THEORETICAL PAPERS AND REVIEWS

Studying Plant Genome Variation Using Molecular Markers

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Abstract—The authors' studies on the organization and variation of plant genome with the use of molecular markers are briefly reviewed with special emphasis on random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), sequence characterized amplified region (SCAR), and cleaved amplified polymorphic sequence (CAPS) markers detected with the use of polymerase chain reaction (PCR). These markers have been demonstrated to be promising for identifying cultivars and determining the purity of genetic strains of pea. Genetic relationships between strains, cultivars, and mutants of pea have been studied. The role of molecular markers in molecular genetic mapping and localizing the genes of commercially important characters of pea has been shown. The possibility of the use of molecular markers for studying somaclonal variation and detecting mutagenic factors in plants during long-term spaceflights is considered. The prospects of using DNA markers for understanding the organization and variability of higher plant genomes are discussed.

INTRODUCTION

Genomes of higher plants are characterized by large sizes and complex organization. The use of molecular methods of DNA analysis is one of approaches to studying these complex genomes. Molecular markers are currently most often used for these purposes. Molecular DNA markers are polymorphic nucleotide sequences dispersed over the genome whose mutations may be detected by PCR-based techniques.

The introduction of molecular markers into biological studies offered new possibilities for studying genetic diversity and determining genetic relationships within and between species.

The use of molecular DNA markers is very promising for detailed chromosome mapping, identifying and cloning genes, and constructing new plant cultivars. Variants of DNA amplification with arbitrary and specific primers allowing the rapid detection of numerous variable loci throughout the plant genome are widely used.

METHODS OF DNA ANALYSIS

RAPD Markers

The random amplified polymorphic DNA (RAPD) method [1, 2] is one of the most widely used approaches for revealing genetic polymorphism in plants. This method is based on polymerase chain reaction (PCR) with arbitrary single primers nine to ten nitrous bases in length with a predominantly G/C composition (60–80%) and relatively low annealing temperatures. Usually, RAPD primers yield three to fifteen amplification products [3]. The DNA regions amplified

by the RAPD method are scattered over the genome; most of them are repetitive nucleotide sequences [2].

This method has the advantage of technical simplicity and rapidness. It requires neither information on the DNA sequence nor large initial amounts of DNA [4, 5]. The method does not use radioactive substances, is inexpensive, and can be automated [6].

ISSR Markers

Beginning from 1994, PCR-based inter simple sequence repeat (ISSR) analysis [7] has been used in molecular marking. Oligonucleotide primers used in PCR consist of repetitive units and the so-called anchor at the 3' or 5' end. The amplified DNA fragments are located within a relatively small region between two microsatellite sequences. The repetitive units comprise one to five nucleotide pairs. The method does not require preliminary information on the DNA sequence analyzed [7, 8]. The PCR using ISSR primers yields a set of intermicrosatellite DNA fragments of various lengths. The ISSR analysis has as high a resolution as the RAPD method while ensuring better reproducibility of the spectrum (since the primer is longer and complementary to the microsatellite region). The method is promising for genomic mapping and marking agriculturally important characters [9–11].

SCAR Markers

Sequence characterized amplified region (SCAR) markers [12] are PCR-based molecular markers originating from individual RAPD fragments but identified with the use of long, specific primers.

To obtain specific SCAR markers, polymorphic RAPD or ISSR fragments are cut out of the gel, cloned, and sequenced. After the sequencing, SCAR primers (usually 20–25 bases in length) are selected for the terminal regions of the fragments.

When RAPD markers are transformed into SCAR markers, a fragment may be amplified that is a dominant marker and exhibits a polymorphism similar to that of the original RAPD marker (i.e., it is amplified only in the same samples as the original RAPD fragment is) [13, 14]. In the case of dominant SCAR markers, when the only fragment is amplified in some DNA samples but not in others, the analysis can be performed in terms of presence/absence. SCAR markers may be codominant if insertions or deletions appear between primer binding sites in one genotype compared to another [15].

These markers were successfully used for identifying and mapping the genes of resistance to various diseases of lettuce [12], tomato [16], and wheat [17].

CAPS Markers

The cleaved amplified polymorphic sequence (CAPS) method [18] belongs to the STS group. The method uses so-called secondary markers, which are flanked by two primers synthesized on the basis of a known DNA sequence (e.g., SCAR fragments). They specifically amplify single DNA fragments; however, polymorphism is observed if these fragments are cleaved by one or several restriction endonucleases. This method was effectively used for obtaining molecular DNA markers in *Arabidopsis*, which proved to be ecotype-specific [19].

The CAPS method has some advantages over random-replicon methods, primarily, codominant inheritance and high reliability. In addition, the use of CAPS markers makes it possible to determine the correspondence between the genetic and physical chromosome maps, because the physical positions of loci of a given type is usually known before their use in genetic analysis. This method is not sensitive to the amount of the DNA template and is inexpensive.

REVEALING PEA GENOME POLYMORPHISM USING DNA MARKERS

To date, the RAPD method is the most common approach to identifying DNA polymorphisms in plants [20–22].

The sets of reaction products, i.e., RAPD spectra comprising DNA fragments of different lengths, allow the researchers to detect differences between the genomes of closely related organisms. These fragments are specific genomic markers and allow polymorphisms to be found in numerous loci over the entire genome. Therefore, it is not surprising that RAPD anal-

ysis reveals more genetic polymorphisms than other methods do.

The RAPD method was used to determine that different cultivated plants differ from one another in genetic variation rate. For example, the degree of DNA polymorphism in various cultivars of cross-pollinators (e.g., maize and sunflower) was found to be higher than in self-pollinators (e.g., barley and soybean) [23]. The RAPD analysis of barley, potato, and plants from the mustard and legume families demonstrated that differences between cultivars were considerably larger than those between species [20–22].

