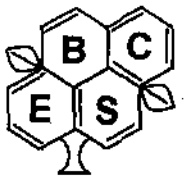

Journal

GENE EXPRESSION PROFILE ANALYSIS FOR MYCORRHIZA INOCULATED WHEAT USING REAL TIME RT-PCR

Mansour A. A., SH. Edris, A. B. Abd EL-Razik and A. Abo-Doma

Department of Genetics, Faculty of Agriculture, Ain Shams University

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ABSTRACT

Roots of most extant plants are able to become engaged in an interaction with arbuscular mycorrhizal (AM) symbiosis. This interaction improves the fitness of both fungal and plant associates. Plant traits like youngest emerged blade (YEB), Number of branches/plant trait, panicles length trait, number of seeds/panicle trait, panicles weight, and root dry weight trait have been considered important for uptake of low-mobility ions such as phosphate. The molecular analysis of plant gene expression profile of calcium dependent protein kinase (CDPKs) and phospho-enol pyruvate carboxylase (PEPC) using RT- real time PCR and cDNA northern blotting were performed using two cultivars of *Triticum aestivum* L. namely; Giza168 (hexaploid) and Sohag3 (tetraploid). We suggested that the inoculation of plant crops cultivated in the farms by arbuscular mycorrhizal (AM) fungi will increase the crop yield.

INTRODUCTION

The arbuscular mycorrhiza (AM) fungus is characterized by the formation of arbuscules (some folds vesicles). The fungus occupies various proportions of the volume of host cells but is separated from the cell cytoplasm by the elaboration of the host plasma membrane forming a fungal membrane and an apoplastic compartment between the fungus and host cytoplasm, (Rasmussen, 2002).

The inoculation with mycorrhiza enhanced the release of organic acids and higher acid phosphatase activity in the rhizosphere which

may be useful for increasing phosphorus acquisition from inorganic and organic phosphorus pools to the plant (Gahoonia and Nielsen, 2004). Hans *et al.* (2004) found that the colonization of plant roots by symbiotic arbuscular mycorrhizal lead to immunolocalization of 1-deoxy-d-xylulose 5-phosphate reducto-isomerase (DXR) which is upregulated in maize roots, shown at transcription level, but is also abundant in leaves and seedlings. Weidmann *et al.* (2004) identified 29 plant genes which were upregulated as response to root inoculation with mycorrhiza in *Medicago truncatula*. Eleven genes coding for plant proteins with predicted functions in transcription were investigated in their relation to early events of symbiotic interactions.

Fester *et al.* (2002) reported that a small protein, designated Myk15, was found to be strongly induced in wheat (*Triticum aestivum* L.) roots which colonized by the arbuscular mycorrhizal comparing to non inoculated plants.

Using an RNA interference–based screen for gene that is involved in root development in *Medicago truncatula*, Ca^{2+}-dependent protein kinase1 (*CDPK1*) gene was identified. It is predicted to encode a Ca^{2+}-dependent protein kinase, which results in a significantly reduction of root hairs and root cell lengths. Inactivation of *CDPK1* is also associated with significant diminution of mycorrhizal symbiotic colonization. Additionally, microarray analysis revealed that silencing *CDPK1* gene alters cell wall and defense-related gene expression. Moreover, *CDPK1* gene is a key component of one or more signaling pathways that directly or indirectly modulates cell expansion or cell wall synthesis in *M. truncatula* (Sergey *et al* ., 2005).

Phospho-enol pyruvate carboxylase (PEPCs) enzyme was previously described to be induced in plant nodules (Vance and Gantt, 1992), and a corresponding gene was found to be transcriptionally activated. A carbonic anhydrase (MtC00156) gene is highly up-regulated at early and late symbiotic stages, as already documented (Coba de la Pena *et al.*, 1997; Galvez *et al.*, 2000), which could relate to the control of osmolarity.

This study aimed to investigate the effect of wheat root inoculation with mycorrhiza on plant growth and productivity and to study the effect of wheat root inoculation with mycorrhiza on the plant gene expression profiles of calcium dependent protein kinase

(*CDPK1*) and phospho-enol pyruvate carboxylase (*PEPCs*) genes using reverse (RT) transcription and real time PCR.

