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RESEARCH PAPER

Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize

Huixia Shou*, Patricia Bordallo and Kan Wang†

Plant Transformation Facility, Department of Agronomy, Iowa State University, Ames, IA 50011-1010, USA

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Abstract

Drought is one of the most important abiotic stresses affecting the productivity of maize. Previous studies have shown that expression of a mitogen-activated protein kinase kinase kinase (MAPKKK) gene activated an oxidative signal cascade and led to the tolerance of freezing, heat, and salinity stress in transgenic tobacco. To analyse the role of activation of oxidative stress signalling in improving drought tolerance in major crops, a tobacco MAPKKK (NPK1) was expressed constitutively in maize. Results show that NPK1 expression enhanced drought tolerance in transgenic maize. Under drought conditions, transgenic maize plants maintained significantly higher photosynthesis rates than did the non-transgenic control, suggesting that NPK1 induced a mechanism that protected photosynthesis machinery from dehydration damage. In addition, drought-stressed transgenic plants produced kernels with weights similar to those under well-watered conditions, while kernel weights of drought-stressed non-transgenic control plants were significantly reduced when compared with their non-stressed counterparts.

Key words: Drought tolerance, *Nicotiana* protein kinase, photosynthesis rate, transgenic maize, *Zea mays*.

Introduction

Drought is one of the most important abiotic stresses affecting crop productivity (Boyer, 1982). It causes 24 million tons of yield loss in maize annually (Heisey and Edmeades, 1999). Drought reduces productivity by inhibiting plant growth and photosynthesis (Taiz and Zeiger, 1998). Reduction in photosynthesis rate under drought conditions is caused by two factors, water deficit and mesophyll cell dehydration. Water deficit causes stomatal closure and thereby decreases intercellular CO_2 concentrations, whereas dehydration of the mesophyll cells damages the photosynthetic machinery (Taiz and Zeiger, 1998). The relationship between photosynthesis rate and crop yield is not always coupled because crop yield is also affected by assimilate partitioning and utilization (Guo *et al*., 2002). However, the positive correlation between them has been reported in several studies (Chandra Babu *et al*., 1985; Fischer *et al*., 1998; Pettigrew and Meredith, 1994; Pooter and Remkes, 1990; Zelitch, 1982).

Under stress conditions, plants generate reactive oxygen species, including hydrogen peroxide (H_2O_2), the superoxide anion, and hydroxyl radicals (Inzé and Van Montagu, 1995). Accumulation of H_2O_2 can, in turn, induce the expression of detoxification and stress protection genes such as heat shock proteins (HSPs), glutathione-*S*-transferases (GSTs), peroxidases, superoxide, and pathogenesis-related proteins, thus protecting plants from stress damages (Kovtun *et al*., 2000). HSPs have been reported to serve as molecular chaperones that participate in ATP-dependent protein unfolding or assembly/disassembly reactions and prevent protein denaturation during stress (Pelham, 1986). Correlations between expression of HSPs and thermotolerance have been found in maize, tomato, and creeping bentgrass (Park *et al*., 1996; Preczewski *et al*., 2000; Ristic *et al*., 1998). GSTs are enzymes that detoxify endobiotic and xenobiotic compounds by covalent linkage of glutathione to hydrophobic substrates. Accumulation of both proteins can reduce damage caused by chilling, heat, and drought, and

* Present address: College of Life Sciences, Zhejiang University, Hangzhou, 310029, China.
† To whom correspondence should be addressed. Fax: +1 515 294 2299. E-mail: kanwang@iastate.edu
Abbreviations: AP, apparent photosynthesis; GST, glutathione *S*-transferase; HSP, heat shock protein; MAPK, mitogen-activated protein kinase; NPK1, *Nicotiana* protein kinase.

protect plants from environmental stresses (Li *et al*., 2003; Ristic *et al*., 1998; Roxas *et al*., 1997; Sabehat *et al*., 1998).

