academicJournals

Vol. 12(27), pp. 4251-4255, 3 July, 2013
DOI: 10.5897/AJB2012.2992
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro screening and molecular genetic markers associated with salt tolerance in maize

Rajurkar Ashish Balkrishna* and Shende Sarika Shankarrao

Department of Agricultural Biotechnology, Marathwada Agricultural University Parbhani, Maharashtra, 431-401 India.

Accepted 14 June, 2013

Salinity is one of the major limitations of plant growth and productivity all over the world. *In vitro* screening with marker assisted selection can greatly facilitate the selection and isolation of useful tolerant lines. The genetic basis of salt tolerance was examined in salt tolerant (EC 558706), moderately tolerant (EC 558719) and salt sensitive (EC 558620) maize genotypes. Callus lines from mature embryos were developed and exposed to culture media containing, 0.5, 1, 1.5 and 2% NaCl concentrations. The treated calli were used to detect DNA based molecular markers associated with salt tolerance by random amplification of polymorphic DNA (RAPD) technique. Ten (10) polymorphic bands were found related to salt tolerance in maize. The analysis revealed A 11 (1200, 600, 300 bp), C 20 (300 bp), OPA 10 (2800 bp), OPA 13 (900, 600, 250 bp), OPX 11 (800 bp), OPI 01 (700 bp) and OPK 20 (700 bp) primers associated with salt tolerance which can be utilized in breeding programme *via*., marker assisted selection and developing salt tolerant genotype by genetic transformation.

Key words: Maize, salt tolerance, molecular marker, RAPD.

INTRODUCTION

Cereal culture is of great economic importance in an agricultural country like India (Zair, 2003). Maize (*Zea mays* L.) is the most important cereal crop of world agricultural economy. Among the cereals and grain crops, maize rank third in production in the world being suppressed only by rice and wheat.

Salinity is one of the major limitations of plant growth and productivity all over the world (Flowers, 2004; Foolad, 2004). Maize is moderately sensitive to salinity and considered as the most salt sensitive of the cereals (Maas and Hoffman, 1977). *In vitro* selection shortens the time considerably for selection of desirable trait under selection pressure without having any environmental influence (Bressam et al., 1985; Rosas et al., 2003). Testing genetic resources for productivity, quality parameters and stress tolerance, field trials are usually time consuming. Molecular variation in tissue culture derived plants has been characterized at DNA level (Jain, 2001). So, the modern one in which natural genetic variation is considered and selection in stressful environment is carried out with molecular marker technology at genetic level is method of choice (Karp et al., 1993).

Various molecular markers *viz*., randomly amplified polymorphic DNA (RAPD) restriction fragment length polymorphism (RFLP), amplified fragment length polymorphisms (AFLP), inter-simple sequence repeat (ISSR) and simple sequence repeat SSR are being used to characterize the induced genetic variation. Amongst these molecular markers, RAPD analysis is a simple, quick, easy to perform and require small amount of DNA (Welsh and McClelland, 1990; Williams et al., 1991).

These benefits justify the frequent application of RAPD technique in genetic variability studies (Mondal and Chand, 2002; Bennici et al., 2003; Feuser et al., 2003). Keeping these considerations in view, the present work was aimed at *in vitro* screening for salt tolerance and to

*Corresponding author. ashu.raj123@gmail.com. Tel: +91 9975051209.

Table 1. List of primers along with their sequence used for RAPD analysis.

S/N	Primer	Primer sequence (5' - 3')	Number of base pairs
1	A11	CCT GTT AGC C	10
2	C 20	ACT TCG CCA C	10
3	OPA 10	GTG ATC GCA G	10
4	OPA 13	CAG CAC CCA C	10
5	OPA 20	GTT GCG ATC C	10
6	OPG 09	CTG ACG TCA C	10
7	OPH 20	GGG AGA CAT C	10
8	OPI 01	ACC TGG ACA C	10
9	OPJ 06	TCG TTC CGC A	10
10	OPK 20	GTG TCG CGA G	10
11	OPM 20	AGG TCT TGG G	10
12	OPX11	GGA GCC TCA G	10

identify molecular markers associated with salt tolerance in maize by RAPD technique.

MATERIALS AND METHODS

Previously screened maize (*Zea mays* L.) genotypes (Rajurkar et al., 2011) *viz*., EC 558620 (salt sensitive), EC 558706 (salt tolerant) and EC 558719 (moderately tolerant) were selected and procured from the National Agriculture Research Program (NARP) Aurangabad, Maharashtra, India.

