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Simple and efficient way to detect small polymorphic bands in plants

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ABSTRACT

There are many ways to detect polymorphism. In this study we use the microsatellite markers to detect the polymorphism for the salt tolerance. This method has been successfully conducted in *Oryza sativa* and *Brassica juncea*. The results are reproducible. In contrast to previous methods, our method is simple and quite accurate for detecting the polymorphic bands. In this study instead of using agarose gel and ethidium bromide staining, we used non-denaturing polyacrylamide gel and a low-cost improved method for silver staining when we compare it to 11 other methods for their ability to detect simple sequence repeat polymorphisms as small as 50 bp in denaturing polyacrylamide gels. All methods detected the same alleles and banding pattern. However, important differences in sensitivity, contrast, time consumption and background were observed.

1. Introduction

In plant breeding, conservation and evolutionary studies, molecular markers such as simple sequence repeats (SSRs) or sequence-tagged microsatellite sites (STMS), are widely used (e.g., genome mapping, marker assisted selection). Efficient methodology is required for mass genotyping [1]. Uses of the radioactive or fluorescent labelled nucleotides are the most efficient way to visualize single strand DNA in polyacrylamide gels. These procedures are expensive, time consuming and require special facilities and it makes them impracticable for most of the tropical countries where sophisticated infrastructures are lacking [2].

The silver-staining of proteins has been used for a wide variety of physical and biological analyses in polyacrylamide gels. In last decade, it has been applied in polyacrylamide gels to detect nucleic acid as silver ions bind to the bases and then under alkaline conditions it can be selectively reduced by formaldehyde. For detecting DNA in PCR-single strand conformation polymorphism analysis, silver staining of nucleic acid is widely used. Many other alternative methods to silver staining for DNA detection have been described previously. However, most of them are not rapid enough [3] because of time consuming steps and involve the changing of solutions repeatedly. It is hard to establish a highly output staining method. Although many simplified methods have been reported before [4–14], they still lack in sensitivity and/or efficiency.

The main objective of this study was to evaluate and standardize a new low-cost method for detecting polymorphism using silver staining using rice (*Oryza sativa*) and Indian mustard (*Brassica juncea*) as a model system. We compared sensitivity of this procedure to the 11 other commonly used procedures [4–14] and optimized the reaction conditions for detection of polymorphism by using denaturing polyacrylamide gels.

2. Materials

2.1. Reagents and equipment

2.1.1. Genomic DNA isolation

CTAB buffer, Microfuge tubes, Mortar and Pestle, Liquid nitrogen, Microfuge, 70% Ethanol (ice cold), Isopropanol, 60 °C water bath, Chloroform: Iso-amyl alcohol (24:1), Water (sterile), Agarose, 6× Loading buffer, 1× TBE solution, Agarose gel electrophoresis system, Ethidium bromide solution.

2.1.1.1. Elution buffer 100 ml. 2.0 g CTAB (Hexadecyl trimethylammonium bromide), 28.0 ml 5 M NaCl, 4.0 ml 0.5 M EDTA pH (8.0), 10.0 ml 1 M Tris-cl (pH 8.0), 1% (*v*/*v*) 2-Mercaptoethanol, 1% (*w*/*v*)

Abbreviations: SSR, simple sequence repeat; TBE, tris borate ethylenediaminetetracetic acid; TEMED, tetramethylethylenediamine.

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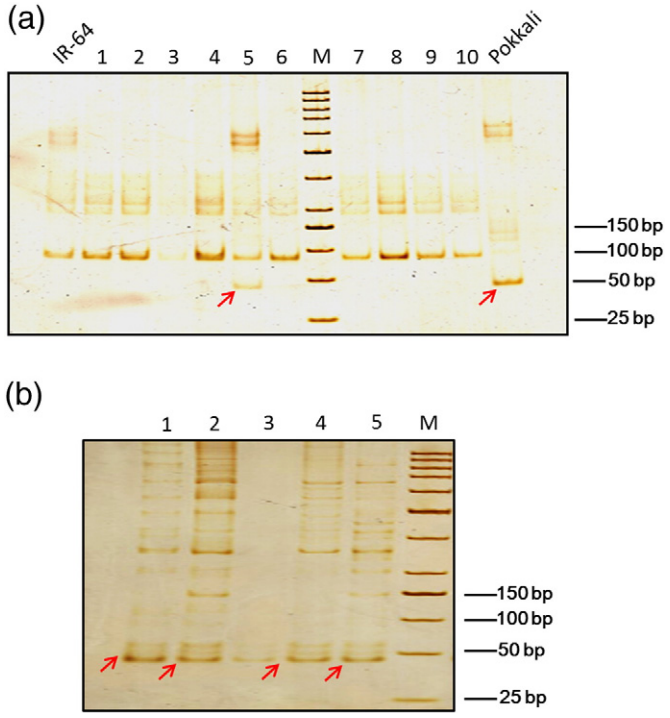

Fig. 1. PAGE of SSR markers of salt tolerant mutant lines of ***Oryza sativa*** and ***Brassica juncea***. (a). PAGE of mutant lines(1–10) of rice amplified with specific SSR marker (details not given) at Tm 64 °C. Pokkali is a wild type salt tolerant cultivar. (b). PAGE of mutant lines (1–5) of *Brassica juncea* amplified with specific SSR marker (details not given) at Tm 66 °C. Lines 1, 2, 4 and 5 are salt tolerant lines. Red arrow indicates polymorphism.

polyvinyl-pyrrolidone (PVP, Mw 10,000). Adjust all to pH 5.0 with HCL and make up to 100 ml with H_2O.