Molecular markers were first used to study the genome of pea, a classic genetic object, in the early 1990s [24]. In our laboratory, DNA markers have been used since the late 1990s [25, 26]. The objects of the first RAPD analysis of plant genomes in Russia were cultivars, strains, and mutants of pea from the collection of the Department of Genetics of Moscow State University with the use of Jacquard's coefficient as a measure of genetic similarity. The divergences between mutants and original cultivars, between different strains, and between cultivars were found to be 9, 28, and 35%, respectively.

In this study, RAPD markers were found that were present in the spectrum of one cultivar and absent in the spectra of all others. These markers can be used for identifying pea cultivars.

Subsequent studies [27, 28] confirmed the high intraspecific polymorphism, i.e., large differences between pea cultivars, strains, and mutants; six cultivar-specific RAPD markers were found that can be used for the certification of pea cultivars.

Our further studies on the genetic polymorphism of the pea genome with the use of DNA markers demonstrated that each pea genotype was characterized by its own specific RAPD spectrum (Fig. 1) [29, 30]. On the other hand, the results showed the absence of polymorphism within each strain.

We performed special experiments to test the inheritance of some polymorphic RAPD fragments (B474#800, B474#550, Pr10#820, Pr10#660, and Pr10#340) characterizing individual genotypes of pea (Figs. 1a, 1b). The RAPD method was used to study the F₁ and F₂ hybrids between plants polymorphic for these fragments. The results of these experiments showed that the RAPD fragments studied were dominant Mendelian characters, which agrees with data reported by other authors [20] and the results of our earlier studies [27].

In order to estimate quantitatively the polymorphism found in the study [30] and the genetic divergence between different strains, cultivars, and mutants of pea, we presented the data in the form of a matrix of the states of 90 binary characters and used them to calculate genetic distances. The mean divergence between different cultivars of pea was 18.7%; the mean divergence between genetically related mutants and culti-

Fig. 1. Amplification products of the DNAs of cultivars, strains, and mutants of pea obtained with the use of primers (a) B474 and (b) Pr10. M, a molecular weight marker, 1.5 kb + 100-bp DNA ladder (Sibenzim); C⁻, negative control. Cultivars: 1, Rannii Zelenyi; 2, Raport; 3, Kapital; 4, Nord; 5, Filbi; 6, Pioneer; 7, Flagman; 8, Nemchinovskii; 9, Korol' Gurmanov; 10, Zhegalovskii; 11, Roza Kraun; 12, Anvend; 13, Izumrud; 14, Viola; 15, Sakharnyi; 16, Bogatyr'; 17, Neosypayushchiysya; 18, Truzhenik; 19, Orus; 20, Uzkobovoyi. Strains: 21, L-1238; 22, L-111; 23, L-25; 24, L-84. Mutants: 25, R-64; 26, Khl-42; 27, Khl-7; 28, R-13; 29, Khl-18; 30, Khl-15; 31, W-2; 32, Khameleon; 33, Lyupinoid; 34, Vio-V.

vars, 7.6%. These data agreed with our previous results [26].

We used the matrix of the differences between all pea strains, cultivars, and mutants examined to construct the tree reflecting the degrees of differences between the RAPD spectra of the object studied (Fig. 2). The unweighted pair-group method with arithmetic averages (UPGMA) was used. As seen from Fig. 2, reliable clusters with high bootstrap values (73–100%) were formed by mutants and original cultivars (cultivar Viola clustered with its mutant Vio-V; cultivar Kapital, with mutants Khl-7 and R-13; and cultivar Rannii Zelenyi, with mutants W-2 and Khl-18). The substantial intraspecific genetic polymorphism found in this study provides a reliable basis for planning effective crosses used in experiments on mapping the pea genome. In addition, RAPD-based genotyping allowed us to detect cases of large genetic differences between cultivars and mutants obtained from them. The finding of differences in polymorphic loci between forms that are so close to one another is of special interest as it indicates the large scale of genetic rearrangements caused in plants by mutagenic factors.

Careful selection of RAPD primers and RAPD fragments for identifying different genotypes of pea, as well as adjustment and standardization of the PCR conditions using the selected primers, made it possible to obtain highly reproducible results and raise the question of the development of inexpensive, rapid, and effective technologies of the certification of pea cultivars, strains, and hybrids with the use of RAPD markers.

Studies on the identification of various genotypes of pea with the use of DNA markers based on PCR with

specific primers are underway in our laboratory. Attempts to use SCAR and CAPS markers in these studies showed that this approach offers even a more precise and effective method of genotype certification than the RAPD method [30, 31].

In contrast to RAPD markers, SCAR and CAPS markers permit detecting chromosome regions that yield electrophoretic spectra with a few distinct bands among the regions that have already been characterized in detail and, in many cases, mapped. In addition, all CAPS and many SCAR markers are inherited as codominant characters, which considerably increases their resolution as the means of genetic analysis.

Sequencing some RAPD fragments of the pea genome demonstrated that a part of the nucleotide sequence of one fragment, B474#550, was homologous to the noncoding region of the chalcone synthase gene of pea. Another part of this fragment is homologous to one of repeats of the pea genome. Comparative analysis of translated sequences demonstrated that the presumptive protein products of the repetitive element were homologous to different proteins of retrotransposons of *Arabidopsis*, rice, and maize [29].

A part of the sequence of another fragment, E16#840, was homologous to different parts of some repeats in the genomes of pea and other legumes. Analysis of amino acid sequences of proteins showed considerable homology between the presumed translation products of the E16#840 fragment and the translation products of retrotransposons of rice, *Arabidopsis*, and wisteria. Judging from these homologies, the E16#840 fragment may be a part of a retrotransposon or a ret-

rotransposon-like nucleotide sequence of the pea genome.

A part of the nucleotide sequence of the R06#1100 fragment was found to be homologous to a part of the polyprotein gene of a retrotransposon of *Medicago sativa*. The search for homology in the databases of protein sequences confirmed that a part of the R06#1100 fragment was most likely to be a part of a pea retrotransposon.

