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Molecular characterization of six released tomato varieties using Inter Simple Sequence Repeat markers

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ABSTRACT

Inter Simple Sequence Repeat (ISSR) markers were used for identification of six tomato varieties. Out of 20 ISSR primers tested, 12 ISSR primers produced 118 detectable fragments, of which 57 (55.9%) were polymorphic across the varieties. Fourteen unique bands specific to the varieties were detected. These may be used as variety specific probes for identification purposes. Genetic relationships among these varieties were evaluated by generating a similarity matrix based on the Dice coefficient and the Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendogram. Both molecular and morphological markers may be used for assessment of the tomato germplasm as selecting genotypes for the variety improvement programme.

Key words: Inter Simple Sequence Repeat (ISSR), *Solanum lycopersicum*, DNA fingerprinting.

INTRODUCTION

Until recently, virtually the progress in both breeding and model genetic systems relied on a phenotypic assay of genotypes. Because the efficiency of a selection scheme or genetic analysis based on phenotypes as a function of the heritability of the trait, factors like the environment, multigenic and quantitative inheritance, or partial and complete dominance often confounded the expression of a genetic trait. Many of the complications of phenotype based assay could be mitigated through direct identification of genotype with DNA-based diagnostic assay. Although morphological markers enabled the detection of genetic variation, it was often disguised by factors in the environment, and minimized by a paucity of discernible morphological markers. Significant advancements in molecular biology have shifted the focus of assessment of biodiversity based on morphological markers to using isozymes and DNA markers (Brown, 2; Bretting and Widrlechner, 1; Karp and Edwards, 6). For this reason, DNA-based genetic markers are being integrated into several plant systems and are expected to play an important role in the future plant breeding. The utility of DNA-based diagnostic markers is determined to a large extent by the technology that is used to reveal DNA-based polymorphisms. Over the last few years, polymerase chain reaction technology has led to the development of several novel genetic assays based on selective DNA amplification (Krawetz, 7; Innis *et al*., 5).

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Tomato (*Solanum lycopersicum* L.), is the second most important vegetable in terms of total production and has worldwide commercial distribution. Hybrid tomato varieties under optimum management give economically higher yield as compared to open-pollinated varieties. Some tomato varieties were developed by the Department of Horticulture, OUA&T, Bhubaneswar having higher yield, better keeping quality and resistance to wilt. In this study attempts were made to characterize these varieties through molecular analysis to identify the variety specific markers.

MATERIALS AND METHODS

Seedlings of six tomato varities, i.e. Utkal Deepti, Utkal Pallavi, Utkal Kumari, Utkal Urbashi, Utkal Raja and Utkal Pragyan were raised from seeds obtained All India Coordinated Vegetable Improvement Project (ICAR), OUA&T, Bhubaneswar. Identification of the varieties was confirmed using the literature published by the university; the morphological characters of the varieties are described (Table 1).

Young leaves were used for isolation of DNA using CTAB method following the protocol of Doyle and Doyle (30) with minor modification. About 1.0 to 1.5 g of young leaves were ground in liquid nitrogen, incubated in CTAB buffer (3% w/v CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4M NaCl, 2% v/v β-mercaptoethanol, 2% w/v polyvinyl pyrrolidine, pH 8.0) for 2 h at 65 °C. The homogenate was then extracted with an equal volume of chloroform: isoamylalcohol (24:1) and centrifuged at 9,000 rpm for 10 min. The upper aqueous layer was recovered and precipitated with pre-chilled isopropanol. The pellet was suspended with Tris-EDTA buffer (pH

Table 1. Morphological characteristics of six tomato varieties released from OUA&T, Bhubaneswar.

Variety	Morphological characters
Utkal Deepti	Determinate type, slightly curling leaf. Fruit round shaped, small nipple present at the blossom end of the fruit, small to medium size fruit with 6 – 8 fruits per cluster and good keeping quality. Matures in 80 – 85 days after sowing. Resistant to bacterial and nematode wilt. It also performs well in saline soil.
Utkal Pallavi	Determinate type, curly leaf. Fruit borne in cluster consisting of 6-12 in number. Medium to small in size, pear shaped, nipple at blossom end, fleshy. Matures in 85 – 90 days after sowing. Resistant to bacterial and nematodes wilt. It also performs well in saline soil. The average yield is 375.75 quintal per ha.
Utkal Kumari	It has indeterminate growth habit. Fruit borne in cluster consisting of 3 – 5 in number, medium large in size, round shaped, uniform red colour at maturity. Matures in 90 – 95 days after sowing. Resistant to bacterial wilt. Average yield is 402 quintal per ha.
Utkal Urbashi	Interminate, tall plant, good foliage cover. Fruit pear shaped, medium large size with slightly green shoulder, good keeping quality, 5 – 8 fruits per cluster. Matures in 100 – 105 days of sowing. It is resistant to bacterial wilt and tolerant to early blight. The average yield is 451.67 quintals per ha.
Utkal Pragyan	: Determinate growth habit, fruit round, deep red in colour, medium in size with a blunt beak at distal end. Fruits in clusters of 5 – 6 in number, pulpy and tolerant to bacterial wilt disease. Matures in 90 – 95 days after sowing. The average yield is 412.22 quintal per ha.
Utkal Raja	Indeterminate growth habit, fruit round, medium large in size and deep red in colour at ripening. Fruits bear in clusters of 4 – 5 in numbers. Tolerant to bacterial wilt disease and matures in 95-100 days of sowing.

