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Optimisation of CTAB and FTA Protocols for Tomato Plant DNA Extraction and Amplification Using SSR

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Abstract

This study was to identify which protocol yielded much genomic DNA and to optimize genomic DNA isolation methods to determine the annealing temperature of SSR markers suitable for PCR amplification. 22 tomato accessions from Western and North Central Nigeria were planted in 4 liter plastic pots containing top soil in a screen house at University of Agriculture Makurdi. FTA card was used to isolate DNA from 14 days old leaves while CTAB method was used to extract DNA from 23 days old leaves of accessions at the Molecular Biology Laboratory of University of Agriculture Makurdi. Genomic DNA from FTA Plant Saver card and CTAB protocol was verified on 2% agarose gel and the quality of DNA was ascertain using Standard DNA ladder of 50kb. 4 SSR primers were used for PCR amplification and the products were run on 2% agarose gel stained with ethidium bromide, resolved for 2 hours at 100V and visualized under UV light. Gel image revealed sharp DNA bands from CTAB protocol unlike the DNA from FTA card, with faintly visible bands. PCR optimization revealed sharp bands with annealing temperature of 55°C, and an extension temperature of 72°C for 35 cycles. Therefore, CTAB protocol and annealing temperature of 55°C is recommended for tomato genomic DNA extraction and amplification with SSR primers.

Keywords

Optimization, CTAB Protocol, Tomato, SSR Primers, FTA Plant Saver Card

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widespread vegetable crops around the world and an annual herbaceous plant belonging to the Solanaceae family [6]. According to Nkiru and Ifenkwe [14], tomato is helpful in the development of local agro- industries.

Molecular markers are important tool for genotype identification and studying the organization and evaluation of plant genome [17], which characterizes variability that is not detectable by simple visual observation and the assessment of genetic diversity within and between populations at molecular level using various laboratory based techniques [12]. Simple sequence repeats (SSRs) being one among the molecular markers are the most widely used molecular

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marker type and are not only very common, but also are hypervariable for numbers of repetitive DNA motifs in the genomes of eukaryotes [23, 21].

Successful extraction of DNA from plant tissues generally has been most successful from freshly field-collected tissue placed into either liquid nitrogen or, more commonly, into silica gel desiccant [19]. The cetyltrimethylammonium bromide (CTAB) method and DNA extraction kits are often reported in literatures [10, 18]. Alternative DNA extraction method using Whatman FTA PlantSaver Cards (Whatman, Maidstone, United Kingdom), with effectiveness for agricultural plant species, entomology, mycology, and other food sciences has been reported [22, 11, 7]. The FTA cards are also useful because the collected samples do not require refrigeration, extensive laboratory expertise, or as many hazardous chemicals as extractions using the CTAB-based technique [19].

According to Aguoru and Olasan [4], the factors that influence the choice of extraction method are: quality of extraction cost of reagent, familiarity of procedures and simplicity of protocol. Many protocols on DNA extraction and purification are rather complicated to use. The simple and cost effective procedures of extracting or isolating DNA cannot be over emphasized in molecular research. The problems encountered in the isolation and purification of DNA include degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions [15]. Also, contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification [16] and improper priming of DNA templates during thermal cycle sequencing [15].

Therefore, it is necessary to identify an efficient protocol for isolation of tomato genomic DNA as well as the optimum annealing temperature for SSR markers to save energy, time and resources when characterizing the genetic diversity of tomato plant using SSR markers.

2. Materials and Methods

2.1. Plant Material and SSR Primers

A total of 22 tomato accessions were sourced from; National Centre for Genetics Resources and Biotechnology Ibadan, Agrotropic Limited, Jos and Local Farmers in Benue State.

The SSR primers used for this study and their forward and reverse sequences are shown in Table 1 below.

Table 1. SSR Primer Codes and their Sequences.

Primer code	Sequences	
T-7	GTTATGGATTCACTTACCGCAAGTT	*F*
	CATTCGTGGCATGAGATCAA	*R*
T-31	GGTAATCAATTTTGAAGCTAAAAGC	*F*
	TGGGAAGAACTCAAGTCAAAAA	*R*
T-34	GTCAACTAGCGCTCCAATCT	*F*
	AAAGGGTTGTGGGAATTGTG	*R*
T-38	GAATTAGAGGGTTTGTGATACCG	*F*
	AAAAAGCTTCCTGGCTAAGAAAT	*R*
T-43	GAGAGAGAGTATGTATGTGCATTTCC	*F*
	TGAAAATTTGTGGTGTTGACG	*R*

Source: Singh *et al.* [20]. *F*= forward, *R*= reverse.

