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IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH MAIZE RESISTANCE TO *Sesamia cretica*

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ABSTRACT

Seed storage proteins of eight inbred lines of maize showing clear differences in their reaction to the pink stem borer Sesamia cretica (four resistant and four susceptible) were biochemically analyzed using sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique in order to identify some molecular marker(s) associated with resistance to S. cretica. High level of polymorphism (60%) was exhibited .Each inbred line was identified by at least one specific band (positive marker). Thus SDS-PAGE technique was successful to discriminate among all tested inbreds and to assign a unique fingerprint for each inbred. The data indicated the presence of one band at 60.1 kDa in all resistant inbreds (MBR 102, CML 135, CML 105 and CML 67) which was associated with maize resistance and another one band of MW 60.0 kDa in all susceptible inbreds (Sd 7, Sd 63, GZ 649 and B 73) which was associated with maize susceptibility to the pink stem borer. These markers could assist the maize breeders to accurately and rapidly select genotypes for resistance to this insect.

Key words: ***Zea mays, Maize, Corn, Sesamia cretica, Pink stem borer, Insect resistance, SDS-PAGE, Molecular marker.***

INTRODUCTION

The first step in designing an efficient breeding program for resistance to a certain insect is to identify sources of resistance, and to determine how plant behavior under insect attack is transmitted from the original parents to the improved cultivars (Pathak 1990). Considerable efforts has been devoted to identifying and developing corn germplasm with resistance to damage by the pink stem borer *Sesamia cretica* through scoring symptoms of plant damage after artificial infestation (Al-Naggar *et al* 2000, Saafan 2003 and Soliman 2003).

Morphological characters have been used to identify plant species, families and varieties. However, they have many disadvantages where they are influenced by the environment and scoring is a time-consuming process. Therefore, it is necessary to find alternatives to this morphologically-based approach by the use of genetic molecular markers (protein and DNA-based) which are becoming widely accepted valuable tools for different breeding

applications (Cooke 1999). Sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) becomes the technique of choice for the identification and characterization of different genotypes, since it is considered as a low cost, reproducible and rapid approach (Laemmli 1970). This technique proved to be successful in discriminating among a number of inbred lines regarding maize resistance to corn borers other than *S. cretica* (Abdel-Tawab 2004). Moreover, breeders can use genetic similarity information to make informed decisions regarding the choice of genotypes to be crossed for the development of populations or to facilitate the identification of diverse parents to be crossed in hybrid combinations in order to maximize the expression of heterosis (Smith *et al* 1990 and Santos *et al* 1994). There are no published studies on using SDS-PAGE for differentiation among maize genotypes for their resistance to the pink stem borer *S. cretica*. The main objective of the present investigation was to identify some molecular marker(s) predicted to be associated with maize resistance to *S. cretica*.

MATERIALS AND METHODS

The seed samples were taken from eight parental maize inbred lines, four of them (MBR 102, CML 135, CML 105 and CML 67) proved highly resistant and the other four (Sd 7, Sd 63, GZ 649 and B 73) proved highly susceptible to attack of the pink stem borer *S. cretica* based on previous experiments (Al-Naggar *et al* 2006). The inbreds MBR 102, CML 135, CML 105 and CML 67 were introduced from CIMMYT, Mexico, B 73 from USA while Sd 7, Sd 63 and GZ 649 were bred in Egypt. Sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used according to the method of Laemmli (1970) as modified by Studier (1973).

Extraction of water-soluble protein:

Ground kernels (0.5 g of each genotype) were inserted into Eppendorf tube, mixed with 1 ml of water-soluble extraction buffer and left in refrigerator overnight then centrifuged for 20 minutes at 12000 rpm at 4°C. Supernatants containing protein fractions were transferred to new tubes and stored at -20°C until used for electrophoresis analysis.

The acrylamide stocks (kept in dark at 4°C) consisted of 30 g acrylamide, 0.8 g bis-acrylamide and dH2O up to 100 ml for resolving gel, 30 g Acrylamide, 0.1 g bis-acrylamide and up to 100 ml dH_2O for stacking gel.