Callus induction

The mature seeds were surface sterilized with 70% ethanol for 2 min and 0.1% mercuric chloride for 15 min. The seeds were soaked in 2,4-dichlorophenoxyacetic acid (2,4-D) (4 mg/l) and swollen mature embryos were removed after 72 h. The plumule and radicle section (2.5 mm) was longitudinally sliced into halves and inoculated on MS basal medium supplemented with 2,4-D.

The induced callus (30 mg) were then transferred on MS media (Murashige and Skoog, 1962) containing 2, 4-D (4 mg/L), and 0.5, 1, 1.5 and 2% NaCl. The cultures were incubated in dark at 25±2°C for 30 days.

DNA isolation and PCR amplification

Genomic DNA was extracted from 30 days old salt treated calli (100 mg) by cetyl trimethyl ammonium bromide (CTAB) method (Saghai-Maroof et al., 1984) with some modifications; the concentration of some chemicals was standardized to get maximum quantity of genomic DNA.

Twelve (12) out of 33 random decamer primers from different sets (A, RC, C, O, Z, OPA, OPC, OPE, OPG, OPH, OPI, OPM, OPR and OPX) of Operon Technology Inc, USA were chosen on the basis of reproducible and scorable amplification products for salinity specific gene expression studies (Table 1).

A 25 µl mixture contained 20 ng of genomic DNA, 1U *Taq* DNA polymerase (Genetix), 1XPCR buffer, 4 mM $MgCl_2$, 0.2 mM of dNTPs (Genetix), and 30 pmol primers. Amplifications were carried out using a 96 thermal cycler (Bio metra) programmed for 40 cycles as follows: initial denaturation at 94°C for 5 min, further denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 1 min and final extension for 8 min at 72°C. The PCR products were resolved at 100 V for 4 hon 1.2% agarose gel prepared in 1x Tris-borate-EDTA (TBE) buffer. Gel was photographed using Gel-Documentation system (ALPHAIMAGER TM 2200).

RESULTS

Callus induction

Swelling of the scutellum region and the appearance of small calli (0.5 to 1 mm diameter) from mature embryos were observed after 10 days. The callus induction in terms of growth was relatively low (Figure 2) at a low level of 2,4-D (2 mg/l), however maximum induction was observed at 4 mg/l 2,4-D. Higher concentration of 2,4-D (5 mg/l) did not significantly change the callus quantity or quality. Hence, it was found that 4 mg/l 2,4-D is optimal concentration for callus induction.

The optimised concentration of 2,4-D (4mg/l) was used in salinity treatments. The salt treatment was measured in terms of callus growth. Callus cultures on salt selection media showed decrease in callus growth with increase in salt concentration (Table 2).

RAPD analysis

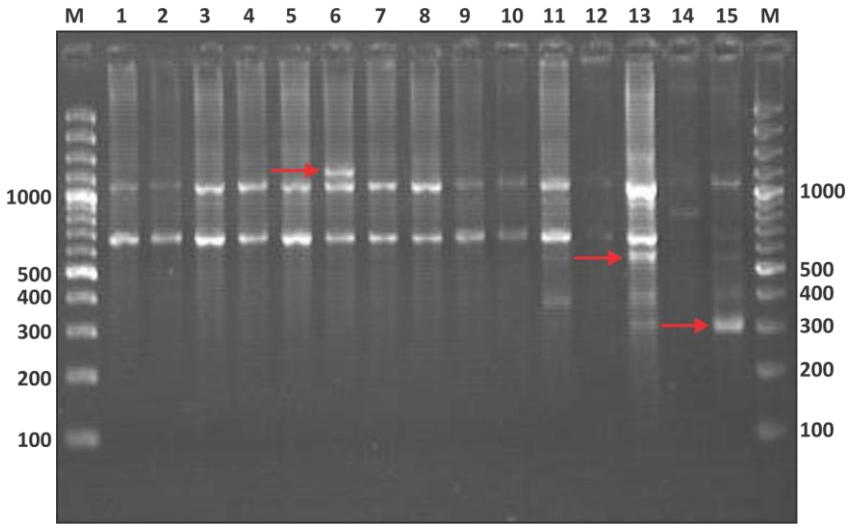
RAPD markers associated with salt stress

The gene expression under salt stress among the selected genotypes was analysed using RAPD markers. Out of the 33 primers, only 12 primers were amplified and developed a total of 333 bands. The lowest number of bands (19) was developed by primer OPK 20, while the highest (45) was developed by OPA 10. Seven primers *viz.,* A 11, C 20, OPA 10, OPA 13, OPX 11, OPI 01 and OPK 20 showed unique bands (Table 3 and Figure 1). A positive marker is a band generated in salt treated calli whereas, negative marker is a band obtained in control. The positive unique bands obtained in salt tolerant calli

Table 2. Effect of salt stress on growth of callus culture.

