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A Feasibility Study for the use of Molecular Markers (RAPDs) for Salinity Tolerance

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A feasibility study was carried out for the use of molecular markers (RAPDs) for salinity tolerance in maize. Polymorphism was sought in two salt tolerant accessions and a small sample of their F_2 progeny using a limited number of random primers. Whilst, a considerable level of polymorphism was found using only 4 primers, to make any conclusive remarks about the suitability of RAPD markers in the genetic analysis of salinity tolerance in maize based on the limited data perhaps would be inappropriate. The RAPD PCR amplifications were extremely sensitive and a single change in the concentration of reaction component and/or thermal cycling parameters appeared to be altering the RAPD patterns. Nevertheless, once optimised, the technique has the potential of providing an effective and convenient method to generate molecular markers, which could be utilised in mark aided selection of complex traits like salinity tolerance.

Key words: RAPD, Salinity, Maize, Molecular markers.

Introduction

Breeding for salt tolerance is often hampered by complexity of the trait, which is affected by a number of interacting plant and environmental factors. The apparent quantitative nature of inheritance of the genes conferring tolerance has further complicated breeding efforts (Flowers *et al* 1997). One approach to facilitate the selection and breeding for complex traits such as salt tolerance is the identification and utilisation of simply inherited genetic markers that are genetically associated with the trait(s) of interest (Stuber *et al* 1992; Dudley 1993 and Foolad *et al* 1995). Molecular markers tightly linked to genes of interest allow the simultaneous selection for several traits introduced from the donor parents following the initial hybridisation, thereby reducing both the number of generations required to introgress a gene and the extent of linkage drag (Dudley 1993). To increase the efficiency of artificial selection for several traits, genetic/molecular markers alone or combined with traditional phenotypic methods can be used through indirect selection or marker assisted selection. Molecular genetic markers are finding increasing applications in plant breeding programmes and genetic studies. They are useful for fingerprinting varieties, establishing phylogenies, tagging desirable genes, determining similarities among inbreds and mapping plant genomes.

Through the development of restriction fragment length polymorphism (RFLP) markers in the early eighties, indirect selection in plant breeding using DNA markers became technically feasible. RFLPs have been used extensively to develop genomic map (Caetano-Anolles *et al* 1991), establish linkages to traits (Flowers *et al* 1997), develop phylogenetic trees (Song *et al* 1988) and tag chromosomes (McGrath *et al* 19990). However detection of RFLPs by Southern blotting is often laborious, time consuming and expensive and requires large amounts of high molecular weight high quality DNA for each individual assay. In addition the RFLP procedure involves the use of radioactive material.

With the introduction of polymerase chain reaction (PCR) technology (Saiki *et al* 1988) several alternate strategies such as minisatellite, microsatellite and random amplified polymorphic DNA (RAPD) has become available to the scientists to generate genetic markers (Caetano-Anolles *et al* 1991 and Williams *et al* 1990).

Because of its simplicity and relatively low cost Random Amplified Polymorphic DNA (RAPD) methodology is well suited to high throughput requirements in plant breeding (Williams *et al* 1990). Moreover, genetic analysis using RAPDs does not require the prior nucleotide sequence of the template, probe libraries, radioactivity or Southern transfers and a universal set of primers can be used for all species. The technique permits simultaneous investigation of multiple loci in a single PCR reaction using very minute quantities of DNA template and the process can be automated (Williams *et al* 1990).

From RAPD analyses of an F_2 population of rice, Haiyuan *et al* (1998) demonstrated that a single major dominant gene

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controls salt tolerance in rice. Foolad and Chen (1998) identified favourable QTLs for salinity tolerance from RAPD marker studies in an interspecific cross of tomato. They suggested greater chances of recovering transgressive segregants for salt tolerance in the progenies, which can be identified through RAPD markers.

The present preliminary studies were undertaken to test the feasibility of using RAPD markers for salinity tolerance. Specifically, polymorphism was sought in two salt tolerant accessions and a small sample of their F_2 progeny using a limited number of random 10-mers.

Materials and Methods

Plant material. Two salt tolerant accessions, Zeol, from Zeneca seeds, UK and Sundance from USA and their F_2 population provided the material for experimentation. The accessions were crossed by artificial hand pollination in a glasshouse during summer 1996. The F_2 population was derived by polycrossing a group of 50 F_1 plants during summer 1997 in isolation in polythene tunnels at Ness Botanical Garden, Wirral, Cheshire, UK.

Extraction of the DNA. DNA of each of the parental accessions and 16 randomly selected F_2 progeny was extracted from the leaves of three-week-old seedlings grown in 8-cm pots in a heated glasshouse. Leaves were harvested from single seedlings of each parent and the F_2 progeny. The phytopure™ DAN isolation Kit (Scotlab) was used for genomic DNA extraction according to the manufacturer's instructions.

