

Genetic Transformation of the *Nicotiana* Protein Kinase (NPK1) Gene Confers Osmotic Tolerance in Egyptian Maize

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Abstract: Maize is an economically important food crop. Environmental stresses, such as water deficit, increased salinity of soil, and extreme temperature, are major factors limiting maize productivity. Biotechnology and especially genetic engineering could offer great benefits by developing inherent engineered tolerance to drought and salt stresses. In recent years, *Agrobacterium*-mediated transformation has been successfully applied in important gramineae species, especially maize. In the present work, we introduced the *NPK1* gene (for drought and salt tolerance) in combination with *bar* gene (for herbicide resistance, as a selectable marker) into elite maize inbred lines using the *Agrobacterium*-mediated transformation, the transformation frequency ranged between 2.7% and 5.2% for the genotypes used. Molecular analysis revealed the presence and integration of the transgenes in the genome of transgenic plants. R1 progeny of transgenic plants were tested *in vitro* for tolerance to osmotic (mannitol) stresses as compared to non-transgenic control plants. Transgenic plants maintained a higher growth and showed increased tolerance to stress conditions compared to non-transgenic plants. Thus, providing strong evidence that the NPK1 may play an important role in the protection of maize against possible water-deficiency stress conditions.

Key words: Maize (*Zea mays* L.), NPK1, *bar*, *Agrobacterium*-mediated transformation and mannitol-stress

INTRODUCTION

Environmental stresses, such as water deficit, increased salinity of soil, and extreme temperatures, are major factors limiting plant growth and productivity. Therefore, a growing interest in crop response to environmental stresses has been raised in the last decades (Blum, 1985 and Tuberosa and Salvi, 2006). Among the various abiotic stresses, drought or water deficit is the most severe limiting factor of plant growth and production worldwide affecting plant growth, development and productivity through alteration of metabolism and gene expression (Boyer, 1982; Leopold, 1990 and Jeanneau *et al.*, 2002). Water is a fundamental constituent of all life, constituting about 90% of the fresh weight in most physiologically active plant cells. If water content falls much below this level, plants exhibit numerous responses at different levels (Christou, 1996; Geadii *et al.*, 2002, Shou *et al.*, 2004a&b and Wang *et al.* 2008). Developmental responses lead to delay of flowering, reduced tiller formation and modified growth habit (Bradford and Hsiao, 1982). Wilting, leaf rolling and decreased stomatal aperture represent water conservation mechanisms. Moreover, reduced transpiration is manifested as a typical physiological change under stress. Furthermore, to ensure their own survival and the prosperit of their offspring, plants have evolved a range of strategies to cope with water stress. One common mechanism is the accumulation of compatible solutes including amino acids such as proline, sugars such as raffinose, sucrose and trehalose and sugar alcohols (Seki *et al.*, 2007). A typical plant response to environmental stresses is the accumulation of oxygen reactive species such as hydrogen peroxide, superoxide anions and hydroxyl radicals (Hare *et al.*, 1998 and Shou *et al.*, 2004a&b) Accumulation of peroxides activates genes encoding protective proteins conferring tolerance to stressors such as osmoprotectants, reactive oxygen scavengers, heat shock proteins and glutathione-S-transferase (Kovtun *et al.*, 2000).

Nicotiana protein kinase 1 (NPK1) is a gene naturally found in tobacco plants and is activated in the presence of peroxides. Under stress conditions, this gene initiates a metabolic cascade of mitogen activated protein kinases (MAPKs). MAPK cascades generally function in response to osmotic stress, which is incurred upon in plants during drought and temperature extremes (Mizoguchi *et al.*, 1996). NPK1 is located upstream of the oxidative pathway and can induce expression of HSPs and GST1 in Arabidopsis and maize (Kovtun *et al.*, 2000).

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Maize is one of the most important crops and it suffers from serious water-deficit stress in cultivated areas in Egypt and worldwide. In the present study, two Egyptian maize inbred lines in addition to the American A188 line were transformed through *Agrobacterium*-mediated transformation system and a standard binary vector harboring the *NPK1* gene for abiotic stress tolerance and the *bar* gene as a selectable marker. Osmotic stress (mannitol) tolerance of transgenic maize plants constitutively expressing NPK1 was also investigated.