MATERIALS AND METHODS

Plant and mycorrhiza source

Two cultivars of *Triticum aestivum* L. namely; Giza168 (hexaploid) and Sohag3 (tetraploid), which were kindly provided by Field Crop Research Institute, A.R.C., Giza, were used to investigate the effects of arbuscular mycorrhiza (AM) as spore suspension isolated from soil of corn field using wet sieving technique (Gerdemann and Nicolson, 1963). Mycorrhiza was kindly supplied by Bio-fertilizer Unit, Faculty of Agriculture, Ain Shams University.

Experimental design

A clean sandy soil was packed in sixteen pots (eight pots for each cultivar). Each pot contained about 9-10 Kg soil and six wheat seeds. The inoculated dosage was 2.5 ml of the inoculum per pot, which contained approximately 250 spores. The study was carried out in a factorial experiment with four replications. Each replicate contained the two tested cultivars under AM treatment and control to investigate the effects of AM as a biofertilizer agent on *Triticum aestivum* growth. Some morphological traits were measured as indicators for plant growth such as shoot length, number of leaves per plant, youngest emerged blade (YEB) leaf, shoot fresh weight (SFW), number of branches/plant, number of panicles/plant, main panicle weight, main panicle length, number of seeds/panicles and root dry weight (RDW).

RNA extraction

RNA was extracted according to Ashoub *et al.* (2006) from plant leaves of the two cultivars under AM treatment and control. RNA quality was examined on denature agarose gel electrophoresis. The yield of the extracted RNA was quantified using spectrophotometer.

Reverse transcription-PCR

RNA of the aforementioned samples was duplicated in two PCR tubes for each sample and reverse transcribed (RT) to produce the first strand of cDNA in the presence of 5 mM $MgCl_2$, 1X PCR buffer, 1 mM dNTPs, 25 u MuLV reverse transcriptase and 4 u RNA-guard

Ribonuclease inhibitor. A 2.5 μl of 20 P mol of CDPKs reveres primer (with the following sequence; AAT TGA TGG CCA TGG CCT GAC TTT C) was added to the mixture of the first tube of each sample and a 2.5 μl of 20 P mol of PEPCs reveres primer (with the following sequence; GCC GGC TTG CTC GTG TCC AT) was added to the other tubes. The final reaction volume was adjusted up to 30 μl in each tube. Reactions were carried out at 42°C for 30 min, followed by a 10 min step at 94°C to denature the enzyme, then was cooled at 4°C.

cDNA northern blotting

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 a 0.5 mM of 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 a 20x of SSC (standard saline-citrate). The membrane was baked at 120°C for 30 min. A probe consisting of a 520 bp PCR amplified fragments of the cDNA from wheat plant was labeled with digoxigenin-dUTP (Roche) according to the manufacturer's instructions. Hybridization was performed overnight at 42°C in Ultra hybridization solution (Ambion) in a probe concentration of 1 ng/mL. The membrane was washed twice in 2x SSC and 0.1% SDS for 5 min and twice in 0.1x SSC and 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.

Real time PCR quantification of cDNA encoding calcium dependent protein kinase (CDPKs) and phospho-enol pyruvate carboxylase (PEPCs)

All PCR processes were performed using commercially available reagents that include a thermostable DNA polymerase, dNTPs, MgCl2, and other salts and buffering agents which necessary for optimum performance. A 1 μl of cDNA of the four aforementioned samples was used as templates in the reaction mix, in a final volume of 25 μl in all assays. Conventional PCR using CDPK1 and PEPC forward and reverse primers [with the following sequences; CDPK1

forward (TGA GTA AGG CCG ACA AGG AGG ATA), reverse (AAT TGA TGG CCA TGG CCT GAC TTT C) and PEPC forward (TGG CCC CAC TCA TCT TGC TAT CTT), reverse (GCC GGC TTG CTC GTG TCC AT)] were employed to define the detection limit of the assay. Cycling was carried out in a Stratagene Mx-3000 Real-time PCR system which allows for the detection of most commercially available dyes including FAM, SYBR® Green I, TET, HEX™, JOE™, VIC™, TAMRA™, TexasRed®, ROX™, Cy5™, Cy3™ and ALEXA Fluor® 350. The system supports 96-well plate format and can perform multiple sub-experiments up to four dyes in the same well.

Bioron product, SYBR® Green I Real Time QPCR (cat No. 119205) master mix for (100 rcs) detection protocol was used in this investigation as described in Bioron manual. Data from fluorescence thresholds were statistically analyzed using Microsoft Excel (Office 2000).

