American Journal of Plant Physiology

ISSN 1557-4539

Academic Journals Inc.

www.academicjournals.com

American Journal of Plant Physiology 2 (2): 99-106, 2007
ISSN 1557-4539

Profiling the Transcriptome in *Capsicum annuum* L. Seeds During Osmopriming

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Abstract: Loss of vigor during seed storage causes a reduction in germinability and seedling establishment. Several strategies have been developed to improve this phenomenon and one of these is osmopriming, which consists of pre-imbibing seeds in a solution containing an inert osmotic agent such as Polyethylene Glycol (PEG). PEG reduces water availability and this situation can provoke that cells in seeds reactivate metabolism but germination will not result. Thus, the beneficial effect of osmopriming is manifested as a fast and uniform germination after PEG is removed. In combination with PEG, several other compounds such as gibberellic acid and nitrate salts can be used in order to improve the vigor of seeds. In order to analyze at molecular level the effect of osmopriming seeds of chili pepper (*Capsicum annuum* cv. caballero) with PEG plus either Gibberellic Acid (GA_3) or KNO_3, a profile of transcripts obtained using Suppression Subtractive Hybridization (SSH) cDNA libraries was obtained. Our results displayed the induced expression of several genes in all treatments evaluated. Some cDNA clones were sequenced and showed high similarity to genes codifying LEA and heat shock proteins, proteinase inhibitors, enzymes involved in DNA replication and proteins with unknown functions. Possible roles of some of these genes in osmopriming of pepper seeds and further germination are discussed.

Key words: Chili pepper, osmopriming, gibberellic acid, KNO_3, SSH

INTRODUCTION

Reproduction through seeds is a prominent feature of higher plants. Seeds are adapted to survive for periods of time under adverse conditions until conditions favorable for seedling establishment are encountered (Soeda *et al*., 2005). From an economic point of view, the quality of dry seeds is important in agriculture, since seeds are often starting material for crop production and crucial for achieving a good harvest. Several aspects of seed quality influence agricultural performance, such as total emergence, the rate and uniformity of emergence, emergence under suboptimal conditions and seed longevity. To improve these aspects several priming treatments have been developed. During priming

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treatments seeds are allowed to take up water and partially start their germination-related processes, but emergence of the radicle is prevented to avoid the loss of desiccation tolerance needed for subsequent drying, storage and marketing of the treated seeds. Priming treatments are used to synchronize the germination of individual seeds (Heydekker *et al*., 1973). Due to priming promotes the initiation of several germination-related processes, generally causes faster germination and field emergence, especially under adverse field conditions (McDonald, 2000). To prevent radicle protrusion, water uptake may either be limited by imbibition in an osmotic solution (osmopriming) instead of water or by restricting the period of germination on water and drying the seeds prior the radicle protrusion (Soeda *et al*., 2005). During osmopriming only a subset of events occurs, in comparison with germination on water, as previously demonstrated at protein level (Gallardo *et al*., 2001).

The seed industry worldwide is in need of tests that can provide information on the physiological quality of seed lots. The expression of certain genes in seeds during maturation and processing, like priming, must result in an altered physiological state and affects seed quality. Several genes encoding to Late Abundant Embryogenesis (LEA) and heat shock proteins, have been identified as induced in primed seeds of *Brassica oleraceae*, Maize and bean (Campos-Álvarez *et al*., 2002; Soeda *et al*., 2005). To understand in more detail at molecular level changes caused by priming treatments on seeds of monocotyledonous and dicotyledonous, several studies have been carried out elsewhere (Campos-Álvarez *et al*., 2002; Gallardo *et al*., 2001; Ruuska *et al*., 2002; Soeda *et al*., 2005). Regarding to osmopriming, it has been demonstrated that seeds are in a metabolically active state and protein and DNA synthesis can be detected within a few hours after imbibition in PEG solutions and this synthesis may continue for several days (Bray *et al*., 1989; Davison and Bray, 1991; Cruz-Garcia *et al*., 1995). Additionally, it has been shown that osmopriming using osmotic solutions combining PEG and compounds as gibberellic acid and nitrate salts, improve the fast and uniformity of germination of beet seeds (Bradford, 1986; Sanchez-Jiménez *et al*., 1997). Although much is known about factors controlling seed maturation and the transition to germination (Holdsworth *et al*., 1999; Peng and Harberd, 2002), little is known about the profiles of global gene expression during osmopriming treatments. Thus, in this study, we used *Capsicum annuum* cv. caballero seeds as a model to analyze the transcriptome when imbibed in osmotic solutions based on PEG combined either with gibberellic acid (GA3) or KNO_3. *C. annuum* is one of the main horticultural crops in México and routinely their seeds have problems when germinating and osmopriming treatments have been used to improve re-vigorate them. Using Suppression Subtractive Hybridization (SSH) we obtained two cDNA libraries from osmoprimed *C. annuum* cv. "caballero" seeds. Expressed sequence tags (ESTs) encoding putative LEA and heat shock proteins, proteinase inhibitors, enzymes involved in DNA replication and proteins with unknown functions were identified. Possible roles of some of these genes during the osmopriming of pepper seeds and further germination are discussed.

