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The impact of over-expression of NPK1 gene on growth and yield of sorghum under drought stress

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***Sorghum bicolor* L. is an important crop in many tropical regions, yet it has received little attention in applying modern biotechnologies for improvement due to transformation difficulties. Drought is among the most important factors limiting sorghum productivity. The *Nicotina* Protein Kinase 1 (*NPK1*) gene confers tolerance to adverse environmental conditions. The present work was conducted to determine the amenability of sorghum genotypes to *Agrobacterium*-mediated transformation with the *NPK1* gene and to characterize the role of that gene in *S. bicolor*. The *NPK1* gene along with the *bar* gene was successfully introduced into two sorghum genotypes: Dorado and SPGM94021. The transformation frequencies were 1.49 and 1.79% for Dorado and SPGM94021, respectively. Stable integration of the transgenes in T_0 and T_1 plants was confirmed through polymerase chain reaction (PCR) and Southern blotting. The *NPK1* gene expression was measured through real-time-PCR. T_1 plants tested *in vitro* for tolerance to mannitol osmotic stress maintaining a higher growth rate and showed increased tolerance to stress conditions compared to the non-transgenic plants. The transgenic sorghum had a significantly higher kernel weight under drought stress conditions than the control plants. Accordingly, the *NPK1* gene might induce a mechanism that protects sorghum plants against possible water-deficiency stress conditions.**

Key words: Abiotic stress related genes, regulatory genes, signal transduction, transgenic sorghum, plant transformation, quantitative real time-polymerase chain reaction (qRT-PCR), drought stress tolerance.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench], a tropical plant of the family Poaceae, is the fifth most important cereal after wheat, rice, maize and barley and plays a unique role in food security and renewable energy (Belton and Taylor, 2004). The crop is well adapted to tropical and subtropical areas throughout the world. Beside its

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principal uses as flour, in the preparation of porridge and unleavened bread, sorghum species are sources of fiber and fuel and are, also, used in the alcohol industry as they are rich in starch. Despite its importance, the yield and quality of sorghum are constrained by a range of biotic and abiotic factors. Nevertheless, abiotic stresses remain the greatest constraints of crop production with approximately 70% estimated yield reduction (Acquaah, 2007).

Drought is one of the priciest factors affecting a huge number of people every year (Wilhite, 2000). Drylands occupy about 41% of the global terrestrial surface and are home to more than a third of the world's population (Mortimore, 2009). Moreover, the increase of potential evapotranspiration and the changes of the precipitation patterns under a warming climate are leading to a growing global aridity and expansion of drylands (Milesi et al., 2010). Therefore, interest in crop tolerance to environmental stresses has been growing since the last few decades (Tuberosa and Salvi, 2006).

Sorghum is a drought tolerant crop and often grows where water stress conditions are expected. However, the yields, under dryland conditions, are severely affected and much less than that of irrigated sorghum (Assefa and Staggenborg, 2010). Several investigations have been conducted on grain sorghum under stress conditions. Seed mass, harvest index, and biomass were among the parameters affected, most severely, by moisture stress (Wenzel, 1999). Genetic engineering is a potential tool for crop improvement. Although grain sorghum is a very important cereal, particularly in the semi-arid tropic areas as a vital source of food for millions of people around the world, sorghum improvement through genetic engineering is progressing slowly. Like other cereal crops, the first fertile transgenic sorghum plants were obtained *via* particle bombardment (Casas et al., 1993). *Agrobacterium*-mediated transformation of sorghum was first reported seven years later (Zhao et al., 2000).

To improve sorghum abiotic stress tolerance, *HVA*1, thaumatin-like protein (TLP), *mtld*, *OsCDPK*-7 and *TPS*1 genes were successfully introduced into sorghum genome (Devi et al., 2004; Gao et al., 2005a; Maheswari et al., 2010; Mall et al., 2011; Yellisetty et al., 2015). In order to make plants more tolerant to stress and restore the cellular balance, transferring a single gene encoding a single specific stress protein, that is, "single-action" genes may not suffice the required tolerance levels. To overcome such constraint, plant transformation with regulatory genes has emerged (Bhatnagar-Mathur et al., 2008).

Nicotina Protein Kinase 1 (*NPK1*) is a regulatory single transduction gene located upstream of the oxidative pathway and can induce expression of HSPs and GSTs (Kovtun et al., 2000; Shou et al., 2004a). Under stress conditions, plants generate reactive oxygen species, including hydrogen peroxides, superoxide anion, and hydroxyl radical (Shou et al., 2004a). Hydrogen peroxide accumulation can initiate the expression of detoxification and stress protection genes, e.g. heat shock proteins (HSPs), glutathione-S-transferases (GSTs), peroxidases and pathogenesis-related proteins, thereby, guarding plants against stress damages (Kovtun et al., 2000). HSPs serve as molecular chaperones in the ATP-dependent protein assembly/disassembly and prevent protein denaturation during stress (Horwich, 2014). GSTs are enzymes that can detoxify endobiotic and xenobiotic compounds through covalent linkage of glutathione to hydrophobic substrates. Activation of these stress genes can reduce damage caused by chilling, heat, and drought; thus, protect plants from environmental stresses (Das and Roychoudhury, 2014; Li et al., 2003), thereby improving the yield potential of the major cereal crops.

The objectives of this study were to utilize *Agrobacterium*-mediated transformation method to develop water use efficient sorghum lines and to evaluate the regenerated transgenic lines at molecular level. An additional goal was to evaluate the first generation of the transgenic lines (T1) for survival to water deficiency *in vitro* and under greenhouse conditions.