The buffers were prepared by the addition of 6 ml Tris (1 M, pH 8.8), 20 ml SDS (10%), 10 ml glycerol, 800 µl EDTA (0.25 M) and up to 100 ml dH_2O, for extraction buffer (0x), 18.15 g Tris, 3.5 ml HCl (conc.)

and up to 100 ml dH_2O for the resolving gel buffer (4x Tris, pH 8.4, 4°C), 12.11 g Tris and up to 100 ml dH_2O for the stacking gel buffer (1M Tris-HCl, pH 6.8, 4°C) and 15.14 g Tris, 72.07 g Glycine, 50 ml SDS (10%) and up to 5 L dH_2O for the run buffer.

The gel preparation was performed in a vertical slab (18 x 16 cm) gel electrophoresis apparatus as marketed by Hoefer (Hoefer SE 600 series CHSI). All glass plates were washed with tap and distilled water, then surface sterilized with ethanol. Spacers of 1.5 mm were used.

The resolving gel (15% acrylamide) was prepared by the addition of 31.66 ml acrylamide stock (for resolving gel), 16.25 ml Tris (4x, pH 8.4) and 16.10 ml dH_2O. This solution was filtrated, and then the following ingredients were added: 750 µl SDS (10%), 500 µl ammonium persulfate (10%) and 100 µl TEMED. This solution was instantly swirled. Two gels were poured simultaneously to a height of 1.5 cm below the bottom of the comb. Gels were overlaid with isopropanol and left to polymerize for at least 1 hour. Isopropanol was removed before the stacking gel was poured. The stacking gel was preposed by addition of 2.66 ml acrylamide stock (for stacking gel), 2.50 ml Tris (pH 6.8) and 14.70 ml dH_2O. This solution was filtrated, and then the following ingredients were added: 250 µl SDS (10%), 100 µl ammonium persulfate (10%) and 40 µl TEMED. This stacking gel solution was quickly poured over the two resolving gels, and 15 well combs were used. Gels were left to polymerize for 45 min before gels were run.

Application of samples

A volume of 50 µl protein extract for each sample was added to the same volume of extraction buffer. B-mercaptoethanol (10 µl) was added to each sample. Samples were boiled for 10 minutes in water bath, then, 10 µl bromophenol blue solution (0.25 g in 100 ml g H_2O) was added to each tube before samples loading (50 µl).

Gel running and staining

Four liters of the run buffer were poured into the running tank to be pre-cooled by flooding cold water (4°C) through cooling tubes. The run buffer was added to the upper tank before running. Gels were run at 100V for 15 minutes and the voltage was raised to 150 V until the dye reaches one inch from the apparatus and placed in plastic tanks, then covered with staining solution. Gels were gently agitated overnight. The staining solution was prepared by the addition of 1 g commassie brilliant blue-R250, 455 m methanol, 90 ml acetic acid (conc.) and 455 ml dH_2O. After removing the

staining solution, gels were covered with a destaining solution composed of 700 ml methanol, 200 ml acetic acid (conc.) and up to 3500 ml dH_2O.

Gels were gently agitated for 1 hour. After removing the solution, a new one was added. This step was repeated several times until gel's background becomes clear to be photographed. Data were scored for computer analysis on the basis of presence or absence of protein bands. To ease computer analysis the protein band was designated as "+" when present in any given genotype, while designated as "-" when absent. Pair-wise comparisons of inbreds, based on the presence or absence of unique and shared polymorphic bands were used to generate similarity coefficients. Genetic similarity (Dice) coefficients among genotypes were estimated according to Sokol and Sneath (1973). The similarity coefficients were then used to construct a dendrogram by UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) using NTSYS-pc (Rohlf 1993).

RESULTS AND DISCUSSION

Fingerprinting for the eight parental inbred lines (Sd 7, Sd 63, GZ 649 and B 73 used as highly susceptible and MBR 102, CML 135, CML 105 and CML 67 used as highly resistant) by using SDS-PAGE water-soluble proteins is shown in Table (1) and Figure (1). Data exhibited a maximum number of 32 bands (ranging from 29.3 to 130.3 kDa), which were not necessarily present in all inbreds, 19 of them were polymorphic. This represented a level of polymorphism of as high as 60 %. The highest number of bands (10 bands) was found in maize inbreds MBR 102, CML 135 and CML 105. The lowest number of bands (8 bands) was found in the inbreds Sd 7, Sd 63, B 73 and CML 67.

