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ORIGINAL PAPER

Electroporation of maize embryogenic *calli* with the trehalose-6-phosphate synthase gene from *Arabidopsis thaliana*

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Abstract Trehalose is a non-reducing disaccharide of glucose that occurs in a large number of organisms, playing an important role in desiccation and heat stress protection. 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 genetically engineer maize with the *Arabidopsis thaliana* trehalose phosphate synthase gene (AtTPS1), involved in trehalose biosynthesis via electroporation. A cassette harboring the AtTPS1 gene under the control of the CaMV35S promoter and the Bialaphos resistance gene Bar as a selective agent was inserted in the plasmid vector pGreen0229 and used to transform maize inbred line Pa91 via electroporation. Fifteen putative transgenic plants (T0 generation) were obtained. Transgene integration in T0 plants was analyzed by Southern-blot analysis. T0 plants had normal phenotypes, although smaller than wild type plants. Contrary to wild type plants, when sexual organs emerged, tassels appeared at least 15 days earlier than ears in the same plant, rendering impossible the self-pollination of the T0 plant. These plants were then crossed with wild type plants and in some cases T1 seeds were obtained. T1 seeds presented deformities, especially the lack of endosperm, but it was still possible to germinate some of these seeds. The so obtained plants were tested by Northern blot but no AtTPS1 gene expression was detected, a fact possibly due to the incomplete insertion of the AtTPS1 gene or an extremely low gene expression level.

Keywords Maize · Electroporation · Trehalose

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Introduction

Three important factors are believed to be determinant for world agricultural production in the twenty-first century: increase in world population 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. It is the most important primary food staple for a high percentage of the world population, in particular in Latin America and Africa. It is also

essential for animal production, being the basis of feeds used in the poultry, pork and dairy industries in many developed countries. Maize is a crop with high water requirements and its selection towards water deficit 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 the 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 has not been 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 an ability in resurrection plants has been associated with the accumulation of trehalose in 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 phosphorylated by the enzyme TPS (trehalose-6-phosphate-synthase) forming trehalose-6-phosphate. The latter is de-phosphorylated by TPP (trehalose-6-phosphate-phosphatase) and trehalose is synthesized. In plants, trehalose is metabolized by the enzyme trehalase (Wingler 2002). Synthesis of TPS is considered to be the key factor in the regulation of trehalose biosynthesis in plants, 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 *Escherichia 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) and Jang et al. (2003) 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 and consequently to genetically engineer (Komari et al. 1998). Its transformation has been achieved mainly through biolistic (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).

We had previously reported that transgenic tobacco plants expressing a gene of plant origin that encodes for trehalose-6-phosphate-synthase (AtTPS1), display a higher level of tolerance to abiotic stress than wild type plants (Almeida et al. 2005). In this work, we report the first attempt to genetically engineer maize with the AtTPS1 gene and describe the main features of the obtained plants in order to contribute to future research on the increase of abiotic stress tolerance in maize.

Materials 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 *Arabidopsis thaliana* trehalose-6-phosphate-synthase (*At*TPS1) gene. Bacteria were cultured on Luria Broth medium (LB—10 g/l triptone, 5 g/l NaCl and 5 g/l yeast extract).

Construction of pGreen0229/35S-AtTPS1

The cDNA of TPS1 gene from *Arabidopsis thaliana* was used to construct pGreen0229/35S-*AtTPS1* to be used for plant transformation. AtTPS1 cDNA was kindly supplied by Henriette Schluepmann (Utrecht University, The Netherlands). Plasmids pJIT60 and pGreen0229 (Hellens et al. 2000) were kindly supplied by Phil Mullineaux (John Innes Centre, Norwich, UK) and their restriction 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 phosphynot-

rycin (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) and were previously described (Almeida et al. 2005). A schematic representation of the construct is depicted in Fig. 1.