MATERIALS AND METHODS

Source of Seeds

The seeds of *Capsicum annuum* cv. "caballero" were produced by SAKATA seed de Mexico, S.A. de C.V. (Tesistan, Jalisco, Mexico).

Chemicals

Polyethylene glycol (PEG) 6000, gibberellic acid (GA_3) and Potassium nitrate were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Osmopriming of Seeds

The seeds of *C. annuum* cv. caballero were first dried at 25°C in an incubator (FELISA, Guadalajara, Jalisco, México), then they were homogenized in a pneumatic sorter (Manufacturing

Company Hoffman, Albany, Or, USA) and finally maintained in flasks at 12% of relative humidity in a chamber (Cenviron, Winnipeg, Manitoba, Canada) until their use in osmopriming experiments. Three different osmopriming treatments were carried out; in each treatment, 10 g of seeds were placed in a Petri dish and then the seeds were imbibed in 10 mL of one of the following osmotic solutions: 1) PEG 6000 (500 ppm) + gibberellic acid (500 ppm), 2) PEG 6000 (500 ppm) + KNO_3 (200 ppm) or 3) PEG 6000 (500 ppm), equivalent to -0.01, -0.1 and -0.06 MPa respectively. The time of imbibition was 9 h and then the seeds were washed with distilled water at 25°C.

Isolation of Total RNA

RNeasy plant mini kit (Qiagen, Hilden, Germany) was used to extract the total RNA from 3 g of osmoprimed seeds. RNA purified by RNeasy column was analyzed for integrity and size by formaldehyde agarose gel electrophoresis and quantification and purity of RNA by $OD_{260/280}$ value.

Synthesis, Amplification and Purification of cDNA

One microgram total RNA of each conditions of osmopriming was used as template to synthesize the first strand of cDNA using the Superscript II Reverse Transcriptase (Life Technologies, Rockville, MD, USA) and the SMART™ PCR cDNA synthesis kit (Switch Mechanism At the 5′ end of RNA Transcript)(Clontech, Palo Alto, CA, USA), then amplified by LD-PCR with 15, 18, 21 and 24 cycles separately and analyzed through 1.2% agarose gel electrophoresis in order to get optimal cycle number which is harvested a suitable amount rather than a superfluous one to build the subtractive library. Placental total RNA was performed as control. CROMA-SPIN 1000 column (Clontech, Palo Alto, CA, USA) was used to purify cDNA.

Isolation of Differentially Expressed cDNA Fragments

Suppression Subtractive Hybridization was conducted using the CLONTECH PCR-Select™ cDNA subtraction kit (Clontech, Palo Alto, CA, USA). The tester [PEG 6000 (500 ppm) + gibberellic acid (500 ppm) or PEG 6000 (500 ppm) + KNO_3 (200 ppm) treatments] and driver [PEG 6000 (500 ppm)] cDNAs were digested with *Rsa* I, a four base-cutting restriction enzyme that yields blunt ends. The tester cDNA fragments were divided into two aliquots and each was ligated separately with adapter 1 and adapter 2 resulting in two populations of tester cDNA. A small amount of each tester population and driver in excess (5 μg) were mixed, heat-denatured and allowed to anneal 8 h at 68°C. The two samples from the first hybridization were combined and annealed with additional freshly-denatured driver cDNA, overnight at 68°C. A primary PCR was conducted to amplify those cDNAs that represented differentially expressed genes. A secondary PCR amplification was conducted using nested primers 1 and 2R to reduce background. The secondary PCR amplification products were electrophoresed and the fragments greater than 250 bp were sliced using a scalpel and purified by QIAEXII Gel extraction kit (Qiagen, Hilden, Germany).