S/N	Genotype	Treatment (%)	Initial weight (mg)	Final weight* (mg) mean± S.D	Proline accumulation (µmol/g of fresh weight)
1	EC 558620	Control	30	115.28±2.15	34.63
		0.5	30	100.89±1.24	69.26
		1.0	30	66.44±3.07	103.89
		1.5	30	42.05±1.15	191.51
		2.0	30	39.17±1.70	519.48
2	EC 558706	Control	30	129.62±2.86	173.16
		0.5	30	120.90±9.34	225.10
		1.0	30	82.22±3.74	432.90
		1.5	30	46.40±3.29	786.14
		2.0	30	40.90±0.84	1038.96
3	EC 558719	Control	30	127.61±2.38	155.84
		0.5	30	115.73±8.33	329.00
		1.0	30	75.07±3.29	363.63
		1.5	30	45.09±1.70	502.16
		2.0	30	40.17±0.39	1021.64

*Weight taken after 25 days of inoculation on different salt concentration medium.



1 - Control of EC558620
2 - 0.5% of EC558620
3 - 1.0% of EC558620
4 - 1.5% of EC558620
5 - 2.0% of EC558620
6 - Control of EC558706
7 - 0.5% of EC558706
8 - 1.0% of EC558706
9 - 1.5% of EC558706
10 - 2.0% of EC558706
11 - Control of EC558719
12 - 0.5% of EC558719
13 - 1.0% of EC558706
14 - 1.5% of EC558706
15 - 2.0% of EC558706
M – Marker / Ladder

Figure 1. RAPD profile of salt treated genotypes with primer A11.

will be used as a RAPD marker for developing salt tolerant genotypes.

DISCUSSION

The determination of absolute tolerance under *in vitro* conditions has rather been impossible because of the complex interactions between plant systems and the environmental factors. However, the development of improved stress tolerant variety appears to be promising. The *in vitro* techniques have become useful in generating novel genetic variability in many seeds as

Table 3. Characteristics of the amplified products obtained by RAPD primers.

S/N	Primer name	Number of bands amplified	Number of unique bands	Size and name of genotype where unique band observed
1	A 11	44	3	1200 bp (EC 558706); 600bp, 300 bp (EC 558719)
2	C 20	36	1	300 bp (EC 558719)
3	OPA 10	45	1	2800 bp (EC 558706)
4	OPA 13	36	3	900 bp, 600 bp (EC 558706); 250 bp (EC 558719)
5	OPX 11	29	1	800 bp (EC 558706)
6	OPI 01	29	1	700 bp (EC 558719)
7	OPK 20	19	1	700 bp (EC 558706)

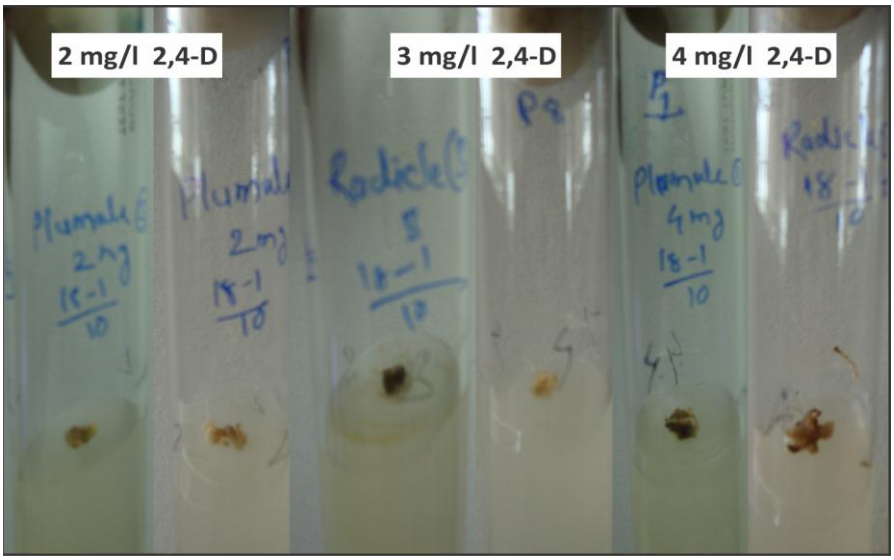


Figure 2. Callus induction on MS media supplemented with different concentrations of 2,4-D (2, 3, 4 mg/l).

well as vegetatively propagated plants (Maluszynski et al., 1996).

