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Salt Tolerance Associations with RAPD Markers in *Hordeum vulgare* L. and *H. spontaneum* C. Koch

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Abstract: Randomly Amplified Polymorphic DNAs (RAPDs) were used to search for markers associated with salt tolerance in barley. Initial screens involved growing 63 cultivated and wild barley genotypes in saline conditions and testing for shoot sodium content along with other physiological traits. From these tests 5 tolerant and 5 non-tolerant genotypes were selected. DNA from the tolerant and non-tolerant genotypes were formed into two contrasting bulks and interrogated using 30 different 10-mer RAPD primers. One primer (P15) produced a 5100 bp band found only in non-tolerant genotypes and additionally produced a 1300 bp product found only in the tolerant group. Primer P10 produced a band specific to tolerant bulk and P22 produced a band specific to the non-tolerant group.

Key words: RAPD markers, salt tolerance, cultivated, wild barleys

INTRODUCTION

Soil salinity is one of the major concern of arid and semi-arid areas of the world, including Iran. The exploitation of tolerant crops is one approach to the problem and barley is considered to be the most salt tolerant cereal crop (Maas *et al*., 1977; Gill and Dutt, 1987). Wide genetic variation exists for salt toleranc in both cultivated (*Hordeum vulgare* L.) and wild (*H. spontaneum* C. Koch) barley germplasm (Pakniyat *et al*., 1997). The wild species is an important source of genetic variation including salt tolerance for breeding programs. It is fully inter-fertile with cultivated barley and is considered the wild ancestor of cultivated barley (Harlan and Zohary, 1966).

Common tests for salt tolerance in barley have involved measuring tissue sodium content, carbon isotope composition ($\delta^{13}c$) and proline content which have been correlated with dry matter content in salinity tests (Forster *et al*., 1994; Pakniyat *et al*., 1997; Pakniyat *et al*., 2003; Greenway and Munn, 1980; Gorham *et al*., 1985; Flowers *et al*., 1977; Wyn Jones *et al*., 1984; Hampson and Simpson, 1990; Schachtman *et al*., 1991).

Genetic markers have frequently used in genetic studies of plants. In barley, these have included isozymes, storage proteins, restriction fragment length polymorpohisms ($RFLP_s$), randomly amplified polymorphic DNA_s ($RAPD_s$) and amplified fragment length polymorphism (AFLP) (Nevo *et al*., 1986; Doll and Brown, 1979; Chalmers *et al*., 1991; Saghai-Maroof *et al*., 1984; Dawson *et al*., 1993; Peterson *et al*., 1994; Sanchez de la Hoz *et al*., 1996; Russel *et al*., 1997; Pakniyat *et al*., 1997).

Association of AFLP markers to salt tolerance in 39 *H. spontaneum* genotypes collected from the Fertile Crescent has been shown by Pakniyat *et al*. (1997). They introduced 12 AFLP markers which showed correlation to salt tolerance in the wild barley genotypes. Here we used RAPDs as a quick and easy method to search for DNA markers associated with salt tolerance in selected cultivated and wild lines contrasting for salt tolerance. In the previous study, Pakniyat *et al*. (2003), used shoot sodium and proline content to screen for salt tolerance in 63 Iranian genotypes of cultivated and wild barley. From this study they selected the most tolerant and most non-tolerant genotypes. RAPD fingerprinting of the individual genotypes and the two contrasting bulks were used to identify markers associated with salt tolerance.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation: A total of 14 wild and cultivated Iranian barley genotypes were selected; 5 most tolerant, 5 most non-tolerant, an an additional group of 4 tolerant genotypes (Table 1). They were a subset of the tolerant and non-tolerant cultivated and wild genotypes selected from 63 tested genotypes (Pakniyat *et al*., 2003).

