Proceedings of the 7th Hungarian Congress on Plant Physiology, 2002
S1-02

Volume 46(3-4):5-7, 2002
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Improvement of wheat abiotic stress resistance via genetic transformation

J Pauk[1*], F Ertugrul[2], T Bartók[1], R Mihály[1], O Kiss[1], L Cseuz[1], D Dudits[2]

[1]Cereal Research Non-profit Co., Szeged, Hungary, [2]Biological Research Centre, Institute of Plant Biology, Szeged, Hungary

ABSTRACT Aldose reductase gene (*ALR)* isolated from alfalfa (MsALR), was constructed into a plant expression vector (pAHC), and then named as pAHALR. Wheat suspension culture was bombarded with this plasmid and the transformed calli were cultured and selected on medium containing 10mg/L Bialaphos (15% glufozinate-ammonium) as a selective agent. For the molecular analysis of putative transgenic samples, PCR, Southern and Western blots were carried out. Also aldose reductase enzyme activity was detected by HPLC only in the transgenic callus samples by the accumulation of sorbitol, which is the end product of aldose reductase catalyzed glucose reduction reaction. Protective function of ALR was verified by malondi-aldehyde analysis under stress condition. The activity of the ALR gene will also be investigated on the transgeneic plants by biochemical and physiological analysis.

Acta Biol Szeged 46(3-4):5-7 (2002)

KEY WORDS

abiotic stress
aldose reductase gene
wheat

Production of transgenic plants carrying certain genes that provide protection under drought conditions are very popular and transgenic plants were produced for improved drought and salt tolerance (Khanna-Copra and Sinha 1998).

Up to now, plant aldose reductase homologue genes had been cloned only from monocot species, and none had been shown to be active with 4-hydroxy-nonenal (Oberschall et al. 2000) identified a full- lenght cDNA (MsALR) encoding an alfalfa homologue of aldose/aldehyde reductase among several stress-induced cDNAs from an embryo-derived library. The isolated protein is composed of 313 aminoacids and shares considerable similarity with aldose and aldehyde reductases of different origins; 43,3% with barely ALR-h protein, 46,2% with human aldehyde reductase, 44,7% with human aldose reductase and 46,1% with pig ALR. MsALR gene was found in tissues of alfalfa plant. The product of the gene was accumulated in cultured alfalfa cells exposed to osmotic shock by PEG heavy metal toxicity ($CdCl_2$), oxidative agents (H_2O_2) and ABA (Oberschall *et al.* 2000).

Physiological function of the enzyme was studied by production of the transgenic tabacco plants by exposing MsALR cDNA under the control of strong constitutive promoter CaMV 35S. Transgenic clones were tested drought conditions and against metal paraquat as an oxidative stress-inducting agent. Transgenic lines, overproducing the aldose/ aldehyde reductase exhibited tolerance under osmotic and oxidative stress conditions (Obereschall et al. 2000). This paper summaries the preliminary results of ALR trans-formation and tests of the transformation products into common wheat (*Triticum aestivum* L.).

*Corresponding author. E-mail: janos.pauk@gk-szeged.hu

Materials and Methods

Plant material, culture media, bombardment and regeneration

Suspension culture of spring "CY-45" wheat (*Triticum aestivum*) line was used in transformation studies. Culture media for the maintenance of initiation explants were obtained from Sigma (M5519) which is commercially available. Other media; AA (Abdullah et al. 1986) and 190-2Cu (Pauk et al. 1991) were prepared from stock solutions. For all tissue culture studies, commercial sterile lab-wares were used. One µm size gold particles were used in bombard-ment experiments. Bialaphos as effective molecule of the herbicide was obtained from Shinyo Sangyo Co., Ltd. Japan. When the suspensions growth rate increased and the cell volume reached to certain quantity, the cells in the suspensions were plated onto solid AA medium containing 2 mg/l 2,4-D and bombarded with pAHALR plasmid by PIG device.

Regeneration

Because of the long selection period for the determination of the transgenic samples, it was not possible to regenerate plantlets directly from transgenic calli. So, an extra hormone treatment was required for the embryogenic calli formation from the transgenic callus material. For this purpose, AA medium including 0.2 - 0.4 mg/l ABA (abscisic acid) and 10 mg/l bialaphos was prepared and some of the calli material were transferred onto this medium.

Polymerase chain reaction (PCR) and Western blot analysis

The calli, which are expected to be transgenic, were tested by PCR. So from the sequence of aldose reductase gene, a specific primer set, which fits the around 975 bp coding sequence of *ALR*, was ordered to be synthesized Then well

developing calli from immature embryos on the MS medium containing 2mg/l 2,4-D and 10 mg/l PPT were chosen and genomic DNA isolation was performed by using Nucleospinplant DNA Isolation Kit.

Western Blot technique is used to detect, in a mixture of proteins or fragments of proteins, those that react with the same antibody. The transgenic calli from the bombardment of cell suspension culture were analyzed by Western Blot analysis. Important details (protein isolation and quantification, detection of protein quantity,transfer of proteins from SDS-gel to PVDF membranes, antibody production and hybridization, vector construction: cloning of ALR into a binary vector etc.,) are found in dissertation of Ertugrul (2002).

