UDC 575: 577.114
Original scientific paper

GENETIC ENGINEERING OF MAIZE TOWARDS DESICCATION TOLERANCE: ELECTROPORATION WITH THE TREHALOSE GENE

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Almeida A.M., S. Araújo, L.A Cardoso, P. Fevereiro, J.M. Torné, and D. Santos (2003): *Genetic engineering of maize towards desiccation tolerance: electroporation with the trehalose gene*. – Genetika, Vol. 35, No. 2, 111-121.

Trehalose is a non-reducing disaccharide of glucose that occurs in a large number of organisms such as bacteria, fungi, nematodes or crustaceans. Trehalose plays an important role in desiccation and heat stress protection, since it has been shown to stabilize proteins and cell membranes under these stress conditions. Trehalose accumulation has proven to be an effective way of increasing drought tolerance in both model plants such as tobacco and important crops such as potato or rice. In this work we aim to improve desiccation tolerance in maize, one of the most agronomical important crops, by increasing trehalose accumulation through transformation with the *Arabidopsis thaliana* trehalose phosphate synthase gene (AtTPS1) is involved in trehalose-6-phosphate synthase and hence on trehalose biosynthesis. A cassette harboring the AtTPS1 gene under the control of the CaMV35S promoter and the Bialaphos re-

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sistance gene Bar as a selective agent (conferring resistance to the PPT) was inserted in the plasmid vector pGreen0229 and used to transform maize inbred line Pa91. Immature zygotic embryos were collected 14-20 days after pollination and embryogenic calli culture were initiated. Embryogenic calli were electroporated with 20μg of plasmid DNA using a Biorad Gene Pulser II at 374 V, for 1 second. Embryogenic calli were electroporated and selected PPT. Eighty putative transgenic plants were obtained and analysed by PCR for the presence of the AtTPS1 gene.

Key words: desiccation tolerance, electroporation, maize, Trehalose

INTRODUCTION

Three important factors are believed to be determinant for world agricultural production in the 21st century: increase in world population, especially in developing nations, continuous scarcity of fresh water available for irrigation and a continuous deterioration of arable land. These three factors combined suggest the urgent need to dedicate considerable effort to the development of means to improve abiotic stress resistance of crop plants (SIEDOW, 2001).

Maize (*Zea mays*) is the third most important crop in the world. According to FAO (2003) in the year 2002 a total of 602,6 x 10^6 Mt were produced worldwide, on an area of 139 x 10^6 Ha. It is the most important primary food staple for a great percentage of the world population, especially in Latin America and Africa. It is also determinant for animal production, being the basis of feeds used for the poultry, pork and dairy industries in many developed countries. Maize is a crop with high water requirements and its selection towards drought tolerance should be regarded as a top priority.

Trehalose (α,α-trehalose or α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide of glucose that commonly occurs in a large range of organisms such as bacteria, fungi, nematodes and crustaceans (ELBEIN, 1974). Trehalose has a capacity to stabilize proteins and membranes under stress conditions, especially desiccation and heat, since it prevents the denaturation of proteins and the fusion of membranes (WINGLER, 2002). Although it has a wide distribution throughout nature, trehalose was not isolated in plants, with the exception of ripening fruits of the Apiacea family and leaves of some resurrection plants such as *Selaginella lepidophylla* (GODDIJN and VAN DUN, 1999). Resurrection plants have the ability to withstand almost complete water loss in their vegetative tissues, being able to remain alive in the dried state for several years and regaining full functionality upon re-hydration (SCOTT, 1999). Such capacity of resurrection plants has been associated with an accumulation of trehalose on plant leaves (ITURRIAGA *et al*., 2000). It can be concluded that engineering trehalose accumulation in important crop plants such as maize could be an important way of increasing their drought and salinity tolerance (ROMERO *et al*., 1997).