Transformation of maize via electroporation

Ten to fourteen days after pollination, kernels were harvested and disinfected (18% solution of commercial bleach for 13 min). After rinsing with double distilled water, 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 (2,4-D), solidified with 2 g/l gelrite and pH 5.8. After a 30-day culture period in the dark at 25°C embryogenic *calli* were formed. They could be maintained for up to 8 months without losing embryogenic ability if transferred every 14 days to fresh medium. Maize embryogenic *calli* were divided in 1.5 mm diameter fragments in buffer EPM^- (0.74 g/l $CaCl_2$, 10 mM Hepes, 0.425 M mannitol, pH 7.2) and underwent preplasmolysis for 3 h. They were later washed with EPM^+ buffer (EPM^- plus 13 g/l sodium glutamate) for 10 min. Approximately 100–200 mg of preplasmolysed *calli* was transferred to an electroporation cuvette with 200 µl of EPM^+ buffer and 20 µg of plasmid DNA. After 1 h of incubation, the electroporation cuvette was cooled on ice for 10 min. Electroporation was done with a Gene Pulser II electroporator (Biorad) at 374 V for 1 s. To wash the *calli* 200–400 µl of N6aph medium was added to the cuvette, immediately after electroporation. Liquid N6aph medium was composed of N6 macro and micronutrients (Chu et al. 1975) supplemented with 0.9 g/l asparagin, 1.38 g/l mM L-proline, 1 mg/l thiamine HCl, 0.5 mg/l nicotinic acid, 100 mg/l casein hydrolysate, 100 mg/l inositol and 5.4 g/l mannitol (pH 5.8). To select the transformation events, *calli* were cultured in medium Mah1VII supplemented with a selection agent. The composition of the Mah1VII medium was as follows: 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 $MgCl_2$, 1 mg/l 2,4-D, 20 g/l sucrose, 36.4 g/l mannitol and solidified with 1.6 g/l gelrite, pH 5.8. The selection agent used was 2 mg/l L-phosphinothricin (PPT). The first step of the cultivation period lasted 14 days, after which *calli* were transferred to Mah1VII medium without mannitol for a period of 8 weeks. All the selection process was done in the dark at 23–25°C.

To regenerate plants, *calli* resistant to PPT were transferred to half strength MS medium solidified with 1.6 g/l gelrite and pH 5.8 and cultured at 23–25°C in a phytotron (16 h light to 8 h dark) for 20 days. Two leaf plantlets were transferred to small pots with soil and undergo a period of 2 weeks on acclimatizing chamber with controlled light and temperature (24°C; 16 h light to 8 h dark photoperiod). They were later transferred to 13 l soil pots in the greenhouse and grown to full maturity.

Genomic DNA extraction and PCR analysis

DNA was extracted from mature leaves of 2 to 4-week-old T0 plants according to the method of Dellaporta et al. (1983). Briefly, tissue was ground with mortar and pestle in liquid nitrogen with quartz sand and frozen samples were mixed with extraction buffer (7 M urea, 0.3 M NaCl, 50 mM Tris–HCl, 20 mM EDTA and 1% sarcosyl) for 10 min at 4°C. Two extractions with chloroform: isoamylic alcohol were performed and 0.3