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Table 1: Fourteen selected barley genotypes formed into tolerant and non-tolerant groups using shoot Na^+ data from Pakniyat *et al.* (2003)

Genotypic identification	Na^+ content mg/g dry weight
Tolerant genotypes	
Vineyard-1 (Hs) *	10.77
Afzal (Hv)**	12.17
Victoria (Hv)	14.83
Plot. No. 21 (Hs)	15.50
Na-cc-4000-123/Walfajre (Hv)	15.75
Non-tolerant genotypes	
Vineyard-2 (Hs)	24.33
Asse/Karoon (Hv)	24.67
Reihane (Hv)	25.17
Star/Jenusa/em//Rihan-03(Hv)	27.00
Plot. No. 41 (Hs)	28.00
Additional tolerant genotypes	
Plot. No. 34 (Hs)	16.20
80-5010/Mona (Hv)	17.77
Valfajre (Hv)	16.90
Black grain (Hv)	17.67

**Hordeum spontaneum*, ***Hordeum vulgare*

Seeds were planted in 2 kg pots in the greenhouse. After 2 weeks DNA was extracted from plant shoots according to CTAB method of Doyle and Doyle (1987) with minor modifications. Young leaf (3.0 g) was ground to fine powder in liquid nitrogen and mixed with 6 mL of CTAB extraction buffer (15 mL TRIS-HCl, 21 mL 5 M NaCl, 3 mL 0.5 M EDTA (pH = 8), 1.5 mL 1% CTAB, 58 mL DH_2O, 1.5 mL mercaptoethanol). The sample was incubated at 60°C for 30 min, mixed with 4.5 mL of chloroform-isoamylalcohol (24:1) and centrifuged at 8000 rpm for 20 min. The aqueous supernant phase was recovered and mixed with 3 mL of isopropanol to precipitate the DNA. The nucleic acid precipitate was recovered with a glass hook and transferred to 70% ethanol for 15 min, centrifuged at 2000 rpm for 2 min and resuspended in 1.5 mL TE buffer (50 mM Tris-HCl (pH = 8) 10 mM EDTA, Chloroform-isoamylalcohol (24: 1), Isopropanol (100%), Ethanol 70%) and Rnase and kept at -20°C before PCR reaction.

Two bulks of 5 non-tolerant genotypes and 5 tolerant genotypes were formed (Table 1) and used in a bulked segregant analysis (Michelmore *et al*., 1991). DNA bulks consisted of 3 μL (15 ng μL^{-1} DNA) from each contributing genotype. The additional tolerant genotypes were used to test further any selected marker from the bulked analysis.

PCR conditions: PCR amplification reactions were carried out as described by Williams *et al*. (1990) with minor modifications. The 25 mL reaction mixture contained 2 mL, 15 ng μL^{-1} DNA, 1×PCR buffer, 1.9 μM $MgCl_2$, 0.4 μM primer (Sinagene, Iran), 0.1 μM each of $dNTP_s$ (Sinagene, Iran) and 0.06 units of Taq DNA polymerase (Sinagene, Iran). Thirty 10-mer oligonucleotide primers used for DNA amplification are (Table 2). Samples briefly centrifuged prior to amplification. DNA amplification reactions were performed in a Technogene Co. thermocycler subjected to following thermal cycles: 1 cycle of 30 min at 94°C followed by 40 cycle of 1 min at 94°C, 1 min at 38°C and 1 min at 72°C. After the final cycle, the samples were incubated for 4 min at 72°C and then held at 4°C prior to analysis. Amplification products were analyzed by gel electrophoresis on 0.7% agarose gels (GIBCO) in 1×TBE buffer. Gels were detected using ethidium bromide and UV light. Each amplification was performed using a single primer (Table 2) and gel scored for the presence and absence of products.

Table 2: Primer nucleotide sequence used to amplify DNA

Primer designation	Sequence 5'-3'	Primer designation	Sequence 5'-3'
P1	ACACAGAGGG	P16	CCTGGGCTTC
P2	CCTCTCGACA	P17	CCTGGGCTTG
P3	TCTCAGCTGG	P18	CCTGGGCCTA
P4	GTGTGCCCCA	P19	CCTGGGCCTC
P5	CCACGGGAAG	P20	TGCCCCGAGC
P6	TCGGCGGTTC	P21	TTCCCCGACC
P7	CTGCATCGTG	P22	GAGGGCGGGA
P8	TGAGCCTCAC	P23	AGGGGCGGGA
P9	TCGGCACGCA	P24	GAGGTCCAGA
P10	CTGCGCTGGA	P25	GGGGGTTAGG
P11	CCATTCCCCA	P26	ATCGGGTCCG
P12	GGTGAACGCT	P27	CCGTGCAGTA
P13	CTCCCTGAGC	P28	TAGCCGTGGC
P14	TTCCGGGTGC	P29	GGCTAGGGGG
P15	GAGCTCGCGA	P30	TACGTGCCCG

