mousa3*

1. Plant pathology department (Genetic branch), Faculty of Agriculture, Damanhour University, Damanhour, Egypt.
manal_moustafa2000@yahoo.com
2. Nucleic acid research department, City for Scientific Research and Technological Applications, New Borg El-Arab City, Alexandria, 21611, Egypt.
asmaameg71@yahoo.com
3. Environmental biotechnology department, Genetic engineering and biotechnology research institute, University of Sadat city, Egypt.
ibrahim.mousa@gebri.usc.edu.eg ibrahim23365@yahoo.com
Tel: 00201112779239 Fax: 0020482601268

Abstract: In the present study, changes in the morphotype of non-regenable (NR) callus to regenable callus (R) of maize (*Zea mays L.*) and the transcript abundances of associated gene were investigated in callus treated with 10% and 20% polyethylene glycol (PEG). Differential display technique reverse transcription polymerase chain reaction was used to study the effect of PEG treatment on two type of maize HiII genotype calli. Seven arbitrary primers were used to study the difference between R and NR callus as a result of PEG treatment. Results indicated that highest variations were shown among the treated samples examined with primers A1, R14, P2 and A13. Six different down regulated genes were observed, two, one and three genes were amplified in control samples with primer A1, R14, and p2, respectively. On the other hand total of 30 up regulated genes were observed in treated samples comparison to control. Nine of these genes were eluted, purified and sequenced. These genes might participate in the transformation of NR to R callus. Also, results revealed that their deduced amino acids, ATFP protein of Zea mays, putative pol protein, Opie2b gag protein, putative gag/pol protein, aminotransferase class III and Putative gag/pol precursor (Zea mays) protein were expressed as a result of PEG treatment. These results suggested that PEG as water regulator may have contributed to change NR calli to R calli by enhancing protein metabolism and its related genes.

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1. Introduction

The transformation of non-regenable (NR) callus to regenable (R) callus has great benefits for tissue culture techniques especially in case of maize genetic manipulation. For classic breeding and tissue engineering strategies, relying on the transfer of one or several genes are limited. Environmental stresses include several factors are well known to have an effect on plant growth, limit crop productivity and seed production (Shinozaki et al., 2003). In the same trend, the application of PEG has become a routine method for stimulating somatic embryo maturation in tissue culture. From our previous research, the yield of mature somatic embryos is dramatically increased due to the poly ethylene glycol (PEG), induced drop in medium upon transition from proliferation to maturation (Abdel-Rahman and Widholm, 2010).

The genetic complexity and multigenic trait of water stress tolerance have great important (Vinocur and Altman 2005). Generally, as all the modification associated with water stress responses affect plant growth and yield (Nonami and Boyer 1989, Davies and Zhang 1991). Little information is available on the effect of PEG on non-embryogenic callus and plantlets regeneration from immature embryos in maize. PEG has non-plasmolyzing osmotic capability, which produces the effect of partial drying tolerance and does not enter the cell (Slama et al., 2007). Also, storage proteins biosynthesis is thought to be regulated by PEG at the post transcriptional level by osmotic

stress-induced translation of storage protein mRNAs accumulated in response to abscisic acid (Lozovaya et al., 2006). Many genes induced by stress have been identified to be essential for stress tolerance, which included genes of lipid metabolism, chloroplast function, free radicals detoxification (Provart et al., 2003). To cope with these constraints and plant induce complex modification such as osmotic adjustment and metabolic pathways that were changed (Bohnert and Sheveleva 1998; Aruna Kumara and De Costa, 2015), many investigations have to be run for the effect of PEG.

At molecular level, many groups of proteins play important roles in response to PEG. Proteins that involved in signal transduction and regulation of gene expression and proteins that participate in stress adaptation were effected (Shinozaki et al., 2003). In our previous studies, a set of metabolic features that are associated with capacity for regeneration of maize plants in tissue cultures was determined by Ulanov et al., 2009. The tissues with high regeneration capacity (R) had low sugar and Gamma-aminobutyric acid concentrations and increased most amino acids concentrations compared to NR callus, indicating an association of these metabolites with the capacity of maize cultured tissue of regenerated plants (Mekonnen et al., 2016).

