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Comparative approach for the isolation of genes involved in the osmotolerance of wheat

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ABSTRACT Improving drought tolerance of wheat is of great agronomical importance. Gene isolation techniques based on expression properties may provide new tools for breeders both in early characterization of new cultivars and in improving drought tolerance via molecular breeding. The main aim of our project is to isolate new drought activated genes which may serve both purposes. As a first step, a subtracted cDNA library was prepared, which represents the difference in the mRNA populations of wheat plantlets grown under 400 mOsm polyethylene-glycol derived osmotic stress versus plantlets grown in optimal conditions. By applying the subtraction approach, the resulted cDNA library becomes enriched in clones of differentially expressed genes including the ones of rare messages as well. This allows us to clone these genes, sequence them and, later, perform *in silico* analysis. Our first results indicate that the resulted library is enriched in clones coding for membrane associated channel proteins as well as abscisic acid and stress responsive ones. **Acta Biol Szeged 46(3-4):49-51 (2002)**

KEY WORDS

wheat
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subtraction
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Under climatic conditions of Hungary, drought is one of the most damaging abiotic stresses, which frequently causes severe loss in crop yield. Since wheat has major agronomical importance in Hungary, traditional breeding aims to develop drought tolerant wheat cultivars, which can ensure good yield even if the vegetation period is interrupted by extended dry periods. Molecular tools for the characterization of the cultivars in respect to drought tolerance may speed up breeding programs. In our "Stress Gene Isolation and Function" workgroup we are undertaking to identify and isolate genes possessing increased expression in response to drought stress. These genes may serve not only molecular markers of drought stress but some of them can increase drought tolerance in plants (reviewed in Holmberg and Bülow 1998; Cushman and Bohnert 2000) as is the case with aldose reductase (Oberschall et al. 2000). Based on previously characterized drought tolerant cultivars (Erdei et al. 1990; Szegletes et al. 2000) we would like to produce set of cDNA clones, which can cover most of the genes that play important role in the drought tolerance. With the help of this gene collection our plant breeder partners will be able to characterize breeding stocks much faster and hopefully easier allowing reduction of the time and labor consuming preliminary field tests. The molecular characterization of the genotypes will be more precise, and so the selection could be more straight-forward.

Materials and Methods

Hydroponic cultures of *Triticum aestivum* L. cv. Kobomugi (drought resistant) and cv. Öthalom (drought sensitive) were grown with, or without polyethylene-glycol 6000 (PEG) according to Erdei et. al. (2002) in order to generate control and stressed plantlets as starting material for messenger RNA purification.

App. 10 g of shoots or roots were flash frozen in liquid nitrogen and grinded to fine powder using mortar and pestle. Extraction buffer containing 4 M guanidium thiocyanate was added and the homogenate was extracted three times with phenol:chloroform 1:1 (pH5.5) to remove proteins and the majority of chromosomal DNA. Crude extract of total cellular RNA was recovered by precipitation with one volume of isopropanol. The pellet was washed with 70% ethanol, dried briefly and solved back in diethyl pyrocarbonate treated sterile water at concentration of 1 mg/ml.

The total RNA was further purified with affinity chromatography: poly(A)$^+$ fraction of the RNA was bound to oligo(dT)-cellulose in high salt buffer, washed extensively and eluted in salt free buffer (Sambrook et al. 1989)

First strand cDNA was synthesized using oligo(dT)$_{12-18}$ and MMLV reverse transcriptase and PCR-based subtraction was performed with the PCR-Select kit according to the manufacturers instruction (Clontech, Palo Alto CA). The resulted double stranded cDNA was cloned into pGemT vector and transformed into *E.coli*. The insert content was tested by colony PCR on randomly picked transformed colonies.

Results and Discussion

Construction of a subtracted library

Poly(A)$^+$ RNA derived from shoots of PEG treated and untreated Kobomugi plantlets were transcribed to double stranded cDNA (dscDNA) The resulted cDNA population covers a wide range in length up to 1.5-2 kb (Fig. 1).

