

DEVELOPMENT OF ISSR MARKERS FOR A BULGARIAN TOMATO BREEDING COLLECTION AIMING TO IMPROVE ANTIOXIDANT COMPOUNDS IN FRUITS

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Abstract

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The Inter-Simple Sequence Repeat marker technique was applied to a set of tomato genotypes that were found to diverge in antioxidant compounds in fruits. As marker-assisted selection is becoming a tool that brings major advantages for breeding programs need is rising to use it to quickly respond to consumer demand. Being part of a system for efficient plant breeding in many crops the aim of the present study was to test Inter-Simple Sequence Repeats as candidate tool for incorporating in a breeding program aiming to improve antioxidant properties of tomato fruits. Application of Inter-Simple Sequence Repeat markers resulted in obtaining adequate representation of every accession from the selected group. This opens the opportunity for using the system in confirming the hybrid nature of the F1 progenies and further accelerating the breeding of genotypes for use as healthy food.

Key words: *Solanum lycopersicum*; molecular markers; Inter-Simple Sequence Repeats; ISSRs

Introduction

Genotype identification is of great importance for seed production and cultivar propagation. In the recent years marker-assisted selection (MAS) is becoming a tool that brings major advantages through allowing for identification of genotypes based on the unique sequences in their genome and following these sequences through the progenies of intercrossed species.

The potential of the molecular techniques for developing “DNA passports” for tomato cultivars and hybrids is discussed in a number of publications (Kochieva et al., 2002a;

Kochieva et al., 2002b; Suliman-Pollatschek et al., 2002; Cooke et al., 2003; He et al., 2003; Tikunov et al., 2003; Park et al., 2004; Frary et al., 2005; García-Martínez et al., 2006). In many of these reports a tendency for decreasing of the genetic diversity in modern cultivars is discussed, the process being particularly notable in the recent years and within the breeding programs for hybrid production (Archak et al., 2002). Testing of 27 cultivars widely grown in India with RAPDs (Archak et al., 2002) demonstrated that the variability within the primitive local and older introduced cultivars is higher than that within the recently developed genotypes. The overall variability in the studied genotypes was rather

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low in spite of the presence of cultivars from both primary and secondary centers of origin of the crop.

RAPDs were applied by Kochieva et al. (2002b) for the analysis of a collection composed of 43 accessions and 10 cultivars from many representatives of the *Solanum lycopersicum* species. The applied 8 RAPD primers allowed grouping of the genotypes according to the known phylogenetic relations. However even after producing 248 polymorphic bands within this group of genotypes obtaining a unique pattern for each of them could not be achieved. The same group (Kochieva et al., 2002a) attempted the application of Inter-Simple Sequence Repeat markers (ISSRs) where the combination of 14 ISSR primers for characterizing 54 genotypes resulted in 304 polymorphic fragments and this extended set was able to produce unique pattern for each of the tested accessions.

ISSR marker system was used by Tikunov et al. (2003) for comparative characterization of *Lycopersicon esculentum*, *Lycopersicon pennellii*, *Lycopersicon cheesmanii*, *Lycopersicon humboldtii*, *Lycopersicon hirsutum*, together with two isogenic lines from *Lycopersicon esculentum*. In this study 9 of the 14 primers used were able to independently differentiate all the species. Authors concluded that in addition to their high reliability, reproducibility and speed of development ISSRs can identify significant number of polymorphisms in tomato, which characteristics are extremely important for the practical applications of a DNA marker system in plant breeding. Similarly ISSR markers were tested on 10 genotypes to demonstrate their relative reproducibility as compared to SSR and RAPD markers (Sanghani and Mandavia, 2013). The same system was used by Henareh et al. (2016) to assess genetic diversity in tomato landraces from Turkey. Furthermore ISSR markers were demonstrated to bare potential in associating with agronomically important traits such as hybrid performance (Hernandez-Ibanez et al., 2014) and drought tolerance (Metwali et al., 2016).