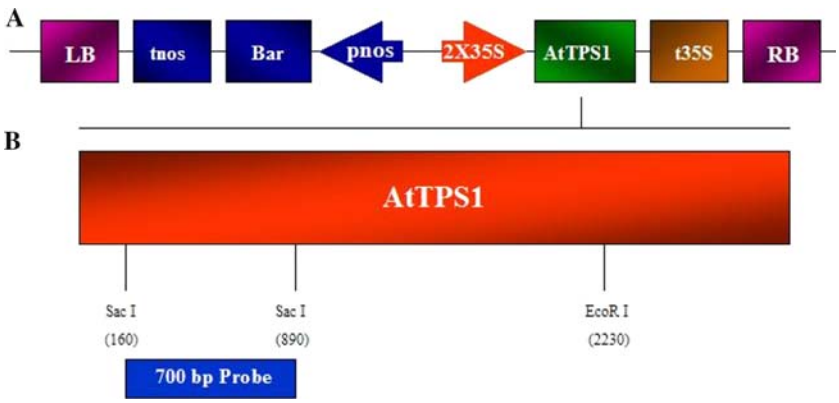

Fig. 1 Plasmid construct pGreen0229/35S-*AtTPS1* (**a**) and diagram of the AtTPS1 gene showing cleavage sites for *Sac*I and *Eco*RI and 700 bp probe location (**b**). *LB* left border, *RB* right border, *t35S* 35S terminator, *AtTPS1* trehalose-6-phosphate-synthase gene (*Arabidopsis thaliana*), *p35S* 35S promoter, *pnos* nos promoter, *tnos* nos terminator, *bar* Bialaphos resistance gene

volumes of 10 M NaOAc were added, followed by 0.6 volumes of cold isopropanol. After precipitation and centrifugation, the resulting pellet was washed with 70% ethanol and resuspended in TE (10 mM Tris–Cl; 1 mM EDTA). RNase was added and following incubation at 37°C for 1 h; DNA samples were quantified by standard agarose gel electrophoresis by comparison with known quantities of λ DNA, and stored at 4°C. Polymerase chain reaction (PCR) was carried out in a thermocycler UNO II (Biometra, Germany) with Taq polymerase (Invitrogen, USA). The following parameters were used: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min. Final extension time of 10 min at 72°C was used. For identification of TPS1 transgenic plants two primers were used: TPS 1 (5′-GAA TTT GAG GCC AGA TGG ATA G-3′) and TPS 2 (5′-TAT CTC AGA CGA AGG GAA TGG T-3′). Primers were designed to obtain a 400 bp amplification band of the AtTPS1 sequence. PCR products were separated on a 2% agarose gel electrophoresis.

Genomic DNA extraction and Southern-blot analysis

DNA was extracted from mature leaves of 2 to 4-week-old T0 plants using the DNeasy plant DNA extraction kit from Qiagen following the manufacturer's instructions. DNA was quantified using the NanoDrop® system (NanoDrop Technologies, Wilmington, DE, USA) and digested overnight with *Eco*RI. Full digestion was monitored on standard 1% agarose gel. If digestion was complete, digested DNA was precipitated with 1/10 volume of 3 M sodium acetate and two volumes of ice-cold absolute ethanol. After centrifugation, pellet was washed with 70% ethanol and dissolved in 40 µl TE. Twenty micrograms of digested DNA was separated in large 1% agarose gels at low voltage.

The gel was incubated in a denaturing solution (0.5 M NaOH and 1.5 M NaCl) under slow agitation for 30 min and subsequently in a neutralizing solution (0.5 M Tris–HCl pH 7.4 and 1.5 M NaCl) for another 30 + 15 min. DNA was transferred onto Hybond-N^+ nylon membranes (Amersham) using a standard blotting procedure with a 10× SSC buffer (300 mM sodium citrate and 300 mM NaCl). Southern blot hybridization was carried out according to Amasino (1986). Briefly, blots were hybridized with a α-^{32}P-labeled denaturated probe (a 700 bp fragment of AtTPS1 gene obtained from digestion with *Sac*I) using the Amersham random primer labeling kit following manufacturer's instructions. Pre-hybridization was carried out in hybridization buffer (0.25 M $NaHPO_4$ pH 7.2; 7% SDS electrophoresis purity reagent; 1 mM EDTA; 0.25 M NaCl; 10% PEG6000 and 0.04% formamide) at 42°C for 60 min, followed by overnight hybridization at the same temperature and buffer. Several washings (15 min each) were conducted using washing buffer (2× SSC; 0.5% SDS) at 42°C. Hybridized membranes were exposed for 7 days at –70°C to X-ray films (Kodak, USA).