In this research, a cDNA fragment was expressed that encodes the kinase domain of *Nicotiana* protein kinase 1 (*NPK1*) in maize under the control of a constitutive promoter. NPK1 is a tobacco mitogen-activated protein kinase kinase kinase (MAPKKK). The catalytic domain of NPK1 specifically activates a *BCK1* (bypass of C kinase)-mediated signal transduction pathway in yeast, indicating that the catalytic function of NPK1 is conserved among different organisms (Banno *et al*., 1993). NPK1 is located upstream of the oxidative pathway and can induce expression of HSPs and GST1 in *Arabidopsis* and maize (Kovtun *et al*., 2000). Activation of these stress genes could protect the photosynthesis machinery of plants from damage caused by drought, thereby improving the yield potential of the major cereal crop, maize.

Materials and methods

Maize transformation

Transgenic maize plants were recovered from both *Agrobacterium*-mediated and particle bombardment transformation methods (Plant Transformation Facility, Iowa State University). Plasmids pSHX002 (Fig. 1A) and pBAR184 (Fig. 1B) were used for co-bombardment transformation. pSHX002 was derived from pBluescript and contains a transgene cassette carrying a modified CaMV 35S promoter (35SC4PPDK), an 800 bp DNA fragment encoding the kinase domain of *Nicotiana* protein kinase (NPK1) (Sheen, 1993), and a nopaline synthase terminator (Depicker *et al*., 1982). The construct pBAR184 (Frame *et al*., 2000) consists of the maize ubiquitin promoter with its intron (Christensen and Quail, 1996) driving the *Streptomyces hygroscopicus* phosphinothricin transferase gene (*bar*; Thompson *et al*., 1987) with the *Agrobacterium tumefaciens nos* terminator. *Agrobacterium* strain EHA101 harbouring the binary vector pSHX004 (Fig. 1C) was used to transform Hi II maize immature embryos as described (Frame *et al*., 2002). The *NPK1* transgene cassette in pSHX004 was identical to that in pSHX002. The selectable marker cassette in pSHX004 contained a 2× CaMV 35S promoter (Odell *et al*., 1985), a tobacco etch virus 5′ untranslated region (TEV; Carrington and Freed, 1990), the *bar* gene, and a soybean vegetative storage protein terminator (Tvsp; Mason *et al*., 1993). Transgenic events achieved by particle bombardment and *Agrobacterium*-mediated transformation were designated as P84 and A4, respectively. Regenerated transgenic plants (R_0) were crossed with either the hybrid Hi II or the inbred line B73 to produce R_1 seeds.

Southern blot and real-time RT-PCR analyses

Leaf genomic DNA was extracted from fresh maize leaf tissue using the cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson, 1980). Ten micrograms of genomic DNA from maize plants were used for Southern blot analysis. *Agrobacterium*-derived A4 events (construct pSHX004) and bombardment-derived P84 events (construct pSHX002) were digested with restriction enzymes *Stu*I and *Eco*RI, respectively, at 37 °C overnight. Digested DNA was separated on a 0.8% (w:v) agarose gel. A 1.8 kb DNA fragment containing the *NPK1* gene and its promoter and terminator (NPK probe 1 in Fig. 1A) was used as a hybridization probe for the P84 events and a 0.8 kb DNA fragment of the coding region of *NPK1* gene (NPK probe 2 in Fig. 1C) was used for the A4 events.

Real-time RT-PCR was conducted to detect the levels of the *NPK1* transcript as described (Shou *et al*., 2004*a*). Briefly, RNA extraction, DNase treatments, and cDNA synthesis were carried out using TRIzol reagent, deoxyribonuclease I, and SUPERSCRIPT™ RNase H-Reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. A Taqman assay was used to detect the amount of *NPK1* cDNA in samples. The assay was conducted by using the iCycler iQ Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's recommendations. All oligonucleotides, including primers and probes were designed using the DNA mfold server of Dr Michael Zuker (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) and Primer3 software (Whitehead