MATERIALS AND METHODS

Plant materials, *Agrobacterium tumefaciens* strain and binary vector

Two *S. bicolor* genotypes, that is, Dorado and SPGM94021, obtained from the Department of Sorghum, Field Crops Research Institute, Agricultural Research Center, Egypt, were sown in the field during the sorghum season and plants have been self-pollinated. Panicles were harvested 10 to 12 days post pollination. *A. tumefaciens* strain LBA4404 containing the standard binary vector pSHX004 (Shou et al., 2004a) was used for sorghum transformation. The binary vector has been introduced into the *A. tumefaciens* strain LBA4404 by direct transformation of the competent cells with the pSHX004 DNA. The vector system, pSHX004 in LBA4404, was maintained on a yeast extract peptone (YEP) medium containing 50 mg/L spectinomycin and 25 mg/L rifampicin. Bacteria cultures, for weekly experiments, were initiated from stock plates stored for up to two weeks at 4°C.

Sorghum transformation

Sorghum panicles were surface sterilized as described in Assem et al. (2014). Immature embryos ranging in length from 1.0 to 1.5 mm were aseptically excised from kernels and prepared for transformation. The *A. tumefaciens* strain LBA4404 was used for the delivery of the binary vector pSHX004 into the dissected immature embryos.

For transformation, *A. tumefaciens* cultures were grown for 2 days at 28°C on YEP medium amended with 100 mg/L spectinomycin and 25 mg/L rifampicin. One loop of the culture was scrapped and suspended in 5 ml of liquid infection medium supplemented with 100 µM acetosyringone. Immature zygotic embryos were washed twice with bacteria-free infection medium, the final wash was discarded and 1 to 1.5 ml of *A. tumefaciens* suspension (OD_{550} = 0.4-0.5) was added to the embryos. The tubes were incubated, in dark, for 10 min at ambient temperature. After

Table 1. Composition of utilized media.

Media	Composition
Infection	4.3 g/L MS salts (Duchefa Biochemie, Prod. No M0221.0050), 0.1 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 1.5 mg/L 2,4-D, 1 g/L vitamin assay casamino acid, 36 g/L glucose, 68.5 g/L sucrose, pH 5.2. Add 100 µM acetosyringone before using.
Co-cultivation	Infection media with reducing sucrose to 20 g/L and glucose to 10 g/L, increasing 2,4-D to 2 mg/L, adding 0.5 mg/L MES, 0.7 g/L L-proline, 10 mg/L ascorbic acid, pH 5.8, 3 g/L phytagel. Adding 100 µM acetosyringone after autoclaving.
Resting	Co-cultivation media with reducing 2,4-D to 1.5 mg/L. Adding 10 g/L PVP and 100 mg/L carbenicellin (After Autoclaving). Without glucose and acetosyringone.
Selection	Resting media with 1.5 mg/L bialaphose (After autoclaving).
R1 (shooting)	4.3 g/L MS salts (Duchefa Biochemie, Prod. No M0221.0050), 0.1 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 0.7 g/L L-proline, 60 g/L sucrose, 10 g/L PVP, pH 5.8, 3 g/L phytagel. After autoclaving add 0.1 mg/L TDZ, 0.25 mg/L ABA, 0.5 mg/L Zeatin, 1 mg/L IAA, 100 mg/L carbenicellin.
R2 (rooting)	2.2 g/L MS including vitamins (Duchefa Biochemie, Prod. No M0222.0050), 20 g/L sucrose, 3 g/L phytagel, pH 5.8. After autoclaving, add 100 mg/L carbenicellin.

infection, embryos were transferred to co-cultivation medium with scutellum side up. Plates were incubated in the dark at 22°C for 4 days after which embryos were transferred to resting medium and incubated at 28°C for one week. The transformation process can be divided into 5 sequential steps: agro-infection, co-cultivation, resting, selection, and plant regeneration. The infection and the co-cultivation media were prepared as described by Zhao et al. (2000), without modification. The compositions of all media utilized in this study are shown in Table 1.

Selection and regeneration

For stable transformation, selection of transformed events was carried out using selection medium containing 1.5 mg/L bialaphos as a selective agent. After 8 weeks on selection medium, survived embryogenic calli were transferred to regeneration medium (R1) for shoot development. Calli-derived shoots were transferred to rooting medium (R2). Cultures of shooting and rooting stages were maintained at 28°C under 16 h photoperiod cool white fluorescent light (75 µmol/m^2/s). Regenerated plantlets have been transferred to soil for further development in the greenhouse. Putative transgenic and non-transgenic plants were subjected to leaf painting when 6 to 7 leaves had fully emerged from the whorl. Solution of the commercial Basta herbicide at concentration of 0.1% was applied to the upper and lower surfaces of the fifth fully emerged leaf.

Polymerase chain reaction (PCR)

PCR analysis was conducted to screen the putatively transgenic T_0 and T_1 plants. Genomic DNA was extracted from putatively transgenic sorghum plants and non-transformed plants, as control, using the cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson, 1980). The forward and reverse primers, 5' TAACAAATGGATGCTGAAGC 3' and 5' CCATCCCAACATAGTGAGAT 3', were used to amplify a 605 bp fragment of DNA containing part of the *NPK1* transgene; whereas the forward and reverse primers, 5' TAC ATCGAGACAAGCACGGTCAACT 3' and 5' ACGTCATGCCAGT CCCGTG 3', were used to amplify a fragment of 484 bp for the *bar* gene. PCR reactions were carried out in a total volume of 25 µl containing 10 ng of genomic DNA, 1x PCR buffer, 3 mM $MgCl_2$, 0.2 µM of forward and reverse primers, 0.24 mM dNTP, and 1.5 U of Taq polymerase. Amplification was conducted in a T100 thermocycler (BIO-RAD, Singapore) using the following program for the *NPK1* fragment: An initial DNA denaturation for 5 min at 94°C followed by 35 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. For the amplification of *bar* fragment, the annealing temperature was increased to 60°C for 1 min.

