Genetic engineering of maize for drought tolerance in Eastern and Central Africa

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Abstract

Maize, the most important staple food crop in East and Central Africa (ECA), is affected by drought – the single most important abiotic factor responsible for up to 70% maize loss. In the year 2008, a project funded to a tune of USD350, 000 by USAID through ASARECA was initiated. The objective of the project was to develop and avail drought tolerant engineered maize genotypes adapted to ECA. This was done using genetic engineering approaches of gene up-regulation, under-regulation (silencing) and drought inducible expression of candidate genes. To date 15 tropical maize genotypes have successfully been transformed with drought conferring genes; Annexinp35, Annat1, NHX1, XvPrx2, XvSAP1, IPT, CBF 1, amiRNA1 and amiRNA3 . The maize genotypes developed using these genetic engineering technologies are being advanced and evaluated in the glass house at Kenyatta University in preparation for drought stress experiments and field trials. The genetically engineered maize lines are: one Ethiopian open pollinated variety (OPV), one temperate line, eight Sudanese genotypes , one Tanzanian OPV and five CIMMYT inbred lines. The research products ready for uptake and utilization are the base sequence of a drought tolerance gene that has been isolated and cloned and optimized protocols for regeneration of maize germplasm adapted to ECA. Seventeen partners from around the globe have been identified and are playing different roles and responsibilities in the development of drought tolerant transgenic maize. Scientists from ECA region are being trained on genetic engineering at MSc. and PhD. Level. To avail drought

tolerant engineered maize genotypes to farmers in ECA region more studies involving the generated maize lines are critical and this will be done by building synergies for up-scaling the utilization of findings of this research.

Background/rationale

Maize is the most widely cultivated cereal covering over 147 million hectares in the world (maize outlook report). It is the third most important food crop for humans after wheat and rice. It is a staple food crop in East and Central Africa that is grown on more than 5.5milion hectares mostly by small and medium scale farmers. Maize provides well over 50% dietary calories with a per capita consumption of about 100kg/year.

Maize productivity has been declining over years due to a number of biotic and abiotic factors. Drought is the single most important abiotic stress responsible for reduced maize productivity in arid and semi-arid areas, leading to up to 70% crop loss. This, coupled with lack of suitable varieties that perform well under insufficient and erratic distribution of rainfall significantly reduce productivity of maize, with grain yield of 1.3tons/ha, compared to the potential of over 10tons/ha.

While conventional breeding has produced some success in development of drought tolerant varieties, complementing it with biotechnological tools to introgress drought tolerance genes into well adapted varieties has been proven to work elsewhere. This project aims at introgressing genes conferring drought tolerance through genetic transformation into local varieties from Sudan, Kenya, Tanzania and Ethiopia that are already popular with the farmers.

The general objective was to develop and avail drought tolerant engineered maize genotypes adapted to ECA with the following specific objectives:

I. Identify, isolate and characterize drought tolerance conferring genes to transform maize germplasms adapted to ECA

II. Access available drought tolerance conferring genes to transform adapted maize germplasms in ECA

III. Subclone accessed drought tolerance conferring genes into suitable plant expression vectors

IV. Optimize tissue culture protocols for regeneration of maize germplasm adapted to ECA

V. Transform maize for drought tolerance

Methodology

I. Identification, isolation and characterization of drought tolerance conferring genes

One gene was identified, isolated, and cloned based on the role of Annexin 1 in drought Stress tolerance in Arabidopsis (Dorota et al., 2009). These Annexin genes act as targets of calcium signals in eukaryotic cells, and play an important role in plant stress responses. Plants that over expresses Annexin genes are more drought tolerant. Maize Annexinp35 cDNA was isolated from plant tissue according to Kranz *et al.*,(1994). The gene was then cloned into the Plant expression vector pCAMBIA1300 and the new gene construct named pCAMBIA-ASAGE35 (The vector was named after ASARECA and GEMADOT project). The gene was then subcloned into pNOV2819 vector for maize transformation.

II. **Access available drought tolerance conferring genes**

The available genes (Table 1) for conferring tolerance to drought were accessed in partnership with relevant donors and agencies, such as the Rockefeller Foundation, the African Agricultural Technology Foundation (AATF) and the Public Sector Intellectual Property Resource for Agriculture (PIPRA) initiative, with focus on drought stress tolerance in maize. Genes were acquired through a material transfer agreement and the freedom to operate license between the partner institution and the department of Biochemistry and Biotechnology, Kenyatta University. The donor institution identified, isolated and characterized drought tolerance conferring genes from naturally drought tolerant plants. Molecular and genomic analyses facilitated the discovery of genes involved in drought stress tolerance. The genes employed include genes encoding functional proteins such as transporters and chaperones as well as those genes encoding regulatory proteins, such as transcription factors and signaling factors.