In the present study, the frequency of callus formation from mature embryos was low (Figure 2). This follows results reported by Wang (1987) in maize. The role of plant growth regulators in cereal tissue culture has been reviewed by Bhaskaran and Smith (1990). In general, auxins usually 2,4-D in the range of 1 to 3 mg/l, are essential for the establishment of embryogenic callus from cereals embryos. The use of 4 mg/l 2,4-D to induce callus formation from maize embryos is a critical factor. The result of this study suggests that it might be possible to improve regeneration from mature embryos by optimizing the compositions of regeneration media for specific genotype. Hung and Wei (2004) obtained 90% frequency of primary calli from mature embryo of maize at 4 mg/l 2,4-D.

Six positive markers *viz.,* A11, C 20, OPA10, OPA 13, OPI 01, OPK 20 were found to be related with salt stress which are in conformity with the results obtained by the Zacchini et al. (1997) in which they found unique band of 550 bp in *Zea mays* calli treated with 1% NaCl. Younis et al. (2007) developed four RAPD markers *viz.,* C02-715, C05-1585, O16-210, Z12-2000 for salt tolerance in sorghum and Abdel-Tawab et al. (1997, 1998) detected one RAPD marker for salt tolerance and three for drought tolerance in maize while they developed two positive and two negative molecular markers for salt tolerance in maize (Abdel-Tawab et al., 2001) using bulk segregation analysis.

The two negative markers *viz.,* OPA 20 and OPX 11 were obtained in control calli. Similar results were also obtained by Younis et al. (2007) and showed 10 negative markers *viz.* C04-2015, C04-1770, C05-300, C05-240, C16-270, C16-200, O12-1100, O16-465, O09-1700, O18-855 in salt sensitive parent of sorghum. Other successful attempts to detect RAPD markers for salt or drought tolerance were reported by Breto et al. (1994) and Rahman et al. (1998) in *Lycopersicon* and rice respectively. RAPD occurs as the simplest and fastest method for detecting a great number of genomic markers in short period of time.

These results suggest that this polymorphism could

not be caused by selection of pre-existing variants in the zygotic embryos population from which the calli were derived, but must be attributed to somaclonal variation occurring during callus formation in response to salt stress.

The results from the present study are in agreement with the detection of genetic variation in response to salt stress with RAPD analysis by Rasheed et al. (2001) and Patade et al. (2005). Rasheed et al. (2001) analyzed genetic variability among salt stress (200 mM NaCl) calli of sugarcane and potato using RAPD technique. Out of six primers used, only two primers enabled the identification of polymorphism among salt tolerant plants of sugarcane whereas Patade et al. (2005) obtained a unique band of 500 bp in salt tolerant regenerated lines of sugarcane.

The RAPD technique has proved very sensitive for genetic variation studies and fastest method for detecting a great number of genomic markers in *in vitro* selected salinity tolerant calli in a short period of time.

Conclusion

In vitro selection shortens the time considerably for selection of desirable trait under selection pressure and has been used for selection of abiotic stresses while the RAPD technique has proved very sensitive for genetic variation studies and fastest method for detecting a great number of genomic markers in *in vitro* selected salinity tolerant calli in a short period of time.

ACKNOWLEDGEMENT

The authors are grateful to the Department of Biotechnology, Government of India, New Delhi, for providing financial support for this study.

REFERENCES

Abdel-Tawab FM, Fahmy Eman M, Baieldin A, Eissa Hala F (1997). Molecular marker for salt tolerance in some inbreds of maize (*zea mays* L.). Arab Univ. J. Agric. Sci., Ain Shams Univ. 5:389-417.

Abdel-Tawab FM, Fahmy Eman M, Baieldin A, Mahmoud Asmahan A, Saleh OM (1998). Development of molecular genetic indices for drought tolerance in some inbreds and hybrids of maize (*Zea mays* L.). 3rd Arab conference, Modern Biotech and Areas of Application in the Arab world, 14-17 December 1998, Cairo, Egypt. p. 641-660.

Abdel-Tawab FM, Rashed MA, EI-Domyati FM, Salem TZ, Azer SA, Khufaga AF (2001). Marker assisted selection for salt tolerance in maize (*Zea mays* L.). Egypt J. Genet. and Cytol. 29:175-181.