In 2003 Cooke et al. studied the applicability of the SSR markers to distinguishability, uniformity and stability (DUS) testing in accordance with the UPOV standards. Analyzing 36 plants from 10 widely used cultivars demonstrated that 9 of the 10 cultivars were homogenic in the 6 loci tested thus meeting the UPOV standards. In spite of fully corresponding to the phenotypic descriptors of the organization 3 of the plants from the last cultivar carried a different allele in one of the tested loci. Current DUS testing standard for tomatoes allows for maximum 1 in 20 plants deviating for some of the characteristics, thus this cultivar would not pass the test if the results from the DNA analyses were equally acceptable to the phenotypic characteristics. Similar or even higher levels

of heterogeneity were observed by Bredemeijer et al. (2002) when constructing the European tomato database. Testing of 500 cultivars in that study resulted in 30% of them expressing some heterogeneity.

The analysis of the available literature indicates that the lack of preliminary information about the Bulgarian genotypes suggests that the initial screening of such collection should be made with ISSR primers. Due to their multilocus assessment capability in a single reaction and the relatively uniform distribution within tomato genome (Tikunov et al., 2003) their application bares sufficient potential of identifying unique profiles for each genotype.

The aim of the present report is to present the results from testing ISSRs as candidate technique for identification of diversity in tomato genotypes within one Bulgarian breeding collection, assembled with the aim of improving the antioxidant content of cultivated varieties.

Materials and Methods

Plant material

Breeding lines 975, 984, 53 β , 1116, 1140 as well as varieties IZK Alia and Plovdivska karotina were used in the present study. All cultivars and breeding lines belong to the *Solanum lycopersicum* species. Accessions are maintained at "Maritsa" Vegetable Crops Research Institute and are used in establishing a breeding program as donors of antioxidative stress ingredients (ascorbic acid, lycopene, beta-carotene, anthocyanins, etc.) and other fruit quality traits (ex. dry matter content). Furthermore, some of these lines proved valuable sources for traits, related to improving agronomic performance as crops.

The accessions used in the present study were homogenized and stabilized for the main approbation traits of the fruits and plants and controlled for the main biochemical compounds of the fruits. Only the plants fully corresponding to the phenotypic descriptors of the UPOV for the cultivar/line were used for DNA extraction. These genotypes were never before tested for their homogeneity with the use of molecular markers.

ISSR analysis

ISSR analysis was performed by testing a number of primers that demonstrated high reproducibility and polymorphism identification in our previous studies (Bojinov and Danailov, 2009). The selection of the final group of 7 primers (Table 1) was based on our previous experience and the screening of the selected primers against the available data on the frequency of tandem repeats in tomato genome.

Table 1
Description of the tested ISSR primers

Primer	DNA sequence	Length (bp)
ISSR 1	(AG) ₈ C+TC	19
ISSR 2	(AG) ₈ C+TG	19
ISSR 3	(GA) ₈ T	17
ISSR 4	(GA) ₈ C	17
ISSR 5	(GA) ₈ YC	18
ISSR 6	(AG) ₈ YT	18
ISSR 7	(GT) ₈ YC	18

PCR reactions were performed in 25 µl volume with the following cycling regime: denaturing at 94°C for 3 min, 40 cycles of 94°C – 1 min, AT – 45 sec, 72°C – 45 sec, followed by final extension of 72°C – 4 min, where AT is the annealing temperature for each primer calculated according to Kochieva et al. (2002a).

PCR products were analyzed through separation in 2% agarose gels and staining with ethidium bromide.

Results and Discussion

DNA extraction procedure resulted in obtaining high quality genomic DNA from all accessions (Figure 1). Between 300 and 500 µg of genomic DNA was recovered in most of the cases. When the quantity or quality of the obtained DNA was considered unsatisfactory the extraction was repeated, so that the above criteria can be met.