RESULTS AND DISCUSSION

All markers were scored for the presence or absence of the amplification products. On average each primer generated four amplification products which were considered to represent distinct genetic loci.

Four primers (P3, P26, P27 and P30) did not produce amplification products, two (P7 and P28) produced vague un-scorable bands and P17 and P20 did not produce any polymorphic band. The remaining 22 primers produced polymorphic bands suitable for analysis, of these 20 produced bands that were specific one or other of the two contrasting bulks. P10 for instance, produced a band (5000 bp) that was present only in the tolerant bulk and P22 generated a band (4000 bp) that was present only in the non-tolerant bulk (Fig. 1). P15 was particularly interesting, as it produced two polymorphic bands: a large band (5100 bp) product that was present only in non-tolerant genotypes and a smaller band (1300 bp) that was present only in salt tolerant genotypes including the additional salt tolerant genotypes (Fig. 2). These bands are therefore useful in screening for salt tolerance in both cultivated and wild barley genotypes and may be the first case where two RAPD markers from a single primer, alternate between extremes for a trait.

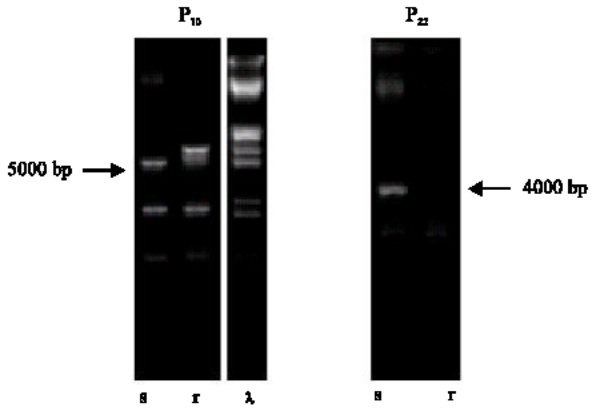

Fig. 1: RAPD banding patterns of bulked DNA for salt tolerant and non-tolerant genotypes using P10 and P22. Note the 5000 bp band in tolerant (r) bulk using P10 and 4000 bp band in non-tolerant (s) bulk using P22

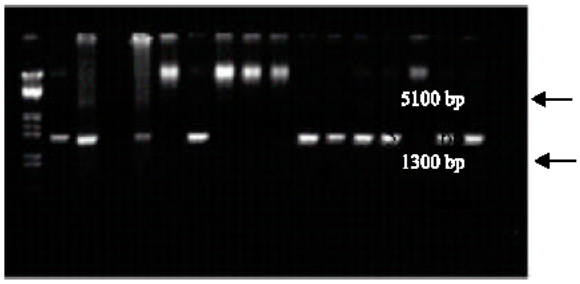

Fig. 2: RAPD banding patterns from DNAs of salt tolerant and non-tolerant barley genotypes using P15. The 5100 bp band is present in non-tolerant genotypes and the 1300 bp band is present in tolerant ones

The results indicated that RAPD technology is a powerful tool in quickly identifying markers in bulked analysis, in this case for salt tolerance. It is hoped that the discovery of markers associated with salt tolerance with aid the identification of the genes involved. One strategy for the P15 products is to sequence the smaller band and search against stress-related ESTs in public databases to identify the gene involved. The strategy can then be repeated for the larger band to see if the products are allelic. Sequence data can also be used to develop more robust PCR primers as diagnostics for salt tolerance. Another strategy is to map the two P15 products to confirm or otherwise genetic co-location.

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