Differential display is a molecular technique that can distinguish between genes that are regulated. Initially described, differential display encompasses several reverse transcription polymerase chain reaction (RT-PCR) techniques that can produce a large number of cDNA fragments from cellular mRNA (Liang and Pardee, 1992). Under different environmental conditions, differential display of mRNA has been widely used to identify genes whose expression levels have been altered. Because of its technical simplicity and lack of requirements for previous genomic information of the species interest (Carginale et al, 2004). Transcriptome profiling of plants response to environmental stresses could be studied using different techniques, serial analysis of gene expression, subtractive hybridization and cDNA microarray (Skorupa et al., 2016).

For understanding the transformation of NR callus with medium containing the non- metabolized compounds such as PEG, that has changed to tissue capable of efficient plant regeneration. In the presence of PEG, the majority of mRNAs were translated and osmotic embryos possessed the entire complement of storage polypeptide. More drought-tolerant varieties must be developed. In the present work, we describe the application of differential display DD-RT-PCR as a powerful and reliable tool that can be used to identify genes that are differentially expressed in case of R and NR calli after using PEG with different concentrations. Also, the expression profiles of genes in Maize callus with PEG treatment was analysed and the functions of differentially-regulated expressed genes were deduced.

2. Materials and Methods

2.1. Treatment of maize HiII callus:-

Maize HiII immature zygotic embryos culli were initiated as described by Armstrong (1994) and were grown on the N6E callus induced medium (Chu et al. 1975) containing N6 salts (2 mg/L 2,4-dichlorophenoxyacetic acid, 3% sucrose, 100 mg/L myo inositol, 2.76 g/L proline, 100 mg/L casein hydrolysate, and 2.5 g/L gelrite), pH 5.8, and 25 µM filter sterilized silver nitrate was added after autoclaving. The cultures were grown in the dark at 27–28°C. Three ages were used in this experiment as young calli (6 months), young calli (one year) and old calli (4 years) as shown in Table 1 and figure 1. The PEG treatment was carried out by placing ten pieces of R and NR calli with each weighing about 100 mg. The growth medium containing different concentrations of PEG (3,350 molecular weight, Sigma, St. Louis, USA) as 10% and 20% (w/v) unless otherwise specified for 21 days was used. The live pieces were transferred to the same fresh medium for twenty one days two more times each. Dead calli with brownish and showed no growth were excluded.

2.2. RNA Extraction and cDNA synthesis:

Treated callus total RNA was extracted using RNA easy kit according to manufacturer's instructions (QIAGEN). The RNA was dissolved in DEPC-treated water, quantities spectrophotometrically and analyzed on 1.2% agarose gel. For cDNA synthesis 3µl from RNA was combined with 5µl of a 2x reverse transcription mixture containing (50 mM 2 Tris HCl (pH 8.3), 4 mM MgCl2, 50 mM KCl, 20 mM dithiothreitol, 1µl dNTPs (4 mM), 1µl oligdT primer (Promega), 13µl of RNAs free water and 1µl (50unit/µl) of Murine Leukemia Virus (MLV) reverse transcriptase and incubated at 37°C for 1 hr, followed one cycle for destruction the enzyme at 75 oC for 10 min. as described by Nishimura, et al. (2015).

2.3. Differential Display for the treated callus:

DDRT-PCR partitions transcripts by synthetizing cDNA and their terminal parts were amplified with primer pairs consisting of an anchored poly (dT)-primer. mRNA differential display were used to study the genetic variation between treated and control maize callus. The cDNA was subjected to second PCR using nine random primers (table 2). The reaction mixture for differential display PCR was carried out in a total volume 25 µl containing 2 µl 2.5 mM dNTPs, 1 µl 10 pmol primer 1.5 µl cDNA, 2.5 µl 10 x buffer with MgCl2, and 0.2 µl Taq DNA polymerase (5 units/µl).