Bands number 28, 29, 7 and 20 with MW of 40.5, 38.1, 108.7 and 58.0 kDa, were found only in the inbreds Sd 7, Sd 63, B 73 and CML 67, respectively and therefore could be considered as single specific bands (single positive markers) for these inbreds.

Bands number 3 (113.7 KDa) and 14 (82.3 kDa) were found only in inbred line GZ 649, while bands number 10 (101.3 kDa) and 24 (46.2 kDa) existed only in the inbred line MBR 102, and bands number 1 (130.3 kDa) and 23 (47.8 kDa) were found only in the inbred line CML 105 as two specific bands (2 positive markers) for these inbreds. The inbred line CML 135 had the maximum number of specific bands (3 positive markers) *i.e.*

Table 1. SDS-PAGE of water soluble protein extracted from the eight maize inbred lines (L1= Sd 7, L2= Sd 63, L3= GZ649, L4= B 73, L5= MBR 102, L6= CML 135, L7= CML 105 and L8= CML 67).

No. Band	MW (KDa)	Inbreds							
		L1	L2	L3	L4	L5	L6	L7	L8
1	130.3	-	-	-	-	-	-	+	-
2	115.6	+	-	+	+	+	+	+	+
3	113.7	-	-	+	-	-	-	-	-
4	113.0	-	+	-	-	-	+	-	-
5	112.7	-	-	-	-	+	-	+	-
6	109.3	-	-	-	-	+	+	+	-
7	108.7	-	-	-	+	-	-	-	-
8	107.4	-	+	-	-	-	-	-	+
9	106.1	+	-	+	-	-	-	-	-
10	101.3	-	-	-	-	+	-	-	-
11	98.3	-	-	-	-	-	+	-	-
12	92.0	+	+	-	+	-	-	-	-
13	90.6	-	-	-	-	+	-	+	+
14	82.3	-	-	+	-	-	-	-	-
15	70.3	+	+	-	+	+	+	+	-
16	66.5	-	-	+	-	-	-	-	+
17	61.1	-	-	-	-	+	+	+	+
18	60.0	+	+	+	+	-	-	-	-
19	59.7	-	-	-	-	-	+	-	-
20	58.0	-	-	-	-	-	-	-	+
21	56.6	-	+	-	+	+	+	+	-
22	54.7	+	-	+	-	-	-	-	-
23	47.8	-	-	-	-	-	-	+	-
24	46.2	-	-	-	-	+	-	-	-
25	44.7	-	-	+	-	-	-	-	+
26	44.2	-	-	-	+	+	-	-	-
27	41.5	-	-	-	-	-	+	-	-
28	40.5	+	-	-	-	-	-	-	-
29	38.1	-	+	-	-	-	-	-	-
30	36.5	-	-	-	-	-	-	+	+
31	32.9	+	-	+	+	-	-	-	-
32	29.3	-	+	-	-	-	+	-	-
No. of total bands		8	8	9	8	10	10	10	8
No. of positive bands		1	1	2	1	2	3	2	1