Cloning and Screening of Subtraction Fragments

Two microliter PCR fragments were ligated with 1 μL pCR2.1-TOPO cloning vector according to the manufacturer′s instructions (Invitrogen, Carlsbad, CA, USA). Two microliter ligation reaction solutions were transformed into 50 μL *Escherichia coli* chemically competent cells strain TOP 10. The transformation culture was plated on Petri dishes containing LB/kamamycin/IPTG/X-gal and the screened white colony for insert fragment. Individual white bacterial transformants were cultured into LB/ampicillin/kanamycin solution and then it was shaken at 37°C overnight and plasmid was screened for inserts presence using *Eco* RI restriction enzyme (Invitrogen, Carlsbad, CA, USA).

Storage of Library

Selected white colonies containing recombinant plasmid were inoculated separately into 5 mL LB/ampicillin/kanamycin solution and it was shaken at 37°C overnight. Then 500 μL of each culture were added into 2 mL cryogenic vial (Corning, Acton, MA, USA) containing 500 μL 100% glycerol and kept at -80°C.

Construction of cDNA Arrays

Each recombinant plasmid was used as template and nested primers 1R and 2R to amplify the insert and then 0.2 μg were spotted into 7×10 cm BrightStar™-Plus positively charged nylon membrane (Ambion Inc, Austin, TX, USA) to construct a 7×7 clones array using Slot manifold (Amersham Biosciences, Buckinhamshire, UK HP7 9NA).

Preparation and Labeling of cDNA Probes and Membrane Hybridization

Replicates of the SSH library were hybridized by Southern analysis (Sambrook *et al*., 1989) either driver or tester cDNA probes. These probes were generated by incorporating fluorescein-11-dUTP using Gene Images CDP-Star random prime labeling module according to the manufacturer´s instructions (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Detection of fluorescein-labelled probes in Southern slot blots was performed using Gene Images CDP-Star detection module (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA), employing anti-fluorescein alkaline phosphatase conjugate and CDP-Star detection reagent.

Northern Blot Analysis

The Northern blot analysis was essentially carried out as mentioned in Anaya-López *et al*. (2005). For each treatment it was used 15 μg of total RNA.

DNA Sequencing and Database Comparison

The nucleotide sequences of differentially expressed fragments were determined using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA). On-line database comparisons were performed using Blastx algorithm (Altschul *et al*., 1990) from National Center for Biotechnology Information (NCBI).

RESULTS

cDNA Libraries

Two subtracted libraries of *C. annuum* seeds (cv. caballero) osmoprimed using either PEG alone or in combination with GA_3 or KNO_3 were obtained using the procedure mentioned before. Each library contained at least 100 non-redundant clones, with DNA fragments sized between 500-800 bp (data not shown).

cDNA Arrays Hybridization

Both cDNA libraries were hybridized in arrays on nylon membranes using different kinds of probes (Fig. 1). In panel A of Fig. 1, is showed a typical result obtained in hybridization assays using as labeled probes cDNA from *C. annuum* seeds osmoprimed with either PEG or PEG+GA_3. In panel B, it is showed the hybridization signal obtained when used cDNA from *C. annuum* seeds osmoprimed with either PEG or PEG+KNO_3. In both cases it was obtained an induced expression in almost all of the clones on both libraries, which confirmed the differential expression of the clones obtained in the subtraction experiments and analyzed in this study.