Quantification of DNA. Nucleic acids were quantified by measurement of optical density (OD) in a Perkin-Elmer Lambda 2 UV/VIS Spectrometer. An absorbance of 1 at 260 nm wavelength corresponds to 50 $\mu g\ ml^{-1}$ for double stranded DNA, 37 $\mu g\ ml^{-1}$ for single stranded DNA and 33 $\mu g\ ml^{-1}$ for oligonucleotides. Contaminating protein absorbs at 280 nm and thus the ratio of absorbance at 260 nm to 280 nm gives an indication of purity. DNA having an OD 260 nm/280 nm of around 1.8 or higher is usually required for most molecular techniques.

RAPD analysis. Primers. RAPD analyses were performed using four random 10 base random primers synthesised by Perkin-Elmer Applied Biosystems UK. The primers were characterised by an arbitrary sequence, while satisfying the imposed condition of 50-70% G+C content and no internal repeats. Sequences of the primers used in these studies are given in Table 1.

Standard polymerase chain reaction. The Polymerase Chain Reaction (PCR) amplifications were performed in a PTC-100™ Programmable Thermal Cycler, in a total volume of 10 µl in 0.5 ml thin walled PCR tubes. *Ampli* Taq® DNA Polymerase (Perkin-Elmer) was used for amplification. The PCR buffer obtained from Perkin-Elmer already contained Magnesium chloride ($MgCl_2$) therefore no additional $MgCl_2$ was added. dNTPs were made into 2.5 mM mixes from different stocks (Boehringer Mannheim). The template DNAs and primers were all dissolved in sterile distilled water (SDW), rather than TE (Tris EDTA), to avoid any inhibitory effect of EDTA. A bottle of SDW which was used successfully in an initial PCR was divided into 1 ml aliquots and stored at 20°C for subsequent use. The PCR mix (90 µl) was constituted as, SDW, make to 90 µl, 10 µl PCR buffer (10x), 8 µl dNTPs mix (2.5 mM stock), 2 µl Primers (0.2 µM), 0.5 µl *Ampli* Taq® DNA Polymerase (2.5 Units/100 µl). The constituents were mixed and dispensed into ten 9 µl aliquots before the addition of 1 µl of DNA template (20 ng/µl) to each tube, and was overlaid with about 1 µl mineral oil.

Protocol for amplification was used as:

Initial template denaturation	94°C	60 sec
Denaturation	94°C	10 sec
Annealing*	29°C	30 sec
Extension	72°C	60 sec

The cycles were repeated 40 times, followed by 5 min. at 72°C for extension at the end.

RAPD products were visualised with ethidium bromide after electrophoresis on 2% (w/v) agarose gels, run at 80-100 volts for a period that allowed the desirable separation. The stained DNA was visualised under UV light and photographed using a video camera.

Results and Discussion

DNA extraction. The DNA yield of the 20 samples examined ranged from 500 to 950 $\mu g\ gram^{-1}$ of fresh tissue. After digestion with RNase OD ratios (260 nm/280 nm) of the DNA samples were between 1.7 and 1.8. The DNA obtained through the Phytopure method was used in RAPD analyses without any further purification or cleaning.

Optimisation of amplification conditions. Four random 10-mer primers were used for PCR amplification and identification of polymorphic markers. During optimisation, several cycling conditions and reaction components were tested in order to obtain an optimal RAPD protocol. By taking into consideration cost and reproducible amplification, the optimal reaction conditions were obtained with 10 µl volume and consisted of 20 ng of template, 5 units of *Ampli*Taq® DNA polymerase, 0.2 µM Primer, 200 µM dNTPs and 1 x PCR buffer ($MgCl_2$ included, from Perkin-Elmer).

Characterisation of RAPDs. The inheritance of RAPD markers was studied by sampling 16 F_2 progenies derived from the cross Zeo 1 x Sundance. The results presented here (Table 1, Fig 1) are limited for practical reasons by the small number of F_2 progeny surveyed with the RAPD procedure using only 4 primers.

Figure 1 shows the RAPD banding patterns of the amplified samples obtained by using four 10-mer primers. Lanes 2 and 3 represents the two parents, Zeo 1 and Sundance respectively and lane 4 to 19 represents the 16 F_2 progenies Lane 1 and 20 represents the 12-Kb ladder used as a marker. None of the 4 decamers gave satisfactory banding in the gels with all the genotypes. The number of amplified fragments ranged from 1 to 7 (Table 1). The size of the amplified fragments that could be scored ranged from 1.6 to 4.0 kb.