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 carried out as described (Almeida et al. 2005).

In this experiment, we have obtained *calli* resistant and sensitive to PPT. Figure 2 show embryogenic *calli* at the end of the selection period. PPT-resistant *calli* (Fig. 2a, b) are easily distinguished from PPT-sensitive *calli* (Fig. 2c, d). The first are bright yellow and display a continuous growth throughout the selection period. On the contrary, PPT-sensitive *calli* are much smaller, white and showing characteristic red spots that indicate necrotic tissue as a consequence of the effect of the selection agent.

In Fig. 3 the regeneration process is presented in detail. Figure 3a depicts plantlets emerging from the embryogenic *calli*. Such plantlets are later transferred to in vitro culture glass jars (Fig. 3b, c), where they are grown until they have two leaves, and later moved to small pots with soil (Fig. 3d, e) in an acclimatizing chamber. They are later transferred to the greenhouse in order to produce seeds (Fig. 3f, g).

We have obtained 16 plants that were regenerated and transferred to the greenhouse. Each plant was obtained from a different callus. Obtained plants were smaller than wild type plants but aberrant phenotypes, such as abnormal leafs, were not detected. This selection process is considered quite efficient, as untransformed plants are unable to grow in selective media due to the high toxicity of the selection agent (D'Halluin et al. 1992). For this reason, we have chosen not to include *Bar* gene expression assays in subsequent molecular biology tests.

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 a control. The results are presented in Fig. 4. As expected, in the wild type plants no amplification band was found (Fig. 4 lane wt). Of the 16 putative transgenic T0 plants tested, a total of 9 showed a fragment of 400 bp (see for example lanes 3 or 7, Fig. 4) sug-

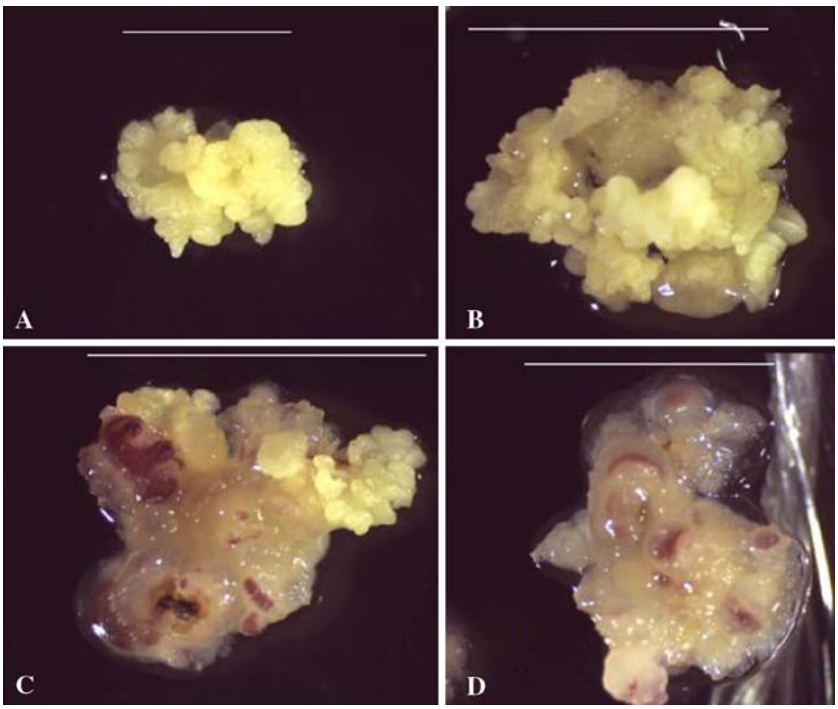

Fig. 2 PPT sensitive and PPT resistant *calli*. PPT resistant *calli* (**a**, **b**) display continuous growth throughout the selection procedure and are yellow. PPT sensitive *calli* (**c**, **d**) are white, having a reduced or null growth and show red and brown necrotic lesions. (**a**, **b**: *bar* 12 mm, **c**: *bar* 11 mm, **d**: *bar* 8 mm)

gesting the presence of the AtTPS1 gene in these T0 maize plants.

