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Full Length Research Paper

Maize breeding for marginal lands: Physiological and molecular approach to decipher response and selection of maize recombinant inbred lines (RILs) under water deficit at early growth stage

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Drought is the major abiotic stress constraining the production of maize (*Zea mays* L.) in the arid and semi-arid areas. Recombinant inbred lines (RILs) with improved performance from the 6th selection cycles were evaluated against drought stress at early growth stages and genetic distance was determined through random amplified polymorphic DNA (RAPD) primers. Significant variation was observed in the rate of water loss (RWL) at 30, 90 and 150 min after excision, relative water content (RWC) and membrane stability among the RILs. In comparison with check variety Azam, 7 RILs maintained a low RWL, 2 had higher RWC and 6 RILs had higher membrane stability, thus showing a higher degree of adaptation to drought stress. The preliminary results showed polymorphism among the drought sensitive and tolerant RILs. The RILs were clustered into three groups on the basis of amplification pattern obtained with 60 RAPD markers. Variation in the genetic makeup of the tolerant RILs was evident from their clustering in different groups, though most were clustered in group III. Furthermore, one RAPD marker could identify maize genotypes, maintaining low RWL from the excised leaves. We are in the process of sequencing the amplified product of this primer and transforming it into sequence characterized amplified region (SCAR) markers for a more reliable marker assisted selection.

Key words: Maize recombinant inbred lines (RILs), drought, physiological markers, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Global climate change is now generally considered to be underway and is expected to result in a long-term trend towards higher temperatures, greater evapotranspiration and an increased incidence of drought in specific regions (Hillel and Rosenzweig, 2002). Under these conditions, research into plant breeding for developing improved crop varieties and management practices to enhance plant water use efficiency and growth, when water is limiting has become increasingly important. As one of the major abiotic stress, drought causes differences between the mean and potential yield, as well as variation from year to year. It has been estimated that crops attain only 25% of their potential yield because of the detrimental effects of environmental stresses (Boyer, 1982). Maize is cultivated worldwide under very diverse climatic conditions and has the largest total annual grain production in the world (590.5 million metric tons, mmt) among the major grain crops. Though maize is essential for global food security, in several key production environments, the

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natural resource base (soil and groundwater) is becoming depleted through compaction, erosion, salinization, net nutrient export and diminishing water supply (Cassman, 1999). In different tropical climates, maize is often exposed to terminal and mid season drought. The genetic improvement for adaptation to drought through the conventional approach of selection for yield and its stability is slow because of the low heritability of yield under stress, the inherent variation in the field and the limitation that there is usually only one crop exposed to drought per year (Ribaut et al., 1996). Although selection of genotypes with increased productivity in drought conditions has been an important aspect of crop breeding, the biological basis for drought tolerance is still poorly understood. Maintenance of high photosynthesis rate (Gummuluru et al., 1989), osmotic adjustment to reduce water loss (Blum, 1989), maintenance of high instantaneous water efficiency defined as the ratio of leaf photosynthesis to transpiration (Morgan and LeCain, 1991), waxy layer on plant surface and deeper roots system are some of the traits found in drought tolerant genotypes.

The viability of using specific morpho-physiological traits as indicators for crop performance under water stress conditions is already proven (Bruce et al., 2002). However, the selected morpho-physiological traits must meet the requirements of cost and speed to be easily used in a breeding program. Several putative traits contributing to drought resistance have been suggested (Fukai and Cooper, 1995). During this experiment, the maize genotypes were evaluated for their drought tolerance potential at the seedling stage using three physiological markers, that is, rate of water loss from excised leaves, relative water content and membrane stability, which are highly correlated with grain yield (Fukai and Cooper, 1995; Bruce et al., 2002; Ferrat and Lovatt, 1999).