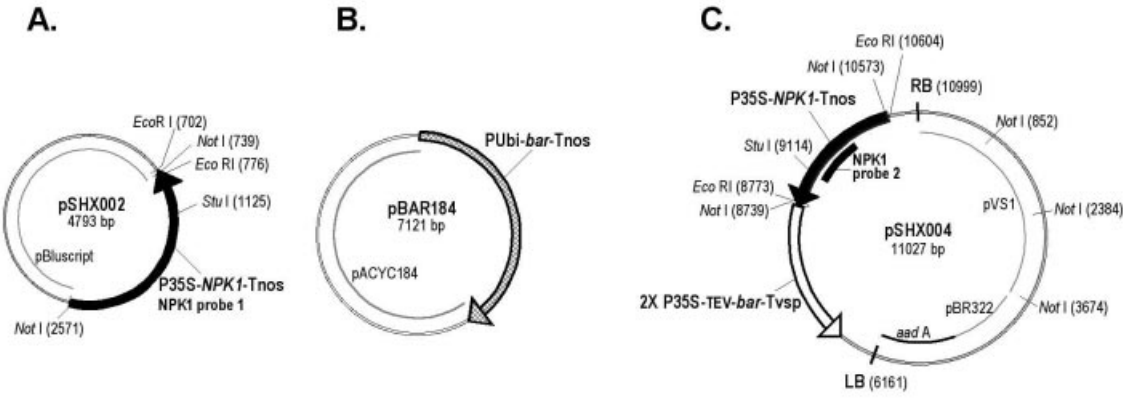

Fig. 1. Constructs for maize transformation. (A) pSHX002 and (B) pBAR184 constructs used for co-bombardment transformation. (C) pSHX004, binary vector construct for *Agrobacterium*-mediated transformation; P35S-*NPK1*-Tnos, a transgene cassette containing a modified 35S promoter, the *NPK1* gene, and the *nos* terminator; PUbi-*bar*-Tnos, a selectable marker cassette containing a maize ubiquitin promoter with its intron, the *bar* gene, and the *nos* terminator; 2× P35S-TEV-*bar*-Tvsp, a selectable marker cassette containing a 2× CaMV 35S promoter, a tobacco etch virus 5′ untranslated region (TEV), the *bar* gene, and a soybean vegetative storage protein terminator (Tvsp); pBluescript and pACYC184, vector backbones; pBR322 and pVS1, origins of replication for *E. coli* and *Agrobacterium*, respectively; *aad*A, a spectinomycin-resistant marker gene for bacterial selection; LB, left border, coordinates 6161–6186; RB, right border, coordinates 10 999–11 024; E, *Eco*RI; N, *Not*I; S, *Stu*I; NPK1 probe 1, 1.8 kb fragment containing the *NPK1* gene with its promoter and terminator; NPK1 probe 2, 0.8 kb fragment containing the *NPK1* gene.

Institute for Biomedical Research in Cambridge, MA, USA). The 18S small-subunit ribosomal RNA gene (GenBank accession number AF168884) served as the endogenous control for RNA quantification. The internal oligonucleotide probes specific for the *NPK1* and 18S rRNA genes were labelled at the 5′ end with FAM and at the 3′ end with BlackHole Quencher-1 or at the 5′end with TAMRA and at the 3′ end with BlackHole Quencher-2, respectively. All primers and probes were synthesized and labelled by Integrated DNA Technologies (Coralville, IA, USA). Serial dilutions of plasmid DNAs from pSHX004 (Fig. 1C), containing the *NPK1* gene and pSHX007 (Shou *et al*., 2002), containing the 18S rRNA gene fragment, were used to generate standard curves for the quantification of *NPK1* and 18S RNA cDNA. The amounts of *NPK1* cDNA detected by the real-time RT-PCR were normalized by dividing them by the corresponding amount of the 18S RNA gene.

PCR screen for transgenic plants

Transgenic plants were selected using PCR analysis. Genomic DNA was extracted from seedling leaf discs using the CTAB protocol (Murray and Thompson, 1980). Primers of GGCTGCAGGAATTC-TCACATGT and GCTCCCGAAGTCATTCTGCA were used to amplify a 646 bp fragment of DNA containing part of the transgene and the *nos* terminator. PCR reactions were conducted in a total volume of 20 µl containing 50–100 ng of genomic DNA, 1× PCR buffer, 3 mM $MgCl_2$, 0.2 µM of forward and reverse primers, 0.2 mM dNTP, and 1.5 U of *Taq* polymerase. The PCR reaction included 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.