RESULTS AND DISCUSSION

Inoculation experiment:

The results present in Table (1) indicated that shoot length trait after 72 days from germination was significantly increased in the inoculated plants by 1.3 and 1.26 folds over the control in G168 and Sohag3 cultivars, respectively. Moreover, number of leaves trait was significantly increased in G168 and Sohag3 cultivars under AM treatment by 2.9 and 1.6 folds respectively, compared with their controls. In addition, the youngest emerged blade (YEB) leaf trait was significantly increased in G168 and Sohag3 cultivars under AM treatment by 1.6 and 1.48 folds respectively, compared with their controls. Finally, shoot fresh weight trait was highly significantly increased in G168 and Sohag3 cultivars under AM treatment by 5.7 and 3.13 folds, respectively, compared with their controls.

Number of branches/plant trait was significantly increased in AM inoculated plants by 2.67 folds compared to its control in G168 cultivar, while it was not affected by AM inoculation in Sohag3 cultivar. Also, number of panicles/plant trait was significantly increased in AM inoculated plants by 2.3 and 1.33 folds over the control plants in G168 and Sohag3 cultivars, respectively.

A significant increase was detected in panicles length trait, where AM inoculated plants showed an increase up to 2.00 and 1.33

folds over the control plants in G168 and Sohag3 cultivars, respectively. Number of seeds/panicle trait was significantly increased in AM inoculated plants up to 4.0 and 1.3 folds over control plants in G168 and Sohag3 cultivars, respectively. Moreover, panicles weight was highly significant increased in AM inoculated plants up to 11.0 and 3.0 folds over the control plants in the G168 and Sohag3 cultivars, respectively. Finally, root dry weight trait was also highly significant increased in AM inoculated plants up to 12.0 and 3.7 folds of the control plants in cultivars G168 and Sohag3, respectively.

Table (1): Six growth-related traits and four yield-related traits in the two tested cultivars of wheat after 72 days from germination:

cultivar		Shoot length	No. leaf/plan	YEB leaf	SFW	No. bran /plat	RDW	No. panicle	Panicle length	Panicle weight	No. seeds / Panicle
G168	control	48.3	5.0	16.7	1.3	1.0	0.067	1.0	3.5	0.27	7.3
	AM	64.3	14.3	27.3	7.4	2.67	0.8	2.3	7.13	2.97	29.3
	Folds	1.3	2.9	1.6	5.7	2.67	12	2.3	2.0	11.0	4.0
Sohag3	control	41.83	5.33	14.33	1.183	1.33	0.125	1.0	2.33	0.25	13.7
	Myco	52.7	8.7	21.3	3.7	1.33	0.47	1.33	3.0	0.77	17.7
	Folds	1.26	1.6	1.48	3.13	1.0	3.7	1.33	1.33	3.0	1.3

The results indicated significant increases in all of the measured traits, especially in RDW and panicle weight traits, in both cultivars but the increases were greater in G168 cultivar than in Sohag3 cultivar. These results indicated the greater response for AM inoculation in cultivar G168 (hexaploid) than in cultivar Sohag3 (Tetraploid).

The effects of mycorrhizal colonization on yield components have been investigated in plant by many authors (Bagyaraj and Sreeramulu, 1982, Buwalda *et al.*, 1984, Rousseau *et al.*, 1994, Nehl *et al.*, 1999, and Harper *et al.*, 2004)

Ahmed and Abo-Ghalia (2008) showed that the inoculation by AM fungi led to the enhancement in the growth, nutrition, productivity and improved the yield quality of wheat plants subjected to short-term water-stress at three critical stages of plant growth (tillering, heading and grain-filling) followed by recovery. The inoculation with AM fungi increased the accumulation of some metabolic products and the activity of antioxidant system on drought

stressed wheat plants (*Triticum aestivum* L cv. Sakha93). The mycorrhizal inoculation significantly elevated the proline content with 72, 51 and 68% more than those of non-mycorrhizal ones when they were subjected to water stress at tillering, heading and grain-filling stages respectively. AM inoculated plants had significantly higher soluble proteins by 35% more than non-AM inoculated plants under well watered conditions. However, under drought conditions, the mycorrhizal treated plants showed higher concentrations of soluble proteins by 58, 29 and 34% more than those in non mycorrhizal treatment ones when the plants exposed to drought treatment followed by recovery at tillering, heading and grainfilling stages of plant growth respectively. The crude protein content of inoculated wheat plants was increased by about 6% under well watered conditions. However, these increases were by 6, 9 and 15%, when these plants were exposed to drought treatment at tillering, heading and grain-filling stages respectively. The total carbohydrate, soluble and insoluble sugar concentrations were increased in mycorrhizal wheat plants subjected to water stress. However, the level of these metabolites was more pronounced when the plants were exposed to water stress at heading stage. The activities of some antioxidant enzymes such as peroxidase (POX) were increased in mycorrhizal plants by 22, 77 and 83% and that of catalase (CAT) by 21, 28 and 31% when these plants were exposed to stress at tillering, heading and grain-filling stages, receptively. The overall results, suggested that mycorrhizal colonization could improve the osmotic adjustment response, enhance its defense system, and alleviate oxidative damage of cell viability of wheat plants.