Traditional breeding programs that depend on phenotypic selection are time-consuming and less efficient (Collard et al., 2005). The value of molecular markers as a complement to phenotyping under several breeding scenarios is largely unquestioned, as demonstrated by the increasing number of successful studies published (Varshney et al., 2006). However, providing useful information about different genes and their closely linked markers involved in a complex trait like drought tolerance for molecular breeding strategies which is a difficult task. Recent advances in genomics and bioinformatics offer real opportunities for dissecting complex traits into their component sub-traits, which will simplify the process of developing the tools necessary to manipulate the underlying genes (Varshney et al., 2005). Many experiments have targeted crop improvement for disease resistance, morphological traits or quality traits (Torres, 2009) and there are still some ways to go before markers can be used routinely and ubiquitously to breed for complex traits, such as tolerance to abiotic stress (Ribaut and Ragot, 2007). The objective of this study is to use different physiological indicators and molecular tools to discriminate between maize genotypes with contrasting response to water deficit stress and to determine the genetic distance between the drought tolerant and susceptible genotypes at molecular level through random amplified polymorphic DNA (RAPD) primers. Such analysis can be used for selecting better genotypes for future breeding program as well as introgression of desirable drought tolerance traits into a single genotype.

MATERIALS AND METHODS

Plant material and stress treatment

Seeds from the 6th selection cycle of twenty two recombinant inbred lines (RILs) and the three open pollinated varieties (OPVs) were obtained from Cereal Crop Research Institute, Pirsabak, Nowshera. Azam, an open pollinated maize variety, known to be moderately drought tolerant was used as a control. Ten seeds from each genotype were planted in plastic pots filled with equal amount (15 kg) of potting material (1:1:1 sand, silt and farm yard manure) in a glass house. Saturation percentage of the soil mixture was calculated to be 35%, therefore each pot was provided 1.93 L of water on every third day. After complete germination, three uniform size plants of each genotype were maintained in plastic pots. Drought stress was imposed by withholding water from one month old seedlings for 20 days. Control plants were regularly provided with water on the third day.

Rate of water loss (RWL)

The rate of water loss from excised leaves was determined as previously reported (Basil et al., 2005; Jenk et al., 1994) with minor modifications. Briefly, the third fully expanded leaf of each genotype was excised at 10 o'clock in the morning and the petiole was sealed with silicon. The detached leaf was immediately weighed in a growth room at 340 $\mu mol.m^{-1}.s^{-1}$ PAR, 30°C and 55% relative humidity to the nearest 0.01 mg for recording initial leaf weight. The leaves were then weighed every 30 min for the next 3 h. Data was obtained from at least nine plants and the average was used as rate of water loss.

Relative water content (RWC)

Relative water content was determined on third leaf of each genotype under control and drought stress condition (Liu and Stützel, 2002). The leaves were immediately weighed with analytical balance to obtain fresh weight (Wf). The leaf samples were then completely immersed in double distilled water and were placed overnight at 4°C in dark to avoid respiratory losses. After 12 h, the samples were blotted dry on filter paper and weighed again to obtain the turgid weight (Wt). The samples were then oven dried at 70°C for 48 h and dry weights (Wd) were obtained. Relative water content was calculated using the following formula:

$$RWC = [(W_f - Wd) / (Wt - Wd)] \times 100$$

Percentage of electrolyte leakage

The electrolyte leakage percentage (%) from the leaf disks was calculated with a conductivity meter (Consort C-931, USA). The initial conductivity (C_i) was measured after subjecting the samples from controlled and drought stressed seedlings to incubation at

25°C in 5 ml de-ionized water for about 3 h with continuous shaking at 100 rpm. The samples were then autoclaved at 121°C for 20 min at 120 psi. Final conductivity (C_f) was measured after the samples had cooled down to 25°C.

