ИЗПОЛЗВАНЕ НА МАРКЕРИ МЕЖДУ ПРОСТИТЕ ПОВТОРЕНИ ПОСЛЕДОВАТЕЛНОСТИ В РАЗРАБОТВАНЕТО НА СЕЛЕКЦИОННА ПОПУЛАЦИЯ ПРИ ДОМАТИТЕ
APPLICABILITY OF INTER-SIMPLE SEQUENCE REPEAT MARKERS IN DEVELOPING TOMATO BREEDING POPULATION

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Резюме

В настоящата разработка са идентифицирани ДНК маркери между простите повторени последователности при родителски форми и при техните F_1 хибриди, различаващи се по отношение на антиоксидантния състав на плодовете. С превръщането на селекцията с помощта на молекулярни маркери в основно средство, носещо съществени ползи за селекционните програми, нараства и нуждата от използването й за постигането на бърз отговор на изискванията на потребителите. Съвременните генни технологии разкриват възможности за ефективното приложение на системи за ефикасна селекция при много култури, включително и при доматите. Целта на изследването е да се изпитат този тип маркери като кандидати за използване в селекцията при доматите. С помощта на тази маркерна система беше постигнато ефективно установяване на хибридния характер на поколението от родителски генотипи домати, кръстосани с цел подобряване на антиоксидантното съдържание на плодовете.

Abstract

The Inter-Simple Sequence Repeat marker technique was applied to parental tomato genotypes and their F_1 hybrids differing in the antioxidant compounds in the fruits. As the marker-assisted selection is becoming a tool that brings major advantages for breeding programs, the need to use it to quickly respond to the consumer demand has greatly increased. Modern genetic technology opens the possibility for effective application of a system for efficient plant breeding in many crops, including tomatoes. The aim of the present study was to test the Inter-Simple Sequence Repeats as a candidate tool to use in Marker-Assisted Selection in tomatoes. The application of the Inter-Simple Sequence Repeat markers allowed for efficient verification of the hybrid origin of the progeny from parental genotypes that were crossed for improving the antioxidant content in tomato fruits.

Ключови думи: *Solanum lycopersicum*, молекулярни маркери, Inter-Simple Sequence Repeats, ISSRs.
Key words: *Solanum lycopersicum*, molecular markers, Inter-Simple Sequence Repeats, ISSRs.

INTRODUCTION

Marker-assisted selection (MAS) is becoming a tool that brings major advantages through speeding up the process of developing improved crops. A number of publications discuss the potential of the molecular techniques for developing "DNA passports" for tomato cultivars and hybrids (Kochieva et al., 2002a; Kochieva et al., 2002b; Suliman-Pollatschek et al., 2002; Cooke et al., 2003; He et al., 2003; Johnson et al., 2003; Tikunov et al., 2003; Adreakis et al., 2004; Park et al., 2004; Frary et al., 2005; García-Martínez et al., 2006). In many of these reports a tendency for reduction 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). Variability was found to be higher within the primitive local and older introduced cultivars than within the recently developed genotypes (Archak et al., 2002) when 27 cultivars widely grown in India were assessed with RAPDs. The overall variability was rather low in spite of the presence of cultivars from both primary and secondary centers of origin of the crop.

Kochieva et al. (2002b) also used RAPDs for the analysis of a collection composed of 43 accessions and 10 cultivars from *Solanum lycopersicum* species. The grouping of the genotypes according to the known phylogenetic relations was achieved through the combining the results from amplifications with 8 RAPD primers. The same authors (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. Their study provided evidence that ISSRs can be effectively used in tomato breeding.

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*. Authors found 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 DNA marker systems in plant breeding.

Cooke et al. (2003) studied the applicability of the SSR markers. Analyzing 36 plants from 10 widely used cultivars they demonstrated that 9 of the 10 cultivars were homogenic in the 6 loci tested. 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 level of heterogeneity.

