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Molecular Characterization of Indian Potato (*Solanum tuberosum* L.) Varieties for Cold-Induced Sweetening Using SSR Markers

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Abstract: Cold-induced sweetening developed during storage of potatoes (*Solanum tuberosum* L.) at low temperature is a crucial factor influencing the processing quality of potato tubers and remains one of the principal concerns of potato processing industry. Developing CIS-resistant genotypes is the most effective method to cope with this stress. In this study, the genetic diversity of 11 Indian potato varieties with different reactions to CIS was assessed using 10 SSR primers. The primers detected a total of 42 alleles arranged in 44 different configurations, among which 37 alleles (88%) were polymorphic. The polymorphic information content (PIC) value of the SSR locus ranged from 0.473 to 0.787 thus indicating a high utility of these markers for study of genetic diversity in potato. A number of polymorphic fragments appeared to be specific to a given sugar-forming group. Primer Sti007 generated one fragment $Sti007_{131bp}$ present only in all the high sugar-forming varieties. The dendrogram derived from Dice's similarity coefficients among the 11 varieties could partially but efficiently differentiate close parents and sugar-forming groups among the varieties. These findings demonstrate the effectiveness of SSR markers to assess the genetic variation among potato cultivars in order to develop molecular markers associated with CIS to improve potato breeding programs.

Keywords: Cold-Induced Sweetening, Potato, *Solanum tuberosum*, SSR, Genetic Diversity

1. Introduction

Potato (*Solanum tuberosum* L.) is the third most important food crop, the most important non-grain food crop and one of the most essential basic vegetable worldwide as well as in Indian subcontinent.In India as in many other potato producing countries, after harvest, potatoes are stored in cold storage (generally at 4ºC) to provide round the year supply to industry and consumers [1]. Despite many advantages offered by cold storage, the tubers under low temperature accumulate considerable amount of sucrose (Suc) which is then converted into glucose (Glu) and fructose (Fru)[2]in a process known as cold-induced sweetening (CIS). During processing like frying or dehydration at high temperature, these potatoes lead to dark-brown-coloured and bitter tasted fries and chips as a result of Maillard reaction between the accumulated reducing sugars and free amino acids (arginine). These darkened chips and fries are unacceptable to consumers and also result in greater amounts of acrylamide production which has been linked to many cancers[3,4].

As CIS remains one of the serious hurdles of potato processing industry, developing CIS-resistant genotypes is one of the frontal areas of research on potato quality all over the world. Unfortunately, despite extensive breeding efforts, no truly CIS-resistant cultivars have been released onto the market, and CIS remains one of the major issues facing the potato-processing industry [5]. The biggest impediment towards development of cold-resistant potato cultivars through conventional breeding is lack of suitable germplasm to be used as parents [6]. The content of RS in potato is governed by the genetic makeup of the genotype and is

influenced by environmental conditions. Potato genotypes can therefore be classified as CIS-resistant or CIS-sensitive [7]. Quantification of genetic diversity present within the cultivars by molecular markers would be of great help to improve selection of CIS-resistant potato genotypes through selection of efficient and diverse combination of parents. Microsatellites or simple sequence repeats (SSRs) marker systems have shown many advantages over other markers based on Polymerase Chain Reaction (PCR), such as ease of analysis, high polymorphism rate, high reliability, co-dominance and transferability among related species [8]. Microsatellites therefore appear as valuable tools for analysing the genetic diversity in potato.

SSRs have been used in the identification of cultivars, fingerprinting and potato genome mapping projects. The relative abundance of SSRs in the EMBL and the GenBank databases of potato and related sequences were reported [9]. (1996). Milbourne *et al*[10] developed 112 SSR markers among which 98 markers showed a high level of polymorphism. Using 65 of these markers, 89 loci in two potato mapping populations were mapped. Ghislain *et al*. [11] developed 48 SSR markers and used them to identify 931 potato germplasm accessions. In addition, they mapped 31 of these markers using mapping populations and developed a new 24 microsatellite locus-specific markers namely Potato Genetic Identity (PGI) kit for fast molecular characterization of potato. For mapping and for genetic characterisation of 30 potato cultivars, 94 SSR markers were developed and 61 of them were used[12]. Barandalla *et al*. [13] used 19 SSR markers to fingerprint 41 local potato cultivars from 10 locations of Tenerife Island. A total of 67 alleles were observed, 12 of them were present in all cultivars. Several accession and group specific alleles were detected. Generally, cultivar groups with identical or related common names showed the same SSR patterns or clustered closely together.