The results obtained indicate that the pea genome contains many retrotransposons. This agrees with published data on a high frequency of retrotransposons in plant genomes [32, 33], including the pea genome [34–36]. The nucleotide sequences of the R06#470, K8#620, E16#840, D6#550, Pr10#340, and R06#1100 fragments are contained in the GenBank database (<http://www.ncbi.nlm.nih.gov>) and are available at accession numbers AY303675, AY303676, AY303677, AY303678, AY303679, and AY303680, respectively.

GENETIC MAPPING USING MOLECULAR MARKERS

Most genetic maps of various plant species are based on morphological markers, the number of which is limited. The accurateness of genetic maps has increased by many times during the past 15 years owing to the use of molecular markers, which reveal polymorphism directly in DNA and permit a rapid analysis of segregation with respect to numerous loci on the basis of the standard genotyping procedure. The maps of linkage groups for more than 30 plant species have been constructed using these markers [37]. DNA markers are used both for creating new genetic maps and for improving the resolution of those already existing [9].

The genetic map of pea is now being studied and corrected. Therefore, we performed a study aimed at the search for, and identification of, new molecular markers and the construction of the genetic map of pea for mapping the loci of qualitative and quantitative characters in two crosses (L-1238 × Chi115 and L-1238 × Flagman). It was important not only to create one more molecular genetic map, but also to combine it with the linkage maps of pea published earlier [38–40].

The analysis of F₂ hybrids with respect to more than 200 polymorphic loci found using the RAPD, ISSR, SCAR, and CAPS methods allowed us to obtain the first genetic map of pea in Russia that contained seven linkage groups. It had a resolution of 12.6 cM and a total length of 1262 cM (Fig. 3). About 46% of the intervals of this map were shorter than 10 cM, and only 17% were longer than 25 cM. This map of linkage groups of the pea genome contains no fewer molecular markers than the maps constructed previously, and permits localizing new loci determining important qualitative and quantitative characters of pea. Some of the markers that we mapped were previously used by other authors [38, 39]; therefore, we compared the genetic

Fig. 2. The tree of all strains, cultivars, and mutants of pea studied constructed by the UPGMA. I–VII, clusters; GD, genetic distance calculated according to Nei and Lee. Numbers are bootstrap values (in percent). Mutants and related cultivars are shaded.

map obtained in this study with versions published earlier [41, 42].

Polymorphism within the introns of the sequenced genes of pea detected by the CAPS (PCR–RFLP) method is being intensely searched for. The set of markers of this type is highly reproducible; hence, it can serve as a universal system for localizing new genes that can be used in any laboratory. If necessary, all cases of nucleotide substitution can be detected in automated systems for rapid genotyping by the microarray method [43]. To date, nine new CAPS markers have been created and localized. They have been integrated into the genetic linkage map along with ten markers from the study [40] (the polymorphism of the strains used with respect to these markers was described for the first time in [31]). The development of the set of CAPS markers for localizing genes throughout linkage group III has been almost completed (unpublished data). The information on all CAPS markers, including the table of their allelic states in different strains and cultivars, is inputted into a database, which facilitates the exchange of information between research groups.

The development of genetic maps of pea saturated with markers serves as the basis for planning applied studies on the genetics and breeding of important cultivated plants. The use of molecular markers for fine genetic mapping of pea chromosomes makes it possible to combine molecular and genetic maps and approach cloning and sequencing of individual genes of pea determining its commercially valuable characters.

MARKING THE GENES DETERMINING COMMERCIALY VALUABLE CHARACTERS

The creation of detailed genetic maps allows the identification of molecular markers closely linked to the genes of qualitative and quantitative characters, such as grain weight, protein content, flowering time, morphological parameters, and resistance to diseases and agricultural pests. The conferring disease and pest resistance is one of the main problems of plant breeding.

One of the applications of molecular markers linked to commercially important plant genes was suggested in the course of studies on lettuce populations segregating with respect to genes of the resistance to powdery mildew. This method is called bulked segregant analysis (BSA) [44]. In this method, mixtures of DNAs obtained from several representatives of segregating plant populations are used as matrices for RAPD analysis. To create two such mixtures, DNAs from several plants phenotypically differing in one binary character and homozygous for the locus determining this character are used; therefore, these mixtures differ from each other only with respect to the genomic region studied. Therefore, all polymorphic products revealed by RAPD analysis must pertain to the DNA region linked to the gene studied. This strategy was used to identify markers linked to locus *nor* (fruit ripening) in tomato [45].

The RAPD method and BSA were used to identify a marker linked to the *shm-1* gene (resistance to mosaic virus) in the pea genome [46] and to find closely linked DNA markers for several genes of symbiogenesis [47].

We used BSA to identify RAPD markers linked to genes *A* (the presence of anthocyan pigmentation), *B* (the color of anthocyan pigmentation), *I* (cotyledon color), and *S* (seed adherence, and two mutations affecting chlorophyll biosynthesis (*chi115* and *xa18*) [48, 49]. In general, BSA is one of the most promising methods permitting the rapid identification of many DNA markers linked to the genes studied, which is an important prerequisite for targeted studying the corresponding loci [50, 51].

In our study, a population of F_2 hybrids comprising 223 plants obtained from crossing the *chi115* pea mutant (mother plants) and the marker strain L-1238 was studied in order to localize the chlorophyll mutation of pea affecting the structure and functions of the genetic apparatus [41]. Originally, BSA using 46 RAPD and 10 ISSR primers was performed to identify the DNA markers linked to the *chi115* gene.

We identified three RAPD markers closely linked to the *chi115* gene (D6#550, K10#830, and K10#950). Another nine RAPD and two ISSR markers belonging to this chromosome region have been identified when studying the DNAs of 130 individual F_2 plants. The treatment of these data using the Mapmaker/EXP 3.0 software with a threshold of $LOD = 3.0$ yielded the genetic map of the region studied (Fig. 4).