Bennici A, Anzidei M, Vendramin GG (2003). Genetic stability and uniformity of *Foeniculum vulgare* Mill. regenerated plants through organogenesis and somatic embryogenesis. Plant Sci. 1-7.

Bhaskaran S, Smith RA (1990). Regeneration in cereal tissue culture: a review. Crop Sci. 30:1328-1336.

Bressom RA, Singh NK, Handa AK, Kononowicz A, Hasegawa PM (1985). Stable and unstable tolerance to NaCl in cultured tobacco cells. In: Freeling M. (ed.) Plant genetics: Proceedings of third annual ARCO Plant cell Research Institute – UCLA symposium on Plant Biology: New Series, A. R. iss, New York: 755-769.

Breto MP, Asins MJ, Carbonell EA (1994). Salt tolerance in *Lycopersicon* species. III. Detection of Quantitative trait loci by means of molecular markers. Theor. Appl. Genet. 88:395-401.

Feuser S, Meler K, Daquina M, Guerra MP, Nodari RO (2003). Genotypic fidelity of micropropogated pineapple (*Ananus comosus)* plantlets assessed by isozyme and RAPD markers. Plant Cell Tiss. Organ Cult. 72:221-227.

Flowers TJ (2004). Improving crop salt tolerance. J. Exp. Bot. 396 (55):307-319.

Foolad MR (2004). Response to selection for salt tolerance during germination in tomato seed derived from P.I. 174263. J. Amer. Soc. Hortic Sci. 121:1006-1011.

Huang XQ, Wei ZM (2004). High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays* L.). Plant Cell Rep. 22:793-800.

Jain SM (2001). Tissue culture-derived variation in crop improvement. Euphytica. 118:153-166.

Karp A, Kresovich S, Bhat KV, Ayad WG, Hodgkin T (1993). Molecular tools in plant genetic resources conservation: A guide to the technologies, IPGRI, Rome, Italy. pp. 278-286.

Maas EV, Hoffman EJ (1977). Crop salt tolerance. Current assessment. Proc. Amer. Soc. Civil Engg. 103:115-134.

Malliszynski M, Szarenko I, Sigurbjornsson B (1996). Haploidy and mutation techniques. In: Jain SM, Sopory SK, and Veilleux RE (Eds). *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordreeht. 1:67-930.

Mondal TK, Chand PK (2002). Detection of genetic variation among micro propagated tea (*Camellia sinesis* (L.) O. Kuntze) by RAPD analysis. In Vitro Cell Bio Plant. 38:296-299.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant. 15:473-497.

Patade VY, Suprasanna P, Bapat VA, Kulkarni UG (2005). Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane. BARC Newsletter Issue no. 273:244-257.

Rahman M, Malik TA, Iqbal MJ, Zafar Y, Alam K, Perveen Z, Rehman S (1998). DNA marker for salinity resistance. Rice Biotech. Quarterly. 35:12-13.

Rajurkar AB, Shende SS, Gadge PJ (2011). Inducing salt tolerance and its effect on growth & germination of maize (*Zea mays* L.) genotypes. Asian J. Bio Sci. 6(1):69-73.

Rasheed SU, Asad M, Zafer S, Waheed RA (2001). Use of radiation and *in vitro* techniques for development of salt tolerant mutants in sugarcane and potato. IAEA-TECDOC. 1227:45-50.

Rosas HG, Garcia SS, Reyes GR, Ontiveros JLR, Villasenor ACR (2003). Preliminary results on *in vitro* selection for tolerance to chloride excess in avacado. Revista Chapingo Serie Horticulture. 9(1):39-43.

Seghai Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984). Ribosomal spacer length polymorphism in Barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA. 81:8014-8019.

Wang AS (1987). Callus induction and plant regeneration from maize mature embryos. Plant Cell Rep. 6:360-362.

Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.

Williams JGK, Kubelik AR, Livak KJ, Raflaski JA, Tingey SV (1991). DNA polymorphism amplified by arbitary primers are useful genetic markers. Nucleic Acid Res. 18:6531-6532.

Younis Rania AA, Ahmed MF, Mervat M, Menshawy EL (2007). Molecular genetic markers associated with salt tolerance in grain sorghum. Arab J. Biotech. 10(2):249-258.

Zacchini M, Marotta A, Agazio M (1997). Tolerance to salt stress in maize callus lines with different polyamine content. Plant Cell Rep. 17:119-122.

Zair I, Chlyah A, Sobounji K, Tittahsen M, Chlyah H (2003). Salt tolerance improvement in some wheat cultivars after application of *in vitro* selection pressure. Plant Cell Tissue Organ Cul.73:237-244.