Drought-stress tests

The field capacity (FC) of soil was measured as followed. A 9.0 l pot with four drainage holes was filled with soil and set in 20 cm standing water overnight. The water-saturated pots then were covered to avoid water evaporation and drained for 4 h. The water content of this treated soil was defined as 100% FC (Samarah, 2000).

All drought-stress experiments were conducted in a greenhouse with 16:8 h photoperiod and temperatures of 28 °C during day time and 21 °C at night. R_1 seeds from *Agrobacterium*-derived events A4-1, A4-2, and A4-9, and R_2 seeds from bombardment-derived event P84-12 were germinated in Universal Mix soil (Hummert™ International, St Louis, USA) and screened for the presence of the transgene by PCR analysis. Ten uniform transgenic maize seedlings from each of the above transgenic events along with ten non-transgenic Hi II control plants were selected and transplanted into 9.0 l, preweighed nursery pots containing the same type of soil. The plants were then divided into two groups. One group of plants was exposed to drought stress (DS) conditions in which the soil water content was kept constant at 25% of FC, while plants in the other group were maintained under well-watered (WW) conditions in which soil FC was kept at 100%. Soil water content was maintained accordingly by weighing and adding water into individual pots daily. Treatments were initiated at the four-leaf stage (Ritchie and Hanway, 1992) and maintained until plants reached maturity.

Apparent Photosynthesis rate

Leaf Apparent Photosynthesis (AP) rate of plants in the drought-stress experiment was measured at midday on the third leaf from the top of the canopy using a Li-Cor 6200 Photosynthesis System (Li-Cor, Inc., Lincoln, NE, USA). Measurements were taken on days 17, 24, 31, and 38 after the initiation of drought treatments. Five plants from each population treated under either WW or DS conditions were measured.

Agronomic traits of treated plants

Plant height and leaf numbers of maize plants were measured at the tasselling stage. Seed number and weight from each of the treated plants were measured after harvesting.

Statistical analysis

Statistical analyses of AP rates and leaf numbers were carried out using an SAS program (SAS Institute Inc., Cary, NC, USA).

Results and discussion

Production of NPK1 *transgenic maize*

Transgenic maize plants were achieved by particle bombardment or *Agrobacterium*-mediated transformation using plasmid pSHX002 (Fig. 1A) or binary vector pSHX004 (Fig. 1C), respectively. The transgene of interest in both constructs was the kinase domain of the *NPK1* gene driven by a constitutive promoter 35SC4PPDK (Sheen, 1993). Southern blot analysis confirmed the presence of the *NPK1* gene in 24 A4 events and 12 P84 events (Shou *et al*., 2004*b*). Transgenic events A4-1, A4-2, A4-9, and P84-12 were selected for the drought tolerance test. The *Agrobacterium*-derived events contained fewer than five copies of the *NPK1* transgene (e.g. A4-1, two copies, A4-2, three copies, and A4-9, four copies), whereas the bombardment-derived event P84-12 carried more than 20 copies of the transgene (Shou *et al*., 2004*b*).

Expression level of the *NPK1* gene was determined by means of real-time RT-PCR analysis. Significant differences at the level of transgene expression were found among different transgenic events (P <0.0001). The relative amounts of *NPK1* transcript for events used in the drought stress test is listed in Table 1. Among the four transgenic events, A4-9 had the highest *NPK1* gene expression. Transgene expression in events A4-1 and A4-2 ranked at the medium level and event P84-12 had the lowest level of transgene expression. The transcript levels detected by real-time RT-PCR were confirmed by northern blot analysis (Shou *et al*., 2004*b*). The lowest expressor, event P84-12, had more transgene copies than the other three events. This observation supports the argument that multiple copies of a transgene may lead to co-suppression and silencing (Dai *et al*., 2001; Fagard and Vaucheret, 2000).