These dscDNAs were digested with the frequently cutting restriction enzyme *Rsa*I. In such a way short fragments of one gene represent this gene several times. The dscDNA population supposed to contain specific (differentially

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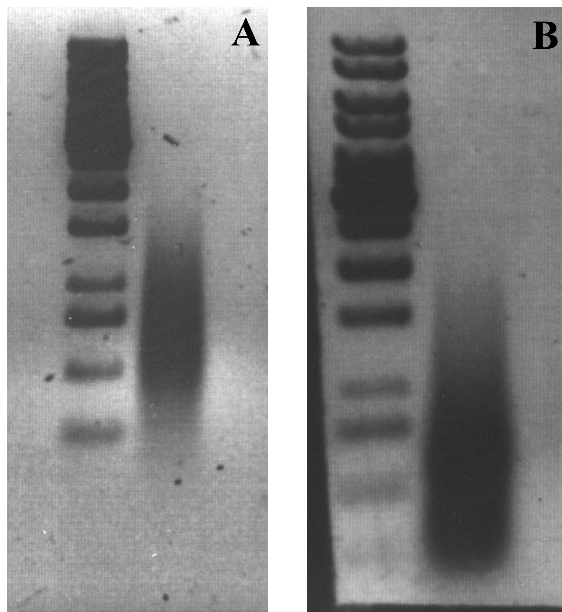

Figure 1. PCR amplification of double stranded cDNA.
ds cDNAs of the *Kobomugi* variety were PCR amplified using the adaptor-primers added during cDNA synthesis. A: PEG treated; B: control. Mw. marker bands are, from the bottom, 250, 500, 750, 1000, 1500, 2000, 2500, 3000 bp respectively.

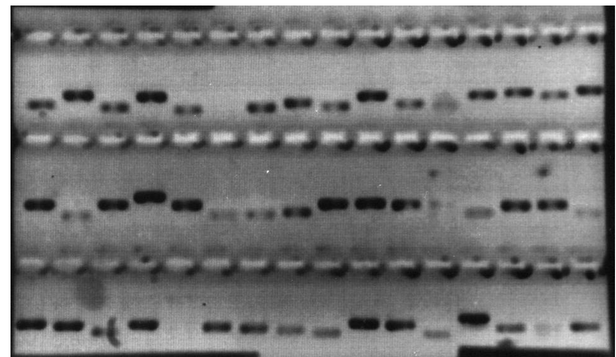

Figure 2. Colony PCR on independent bacterial colonies of the subtracted library.
The PCR products were electrophoresed on 1.5% agarose gel indicating that the majority of the colonies carry the expected 150-200 bp long cDNA inserts.

expressed) transcripts is defined as a "tester" and the control ds cDNA as a "driver". In our combination the tester derived from the PEG treated plantlets.

After *Rsa*I digestion generating blunt ended fragments the "tester" dscDNA was divided into two portions and each was ligated with a different dscDNA adaptor. This step allows later the selective amplification by PCR of the differentially expressed sequences. The "driver''s blunt ends were not adapter ligated.

The subtraction technique is based on two hybridization steps. In the first each denatured tester is hybridized with the denatured driver and as a result enrichment of differentially expressed sequences is achieved. The second hybridization helps the further enrichment by addition of fresh denatured driver dscDNA to the mixed testers. The last step is a selective PCR amplification, that can amplify those fragments only which bear the two different adaptors.

The subtracted and amplified dscDNA fragments were successfully ligated into pGemT plasmid vector. The ligate was transformed into *E.coli* and app. 1000 independent bacterial colonies were obtained.

Control of the subtracted library and analysis of the first isolated clones

cDNA inserts of randomly taken colonies were PCR amplified using the adaptor primer pair of the subtraction. More than 95% of the colonies carried plasmid with the expected size (150-200 bp) insert (Fig. 2).

Plasmid DNAs of 32 clones were purified and were subjected to sequence determination. Quarter of them coded for ribulose-1,5-bisphosphate-carboxilase (Rubisco) small subunit, the most abundant mRNA of green tissues. Another quarter of the 32 clones did not give readable sequence.

Half of the clones yielded useful sequence information that was compared to sequence databases. Several clones similar to known stress related genes were found. Among them, there is an orthologue of an abscisic acid (ABA) induced gene, another is related to cold and salt stress, while we found an ABA induced membrane protein (aquaporine) homologue as well. There was a cDNA of a lipid transfer protein, which is also membrane associated and frequently found in cDNA libraries of stressed plants.

Furthermore several clones coded for ribosomal proteins and other components of the translation machinery or photosynthesis.

Five clones exhibit similarity to unknown hypothetical proteins and in some cases, homology was not found to any known sequence.

The relatively high frequency of ABA/stress related clones indicates that the constructed cDNA library is suitable for further, larger scale investigations, the subtraction seems to be successfull. However more detailed expression studies of these clones are needed in order to judge the efficacy of the subtraction procedure.

Currently, a four way reverse northern comparison is in progress that will give information about the relative transcript levels of the above clones both in Kobomugi and Öthalom cultivars under normal and PEG-stressed regime as well.

Acknowledgements

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