Southern blotting

Ten micrograms of genomic DNA from sorghum plants were used for Southern blot analysis. DNA from T_0 and T_1 *Agrobacterium*-derived events and control plants were digested with the restriction enzyme *Eco*RI, at 37°C overnight. Digested DNA was separated on a 0.8% (w:v) agarose gel and transferred by alkaline transfer method onto a positively charged nylon membrane (Boehringer, Mannheim, Germany) and then cross-linked to the membrane by UV irradiation. A fragment (605 bp) of the *NPK1* gene sequence, generated by PCR, was DIG-labeled and used as a probe. The membrane was hybridized overnight at 68°C with the labeled probe. The hybridization signals were detected by the colorimetric method. The labeling and signal detection were performed using the random priming DNA labeling and detection kit (Roche Cat. No.11093657910).

Quantitative Real-Time PCR

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen,

Table 2. Summary of transformation experiments of two sorghum genotypes using pSHX004 harboring the *NPK1* gene.

Genotype	No. of embryos on Co-cultivation	No. of survived embryogenic calli after 8 weeks of selection	No. of regenerated plantlets	No. of plantlets (+PCR)	Transformation frequency (%)
Dorado	535	151[b]	14[a]	8[a]	1.49[a]
SPGM94021	334	125[a]	11[a]	6[a]	1.79[a]

Numbers with the same letters do not differ statistically between themselves at the level of 5% probability.

Hilden, Germany) following manufacturer's instructions. After removing DNA using TURBO DNAse following the manufacturer's instructions (Life Technologies), total RNA samples were quantified using the Qubit RNA assay (Life Technologies). One step-Taqman assay was used to detect the amount of *NPK1* mRNA in the samples. The 18S small-subunit ribosomal RNA gene was used as the endogenous control for RNA quantification. *NPK1* primers and probe were synthesized and labeled by Life Technologies (Carlsbad, CA). Real-time PCR *NPK1* primers and probe sequences were obtained from Shou et al. (2004b) and 18S rRNA TaqMan assay mix was obtained from Life Technologies. Real-time PCR was carried out using the 7500 Fast Real-Time PCR instrument (Life Technologies). Reactions were conducted in a total volume of 20 µl containing 100 ng of sorghum total RNA, 10 µl of 2X master mix from the TaqMan RNA-to-CT 1-step Kit (Life Technologies), 0.4 mM of each primer, 0.2 mM of probe, and 0.5 µl of reverse transcriptase from the same kit. PCR conditions were as follows: Reverse transcription on 48°C for 15 min, heat activation at 95°C for 10 min, followed by 40 amplification cycles (at 94°C for 30 s, annealing at 57°C for 1 min and extension at 72°C for 1 min). All samples were tested in triplicate. To eliminate the possibility of inherent variation in the amount of starting material between samples, the amounts of *NPK1* mRNA detected by real-time RT-PCR were normalized by dividing them with their corresponding amounts of 18S rRNA**.**

Preliminary evaluation of transgenic plants under water-deficit conditions

The performance of the transgenic plants expressing the *NPK1* gene was evaluated under mannitol stress conditions using seeds from the two transgenic lines Dorado and SPGM94021. Drought tolerance assay was applied as described in Kim et al. (2014) with modifications. Briefly, T_1 seeds from each transgenic line and non-transgenic lines were surface-sterilized and germinated for four days in the dark at 25°C on half-strength MS basal medium containing 1% sucrose with or without 5 mg/L bialaphos for transgenic and non-transgenic plants, respectively. Germinated seedlings were then transferred to a half-strength MS containing 400 mM mannitol medium and maintained at 28°C under 16 h photoperiod cool white fluorescent light (75 µmol/m^2/s). These experiments were replicated three times. The response of young seedlings to stress conditions was analyzed after 6 days. The parameters of shoot length, root length and fresh weight were scored for twenty randomly selected bialaphos resistant seedlings and plantlets were transferred to a soil mixture composed of peatmoss: soil: sand (1:1:1) in pots of 10 cm diameters. Plantlets were grown in greenhouse at 28°C and 16/8 h (light/darkness) photoperiod. Plantlets were grown under well-watered conditions of 100% field capacity for the first three weeks. Soil water content was maintained by weighing and adding water into pots, daily. After three weeks of well irrigation, water withholding was applied for 11 to 13 days (depending on the weather) until leaves wilted, at which point the plants were irrigated. Repeated drought cycles were imposed until plants reached anthesis. The yield potential was evaluated by counting the number of kernels per panicle and weighing 100 grains harvested from the transgenic and control plants grown under drought stress conditions in the green house.

Statistical analysis

Three replicates of each experiment were analyzed for significant difference between treatments. For transformation experiments, to eliminate the variation in the initial number of transformed embryos, the percentage values of each replicate were compared in both genotypes. For the preliminary evaluation of T1 plants, the mean values of each parameter from twenty randomly selected plants for each replicate were compared relatively to the other genotype and to the nontransgenic control. Significance was determined by the analysis of variance (ANOVA) employing ASSISTAT software version 7.7 using a complete randomized design with repetition and the difference between the means were compared using Tukey test at a level of 5% probability.