PCR amplification was performed in a PCR for one cycle at 95oC for 5 min. then 34 cycles were performed as follows: 30 s at 95oC for denaturation, one min at 45oC for annealing, two min at 72oC for elongation, ten min. at 72oC for final extension. The reaction mixtures were held at 4oC. About 2 µl of loading dye was added prior to loading of 10 µl per gel pocket. Electrophoresis was performed at 100 Volt with 0.5 x TBE buffer in 12% polyacrylamide gel and then the gel was stained in 0.5 µg/ml (w/v) ethidium bromide solutions for only one minutes and destained in deionized water for about ½ h. Finally the gel was visualized and photographed by using gel documentation system (Chen et al., 2005).

2.4. Nucleotide sequence and sequence analysis

Unique PCR-amplified cDNA fragments from treated callus were selected and considered significantly up-regulated. The selected cDNA bands extracted and eluted from the gel using Gel Extraction Kit (Fermentas, EU). Purified DNA was sequenced by using Big Dye_ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were analyzed and compared to The Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA sequences were compared to protein database using a translated nucleotide (BLASTx) and were named according to the best BLAST match. The Limoniastrum DNA sequences obtained in this work were submitted to the GenBank the accession numbers of which PCR-amplified cDNA fragments were separated as described by Busby (2013).

3. Results and Discussion

3.1. Somatic embryogenesis and expression profiling

Maize HiII callus that had been resulted in tissue culture for 6 months, one and four years were divided into R and NR based on morphology (Table 1). R callus was yellowish, fast growing and able to grow on medium containing PEG or not. While NR callus was watery, white slow growing and did not grow well on medium without PEG as shown in figure (1). Exposure of NR young maize cultured tissues to medium containing PEG changed their morphology and caused NR HiII callus to become R as was reported by Abdel-Rahman and Widholm (2010).

Figure 1 showed that the effect of PEG and increases regeneration ability of NR callus and enhance mechanism that could involve the osmoticum effects on the cell wall mechanical properties. Dehydration caused by application of non- penetrating osmoticum over long culture period could induce tissue metabolic responses resulting in physical and biochemical changes in the cell walls, which are known to be largely involved in control of growth and morphology of plant and tissue (Humphrey et al., 2007, Lygin et al., 2012).

3.2. Gene expression patterns following induction of somatic embryogenesis

Extracted mRNA from 11 treatments and its control was used to synthesize cDNA and was subsequently subjected to PCR differential display analysis using different primers (Table 2). Seven arbitrary primers were used to detect increased gene expression in treated calli. The major observation was an increase in genetic variations between controls and treated calli as shown in Figure 2. The highest variations were shown among the treated samples examined with primers OPA01, OPR14, P2 and OPA13. For primer OPA01, 10% NR form young calli, showed strong bands than 10% NR in one year and old calli. Also, young calli showed more bands than one year and old callus. Also, unique band appeared about 1400bp in young calli compared with 10% NR both of one year and old calli (Figure 3).

Otherwise, for primer OPA13, there was a band for old calli in control and 10% NR but disappeared in old callus of 10% NR and 20% NR. There were three common bands in young calli (control, 10% NR, 20 % NR, and 10%R) but two bands showed in young calli 20% R callus. Also, there was a strong band in one year 10% reg. and firm bands in both of one year (10% NR., 20% reg. and 20 % NR.). There was a band in old calli (control and 10% reg.) but did not shown in old calli (10% NR. and 20 % NR). For primer OPR14, new bands were appeared in one year 10% NR, 20 % reg. and 20 % NR.). But for primer 2 new bands were shoed in one year (20% R and 20% NR) (as seen in Figures 2, 3, 4, 5, 6, 7 and 8).

Six different down regulated genes were observed, two, one and three genes were amplified in control samples with primer A1, R14, and p2, respectively (Table 3). On the other hand total of 30 up regulate genes were observed in treated samples comparison to control. The expression patterns of a number of genes were stage-specific. Such genes might make good markers to track the progress of calli development and differentiation. Nine of these genes were eluted, purified, sequenced and sequence analyses were performed. Results of sequence and expression analysis of nine genes were summarized in Table (3). Only JX051363, JX051368 and JX051369 were identical to maize accessions. Coding for ATFP4,

Putative GAG-POL precursor and Retrotransposon Opie-2 with similarity 63%, 63% and 50% respectively were conducted. High similarity (95% and 86%) was showed for accessions JX051364

and JX051365 coding for Class III aminotransferase. While low similarity was noticed for accessions JX051370 coding for Transporter ATP- binding protein 43%.