The analysis of the available literature indicates that for the purposes of marker-assisted selection (MAS) in tomatoes ISSR markers bare great potential. Due to their multilocus assessment capability in a single reaction and the relatively uniform distribution within tomato genome (Tikunov et al., 2003) they can be effectively used to produce unique profiles for each genotype. This is an essential first step in applying the technique in a breeding scheme.

The aim of the present report is to present the results from testing ISSRs as candidate technique for assisting breeding efforts in a Bulgarian tomato breeding scheme, initiated with the aim of improving the antioxidant content of cultivated varieties.

MATERIALS AND METHODS

Plant material

Parental genotypes "IZK Alia", "Plovdivska karotina" (PK) and "L 1116" used in present study belong to the *Solanum lycopersicum* species. They are maintained at "Maritsa" Vegetable Crops Research Institute and are used in breeding programs as donors of antioxidative stress ingredients (ascorbic acid, lycopene, beta-carotene, etc.) and other fruit quality traits (i.e. dry matter content).

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 respective line were used for DNA extraction. DNA was extracted from a total of 56 plants from parental genotypes and 13 F_1 individuals and used to perform marker analyses. The parental genotypes were never before tested for their homogeneity with the aid of molecular markers.

ISSR analysis

ISSR analysis was performed by testing a number of primers that demonstrated high reproducibility and capacity for 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.

PCR reactions were performed in 25 µl volume with the following cycling regime: denaturing at

Таблица 1. Описание на използваните ISSR праймери
Table 1. Description of the tested ISSR primers

Primer Праймер	DNA sequence ДНК последователност	Length (bp) Дължина (нд)
ISSR 1	$(AG)_8C+TC$	19
ISSR 2	$(AG)_8C+TG$	19
ISSR 3	$(GA)_8T$	17
ISSR 4	$(GA)_8C$	17
ISSR 5	$(GA)_8YC$	18
ISSR 6	$(AG)_8YT$	18
ISSR 7	$(GT)_8YC$	18

94^0C for 3 min, 40 cycles of 94^0C – 1 min, AT – 45 sec, 72^0C – 45 sec, followed by final extension of 72^0C – 4 min, where AT is the annealing temperature for each primer calculated according to Kochieva et al. (2002a).

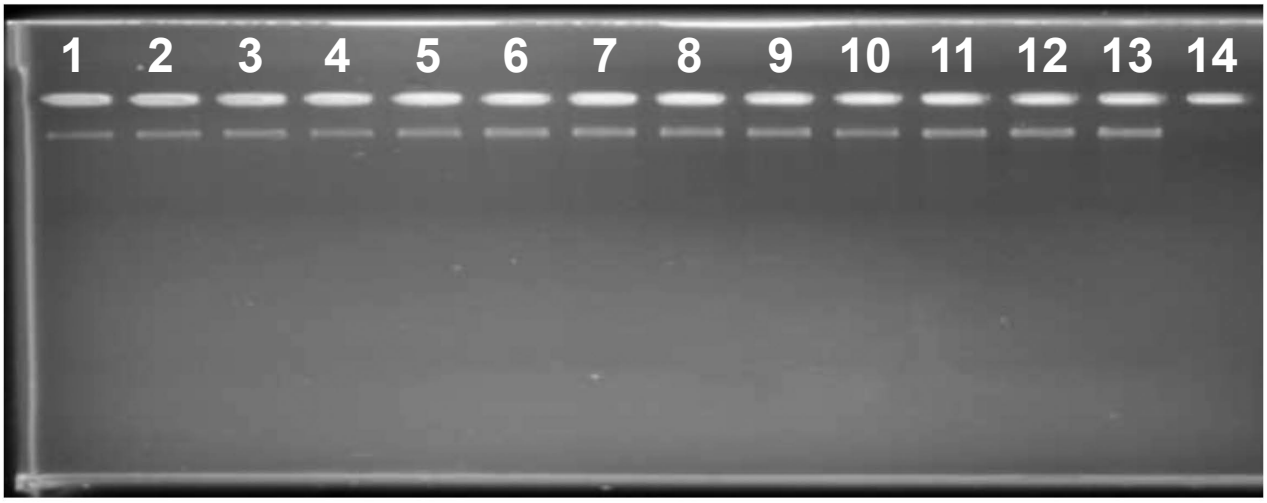
PCR products were analyzed through separation in 2% agarose gels and staining with ethidium bromide.