RESULTS

Sorghum transformation and regeneration

Agrobacterium-mediated transformation was utilized to develop transgenic sorghum plants with low transgene copy number. Two sorghum genotypes; Dorado and B-SPGM49021 were transformed with the standard binary vector pSHX004 in LBA4404. The transgene of interest was the kinase domain of the *NPK1* gene driven by a constitutive promoter (35S C4PPDK). The transformation data (Table 2) were obtained from three independent experiments for sorghum genotype Dorado and two independent experiments for genotype SPGM94021.

Most of the infected immature embryos showed normal growth on co-cultivation and resting media, although some embryos failed to grow after transformation. Selection stage was initiated by placing the embryo-derived calli on selection media (callus induction media containing 1.5 mg/l bialaphos). After two months of selection, survived embryogenic calli were used for shoot regeneration (Figure 1). Transgenic plants grew normally in the greenhouse like non-transgenic plants and showed normal fertility. The transformation frequencies based on PCR screening of

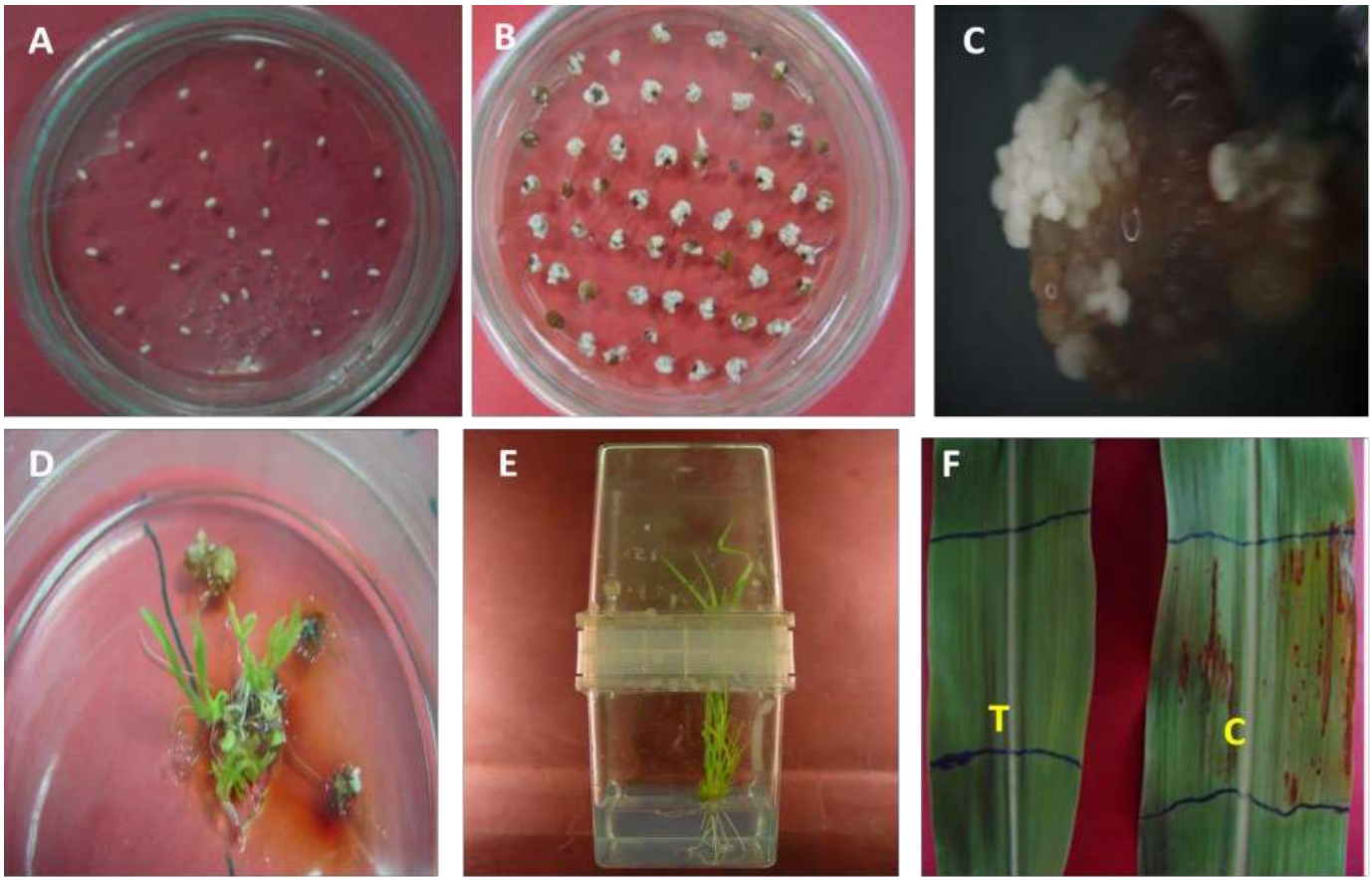

Figure 1. Different stages of transformation. (a) Immature embryos after *Agrobacterium* infection. (b&c) Embryogenic calli on selection medium. (d) Shoots formation on regeneration medium. (e) Putative transgenic sorghum plantlets on rooting medium. (f) Basta herbicide painting of transgenic "T" and non-transgenic control "C" leaves.

the putatively transgenic plantlets were 1.79 and 1.49% for B-SPGM94021 and Dorado, respectively. Transformation frequency was calculated as the number of regenerated PCR-positive plantlets/number of agro-infected embryos × 100. Leaf painting assay indicated improved tolerance of the transgenic plants to Basta herbicide as compared to the non-transgenic plants which showed wilting at the painted area (Figure 1f). Transgenic T_0 plants were harvested and T_1 seeds of both genotypes were grown in the bio-containment greenhouse to test the inheritance of the transgenes.