2.2. FTA Plantsaver Card DNA Extraction Protocol

Genomic DNA of the various accessions was extracted by adapting the protocol of Agurou *et al*. [5] for DNA isolation from eggplant using the FTA Plantsaver card with slight modifications as outlined below:

1. Each of the four squares of the card was divided into two, resulting to eight squares (figure 1A) and each was labelled accordingly
2. 14 day old leaves of the various accessions where placed on the card and overlayed with Parafilm and gently pressed by a side-to-side rolling with ceramic pestle until sufficient greenish sap was transferred to the paper, visible underneath.
3. The parafilm was removed and the card air dried for 24 hours at room temperature.
4. The Harris punch was used to cut 2 discs of 2 mm diameter from each of the sample into 2 ml eppendorf tube
5. 200 µl of 70% ethanol was added to the tubes(s) and allowed to soak for 5 minutes and vortexed for 30 seconds before discarding the liquid gently; leaving the discs in the tube and this step was repeated.
6. 200 µl of FTA purification reagent was added to the tube(s) and allowed to soak for 5 minutes and vortexed for 30 seconds before discarding the liquid gently, leaving the discs in the tube, this step was repeated.
7. The two discs in each tube were transferred into a fresh tube using micropipette tips to facilitate drying for about 1:30 minute and thereafter stored in the fridge.

2.3. CTAB DNA Extraction Protocol

Genomic DNA was isolated according to Doyle and Doyle [8] CTAB DNA extraction protocol with slight modifications as outlined below

i. 23 day old leaves of accessions were collected and preserved in a plastic containers containing silica gel

(figure 1B) for 7 days.

ii. About 1 g of the collected samples was ground into a fine powder using ceramic mortar and pestle.

iii. The powder samples were transferred into a 2 ml eppendorf tube and immediately, 800 µl of CTAB extraction buffer (200 mM Tris-HCl, pH 7.5; 50 mM EDTA, pH 8.0; 2 M NaCl; 2% CTAB; 1% β-mercaptoethanol) was added and the solution was mixed by shaking the tubes.

iv. The solution was incubated in a water bath at 60°C for 30 minutes. During this period, the tubes were shaken once by gently inverting them a few times.

v. The tubes were then removed from water bath, gently tapped and centrifuged at 3500 rpm for 10 minutes.

vi. About 450 µl of the aqueous phase was transferred into new tubes, and 500 µl chloroform-isoamylalcohol (24: 1) was added to the samples to form emulsion by shaking the tubes with hands.

vii. The solutions were centrifuged for 10 minutes at 3500 rpm.

viii. The supernatant of the solution was pipetted into 1.5 ml tubes and the process in step vi repeated.

ix. The upper aqueous layer was transferred to fresh tubes and 600 µl of 100% ice-cold isopropanol (2-propanol) was added and the tubes gently inverted about 30 times.

x. The tubes were put in the freezer at -20°C for 1 hour. Thereafter, the solution was centrifuged for 20 minutes at 3500 rpm to form a pellet at the bottom of the tube and the supernatant discarded carefully to avoid losing the pellet with DNA.

xi. 400 µl of cold 70% ethanol was added to the tubes with the pellets and centrifuged for 15 minutes at 3500 rpm and the ethanol decanted. This process was repeated.

xii. The pellets of the various samples were air dried for 24 hours at room temperature.

xiii. The pellets were re-suspended with 100 µl of TE.

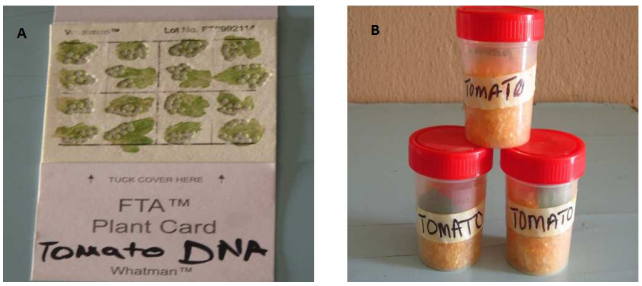

Figure 1. A = Sample image of FTA Card showing Plant sap of tomato accessions collected and discs punched out for DNA extraction. B = Sample image of tomato accessions collected in silica gel for CTAB DNA extraction.