Table 1: Partners that supplied the drought conferring technologies

Partner	Role and responsibility
Institute of Biochemistry and Biophysics, Poland	Provision of Annnat 1 Geneexpression vector
University of Nevada	Provision of NHX1, CBF1 and CBF3 gene expression vectors
Syngenta	Provision of pNOV2819 plant gene exspression vector
University of California	Provision of IPT gene expression vector
University of Cape Town	Provision of XvPrx2, XvSap1 and XvG6 gene expression vectors and promoters.
Ghent University	Provision of amiRNA gene expression vector
IOWA State University	Provision of PTF102 plant expression vector

III. Subcloning drought tolerance conferring genes into suitable plant expression vectors

Eight accessed genes and one isolated gene were cloned into suitable plant expression vectors. *CBF1* gene was obtained from pCAMBIA-CBF1 gene construct that has hygromycin (an antibiotic) resistance gene for selection in plants. Using PCR cloning, the gene was cloned into pNOV-2819 that has PMI gene for plant selection on mannose. The amiRNA PCR genes were subcloned between the Ubiquitine promoter and TNos terminator in Ubi/NC1300 vector. The amiRNA expression cassette was then removed as an *HindIII/ SpeI* fragment and subcloned into pNOV2819. Cotransformation vectors containing the

selectable marker TDNA and the TDNA for the gene of interest (amiRNA) for production of plants free of marker genes were constructed essentially using methods described by Sambrook et al, (1989). IPT gene was obtained in construct carrying hygromycin resistance gene as plant selectable marker. The *SARK::IPT:NOS* expression cassette was sub-cloned into plant expression vector pNOV2819. This was accomplished by engineering for *AscI* and *HindIII* restriction sites on up- and downstream ends of the expression cassette, respectively, through PCR using high fidelity Taq polymerase. The fragment was then ligated into pNOV2819 that had been digested with the same enzymes. Maize was then transformed with this gene. *PMI* gene was used as plant selectable marker with mannose as the selective agent (Negrotto et al., 2000). Annexin1 gene was removed from pROK2 vector as a *Kpn1*/*BamH1* fragment and then engineered into Ubi/NC1300 vector. Annat1 gene cassette was removed from this resultant vector and ligated to PNOV2819 vector to form pNOV2819-Annat1. *PMI* gene was removed from PNOV2819 as a *Sal1/Kpn1* fragment and placed into pCAMBIA-NHX1 vector. The new construct was named pCAMBIA-NHX1-PMI.

Xerophyta genes were harbored in pTF 101.1 which has *bar* gene as plant selectable marker. Sub cloning of the xerophyta genes into pNOV2819 vector was achieved by introducing *HindIII* and *PacI* restriction sites on the ends of the expression cassettes of the genes. Subsequently, the expression cassettes for XvPrx2, XvSap1 and XvG6 were then singly subcloned between the the same restriction sites on pNOV2819 backbone. The constracts were then immobilized into *Agrobacterium tumefaciens* strain EHA101 or LBA4404 which was used to transform immature zygotic embryos of maize.

IV. **Optimizing tissue culture protocols for regeneration of maize germplasm**

Adapted maize germplasm were obtained from national and regional maize breeding programs in Ethiopia, Kenya, Sudan and Tanzania, as well as from CIMMYT. The germplasm were bulked in the respective countries for, while others were grown at Kenyatta University where regeneration and transformation experiments were initiated and procedures optimized. Different explants were used for callus initiation, subsequent somatic embryogenesis and regeneration according to procedures already in use in the laboratories of Prof. Jesse Machuka (Rasha *et al*., 2008, Ombori et al., 2008, Oduor et al.,2006), Dr. Kan Wang at Iowa State University (ISU),

Ishida (Ishida et al., 2007) and Ames (Frame et al., 2002). Parameters that had been optimized for temperate maize regeneration were varied depending on genotypes in order to obtain optimal regeneration efficiency for ECA germplasm. Attempts were also made to obtain regeneration from seed calli, leaf disks, and young shoot meristems (Rasha et al.,in press). Optimization of regeneration protocols for CIMMYT inbred lines, Tanazanian and Ethiopian OPVs, was achieved using immature zygotic embryos, obtained 14-16 days after pollination depending on genotypes. All media used were based on MS basal salts (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965). Immature embryos 1-1.5mm in size were excised from maize kernels under sterile conditions and incubated on callus induction medium (CIM) supplemented with either 1 to 2.5 mg/l 2,-4-D and 10 mg/l Silver nitrate (AgNO3). In this study only 2, 4-D was used because of its availability, low cost and it plays a great role in maize embryogenic callus induction. Induced somatic embryos from Embryogenic calli (EC) were matured in embryo maturation medium amended with 6% sucrose. Plants were regenerated from matured somatic embryos as described by Ombori et al., 2008. Plantlets were hardened and acclimatized as described by Ombori et al., (2008). Optimization of tissue culture protocols for Sudanese maize was achieved using different sources of explants which include mature embryos, shoot tips and leaf segments. The protocols were optimized using different concentrations of 2, 4-D ranging between 0 and 10mg/L.