Analysis of the transgenic plants

PCR analysis (Figure 2) revealed the presence of the expected bands for *bar* and *NPK1* amplicons at 484 and 605 bp, respectively, in 14 out of a total of 25 individual events of the genotypes Dorado and SPGM94021 with no T-DNA truncation. Similarly, PCR analysis was conducted on the T_1 plants. As shown in Figure 2c, bands corresponding to the expected size of 605 bp for the *NPK1* gene were observed in some of the progeny, thus, confirming the inheritance of the transgene.

To confirm the integration of the transgene, Southern blot analysis of T_0 and T_1 transgenic plants for *NPK1* gene was conducted. The results confirmed the stable integration of the *NPK1* gene into the sorghum genome as well as its transmission to the T_1 plants (Figure 3). The estimation of transgene copy number revealed the presence of fewer than five copies of the *NPK1* transgene, e.g. two copies in sample No. 2, four copies in sample No. 8, and one copy in sample No. 9.

Further analysis of the putative transgenic plants using a quantitative real-time RT-PCR analysis indicated all the tested transgenic events expressed the *NPK1* gene, although they had different expression levels. Table 3 summarizes the results of the molecular evaluation of 6 independent transgenic events, 3 belonging to the Dorado genetic background while the other 3 were generated from transforming the SPMG94021 background. All 6 events were positive in PCR evaluation and in all, except for event 14 (not tested),

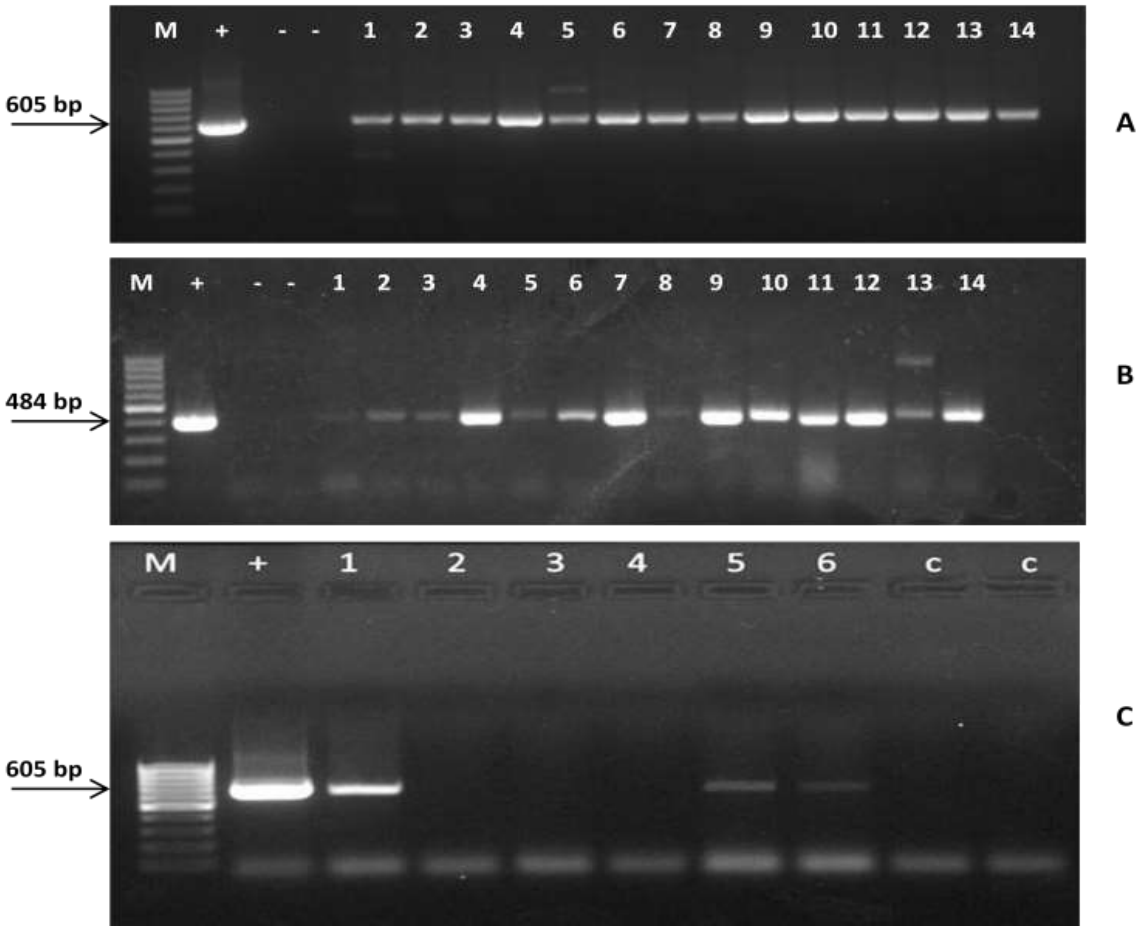

Figure 2. PCR verification of the transgenes in sorghum plants. Verification of *bar* gene (a) and *NPK1* gene (b) into putative transgenic T_0 plants. "M" Molecular weight size marker 100 pb; "-" negative control of un-transformed plant. "+" positive control of the plasmid pSHX004; Lanes 1-16 DNA samples from individual events of sorghum genotypes Dorado (1, 2, 3, 4, 6, 12, 13, 14, and 15) and SPGM94021 (5, 7, 8, 9, 10, 11, and 16). (c) Verification of the *NPK1* gene into transgenic T_1 plants. Lanes 1-6 DNA samples from Dorado (1-3) and SPGM94021 (4-6) T_1 plants.

the band specific for the *NPK1* transgene were detected in Southern blotting. Relative Quantification (RQ) represents the values of accumulated mRNA of the transgene compared to the endogenous gene of the calibrator sample (control non-transgenic) valued as 1.