If the LOD -score threshold was decreased to 2.00, several DNA markers linked to the *chi115* gene appeared to be associated with the *b* marker gene located in linkage group III and determining the color of the flower [38]. To prove that the markers belong to linkage group III, we used three STS markers preliminarily localized to this chromosome [40]. For these markers (*Sodmt*, *TubA1*, and *Rb*), polymorphism with respect to the *Ksp22I*, *TaqI*, and *RsaI* restriction endonuclease sites, respectively, was found. The codominant inheritance of these markers was confirmed. The data on CAPS segregation were used for the genetic analysis of the population of F_2 hybrids. The analysis demonstrated that all the three markers were linked to the *chi115* gene and two of them (*Rb* and *TubA1*) were linked to one another, which agrees with published data (Fig. 4).

All these data taken together allow us to conclude that the *chi115* gene is located in the upper arm of linkage group III and can be included into the genetic map of pea chromosomes. This mutation, which is interesting per se in terms of studying the genetic control of photosynthesis, can be also used as a new marker of the pea genome in further studies on genetic mapping. The given fragment of linkage group III covers about 137.6 cM, the mean distance between markers being 8.1 cM.

The above data allowed the comparison the genetic lengths of individual fragments of linkage group III with the consensus map of pea chromosomes [38]. In addition, the results obtained were important as a prerequisite for the positional cloning of the gene involved in the control of chlorophyll synthesis. These data also made it possible to begin studies on localizing the genes of quantitative characters in this region.

Cheghamirza [42] performed this study in our laboratory demonstrating that linkage group III of pea contained several loci for quantitative characters (stem height, seed number, and seed weight). Thus, the results of our studies show the importance of molecular markers for the detailed mapping of pea chromosomes and their role in the construction of an integrated molecular genetic map of chromosomes of this plant.

STUDYING DNA POLYMORPHISM IN CALLUS CULTURES AND REGENERANTS OF PEA AND MAIZE

The genetic variation expressed in *in vitro* cultures of plant cells and tissues is termed somaclonal varia-

Fig. 3. The map of linkage groups of pea based on the results of genetic analysis of the F_2 (Chl115 $\times$ L-1238) population with the use RAPD, SCAR, ISSR, and CAPS markers and genes of morphological characters. Distances are indicated in cM.

Fig. 4. The map of the region of linkage group III of pea constructed based on the results of marking the *chi115* mutation in the F_2 (*Chi115* $\times$ *L-1238*) population. Distances are indicated in cM.

tion. This variation has been found in many plant species and is a source of genetic diversity [52, 53].

The results of several studies have demonstrated that somaclonal variation is hereditary [54–56]. Apparently, the mechanisms of somaclonal variation are related to disturbance in intercellular interactions. Various genomic changes accompanying this process have been described, the most common of them being chromosome aberrations, transpositions of transposable elements, and the rearrangements of the ratio between repetitive and unique nucleotide sequences [57].

Researchers performing traditional genetic analysis of regenerant plants identify only the changes in genetic material that lead to noticeable phenotypic changes. We may assume that the true genetic variation in *in vitro* cultures is substantially larger than the observed one.

The main problem with the studies on somaclonal variation is the necessity to develop reliable methods for determining significant genetic differences between regenerants and the corresponding original strains.

We used the RAPD method for studying genetic polymorphism in the offspring of pea regenerants (somaclones) isolated from callus cultures [58]. Figure 5 shows the RAPD spectra of different strains of pea and somaclones obtained from cultivar *Rannii Zelenyi*. The data on polymorphism were treated by the UPGMA to construct a tree reflecting the degrees of differences between the genotypes of plants studied (Fig. 6). The divergences between pea strains originating from different cultivars and between different somaclones originating from the same cultivar estimated by Jacquard's coefficient were 23–38%.

The high genetic polymorphism that we found in the offspring of regenerants isolated from callus cultures of the same cultivar indicate considerable genetic changes that may have been appear during *in vitro* culturing of cells and tissues. This finding motivated us to study somaclonal variation in more detail with the use of molecular markers. In subsequent studies on pea cell cultures, we used calluses of different pea genotypes cultured for long periods of time (more than a decade). We obtained primary regenerant plants from these calluses and thoroughly analyzed their differences from the original plants. In these regenerants (the R_0 generation), we found changes affecting both qualitative and quantitative characters [59, 60].

Studying regenerants with the use of molecular markers (RAPD and ISSR) demonstrated that the degree of genetic changes depended on the original genotype. For example, even the genetically close strains R-9 and W-1 obtained from the same cultivar (*Rannii Zelenyi*) differed in genetic divergence. This value was no higher than 1% in W-1 regenerants and varied from 0.7 to 5% in R-9. In regenerants of cultivar *Viola*, the divergence was as high as 15%.

We also found considerable genetic variation, i.e., differences at the DNA level, between the offspring of regenerant plants (somaclones) and the original strain, in regenerants of maize [61, 62]. We used RAPD and ISSR markers to determine genetic differences between regenerants obtained from callus cultures and the original maize strain A188. Analysis of polymorphism with the use of 38 RAPD and 10 ISSR primers showed that the differences between the somaclones studied and the original strain varied from 6.5 to 23%. The regenerants obtained from callus cultures formed two clusters according to their origin. We found that the regenerants isolated after eight months of callus culturing exhibited larger differences from the original strain and from one another than the regenerants obtained from two-month-old callus cultures.

Our study on the molecular markers of regenerants was the first to identify specific RAPD and ISSR markers each of which was present in only one somaclone,

but not in other somaclones or the original strain of maize. Six specific fragments were used to create SCAR markers. Five SCAR markers entirely confirmed the specificity of polymorphism obtained using arbitrary primers [62, 63].