Trehalose synthesis is a two-step pathway. Two molecules of glucose are joined and phosphatyzed by the enzyme TPS (trehalose-6-phosphate-synthase) forming trehalose-6-phosphate. The latter is dephosphatyzed by TPP (trehalose-6-

phosphate-phosphatase) and trehalose is synthesized. Trehalose is degraded by the enzyme trehalase (WINGLER, 2002). Synthesis of TPS is considered to be the key factor in the regulation of trehalose biosynthesis, since endogenous phosphatases can replace TPP in trehalose synthesis as demonstrated by ROMERO *et al*. (1997). In baker's yeast (*Saccharomyces cerevisae*), TPS1 gene encodes for TPS enzyme (ROMERO *et al.*, 1997), while in *Eshericshia coli* is the otsA gene (GODDIJN *et al*., 1997).

Trehalose accumulation in both model and crop plants through genetic engineering has been described. GODDIJN *et al*. (1997) using the otsA gene from *E. coli*, involved in trehalose synthesis, successfully transformed both tobacco and potato plants. Using a construct with the TPS1 gene from *S. cerevisae*, ROMERO *et al*. (1997) and YEO *et al*. (2000) engineered transgenic tobacco and potato plants respectively. GARG *et al*. (2002) improved resistance to several abiotic stresses in rice using the otsA gene from *E. coli*.

Maize, as other monocots, is considered a difficult plant to regenerate through somatic embryogenesis and consequently to genetically engineer (KOMARI *et al*., 1998). Its transformation has mainly been achieved through Biolistics (ZHONG *et al*., 1996; BOHOROVA *et al*., 1999) and to a less extent by *Agrobacterium* mediated transformation (GOULD *et al*., 1991; ISHIDA *et al*., 1996; FRAME *et al*., 2002). Nevertheless, other non-conventional methods previously described, such as silicone carbide whiskers (WANG *et al*., 1995) or tissue electroporation (D'HALLUIN *et al*., 1992) demonstrated to have encouraging results (THOMPSON *et al*., 1995).

The objective of our work is to make a contribution to the increase of drought resistance in maize by increasing trehalose accumulation by transformation via electroporation of maize embryogenic calli with a gene of plant origin that encodes for trehalose-6-phosphate-synthase. In this paper, we describe the DNA construct and the process of electroporation of maize embryogenic calli used at the laboratories involved in this research project. Short preliminary results are presented as well as considerations and future prospects.

MATERIAL AND METHODS

Plant material and Bacterial strains - Maize (*Zea mays* L.) Pa91 inbred line was used. It is a yellow dent market class, with interesting agronomical traits and can be regenerated via somatic embryogenesis (D'HALLUIN *et al*. 1992). *Escherichia coli* strain DH5α was the bacterial host of every plasmid used for cloning the *At*TPS1 gene. Bacteria were cultured on Luria Broth medium (LB – 10g/l triptone, 5g/l NaCl and 5g/l yeast extract).

Construction of pGreen0229/35S-AtTPS1 - For construction of vector pGreen0229/35S-AtTPS1 to be used for maize transformation, the cDNA of TPS1 gene from *Arabidopsis thaliana*, accession number Y08568, was used. *At*TPS1 cDNA was kindly supplied by Henriette Schlepmann (Biology Faculty, Utrecht University, the Netherlands). Plasmids used were pJit60 and pGreen0229 (HELLENS *et al*., 2000) and were kindly supplied by Phil Mullineaux (John Innes

Centre, Norwich, U.K.) and their maps are available at http://www.pgreen.ac.uk. Plasmid pGreen0229 contains a cassette with the Bialaphos resistance gene Bar as a selective agent, that confers resistance to phosphynotrycin (PPT) and the herbicide BASTA (D'HALLUIN *et al*., 1995). Plasmid pJIT60 contains the 35S promoter with double enhancer regions. Standard cloning and plasmid manipulation procedures were used (SAMBROOK *et al*., 1989).