2.1.1.2. 1 M Tris pH 8.0. Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust pH to 8.0 by adding 42 ml of concentrated HCL. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 1 l with H_2O. Sterilize using an autoclave.

2.1.2. Polymerase chain reaction using microsatellite markers

10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.4 μM 20 mer primer, 1 unit Taq DNA polymerase, 20 ng template DNA.

2.2. Non-denaturing polyacrylamide gel electrophoresis for the separation of the bands

2.2.1. Buffer and solutions

Acrylamide: bisacrylamide (29:1) (% *w*/*v*), Ammonium persulfate (10% *w*/*v*), Ethanol, KOH/methanol, 6× Gel-loading buffer, 5× TBE Electrophoresis buffer, TEMED.

2.2.2. Special equipment

Electrophoresis apparatus, glass plates, combs and spacers, gel-sealing tape, micropipette with drawn-out plastic tip, petroleum jelly and syringe.

2.3. Detection of the polymorphism in non-denatured polyacrylamide gel by silver staining

Acetic acid (3% *v*/*v*), Developer — Dissolve 30 g of sodium carbonate in a final volume of 1 l of distilled H_2O. Ethanol (10% *v*/*v*), Formaldehyde (37% *v*/*v*), Nitric acid (0.7% *v*/*v*), Silver nitrate (0.2% *w*/*v*) freshly prepared and Gel scanner.

3. Procedure

3.1. Legend

* ***ATTENTION***
* ***HINT***
****REST***
The study comprised of four main steps (Fig. 2):

1. Genomic DNA isolation.
2. Polymerase chain reaction using microsatellite markers.
3. Non-denaturing polyacrylamide gel electrophoresis for the separation of the bands.
4. Detection of the polymorphism in polyacrylamide by silver staining.

3.1.1. Genomic DNA isolation

Extraction of DNA from 10 DAG seedlings was isolated from salt tolerant and salt sensitive wild type variety of *O. sativa* and *B. juncea* as described by [15–16].

3.1.2. Polymerase chain reaction using microsatellite markers

* ***HINT***: *Annealing temperature (Tm) can be adjusted according to primer set.*

Amplification reactions were carried out in a volume of 20 μl. Reaction mixtures contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.4 μM of 20-mer primer, 1 unit Taq DNA polymerase and approximately 20 ng of template DNA. The amplification was carried out using a thermocycler. The thermal cycler was programmed to one cycle of 4 min at 94 °C for the initial strand separation, followed

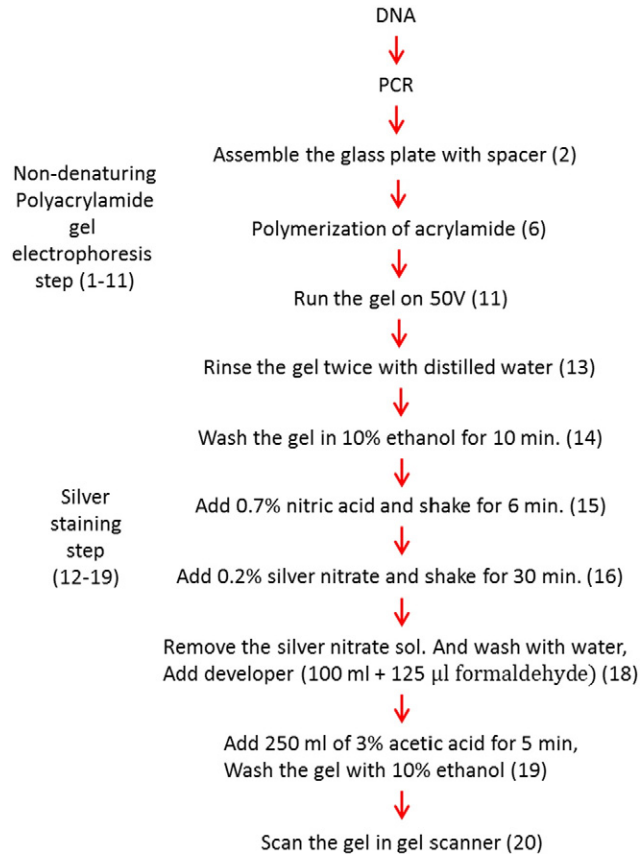

Fig. 2. Flow diagram to illustrate the major steps of the way to assess the small polymorphic bands. Each step, which is shown in parentheses, corresponds to the step in the Procedure section.