The banding pattern generated by the primer OPA01displayed monomorphic profiles or very low level of variation, unsuited to the discrimination of genotypes (Fig 1a). The primer OPA13 revealed polymorphism (Fig 1b), although it did not work in one of the parents, Zeo 1 (lane 2) and three F_2 samples (lanes 9, 11, 13). It generated one monomorphic and 6 polymorphic bands. A heteroduplex band was observed in the F_2 sample numbers, 13, 15 and 16 (lane 15, 18, 19), showing condominant nature of the marker.

The amplification of the DNA samples with the primer OPB08 was very poor; it did not work in about 50% of the samples including the two parents. However, it can been seen from Fig 1c that OPB08 revealed polymorphism among the F_2 progeny. Seven polymorphic bands were scored in the 8 samples that worked. Whilst both parents did not work, the heteroduplex bands were observed in the F_2 progeny (lane 9, 12, 13, 18) suggesting codominance for the marker.

The Primer OPC06 worked in one of the parents Sundance and 13 samples of the F_2 population (Fig 1d). The banding pattern generated by the primer showed polymorphism and six bands were scored for polymorphism. A dominant band can be clearly observed in the population and heteroduplex band was also observed in samples 6 and 12 (lane 9, 16).

Genomic analysis of RAPDs has been well documented in a wide variety of species including plants (Welsh *et al* 1991). The methodology is very flexible since, by changing the amplification conditions a single primer can conveniently amplify from few to numerous different sequences to produce amplification fingerprinting (Caetano Anolles 1991). RAPD markers used by Marsan *et al* (1993) for genetic analysis revealed extensive DNA polymorphism in maize. In their studies about 75% of the tested markers generated discrete amplification products and one third of these produced bands polymorphic between the two maize lines used. On the basis of their data they (Marsan *et al* 1993) suggested that RAPD analysis is suitable for the construction of genetic maps in maize.

The four primers used in the present studies, for generating RAPD patterns were chosen based upon their consistency of amplification strength assessed by Fritsch *et al* (1993), who characterised 480 primers (10-mer) for RAPD analysis in flowering plants.

Based on a limited data obtained from the present results it is not possible to make any conclusive remarks about the suitability of RAPD markers in the genetic analysis of salinity tolerance in maize. However, the level of polymorphism obtained using only 4 random primers is encouraging, compared with the 40 primers tested by Huff (1979) for investigating inter- and intra-cultivar variation in perennial rye grass of the 40, only 2 generated polymorphic information. Similarly in a study involving much larger screening of primers, Bai *et al* (1997), found that of 420 primers, only 36 could be used to assess genetic diversity amongst populations of North American ginseng *(Panax quinquefolius).* In the current endeavour, all the primers revealed at least some degree of polymorphism in the samples that worked. Some samples did not work even with different amplification conditions. The failure of PCR amplification in these samples may be attributed to poor quality of the template. Kepinski (1997) also experienced such problem during his DNA profiling studies in *Anthoxanthum odoratum.* Fritsch *et al* (1993) found that they could often improve amplification strength and consistency by simply furthering the purification of the DNA template. The number and size of the DNA fragments generated strictly depends on the nucleotide sequence of the primer used and the source of the DNA (Ferreira and Keim 1997), and 100% matching between primer and template DNA was found necessary to obtain amplification in maize (Marsan *et al* 1993). Therefore, a minor mismatch between the template and the primer might have been the cause of amplification failure in these maize samples.

Table 1
Sequence of primers used in RAPD analyses, annealing temperature and number of bands

Name	Sequence (5' to 3')	Annealing temperature	Number of bands	
			Monomorphic	Polymorphic
OPA01	CAGGCCCTTC	29°C	1	1
OPA13	CAGCACCCAC	29°C	1	6
OPB08	GTCCACACGG	29°C	-	7
OPC06	GAACGGACTC	27°C	1	4