A cDNA fragment of 2970 bp corresponding to the AtTPS1 was excised with *Spe*I and *Nco*I from its original cloning plasmid pGEMT, filled-in and ligated to dephosphorylated vector pJIT60 previously linearized with *Sma*I. The ligation reaction was performed as reported in SAMBROOK *et al*. (1989) for the ligation of blunt-end termini. Plasmid vector and *AtTPS*1 fragment were used in 1:1 and 1:3 molar ratios (Vector:Insert). An aliquot of the ligation product was used to transform CaCl2 competent *Escherichia coli* DH5α cells. The presence of recombinants with *AtTps1* sequence in a sense or antisense orientation was scored by *Ecor*I digestion and electrophoresis on a agarose gel. Recombinants with *AtTps1* in sense orientation should generate a 500 bp fragment, whereas those with *AtTps1* in an antisense orientation should generate a 3000 bp fragment. The cassette 2x35S-AtTPS1-t35S was excised from pJIT60 with *Kpn*I and *Sph*I restriction enzymes, filled-in and ligated to the plant transformation vector pGreen0229 previously linearized with *Kpn*I/*Eco*RV. The ligation reaction was performed as reported in SAMBROOK *et al*. (1989), using pGreen0229 vector and 2x35S-*AtTps1*-t35S fragment in molar ratios of 1:1 and 1:3 (Vector:Insert). Recombinant bacterial clones were detected by restriction with *EcoR*I and *Kpn*I. The final construct has a unique restriction site for *Kpn*I and two restriction sites for the *EcoR*I enzyme. Digestion of the selected plasmid with *Kpn*I should linearise it producing a 8 Kbp band. Digestion with *EcoR*I should generate a 500 bp band.

A schematic representation of the construct is depicted on Fig. 1.

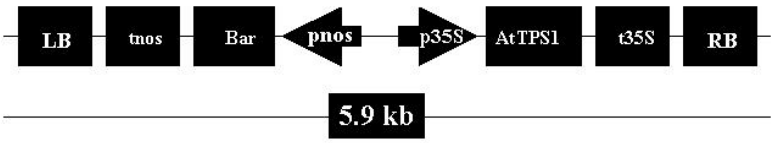

Fig. 1. Plasmid construct. LB – Left Border; RB – Right Border; t35S – 35S Terminator; AtTPS1 – Trehalose Phosphate Synthase gene (*Arabidopsis thaliana*); p35S –35S promoter; pnos – nos promoter, tnos – nos terminator; Bar – Bialaphos resistance gene

Transformation via electroporation of maize calli - The first step in the electroporation procedure is the establishment of an embryogenic calli culture. Fourteen to 21 days after pollination, kernels were harvested and disinfected (18 % Solution of commercial bleach for 13 min). After rinsing with double distilled wa-

ter, immature embryos were extracted from the kernel and cultured on L9 induction medium: N6 (CHU *et al*., 1975) Macro and micro nutrients, MS vitamins (MURASHIGE and SKOOG, 1962), supplemented with 100 mg/l Casein Hydrolysate, 0.69 g/l L-proline, 0.5 g/l 2-(N-Morpholino)ethanesulfonic acid monohydrate (MES), 2.5 ml/l Silver thiosulfate, 20 g/l sucrose, 2 mg/l 2,4-Dichlorophenoxyacetic acid (2.4D), solidified with 2 g/l gelrite and pH 5.8. After a 30-day culture period in the dark at 25ºC embryogenic calli will be formed. They can be maintained for up to 8 months without loosing embryogenic ability if re-cultured every 14 days.

Subsequently, calli are divided in 1.5 mm diameter fragments in buffer EPM^- (0.74 g/l Ca Cl_2, 10 mM Hepes, 0.425 M Manitol, pH 7.2) and undergo preplasmolysis for 3 hours. They are later washed with EPM^+ Buffer (EPM^- plus 13 g/l Sodium glutamate) for 10 minutes. Approximately 100 to 200 mg preplasmolysed calli are transferred to on an electroporation cuvette with 200 µl of EPM^+ buffer and 20 µg of plasmid DNA. After one hour of incubation, the electroporation cuvette is cooled on ice for 10 min. Electroporation is done on a Gene Pulser II electroporator (Biorad) at 374 volts for 1 second. Immediately after electroporation and in order to wash the calli, 200-400 µl N6aph medium are added to the cuvette. Liquid N6aph medium is composed of N6 macro and micronutrients (CHU *et al*., 1975) supplemented with 0.9 g/l Asn, 1.38 g/l mM Pro, 1 mg/l Thiamine HCl, 0.5 mg/l Nicotinic Acid, 100 mg/l Casein hidrolysate, 100 mg/l Inositol and 5.4 g/l Manitol (pH 5.8).