8.0). The crude DNA was treated with RNase and incubated for 30 min. at 37 °C and again extracted with 1 volume phenol and subsequently with chloroform: isoamylalcohol (24:1). The supernatant were collected and precipitated with 3M sodium acetate and pre-chilled ethanol. The DNA pellet was washed with 70% ethanol, dried and re-suspended in Tris-EDTA buffer. The high molecular weight DNA was checked for quality and quantity electrophoretically using 0.8% agarose gel against a known amount of λ-DNA taken as standard.

Twenty synthesized ISSR primers (M/s Bangalore Genei, Bangalore) were initially screened using one variety Utkal Deepti to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products within the genotypes. To ensure reproducibility, the primers generating weak or complex patterns were discarded. A few well-amplified fragments that were not reproducible across two replicates of DNA extraction were also discarded. For ISSR study, the initial optimization of PCR was done including concentration of template DNA, primer, $MgCl_2$, number of PCR cycles and annealing temperature. The PCR reaction had a total volume of 25 µl containing 20 ng template DNA, 100 mM each dNTPs, 20 ng of oligonucleotides synthesized primer (M/s Bangalore Genei, Bangalore), 2.5 mM $MgCl_2$, 1 × *Taq* buffer [10 mM Tris-HCl) pH 9.0, 50 mM KCl, 0.01% gelatin] and 0.5 U *Taq*DNA polymerase (Bangalore Genei, Bangalore). DNA amplification was performed in a PTC-100 thermal cycler (M J Research, USA) programmed for a preliminary 5 min. denaturation step at 94°C, followed by 40 cycles of denaturation at 94 °C for 20 sec., annealing temperature depending on the primer (50 to 56 °C) for 30 sec. and extension at 72 °C for 45 sec., finally at 72 °C for 5 min. Amplification products were separated alongside a low range molecular weight marker (Bangalore Genei, Bangalore) on a 2% (w/v) agarose gel electrophoresis in 1 × TAE (Tris acetate-EDTA) buffer stained with ethidium bromide and visualized under UV light. gel photographs were scanned through Gel Doc System (GelDoc 2000, BioRad, California, USA) and the amplification product sizes were evaluated using the software Quantity one (BioRad, California, USA).

Clearly defined ISSR bands that behaved as dominant markers were scored for the presence (1) or absence (0) for all the species/cultivars and entered into a data matrix. The genetic relationships among the species/cultivar were determined by calculating the Dice's coefficient, estimated as $S = 2N_{AB} / N_A + N_B$. Where N_{AB} is the number of amplified product common to both A and B. N_A and N_B corresponds to number of amplified product in A and B, respectively.

RESULTS AND DISCUSSION

Twenty ISSR primers were used to assess the six varieties of tomato. Fifteen, out of the 20 primers generated clear multiplex banding profiles, among which six primers IG-01 (AGGGCTG GAGGAGGGC), IG-02 (AGAGGTGGGCAGG TGG), IG-03 (GAGGGTGGAGGATCT), IG-06 (GACAGATAGACAGATA), IG-10 [$(AC)_8T$] and IG-14 [$(GA)_8T$] produced the best ISSR profiles. However, the rest 8 primers produced either smear with bands or no products at all. Modification on the annealing temperature, primers concentration, $MgCl_2$ and template concentration did not improve the patterns. In addition, the results also showed that most of the primers based on GA/AG and GT/TG dinucleotides core repeats generated good banding profiles. The amplification of ISSR markers was consistent across two replicate DNA extractions from three samples, with 98% of scorable and reproducible fragments. This was in concordance with previous studies on tomato and cucumber (Temiesak *et al*., 11). A higher concentration of $MgCl_2$ (2.5 mM) gave best results which may be due to non-specific amplification because of reduced enzyme fidelity (Hofkins and Hilton, 4). The concentration of $MgCl_2$ affected the specificity and yield of reaction by increasing the stringency of primer annealing or had a direct effect on *Taq* polymerase (Saiki, 9: Rom *et al*., 8; Singh *et al*., 10).

