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Upgraded Horizontal Polyacrylamide Gel Units for DNA Marker Genotyping

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Abstract

DNA marker technology represents a potential tool for molecular breeding. The successful deployment of this technology depends on availability of reliable and simple genotyping platforms. But, most of the lab which dealing with molecular breeding has poor lab facility and they rely on horizontal agarose gel electrophoresis for genotyping. The poor resolution provided by this method in terms of allele differences is the major setback for research community. Therefore, we tried to introduce a simple method for upgrading the existing horizontal agarose units into horizontal polyacrylamide gel electrophoresis (H-PAGE) units with few amendments. This method can provide higher resolution with less effort and allows easy genotyping for different markers systems as compared to vertical PAGE units. In-order to reduce time, we employed fast staining methods such as ethidium bromide and fast silver staining for 20 min. The performance of modified unit was checked by separating and scoring SSR, SRAP and TRAP markers on 30 pigeon pea genotypes. Number of bands scored and size range observed for each marker technique was comparable to earlier results as reported for vertical PAGE based genotyping. Therefore, these H-PAGE units could be a valuable tool for molecular breeding.

Keywords: Horizontal Polyacrylamide Gel, Marker Genotyping

1. Introduction

Sequencing projects in various crops have generated huge ample of genomic data. The data stored in public databases have provided new directions for marker technology by initiating gradual shift in type of PCR based techniques commonly used in plant science[1]. Through bioinformatics tools, number of gene or genomic based marker techniques were developed such as viz., cleaved amplifed polymorphic sites (CAPS), derived cleaved amplifed polymorphic sites (dCAPS), expressed sequence tag-simple sequence repeats (EST-SSR), simple sequence repeats (SSR), sequence related amplifcation polymorphism (SRAP), targeted region amplifcation polymorphism (TRAP), sequence specific amplifcation polymorphism (SSAP) and inter-primer binding site amplifcation (iPBS). These techniques were used successfully in many crops for genetic diversity study, construction of linkage maps, mapping and tagging of genes/QTL's, association mapping and comparative genomics[2–11]. In-order to get reliable genotyping information in terms of allele/fragment polymorphism, most of these techniques depends on either capillary or high resolution polyacrylamide gel electrophoresis. But, due to high expenses with capillary or technical difficultly in casting and handling vertical PAGE units, many researchers go for horizontal agarose gel electrophoresis for separation of markers. Some researcher even tried specialized agarose like super fine agarose and metaphor to get higher resolution for marker separation. But, these agarose were found to be very costlier than normal agarose and provides less resolution than polyacrylamide[12]. Polyacrylamide gels even though provide relatively high resolution, but require expensive vertical gel units and also tedious steps to cast the gels.

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Therefore, horizontal agarose gel electrophoresis was found very handy for many researchers. However, this method provides less information due to low resolution of marker separation. Therefore, for successful molecular breeding research require a simple genotyping platform that suits for various molecular marker techniques and provide more information. With this background, here we tried to introduce a simple method for upgrading routine horizontal agarose units into H-PAGE units. This unit can provide high resolution and easy to perform as that of horizontal agarose gel electrophoresis.

2. Materials and Methods

2.1 Electrophoresis unit

For upgradation, we used horizontal agarose unit of 22 x 30 cm (LxW) dimension (Maxi sub system, Bangalore GeNei, India). In order suit this unit to perform H-PAGE, following accessories were arranged i). Single glass plate of 17 x 20 cm, with 3 mm thickness was used as covering plate to cover 20 x 20.5 cm gel casting tray ii). Two sided tape with 20 x 1 cm, with 2 mm thickness was used as a spacer iii). Small glass piece of 20 cm x 0.9 cm, with 3 mm thickness was used for holding comb iv). 40 wells comb, with 2 mm tooth width and 1 mm thickness was used for casting wells v). Rain repellent solution (Clear Vue®, Turtle Wax Europe B.V) was used to treat outer and inner surface of casting tray and covering glass plate. The detail steps followed for performing H-PAGE was shown in figure 1A-L.