+ = Band presence - = Band absence

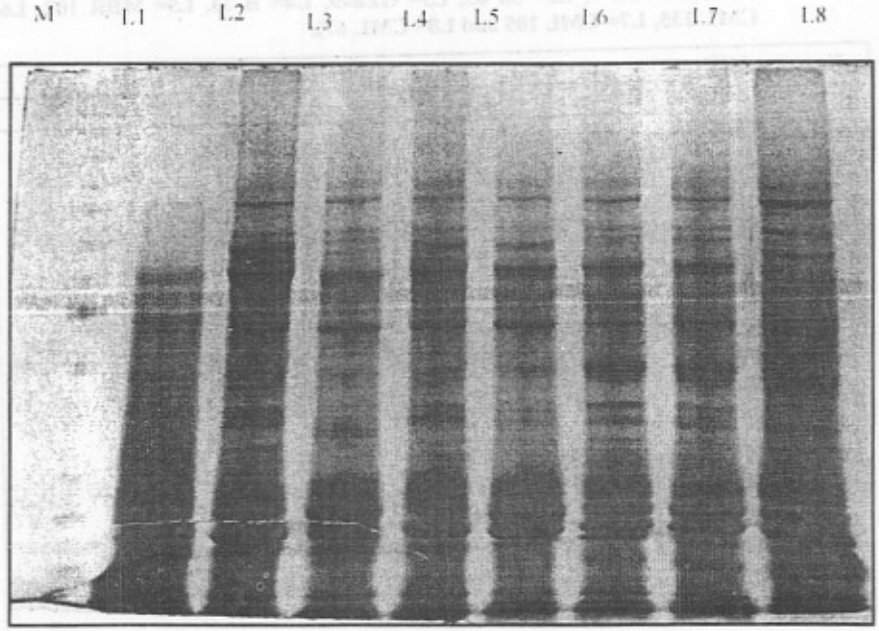

Fig. 1. SDS-PAGE of total protein of the eight maize inbred lines (M= marker, L1= Sd 7, L2= Sd 63, L3= GZ649, L4= B 73, L5= MBR 102, L6= CML 135, L7= CML 105 and L8= CML 67).

bands number 11 (98.3 kDa), 19 (59.7 kda) and 27 (41.5 kDa). All these bands are considered as positive specific markers for these inbreds.

Band number 2 (115.6 kDa) was absent only in the inbred Sd 63, so it could be considered as a negative marker for this inbred. The data indicated the presence of one band at 61.1 kDa as a positive genetic marker associated with maize resistance to *S. cretica*, because it appeared in all resistant inbreds (Table 1). Data also indicated the presence of one band with MW of 60.0 kDa as a positive genetic marker supposed to be associated with maize susceptibility to the same insect. These results confirmed that for the first time SDS protein banding patterns for water soluble fraction of seed storage proteins was successful in generating biochemical genetic markers related to maize resistance to *S. cretica*. Our results agreed with those obtained by Abdel-Tawab (2004) who found a positive genetic marker associated with maize resistance to corn borers

other than *S. cretica* at 102.5 kDa and another one at 9.1 kDa as a positive genetic marker associated with maize susceptibility to such borers.

The data of SDS-PAGE were loaded to the computer program (SPSS Windows version 10) to get a dendrogram for genetic distance and similarity matrix as shown in Table (2) and Figure (2). The highest similarity (90 %) appeared among the inbred pairs (Sd 7 and GZ 649) and (B 73 and CML 67), while the lowest similarity (12 %) appeared between inbred pairs (GZ 649 and MBR 102) and (GZ 649 and CML 135). The mean value of genetic similarity was 54.6 %.

Table 2. Similarity matrix among the eight maize inbred lines based on SDS-protein analysis (L1= Sd 7, L2= Sd 63, L3= GZ649, L4= B 73, L5= MBR 102, L6= CML 135, L7= CML 105 and L8= CML 67).

Inbred	L1	L2	L3	L4	L5	L6	L7
L2	0.37						
L3	0.9	0.50					
L4	0.75	0.62	0.62				
L5	0.25	0.37	0.12	0.80			
L6	0.50	0.66	0.12	0.75	0.75		
L7	0.75	0.62	0.62	0.25	0.15	0.25	
L8	0.50	0.80	0.87	0.90	0.75	0.50	0.25