MATERIALS AND METHODS

Binary vector and *Agrobacterium* strain:

The binary vector pSHX004 (Shou *et al* 2004 a&b), has been used in the present study. This binary vector (Fig.1) contains a transgene cassette carrying a modified CaMV 35S promoter ((35S C4PPDK), an 800bp DNA fragment encoding the kinase domain of *Nicotiana* protein kinase (NPK1) and a nopaline synthase terminator (T-nos). In addition pSHX004 contains a selectable marker cassette comprised of a 2xCaMV 35S promoter, a tobacco etch virus 5' untranslated region, the *bar* gene and a soybean vegetative storage protein terminator. The binary vector has been introduced into the *Agrobacterium* strain EHA101 by direct transformation of the competent *Agrobacterium* cells with the pSHX004 DNA.

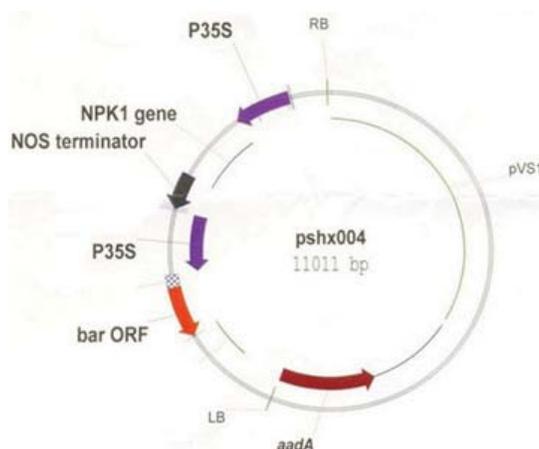


Fig.1: The binary vector pSHX004.

Plant material:

Two Egyptian genotypes, i.e. Gz 639, Gz 649 and the American line A188, were sown in the field during the maize season and plants have been self pollinated. Ears were harvested 10-15 days post pollination and immature embryos were excised to be used in transformation experiments.

Maize Transformation:

Maize ears were surface sterilized by soaking for 20 min in 2.6% sodium hypochlorite (prepared from commercial bleach) containing 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate). The surface sterilized ears were washed three times with sterile distilled water and immature embryos were aseptically dissected and prepared for transformation.

The *Agrobacterium* strain (EHA101) was used for the delivery of the binary vector pSHX004 into the dissected immature embryos. *Agrobacterium tumefaciens* cultures were grown for 2 days at 28°C on YEP medium amended with 100 mg/l spectinomycin and 50 mg/l kanamycin. One loop of the culture was scrapped and suspended in 5 ml of liquid infection medium supplemented with acetosyringone.

For transformation, immature zygotic embryos were washed twice with bacteria-free infection medium, the final wash was removed and 1-1.5 ml of *A. tumefaciens* suspension (O.D. 0.4) was added to the embryos. The tubes were incubated for 5 min at room temperature. After infection, embryos were transferred to co-cultivation medium. Plates were incubated in dark at 22°C after which embryos were transferred to resting medium and incubated at 28°C for one week. The infection, cocultivation and resting media were used according to Frame *et al.*, (2002).

Selection and Regeneration:

For stable transformation, selection of transformed events for the *bar* gene has been carried out using N6-based callus induction medium supplemented with 250 mg/l cefotaxime. The selection medium contained bialaphos (the active ingredient of herbicide Herbiace[®]) as a selective agent. The concentration of the selective agent started with 1.5 mg/l for 2 weeks and increased in the subsequent subcultures to 3.0 mg/l. For regeneration, embryogenic type I or type II calli were transferred to regeneration medium for development of shoots and roots. The regeneration medium also contained the selective agent. Regenerated plantlets have been transferred to soil for further development in the greenhouse. Putative transgenic and non-transgenic plants have been painted using the herbicide basta (0.1%).

PCR Screening for Transgenic Plants:

Genomic DNA was extracted from putatively transgenic plantlets and non-transformed plants as control using the CTAB protocol (Murray and Thompson, 1980). Primers of 5' GGCTGCAGGAATTCTCACATGT 3' and 5' GCTCC CGAAGTCATTCTGCA 3' were used to amplify a 646 bp fragment of DNA containing part of the NPK1 transgene and the nos terminator. While, primers bar-1 (forward) 5' TAC ATCGAGACAAGCACGGTCAACT 3', and bar-2 (reverse) 5' ACGTCATGCCAGT CCCGTG 3'. were used to amplify a fragment of 484 bp for the *bar* gene. PCR reactions were conducted in a total volume of 25 µl containing 50 ng of genomic DNA, 1x PCR buffer, 3 mM MgCl₂, 0.2 mM of forward and reverse primers, 0.2 mM dNTP, and 1.5 U of Taq polymerase. The PCR temperature profile included an initial DNA denaturation for 3 min at 94 °C followed by 30 amplification cycles (94 °C, 1 min; 55 °C, 1 min, 72 °C, 1 min) and a final extension step at 72 °C for 10 min.