DNA extraction

Approximately 100 mg young and fresh leaves were collected from plants in the green house and put in eppendorf tubes and subsequently dropped in liquid nitrogen to freeze the tissue. In the laboratory, the plant material was crushed with a knitting needle to a fine powder. To the crushed plant material was added 500 µl DNA extraction buffer (1% SDS, 100 mM NaCl, 100 mM Tris HCl pH 8.0, 50 mM EDTA pH 8.5) in each eppendorf tube and mixed well with hand shaking. Equal volume (500 µl) of phenol : chloroform : isoamylalcohol (ratio of 25:24:1) was then added and shaken until a homogenous mixture was obtained. Samples were then centrifuged at 14000 rpm for 5 min. The aqueous phase was transferred to a fresh tube. One-tenth volume (500 µl) of 3 M sodium acetate (pH 4.8) and equal volume (500 µl) isopropanol was added in the tube and mixed gently to precipitate the DNA. Samples were then centrifuged at 14000 rpm for 5 min to obtain the DNA pellet. After pouring the supernatant, the pellet was washed with 70% ethanol and dried at room temperature for an hour and re-suspended in 40 µl TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). To remove RNA, DNA was treated with 40 µg RNAse-A at 37°C for 1 h. After RNAse treatment, DNA samples were stored at 4°C. For polymerase chain reaction (PCR), a 1:4 dilution of DNA was made in doubled distilled, deionized and autoclaved water.

Polymerase chain reaction

The PCR was carried out using protocols of Devos and Gale (1992) with modifications. For PCR, primers RAPD were used. PCR reaction was carried out in 16 µl reaction containing 50 to 100 ng total genomic DNA template, 0.25 µM of each primer, 200 µM of each dATP, dGTP, dCTP, dTTP, 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase. Amplification conditions for RAPD primers was an initial denaturation step of 4 min at 95°C followed by 40 cycles each consisting of a denaturation step of 1 min at 94°C, annealing step of 1 min at 33°C and an extension step of 2 min at 72°C. The last cycle was followed by 10 min extension at 72°C. All amplification reactions were performed using a GeneAmp PCR System 2700 (Applied Biosystem) programmable thermocycler. The amplification products was electrophoresed on 1.5% agarose TBE gel, and visualized by staining with ethidium bromide under ultra-violet (UV) light.

Determination of genetic distance

Data generated from RAPD analysis was used to produce a binary data matrix by scoring the amplified fragment as present (+) or absent (-). A fragment was considered polymorphic if present in at least one genotype and absent in others. This binary matrix data was used to construct a dendrogram for cluster analysis using the unweighted pair group method of arithmetic averages (UPGMA) and the Genetyx Win 5.1 Software. For the construction of dendrogram, the +/- data was replaced with adenine and cytosine, respectively.

Statistical analysis

The experiment was conducted in completely randomized design with three replications. Analysis of variance (ANOVA) was used to determined significance between the means of different genotypes. When significant differences were noted, least significance difference (LSD) test was used to determine where the differences exist. All the statistical procedures were performed using MSTATC computer program.

RESULTS AND DISCUSSION

Water stress is an important limiting factor at the initial phase of plant growth and development. During this study, the effect of water deficit on the physiological performance of different maize RILs and established varieties was evaluated at early growth stages and the genetic distance between lines contrasting in response was determined using RAPD primers. Genetic variability among plants could only be observed after exposure to water deficit (Burke, 2001; Jones, 2007). The differential expression of genes involved in physiological phenomenon such as maintenance of relative water content, osmotic adjustment, photosynthetic rate and water use efficiency enables certain genotypes to adopt better to water deficit when compared with others (Bruce et al., 2002). The RILs used during the present study also exhibited significant variation in RWL, RWC and multiple selection index (MSI) under water deficit conditions at the early growth stages.

RWL from excised leaves and RWC

The first response of plants to a decrease in water availability is to avoid a low water potential, which is achieved either by closing the stomata to reduce transpiration or increase the concentration of compatible solutes (Jones, 2007). This in turn depends on the relative fluxes of water through the plant within the soil-plant-atmosphere continuum. Therefore, the short term response of plants to either a decline in leaf turgor and/or water potential due to drought stress is to reduce the rate of transpiration mostly by closing the stomata, indicating a close link between stomatal responses and leaf water status (Mansfield et al., 1990). Significant differences were noted in the water lost through transpiration from leaves of the RILs and check varieties after 30, 90 and 150 min of detachment (Table 1). The average water loss from the detached leaves of 6, 14 and 24% after 30, 90 and 150 min of excision was in accordance with those previously reported (Hu et al., 2010). After 30 min of excision, minimum loss of water from leaves was found in genotype J158-1 (28.57 mg H_2O g^{-1} FW). After 90 and 150 min of excision, however, the genotype J175-1 had minimum loss of water from the leaves (67.30 and 135.07 mg H_2O g^{-1} FW), followed by J175-2 (82.30 and 140.60). Maximum water loss, on the other hand, was recorded in the

Table 1. RWL at different time points after excision, RWC and electrolyte leakage of the RILs and check varieties under control and drought stress conditions. Means with at least one common letter are not significantly different at 0.05% level of probability according to LSD test.