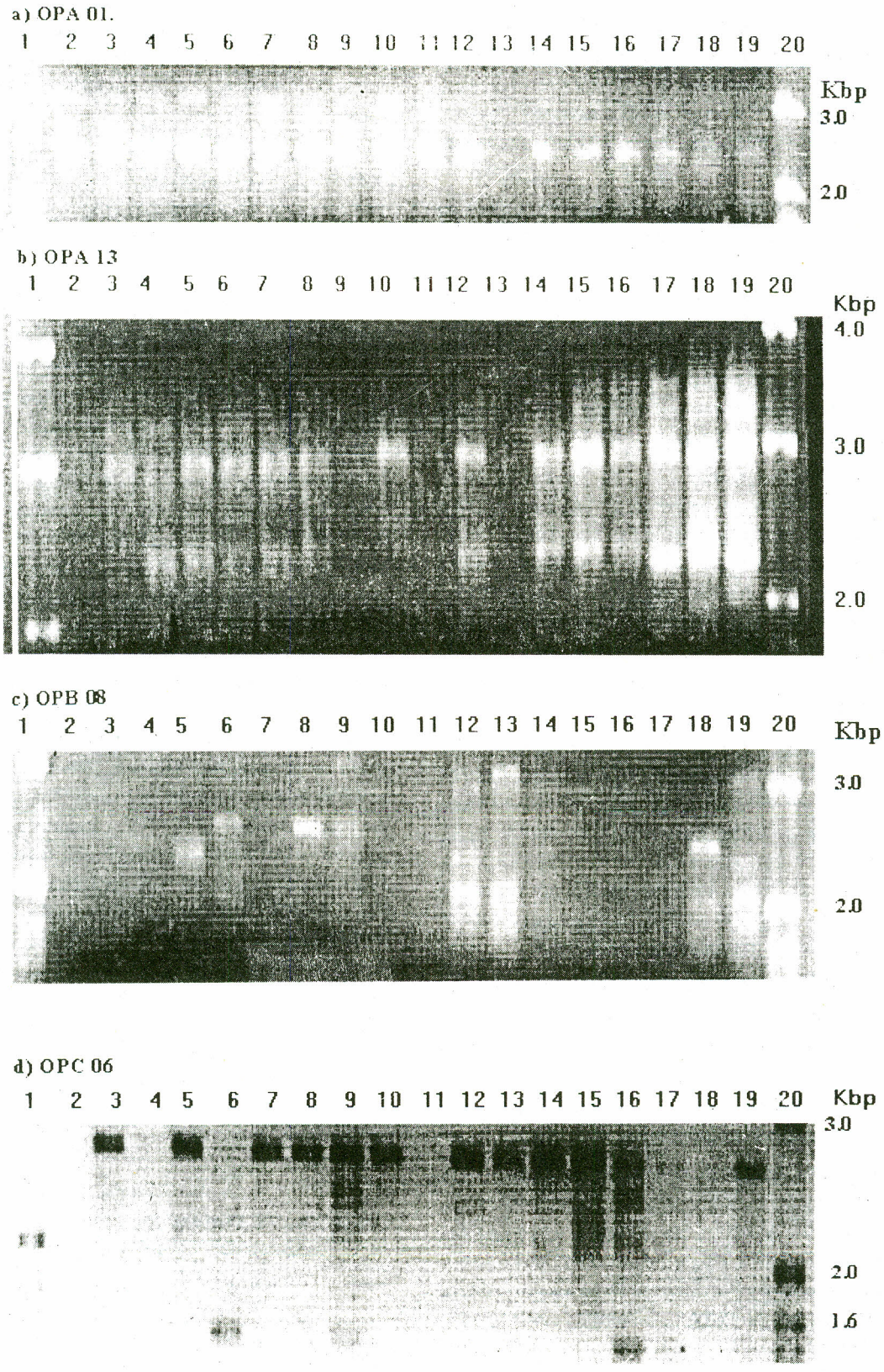

Fig 1. RAPD patterns of F_2 population of a cross between two salt tolerant accessions, using different primers.
Legends (Fig, a-d). Lanes 1 and 20, Marker; Lane 2, Parent 1 (Zeo 1); Lane 3, Parent 2 (Sundance); Lanes 4 to 19, F_2 progeny; Kbp, Kilo base pairs.

RAPD markers are inherited in a Mendelian fashion, but unlike other DNA markers, such as RFLP which are codominant, RAPDs are commonly inherited as dominant markers, where the presence of a particular band is dominant and its absence is recessive (Tingey and del Tufo 1993). Codominant markers are comparatively rare (Schulz *et al* 1994). Whilst, one of the parents Zeol did not work, heteroduplex bands which seem to be non parental were observed in the profiles generated by the primers, OPA13, OPB08 and OPC06, which are associated with codominant RAPDs. Davis *et al* (1995) also identified codominant RAPD markers in chickpea and strawberry populations mapped and suggested that these markers are valuable because of their genetic information content. According to Cai *et al* (1994) these non-parental heteroduplex bands could originate from intra-allelic interaction at an amplifiable region.

Consistency and reproducibility of RAPD patterns has been questioned by many molecular biologists and the sensitivity of the RAPD technique to change in experimental parameters is well known (Devos and Gale 1992; Munthali *et al* 1996 and Reidy *et al* 1992). Several cycling conditions and reaction components were tested in these studies in order to obtain an optimal RAPD protocol. The RAPD PCR amplifications were extremely sensitive and a single change in the concentration of reaction component and/or thermal cycling parameters appeared to be altering the RAPD patterns significantly. The sensitivity of PCR amplifications is perhaps not surprising because PCR amplifications are temperature dependent and any variation in PCR components would not provide the optimum temperature required for the denaturation and annealing, thereby resulting into an altered product. Nevertheless, once optimised, the technique has the potential of providing an effective and convenient method to generate molecular markers, which could be utilised in marker aided selection of complex traits.

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