Southern Blotting:

Ten micrograms of genomic DNA from maize plants were used for Southern blot analysis. DNA from *Agrobacterium* derived events and control plants were digested with the restriction enzyme *EcoRI*, at 37°C overnight. Digested DNA was separated on a 0.8% (w:v) agarose gel and transferred onto a nylon membrane by alkaline transfer method. The membrane was hybridized overnight at 68°C with ³²P-labelled probe. A 1.8 kb DNA fragment containing the NPK1 gene and its promoter and terminator was used as the hybridization probe. The membrane was then washed with 0.1xSSC and 0.1 % SDS solutions at 50°C for 1 h and the radioactive signals detected by autoradiography.

Evaluation of Transgenic Plants under Water-deficit Conditions:

The performance of transgenic plants expressing the NPK1 gene was evaluated under mannitol-stress conditions using R1 plants from the two transgenic lines Gz639 and Gz649. Seeds of non-transgenic plants were used as controls. Fifty R1 seeds from each transgenic line and non-transgenic line were surface-sterilized and germinated for four days in the dark at 25 °C on half-strength MS basal medium (1/2 MS) containing 1% sucrose with or without 5 mg/l bialaphos for transgenic lines, and non-transgenic plants, respectively. Seedlings were then transferred to 1/2 MS plus 200 mM mannitol medium and grown under light at 25°C. These experiments were replicated four times. The response of young seedlings to stress conditions was analyzed after 6 days. The data was collected and plants were transferred to a soil mixture composed of 1:1 (v/v) peat:perlite for further growth and development in the greenhouse.

RESULTS AND DISCUSSION

Maize Transformation:

In the present study, *Agrobacterium*-mediated transformation system has been used in order to recover transgenic maize plants with better integration of transgenes. Two Egyptian maize lines, Gz 639 and Gz 649 and the American line A188 have been transformed using a standard binary vector. The transgene of interest was the kinase domain of the *NPK1* gene driven by a constitutive promoter (35S C4 PPKK).

The transformation results with pSHX004 in EHA101 from four independent experiments for each maize genotype are presented in Table 1.

Infected immature embryos showed normal growth on cocultivation and resting media without selection with the loss of some embryos that failed to grow after transformation. Subculturing the growing embryogenic calli on different selection media resulted in the selection of putatively transformed embryogenic calli. Surviving calli were used for shoot regeneration after 2 months of selection (Fig.2). Transgenic plants grew normally in the greenhouse similar to non-transgenic plants and showed fertility. The highest transformation

frequency after 2 months of selection was 5.2 % for A188, while it was 3.6 % for Gz 639 and 2.7 % for Gz 649. Herbicide painting of putative transgenic and non-transgenic plants showed tolerance of transgenic plants to the herbicide in comparison to non-transgenic plants which showed wilting at the painted areas (Fig 2e). Transgenic T₀ plants were harvested and T₁ seeds of each line were grown in the biocontainment greenhouse to test the segregation and inheritance of the transgenes.

Table 1: Summary of transformation experiments of the three maize genotypes Gz 639 and Gz 649 and A188 using pSHX004 harboring the NPK1 gene.

Line	No. of embryos	No.Calli 2w	Selection			No. of Plantlets	Transformation Frequency %
			4w	6w	8w		
A188	289	273	202	153	38	15	5.2
Gz639	330	310	250	48	23	12	3.6
Gz649	332	300	226	35	18	9	2.7

Transformation frequency after the 2nd month of selection measured as bialaphos resistant calli divided by the total number of infected immature embryos, ×100



Fig. 2: Different stages of tissue culture. a) Immature embryos after *Agrobacterium* infection. b&c) shoot formation on regeneration medium. d) Putative transgenic maize plantlet on rooting medium. e) Basta herbicide painting of transgenic (T) and control (C) plants.

In this context, Shou *et al.* (2004a&b) reported that transgenic maize plants were achieved by particle bombardment or *Agrobacterium*-mediated transformation using plasmid pSHX002 or binary vector pSHX004, respectively. The transgene of interest in both constructs was the kinase domain of the *NPK1* gene driven by a constitutive promoter 35SC4PPDK (Sheen,1993). Average transformation efficiencies were 7.1% for the bombardment method and 3.3% for the *Agrobacterium* method. R1 seeds were obtained by out-crossing the transformants with untransformed HiII or inbred line B73. Eighty-three percent (20 out of 24) and seventy-three percent (eight out of 11) of the *Agrobacterium*-derived and bombardment-derived events, respectively, segregated with a 1 to 1 ratio as expected for the transgene insertion at a single dominant locus. Moreover, Frame *et al.* (2006) reported success in generating transgenic plants and progeny from three maize inbred lines (B104, B114, and Ky21) using an *Agrobacterium*-mediated standard binary vector system to target maize immature embryos. The recovered transgenic plants were analyzed for transgene integration, expression, and transmission. The average transformation frequencies of 6.4% (for B104), 2.8% (for B114), and 8% (for Ky21) were achieved using MS salts.