RESULTS AND DISCUSSION

DNA extraction procedure resulted in obtaining high quality genomic DNA from the three accessions (Fig. 1). Between 300 and 350 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 these criteria can be met.

Due to reasons discussed above our system for screening was based on Inter-Simple Sequence Repeat markers (ISSRs). We used these markers to screen the parents to verify the capacity of the chosen marker system to reveal sufficient number of polymorphisms in the cross.

The evaluation of the existing heterogeneity within each of the parental lines was performed



Фиг. 1. *Резултати от екстракцията на геномна ДНК от млади листа на F_1 хибридите. Във всеки старт е поставен 10 µl от разтвора на екстрахираната ДНК и продуктите са оцветени с етидиев бромид.*
Стартове 1-10 – геномна ДНК от индивидуални растения от кръстоската ПК х Л 1116.
Стартове 11-13 – геномна ДНК от индивидуални растения от кръстоската ИЗК Аля х Л 1116.
Старт 14 – празна контрола
Fig. 1. *Results from extracting tomato genomic DNA from young leaves of F_1 hybrids. Each lane is loaded with 10 µl of the final solution of extracted DNA and stained with ethidium bromide.*
Lanes 1-10 – genomic DNA from individual plants from the PK x L 1116 cross. Lanes 11-13 – genomic DNA from individual plants from IZK Alia x L 1116 cross. Lane 14 – empty control

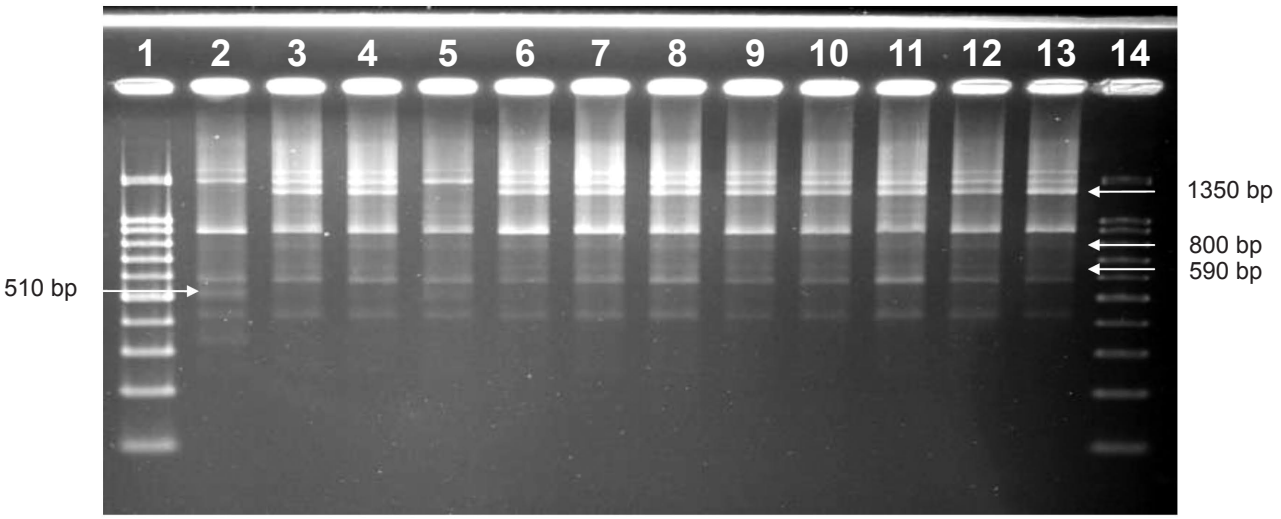
through testing each of the selected parental plants with all 7 ISSR primers and looking for appearance of uncommon bands.