Genotype	RWL (mg $H_2O.g^{-1}$ FW)			RWC (%)		Electrolyte leakage (%)	
	30 min	90 min	150 min	Control	Drought	Control	Drought
J 52-1	40.86 d	134.97 i	247.13 k	87.74	44.68 l	12.43	56.43 o
J 52-2	103.41 m	139.40 j	261.90 l	89.49	42.43 n	11.57	56.97 o
J 54-151	101.42 m	246.70 o	454.70 q	88.04	35.99 p	12.47	64.17 r
J 152-2	64.57 i	175.97 l	261.00 l	88.17	59.14 b	9.27	40.33 j
J 158-2	28.57 a	125.00 g	182.83 d	87.41	62.66 a	9.67	30.00 d
J 175-1	37.62 c	67.30 a	135.07 a	89.48	62.86 a	10.23	24.27 a
J 175-2	45.24 ef	82.30 b	140.60 ab	88.33	57.30 c	11.20	24.83 a
J 175-3	48.90 g	83.77 b	160.80 b	89.3	55.97 de	10.13	26.90 b
J 181-1	43.03 e	127.63 h	227.50 i	87.48	51.92 g	13.67	28.27 c
J 181-2	45.96 f	113.53 e	210.43 g	87.52	55.32 f	14.07	31.10 f
J 181-4	88.70 l	119.03 f	219.03 h	90.41	55.54 ef	12.97	36.53 g
J 185-2	57.64 h	197.83 n	352.60 o	92.74	40.49 o	9.70	45.77 l
P 28-2	77.97 k	102.50 d	170.50 c	86.85	56.11 d	11.30	30.50 de
P 28-3	153.20 n	157.17 k	247.23 k	86.52	49.45 i	9.50	46.37 m
P 34-2	43.67 e	258.67 p	365.33 p	89.28	40.49 o	11.73	61.63 p
P 43-2	40.55 d	113.13 e	193.43 e	89.84	42.47 n	10.57	31.37 f
P 56-6	68.57 j	90.43 c	171.30 c	86.18	46.45 j	10.17	31.13 f
P 124-1	45.51 f	136.63 i	240.80 j	90.26	50.85 h	10.40	46.60 m
P 124-2	56.60 h	137.43 i	250.73 k	85.55	55.02 f	12.00	38.53 i
P 124-3	67.66 j	123.03 g	200.30 f	89.55	45.83 k	10.43	37.40 h
P 124-5	67.58 j	156.87 k	270.23 m	91.23	43.91 m	12.77	54.27 n
P 124-6	51.89 gh	183.10 m	334.97 n	86.71	33.95 q	12.10	63.97 q
Jalal	30.65 b	140.57 j	242.90 jk	88.37	40.72 o	12.30	41.73 k
Pahari	47.21 fg	112.67 e	205.73 g	89.29	44.67 l	11.37	45.10 l
Azam	51.53 gh	120.63 f	198.43 ef	90.32	59.15 b	11.90	30.67 e

genotype P28-2 after 30 min (153.20 mg H_2O g^{-1} FW), and J54-151 had maximum water loss after 90 and 150 min (246.70 and 454.70 mg H_2O g^{-1} FW). Thus, it can be inferred from the data that the genotypes had responded differently to the disruption of water supply and a genetic variation does exist in the genotypes response to drought stress. After the 150 min period, six RILs were found to maintain a lower RWL when compared to the check variety Azam.