Analysis of Transgenic Plants:

PCR analysis revealed the presence of both transgenes (*bar* and *NPK1*) in most of the events tested as illustrated in Fig.3. Southern analysis of R0 and R1 transgenic plants for *NPK1* using the single-cutting restriction enzyme *EcoRI* confirmed the stable integration of the *NPK1* gene without rearrangements into the maize genome (Fig.4). The *Agrobacterium*-derived events contained 1-5 copies of the *NPK1* transgene.

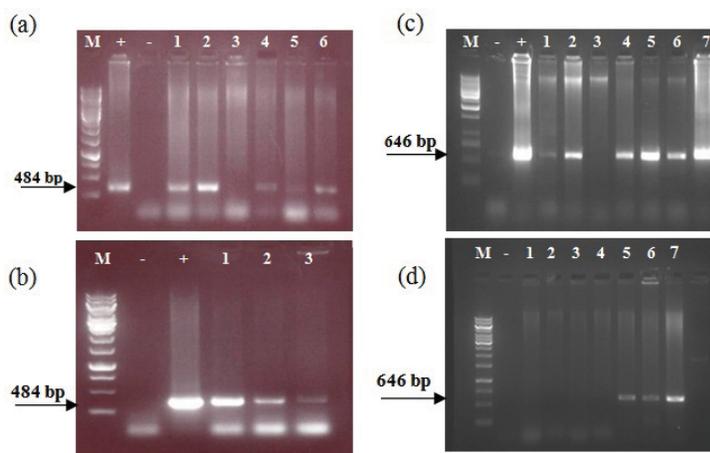


Fig. 3: PCR reactions carried out for *bar* (a&b) and *NPK1* (c&d) genes on DNA samples of putatively transgenic plantlets of T₀ (a&c) and T₁ (b&d). (M) molecular weight size marker 1kb; (-) negative control of un-transformed plant. (+) Positive control of the plasmid pSHX004; Lane 1-7 DNA samples from individual events of inbred line Gz639 and Gz649.

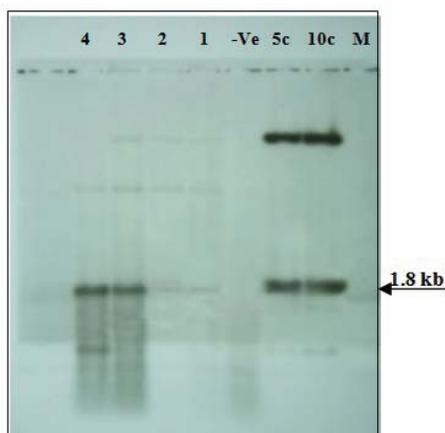


Fig. 4: Southern blot analysis of maize T₀ transgenic plants. (M) Molecular weight size marker; (10c & 5c) 10 and 5 copies of the plasmid pSHX004; (-Ve) negative control; (1-4) samples of transgenic T₀ plants.

All T₁ plants were morphologically normal as compared to the non-transgenic plants of the respective inbred line (Gz 639 and Gz 649). Table (2) represents a comparison of the weight of transgenic and non-transgenic seeds obtained from T₁ plants. The results revealed a slight decrease in the weight of 100 seeds in transgenic plants. This could be attributed to the effect of transformation process and selection pressure. In this context, Shou *et al.*, (2004a) studied the effect of NPK1 gene expression on agronomic traits under drought stress and found that the percentage decrease in kernel weight due to drought stress in transgenic maize plants was much less than in the non-transgenic Hi II control, suggesting that the NPK1 transgenic maize may have higher yield potential than that of the non-transgenic plants under drought conditions.

Table 2: Weight of 100 seeds of NPK1 transgenic and non-transgenic plants.

Ear number	Weight of 100 NPK1 transgenic seeds	Weight of 100 non-transgenic seeds
1	22.42	31.51
2	22.3	31.51
3a	24.62	31.51
3b	22.81	31.51

Stress-tolerance in NPK1 Expressing R1 Plants:

Addition of mannitol to soil or irrigation water causes plant symptoms similar to water-deficit because it affects the availability of water to plants by increasing the osmotic pressure outside of cells (Rumpho *et al.*, 1983).