Plant height and leaf number of R_1 plants from 22 transgenic events were evaluated under field growth conditions. For the traits evaluated, no significant difference was measured between transgenic and non-transgenic control plants (Shou *et al*., 2004*a*), indicating that expression of the *NPK1* gene had no negative effect on the growth of maize plants. NPK1 is a member of MAPKKK family that plays critical roles in cytokinesis, nuclear localization, auxin signalling transduction, and the oxidative stress signalling pathway (Ishikawa *et al*., 2002; Kovtun *et al*., 1998, 2000). Suppression and overexpression of NPK1 in

Table 1. *Effect of drought on Apparent Photosynthesis (AP) rate (μmol m^{-2} s^{-1}) of transgenic and non-transgenic maize plants*

Events	RNA levels of the transgene (mean ±SD)	AP rate (mean ±SD)[a]		AP rate decrease (%)
		Well-watered treatment	Drought treatment	
Transgenic event				
A4-1	0.12±0.03	17.56±3.9 ab	14.22±6.1 abc	19.0%
A4-2	0.20±0.06	17.15±4.4 ab	14.79±5.3 ab	13.8%
A4-9	0.54±0.02	18.02±6.6 a	17.07±6.2 ab	5.3%
P84-12	0.03±0.02	17.27±6.8 ab	13.05±6.1 bc	24.4%
Non-transgenic control				
Hi II	0	17.97±2.4 a	9.53±4.9 c	47.0%

[a] From a total of 20 measurements. Means with the same letter are not significantly different (P <0.05).

tobacco have resulted in some detrimental effects on cell division, embryogenesis, and seed development (Kovtun *et al*., 1998). In this maize study, although no abnormal transgenic plants were observed, the transformation efficiency for both *Agrobacterium*-mediated and particle bombardment transformations were reduced (about 30%) compared with other constructs with the same vector backbone (H Shou, unpublished results). The reduced transformation efficiency could be due to death of the high NPK1 expressors during the transformation process. It is likely that the transgenic maize events generated in this experiment had relatively low expression of the transgene.

Effect of NPK1 *gene expression on Apparent Photosynthetic (AP) rate under drought stress*

To evaluate the drought tolerance performance of *NPK1* transgenic maize, four transgenic events A4-1, A4-2, A4-9, and P84-12 and the Hi II negative control were included in the drought test. Events A4-1 and A4-2 represented the medium level of *NPK1* gene expression. A4-9 and P84-12 were the representatives of high and low expressors, respectively (Table 1). Plants exposed to drought stress were compared with those under well-watered conditions for morphological traits and photosynthesis rate. Non-transgenic Hi II plants were also included as negative controls under both treatments.

Under well-watered conditions no significant difference in AP rate between the transgenic plants and non-transgenic Hi II control was observed (Table 1). The average AP rate ranged from 17.15–18.02 μmol m^{-2} s^{-1} in all plants. Reduced AP rates were observed in all plants subjected to drought stress (with water supply at 25% of FC). In the four transgenic lines, the reduction in AP rates ranged from 5.3% to 24.4% and was not significantly different from their well-watered counterparts. The non-transgenic control Hi II, on the other hand, had a drastic reduction (47%) in AP rate that was significantly lower than the well-watered Hi II plants. The AP rates of transgenic events A4-2, and A4-9 were significantly higher than that of Hi II under drought conditions. The other transgenic lines, A4-1 and P84-12 had higher AP rates than the control, but did not reach a significant level. As can be seen in Table 1, the photosynthetic performance of the transgenic lines and their relative amounts of transgene transcripts are correlated. Transgenic event A4-9, that had the highest AP rate under drought conditions, also had the highest RNA level of the transgene. The AP rate of the lowest expressor P84-12, on the other hand, did not differ significantly from that of Hi II under drought conditions. The decrease of the AP rate under drought conditions was negatively correlated with *NPK1* transcript level (R^2=0.94). These results suggest that constitutive expression of the *NPK1* gene in maize protected the photosynthetic machinery of the plant under drought stress.