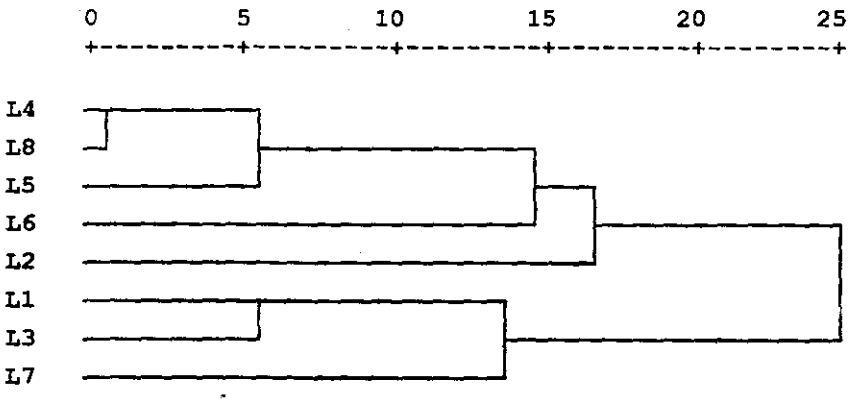

Fig. 2. Dendrogram of the genetic distances among the eight maize inbred lines based on protein analysis (L1= Sd 7, L2= Sd 63, L3= GZ649, L4= B 73, L5= MBR 102, L6= CML 135, L7= CML 105 and L8= CML 67).

The dendrogram (Figure 2) separated the eight parental lines into two clusters, one involved inbreds B 73, CML 67, MBR 102, CML 135 and Sd 63, while the other involved the remaining inbreds (Sd 7, GZ 649 and CML 105). However, inbreds B 73 and CML 67, which belong to the first cluster, were more related to each other. In addition, inbreds Sd 7 and GZ 649, in the second cluster were closely related.

In general, protein electrophoresis system used in this study was successful to discriminate among all tested inbreds and to assign for each of them a unique fingerprint. In this regard, Abdel-Tawab *et al* (1989) reported that the protein electrophoresis bands could be considered as a useful tool for the identification of maize inbreds. In addition, Gorinstein *et al* (1999) confirmed that electrophoresis patterns of the protein fractions are directly related to the genetic background of the protein. Moreover, Nivio and Magoja (1998) stated that biochemical traits are suitable for use in cluster analysis and reveal considerable variation among maize lines.

Results of this study reveal for the first time the presence of a positive genetic marker (PUM) for identifying resistant maize genotypes to the pink stem borer *S. cretica*. This should promote further work in the future to investigate this finding in more details by using DNA-based molecular markers techniques.

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التعرف على واسمات جزيئية مصاحبة لمقاومة الذرة الشامية لدودة القصب الكبيرة.

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تم إجراء التفريد الكهربى للبروتينات القابلة للذوبان فى الماء المختزنة فى الحبوب بطريقة SDS-PAGE لثمانية سلالات مربأة داخلياً من الذرة الشامية تظهر إختلافاً واضحاً فى مقاومتها الحقلية لدودة القصب الكبيرة Sesamia cretica (أربعة منها مقاومة MBR 102, CML 135, CML 105, CML 67 والأربعة الأخرى حساسة Sd 7, Sd 63, GZ649, B 73) بهدف رئيسى هو التعرف على حزم بروتينية مميزة للتراكيب الوراثية المقاومة للحشرة. أمكن الحصول على عدد ٣٢ حزمة band منها ١٩ حزمة (٦٠%) ظهرت فى سلالتين او أكثر مما يدل على وجود درجة عالية من التباين بين السلالات المختبرة. و قد أمكن الحصول على حزمة واحدة موجبة على الأقل مميزة لكل سلالة من السلالات الأبوية الثمانية مما يدل على نجاح طريقة التفريد الكهربى على مستوى البروتين الذائب فى الماء فى التمييز بين السلالات المختبرة. اشتركت كل السلالات المقاومة لحشرة دودة القصب الكبيرة فى ظهور حزمة بروتينية واحدة موجبة (ذات وزن جزيئى ٦١,٦ ك دالتون) اعتبرت أنها واسمة جزيئية خاصة بصفة المقاومة و كذلك اشتركت كل السلالات المصابة فى ظهور حزمة بروتينية أخرى (ذات وزن جزيئى ٦٠,٠ ك دالتون) اعتبرت أنها واسمة جزيئية خاصة بصفةالإصابة. هذه الواسمات يمكن أن تساعد مربى الذرة الشامية فى التعرف الدقيق و السريع على التراكيب الوراثية المقاومة لدودة القصب الكبيرة.

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