Gene expression patterns in maize hybrid line HiII were profiled for somatic embryogenesis in a regeneration proficient of treated calli. The identify genes that might be used as developmental markers or targets to optimize regeneration steps for recovering maize embryogenic calli is required. Six different down regulated non-redundant genes were observed and cDNA sequences using mRNA differential display. We determined the relative expression profile of each gene in both tissues of maize at three different growth ages (R and NR) calli. For discovering genes whose expression is altered under particular conditions, DD-PCR is one of the major tool. This technique has the great advantages for detecting changes in the patterns of expressed genes among multiple samples being compared. Also, without the need of any prior knowledge of genomic information of the species of interest, gene expression markers have been used more widely in recent years to characterize embryonic lines and to describe embryo development (Chugh and Khurana 2002; Balakrishnan et al., 2005; Che et al., 2006).

The presence of PEG, while crystalloid protein mRNAs abundance and profiles were similar to those of zygotic embryos, the optimal PEG concentration (7.5 % {w/v}) resulted in the R calli production of twice as many embryos, compared to the control that was used. These results could be indicated the promoting effects of PEG on the embryogenic root and shoot differentiation and explain the activation of lipid biosynthesis, the substantial increase in the maturation frequency of white spruce embryos by PEG application (Huang, and Song, 2013; Wang et al., 2015). PEG has also dramatically modified the primary metabolism via decreasing sugar concentrations several- fold which could result from intensification of respiratory activity due to higher energy requirements to support morphological changes in NR callus. PEG caused the increase in concentrations of aspartate and aspragine, glutamate, glycine and proline; however, contents of GABA, alanine and pyroglutamic acid decreased after PEG treatment in NR callus. Changes in amino acids could relate to their roles as osmoprotectants, substrates for protein synthesis, which could support different enzymatic activities during morphological changes and account for storage products in embryogenic tissues (Lygin et al., 2012).

Also, PEG looks like environmental stresses such as drought, salinity and temperature (Meng, et al., 2016) include several factors that could be used on plant growth, enhancement tissues and seed production (Liu, et al., 2015). So, these results could be suggested that PEG as water regulator may have contributed to change NR calli to R calli by enhancing protein metabolism and its related genes.
[1].

Table 1. Calli types that used and treated with PEG

No	Age	Treatment
1	Young (6 months)	Control
2		10% non-regenable (NR)
3		20% non-regenable (NR)
4		10% regenable (R)
5		20% regenable (R)
6	One year	Control
7		10% regenable (R)
8		10% non-regenable (NR)
9		20% regenable (R)
10		20% non-regenable (NR)
11	Old (4 years)	Control
12		10% regenable (R)
13		10% non-regenable (NR)
14		20% non-regenable (NR)

Table 2. Primers of mRNA maize differential display

primers	Sequence 5′→ 3′
OPA 01	CAGGCCCTTC
OPA 05	AGGGGTCTTG
OPA 07	GAAACGGGTG
OPA 13	CAGCACCCAC
OPR 14	CCAGCCGAAC
P2	GGGTAACGCC
P3	TCGGCGATAG

Table 3. Predicted function and similarity of differentially expressed sequence tags (ESTs) from interaction between Callus and PEG.

cDNA	Accession Number	Length bp	genes	Similarity	function
1	JX051362	670 bp	Antibiotech biothenthesis monooxygenase	51%	
2	JX051363	324 bp	ATFP4 [Zea mays]	63%	Metal ion binding
3	JX051364	520 bp	Class III aminotransferase	95%	
4	JX051365	591 bp	Class III aminotransferase	86%	
5	JX051366	307	gag/pol polyprotein	53%	
6	JX051367	302	Putative propol	71%	Ribonuclease H activity
7	JX051368	633 bp	Putative GAG-POL precursor [Zea mays]	63%	
8	JX051369	712 bp	Retrotransposon Opie-2 [Zea mays]	50%	
9	JX051370	407 bp	Transporter ATP- binding protein	43%	