Recently, microsatellites have been extensively used to screen potato germplasm[14-17]. Indian potato varieties have been analysed using SSR markers[18]while Tiwari *et al*. [19] validated the genetic diversity of the 77 Indian Andigena potato core collections using the PGI kit previously developed [4]. Datir[20] examined the allelic variation for SSRs markers including candidate genes associated with carbohydrate metabolism in potato progenies. However, little is known about genetic diversity of Indian potato cultivars as related to carbohydrate metabolism under cold storage conditions, hence the present work was planned to characterize the genetic diversity among potato varieties differing in their tolerance/sensitivity to CIS by using SSRs markers.

2. Materials and Methods

2.1. Plant materials

Tubers of 11 potato (*Solanum tuberosum* L.) highly cultivated varieties were obtained from Main Vegetable Research Station, Anand Agricultural University, Anand. Our previous studies[21]on the basis of degree of sugar increase during storage as reflected by the hexoses:sucrose ratio, reducing sugar content and acid invertase activity, could grouped the 11 potato varieties as low sugar-forming varieties (Kufri Jyoti, Kufri Surya, Kufri Sutlej, Kufri Sadabahar, Kufri Himsona) or high sugar-forming varieties (DSP 287, DSP 186, Kufri Chipsona-3, Kufri Lauvkar, Kufri Bahar, Kufri Badshah).

2.2. Genomic DNA Extraction

Genomic DNA was extracted from young leaves following a previously described procedure [22]. Potato tubers were grown in pots and young leaves were collected from 15 day-old plants. Leaf tissue was processed by freezing with liquid nitrogen and ground to fine powder using a mortar and pestle. Approximately 100 mg of fine powder were transferred to 2 mL micro-centrifuge tube and 600 µL of 65°C-preheated modified CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 2% (w/v) CTAB and 20 mMEDTA) was added. Tubes were vortexed for 10 s and incubated for 90 min in water bath at 65°C. Afterward, the tubes were allowed to cool at room temperature for 5 min. A volume of 700 µLchloroform:isoamylalcohol (24:1) was added to each tube and the tubes were gently mixed for 5 to 10 min. The mixtures were centrifuged at 4°C for 10 min at 8000 g and 600 µl of upper aqueous layer were transferred to a clean 1.5 ml micro-centrifuge tube. The DNA was then precipitated by adding 600 µL of isopropanol and the tubes left overnight at -20°C. To pellet the DNA, the tubes were centrifuged at 5000 g for 2 min at 4°C. The supernatant was decanted and the DNA pellet was washed with 600 µL of 70% ethanol. The tubes were centrifuged at 3000 g for 2 min at 4°C. The ethanol was carefully aspirated and the DNA pellets were dried by leaving the tubes open for 15 min at room temperature. DNA pellet was re-suspended in 100 TE buffer (10 mM Tris-HCl pH 8.0, 1 mMEDTA) and stored at -20°C. The quality and quantity of the DNA were assessed using (1%) agarose gel electrophoresis[23] and NanoDrop spectrophotometer (Thermo Scientific).

2.3. SSR Analysis

Genetic diversity among the varieties was screened using 10 potato SSRs primers selected from similar previous studies [11,20](Table1). For PCR reaction, the reaction mixture (10 µL) contained 0.5 µL 10 µM of each forward and reverse primer, 0.25 µL *Taq* DNA polymerase (5 U/µL) (KapaBiosystems), 2 µL 10X Dream Taq Green Buffer (Thermo Scientific), 0.6 µL dNTPs (2.5 mM each) (Genei^{TM}), 1 µL template DNA (approximately 50 ng) and 5.15 µL sterilized deionized water. All the PCR reactions were carried out in 200 µL thin walled PCR tubes. The tubes containing the reaction mixtures were gently spinned in a microfuge and the amplification was carried out in a Bio-Rad MJ Mini^{TM} thermal cycler using the following programme: 7 min as initial denaturation step at 94°C, followed by 40 cycles of 45 s denaturation at 94°C, 45 s annealing at 57 to 62°C

(depending upon SSR primer-pair), and 1 min extension at 72°C plus a final extraction step of 7 min at 72°C. Amplified products were separated by electrophoresis on a 3% agarose gel stained with 0.5 µg/mL ethidium bromide using 1X TBE buffer for 60 min. DNA Ladder (O'rangeRuler^{TM} 100 bp + 500 bp, Thermo Scientific) was used as a molecular size standard. All SSR-PCR reactions were repeated at least twice.