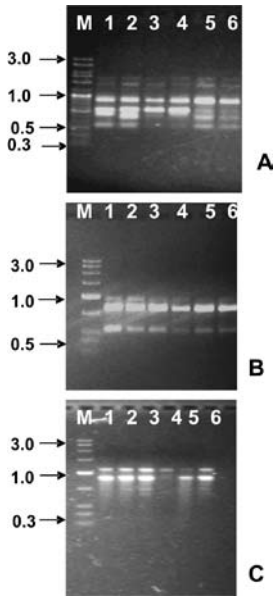

Fig. 1. ISSR banding pattern obtained in six new varieties of Solanum lycopersicum using synthesis primer IG-02 (A), IG-03 (B) and IG-13 (C). M - low range DNA marker. 1 - Utkal Deepti; 2 - Utkal Pallavi; 3 - Utkal Kumari; 4 - Utkal Urbashi; 5 - Utkal Pragyan, 6 - Utkal Raja.

The six selected primers showed highly polymorphic banding profiles. Furthermore, each of these primers produced fingerprint profiles unique to each of the varieties. Therefore, each primer can be used separately to identify these varieties. ISSR amplification for all samples resulted in multiple band fingerprint profiles for the selected ISSR primers (Fig. 1). The average number of scorable fragments per primer was 8, with a range from 4 to 5, while the average number of polymorphic fragments per primer was four. Out of the 69 scorable fragments, 24 were polymorphic revealing 34.8% polymorphism among the six varieties studied (Table 2). Four ISSR loci were recorded as germplasm specific as they occurred in six varieties. These may be used as variety specific probes for identification purposes. Primers based on GA/AG and GT/TG dinucleotides core repeats generated good profiles, which seem to indicate that the more frequent microsatellite in tomato contains the repeated dineucleotides (AG/GA)n and (GT/TG)n**.** These results are in accordance with the SSR assay. ISSR techniques have high reproducibility, possibly due to the use of longer primers (16 to 25 mers) compared to the RAPD primers (Williams *et al*., 13), which permits the subsequent use of a higher annealing temperature, leading to high stringency. Temiesak *et al.* (11) reported the varietal identification of tomato and cucumber using RAPD markers. They found twelve primers having good profile out of 20 primers tested. They also reported variations amongest these closely related varieties. In the present study there was a distant variation in DNA amplification in six tomato varieties released by OUA&T, Bhubaneswar. Matrix calculations revealed varieties in the index value from a minimum of 0.55 to a maximum of 0.91 between “Utkal Deepti” and “Utkal Urbashi” (Table 3); all other varieties showed different intermediate levels of similarity.

In the present study, 8 ISSR loci that were specific to six tomato varieties were identified. The frequency of specific fragments was within 2% margin of error detected in the repeatability assay. These may be used specific probes for identification varieties. The results showed the genetic relationship among the varieties, inferred by ISSR markers, were in accordance with their morphological characters. “Utkal Deepti” and “Ukal Pallavi” were morphologically very similar like leaf curling, nipple present at the blossom end, resistance to bacterial and nematode wilt and maturity within 85 -90 days. These morphological characters had also closely resemblance with variety “Utkal Kumari”. The variety “Utkal Urbashi” fell in a single cluster having 65% similarity with other five varieties. The major morphological characters exhibiting the plant height, good foliage cover, fruit maturity (100-105 days) and resistance to bacterial wilt and tolerant to early blight.

Table 2. ISSR primers (12) used for DNA fingerprinting of six tomato varieties.

Primer code	Primer sequence ('5-3')	Total No. of bands	No. of polymorphic bands	Percentage of polymorphism	Bands range
IG-01	AGGGCTGGAGGAGGGC	06	02	33.3	0.3 - 2.0
IG-02	AGAGGTGGGCAGGTGG	08	03	37.5	0.4 - 2.5
IG-03	GAGGGTGGAGGATCT	05	04	80.0	0.3 - 1.0
IG-06	GACAGATAGACAGATA	04	01	25.0	1.1 - 2.0
IG-09	$(AG)_8C$	06	02	33.3	0.3 - 2.1
IG-10	$(AC)_8T$	05	03	60.0	0.5 - 2.0
IG-11	$(AC)_8G$	06	01	16.6	0.3 - 1.0
IG-13	$(GA)_8A$	05	02	40.0	0.8 - 1.5
1G-14	$(GA)_8T$	07	01	14.3	0.6 - 2.4
IG-15	$(AT)_8G$	06	02	33.3	0.5 - 3.0
IG-22	GACACGACAC	05	01	20.0	0.6 - 2.1
IG-23	$(GA)_8C$	06	02	33.3	0.2 - 1.5
Total	-	69	24	34.78	0.2 - 2.5

Table 3. Similarity coefficients in six new tomato varieties using ISSR markers.

UD	UP	UK	UU	UP	UR
UD	1.00				
UP	0.91	1.00			
UK	0.80	0.80	1.00		
UU	0.55	0.59	0.70	1.00	
UP 0.66	0.66	0.68	0.70	1.00	
UR	0.68	0.73	0.66	0.64	0 . 7 5
1.00					

Utkal Deepti (UD), Utkal Pallavi (UP), Utkal Kumari (UK), Utkal Urbashi (UU), Utkal Pragyan (UP), Utkal Raja (UR)

In conclusion, it can be said that ISSR markers can aid in identification of crop varieties quickly, which could be reliable and reproducible.

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