Since significant differences in rate of water loss were obtained among the genotypes, the RWC was then measured to identify genotypes that could maintain a high water potential after exposure to drought. Significant differences in the RWC of the RILs and check varieties were obtained under water stress conditions only (Table 1). Under irrigated conditions, the RWC was from 85.55 to 92.74%. When challenged with drought stress, there was a rapid decline in the RWC of all the genotypes. The three check varieties: Jalal, Pahari and Azam had RWC of 40.72, 44.66 and 59.15%, respectively. These values indicate that relative to control, the RWC in each check variety had decreased by 54, 50 and 35% under drought stress conditions. Mean values of the data indicated that the highest RWC of 62.86% was obtained in J175-1 followed by J158-2 with 62.66%. In contrast, minimum RWC of 33.95% was recorded in P124-6. Only two RILs could maintain a RWC better than the check variety Azam.

Water loss from the leaves mainly depends on stomatal opening. The stomatal behavior of plants in drying soil is regulated by long distance signals provided by plant hormones such as abscisic acid (ABA), xylem sap pH and inorganic ions that provide the shoot with some measure of water availability (Davies et al., 2002). Beside these factors, root development, production of compatible solutes, modulation of reactive oxygen species (ROS) production and scavenging also determines the RWC of the cells under dehydration stress. The genetic differences among the RILs to maintain a low steady state of ROS, production of compatible solutes and root develop-

ment could explain the difference between the RWL and RWC.

Membrane damage in the RILs and check varieties

When the membrane damage was determined by measuring the extent of electrolyte leakage from each genotype, no significant differences were seen under control conditions (Table 1). Significant differences were noted in the amount of electrolyte leakage from the various maize genotypes under drought stress conditions. Mean values of electrolyte leakage from different maize genotypes showed that under control conditions, electrolyte leakage was between 9.26 and 14.06 % in the maize genotypes. Electrolyte leakage from the check varieties Jalal, Pahari and Azam under well watered condition was 12.30, 11.36 and 11.90%, respectively. When the plants were exposed to drought stress, an increase in electrolyte leakage was noted, which is indicative of higher membrane damage. Minimum electrolyte leakage of 24.26% under drought stress conditions was recorded in J175-1, followed by J175-2 with electrolyte leakage of 24.83%. Maximum electrolyte leakage of 64.16%, on the other hand, was recorded in J54-151. The check varieties Jalal, Pahari and Azam had electrolyte leakage of 41.73, 45.10 and 30.66%, respectively, under drought stress condition. This difference of membrane stability among the genotypes could be due to variation in lipid peroxidation. The variation in lipid peroxidation is indicative of the genotypes capacity to scavenge ROS produced as a conesquence of drought. Changes in the different physiological processes as a result of decrease in RWC induced production of ROS. ROS can cause extensive peroxidation and de-esterification of membrane lipids, as well as lead to protein denaturation and mutation of nucleic acids (Bowler et al., 1992). Induction of ROS scavenging enzymes and low steady state of H_2O_2 in maize genotypes tolerant to drought stress has already been reported (Helal and Samir, 2008). Similarly, the transcripts of some of the antioxidant genes such as glutathione reductase (GR) or the ascorbate peroxidase (APX) is higher during the recovery of water deficit period and may play a role in the protection of cellular machinery against photo-oxidation by ROS (Ratnayaka et al., 2003).