2.4. Electrophoretic Assessment of Genomic DNA Presence

Presence of DNA from both extraction methods was assessed by gel electrophoresis using 0.8% agarose gel stained with ethidium bromide and 50 kb AccuPower® DNA ladder.

2.5. DNA Polymerase Chain Reaction (PCR)

AccuPower® DNA Premix was used for PCR reactions. 20 µl of water was added to each PCR Premix tube and each was reduced by half into a new PCR tube. 2 µl of the liquid template DNA, 1.5 µl of both the forward and reverse Primers were added to the 10 µl of the premix, given a grand total of 15 µl which were spun and loaded in the Bio-Rad thermal cycler. The protocol used is given below.

2.6. PCR Protocol

PCR amplification was set for 35 cycles; annealing temperature (T_m) used ranged from 50°C to 55°C. The PCR protocol was optimized by repeated reaction until a sharp amplification product on 2% agarose gel was obtained.

2.7. Electrophoretic DNA Separation on Agarose Gel

6 g of agarose powder was measured into a 500 ml conical flask. 300 ml of TAE Buffer was added and boiled using a microwave oven to ensure homogeneity, resulting to 2% agarose gel. 3 ul of 10 mg/ml Ethidium bromide (EtBr) solution was added and allowed to cool to down to a level for hand handling. The conical flask was covered with a cork to prevent inhalation of the hazardous Ethidium Bromide. A gel comb with 20 teeth, set at 1.5 mm apart, with each tooth designate for a well was placed in the plate. The gel was poured and allowed to set for about 30 minutes.

Thereafter the PCR products including 50 kb DNA ladder were loaded into individual wells in 1X TBE and resolved for 2 hours at 100 V.

2.8. DNA Visualization

The banding pattern of the samples resolved on 2% agarose gel was viewed on a UV Bench top trans-illuminator and the gel image was captured using a digital camera according to a similar technique used by Kadry and Gamal [9].

3. Results and Discussion

Results of the genomic DNA extracted using FTA plantsaver card showed faint and fairly visible genomic DNA bands when viewed with the UV-trans-illuminator (figure 2) and that of CTAB showed visible bands (figure 3). This study revealed that CTAB protocol is the better isolation method for tomato plant as

it yielded high DNA concentration. This was evidenced by sharp Genomic DNA bands on agarose gel. The variation exhibited between FTA card and CTAB indicated that, FTA card extracted DNA contained a less fragmented DNA when compared to CTAB methods. Siegel *et al.* [19] made similar conclusion. The differences in DNA concentration for CTAB and FTA card-extracted from accessions could have been due to the differences in amount of leaf tissue used during extraction. For CTAB, about 1 g of leaf tissue was used while for FTA card-extracted samples, the amount of DNA produced depended on the concentration and amount of fluid transferred from the leaves to the card. It may probably be that, FTA card method may not work well for tomato plant as compared to other plants. Padmalatha and Prasad [15] reported that, isolation and purification of DNA may encounter problems such as degradation of DNA due to endonuclease, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenol and other secondary metabolites which directly and indirectly interfere with the enzymatic reactions. These may be why no visible genomic DNA from the FTA card was observed on UV-Trans-illuminator. However Siegel *et al.* [19] in their comparison of FTA and CTAB DNA extraction from non-agricultural taxa reported that the FTA card extraction method yielded less concentrated, but also less fragmented samples than the CTAB-based technique. They reported that the card-extracted samples provided DNA that could be successfully amplified and sequenced. This result affirmed the use of CTAB for tomato DNA extraction as previously reported by several workers [20, 3, 21].

Figure 4 revealed gel image on agarose gel resolution of PCR products from amplification of tomato genomic DNA using SSR markers. The gel images show sharp amplification at optimized annealing temperature of 55°C and an extension temperature of 72°C as revealed via electrophoretic separation on agarose gel.