Table 1. *Polymorphism detected by 10 SSR primers in 11 potato varieties.*

Sr. No.	Primer	Annealing Temp. (°C)	No. of configurations	No. of fragments	No. of polymorphic fragments	Polymorphism (%)	Detected products sizes (bp)	PIC
1	Sti060	62.0	6	3	3	100	193 173 165	0.594
2	STWaxy2	61.5	2	3	3	100	246 228 217	0.473
3	inh2α	61.5	4	4	3	75	145 138 127 100	0.656
4	Sti007	58.0	5	4	4	100	138 131 127 118	0.717
5	SSR5148	61.0	4	4	3	75	512 506 457 174	0.678
6	Sti021	58.0	4	4	3	75	245 230 209 103	0.607
7	STM3009	62.0	4	5	4	80	264 169 162 149 140	0.730
8	STM0019	57.0	7	6	6	100	258 239 222 206 184 172	0.787
9	Sti014	58.0	3	3	2	66.67	135 122 100	0.664
10	Sti028	59.0	5	6	6	100	218 203 192 185 112 103	0.726
Total	**10**	**-**	**44**	**42**	**37**	**88**	**42**	**-**

2.4. Statistical Analysis of SSR Data

Most of the loci in potato, a clonally propagated plant, are expected to be heterozygous. Therefore, an SSR marker amplifying a single locus can yield up to four bands in potato, a tetraploid species. Moreover, some bands could appear as more than one copy and consequently as denser than others. This fact, also known as dosage effect, makes the bands difficult to analyze and decrease the reliability of the DNA analysis [24]. In order to overcome this problem, potato researchers prefer scoring the configuration of all bands as a dominant marker rather than scoring each band separately[10,12]. In the present study the analysis of SSR data was performed as described by previous authors [19]. A data matrix of SSR alleles was constructed on the basis of presence (1) or absence (0) of bands of the amplified DNA fragments. A variety was assigned a null allele where an amplification product could not be detected and so not considered in the analysis. Number of alleles, allele sizes and polymorphic information content (PIC) of each SSR were determined for the 11 varieties. The PIC of each SSR marker was calculated according to the formula of Nei[25]: $PIC=1-\Sigma(Pi^2)$, where Pi is the frequency of the i^{th} allele of a marker detected in all varieties. Genetic diversity analysis was performed with the program NTSYSpc 2.21 [26]. A similarity matrix was calculated by Dice's similarity coefficient and the dendrogram was generated using UPGMA clustering method.

3. Results and Discussion

Ten SSR primers were used to characterize the diversity among the 11 potato varieties. The number of detected SSR alleles among the 11 varieties ranged between 17 (K. Sutlej) and 25 (K. Sadabahar). In total, 44 configurations were obtained, ranging from 2 configurations (primer STWaxy2) to 7 configurations (primer STM0019). A total of 42 SSR alleles were detected with the number of allele per locus varying from 3 (Sti060, STWaxy2 and Sti014) to 6 (STM0019 and Sti028). The 37 polymorphic loci (88%) showed varying degree of polymorphism and only 5 loci (12%) were monomorphic. They included $inh2\alpha_{100bp}$, $SSR5148_{157bp}$, $Sti021_{103bp}$, $STM3009_{264bp}$, and $Sti014_{100bp}$. The highest PIC value of SSR locus was observed in STM0019 (0.787) and the lowest was obtained from STWaxy2 (0.473) (Table 1). Chimote*et al.*[18] analysed 32 commercial Indian potato varieties using 4 multi-loci SSR. They generated a total of 123 alleles with the mean number of alleles per variety being 34 and 24 alleles (19.5%) were highly polymorphic.Tiwari *et al*. [19] used the 24 microsatellite markers from the PGI kit [11] to validate the genetic diversity of the 77 Indian Andigena potato core collections. In total, 214 SSR alleles were detected in the core collection, out of which 208 alleles were polymorphic with absolute frequencies between 2 to 58. The PIC values of SSR loci ranged from 0.61 to 0.90. Datir[20] examined the allelic variation for 24 SSRs markers with known position on the potato genetic map including candidate genes associated with carbohydrate metabolism in 305 progeny. All the 24 markers were found to be polymorphic and a total of 84 alleles were produced. The number of different bands produced by each SSR marker varied from two to six bands with average of 3.5. In this study only 12% of the SSR alleles was found to be monomorphic, thereby indicating a high utility of these markers in studies on potato genetic diversity [14-20].