After cloning the polymorphic fragments, their primary nucleotide sequences were analyzed. The most interesting results were obtained when analyzing the nucleotide sequences of the M-10 SCAR fragment in ten somaclones of maize [62]. We found that this fragment contained the well-known retrotransposon Opie-1 5'-LTR and a part of the gene corresponding to the mRNA of ribosomal protein L26. The M-10 sequence homologous to this protein had a 23-bp deletion causing a reading-frame shift; in addition, there was a discrepancy between the sequences of the fragment and the protein at the end of the coding region [64].

When comparing the nucleotide sequences of SCAR markers QR and QR-A characteristic of the somaclones and the original strain of maize, respectively, a long deletion was found in QR. The analysis of the nucleotide sequences of the peroxidase gene region in the R11 somaclone and the original strain A188 demonstrated the presence of point mutations.

These results indicate that in vitro culturing of cells is often accompanied by mutations affecting the structure of retrotransposons. These mutations have no distinct phenotypic expression but may disturb the control of expression of many genes.

To obtain information on the nucleotide sequences of RAPD markers of pea, we cloned and sequenced three fragments of the Ch-6 somaclone. The analysis of the nucleotide sequence demonstrated an 80% homology between the B474#450 fragment and a 5180-bp region of the pectin methylesterase gene of pea [48].

The results of our studies on the molecular marking of pea and maize regenerants agree with data of other authors. For example, Brown *et al.* [65] used RAPD analysis to study genetic differences between wheat plants obtained from tissue cultures and plant grown from seeds. They found differences both between cell cultures at different stages of culturing and between phenotypically similar regenerants. The same research group performed the RAPD analysis of protoplasts isolated from transformed tobacco plants. They found that a unique spectrum of amplified fragments corresponded to each protoplast. These studies demonstrated that RAPD analysis can be used for genetic characterization of individual plant genomes even in single cells.

The RAPD method was also used for determining the genetic variation of somaclonal variants of tomato grown on nutrient media differing in hormone content [66]. The clones grown on different media exhibited considerable polymorphism. The polymorphism was larger in the regenerants obtained calluses that had been cultured for a long time (more than two years) than in those obtained from young embryonic cultures. There-

Fig. 5. The RAPD spectra of (1–6) somaclonal variants and (7–10) strains of pea. M1 and M2 are the phage λ DNA digested by *Pst*I and *Msp*I, respectively.

fore, it was concluded that a cell culture was a highly mutable system.

Thus, studies on pea and maize cell cultures yielded new data on the methods for maintaining and studying plant morphogenic calluses cultured for long periods of time, which retain a high regeneration capacity for many years. It was demonstrated that DNA markers (RAPD and ISSR) may serve as molecular tools for rapid and sufficiently reliable detection of genetic differences between the original plants and regenerants obtained from calluses cultivated for long periods of time. First specific SCAR markers were identified that were characteristic of each individual somaclone of pea and maize, which will help to understand the mechanisms of somaclonal variation in plants [67].

Since molecular markers can reveal changes in the plant genome when culturing cells in vitro [26, 60, 62], we used these markers to analyze genomic variation in plants growing at orbital stations during long-term spaceflights.

We preliminarily estimated the possibility of long-term culturing of higher plants under spaceflight conditions studying the effects of microgravity and cosmic rays on plant genomes. For this purpose, we selected, from the genetic collection of pea at the Department of Genetics, two marker dwarf strains suitable for growing in the “greenhouse” at the International Space Station. The tests performed there made it possible to obtain three successive generations of “space” plants, which was the first such succession ever obtained under

Fig. 6. The tree of pea strains studied constructed by the UPGMA on the basis of the results of RAPD analysis. * Somaclonal variants obtained from the Rannii Zelenyi cultivar (EG); Curl, Ch-6, W-1, R-1, and R-9; Cal is the callus of Rannii Zeleyni pea; L-1238, L-108, and M-10 are marker strains of pea; D_j is Jacquard's distance.

microgravity. Part of seeds was returned to Earth to estimate the effect of a long-term spaceflight on plant genomes.

CONCLUSIONS

The data on the use of PCR-based DNA markers reviewed here show that this approach is increasingly widely used due to its simplicity and efficiency. DNA markers are used to determine genetic relationships between closely related plant species, which is a good supplement to traditional methods.

These markers are very effective for intraspecific comparison between different genomes and make it possible to detect genetic differences between varieties, cultivars, strains, and even individual plants of the same species. The detection of intraspecific differences at the level of DNA markers is of great practical importance for characterization of different strains and obtaining heterosis forms of plants.

The use of DNA markers offers an insight into the mechanisms of somaclonal variation. The considerable changes that many researchers have found in the RAPD spectra cultured plant cells and regenerants obtained from these cells demonstrate a high mutability of cell cultures.

Molecular DNA markers are also successfully used for filling up gaps in genetic maps. Improvements in the DNA marker technique offer new possibilities of the identification and analysis of commercially important genes determining valuable qualitative and quantitative characters of plants.

To summarize our brief review, it should be noted that PCR-based molecular markers penetrated many

fields of genetic and breeding research and, along with other approaches currently employed by genomics, form the methodical basis of studying the structural and functional organization and variability of plant genomes.