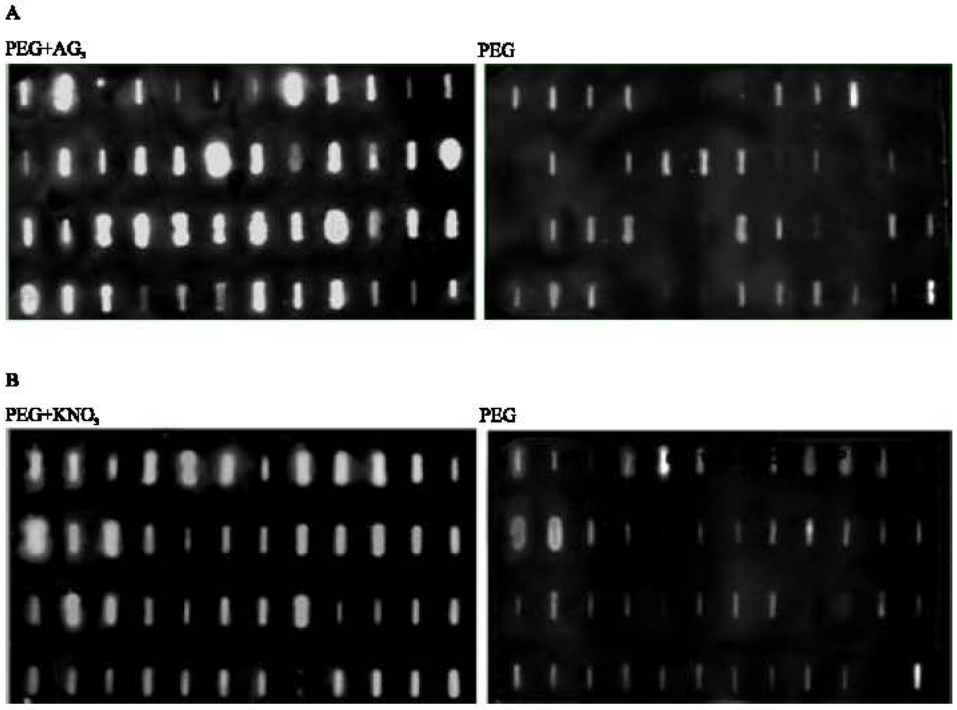

Fig. 1: Differential hybridization patterns of cDNA clones from the two libraries obtained from different osmopriming treatments of *C. annuum* cv. caballero seeds. Panel A, typical hybridization of cDNA clones from the library subtracted in the seeds treatment with PEG and PEG+GA_3; in each section is indicated the probe used in the assay. Panel B, the same than panel A, but using arrays of clones obtained from subtraction between PEG and PEG+KNO_3 treatment; in each section is also indicated the probe used in the assay

Sequence and Homology Analyses of SSH Clones

Once confirmed the differential expression of the majority of the clones on both libraries, several of them based on their most differential expression in the hybridization arrays analysis were selected in order to obtain their sequence and further searching for homologies in the Genbank. Table 1 displays the highest homology found for the sequenced clones from the subtracted library obtained in the osmopriming with PEG+GA_3. On the whole is shown that sequences can be classified in the categories of LEA and heat shock proteins, proteinase inhibitors, proteins involved in replication events and unknown functions. Table 2, shows the homologies of the sequenced clones of the library from PEG+KNO_3. Similar to data from Table 1, some cDNA clones from the library obtained with PEG +KNO_3 can be classified in the same categories based on putative roles found on Genbank comparisons.

Additionally, in Table 2, it can be shown that some clones showed homology to seed storage proteins and enzymes involved in general metabolic roles in cells. Thus, in general, sequences from both libraries analyzed in this study, displayed homologies to proteins with similar roles in plant cells, indicating that likely, osmoprimed treatments evaluated in the work, induced a similar transcript pattern in order to improve the vigor of germination in *C. annuum* cv. caballero seeds.

Table 1: Highest homology of cDNA sequences of clones from the library obtained in PEG+GA_3 treatment of *C. annuum* seeds cv. caballero

Groups	Clone	Homology
Lea proteins		
	10	LEA protein (*Arabidopsis thaliana*)
	16	LEA protein (*Glycine max*)
Heat shock proteins		
	74	Small heat stress protein class CIII (*Lycopersicon peruvianum*)
Proteinase inhibitors		
	31	Proteinase inhibitor, insect injury-induced proteinase inhibitor (*Nicotiana tabacum*)
Replication and signal transduction		
	26	Coatomer-like protein beta prime subunit (*Naegleria gruberi*)
	29	RNA binding/nucleic acid binding (*A. thaliana*)
	32	Coatomer-like protein beta prime subunit (*Naegleria gruberi*)
	36	Coatomer-like protein beta prime subunit (*Naegleria gruberi*)
	39	Notch homolog 4 (*Drosophila melanogaster*)
	48	Splicing factor-like protein (*A. thaliana*)
	59	RNA binding/nucleic acid binding (*A. thaliana*)
	102	Coatomer-like protein beta prime subunit (*Naegleria gruberi*)
Unknown function		
	2	Unnamed protein product (*Candida glabrata*)
	17	Hypothetical protein
	20	Hypothetical protein
	46	Unnamed protein product (*Saccharomyces cerevisiae*)
	54	Hypothetical protein
	60	Hypothetical protein
	61	Hypothetical protein
	63	Hypothetical protein
	65	Similar to olfactory receptor 52D1 (*Bos taurus*)
	81	Hypothetical protein (*Neurospora crassa*)