The protocol by Ishida et al, (2007) was also applied to 15 inbred lines obtained from CIMMYT to assess the efficacy of the protocol in promoting plant regeneration from the genotypes.

V. Transformation of maize for drought tolerance

Several *Agrobacterium tumefaciens* strains, such as LBA4404 and EHA101 were used. *A.tumefaciens* strains used contained standard binary vectors with right and left T-DNA border fragments, an origin of replication and a resistance marker gene for bacterial selection. These strains were supplied from Iowa state university and Syngenta. This was possible due to Material Transfer Agreements (MTA) that have been signed between Kenyatta University (KU) and ISU and between KU and syngenta for use of vectors for optimization of transformation procedures. Reporter gene constructs used were supplied by ISU for protocol development. Various promoters, notably maize double *CaMV 35S, CMPS, ubiquitin XvpSap1* and SARK were used to drive genes including *bar*, phoshomannose Isomerase (*PMI*) and the gus reporter gene.

Several ECA adapted maize genotypes were tested to establish their transformability. This was done based on empirical assessment of factors that affect transformation in monocots (Koichi et al., 2003).

Maize ears with immature embryos of appropriate size were harvested between 12-16 days after pollination depending on the variety (Ishida *et al*., 2007). The ears were dehusked and surface sterilized in a tissue culture hood. The top half of kernels was cut with a sterile scarpel and the embryos excised using a spatula. These the embryos were then transformed with different genes for conferring tolerance to drought with the help of *Agrobacterium* strain *EHA101 or LBA4404* and transferred to resting media to form callus. The calli were selected on mannose containing media (Negrotto *et al*., 2000) and surviving calli regenerated into plants that were hardened according to the protocol by Ishida *et al*., (2007).

Results

I. Subcloning drought tolerance conferring genes into suitable plant expression vectors

Eight accessed genes and one isolated gene were cloned into suitable plant expression vectors (Table 2).

Table 2: Genes subcloned into plant expression vectors and the vectors developed

Gene	Expression vector developed
AnnexinP35 Annexinp35 Annat1	pNOV-ASAGE35 PCAMBIA-ASAGE35 PCAMBIA-Annat1
NHX1	pCAMBIA- NHX1- PMI/
CBF1 gene	pNOV2819-CBF1
AmiRNA3	pNOV2819-Ubi/amiRNA3, pSMF03.1, pSMF03.2, pSMF04.0
amiRNA1	pNOV2819-Ubi/amiRNA1
Annat1	pNOV-Annat1

XvPrx2	pNOV2819-XvPrx2
XvG6	pNOV2819-XvG6
IPT	pNOV2819-IPT-L-KU-PTL

II.**Optimizing tissue culture protocols for regeneration of maize germplasm**

The protocols for regeneration of several tropical genotypes were optimized. Using the procedure described in the materials and methods above, all four Tanzanian OPVs were successfully regenerated with Staha and Situka M-1 being the most regenerable genotypes (Seth et al., in press). Further, five CIMMYT tropical maize inbred lines were evaluated among which CML216, CML395 and CML442 were highly regenerable (Bedada et al. in press). Among six Ethiopian maize genotypes evaluated for regeneration ability using MS medium supplemented with four levels of 2,4-D, Melkassa-2, Melkassa-6Q and [CML387/CML176]-B-B-2-3-2-B[QPM] were found to be the best regenerable ones using immature zygotic embryo explants (Bedada et al. 2011)

Among the Sudanese genotypes IL3 and Hudiba-2 were found to be the most regenerable genotypes with regeneration frequency of 75.8% and 61.7%, respectively (Rasha et al 2008).
The protocol by Ishida et al (2007) was identified as useful for obtaining plants from three genotypes obtained from CIMMYT (TL03B 6754A-20 (18), CML 216 and TL03B 6757-68 (13). The protocol was found to promote embryogenic callus formation at a frequency of between 54 and 76 % on the three genotypes. The protocol also promoted regeneration of between 3 and 5 plants per callus from the three genotypes.