Evaluation of T_1 plants under drought stress conditions

In the present investigation, transgenic seeds were recovered from both sorghum cultivars, Dorado and SPGM94021. A preliminary kill curve experiment has been carried out with control seedlings of both genotypes on 200, 400 and 600 mM mannitol. Results revealed that 400 mM concentration of mannitol was very critical for control seedlings (Zamzam, 2014). Thus, a culture medium containing 400 mM mannitol was employed to evaluate the performance of T_1 transgenic seedlings expressing *NPK1* gene.

As shown in Table 4 and Figure 4, transgenic plants maintained a relatively higher growth rate than that of the non-transgenic plants, under drought stress conditions. Significant differences in shoot length and fresh weight were found between non-transgenic and their counterparts' transgenic plants expressing the *NPK1* gene. However, the reduction in root lengths was not significant at 5% probability. In the greenhouse, three-week-old control and transgenic plants were subjected to water withholding. Normally, under our greenhouse conditions, the non-transgenic sorghum plants are usually irrigated every 6 to 7 days. In this experiment, watering was stopped for 11 to 13 days. The transgenic plants displayed a higher capacity of recovering after re-irrigation compared to the non-transgenic plants. Moreover, the weight of 100 grains

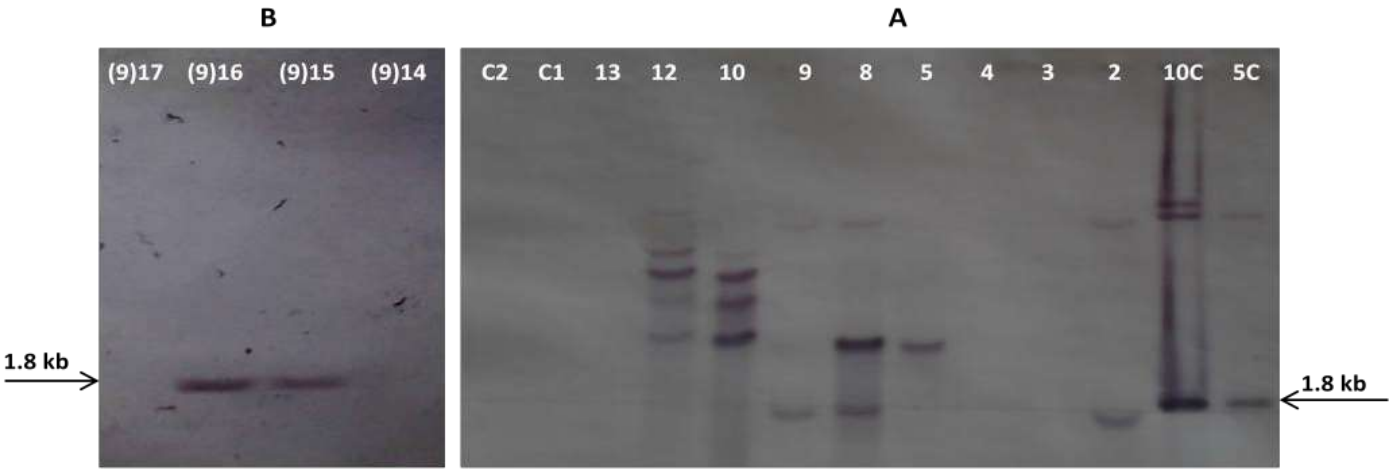

Figure 3. Southern blot analysis of transgenic sorghum plants. (a) T_0 plants probed with PCR product of the *NPK1* gene: Lanes 5C & 10C, five and ten copies of the plasmid pSHX004; lanes C1 & C2, negative control of untransformed Dorado and SPGM94021 plants, respectively; lanes 2, 3, 4, 12 and 13, samples of putative transgenic T_0 events of the genotype Dorado; lanes 5, 8, 9 and 10, samples of putative transgenic T_0 events of the genotype SPGM94021. (b) T_1 plants probed with PCR product of the *NPK1* gene: Lanes (9)14 - (9)17, four plants from the T_1 progeny of SPGM94021 transgenic event No. 9.

Table 3. Summary of the molecular evaluation of some *NPK1* transgenic events.

Genotype	Code	RQ	PCR	Southern blot
Dorado	2	0.022	+	+
Dorado	12	1.608	+	+
Dorado	14	2.019	+	Not tested
SPGM94021	9	2.324	+	+
SPGM94021	8	0.102	+	+
SPGM94021	5	0.523	+	+

Table 4. Effect of drought stress on the growth of seedlings and the yield potential of *NPK1* expressing transgenic sorghum.

Genotype	Fresh weight (g)	Shoot length (cm)	Root length (cm)	Weight of 100 grains (g)	No of grains per panicle
Dorado (NPK1)	0.18^{a}	9.40^{a}	10^{a}	2.21^{a}	1098.33^{a}
Dorado (control)	0.12^{b}	6.63^{ab}	8.76^{a}	1.62^{bc}	1076.66^{a}
SPGM94021 (NPK1)	0.14^{ab}	8.46^{ab}	8.83^{a}	1.97^{ab}	973.33^{a}
SPGM94021 (control)	0.10^{b}	5.81^{b}	7.1^{a}	1.22^{c}	970.00^{a}

Numbers with the same letters do not differ statistically between themselves at the level of 5% probability. Data of fresh weight, shoot length and root length were collected 10 days after seed germination (4 days on bialaphos and 6 days on mannitol stress).

and the number of grains per panicle were used as physiological parameters for yield potential under water-deficit conditions. Kernels were collected from both transgenic plants and few recovered control plants after repeated exposure to drought stress under greenhouse conditions. The transgenic sorghum plants had a significantly higher mean of grain weight, under drought stress conditions, than the control plants.