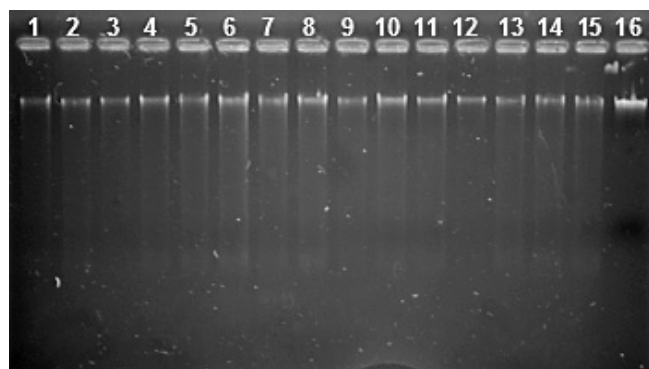


Fig. 1. Results from extracting tomato genomic DNA from young leaves of selected genotypes. Each lane is loaded with 10 µl of the final solution of extracted DNA and stained with ethidium bromide

lanes 1-7 – individual plants from Line 984,
lanes 8-14 – individual plants from Line 975,
lanes 15-16 – individual plants from line 1140

Due to reasons discussed above our system for screening was based on Inter-Simple Sequence Repeat markers (ISSRs). We screened individual plants from each of the accessions (Figure 2) to verify the capacity of the chosen marker system to reveal sufficient number of polymorphisms within each of them. As Figure 2 demonstrates some of the ISSR primers produced few polymorphic bands, which were not sufficient to identify every plant from the accession. This was expected as tomatoes are self-pollinating crop and thus not much heterogeneity is expected within each genotype.

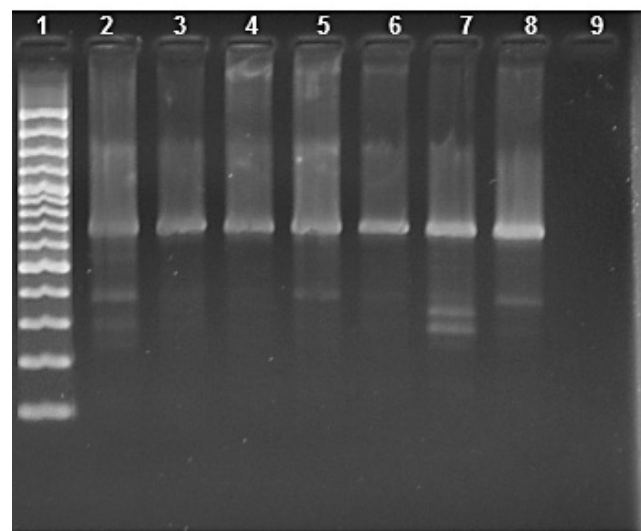


Fig. 2. Revealing heterogeneity within accessions with primer ISSR2

lane 1 – standard sized DNA with bands of 500 and 1000 bp appearing as strong signals, lanes 2-8 – products from PCR reactions with individual plants of line 21β, lane 9 – control

The results from this initial screening demonstrated however the capacity of the selected marker system to differentiate even individual plants within the accessions. The figure represents the results obtained with 7 plants from one of the genotypes.

The above results were obtained by using genomic DNA extracted from single plants for each of the accessions. To verify the usability of individual bands as specific for any genotype bulked DNA samples should be preferably used. However the use of bulked DNA samples obscures the levels of heterogeneity within each genotype and thus can jeopardize the reproducibility of the results if heterogenic material is included in the sample. The chances of such heterogeneity occurring in each sample are not negligible as discussed by Cooke et al. (2003) and Bredemeijer et al. (2002). With no preliminary DNA data on the genotypes used in our study this possibility had to be examined.

The evaluation of the existing heterogeneity within each of the cultivated genotypes was performed through testing each of the selected 7 plants per accession with all ISSR primers and looking for appearance of uncommon bands. Interestingly none of the genotypes showed complete uniformity of the profiles of individual plants (as would be expected from strictly self-pollinating species). In all of the genotypes at least one plant demonstrated differences in the obtained profiles. As demonstrated on Figure 3 plant No. 4 (Lane 5) had the band at about 380 bp missing which band is present in all other plants from the same accession. On the other hand a band at 410 bp appears as relatively strong signal in that plant which is uncommon to other representatives of the same accession.