Phylogenetic analysis of the genotypes

Phylogenetic analysis is an appropriate method for the interpretation of all possible relationships among a large group of genotypes. Sixty RAPD primers were used in the present experiment and a dendrogram was constructed on the basis of amplification pattern of these primers (Figure 1). The dendrogram revealed significant genetic divergence and the results were consistent with the drought tolerance potential of the different genotypes. The genotypes could be divided into three broad groups on the basis of the amplification pattern. Group I comprised of seven genotypes: Pahari, J181-2, P56-6, P124-2, J52-2, J54-151 and P124-6. This group can be further divided into two sub-groups. Group IA composed of Pahari, J181-2, P56-6 and P124-2 while Group IB inclu-ded J52-2, J54-151 and P124-6. Group II comprised of seven genotypes: P124-2, P124-3, P34-2, P124-1, J158-2, P28-2 and Jalal. Group II can also be divided into two sub-groups. Group IIA included P124-2, P124-3, P34-2, P124-1 and J158-2, while Group IIB included P28-2 and Jalal. Group III included maximum number of ten genotypes: J152-1, J181-1, P43-2, P28-3, Azam, J175-2, J181-4, J52-1, J175-2 and J175-1. This group can also be divided into two sub-groups. Group IIIA included J152-1, J181-1, P43-2, P28-3, Azam, J175-2 and J181-4, however the last named genotype was very distantly related with this subgroup. Group IIIB included J52-1, J175-2 and J175-1 (Figure 1). A comparison with the physiological indicators (Table 1) showed that Group III mostly included the drought tolerant genotypes. Drought tolerance is a complex trait and many genes are involved in making plants to be capable of avoiding this stress. Thus, the inclusion of drought tolerant genotypes in different groups is not unexpected.

Association between RAPD marker GLA-11 and RWL

The banding pattern obtained using primer GLA-11 (Genelink™, USA) is presented in Figure 2. A total of 64 bands were amplified in 25 genotypes giving an average of 2.56 bands per genotype. The bands amplified using this primer ranged from 750 to 2300 bp. All genotypes showed various levels of genetic polymorphism for the loci detected using primer GLA-11. Maximum number of five loci were amplified in the RIL J54-151, whereas a single loci could be amplified in RIL P124-1. Two bands (2320 and 1900 bp) were monomorphic and amplified in all the genotypes. Two bands (~1100 and 560 bp) were amplified in two and six genotypes each. A 1500 bp band was the most polymorphic and was missing in genotypes J52-1, J52-2, J54-151, J152-2, J181-1, J181-2, J181-4, J185-2**,** P28-3, P34-2, P124-1, P124-2, P124-5, P124-6 and Jalal. All these genotypes have a relatively high RWL at each time after detachment. Thus, this RAPD primer can be an indicator of selecting genotypes on the basis of RWL in future breeding programs. Polymorphism in amplified product of RAPD primers have previously been between freezing tolerant and susceptible clones of Euclyptus (Keil and Griffith, 1994). However, because of the low reproducibility of the RAPD primers (Jones et al., 1997), we are in the process of obtaining sequence information about the amplified product of this RAPD marker. The information will be used to design specific primers for development of sequence characterized amplified region (SCAR) marker. The SCAR markers can be used for a

