Detected Length Polymorphism Shows the Same Characteristics for AFLP and ISSR when Amaranth Gamma-Radiation Mutants are Evaluated

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Abstract
The information characteristics of AFLP and ISSR techniques were compared in the analysis of length polymorphism of gamma-treated mutant lines of *Amaranthus cruentus* and *Amaranthus hypochondriacus* x *Amaranthus hybridus*. Both of the techniques are widely used in the manner of universal marker system suitable for the analysis of any plant species, but differ in the time needed for the analyse and costs. Binary data matrices were used for statistical analyses. Cluster analysis was performed in UPGMA method using Euclidean distance to construct dendrograms and principal component analyses was used for generated data, too. Comparison of techniques was made on the graphs obtained from result of PCA and dendrograms that were obtained from results of cluster analysis. Matrix comparisons of Mantel test, for the correspondence of the similarity matrices was performed for the null hypothesis that there is no association between similarity matrices. In the analyzed set used in this study, ISSR primers produced 59% polymorphic bands, while AFLP primers produced 48% polymorphic bands and both of techniques were similar in their obtained information characteristics.

Keywords: AFLP, amaranth, comparison, ISSR,

1. Introduction

Amaranthus is a widely distributed genus with approximately 70 species that are known as weeds, nutritive vegetable or ornamentals. Some of them posses a high potential in human nutrition [1]. Today, it is interesting as a crop not only in Central and South America, its centre of origin, but worldwide [2-4]. In Europe, cultivation of amaranth is actual for Austria, Czech Republic, Slovak Republic, Germany, Poland, Italy, Slovenia and Russia [5]. Gajdošová et al. [6-10] was applied the radiance mutagenesis for creating an amaranth line with increased weight of thousand seeds. Gamma rays is most effective technique for preparing of plant mutants as to have the potential to produce high rate of mutation changes [11-13]. Mutational breeding is used as a great source of generating variability of qualitative and quantitative traits of amaranth. The positive correlation was reported among gamma irradiant and germination and plant habitus and length of the root system [14]. Gamma radiation was applied for producing of amaranth genotypes with higher yield, tolerance to *Sclerotinia* sp. and producing seeds different in colour and size as its parent plants [15]. This method was used to produce *Amaranthus tricolor* tolerant to aridity

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and higher regeneration capacity after stress condition [16].

Different types of DNA markers were applied in the evaluation of genetic diversity of plants [17]. DNA markers are intergenic sequences or genes that are used to define and interpret the sequential genetic variability in organisms [18-19]. Some of them are suitable to be used universally. These are mainly RAPD, ISSR, AFLP or iPBS markers. All of them were applied successfully before in the genetic polymorphism analysis of genomically not well described plant species [20-21].

The aim of the presented study was to evaluate the results of two DNA markers techniques – AFLP and ISSR in a manner to compare their ability to characterize genome changes in amaranth radiant mutant lines. Genetic variability of *Amaranthus cruentus*, genotype Ficha and hybrid K-433 (*Amaranthus hybridus* L. × *Amaranthus hypochondriacus* L.) was analysed comparing them to controls (without gamma rays treatment).

2. Materials and methods

Biological material

Amaranthus hybridus lines of Ficha variety positively selected for increased weight of thousand seeds (coding names: C15, C26, C27, C82, C236) and an untreated control (coding name: A) together with the *A. hybridus* × *A. hypochondriacus* K-433 lines (coding names: D54, D279 and D282) and an untreated control (coding name: B) were used in analysis. This plant material is originated in seeds, that was treated by gamma radiation of the dose of 175 Gy.

Extraction of total genomic DNA, AFPL and ISSR analysis

Young seedlings of amaranth were obtained in pots. DNA was extracted from fresh leaves using the DNeasy® Plant Mini kit (Quiagen) according to the manufacturer´s instructions. Quality and quantity of extracted DNA was analysed using Nanodrop Nanophotometer™ (Implen). ISSR reactions were performed using 50ηg of template DNA. Primers used for the reactions were as follow: $(GTG)_3GC$; $(GT)_6CC$; $(CTG)_3GC$; $(GATA)_2(GACA)_2$; $(GACA)_4$. AFLP procedure was performed according to the modified AFLP Plant Mapping Protocol (Applied Biosystems, Foster City, USA) using the AFLP Kit for Regular Plant Genomes (Applied Biosystems). The basic principle of analysis was as reported before [22].