Osmotic stress treatment of transgenic and control calli with PEG 6000 and NaCl

The protective function of the *ALR* was determined by the treatment of control and transgenic calli derived from CY-45 cell suspension culture. The *ALR* positive callus material verified by PCR and Western Blot analyses (15, 37, 68, 91, 92, 120) were subjected to 14% PEG 6000 and 200 mM NaCl treatments. 0,4 g callus material from each line and also from control was placed onto filter membrane which was overlaid onto AA medim (2 mg 2,4-D/l) containing 14% PEG 6000 and 200 mM NaCl separetely. From each sample triple replicates were prepared and every week changes in callus weight were determined.

Resuls and Discussion

A great attention is paid for the development of new varieties, which are resistant to different stresses, either biotic or abiotic. In this study, development of stress resistant transgenic wheat plants were tried to obtain by micro-projectile particle bombardment. Transformation was carried out with *ALR* gene whose protection mechanisms were first shown in animal cells and recently, also in plant cells, although there are very limit studies in the literature.

PCR Analysis in Cell Suspension-derived Transgenic calli

PCR analysis was applied also for the transgenic callus material obtained from the transformation of cell suspension-derived calli. Independent callus lines numbered as 15, 37, 68, 91, 92 and 120. The numbers were given according to bombardment order of the samples and they were accepted as separate lines although they were coming from the same cell suspension of CY-45. After PCR, the band belongs to *ALR* was appeared at 975 bp size in all transgenic samples and in plasmid, while it's not present in the control callus material.

Southern Blot Analysis

Integration of the gene, *ALR*, into the plant genome was verified with Southern blot analysis. Donor samples were digested with BamHI and SacI and resulted fragment which belongs to gene *ALR* was labeled as probe with the supplies of detection kit. Probe concentration estimation was done to find out the minimum quantity of probe, which is enough for the detection of positive samples. Starting from 2 ng/µl to 0.2 pg/µl, ten times serial dilutions were carried out for the sample and the standard control of the kit. After the detection steps, the concentration of probe was identified as 0.2 ng/µl.

The samples for Southern blot analysis were prepared by PCR. Genomic DNA isolation was performed from the transgenic samples obtained from the bombardment of wheat immature embryo. Since we could not get any signal from the direct application of the digested genomic DNA, we first amplified the gene *ALR* on genomic DNA with PCR and we hybridize this amplified DNA with the probe obtained from the digestion of the plasmid. After running the gel, the samples were transferred to the nitrocellulose membrane. The membrane hybridized with DIG-labeled probe and in detection steps, color development was completed and the bands appeared in dark brown colors which shows the integration of the gene into plant genome.

Western Blot Analysis

Expression of the gene at the protein level was verified by Western Blot Analysis. Total protein isolation was done from the transgenic callus samples, obtained from the bombardment of cell suspension culture with pAHALR and from the regenerated plantlets. Different lines of transformed samples were subjected to Western analysis and in positive samples around 36 kD size protein belonging to *ALR* was detected which was absent in negative control and in some other samples which were not transgenic.

Sorbitol Detection by HPLC

Being an osmolyte, the effect of sorbitol under osmotic stress conditions (drought, salt and cold) is very critical to maintain the osmotic balance and the cell volume. Since the gene, *ALR*, was constructed under a highly active ubiquitin promoter from maize, we expected to see the accumulation of sorbitol in transgenic samples. Total carbohydrate was isolated from the control and transgenic samples and they were analyzed by HPLC. Among the transgenic lines some high sorbitol producers were found.

Osmotic stress treatment of transgenic and control calli with PEG 6000

The *ALR* positive callus materials which were confirmed after the molecular tests, PCR and Western Blot analysis, were subjected to osmotic stress with PEG 6000. PEG, is the

main chemical preferred for the *in vitro* osmotic stress treatments compared to other osmolites such as; mannitol, NaCl and sugars. In the analysis, PEG 6000 was added into the culture media at 14% concentration. Transgenic samples (15, 37, 68, 91, 92, 120 lines) were obtained from the bombardment of the suspension culture, were used. There was a significant difference between control and transgenic samples especially after two weeks treatment. Among the all transgenic samples, 15 seems more responsive one to the osmotic stress, while 37 followed the same trend with control sample. The starting quantity of 15 has been increased almost four times at the end of one month. Other samples showed a similar growth tendency and their starting quantity has been nearly tripled after four weeks.

Osmotic Stress Treatment of Control and Transgenic Calli with NaCl

The same procedure of osmotic stress treatment was performed with NaCl 0.4 g of control and transgenic callus samples were placed on AA medium containing 200 mM NaCl that is known as the maximum concentration to be applied in wheat. The changes in callus weight in all samples were reported every week in three replicates.

The significant response against NaCl was observed only in S15 and S68 transgenic samples. Although an increase was identified in other samples, these are not noteworthy. They almost followed the same trend with the control sample. In S15, the amount of callus was doubled after 4 weeks of treatment. The results in percent increase in callus amount with respect to starting quantity for every week. This result lets to evaluate the results more clearly. For example, every week, the increase in callus quantity was reported more than 20% in S15. Different from PEG 6000 effect on callus, during treatment, cells were dehydrated because of high NaCl and they were observed as small isodiametric cells. The side of control sample faced with the medium damaged extremely and necrosis was observed in a spreading manner.

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