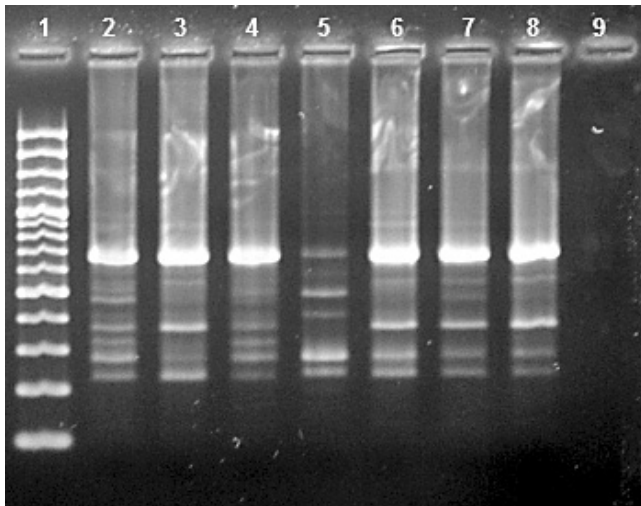


Fig. 3. Revealing heterogeneity within accessions with primer ISSR 1

lane 1 – standard sized DNA with bands of 500 and 1000 bp appearing as strong signals, lanes 2-8 – PCR products from individual plants of line 1140, lane 9 – control

As expected testing 7 ISSR primers resulted in revealing different levels of polymorphism within accessions. However the use of the 3 ISSR primers that together produced most polymorphic bands (55 in total) resulted in obtaining clustering of the tested set of plants that corresponds well to their initial designation (Figure 4). In general the grouping of the plants corresponds to their designated accession with occasional individuals appearing moved to a closely related cluster. Notable exceptions are the accessions “IZK Alia”, Line 1116, and Line 984 where plants were separated in two distinguished groups. These exceptions would have inspired doubts in the applicability of our system if they were not corresponding to the metabolite profiles of the same plants (our unpublished data). However we have found these no-