DISCUSSION

Regulatory genes are very important research interest. Transcription factors represent an important category of regulatory genes. Many genes involved in stress response can be simultaneously regulated by a single gene encoding stress inducible transcription factor (Kasuga et al., 1999). While the expression of *ZmDof1* transcription

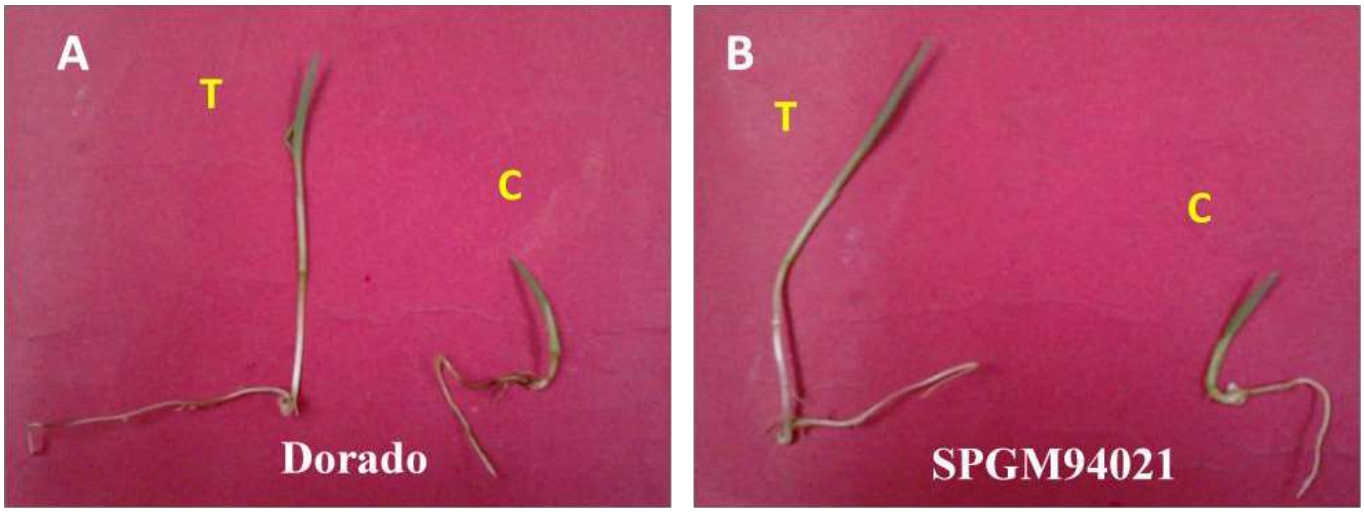

Figure 4. The effect of mannitol osmotic stress on the growth of sorghum seedling. (a) Dorado and (b) SPHM94021: transgenic "T" versus control "C".

factor in sorghum and wheat has activated the primary target, phospho*enol*pyruvate carboxylase (PEPC), which leads to the down-regulation of genes involved in photosynthesis and the functional apparatus of chloroplasts, and negatively impacted photosynthesis, and biomass (Peña et al., 2017). These results indicated that transcription factor strategies for crop improvement need to consider the downstream targets of the genetic elements to be introduced. Another category of regulatory genetic factors is stress signaling genes. Components of one signal transduction pathway may be shared by various stresses such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki, 1999). Moreover, manipulation of signaling factors can control a broad range of downstream targets and result in superior tolerance to multiple stresses (Umezawa et al., 2006). Mitogen activated protein kinase (MAPK) cascade is an evolutionarily conserved signal transduction module involved in plant abiotic stress tolerance (Xiong and Zhu, 2001). The *NPK1* gene, a tobacco mitogen-activated protein kinase kinase kinase (MAPKKK), activated an oxidative signaling cascade and resulted in cold, heat, salinity and drought tolerant transgenic plants (Kovtun et al., 2000; Shou et al., 2004a).

Sorghum transformation and regeneration

To improve drought tolerance and validate the hypothesis that expression of *NPK1* gene in sorghum would improve drought tolerance in sorghum, two genotypes: Dorado and SPGM94021 were transformed with the pSHX004 construct. To the best of our knowledge, this is the first report of transforming sorghum plants with the *NPK1* gene. To select the transformed calli, the herbicide-resistant gene, *bar* (Thompson et al., 1987) was employed as a selectable marker. Selection of bialaphos-resistant callus is often conducted at 2 to 3 mg/L bialaphos (Casas et al., 1993; Frame et al., 2002; Grootboom et al., 2010). Bialaphos, as selection agent, has been found to hinder regeneration capacity of sorghum (Casas et al., 1993; Gao et al., 2005a; Lu et al., 2009; Grootboom et al., 2010). In the present investigation, bialaphos imposed a severe selection on sorghum callus. To sustain callus regenerability, bialaphos concentration was not increased beyond 1.5 mg/L. This is because non-transgenic plantlets may escape the *bar* selection system (Gao et al., 2005b).