Using microarray analysis in a separate study (Shou *et al*., 2004*a*), up-regulation of a small HSP gene by 2.6-fold and 6.85-fold was detected in the transgenic maize events A4-1 and A4-9, respectively, compared with their non-transgenic segregants. The evidence that constitutive expression of NPK1 induced expression of the small HSP protein in transgenic maize is particularly interesting, because increasing amounts of data have shown that HSPs plays an important role in thermotolerance (Maestri *et al*., 2002). Small organellar HSPs associate with membranes under heat stress and protect photosynthetic electron transport (Debel *et al*., 1995). A small chloroplast HSP has been reported to protect thermolabile photosystem II and whole-chain electron transport during heat stress (Heckathorn *et al*., 1998). These experimental results show that transgenic maize expressing NPK1 maintained a higher photosynthesis rate than the non-transgenic control under drought conditions. The elevated expression of the HSP gene observed in these NPK1 transgenic lines may be involved in protecting photosystems under the imposed drought stress.

Effect of NPK1 *gene expression on agronomic traits under drought stress*

Drought stress delayed maturation of the maize plants in this study. However, the effect of drought stress on maturation in transgenic plants differed from that of the negative control. The average days to maturity were

extended by 14 d for transgenic events and 11 d for Hi II negative control. The compensation mechanism by which plants adjusted to water stress may have been attributed to the prolonged days to maturity. Under drought condition, NPK1 transgenic plants extended three more days to maturity compared with the non-transgenic control plants. It is possible that the transgene expression has increased the adaptation ability in transgenic maize plants although the mechanism is yet to be understood.

It was also observed that, under drought stress, the final leaf number of transgenic maize increased whereas that of the non-transgenic control (Hi II) remained constant (Table 2). NPK1 transgenic plants produced an average of 19.6 leaves under drought conditions, which were 0.9 leaves more than they had under well-watered conditions. This increase of leaf number under drought conditions was statistically significant in transgenic event A4-2, while there was no significant difference in the other three transgenic events. Final leaf number is a trait that is highly genetically controlled; rarely changing under different environmental conditions. Photoperiod can affect the final leaf number in wheat because the days to maturity were affected by photoperiod (Pararajasingham and Hunt, 1996). The observed phenomenon that NPK1 maize lines had increased leaf number under drought stress, may be attributed to the delayed maturation of these transgenic plants.

Effect of NPK1 *gene expression on yield components under drought stress*

Kernel weight of both transgenic and non-transgenic plants was decreased by the drought treatment except for event A4-1, whose kernel weight remained the same as its well-watered counterpart (Table 3; Fig. 2). The kernel weight of Hi II non-transgenic plants had the largest decrease, 44.4%, whereas kernel weight decrease in the three transgenic lines A4-2, A4-9, and P84-12 were 17.24%, 22.22%, and 34.29%, respectively. A slight increase in the kernel weight of event A4-1 was observed. Interestingly, the drought-treated plants set similar numbers or more kernels than the well-watered plants in control and transgenic plants, respectively (Table 3). This may be due to the fact that these plants were hand-pollinated using pollen from unstressed plants.

Because factors other than leaf photosynthesis rate, such as leaf area and the allocation of photoassimilate, also affect final yield, the correlation between yield and photosynthesis rate is not always consistent. The results shown here, 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, suggest that the NPK1 transgenic maize may have higher yield potential than that of the non-transgenic plants under drought conditions. The fact that drought-stressed plants set a similar or higher number of seeds than the non-stressed transgenic and non-transgenic control plants could be attributed to their pollen sources. The asynchrony between male and female flowering caused by drought stress made self-pollination in these drought-treated plants almost impossible. To ensure the seed set, pollen from non-stressed maize plants was used to pollinate the stressed plants. This practice may have masked the effect of drought damage on the seed set. The hybrid background of transgenic plants may further complicate the assessment. The maize genotype used for this transgenic work was Hi II, an A188×B73 hybrid that is amenable to tissue culture and transformation (Armstrong *et al*., 1991). The segregation of genetic elements involved in yield potential or stress-tolerance may influence their performance. With this in mind, in this study, the comparison was made not only between transgenic and non-transgenic plants in the drought treatment, but also between drought-stressed plants and their non-stressed siblings in the well-watered treatment.