The use of the 7 ISSR primers produced a total of 35 polymorphic 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 three genotypes at least one plant demonstrated differences in the obtained profiles (data not shown).

The observed polymorphisms between the plants from each genotype 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 is impossible with the classical breeding approaches that were used to develop parental genotypes. The capacity of the ISSR markers to identify such heterogeneity opens up the possibility to further homogenize parental lines, converting them to genuinely true-breeding ones.

It is however questionable whether attempting to eliminate any such heterogeneity with the use of molecular markers is of any practical use as in most cases it could not be associated with any phenotypic effects. Whether it would be possible to link the identified marker diversity in differing plants from our study to the observed variation in metabolite profiles is a matter of further studies that would require *inter alia* a verification of the differences in metabolite profiles under different growing conditions.

On the other hand the demonstrated capacity of the ISSR markers 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 introduced in the breeding programs has to be reliable, efficient, fast and easy to implement. The application of ISSRs in our study resulted in identifying the F_1 plant No. 3 (Fig. 2, lane 5) as a non-hybrid individual. The conclusion that it is rather an individual, originating from self-pollination of the P_1 parent was based on the presence in its profile of the band of 510 bp that was present in the P_1



Фиг. 2. *Резултати от тестването на родителите и F_1 хибридните растения с праймер ISSR 3. Подредбата на генотипи на гела е, както следва: Старт 2 – Родител 1 (ПК), Стартове 3-12 – F_1 хибридни растения. Старт 13 – Родител 2 (Л 1116). Стартове 1 и 14 – ДНК със стандартни размери. Стрелките сочат ивиците, показващи полиморфизъм между родителските форми*

Fig. 2. *Results from testing parents and F_1 hybrid plants with primer ISSR 3. Genotypes are ordered on the gel as follows: Lane 2 – Parent 1 (PK), Lanes 3-12 – F_1 hybrid plants. Lane 13 – Parent 2 (L 1116). Lanes 1 and 14 – standard sized DNA. Arrows indicate bands that are polymorphic between the parents*

parent and not present in P_2 parent. Furthermore, the same individual lacks the bands of 590, 800 and 1350 bp that are present in P_2 and not in P_1. Of course that plant may have originated from a kind of (modified) parthenogenesis or as a result from some other reproductive mechanism but determining what particular mechanism resulted in its appearance is beyond the scope of the present paper and therefore will not be further discussed.

Our results demonstrated that the DNA profiling of the F_1 hybrid plants that allow identification of the non-hybrid individual and the confirmation of the hybrid nature of other individuals could be achieved with the use of a single ISSR primer (ISSR 3 in the case, presented on Fig. 2). The ISSR marker system is very well suited for use in breeding programs as it requires small amount of initial genomic DNA, uses simple application protocol and produces reliable results (Penner et al., 1993; Rajput et al., 2006). The results from this study demonstrated the capacity of the selected marker system to differentiate not only the parental lines, but some of the individual plants within parental lines, too. Furthermore, the application of the ISSR system allowed the identification of a single deviating plant from the F1 hybrid population, which deviation was later confirmed phenotypically.

Our study confirms the applicability of this system in tomato breeding as we demonstrated how the use of a single primer can result in early confirmation of the hybrid nature of the F_1 individuals as well as the detection of the ones of non-hybrid origin. This can be of great help as the F_1 populations in tomatoes are commonly established through transplanting and such early detection of deviating individuals can save expensive experimentation space and reduce needs for qualified labor in the early stages of the breeding program. The effects are multiplied for the cases, where the deviating characteristics are inherited as recessive traits and thus their earliest identification by phenotyping is possible only in the F_2 generation.

CONCLUSIONS

The results presented in this paper demonstrate that ISSR markers can efficiently differentiate tomato genotypes even within highly homogenous accessions. The application of ISSRs revealed not only sufficient polymorphisms between parental genotypes, 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 accessions like the self-pollinating cultivars from *Solanum lycopersicum* species. It was further demonstrated how the system can be efficiently used to confirm the hybrid origin of the F_1 plants and to identify individuals of non-hybrid origin. 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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