Analysis of the transgenic plants

Further screening was conducted through PCR analysis, the transformation frequency was calculated and found to be within the cited range (Emani et al., 2002; Gao et al., 2005b; Zhao et al., 2000; Howe et al., 2006) from 0.2 to 4.5%, even though these reports were on highly regenerable and transformable genotypes, e.g. P898012 and TX430. The low transformation frequency may attribute to the detrimental effects of the *NPK1* gene. In this respect, Kovtun et al. (1998) reported that overexpression of *NPK1* gene causes detrimental effects on embryogenesis. Moreover, the constitutive expression of a calcium dependent protein kinases, *Os*CDPK-7, in sorghum induced apoptotic cell death in transgenic leaves (Mall et al., 2011). Further supporting this hypothesis is that the regeneration frequency for these genotypes is as high as 22 and 16% for Dorado and SPGM94021, respectively (Assem et al., 2014). Also, the *Agrobacterium*-mediated transformation

frequency of Dorado with similar construct, pTF102, was as high as 3.6% (Zamzam, 2014). In this respect, slight reduction in the transformation frequency with the pSHX004 construct compared to pTF102 was also reported in maize (Shou et al., 2004b). Taken together, the reduction in transformation frequency with pSHX004 construct in this study may be attributed to the death of the high *NPK1* expressers during the different transformation stages.

To further confirm the transgenic plants, Southern blotting was employed. Digesting the pSHX004 plasmid with the restriction enzyme *Eco*RI would liberate the *NPK1* gene from the T-DNA giving one band at the size of 1.8 Kb. Different Southern hybridization patterns seen with *Eco*RI-digested DNA implied that the transgenic lines resulted from independent transformation events. The number of hybridization bands ranged from one to four. The presence of more than one band and of bands larger than 1.8 Kb may be attributed to the occurrence of rearrangement or to duplication or amplification of transgenes. Duplication or amplification of the transgene was reported by Spencer et al. (1992) in maize and Cannell et al. (1999) in wheat. The transgene copy number in the recipient genome is an important determinant of the transgene expression and field performance of the transgenic plants. *Agrobacterium* is a potent transformation tool with the advantage of producing transgenic plants with low transgene copy number. In the present study, the low transgene copy number is consistent with other investigations using *Agrobacterium* on cereals (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Zhao et al., 2000; Gao et al., 2005a; Lu et al., 2009).

The expression of the *NPK1* transgene is the key of this investigation. Suppression and overexpression of *NPK1* in tobacco have resulted in some detrimental effects on cell division, embryogenesis, and seed development (Kovtun et al., 1998). In this study, although no abnormal transgenic plants were observed, the transformation efficiency was rather low. It is likely that only transgenic sorghum events with relatively low expression of the transgene were generated in this study. Although, quantitative real time PCR analysis revealed high expression of the *NPK1* transgene in some T_0 plants, that is, events No. 9, 12 and 14, low expression was found in other events (event No. 2 and 8). Transgenic event number 2 was almost silenced and it is likely that the T-DNA was inserted in a heterochromatin region of the chromosome and had low access to the RNA polymerase. In this respect, gene silencing has been previously reported in transgenic sorghum (Casas et al., 1993, 1997; Zhu et al., 1998; Able et al., 2001; Emani et al., 2002). Events 9 and 12 will be the main candidates for carrying out further investigation of the effects of overexpressing the *NPK1* gene on field performance. On the other hand, examining the field performance of event 2 may shed the light on the effects of attenuating the expression of this gene.

Evaluation of T_1 plants under drought stress conditions

The working hypothesis of this study was that expression of *NPK1* gene in sorghum would improve drought stress tolerance. To investigate this hypothesis, transgenic plants were evaluated for growth and yield potential under drought stress. Mannitol has been used to evaluate plant tolerance to drought stress. Mannitol affects the availability of water to plants by increasing the osmotic pressure outside the cells and causes plant symptoms like that of water-deficit (Rumpho et al., 1983). In this investigation, the relative reduction in the growth parameters: fresh weight and shoot length of the non-transgenic seedlings, under mannitol stress, suggests that the presence of the *NPK1* gene in transgenic sorghum plants aids in increasing the *in vitro* osmotic stress tolerance. The relative improvement in the growth of the transgenic plants, under mannitol stress, may be explained by the reduced sensitivity of transgenic cells to stress conditions. In this respect, Kovtun et al. (2000) reported that the ANP1, a class of MAPKKKs from *Arabidopsis* can be induced specifically by H_2O_2 and can activate a specific class of stress-induced MAPKs. The activated MAPK cascade activates stress-response genes that protect plants from diverse environmental stresses.

Moreover, Shou et al. (2004a) reported that the expression of *NPK1* in transgenic maize enhanced drought tolerance, suggesting that *NPK1* induced a mechanism that protected plants from dehydration damages. Similar results were, also, described, in Maize, by Muoma and Ombori (2014).

Over-expression of *NPK1* was found to impose detrimental effects on seed development in tobacco and the number of the defective seeds correlated with the level of the transgene expression, suggesting that seed phenotype was due to transgene expression and enhanced NPK1-dependent MAPK activity (Kovtun et al., 1998). In this study, we did not notice any reduction in seed germination or defect in seed development. Contrarily, under drought conditions, the transgenic plants had a higher means for grain weight despite the provision of equal amount of water. The relatively low expression of the NPK1 in this study may explain the normal development of seeds. The increased weight of transgenic kernels under drought condition in this study is consistent with the results of Shou et al. (2004a). Taken together, the results on the mannitol tolerance and the yield potential of transgenic sorghum plants expressing *NPK1*, demonstrate 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 sorghum inbred lines for osmotic tolerance.

CONFLICT OF INTERESTS

The authors have not declared any conflict interests.

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