In an attempt to identify molecular markers associated with CIS, it was observed that some polymorphic loci appeared to be specific to a given sugar-forming group. Primer inh2α generated a 127bp fragment present only in two high sugar-forming varieties DSP 287 and K. Chipsona-3. Similarly, a 140 bp fragment produced by STM3009 was found only in high sugar-forming varieties DSP 287 and K. Bahar. Also, fragment $STM0019_{172bp}$ was generated only by the two DSP varieties. On the other hand, band $STM3009_{169bp}$ was amplified only in two low sugar-forming varieties K. Surya and K. Himsona. More interestingly, primer Sti007 generated one fragment $Sti007_{131bp}$ presented in all the high

sugar-forming varietiesonly. Earlier researchers [22] reported a specific fragment $STI57_{209bp}$ generated by SSR primer, who was detected in all the early blight resistant potato cultivars, in addition to one moderate cultivar, but absent in all the susceptible cultivars. Besides, this analysis also detected some of the null alleles (or missing values) because it was difficult to separate non-amplification due to experimental errors from null alleles (Primer Sti028 with DSP 287). Null alleles (or missing values) in SSR analysis of Indian core potato collection were also reported[19].

The pair-wise genetic similarity among the potato varieties ranged between 0.390 (DSP 287 and K. Himsona) and 0.889 (K. Lauvkar and K. Bahar) (Table 2), with an average similarity of 0.671. It was observed that high genetic similarities were obtained among close parents like DSP 287 and DSP 186 (0.714). Figure 1 shows a dendrogram of 11 varieties based on the total SSR polymorphism at the Dice's similarity coefficient value. Setting the cut-off point of similarity coefficient at 0.671, three main clusters were distinguished, each of which were further split into different subclusters. The first cluster grouped the two DSP varieties; the second cluster contained a single variety (K. Surya); the third cluster was subdivided in subclusters with varying levels of grouping. The close-related and high sugar-forming varieties DSP 287 and DSP 186 were grouped together, whereas more divergences were found between K. Sutlej and its parent K. Bahar as well as K. Badshah and its parent K. Jyoti. On the other hand, high sugar-forming varieties K. Lauvkar and K. Bahar showed highest similarities to each other, as well as low sugar-forming varieties K. Sutlej, K. Jyoti and K. Himsona were grouped together in the same subcluster. Also the two others low sugar-forming varieties *viz*., K. Surya and K. Sadabahar could be segregated from the high sugar-forming varieties in their respective subclusters. The dendrogram clusters were generally in good agreement with previous classification of the varieties. Chimote *et al.*[18] reported that all the 32 varieties of Indian potatoes could be clearly grouped into 3 major and 5 minor clusters in the dendrogram. Tiwari *et al*. [19] obtained a SSR-based dendrogram revealing 8 main clusters including 26 single accessions at Dice's similarity coefficient value of 0.37. None of the accession showed full similarity with any other accession, the maximum similarity was 0.83. Datir [20] found 5 markers which were candidate genes operating in carbohydrate metabolism in potato tubers. Association analysis between five phenotypic components of CIS (crisp colour, Glu, Fru, Suc and dry matter content) evaluated at five storage treatments at 6°C and the microsatellites revealed that 22 markers (92%) which accounted for a total of 60 alleles showed significant associations with at least one of the phenotypic components of CIS. The majority of the associations were recorded after cold storage at 6°C and reconditioning periods.

Genetic diversity is a key to progress in crop improvement programmes. The best way to increase the diversity of crops is to exploit the germplasm stored in the gene banks by introduction into the crop breeding programs[19].Using this basic information, it may be possible to explore the genetic diversity linked to carbohydrate metabolism in potato varieties more effectively with population genetics based approaches, such as association mapping and SNP markers.