Such results indicate a transformation efficiency of 50%. 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 ratio 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 referred as sufficient some authors recommend higher PPT concentrations, of up to 5 mg/l (D'Halluin et al. 1994).

Integration of the AtTPS1 gene was detected by Southern blot (Fig. 5). Genomic DNA of wild type and T0 maize plants was digested with the enzyme *Eco*RI and probed with a 700 bp *Sac*I fragment of the AtTPS1 gene. The enzyme *Eco*RI cleaves once in the AtTPS1 gene, in the 2,230 bp position, and once in the MCS of the construct pGreen0229/35S-*AtTPS1*, generating an internal fragment of approximately 3,000 bp, corresponding to the 2,230 bp from the AtTPS1 gene and 800 bp from the 35S promoter (Fig. 1). The 700 bp fragment of the AtTPS1 gene used as probe should hybridize to the 3,030 bp fragment only (see probe localization in Fig. 1). Four T0 plants presented AtTPS1 hybridising bands: one plant showed a band of 2,700 bp, two showed a band of 2,300 bp and a last plant, a band of 1,600 bp). The fact that, in the four plants found to have integration of the transgene, bands of smaller size than the expected 3,000 bp were detected might be an indication that the gene was not fully integrated. This could be interpreted as a consequence of the electroporation process that similarly to particle bombardment might lead to the integration of disrupted fractions of the insert DNA.

The same T0 plants were analyzed for AtTPS1 gene expression by Northern blot (data not shown). No band was visible in any of the plants, thus suggesting the absence of AtTPS1 gene expression or alternatively a very low level of expression at the T0 generation that was impossible to detect under the conditions used for this Northern-blot analysis (20 μg of total RNA were used).

All plants transferred to the greenhouse flowered normally; however, male and female inflorescences emerged asynchronously (tassels appeared 2–3 weeks earlier than ears). For this reason, and with one exception, self-pollination was impossible, and we had to cross T0 plants among them or alternatively, with wild type plants. Seeds resulting from such crosses are shown in Fig. 6.

As presented in Fig. 6d and e, cobs from T0 plants crossed either with other T0 plants or with wild type plants showed small number of seeds when compared