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REFERENCES

1. Welsh, J. and McClelland, M., Fingerprinting Genomes Using PCR with Arbitrary Primers, *Nucleic Acids Res.*, 1991, vol. 18, pp. 7213–7218.
2. Williams, J.G.K., Kubelik, A.R., Livak, K.J., *et al.*, DNA Polymorphisms Amplified by Arbitrary Primers Are Useful As Genetic Markers, *Nucleic Acids Res.*, 1990, vol. 18, pp. 6531–6535.
3. Weeden, N.F., Timmerman, G.M., Hemmat, M., *et al.*, Inheritance and Reliability of RAPD Markers, *Appl. RAPD Tech. Plant Breed.* (Minneapolis, United States), 1992, pp. 12–17.
4. Karp, A., Kresovich, S., Bhat, K.V., *et al.*, Molecular Tools in Plant Genetic Resources Conservation: A Guide to the Technologies, *IPGRI Technical Bull.*, Rome: Int. Plant Genet. Resources Inst., 1997, no. 2.
5. Virk, P.S., Zhu, J., Newbury, H.J., *et al.*, Effectiveness of Different Classes of Molecular Markers for Classifying and Revealing Variations in Rice (*Oryza sativa*) Germplasm, *Euphytica*, 2000, vol. 112, pp. 275–284.
6. Waugh, R. and Powell, W., Using RAPD Markers for Crop Improvement, *Trends Biotechnol.*, 1992, vol. 10, pp. 186–191.
7. Zietkiewicz, E., Rafalski, A., and Labuda, D., Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification, *Genomics*, 1994, vol. 20, pp. 176–183.
8. Bornet, B. and Branchard, M., Nonanchored Inter Simple Sequence Repeat (ISSR) Markers: Reproducible and Specific Tools for Genome Fingerprinting, *Plant Mol. Biol. Rep.*, 2001, vol. 19, pp. 209–215.
9. Malyshev, S.V. and Kartel', N.A., Molecular Markers in Genetic Mapping in Plants, *Mol. Biol.* (Moscow), 1997, vol. 31, no. 2, pp. 197–208.
10. Galande, A.A., Tiwari, R., Ammiraju, J.S.S., *et al.*, Genetic Analysis of Kernel Hardness in Bread Wheat Using PCR-Based Markers, *Theor. Appl. Genet.*, 2001, vol. 103, pp. 601–606.
11. Irzykowska, L., Wolko, B., and Swickicki, W.K., Interval Mapping of QTLs Controlling Some Morphological Traits in Pea, *Cell. Mol. Biol. Lett.*, 2002, vol. 7, pp. 417–422.
12. Kesseli, R.V., Paran, J., and Michelmore, R.W., Efficient Mapping of Specifically Targeted Genomic Regions and the Tagging of These Regions with Reliable PCR-Based Genetic Markers, in *Application of RAPD Technology to*