PCR data processing and statistical analysis

Obtained electropherograms were evaluated and binary matrix was constructed on the base of the peaks presence (1) or absence (0). Only polymorphic fragments were used for next analyses, the monomorphic amplicons were not included. Similarity indices (SI, Similarity Index) - Jaccard's coefficient (GD_{JC}) [23] were calculated from binary matrix. Binary data matrices of ISSR and AFLP markers were used for statistical analyses. Cluster analysis was performed via unweighted pair-group method (UPGMA) using Euclidean distance to develop dendrograms. Principal component analyses (PCA) was performed on data generated from each marker type. Comparison (ISSR vs. AFLP) was made on the graphs obtained from result of PCA and dendrograms that were obtained from results of cluster analysis. In addition, matrix comparisons of Mantel test [24], for the correspondence of the similarity matrices (ISSR vs AFLP), was performed by means of MXCOMP procedure of NTSYS-pc for the null hypothesis that there is no association between similarity matrices. To obtain the significance level, 250 permutations were performed. Cluster analysis and principal component analysis (PCA) were conducted using STATISTICA Cz software ver. 10, StatSoft, Inc. (2011). Mantel Z test by NTSYS pc version 2.1 [25].

3. Results and discussion

The variability of genomes is an integral part of life. Spontaneous changes in genomes that exist in nature are combined with aimed mutagenesis by different physical factors or chemicals by humans. The effects of spontaneous and aimed mutagenesis are always unpredictable, random and multidirectional. Here, the comparison of two different marker systems was performed in assessing the diversity of the genome of amaranthus species after treatment of ionizing gamma radiation and subsequently selecting the lines of interest. The biological material used to evaluate the variability of the genome was the *Amaranthus cruentus* L. and the K-433 interspecific hybrids (*Amaranthus hybridus* L.x *Amaranthus hypochondriacus* L.. The variability

of the control genome and the irradiated samples of the amaranth was analyzed on the basis of restriction sites (AFLP, Amplified Fragment Length Polymorphism) and microsatellite polymorphism (ISSR, Inter Single Sequence Repeats).

Both of the techniques used in this study was previously reported as to have the potential to distinguish the gamma-radiant mutants of *Amaranthus cruentus*, L. and *Amaranthus hybridus* L. × *Amaranthus hypochondriacus* L. [26-29]. Both of them are still used widely in plant genetic resources evaluation but are very different in economic characteristics. That is why some authors have reported a comparison of the result both of the techniques for the purposes of length polymorphism evaluation [30].

In the present study, the genetic relationships among gamma-radiated mutants and their non-radiated controls were evaluated for amaranth and the comparison of obtained AFLP and ISSR data was performed.

AFLP method was used to analyse length polymorphism in the genome of amaranth in M7 generation after gamma rays treatment. Digestion was realized using endonucleases *Mse*I and *EcoR*I. This combination, when *Mse*I cleavage site is T*TAA and *EcoR*I clevage site is G*AATTC was chosen because of very high cleavage frequency of both of them [31]. The level of DNA polymorphism for individual AFLP primer combinations was different for *A. cruentus* when comparing it to *A. hybridus* L. × *A. hypochondriacus* L. Higher variability was obtained for the line K-433 of interspecies hybrid (*A. hybridus* L. × *A. hypochondriacus* L) based on the clustering of controls and gamma-radiated mutants. Positioning of controls in dendrogram (figure 1) was in the subclusters of its breeding lines for AFLP profiles.

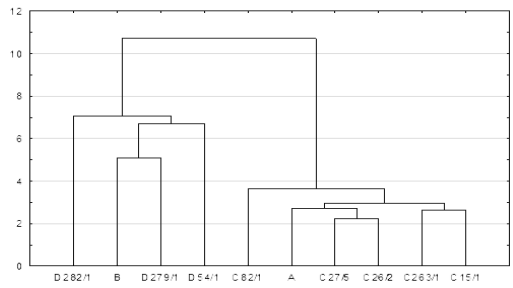

Figure 1. Dendrogram of AFLP amplicon profiles of analysed amaranth accessions

Total number of 352 amplified fragments levels were obtained and 169 of them were polymorphic, what show 48 % of polymorphic bands. Polymorphic information content for AFLP data of gamma-treated and control amaranth accessions was 0,997.

Much higher number of unique loci was detected for K-433 hybrid of *A. hybridus* L. × *A. hypochondriacus* L. accessions. Very concrete numbers of unique fragments for individual accessions are listed in table 1.

Table 1. Number of unique fragments detected for accessions of *A. cruentus* and *A. hybridus* L. × *A. hypochondriacus* L.

Accession	Total number of fragments	Number of unique fragments	Percentage of unique fragments (%)
C15	265	1	0.38
C26	262	2	0.76
C27	263	0	0
C82	259	1	0.39
C236	266	1	0.38
Ficha control	261	1	0.38
D54	287	0	0
D279	258	0	0
D282	291	18	6.2
K-433 control	258	2	0.78

As ISSR fingerprints results show (figure 2), all the mutant lines of the Ficha genotype (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0,16 and their ISSR profiles are more similar to the Ficha than those of K-433 mutant lines.