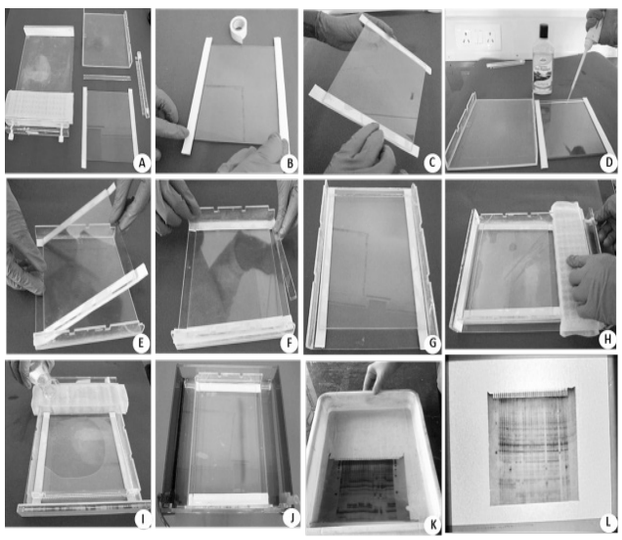

Figure 1. Steps for assembling, casting, separation and staining of PCR products on H-PAGE A. Materials used for up gradation *viz*., gel casting assembly, casting tray, small glass piece, covering glass plate, double sided tape and comb; B. Pasting of double sided tape on vertical edges of the top covering glass plate; C. Top glass plate with spacers extended at top and bottom side; D. Treating with rain repellent solution; E. Placing of top glass plate on to the casting tray; F. Placing of glass piece to create space for inserting comb; G, H & I. Assembling and pouring of gel from the bottom side of covering plate; J. Separation of PCR products; K. Staining of gel using fast silver staining method; L. Imaging and scoring of gel under white lamp.

2.2 Assembling of Gel Casting Tray

The materials used and steps followed for assembling gel casting tray was shown in Figure 1A-H. Vertical edges of covering glass plate (17 x 20 cm) were pasted with one side glue of two sided tape (20 x 1 cm). That will extended 1 cm at top and 2 cm at bottom side of plate (Figure 1B & C). Then outer surface of gel casting tray and inner surface of covering glass plate were treated with 300 µl of repellent solution (Figure 1D). The covering glass plate was placed on casting tray such that 2 mm space created due to spacers (Figure 1E). Small glass piece of 20 x 0.9 cm, was placed on top 1 cm extended side of spacer to create 1 mm space for inserting comb (Figure 1F). Finally, gel casting tray was assembled into the gel casting platform as shown in Figure 1G & H. The 2 cm space left at bottom side of covering plate was used for pour polyacrylamide gel solution.

2.3 Preparation and Casting of 6% Polyacrylamide Gel

In-order to cast 6% (w/v) non-denaturing polyacrylamide gel of 20 x 18 cm and 2 mm thickness. 80 ml of gel solution was prepared, which constituted acrylamide/bis-acrylamide mix (40%), 1X TBE buffer, 15% (w/v) ammonium persulfate and 0.14% (w/v) TEMED. Once incorporation of appropriate volume of ammonium persulfate and TEMED, the gel solution was mixed and poured directly between glass plates from bottom side of the covering plate. Comb was placed on top side of the glass plate in a space left for inserting comb (Figure 1I). Finally, gel was allowed to polymerize at room temperature for 30 min.

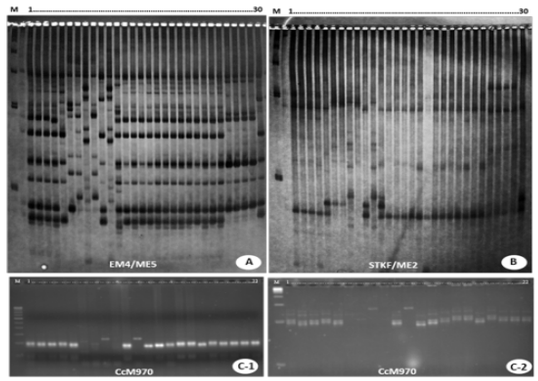

Figure 2. Representative amplification profile for different markers systems on 6% H-PAGE A. SRAP using primer EM4/ME5; B. TRAP using primer STKF/ME2; C-1 & C-2. SSR using CcM970 primer separated on agarose and on H-PAGE. Lanes: M: 100 bp ladder; 1-30 & 1-22 indicates number pigeonpea genotypes used for genotyping.