After electroporation, calli undergo a selection process. They are cultured on Mah1VII medium: N6 basal medium (CHU *et al*., 1975) supplemented with 100 mg/l Casein Hydrolysate, 0.69 g/l L-proline, 0.5 g/l MES, 2.5 ml/l Silver thiosulfate, 0.75 g/l $MgCl_2$, 20 g/l sucrose, 1 mg/l 2.4D, 1 mg/l L-Phosphinothricin (PPT – selection agent), 36.4 g/l Manitol and solidified with 1.6 g/l gelrite, pH 5.8. The first step of the cultivation period lasts 14 days, after which they are transferred to Mah1VII medium without Manitol for a period of 8 weeks. All the selection process is done in the dark at 23-25 ºC.

To regenerate plants, calli resistant to PPT are transferred to half strength MS medium (MURASHIGE and SKOOG, 1962), solidified with 1.6 g/l gelrite and pH 5.8, and cultured at 23-25 ºC in a phytotron (16h light to 8 h dark) for 20 days. Plantlets begin to emerge from the calli and when they have 2 leaves they are transferred to small pots with soil and undergo a period of 2 weeks on acclimatizing chamber with controlled light and humidity. They are later transferred to 13 l soil pots in the greenhouse and grown to full maturity.

Genomic DNA extraction and PCR analysis - Total DNA was extracted according to the method described by DELLAPORTA *el al*., (1983). Polymerase Chain Reaction (PCR) was followed in a UNO II (Biometra, Germany) thermal cycler with Taq polymerase (Invitrogen, USA). The following parameters were used: 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 58ºC for 1min, 72ºC for 1 min. Final extension time of 10 minutes at 72ºC was used. For identification of TPS1 transgenic plants primers AtTPS L (5' – GAA TTT GAG GCC AGA TGG ATA G-3') and TPS 2 (5' –TAT CTC AGA CGA AGG GAA

TGG T-3') were used. These primers amplify a 400 bp fragment of the AtTPS1 sequence. PCR products were separated by electrophoresis on a 2 % agarose Gel.

RESULTS AND DISCUSSION

Cloning of the cassette 35S-AtTPS1 into vector pGreen0229 to be used for maize transformation was successful as confirmed by enzymatic restriction (data not shown).

In figure 2 (A to D), the regeneration process is presented. Figure 2 A depicts plantlets emerging from the embryogenic calli. Such plantlets are later transferred to in vitro culture glass jars (2B) where they are grown until they have two leafs, being later changed to small pots with soil (2C) and later transferred to the greenhouse (2D).