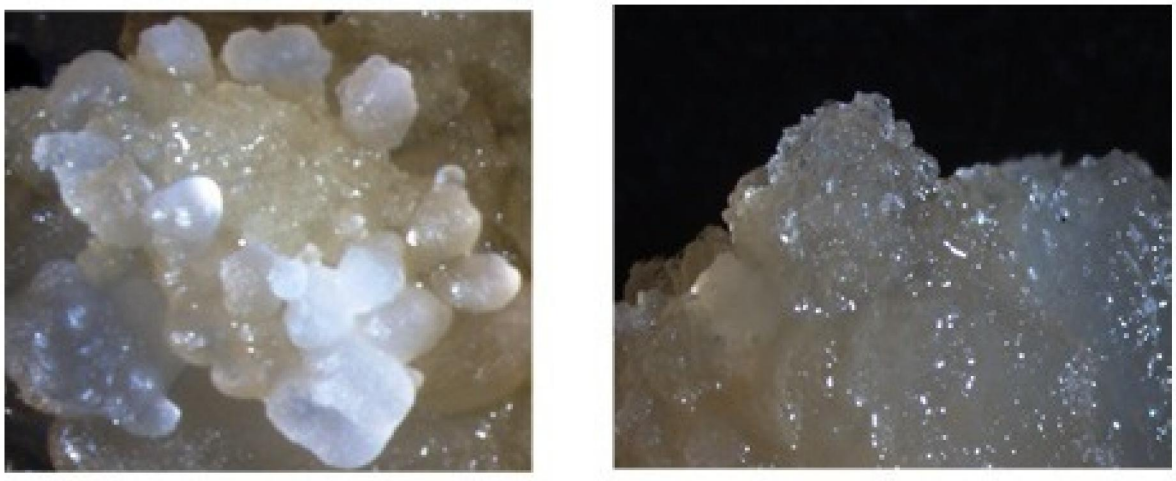
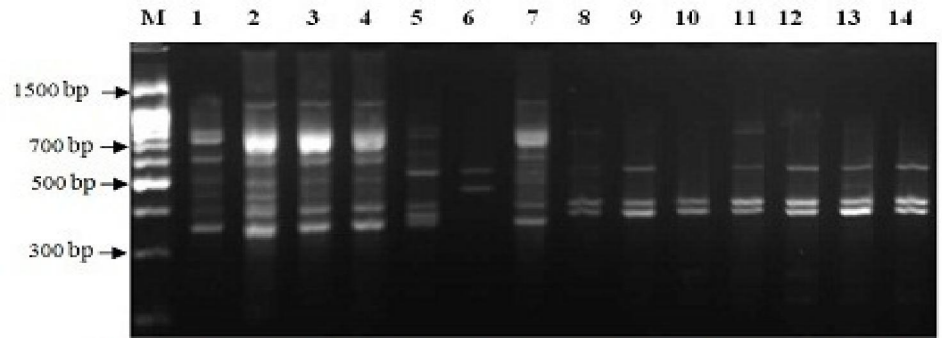
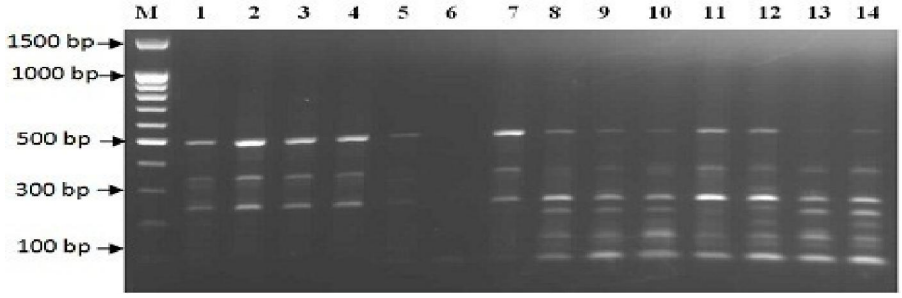