- Plant Breeding: Joint Plant Breeding Symposia Series*, Minneapolis, Minn., 1993, pp. 31–36.
13. Tiwari, K.R., Penner, G.A., and Warkentin, T.D., Inheritance of Powdery Mildew Resistance in Pea, *Can. J. Plant Sci.*, 1997, vol. 77, pp. 307–310.
 14. Zijlstra, C., Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* Based on SCAR-PCR: A Powerful Way of Enabling Reliable Identification of Population or Individuals That Share Common Traits, *Eur. J. Plant Physiol.*, 2000, vol. 106, pp. 283–290.
 15. Deng, Z., Huang, S., Xiao, S.Y., and Gmitter, F.G., Jr., Development and Characterization of SCAR Markers Linked to the Citrus Tristeza Virus Resistance Gene from *Poncirus trifoliata*, *Genome*, 1997, vol. 40, pp. 697–704.
 16. Dax, E., Livneh, O., Auskevicius, E., *et al.*, A SCAR Marker Linked to the ToMV Resistance Gene, *Tm22*, in Tomato, *Euphytica*, 1998, vol. 101, pp. 73–77.
 17. Laroche, A., Demeke, T., Gaudet, D.A., *et al.*, Development of a PCR Marker for Rapid Identification of the *Bt-10* Gene for Common Bunt Resistance in Wheat, *Genome*, 2000, vol. 43, pp. 217–223.
 18. Konieczny, A. and Ausubel, F.M., A Procedure for Mapping *Arabidopsis* Mutations Using Co-Dominant Ecotype-Specific PCR-Based Markers, *Plant J.*, 1993, vol. 4, pp. 403–410.
 19. Whitkus, R., Doebley, J., and Lee, M., Comparative Genome Mapping of Sorghum and Maize, *Genetics*, 1992, vol. 132, pp. 119–1130.
 20. Sivolap, Yu.M. and Kalendar', R.N., Genetic Polymorphism of Barley As Revealed by PCR with Arbitrary Primers, *Russ. J. Genet.*, 1995, vol. 31, no. 10, pp. 1155–1161.
 21. Oganisyan, A.S., Kochieva, E.Z., and Ryskov, A.P., Fingerprinting of Potato Species and Cultivars by the RAPD-PCR Method, *Russ. J. Genet.*, 1996, vol. 32, no. 3, pp. 391–394.
 22. Weising, K., Nybom, H., Wolff, K., and Meyer, W., *DNA Fingerprinting in Plants and Fungi*, Boca Raton, FL: CRC, 1995.
 23. *Ispol'zovanie PTsR-analiza v genetiko-seleksionnykh issledovaniyakh. Nauchno-metodicheskoe rukovodstvo* (Use of PCR Analysis in Genetics and Breeding: A Theoretical and Methodical Handbook), Sivolap, Yu.M., Ed., Kiev: Agrarna Nauka, 1998.
 24. Ellis, T.H.N., Hellens, R.P., Turner, L., *et al.*, On the Pea Linkage Map, *Pisum Genet.*, 1993, vol. 25, pp. 5–12.
 25. Kokaeva, Z.G., Valiejo-Roman, K.M., Bobrova, V.K., *et al.*, Application of Random Amplified Polymorphic DNA Technique for Detection of Polymorphism among Somaclonal Variants of Pea, *Plant Genome IV: The Int. Conf. of the Status of Plant Genome Research*, San Diego, 1996, p. 41.
 26. Kokaeva, Z.G., Bobrova, V.K., Petrova, T.V., *et al.*, Genetic Polymorphism of Pea Cultivars, Lines, and Mutants Detected by RAPD Analysis, *Rus. J. Genet.*, 1998, vol. 34, no. 6, pp. 632–637.
 27. Kokaeva, Z.G., Koveza, O.V., Bobrova, V.K., *et al.*, Use of RAPD Analysis for Genotype Identification in Pea, *Tezisy dokladov 2-i mezhdunarodnoi nauchnoi konferentsii "Biotehnologiya v rastenievodstve, zhivotnovodstve i veterinarii"* (Proc. 2nd Int. Conf. Biotechnology in Plant Industry, Animal Breeding, and Veterinary), Moscow, 2000, pp. 80–81.
 28. Kokaeva, Z.G., Koveza, O.V., Chegmirza, K., *et al.*, Detection of DNA Polymorphism in Various Genotypes of Pea with the Use of the Polymerase Chain Reaction, in 2nd Int. Iran and Russia Conf. "Agriculture and Natural Resources," Moscow, 2001, pp. 8–9.
 29. Koveza, O.V., Identification, Cloning, and Analysis of Molecular Markers of the Pea Genome, *Cand. Sci. (Biol.) Dissertation*, Moscow: Inst. Gen. Genet., 2003.
 30. Koveza, O.V., Kokaeva, Z.G., Kononov, F.A., and Gostimsky, S.A., Detection and Mapping of Polymorphic Markers of the Genome of Pea *Pisum sativum* L., *Rus. J. Genet.*, 2005, vol. 41, no. 3, pp. 341–348.
 31. Kononov, F.A., SARS Markers with Degenerate Primers and Their Potential in Mapping Plant Genomes, *Tezisy dokladov 11-i mezhdunarodnoi konferentsii studentov, aspirantov i molodykh uchenykh "Lomonosov-2004"* (Proc. 11th Int. Conf. of Undergraduate and Postgraduate Students and Young Researchers "Lomonosov-2004"), Moscow, 2004, pp. 69–70.
 32. Nouzova, M., Neumann, P., Navratilova, A., *et al.*, Microarray-Based Survey of Repetitive Genomic Sequences in *Vicia* spp., *Plant. Mol. Biol.*, 2001, vol. 45, pp. 229–244.
 33. Ko, J.-M., Do, G.-S., Suh, D.-Y., *et al.*, Identification and Chromosomal Organization of Two Rye Genome-Specific RAPD Products Useful As Introgression Markers in Wheat, *Genome*, 2002, vol. 45, pp. 157–164.
 34. Kato, A., Sriprasetsak, P., Seki, H., *et al.*, Functional Analysis of Retrotransposons in Pea, *Plant Cell Physiol.*, 1999, vol. 40, pp. 933–941.
 35. Pearce, S.R., Knox, M.R., Ellis, T.H.N., *et al.*, Pea *Ty1-copia* Group Retrotransposon: Transpositional Activity and Use As Markers to Study Genetic Diversity in *Pisum*, *Mol. Gen. Genet.*, 2000, vol. 263, pp. 898–907.
 36. Neumann, P., Nouzova, M., and Macas, J., Molecular and Cytogenetic Analysis of Repetitive DNA in Pea (*Pisum sativum* L.), *Genome*, 2001, vol. 44, pp. 716–728.
 37. Tanksley, S.D., Ganai, M.W., and Martin, G.B., Chromosome Landing: A Paradigm for Map-Based Gene Cloning in Plants with Large Genomes, *Trends Genet.*, 1995, vol. 11, no. 2, pp. 63–68.
 38. Weeden, N.F., Ellis, T.H.N., Timmerman-Vaughan, G.M., *et al.*, A Consensus Linkage Map for *Pisum sativum*, *Pisum Genet.*, 1998, vol. 30, pp. 1–4.
 39. Laucou, V., Haurogne, K., Ellis, N., and Rameau, C., Genetic Mapping in Pea: 1. RAPD-Based Genetic Linkage Map of *Pisum sativum*, *Theor. Appl. Genet.*, 1998, vol. 97, pp. 905–915.
 40. Brauner, S., Murphy, R.L., Przyborowski, J., *et al.*, STS Markers for Comparative Mapping in Legumes, *J. Am. Soc. Hort. Sci.*, 2002, vol. 127, pp. 616–622.
 41. Chegmirza, K., Koveza, O.V., Kononov, F.A., and Gostimsky, S.A., Identification and Mapping of *chi115* Gene and DNA Markers Linked to It in Pea (*Pisum sativum* L.), *Rus. J. Genet.*, 2004, vol. 40, no. 7, pp. 737–742.
 42. Chegmirza, K., Molecular Genetic Mapping of Qualitative and Quantitative Trait Loci in Pea, *Cand. Sci. (Biol.) Dissertation*, Moscow: Inst. Gen. Genet., 2004.
 43. Flavell, A.J., Bolshakov, V.N., Booth, A., *et al.*, A Microarray-Based High Throughput Molecular Marker Geno-