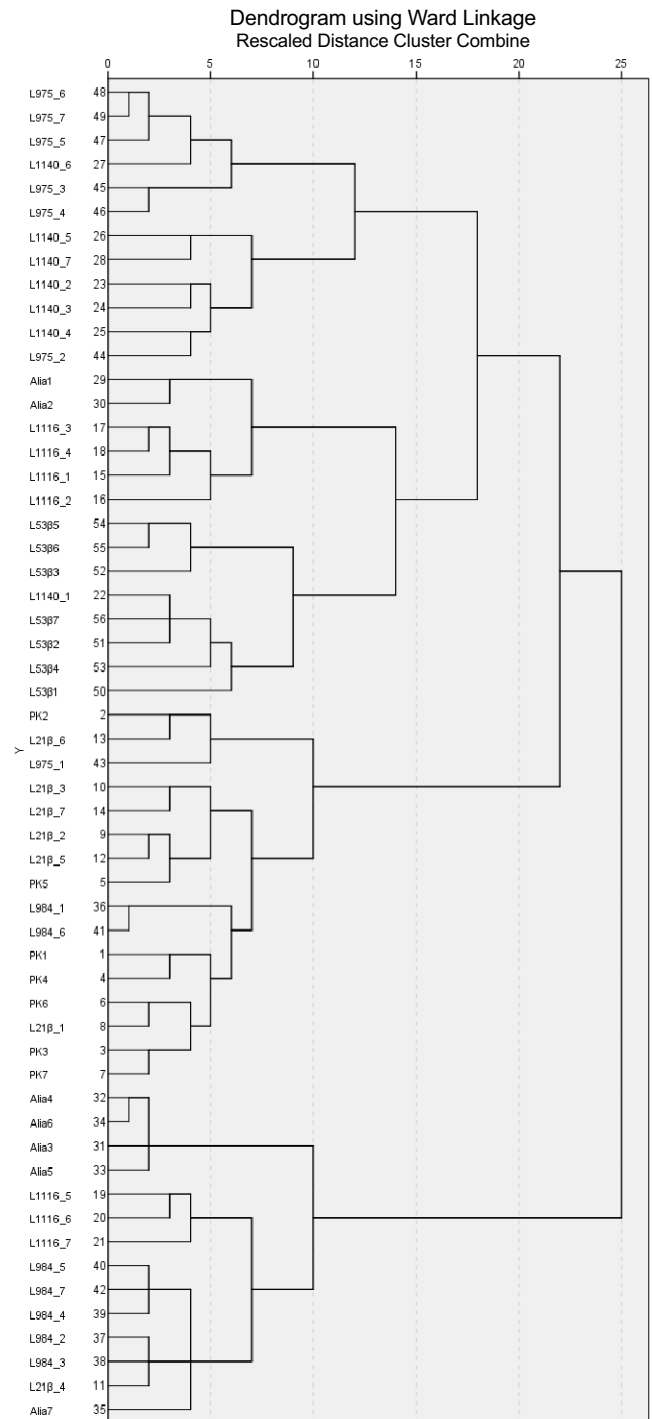


Fig. 4. Dendrogram of genotype distribution based on 55 polymorphic bands produced by three ISSR primers

table exceptions in DNA profiles to correspond well to the deviations in metabolite profiles (antioxidant compounds in fruits) of the same plants.

The observed differences between the plants in each variety that appeared identical in their phenotypic characteristics at the time of initial selection can be attributed to a number of reasons. One possible explanation of the observed disagreement between the phenotypic homogeneity and the identified genotypic heterogeneity is that the heterozygous loci are unrelated to the traits the genotypes are selected for during the breeding process. On the other hand Bredemeijer et al. (2002) suggested that such results can be explained by the presence of residual heterogeneity mainly in the non-coding sequences and/or within the quantitative trait loci (QTLs) with very small phenotypic effect. Eliminating such residual heterogeneity would be impossible with the classical breeding approaches.

It is questionable whether attempting to eliminate any such heterogeneity with the use of molecular markers is of any practical use as in most cases they could not be associated with any phenotypic effects. Whether in the cases of differing plants from Line 1140, Line 984 and IZK Alia the identified marker diversity could be linked to the observed variation in metabolite profiles is a matter of further studies.

The demonstrated capacity of the ISSRs to identify the presence of such heterogeneity in the studied material provides striking evidence in support of the careful selection of the marker system. The marker system of choice that could be used in the breeding programs has to be reliable, efficient, fast and easy to implement. In cases where a single plant deviates from the group of 7 of the same genotype discarding that plant can easily result in eliminating the heterogeneity from the selected genotype. The other 6 plants can be safely used to produce pure material for seed multiplication of that genotype. However in cases where more than one plant appears with a different profile (the cases in Figure 2 and Figure 3) the selection of the representative set of bands for a particular genotype might be difficult, especially when the plants appear divided into two more or less equal groups (like was the case with cv. Plovdivska karotina, data not presented). In such cases the question what should be considered "significant deviation" arises and whether a representative profile for that particular variety can be produced so that it can be distinguished from other varieties.

Conclusions

The results presented in this paper demonstrate that ISSR markers can efficiently differentiate tomato genotypes even within phenotypically highly homogenous accessions. Ap-

plication of ISSRs revealed not only sufficient polymorphisms between different accessions, but was capable of identifying deviating plants within individual accessions. This demonstrates the power of the proposed technique to resolve differences even within presumably highly homogenous genotypes like the self-pollinating cultivars from *Solanum lycopersicum* species. As evidenced in obtained clustering (Figure 4) the ISSR markers are capable of not just identifying diversity in the studied accessions, but are apparently baring the potential to efficiently identify individuals with differing metabolite profiles within varieties. The ease of use and the high reliability of the system make it very good candidate for practical application in tomato breeding programs.

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