In the present study, transgenic seeds were recovered from the two Egyptian lines, Gz 649 and Gz 639. A preliminary kill curve experiment has been carried out with control seedlings of Gz 649 and Gz 639 on 100, 200, 300, 400 mM mannitol and results showed that the 200 mM concentration of mannitol was very critical for control seedlings (Data not shown).

The performance of transgenic plants expressing NPK1 gene was evaluated for stress tolerance under mannitol conditions using transgenic R₁ plants. Seeds of non-transgenic plants were used as controls in stress tolerance experiments. Twenty R₁ seeds from each transgenic and non-transgenic line were surface-sterilized and germinated for four days in the dark at 25°C on half-strength MS basal medium containing 1% sucrose and 5 mg/l bialaphos (for transgenic lines), and without bialaphos (for non-transgenic plants). Seedlings were then transferred to 1/2 MS plus 200 mM of mannitol medium and grown under light at 25°C. This experiment was replicated four times. The response of young seedlings to stress conditions was analyzed after 6 days on mannitol containing medium. Data was collected (Table 3) and plants were transferred to soil for further growth and development in the greenhouse (Fig.5).

Table 3: *In vitro* effect of mannitol-stress on the growth of young seedlings of transgenic maize lines. Average length of shoot, root, and average fresh weight of the plants.

Line	Shoot length (cm)		Root length (cm)		Fresh Weight (gm)	
	MS	Mannitol	MS	Mannitol	MS	Mannitol
Gz 649	25	23	31	27	1.6	1.3
Gz 639	24	21	29	28	1.1	1.2
Control	22	5	27	13	1.3	0.49

Data were collected 10 days after seed germination (4 days in selection and 6 days in mannitol stress).

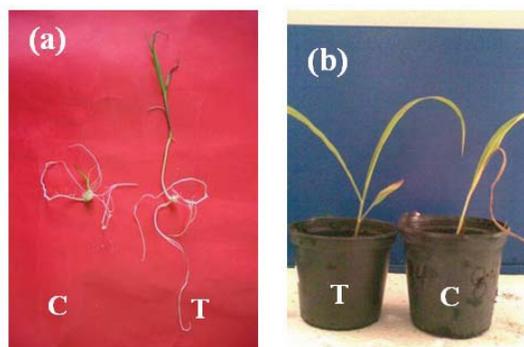


Fig. 5: Transgenic (T) and control (C) plantalets under mannitol stress (a) and in pots (b).

In our experiments, transgenic plants maintained a higher growth rate than non-transgenic plants, under mannitol osmotic stress. There were significant differences in wilting, the death of old leaves, and the necrosis of young leaves of non-transgenic versus transgenic plants expressing *NPK1*. This indicates that the presence of the *NPK1* gene in transgenic maize plants confers increased *in vitro* tolerance to osmotic stress. In this context, it has been reported that the ANP class of MAPKKs from *Arabidopsis* can be induced specifically by H₂O₂ and can activate a specific class of stress-induced MAPKs (ANP, *Arabidopsis* NPK1-like protein kinase and NPK is a *Nicotiana* protein kinase). The activated MAPK cascade plays a dual role in regulation of gene expression: it activates stress-response genes that protect plants from diverse environmental stresses, and it represses auxin-inducible promoters. Thus, the ANP mediated MAPK cascade represents a molecular link between oxidative stress and the plant growth hormone auxin (Kovtun *et al.*, 2000). Moreover, Shou *et al.*, (2004a) reported that expression of NPK1 induced a set of stress genes such as GST, HSPs and PR1, indicating that active NPK1 switched on the oxidative signaling cascade in transgenic maize as well. Moreover, seed germination and early seedling growth are critical stages in crop production, as they indirectly affect crop stand density and grain yield (Gelmond, 1978). On the other hand, in accordance with our results, Maqbool *et al.* (2002) demonstrated the transformation of three oat (*Avena sativa* L.) cultivars with plasmids containing

linked (*hva1-bar*) and non-linked (*gus*) genes. R2 progeny of five independent transgenic lines was tested *in vitro* for tolerance to osmotic (salt and mannitol) stresses. As compared to non-transgenic control plants, transgenic plants maintained a higher growth and showed significantly increased tolerance to stress conditions suggesting that the HVA1 protein may play an important role in the protection of oats against salinity and possible water-deficiency stress conditions.

Our results on the production of mannitol tolerant transgenic maize plants expressing *NPK1* demonstrated that *NPK1* gene might play a role in the protection of plants under water deficiency-stress conditions. Therefore, the *NPK1* gene can be successfully used for genetic improvement of Egyptian maize inbred lines for osmotic tolerance.

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