Result on optimization as revealed in this study shows that; 35 cycles is required for optimization of SSR primers under the following thermal conditions; initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 Sec, annealing at 55°C for 30 Sec, extension at 72°C for 1 min. and final extension at 72°C for 4 min. holding temperature at 4°C, ɑ (infinity). This result is in ranged to the findings by Singh *et al.* [20] who used the following thermal profile for amplification of tomato DNA when he characterized tomato genotypes using morphological and molecular markers: 1 cycle of 4 min. at 94°C (initial denaturation) followed by 45 cycles of 14 sec. at 94°C (denaturation), 45 sec (prime annealing at temperature according to the primer sequence ranging from 51.4 to 56.1°C) and 90 sec at 72°C (primer elongation and finally a cycle of 4 min at 72°C (final extension). Afsana *et al.* [2], carried out molecular diversity analysis of some Chilli (*Capsicum* spp) genotypes using SSR markers revealed; pre-denaturation with 95°C for 4 min., denaturation with 95°C for 40 sec., annealing at 50-60°C for 33 sec., extension at 72°C for 40 sec. and final extension at 72°C for 5 min. continuing for 31 cycles.

All the annealing temperature used by other published work on SSR primers had an annealing temperature ranging from 50 to 60°C, the optimum annealing temperature was not specify for easy and quick result when the primers annealing temperature of a particular SSR primer is not known.

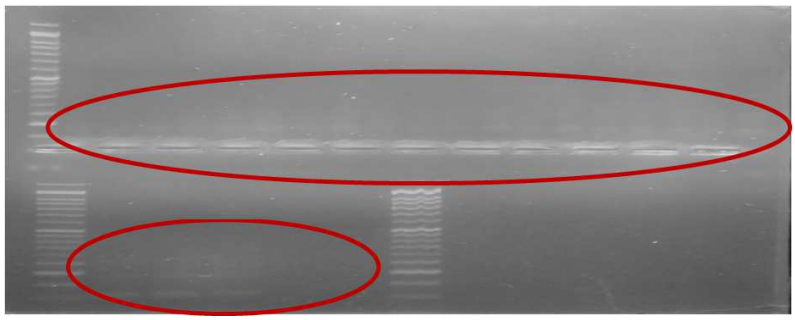

Figure 2. Tomato Genomic DNA as isolated using FTA card.

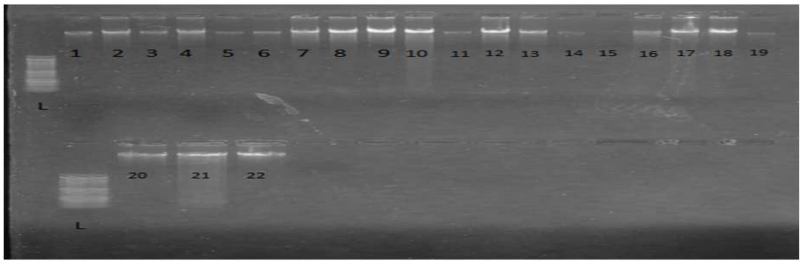

Figure 3. Tomato Genomic DNA as isolated using CTAB.

Legend: L = DNA Ladder. 1-22 = Tomato accessions

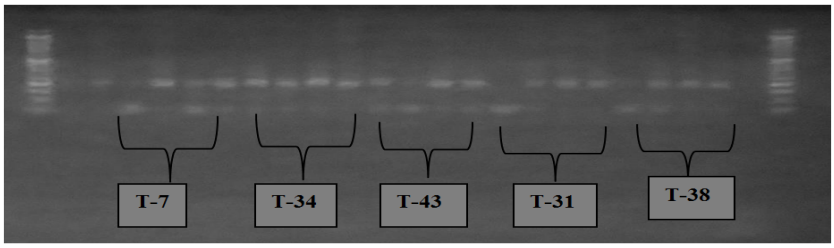

Figure 4. Sample gel image showing amplification of DNA from tomato accessions with Primer T-7, T-34, T-43, T-31 and T-38 at T_m of 55°C. Amplified DNA was extracted using CTAB extraction method. Legend: L = 50kb DNA Ladder.

4. Conclusion

CTAB method of DNA extraction was the better method of tomato DNA extraction in this study as it yielded more quantity of genomic DNA and could be an important tool for assessing and characterizing plant population genetics especially tomato accessions within and between species.

For optimization of PCR using tomato SSR primers, it is important to use the following thermal profile: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 Sec, annealing at 55°C for 30 Sec, extension at 72°C for 1 min. and final extension at 72°C for 4 min. holding temperature at 4°C, ∞ (infinity) for effective and sharp amplification of the product to save time and resources.

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