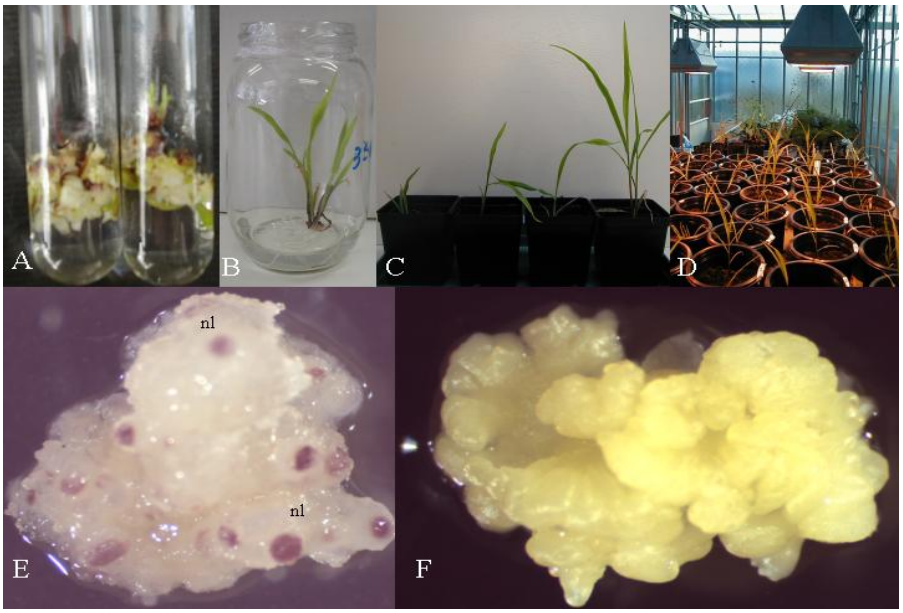

Fig. 2. Selection of embryogenic *calli* and regeneration of plants. A – Embryogenic *calli* with plantlets emerging; B – Plant excised from embryogenic *calli*; C – Plants at several different ages in soil pots in the acclimatizing chamber; D – Plants in greenhouse; E – PPT-sensitive *calli* with dark red necrotic lesions (nl); F – PPT-resistant *calli* with dark yellow color and absence of necrotic lesions

Nine hundred calli were transferred to selection medium after electroporation (Table 1). Of these, the majority (684 calli, 76 %) was susceptible to PPT and consequently discarded, while 216 calli (24%) showed a phenotype of resistance to PPT. Figure 2F and 2E show embryogenic calli at the end of the selection period. PPT resistant calli (Fig. 2F) are easily recognized from PPT-sensitive calli (Fig. 2E). The first have a strong yellow color and display a

continuous expansion throughout the selection period. On the contrary, PPT-sensitive calli are much smaller, show a white color and characteristic red spots that indicate necrotic tissue as a consequence of selection pressure.

Table 1 – Electroporated calli and plants regenerated

	Electroporated *Calli*	PPT Resistant *Calli*	PPT Sensitive *Calli*	Plants Obtained	Flowering plants
Number	900	216	684	80	70
Percentage	100	24	76	37	87.5

Of the 216 PPT-resistant calli, a total of 80 plants were obtained. Most of the plants had an apparently normal phenotype although smaller in size than wild type plants. Although not reported in the rice transformation assays described by GARG *et al*. (2002), stunted growth seems to be frequent in plants transformed with TPS1 genes under the control of 35S promoter as indicate the results of GODDIJN *et al*. (1997) in tobacco and potato, ROMERO *et al*. (1997) in tobacco and YEO *et al*. (2000) in potato. Similarly to stunted growth, shorter and lancet-shaped leaves have also been reported (YEO *et al*., 2000), however in our assay, none of the obtained plants showed any of these phenotypes.

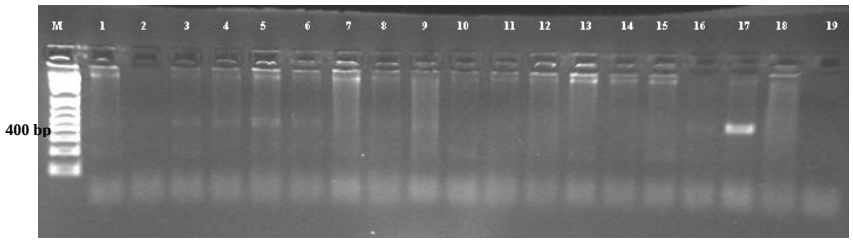

Fig. 3. PCR analysis of putative transgenic maize plants using TPS1 and TPS2 primers. M: Molecular weight DNA ladder 1kb+ (Invitrogen); Lanes 1 and 2: Control wild type plants; Lanes 3 to 19: putative transgenic maize lines. A 400 bp amplification band is visible for maize lines in lanes 3 to 9, 16 and 17

A total of seventy plants flowered, which represents a considerably high percentage of flowering plants (87.5 %). Despite the high flowering percentage, it was extremely difficult to obtain seeds by self-pollination. Male (tassels) and female (ears) inflorescences appeared with a 2 to 5 five-week interval and when pollinations occurred, pollen was no longer viable. As a consequence, few plants had ears with seeds; nevertheless a considerable number of plants produced seeds at the tassel. Desynchronized male and female inflorescences might be related to stress at the flowering season or it can be derivative from a stress caused by the transfer of the plants from *in-vitro* conditions to the greenhouse.