Table 2: Highest homology of cDNA sequences of clones from the library obtained in PEG+KNO_3 treatment of *C. annuum* seeds cv. caballero

Groups	Clone	Homology
Storage proteins	73	11S globulin seed storage protein (*Amaranthus hypochondriacus*), 12S seed storage protein (*A. thaliana*)
Proteinase inhibitors	23	Insect injury-induced proteinase inhibitor (*Nicotiana tabacum*), proteinase inhibitor PSI-1.2, proteinase inhibitor II (*Lycopersicon esculentum*)
General metabolism		
	35	Putative peptide methionine sulfoxide reductase (*Oryza sativa* cv. Japonica)
	71	Oleosin 18.2 kDa
	87	Hydro-lyase (*A. thaliana*)
Replication events		
	22	Putative RNA helicase
	24	ATP dependent RNA helicase
	74	Coatomer-like protein beta prime subunit (*Naegleria gruberi*)
	85	DNA primase
	96	Gag/pol polyprotein (*A. thaliana*)
Unknown function		
	3	Hypothetical protein (*O. sativa* cv. Japonica)
	7	Hypothetical protein (*D. discoideum*)
	10	ORF79 (*Agrotis segetum*)
	21	Unknown protein (*O. sativa* cv. Japonica)
	26	Unknown protein (*O. sativa* cv. Japonica)
	43	Acidic 82 KDa protein-like (*O. sativa* cv. Japonica)
	66	Hypothetical protein (*Plasmodium falciparum*)
	69	Hypothetical protein (*Ustilago maydis*)
	93	Unnamed protein product

DISCUSSION

Capsicum annuum cv. caballero seeds are an important resource in order to get a good harvest of pepper crops in México. However, they have had some problems to germinate vigorously and several osmopriming treatments have been proposed to improve this phenomenon. The use of PEG 6000 alone or in combination with either GA_3 or KNO_3, has shown to be treatments that improve vigor in germination of the mentioned seeds. Thus, in order to investigate at molecular level some genes that could be important in activating germination when using the osmotic agents mentioned before on *C. annuum* cv. caballero seeds, we study the transcriptome of these seeds during osmopriming. The use of Subtractive Suppression Hybridization (SSH) libraries permitted us to analyze selected transcripts that only overexpressed or induced when the seeds were osmoprimed either with GA_3 or KNO_3 in addition to PEG 6000. Present results displayed that several categories of genes as: LEA and heat shock proteins, proteinase inhibitors, proteins involved in replication events and unknown functions are represented in both SSH libraries. These results suggest that both osmopriming treatments induced similar transcript patterns that finally somehow cause improvement in germinability capacity of *C. annuum* cv. caballero seeds. The controlling influence of plant hormones on the transition between embryo and seedling was established many years ago and gibberellic acid and some osmotic solution (as nitrate salts) can promote germination (Holdsworth *et al.*, 1999; Soeda *et al.*, 2005). In osmopriming treatments the seeds germination is synchronized (Heydekker *et al.*, 1973). In the two osmopriming treatments used in this study, several germination-related processes were initiated, but radicle protrusion was prevented. Because of during germination several events occurs such as the activation of respiration (Bewley and Black, 1994), the repair of macromolecules (Osborne, 1993), reserve mobilization (Gallardo *et al.*, 2001), reinitiation of the cell cycle (De Castro *et al.*, 1995; Vasquez-Ramos and Sánchez, 2004) and weakening of covering structures to allow radicle protrusion (Groot and Karssen, 1987), the genes identified in this work are in concordance with these previous reports. Interestingly, some of the strongest induced genes in our study corresponded to ones encoding to LEA proteins, which have been demonstrated previously their expression in the seed following the desiccation stage after embryo maturation in all angiosperms and in vegetative tissues during osmotic stress provoked by cold, salt and drought (Aguado-Santacruz, 2006; Goyal *et al.*, 2005). Finally, molecular cloning of several genes identified in this work raises the possibility of manipulating their functions for agricultural benefit.

ACKNOWLEDGMENTS

We thank to FOMIX-GTO-CONACYT (GTO-2005-C02-52), for partially financial support of this work.

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