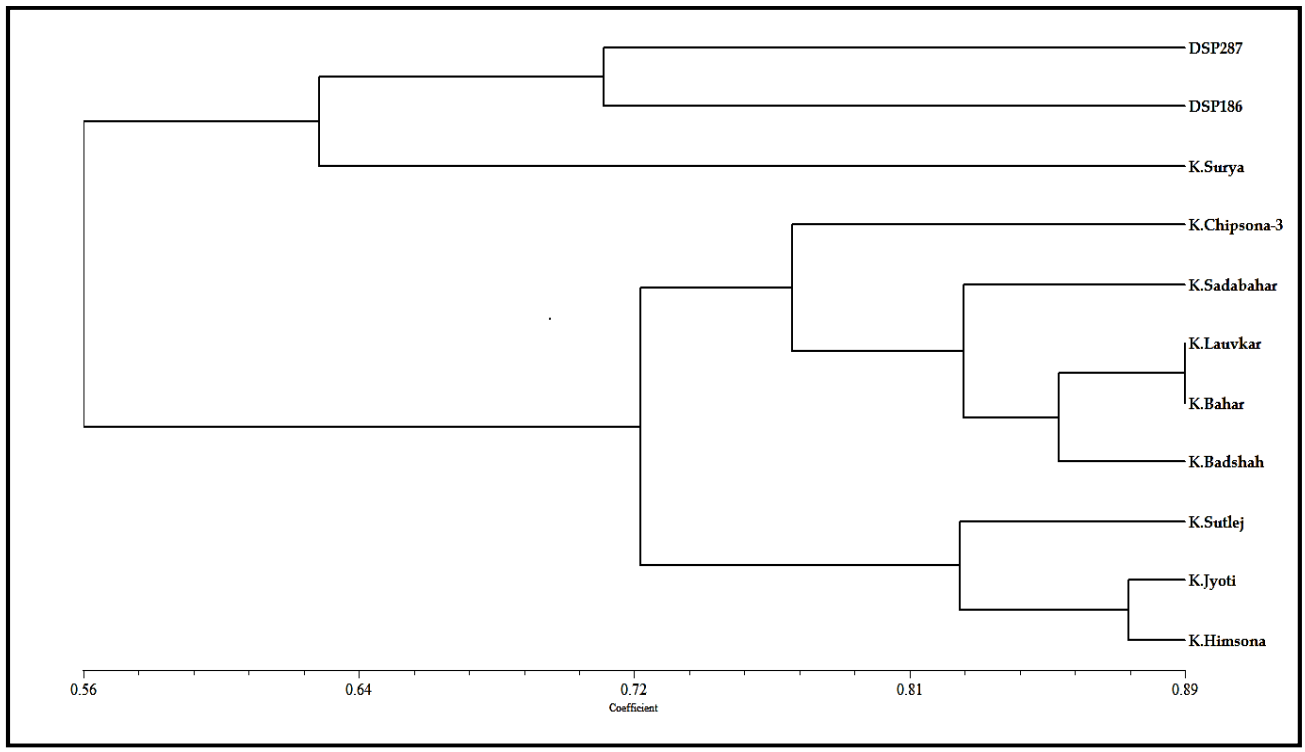

Figure 1. *Dendrogram based on SSR markers of the eleven potato varieties.*

Table 2. *Dice's similarity coefficients based on SSR analysis.*

Potato varieties	DSP 287	DSP 186	K. Surya	K. Chip-3	K. Sutlej	K. Sadabahar	K. Jyoti	K. Lauvkar	K. Himsona	K. Bahar	K. Badshah
DSP 287	1.000										
DSP 186	0.714	1.000									
K. Surya	0.591	0.667	1.000								
K. Chip-3	0.522	0.500	0.696	1.000							
K. Sutlej	0.410	0.486	0.513	0.732	1.000						
K.Sadabahar	0.638	0.533	0.596	0.735	0.619	1.000					
K. Jyoti	0.524	0.600	0.667	0.818	0.865	0.756	1.000				
K. Lauvkar	0.489	0.512	0.578	0.766	0.750	0.833	0.744	1.000			
K. Himsona	0.390	0.462	0.634	0.698	0.778	0.727	0.872	0.762	1.000		
K. Bahar	0.591	0.571	0.591	0.783	0.667	0.851	0.762	0.889	0.732	1.000	
K. Badshah	0.605	0.683	0.605	0.800	0.684	0.783	0.780	0.818	0.650	0.884	1.000

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