In response to different stresses, plants accumulate active oxygen species (AOS) including hydrogen peroxide (H_2O_2). While large amounts of H_2O_2 accumulation lead inevitably to programmed cell death, relatively small amounts of H_2O_2 modify gene expression in ways that enhance plant defence responses (Pastori and Foyer, 2002). Kovtun *et al*. (2000) showed that constitutive expression of an *NPK1* orthologue, an *Arabidopsis* MAP kinase kinase

Table 2. *Effect of drought on final leaf number of transgenic and non-transgenic maize*

Events	Total leaf number (mean ±SD)[a]		Leaf number increase (%)
	Well-watered	Drought	
Transgenic event			
A4-1	18.2±0.8 c	18.8±1.3 bc	3.3
A4-2	18.8±0.8 bc	20.6±1.1 a	9.6
A4-9	18.4±1.1 c	19.0±0.7 bc	3.3
P84-12	19.2±0.5 bc	19.8±0.8 ab	3.1
Non-transgenic check			
Hi II	18.4±1.3 c	18.0±1.0 c	–2.2

[a] Means with the same letter are not significantly different (P <0.05).

Table 3. *Effect of drought on morphological traits of trangenic and non-transgenic maize*

Events	Kernel number (mean ±SD)		Kernel number increase (%)	Kernel weight (g) (mean ±SD)		Kernel weight decrease (%)
	Well-watered	Drought		Well-watered	Drought	
Transgenic event						
A4-1	134.0±41.3	140.3±34.4	4.7	0.22±0.04	0.23±0.07	–0.05
A4-2	99.3±38.9	118.0±20.1	18.8	0.29±0.10	0.24±0.03	17.24
A4-9	131.5±12.9	188.5±50.2	43.3	0.27±0.00	0.21±0.07	22.22
P84-12	93.0±5.8	162.0±42.3	74.2	0.35±0.05	0.23±0.01	34.29
Non-transgenic control						
Hi II	170.5±33.1	172.3±33.1	1.1	0.27±0.03	0.15±0.05	44.44

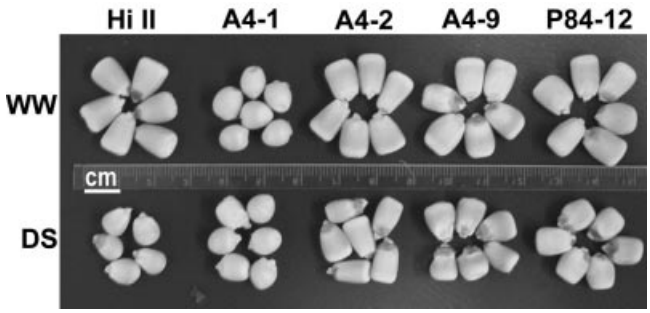

Fig. 2. Kernel appearance of transgenic (A4-1, A4-2, A4-9, P84-12) and non-transgenic (Hi II) plants under well-watered (WW) and drought-stressed (DS) conditions.

kinase (ANP1), mimicked the H_2O_2 signal to activate the oxidative signalling cascade. As with H_2O_2, overexpression of ANP1 induced the expression of many other genes, including a small heat shock protein (HSP18.2) and glutathione *S*-transferase (GST6). Similarly, expression of *NPK1* induced a set of similar stress genes, such as *GST*, *HSP*s, and *PR1*, indicating that active NPK1 switched on the oxidative signalling cascade in transgenic maize as well (Shou *et al*., 2004*a*). The NPK1 expressing transgenic maize plants also displayed enhanced freezing tolerance (Shou *et al*., 2004*a*). Two transgenic events (A4-9 and A4-15) survived longer under subzero temperatures and was able to withstand up to 2 °C colder temperature than non-transgenic control plants. In this study, transgenic maize plants showed an increase in drought tolerance including higher AP rates, higher final leaf numbers, and higher kernel weights compared with negative controls. It has been demonstrated that tolerance to drought stress in maize may be improved through the constitutive expression of a MAPK gene that activates the oxidative signalling pathway.

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