III. Transformation of maize with the constructed plant expression vectors

Fifteen maize genotypes were transformed with nine (9) different drought conferring genes. amiRNA1 construct for silencing of PARP gene were successfully engineered into one CIMMYT inbred line (CML216) using the PNOV2819-amiRNA vectors at a transformation frequency of between 5-11%. Additionally, three CIMMYT inbred lines (TL03B 6754A-20 (18), TL03B 6757-68 (13) and CML 216) have been transformed with Cotransformation vectors bearing amiRNA3 gene construct for silencing of the maize PARP gene. Using the

Cotransformation vectors, the average transformation frequencies of 10%, 13%, 15% were obtained for inbreds TL03B 6754A-20 (18), TL03B 6757-68 (13) and CML 216, respectively. Eight Sudanese maize genotypes (IL3, STR149, Giza-2, PR5655, IL15, IL16, Hudiba-1 and IL43) were transformed with three genes conferring drought stress tolerance, (Annat1, Annexin p35 and NHX1) and the transformation frequency ranged between 0.5% and 34.8%

Two genotypes were transformed with CBF1 gene construct. A188 gave a transformation frequency mean of between 35 % and 69% while transformation frequency mean of CML 216 ranged from 19 to 39%. One Tanzanian OPV (Staha) and one CIMMYT inbred line (CML144) have successfully been transformed using Xvprx2 gene. The transformation frequency for these lines has ranged from 21 to 24%. Two CIMMYT inbred lines, CML216, CML395 and one Ethiopian OPV Melkassa-2 were transformed with IPT gene with transformation frequency and efficiency ranging from 0.0 to 33 and 70 to 100 per cent, respectively.

Conclusion

Tropical maize genotypes have been genetically engineered for drought tolerance by scientists from ECA region at Kenyatta University in Kenya. This was possible through: optimization of regeneration protocols; identification, isolation, sequencing, and acquisition of genes; development of plant expression vectors. More work is needed to advance the transformed genotypes for stress experiments and building synergies for up-scaling the utilization of findings of this research is very critical.

Acknowledgement

A number of partners were involved in this project (Table 3). Financial support for the project was provided by USAID through ASARECA.

Table 3: Partners and their roles

Partner	Role and responsibility	**Contact details**
Institute of Biochemistry	Provision of Annnat 1 Gene	**Dr. Dorota Kanopka-Postupolska,** Institute of Biochemistry and Biophysics, Polish Academy of Sciences,

and Biophysics, Poland	construct	02-106 Warsaw, Poland. E:mail: konopka@ibb.waw.pl
Kenyatta University and ARC Biosafety and Biotechnology Research Center	Provision of AnnexinP35 gene construct	**Rasha Adam, Biosafety and Biotechnology research center**, Khartoum, shambat P. O. Box 126. E:mail: rasho3310@yahoo.com
University of Navada	Provision of NHX3, CBF1 and CBF3 gene vectors	**Dr. Ron Mittler**, Department of Biochemistry and Molecular Biology, University of Navada, mail. Stop 200; Reno, NV 89557
CIMMYT	Provision of Maize Germplasm	**Dr. Dan Makumbi,**Global Maize Program, CIMMYT, ICRAF HOUSEUnited Nations avenue, Gigiri,P.O. box 1041 village market -00621, Nairobi , Kenya, Tel: 254 20 722 4615, d.makumbi@cgiar.org
University of California	Provision of IPT gene	**Dr. Eduardo Blumwald,** Department of Plant Sciences - Mail Stop 5, University of California, One Shields Ave, Davis, CA 95616, Email: eblumwald@ucdavis.edu, Tel Office: 530-752-4640, Fax: 530-752-2278
University of Cape Town	Provision of XvPrx2 and XvG6 genes and promoters. Technical backstopping	**Jennifer Thomson, PhD.**Department of Moleculand Cell Biology, University of Cape Town, Private Bag, Rondebosch 7701, South Africa, Fax: +27 (0) 21 689 7573, Telephone: +27 (0) 21 650 3256, E-mail: jennifer.thomson@uct.ac.za
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KARI	Research supervision and Collaboration in conducting and facilitating field trials	**Dr. James Gethi,** KARI-Katumani, Tel. +254-44-20330, +254-721-831166,Email : jgethi@wananchi.com
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ARC	Provision of maize germplasm and Field trials Conducting and facilitating field trials	**Rasha Adam, Biosafety and Biotechnology research center**, Khartoum, shambat P. O. Box 126. E:mail: rasho3310@yahoo.com
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IOWA State University	Provision of PTF102 construct	**Kan Wang,** Iowa State University, Department of agronomy, Plant transformation facility, Email: kanwang@iastate.edu, Fax: 515 294 2299
Institute of Biochemistry and Biophysics, Poland	Provision of Annnat 1 Gene construct	**Dr. Dorota Kanopka-Postupolska,** Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland, E:mail: konopka@ibb.waw.pl

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