Eighteen plants derived from PPT-resistant calli were tested by PCR to have a first confirmation of integration of the AtTPS1 gene. Wild type Pa91 plants

were used as control. The results are presented in Fig. 3. As expected, in wild type plants no band was found (Fig. 3, lanes 1 and 2). Of the putative transgenic T0 plants tested, a total of 10 showed a fragment of 400 bp (see for example lanes 5 or 17, Fig. 3) suggesting integration of the AtTPS1 gene. Such results indicate a selection efficiency of 55 %. This percentage of transformation is substantially lower than the one described by D'HALLUIN *et al*. (1992) that reported a rate of success of 87 % transformation (positive plants / tested plants). In the same assay, the rate of plants obtained per resistant calli selected was approximately 11%, clearly contrasting with those registered in our experiment (40 %).

Such results point out that the selection efficiency of the transformation system here described might be considered low and room for improvement should be implemented. A first step would be the increase of PPT concentration during the selection phase. In fact, although a PPT concentration of 1 mg/l is recommended as sufficient (LAURSEN *et al*., 1994) since PPT based selection systems tend to be more efficient than those based on kanamycin, some authors recommend higher PPT concentrations, of up to 5 mg/l (D'HALLUIN *et al*., 1994). Phosphynotrycin sensitive calli are clearly distinguished from PPT-resistant *calli* by visual identification (see Fig. 2E and 2F). Despite the length of the selection period (approximately 2 months), our results possibly imply that some escapes might have passed the selection phase. Being so, an alternative towards inclusion of the selection agent in the regeneration media in similar concentrations to those of the selection phase might be pointed out. Subsequent analysis are however required for a full characterization of gene integration in the putative transgenic plants (Southern blot) and AtTPS1 gene expression (northern blot). Physiological evaluation (by comparison with wild type maize plants), of T1 plants resulting from confirmed T0 transgenic plants is equally essential.

Although reported by GOULD *et al*. (1991) and ISHIDA *et al*. (1996), maize is considered refractory to *Agrobacterium*-mediated transformation. Therefore, the use of systems based on particle bombardment is often the solution for monocot transformation, namely maize. A viable alternative to particle bombardment can be electroporation. This method uses a cheaper, easier and more available technology, since electroporators, contrary to particle acceleration devices, are commonly found in most molecular biology laboratories that work on bacteria transformation and are easier to operate. Previous results have shown that the electroporation method can be used to efficiently generate transgenic maize plants (D'HALLUIN *et al*., 1992). The results obtained in our experiment equally demonstrate that the system can be used to obtain putative transgenic plants transformed with Trehalose-6 phosphate-synthase gene (TPS1). To our knowledge, it is the first time that a TPS1 gene of plant origin (*A. thaliana*) is used in plant transformation, namely in maize. Our results also show that the construct designed can be used for transformation of maize and other plants with promising results. They finally show that the selection and regeneration systems used can be considered effective, despite the need for improvement concerning limiting the number of escapes in the selection process.

Acknowledgements - Authors acknowledge scholarships and financial support from Fundação para Ciência e a Tecnologia, PRAXISXXI/BD/21270/99 (A.M. Almeida), SFRH/BPD/1557/00 (D. Santos) and SFRH/BD/5225/01 (S. Araújo). Authors would like to thank Henriette Schleppmann and Phil Mullineaux for supplying respectively the AtTPS1 gene and the pGreen 0229 plasmid and colleagues Mireya Santos (IBMB – Barcelona), Jorge Paiva and Alexandre Campos (ITQB – Oeiras), for their valuable help in experimental and greenhouse work, and finally to Zoran Visak for the kind abstract translation.

Received December 15th, 2003
Accepted December 29th, 2003

REFERENCES

BOHOROVA N., W. ZHANG, P. JULSTRUM, S. McLEAN, B. LUNA, R.M. BRITO, L. DIAZ, M.E. RAMOS, P. ESTANOL, M. PACHECO, M. SALGADO, and D. HOISINGTON (1999): Production of transgenic tropical maize with cryIAb and cryIAc genes via microprojectile bombardment of immature embryos. Theor. Appl. Genet., *99*, 437-444.