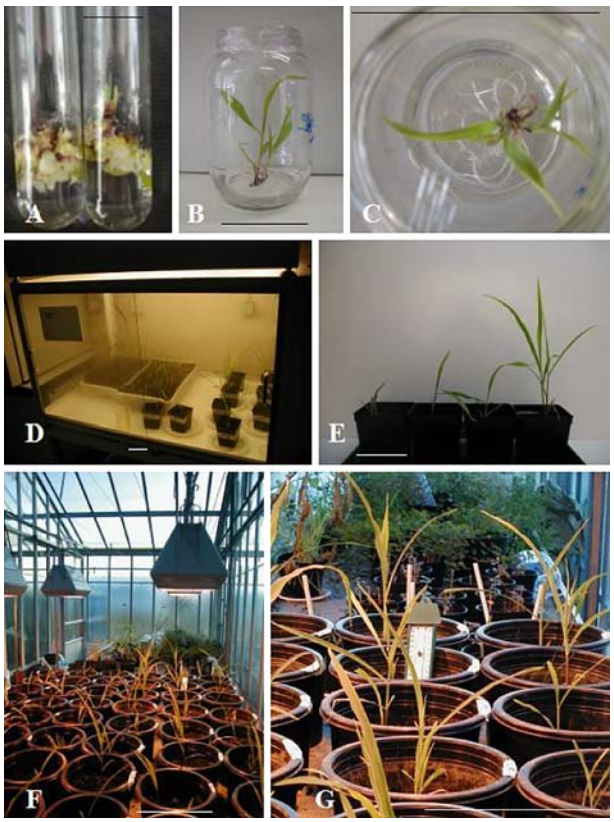

Fig. 3 Regeneration of Pa91 maize plants from electroporated *calli*. **a** Plants regenerating from embryogenic *calli*; **b**, **c** two aspects of isolated plants developing in vitro; **d** plants in pots with soil in the acclimatizing chamber; **e** plants of several ages in the acclimatizing chamber; **f**, **g** two aspects of *calli*-regenerated plants growing in the greenhouse. **a**: *bar* 2.5 cm, **b**, **c**: *bar* 7.5 cm, **d**, **e**: *bar* 10 cm, **f**, **g**: *bar* 25 cm

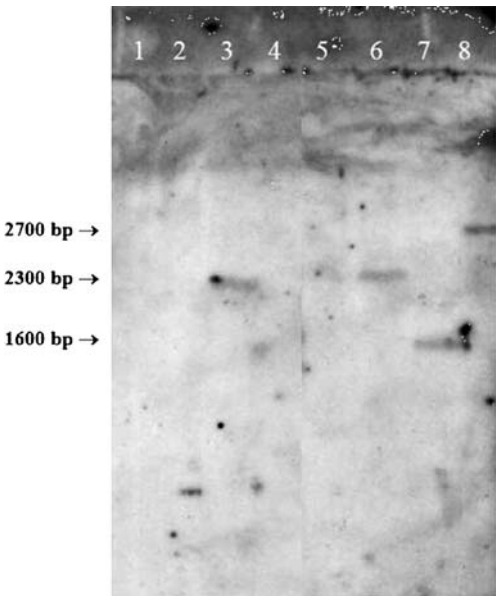

Fig. 5 Southern blot of genomic DNA from wild type (*lanes 1, 2*) and putative transgenic T0 plants (*lanes 3–8*). DNA was digested with *Eco*RI and hybridised with ^{32}P labelled probe (a 700 bp fragment of AtTPS1 gene)

to wild type cobs such as the one depicted in Fig. 6c. Seeds from such cobs were smaller than wild type seeds and presented a reduced amount of endosperm (see Fig. 6a, b). Plants obtained in this experiment showed to have deficiencies regarding synchronization of male and female flowering and at the level of seed formation. Such seed deficiencies have not been reported in other experiments of maize transformation by electroporation (D'Halluin et al. 1992) and were never noticed in another maize transformation experiments using the Pa91 variety and the *bar* gene, as well as in other maize transgenic plants growing simultaneously to ours in the greenhouse. Sterility and seed formation-related problems have been associated to transformation with trehalose synthesis genes (Romero et al. 1997; Goddijn et al. 1997). Although it has been speculated that monocots, namely rice, would be less sensitive (Jang et al. 2003) to trehalose metabolism changes, it has been demonstrated that maize's inflorescence architecture and hence seed deposition is strongly affected by trehalose metabolism (Satoh-Nagasawa et al. 2006), hence consubstantiating our results.

All T1 seeds obtained were germinated in small pots with soil. Most of the seeds, especially those with little

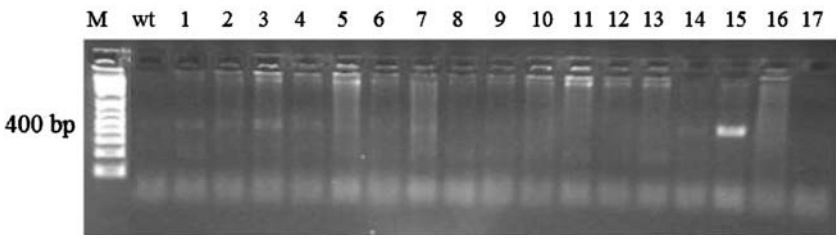

Fig. 4 PCR analysis of putative transgenic maize plants using TPS1 and TPS2 primers. *M* molecular weight DNA ladder 1 kb+ (Invitrogen); *lanes wt* control wild type plants; *lanes 1–14; 16 and 17* putative transgenic maize lines; *lane 15* positive control (construct harboring the AtTPS1 gene). A 400 bp amplification band is visible for maize lines in lanes 1–7 and 14