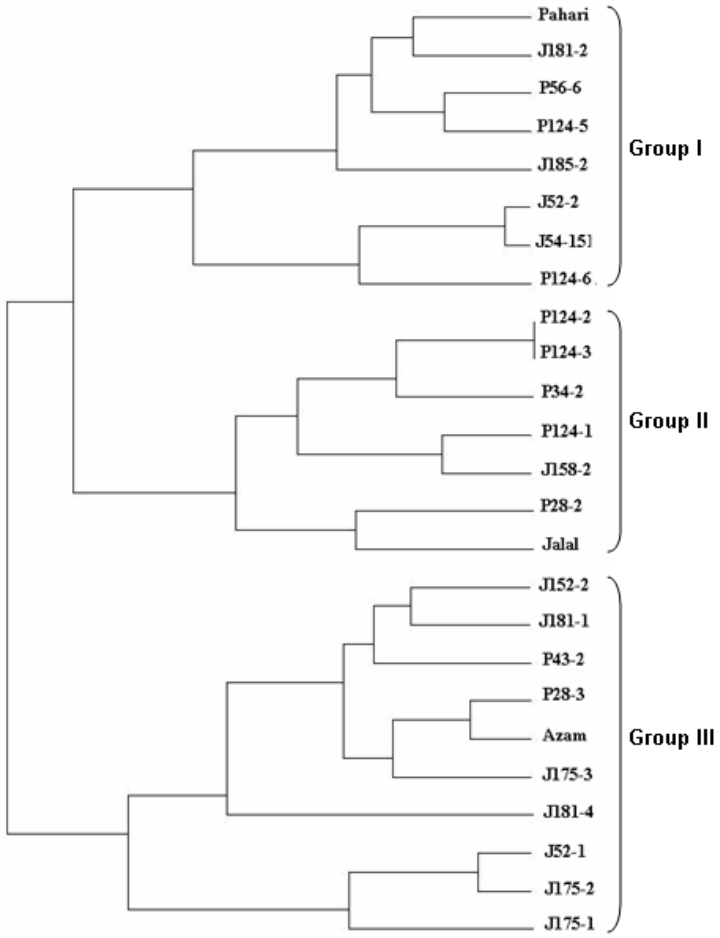

Figure 1. Dendrogram showing the genetic relationship among 25 maize genotypes revealed by UPGMA cluster analysis based on RAPDs through Genetyx Win Software.

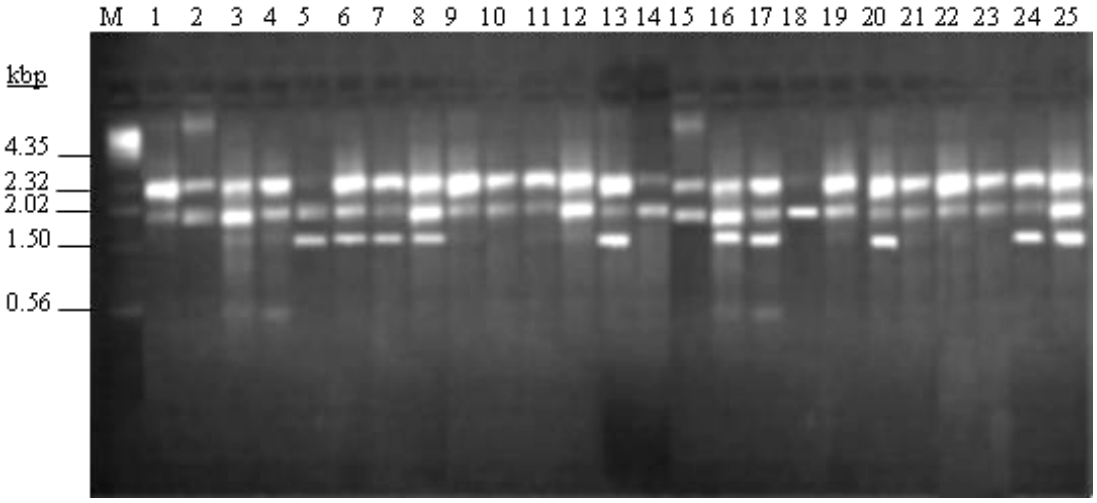

Figure 2. PCR profile of 25 genotypes of maize cultivars using RAPD primer GLA11. The white arrow show the polymorphic band amplified in the genotypes with a low RWL after 150 min of excision. 1, J52-1; 2, J52-2; 3, J54-151; 4, J152-2; 5, J 158-2; 6, J175-1; 7, J175-2; 8, J175-3; 9, J181-1; 10, J181-2; 11, J181-4; 12, J185-2; 13, P28-2; 14, P28-3; 15, P34-2; 16, P43-2; 17, P56-6; 18, P124-1; 19, P124-2; 20, P124-3; 21, P124-5; 22, P124-6; 23, Jalal; 24, Pahari; 25, Azam. M, molecular weight marker.

more reliable selection of genotypes with improved water relations under drought stress condition.

Abbreviations

RAPD, Random amplified polymorphic DNA; RILs, recombinant inbred lines; **OPVs,** open pollinated varieties; **RWL, rate of water loss; RWC, relative water content; Wf,** fresh weight; **Wt,** turgid weight; **Wd,** dry weights; C_i, initial conductivity; C_f, final conductivity; **PCR,** polymerase chain reaction; **MSI,** multiple selection index; **ABA,** abscisic acid; **ROS,** reactive oxygen species; **GR,** glutathione reductase; **APX,** ascorbate peroxidase; **SCAR,** sequence characterized amplified region.

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