Real Time PCR to quantify *CDPKs* and *PEPCs* genes expressions

The same cDNAs of the aforementioned samples used in northern blotting were used to perform the more accurate quantification technique (Real Time PCR) to analyze *CDPKs* and *PEPCs* genes expressions in control and AM inoculated plants of Giza168 and Sohag3 wheat cultivars.

The results in Table (2) and Figure (1) indicated that the concentrations of *CDPKs* gene expression of Sohag3 and Giza168 cultivars were increased from 3100 and 9500 in their controls to 5900 and 13100 in the AM inoculated plants, respectively, indicating the increase of *CDPKs* gene copy number in the treated plants by 1.903

fold more than the controlled Sohag3 and 1.378 fold over the controlled Giza168. The increase of CDPKs enzyme concentration in the plant in response to AM inoculation revealed a prior increase of *CDPKs* gene expression. This enzyme plays a key role in the cytoplasmic Ca^{2+} elevations in many plant physiological processes and its accumulation in the plant in response to AM inoculation (Mori *et al.*, 2006; Songzi and Qi 2008). Ca^{2+} plays an enormous role in the regulation of plant growth and development (Peter 2005).

Table (2): Concentrations of cDNAs encoding for *CDPKs* gene as detected by florescent dye for the two tested cultivars under control and AM inoculation as assessed using Real Time-PCR protocol

Cultivar	Real Time-PCR reading	Folds
Control Sohag 3 control	3100	1.903 folds over the control
Treated Sohag 3 treated	5900	
Control Giza 168 control	9500	1.378 folds over the control
Treated Giza 168 treated	13100	

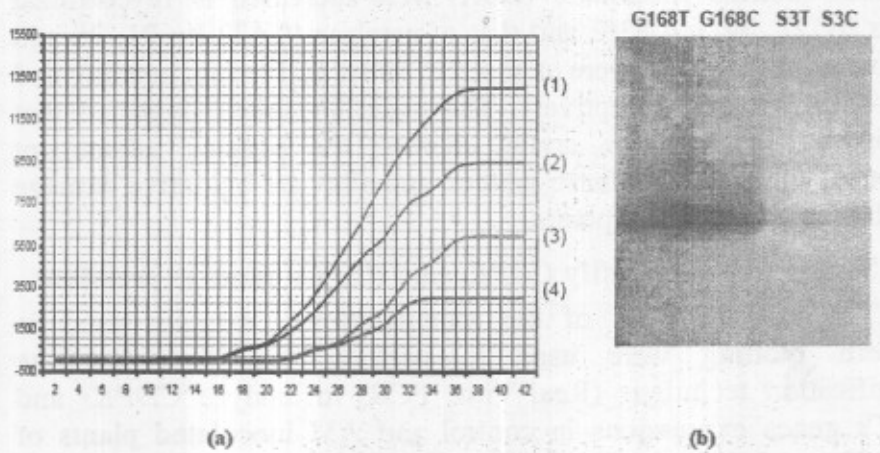

Figure (1): (a) Chart for the concentrations of cDNA encoding for *CDPKs* as detected by florescent dye for the two tested cultivars under control and AM inoculation as assessed using Real Time-PCR protocol (1) treated Giza168, (2) controlled Giza168, (3) Treated Sohag3, (4) controlled Sohag3. (b) Northern blotting profile of the *CDPKs* gene in the treatment and control of Giza186, and Sohag3 cultivars.
- G168T: treated Giza168, G168C: controlled Giza168, S3T: treated Sohag3, S3C: controlled Sohag3.