- typing Method: The Tagged Microarray Marker (TAM) Approach, *Nucleic Acids Res.*, 2003, vol. 31, e115 (electronic version).
44. Michelmore, R.W., Paran, J., and Kesseli, R.V., Identification of Markers Linked to Disease Resistance Genes by Bulk Segregant Analysis: A Rapid Method to Detect Markers in Specific Genomic Regions Using Segregating Populations, *Proc. Natl. Acad. Sci. USA*, 1991, vol. 88, pp. 9828–9832.
 45. Giovannoni, J.J., Wing, R.A., Ganai, M.W., and Tanksley, S.D., Isolation of Molecular Markers from Specific Chromosomal Intervals Using DNA Pools from Existing Mapping Populations, *Nucleic Acid Res.*, 1991, vol. 19, pp. 6553–6558.
 46. Timmerman, D.M., Frew, T.J., Miller, A.L., *et al.*, Linkage Mapping of *sbm-1*, a Gene Conferring Resistance to a Pea Seed-Borne Mosaic Virus, Using Molecular Markers in *Pisum sativum*, *Teor. Appl. Genet.*, 1993, vol. 95, pp. 609–615.
 47. Temnykh, S.V., Mapping of Genes That Control Symbiotic Nitrogen Fixation in Pea, *Cand. Sci. (Biol.) Dissertation*, Novosibirsk: Inst. Cytol. Genet., 1994.
 48. Kokaeva, Z.G., Bobrova, V.K., Gostimsky, S.A., and Troitskii, A.V., Inheritance and Characterization of RAPD Markers Revealed in Somaclonal Variants of Pea, *Dokl. Akad. Nauk*, 2000, vol. 372, no. 4, pp. 565–567.
 49. Cheghamirza, K., Koveza, O.V., Konovalov, F.A., and Gostimsky, S.A., Identification of RAPD-Markers and Their Use for Molecular Mapping in Pea, *Cell. Mol. Biol. Lett.*, 2002, vol. 7, pp. 649–655.
 50. Penner, G.A., Bezte, L.J., and Leisle, D., Identification of RAPD Markers Linked to a Gene Governing Cadmium Uptake in Durum Wheat, *Genome*, 1995, vol. 38, pp. 543–547.
 51. Subudhi, P.K., Borkakati, R.P., Virmani, S.S., and Huang, N., Molecular Mapping of a Thermosensitive Genetic Male Sterility Gene in Rice Using Bulk Segregant Analyses, *Genome*, 1997, vol. 40, pp. 188–194.
 52. Larkin, P.J. and Scowcroft, W.R., Somaclonal Variation—A Novel Source of Variability from Cell Cultures for Plant Improvement, *Theor. Appl. Genet.*, 1981, vol. 60, pp. 197–241.
 53. Skaukroft, U.R., in *Mobil'nost' genoma rastenii* (Mobility of the Plant Genome), Moscow: Agropromizdat, 1990, pp. 228–260.
 54. Gostimsky, S.A., Bagrova, A.M., and Ezhova, T.A., Detection and Cytogenetic Analysis of the Variation Arising during Regeneration of Plants from Cultured Tissues of *Pisum sativum*, *Dokl. Akad. Nauk SSSR*, 1985, vol. 283, no. 4, pp. 1007–1011.
 55. Ezhova, T.A., Bagrova, A.M., and Gostimsky, S.A., Study of the Heritability of Somaclonal Changes in *Pisum sativum* Regenerants, *Genetika* (Moscow), 1989, vol. 25, no. 5, pp. 878–885.
 56. Bagrova, A.M., Ezhova, T.A., Khartina, G.A., and Gostimsky, S.A., Generation of Long-Term Morphogenic Callus Cultures and Analysis of the Somaclonal Variation in Regenerants of Grain and Vegetable Cultivars of Pea, *Vestn. Mosk. Univ., Ser. 16: Biol.*, 1991, no. 1, pp. 28–33.
 57. Frenkel'-Konrat, Kh., Geizen, G., Diz, P., *et al.*, *Mobil'nost' genoma rastenii* (Mobility of the Plant Genome), Moscow: Agropromizdat, 1990.
 58. Kokaeva, Z.G., Bobrova, V.K., Valiejo-Roman, K.M., *et al.*, RARD Analysis of the Somaclonal and Among-Cultivar Variations in Pea, *Dokl. Akad. Nauk*, 1997, vol. 355, no. 1, pp. 134–136.
 59. Kuznetsova, O.I., Ash, O.A., Khartina, G.A., and Gostimsky, S.A., RARD Analysis of Somaclones in Pea *Pisum sativum* L., *Biotehnologiya ovoshchnykh, tsvetochnykh i malorasprostranennykh kul'tur. Sbornik nauchnykh trudov mezhdunarodnoi nauchno-prakticheskoi konferentsii* (Biotechnology of Vegetables, Flowers, and Rare Cultivated Plants: Proc. Int. Conf.), Moscow, 2004, pp. 161–169.
 60. Kuznetsova, O.I., Ash, O.A., Khartina, G.A., and Gostimsky, S.A., RAPD and ISSR Analyses of Regenerated Pea *Pisum sativum* L. Plants, *Rus. J. Genet.*, 2005, vol. 41, no. 1, pp. 60–65.
 61. Osipova, E.S., Troitskii, A.V., Dolgikh, Yu.I., *et al.*, Molecular Genetic Analysis of Maize Somaclones, *Tezisy konferentsii "Gorizonty fiziko-khimicheskoi biologii"* (Proc. Conf. "Frontiers in Physico-Chemical Biology), Pushchino, 2000, p. 132.
 62. Osipova, E.S., Koveza, O.V., Troitskii, A.V., *et al.*, Analysis of Specific RAPD and ISSR Fragments in Maize (*Zea mays* L.) Somaclones and Development of SCAR Markers on Their Basis, *Rus. J. Genet.*, 2003, vol. 39, no. 12, pp. 1412–1419.
 63. Osipova, E.S., Kokaeva, Z.G., Troitskii, A.V., *et al.*, RARD Analysis of Maize Somaclones, *Rus. J. Genet.*, 2001, vol. 37, no. 1, pp. 80–84.
 64. Osipova, E.S., Changes of DNA Markers (RARD, ISSR) in Somaclonal Variation in Maize, *Cand. Sci. (Biol.) Dissertation*, Moscow: Inst. Plant Physiol., 2004.
 65. Brown, P.T.H., Lange, F.D., Kranz, E., and Lorz, H., Analysis of Single Protoplasts and Regenerated Plants by PCR and RAPD Technology, *Mol. Gen. Genet.*, 1993, vol. 237, pp. 311–317.
 66. Bogani, P., Simoni, A., Lio, P., *et al.*, Genome Flux in Tomato Cell Clones Cultured in Vitro in Different Physiological Equilibria: II. A RAPD Analysis of Variability, *Genome*, 1996, vol. 39, pp. 846–853.
 67. Osipova, E.C., Troitskij, A.V., Dolgikh, Yu.I., *et al.*, SCAR-Marker Creation for Maize Somaclones Using Some Specific RAPD and ISSR Fragments, *Maize Genet. Cooperation Newslett.*, 2003, vol. 77, pp. 53–55.