Fig. 6 Seeds and cobs of wild type and T0 maize plants. **a** Wild type seeds (*left*) and T1 seed (*right*); **b** detail of **a**, note smaller size and lack of endosperm in T1 seeds (*right*) as opposed to wild type seeds (*left*); **c** wild type maize cob; **d**, **e** cobs of T0 plants, note the small number of seeds on the cobs of the putative transgenic plants. All *bars* 2 cm

endosperm were not viable and hence failed to germinate. Plantlets from seeds that germinated were allowed to grow until a three to four-leaves stage and then tainted with a 2% PPT solution (v/v). Such solution is lethal to wild type plants and leads to necrosis and death of leaf tissues in contact with PPT and subsequently of the plant itself as shown in Fig. 7a and b.

We have obtained five T1 plants that showed to be tolerant to PPT tainting and those are shown in Fig. 7c at a later stage of development. As can be observed, PPT spraying did not affect the development of the plants that grew normally.

A Northern-blot analysis of such T1 putative transgenic plants was also performed (data not shown) but no band was visible that could indicate AtTPS1 gene expression, even after a prolonged exposure of the membrane. Although reported by Gould et al. (1991) and Ishida et al. (1996) that maize is not refractory to *Agrobacterium*-mediated transformation, this transformation process is not generally used to generate maize transformants. Therefore, the use of systems based on particle bombardment is often the solution for monocot transformation, namely for maize. A viable alternative to particle bombardment can be electroporation. This method uses a cheaper, easier and more available technology, since electroporators, contrary to biolistic devices, are commonly found in most molecular biology laboratories that work on bacteria transformation and are easier and cheaper to operate and it was shown that electroporation can be used to efficiently generate transgenic maize plants (D'Halluin et al. 1992).

The obtained plants demonstrate to have three phenotypic characteristics that allow the differentiation from wild type plants: they display a tolerance to the herbicide PPT, are smaller in size and present non-synchronization of male and female flowering with

Fig. 7 PPT tolerance and sensitivity in maize leafs. **a** Leaf of transgenic line after 2% PPT spraying showing evident resistance to the herbicide; *bar* 4 cm. **b** Leaf of wild type plant after tainting; *bar* 5 cm. **c** T1 maize plants that survived PPT tainting growing in the greenhouse in order to obtain T2 seeds (*front row*). *Bar* 45 cm

deformities in the cobs and seeds. These three facts and the support of the results from the Southern-blot analysis of the plants of the transformation experiment confirm that transgenic maize plants were obtained expressing the Bar gene and hence showing tolerance to PPT. However, the Northern-blot analysis revealed lack of AtTPS1 expression in the T1 plants. Such lack of expression was still found when we used 40 μg of total RNA from the T1 plants instead of the 20 μg used for the T0 plants and the membrane exposition time was longer than the one used for T0 plants. Lack of expression could be due to incomplete transfer of the full AtTPS1 gene, a fact supported by the presence of smaller bands than the expected in the Southern-blot analysis, or to the lack of recognition of the 35S promoter.

To our knowledge, this is the first report of an experiment towards genetic engineering of maize with genes that code for proteins involved in trehalose biosynthesis is made. Our results seem to demonstrate that transgenic plants were obtained, rendering interesting future physiological studies of transgenic maize tolerance to abiotic stresses, by comparison with wild type plants. Another future step would be to conduct other approaches using this transformation methodology, namely the use a construct with a promoter more easily recognized in monocots such as the Ubi1 (*Zea mays* ubiquitin promoter).

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