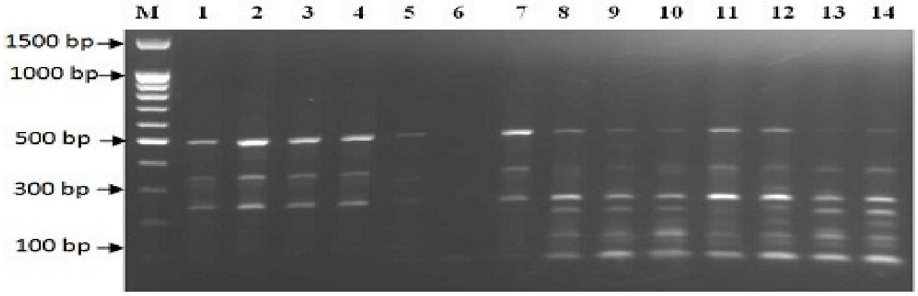
Figure 1: Types of Maize calli Regenable (left) and Non-regnable (right) before PEG treatment.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1500 bp
700 bp
500 bp
300 bp

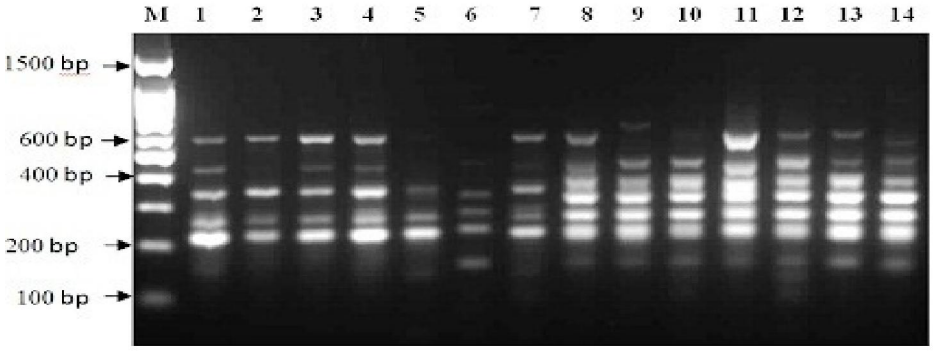
(2A)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1500 bp
1000 bp
500 bp
300 bp
100 bp

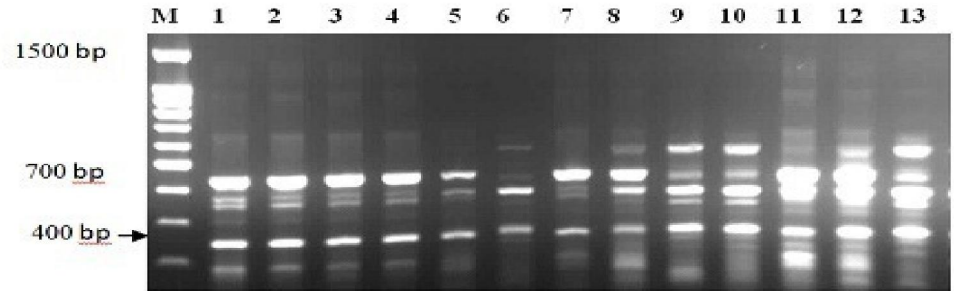
(2B)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1500 bp
1000 bp
500 bp
300 bp
100 bp

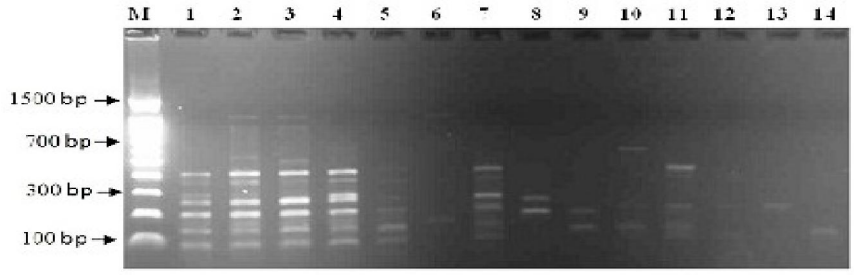
(2C)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1500 bp
600 bp
400 bp
200 bp
100 bp