2.4 PCR Amplification

The diluted DNA samples of 30 pigeonpea genotypes were used for genotyping. PCR amplification were performed with minor modifications as described for SSR, SRAP and TRAP[13–15]. For SSR, 10 µl reactions volume constituted 10 ng genomic DNA, 1 µl 10X PCR buffer (15 mM of $MgCl_2$), 1 mM dNTP mix, 10 µM of forward and reverse primer and 0.2 µl Taq DNA polymerase (3U/ µl). PCR program: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension of 72°C for 7 min. Similarly for SRAP, 10 µl of reaction volume comprised of 1 µl genomic DNA (20 ng/µl), 1 µl 10X PCR buffer, 2 mM dNTP mix, 10 µM each forward and reverse primer and 0.3 µl Taq DNA polymerase (3U/ µl). PCR program: initial denaturation at 94°C for 5 min, followed by 5 cycles of 94°C for 1min, 35°C for 1 min and 72°C for 40 sec, in the following 35 cycles, the annealing temperature was increased to 50°C, with a final extension of 72°C for 7 min. For TRAP, 10 µl reaction volume with 1 µl genomic DNA (20 ng/µl), 1 µl 10X PCR buffer (with 25 mM of $MgCl_2$), 2 mM dNTP mix, 1 µl of 10 µM forward and reverse primer and 0.3 µl Taq DNA polymerase (3U/µl) was used. PCR program: initial denaturation at 94°C for 4 min, followed by 5 cycles of 94°C for 45 sec, 35°C for 45 sec and 72°C for 1 min, in the following 35 cycles, the annealing temperature was increased to 52°C, with a final extension of 72°C for 7 min. Before separation of PCR product of SRAP and TRAP markers. Samples were denatured by adding 3 µl of formamide gel loading dye and incubated at 95 °C for 10 min.

2.5 Separation of PCR Products

After polymerization, comb was gently removed from the gel without disturbing wells as well as small glass piece used for holding comb. The gel along with covering plate was placed in running tank and 1X TBE running buffer was poured into tanks up to the level of top covering glass plate (don't submerge the covering plate). Now, by using 1 ml pipette all the wells were filled with running buffer. Finally, by mixing 3 µl of gel loading dye to 10 µl of PCR products, 8 µl of sample was load for SRAP and TRAP after denaturation, and 4 µl of sample for SSR. The gel was run in 1X TBE buffer at 180 V for 5 hrs and stopped when backward dye (xylene cyanol) reaches to 4/4th of the gel for SRAP and TRAP, 3/4th of the gel for SSR (Figure 1J). After completion of run, top covering glass plate was separated from the gel with the help of plastic wedge. Finally, gel was slowly slided in to gel staining container by squeezing water with the help of squeeze bottle.

2.6 Staining and Scoring of Gel

In order accomplish quick staining of gels two different staining methods were followed. For SSR, ethidium bromide staining method was used[16]. The gel was stained by submerging in 300 ml of distilled water mixed with 15 µl ethidium bromide (10 mg/ml) for 15 to 20 min in dark. After staining, left over solution was stored in the dark bottles and reused for 3 times. Gel was visualized and photographed in gel documentation unit (Alpha Innotech, India). For SRAP and TRAP markers, fast silver staining method was employed as described by Benbouza et al[17]. It involved the following steps: after electrophoresis, gels were washed in 200 ml cold (10-12°C) fixing solution (10% absolute ethanol, 0.5% acetic acid) for 5 min. Washed gels were soaked for 6-7 min at room temperature (22-24°C) in a 200 ml solution of 0.15% $AgNO_3$, 200 µl 37% HCOH. Gels were rinsed quickly (10-15 s) once with 200 ml distilled H_2O. They were then developed by soaking them at room temperature (22-24°C) in a 200 ml developing solution (1.5% NaOH, 300 µl 37% HCOH) until the bands appear with a sufficient intensity (3-5 min). When the desired intensity was achieved development was stopped by impregnating the gel in a 200 ml stop solution (10% absolute ethanol,

0.5% acetic acid) for 2 min (Figure.1K). Finally, gel images were taken and bands were scored manually on white lamp (Figure 1L). The number of bands scored for each marker system were shown in Table 1.