In the case of the *PEPCs* gene (Figure 2 and Table 3), the concentration of *PEPCs* gene expression was increased from 1500 in the control to 3000 in the treated plants with 2 folds over of the control in Sohag3 cultivar. While in Giza168 cultivar, the concentration was increased from 2900 in the control to 3500 in the treated plants with 1.206 folds over its control. The *PEPCs* gene has cardinal roles in the photosynthetic assimilation of atmospheric CO_2 in green plants. The copy number of its RNA was increased in the AM inoculated plants than the control. PEPCs has important functions in non photosynthetic tissues of all plants, e.g., replenishment of tricarboxylic acid cycle intermediates, seed formation, germination, and fruit ripening (Lepiniec *et al.*, 1994). The enzyme also has a special role in stomatal opening of guard cells and in the root nodules of N2-fixing legumes (Zhang *et al.*, 1995 and Outlaw *et al.*, 2002).

As a conclusion, these results explain the reasons why the inoculation with AM increases the plant yield and enhances the expression of different plant genes including *CDPKs* and *PEPCs* genes which improves the plant growth and development.

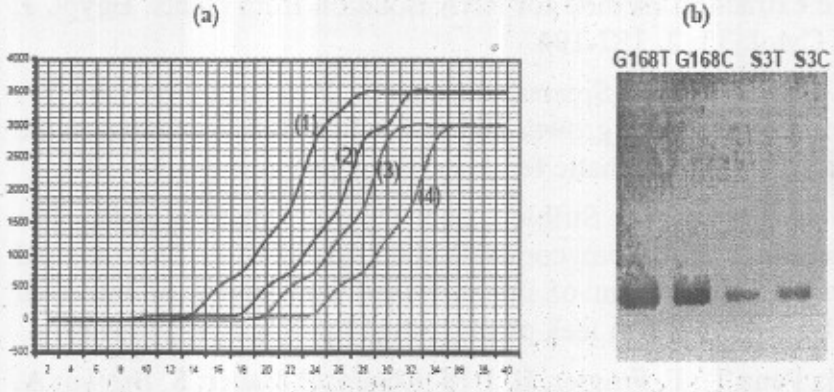

Figure (2): (a) Chart for the concentrations of cDNA encoding for PEPCs as detected by florescent dye for the two tested cultivars under control and AM inoculation as assessed using Real Time-PCR protocol (1) treated Giza168, (2) controlled Giza168, (3) Treated Sohag3, (4) controlled Sohag3. (b) Northern blotting profile of the PEPCs gene in the treatment and control of Giza186, and Sohag3 cultivars.

- G168T: treated Giza168, G168C: controlled Giza168, S3T: treated Sohag3, S3C: controlled Sohag3.

Table (3): Concentrations of cDNAs encoding for *PEPCs* gene as detected by florescent dye for the two tested cultivars under control and AM inoculation as assessed using Real Time-PCR protocol

Cultivar	Real Time-PCR reading	Folds
Control Sohag 3 control	1500	2 folds over the control
Treated Sohag 3 treated	3000	
Control Giza 168 control	2900	1.206 folds over the control
Treated Giza 168 treated	3500	

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تحليل التعبير الجيني لنبات القمح المعامل بفطر الميكروهيزا بأستخدام تكنيك Real time RT-PCR

أحمد عبد الخالق محمد منصور ، شريف إدريس ، أشرف بكرى عبد الرازق ، أحمد حسين أبو دومه

قسم الوراثة – كلية الزراعة – جامعة عين شمس – شبرا الخيمة – القاهرة – مصر

الجذور النامية لمعظم النباتات يمكنها أن تتفاعل مع فطر الأربيوسكولار ميكروهيزا (AM) و هذا التفاعل يحسن من صلاحية و عمل كل من الفطر و النبات . بعض الصفات المورفولوجية للنبات مثل طول الجذور و نسبة وزن او طول المجموع الخضرى على المجموع الجذرى و كذلك كثافة و طول و قطر الياف الجذر و التى تعتبر صفات مهمة لإمتصاص الأيونات بطيئة الحركة مثل الفوسفات . التحليل الجزيئى لتباين التعبير الجينى فى النبات للجين الخاص بإنتاج الكالسيوم المعتمد على انزيم البروتين كينيز (CDPKs) و كذلك الجين الخاص بإنتاج مركب الفوسفور كابوكسلاز (PEPCs) بإستخدام تكنيك تفاعل البلمرة المتسلسل (Real Time-PCR) و عمل northern blotting لهذين الجينين لصنفى القمح احدهما سداسى الجينوم مثل جيزة 168 و الأخر رباعى مثل سوهاج 3 . نحن نؤكد أن عدوى محاصيل الحبوب أثناء زراعتها بفطر (AM) اربيوسكولار ميكروهيزا سوف يؤدى الى زيادة محصول الحبوب .