CHU C.C., C.C. WANG, C.S. SUN, C. HSU, K.C. YIN, C.Y. CHU, and F.Y. BI (1975): Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin (Beijing) *18*, 659-668.

D'HALLUIN K., E. BONNE, M. BOSSUT, M.D. BEUCKLEER, J. LEEMANS (1992): Transgenic maize plants by tissue electroporation. The Plant Cell, *4*, 1495-1505.

D'HALLUIN K., E. BONNE, M. BOSSUT, and R. le PAGE (1994): Transformation of maize via tissue electroporation. Methods in Molecular Biology 111 Edited by R.D. HALL, Humana Press, Totowa (NJ), USA, 367-373.

D'HALLUIN K., M. de BLOCK, J. DENECKE, J. JANSSENS, J. LEEMANS, A. REYMAERTS, and J. BOTTERMAN (1995): The bar gene as selectable and screenable marker in plant engineering. Recombinant DNA methodology II, Academic Press, 157-168

DELLAPORTA S., J. WOOD, and J. HICKS (1983): A plant DNA minipreparation, version II. Plant Mol. Bio. Rep., *1*, 19-21.

ELBEIN A.D. (1974): The metabolism of $_{\alpha,\alpha}$-Trehalose. Adv. Carbohydr. Chem. Biochem., *30*,227-256.

F.A.O. (2003): Statistical database. http://www.fao.org/waicent/portal/statistics_en.asp .

FRAME B., H. SHOU, R.K. CHIKWAMBA, Z. ZHANG, C. XIANG, T.M. FONGER, S.E. PEGG, B. LI, D.S. NETTLETON, D. PEI, and K. WANG (2002). Agrobacterium tumefaciens mediated transformation of maize embryos using a standard binary vector system. Plant physiology, *129*, 13-22.

GARG A.K., J.K. KIM, A.P. RANWALA, Y.D. CHOI, L.V. KOCHIAN, and R.J. WU (2002): Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc. Nat. Acad. Sci., *99* (25), 15898-15803.

GODDIJN O.J. and K. van DUN (1999): Trehalose metabolism in plants. Trends in Plant Science, *4* (8), 315-319.

GODDIJN O.J., T.C. VERWOERD, E. VOOGD, R.W. KRUTWAGEN, P.T. de GRAAF, J. POELS, K. van DUN, A.S. PONSTEIN, B. DAMM, and J. PEN (1997): Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant physiol., *113*, 181-190.

GOULD J., M. DEVEY, O. HASEGAWA, E.C. ULLIAN, G. PETERSON, and R.H. SMITH (1991): Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. Plant physiology, *95*, 426-434.

HELLENS R.P., E.A. EDWARDS, N.R. LEYLAND, S. BEAN, and P.M. MULLINEAUX (2000): pGreen, a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Bio., *42*, 819-832.

ISHIDA Y., H. SAITO, S. OHTA, Y. HIEI, T. KOMARI, and T. KUMASHIRO (1996): High efficient transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology, *14*, 745-750.

ITURRIAGA G., D.F. GAFF, and R. ZENTELLA (2000): New desiccation tolerant plants, including a grass in the central highlands of Mexico, accumulate trehalose. Australian Journal of Botany, *48*, 153-158.

KOMARI T., Y. HIEI, Y. ISHIDA, T. KUMASHIRO, and T. KUBO (1998): Advances in cereal gene transfer. Current opinion in plant biology, *1*, 161-165.

LAURSEN C.M., R.A. KRZYZEK, C.E. FLICK, P.C. ANDERSON, and T.M. SPENCER (1994): Production of fertile transgenic maize by electroporation of suspension culture cells. Plant Mol. Bio., *24*, 51-61.

MURASHIGE T., and F. SKOOG (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., *15*, 473-497.

ROMERO C., J.M. BELLÉS, J.L. VAYÁ, R. SERRANO, and F.A. CULIÁÑEZ-MACIÀ (1997): Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta, *201*, 293-297

SAMBROOK J., E.F. FRITSCH, and T. MANIATIS (1989): Molecular cloning: a laboratory manual, 2nd Edition, Cold Spring Harbor Laboratory Press.