Table 1. Number of bands scored and polymorphism observed for different marker systems on H-PAGE.

SL.No	Markers system	Primer name	Size range of bands (bp)	TNB	NPB	% P
1	SSR	CcM970	170-270	5	5	100
2	SRAP	EM4/ME5	60-700	27	26	96.2
3	TRAP	STKF/ME2	100-550	29	29	100

Note* TNB, Total number of bands scored; NPB, Number of polymorphic bands observed; % P, Per cent of polymorphism.

3. Results and Discussion

The method adopted for upgardation of unit was very simple. Only few accessories were used, which can be easily custom designed and purchased at cheaper costs. Covering glass plate and small glass piece were custom designed for $ 5. Two sided tape was procured at less than a dollar, which is commonly used for exhibiting posters in conferences. One time used tape as spacers was sufficient to cast at least 10-15 gels. Rain repellent solution was purchase at $ 8/250 ml, which is commonly used in cleaning car glasses. Treating of tray and covering glass plate with rain repellent will avoid sticking of gels. Therefore, one can easily upgrade their existing units with these accessories at cheaper costs. As every technique has advantages associated with few limitations. Performing of H-PAGE is very simple in terms of pouring, casting of gels, loading and separation of samples compared to vertical units. Since, 2 mm thick PAGE gels were used for separation of amplification products. These gels can be easily handled for ethidium bromide or silver staining methods. The main limitation lies with H-PAGE is that, it takes longer separate time (5 hrs) compared to vertical PAGE units (3 hrs). This may be due to fact that separations of bands were not towards gravity and higher percentage of gel used. It demands fairly good electrical conductivity for separation of bands. We recommend to use fresh running buffer for each run, which should be filled up to edge of covering plate. It will helps to focus the electrical flow in the gel for good separation and resolution of bands.

In order to reduce the total time taken for H-PAGE, we combined easy and fast staining methods as described for PAGE gels by Raymer and Smith[16] and Benbouza et al[17]. Due to high sensitivity and use of very simple chemicals silver staining methods are most widely used for staining of polyacrylamide gels[18]. Since, for multilocus profiling techniques many bands were visualized and need to score manually. For SRAP and TRAP markers, gel images were obtained after silver staining. Whereas, for allele specific markers such as SSR images were obtained after ethidium bromide staining. A clear allelic difference was observed for SSR on H-PAGE compared to agarose (Figure. 2 C-1, C-2). The obtained results from gel images clearly demonstrated H-PAGE has provided higher separation and degree of resolution to distinguish alleles/fragments for different markers systems used (Figure 2 A-B, C-2). As H-PAGE is technically less demanding high-throughput marker genotyping is possible by adopting this method. For SSR, multiplexing can be done by loading two or three amplification products for the different allele sizes in the same well as suggested by Wang et al[12]. For multilocus techniques, such as SRAP and TRAP markers, 27 and 29 bands were observed respectively on H-PAGE. These results were comparable to results as observed on vertical units. For SRAP, 23 bands or 10-20 bands per primer combinations were reported[14,19–21]. For TRAP, 4 to 21 bands or 33 bands per primer combinations were reported[22,4]. A wide range of scorable bands were observed for TRAP (100-550 bp), SRAP (60-700 bp) and SSR (170-270 bp) on H-PAGE. Results are comparable to

as observed for TRAP markers, 60 to 550 bp in sugarcane, SRAP markers 50 to 2000 bp in brassica, SSR markers 70 to 590 bp in potato[23–25].

4. Conclusion

Owing the above facts of H-PAGE, we have successfully demonstrated the method for upgrading and ease of genotyping in pigeonpea cultivars. These simplified units could be a valuable tool for many researchers with poor lab facility. It can broaden the scope for exploiting all the available marker systems in molecular breeding.

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