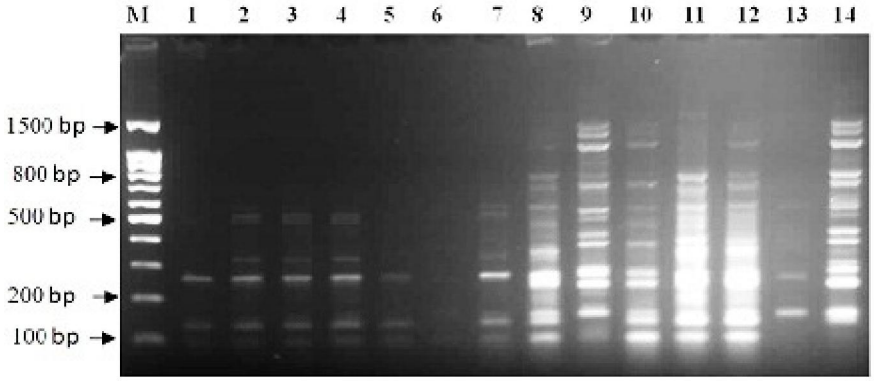
(2D)



(2E)



(2F)



(2G)

Figure 2. Temporal patterns of Zea maize transcript expression corresponding to cDNA clones isolated from different types of calli upon a three selections treatment with PEG. Seven primers were used (2a) OPA 01, (2b) OPA 05, (2C) OPA 07, (2D) OPA 13, (2E) OPR14, (2F) Primer 2, and (2G) Primer 3. 10 mg of total RNA from calli were loaded in each lane. Ethidium bromide staining shows the RNA sample loading. The data are from a representative experiment that was repeated with identical results. Lane as M (1KB marker), 1-5 young calli, 6-10 one year calli and 11-14 old calli.

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References:

[2]. Abdel-Rahman MM, Widholm, JM. Maize tissue culture plant regeneration ability can be improved by poly ethylene glycol treatment. In vitro Cell. Dev. Biol. Plant (2010)46, 509-515.

[3]. Armstrong CL. Regeneration of plants from somatic cell cultures: applications for *in vitro* genetic manipulation. In: Freeling M. and Walbot V. (eds) The maize handbook. Springer, New York, (1994) pp 663-671

[4]. Aruna Kumara UM, De Costa DM. Analysis of differentially expressed gene profiles in a resistant banana cultivar after infection by *Colletotrichum musae*. Physiological and Molecular Plant Pathology (2015) 92, 88-100

[5]. Balakrishnan V, Ursula H, Birger K, Petr K. Conversion of cDNA differential display results (DDRT-PCR) into quantitative transcription profiles. BMC, Genomics (2005) 6, 51-62.

[6]. Bohnert HJ, Sheveleva E. Plant stress adaptations – making metabolism move, Curr. Opin, Plant Biol. (1998) 1, 267-274.

[7]. Busby MA, Stewart C, Miller CA, Grzeda KR, Marth GT. Scotty: a web tool for designing RNA-Seq experiments to measure differentia gene expression. Bioinformatics (2013) 29, 656–657.

[8]. Carginale V, Maria G, Capasso C, Ionata E, La Cara F, Pastore M, Bertaccini A, Capasso A. Identification of genes expressed in response to phytoplasma infection in leaves of *Prunus armeniaca* by messenger RNA differential display. Gene (2004) 332, 29–34.

[9]. Che P, Love MT, Frame RB, Wang K, Carriquiry LA, Howell HS. Gene expression patterns during somatic embryo development and germination in maize HiII callus cultures. Plant Mol Biol (2006) Dol10.1007/511103-006-9013-2.

[10]. Chen Y, Shin YK, Bassham DC. YKT6 is a core constituent of membrane fusion machineries at the arabidopsis trans-Golgi network, J. Mol. Biol. (2005) 350: 92–101.

[11]. Chu CC, Wag CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci Sin.* (1975) 18, 659-668.

[12]. Chugh A, Khurana P. Gene expression during somatic embryogenesis recent advances. Curr Sci. (2002) 83,715-730.