SCOTT P. (1999): Resurrection plants and the secrets of eternal leaf. Annals of Botany, *85*, 159-166.

SIEDOW J.N. (2001): Feeding ten billion people, three views. Plant Physiology, *126*, 20-22.

THOMPSON J.A., P.R. DRAYTON, B.R. FRAME, K. WANG, and J. DUNWELL (1995): Maize transformation utilizing silicon carbide whiskers: a review. Euphytica, *85*, 75-80.

WANG K., P. DRAYTON, B. FRAME, J. DUNWELL, and J. THOMPSON (1995): Whisker mediated plant transformation: an alternative technology. In Vitro Cell Dev. Biol., 31, 101-104.

WINGLER A. (2002): The function of trehalose biosynthesis in plants. Phytochemistry, *60*, 437-440.

YEO E.T., H..B KWON, S.E. HAN, J.T. LEE, J.C. RYU, and M.O. BYUN (2000): Genetic engineering of drought resistant potato plants by introduction of the trehalose-6-phosphate synthase (TPS1) gene from Saccharomyces cerevisae. Mol. Cells, *10*, 263-268.

ZHONG H., B. SUN, D. WARKENTIN, S. ZHANG, R. WU, T. WU, and M.B. STICKLEN (1996): The competence of maize shoots meristems for integrated transformation and inherited expression of transgenes. Plant physiology, *110*, 1097-1107.

GENETSKI INZINJERING KUKURUZA ZA POSTIZANJE OTPORNOSTI NA DESIKACIJU: ELEKTROPORACIJA PUTEM GENA TREHALOZE

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Izvod

Trehaloza je ne-redukujuci disaharid glukoze koji se javlja u velikom broju organizama kao sto su bakterije, gljivice, nematode ili rakovi. Ona igra važnu ulogu u desikaciji i zaštiti od termičkog stresa jer se pokazalo da stabiliše proteine i ćelijske membrane pod ovim stresnim uslovima. Akumulacija trehaloze se dokazala kao efektni način povećanja otpornosti na sušu kako kod model-biljaka kao sto je duvan tako i kod važnih useva kao što su krompir i pirinač. U ovom radu cilj nam da pojačamo otpornost na desikaciju kod kukuruza, jednom od najvažnijih useva u agronomiji, povećanjem akumulacije trehaloze kroz transformaciju sa *Arabidopsis thaliana* trehalose phosphate synthase-genom (AtTPS1) koji je uključen u biosintezu trehaloze. Kaseta koja sadrži AtTPS1 gen pod kontrolom CaMV35S promotera i Bialaphos resistentni gen Bar kao selektivni agens (koji daje otpornost PPT-u) ubačena je u plazmidni vektor pGreen0229. U pogledu biljnog materijala, koristili smo kukuruz sorte Pa91. Nesazreli zigotni embrioni su sakupljeni 14-20 dana nakon polinacije i inicirane su embriogene calli-kulture. One su kasnije elektroporisane sa 20mg plazmidne DNA koristeci Biorad Gene Pulser II na 374 V, u trajanju od 1 sekunde, prema metodi D´Halluin-a i sar. Elektroporisane calli-kulture su držane u selektovanom medijumu (N6 sa 1mg/l PPT-a) na 25°C, u tamnim uslovima, za period od osam dana. Embrioni su tada regenerisani u MS/2 medijumu slobodnom od hormona na 25°C u režimu 16h svetla/8h tame. Kada su biljke dostigle stadijum od 3-4 lista premestene su iz *in vitro* uslova u saksije sa zemljom i držane u aklimatizovanoj komori najmanje 14 dana. Nakon toga postavljene su u saksije u zelenoj bašti. Ukupno je dobijeno 80 trans-genetskih linija. DNA uzorak je ekstrahovan a ove linije su analizirane koristeći PCR pojačanje sa prajmerima za AtTPS1.

Primljeno 15. XII 2003.
Odobreno 29. XII 2003.