[13]. Davies WJ, Zhang J. Root signals and the regulation of growth and development of plants in drying soil, Annu. Rev. Plant Physiol. Plant Mol. Biol. (1991) 42, 55–76

[14]. Fry SC. Cross- linking of matrix polymers in the growing cell walls of angiosperms. Annu. Rev. Plant Physiol. (1986) 37, 165-186,

[15]. Hatfield RD, Ralph J, Grabber JH. Cell wall cross- Linking by ferulates and diferulates in grasses. J. Sci. Food Agric. (1999) 79, 403-407

[16]. Huang H, Song S. Change in desiccation tolerance of maize embryos during development and germination at different water potential PEG-6000 in relation to oxidative process. Plant Physiology and Biochemistry (2013) 68, 61-70

[17]. Humphrey TV, Bonetta DT, Gorting DR. Sentinels at the wall: cell wall receptors and sensors. New Phytol. (2007) 176, 7-21.

[18]. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science (1992) 157, 967-971.

[19]. Skorupa M, Gołębiewski M, Domagalski K, Kurnik K, Abu Nahia K, Złoch M, Tretyn A, Tybursk J. Transcriptomic profiling of the salt stress response in excised leaves of the halophyte *Beta vulgaris* ssp. *maritima.* Plant Science(2016) 243, 56–70.

[20]. Liu B, Su S, Wu Y, Li Y, Shan X, Li S, Liu H, Dong H, Ding M, Han J, Yuan Y. Histological and transcript analyses of intact somatic embryos in an elite maize (*Zea mays* L.) inbred line Y423. Plant Physiology and Biochemistry (2015) 92, 81-91.

[21]. Mekonnen D W, Flügge U, Ludewig F. Gamma-aminobutyric acid depletion affects stomata closure and drought tolerance of Arabidopsis thaliana. Plant Science (2016) 245, 25-34.

[22]. Lozovaya V, Ulanov A, Lygin A, Duncan D, Widholm MJ. Biochemical features of maize tissues with different capacities to regenerate plants. Planta (2006) 224, 1385-1399.

[23]. Lygin A, Abdel-Rahman MM, Ulanov A, Widholm J, Lozovaya V. Poly ethylene glycol treatment promotes metabolic events associated with maize callus morphogenic competence. Phytochemistry (2012) 82, 46-55.

[24]. Meng S, Zhang C, Su L, Li Y, Zhao Z. Nitrogen uptake and metabolism of *Populus simonii* in response to PEG-induced drought stress. Environmental and Experimental Botany (2016) 123, 78–87.

[25]. Nishimura K, Yokokawa K, Hisayoshi T, Fukatsu K, Kuze I, Konishi A, Mikami B, Kojima K, Yasukawa K. Preparation and characterization of the RNase H domain of

Moloney murine leukemia virus reverse transcriptase. Protein Expression and Purification (2015) 113, 44–50.

[26]. Nonami H, Boyer JS. Turgor and growth at low water potentials. Plant Physiol (1989) 89, 798-804.

[27]. Provart NJ, Gil P, Chen W, Han B, Chang HS, Wan X, Zhu T. Gene expression phenotypes of Arabidopsis associated with sensitive to low temperatures. Plant Physiol. (2003) 132, 893–906.

[28]. Shinozaki K, Yamaguchi-Shinozaki K, Seki M. Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. (2003) 6, 410–417

[29]. Slama I, Ghnaya T, Hessini K, Messedi D, Savour´e A, Abdelly C. Comparative study of the effects of mannitol and PEG osmotic stress on growth and solute accumulation in *Sesuvium portulacastrum*. Environmental and Experimental Botany (2007) 61, 10–17

[30]. Ulanov A, Lygin A, Duncan D, Widholm J, Lozovaya V. Metabolic effects of glyphosate change the capacity of maize culture to regenerate plants. J. Plant. Physiol. (2009) 166, 978-987

[31]. Vinocur B, Altman A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations, Curr. Opin. Biotech. (2005) 16, 123–132

Wang H, Wang W, Zhan J, Huang W, Xu H. An efficient PEG-mediated transient gene expression system in grape protoplasts and its application in subcellular localization studies of flavonoids biosynthesis enzymes. Scientia Horticulturae (2015) 191, 82–89.

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