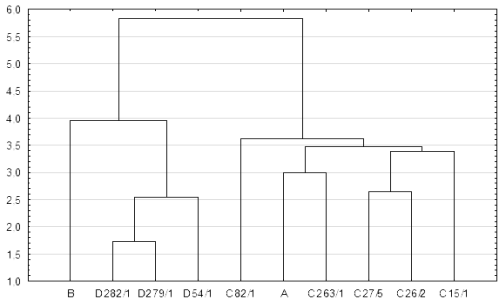

Figure 2. Dendrogram of ISSR amplicon profiles of analysed amaranth accessions

The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0.22 but as the dendrogram shows, are more distinct to their control genotype as a whole, as those of the Ficha

mutant lines. The two major clusters were observed at a dissimilarity coefficient of a 0.38, what means, that the used primers have generated two relatively different sets of fingerprints, one for the Ficha and its mutant lines and one for the K-433 and its mutant lines.

Results from PCA of AFLP markers (figure 3) showed that Amaranth samples C15/1, C26/2, C27/5, C82/1 and C263/1 can be grouped together with sample A and can be separated from the rest by using the PCA axis 1. Second group of accessions (D54/1, D279/1, D282/1 and B) were clustered together in the dendrogram obtained from cluster analysis via UPGMA. Level of similarity is lower than in the case of group C samples and their separation is done by PCA axis 2. The PCA axis 3 is separating sample B from group of D samples.

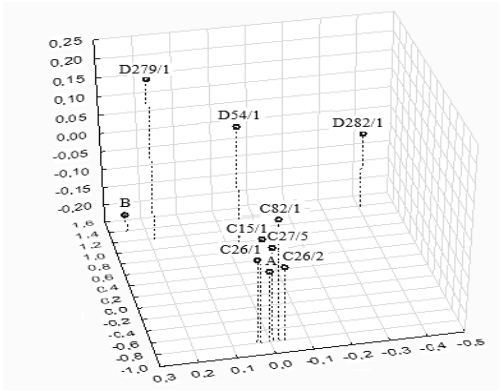

Figure 3. Graph of PCA for AFLP data of analysed amaranth accessions.

Cluster analysis based on ISSR markers (figure 4) is similar to clustering of AFLP markers. PCA is separating group of samples C15/1, C26/2, C27/5, C82/1 and A with D54/1, D279/1, D282/1 and B by axis 1. Axis 2 and 3 is dividing samples of groups C and D with each other. Unlike PCA based on AFLP markers has group of C genotypes good resolution.

Results from similarity matrices comparisons via Mantel Z test showed that there was a good correlation between the similarity matrices of AFLP and ISSR-RAPD ($r = 0.86$, $P < 0.001$). Having a good correlation between the similarity matrices of AFLP and ISSR markers was concordant with the results of analyses of both via PCA UPGMA cluster analysis with the matrix correlation value 0.86.

Molecular markers approach was successfully used to understand intra- and inter- specific genetic diversity and/or evolutionary relationships in amaranths [32-38].

In this study, ISSR markers were applied to distinguish mutant lines of two species of the amaranth - *Amaranthus cruentus* and *A. hypochondriacus* x *A. hybridus* mutant lines. Lee et al. [36] have reported the potential of microsatellite based markers for the *A. hypochondriacus* assessment, when they successfully amplified in all the tested species 12 loci and demonstrated the applicability of these markers for the study not only of intra-, but for inter- specific genetic diversity of amaranth, too.

ISSR is a source of genetic markers that have higher reproducibility than previously standardly used RAPDs [39-40].

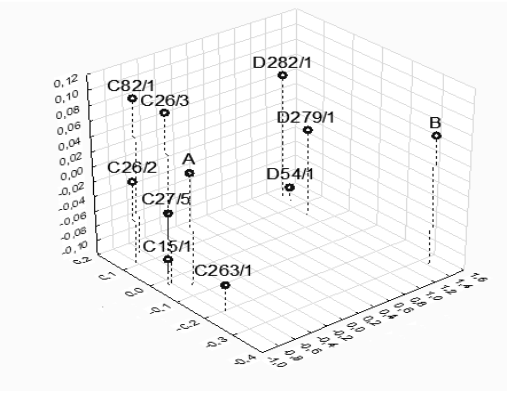

Figure 4. Graph of PCA for ISSR data of analysed amaranth accessions.

Along with the RAPD technique, both of them are still preferred method for fast primary identification of genotypes because they are relatively inexpensive, utilizes arbitrary primers, and randomly samples a potentially large number of loci in a less complex pattern than other PCR based markers [41-42].

The AFLP method used here, detected the length polymorphism in the genome when using restriction digestion that was performed with two restriction endonucleases *Mse*I and *EcoR*I. The combination of the restriction endonucleases *Mse*I and *EcoR*I was selected on the base of high frequency of using these two restriction endonuclease combinations and the similarity of their different frequency occurrence. It is reported that *Mse*I cleavage sites occur multiple times in the genome compared to *EcoR*I, due to their different number of recognition nucleotides. Preselective amplification and selective

amplification was used to reduce the number of fragments after restriction digestion. If the G/C base content of the genome is greater than 65%, *Mse*I will not provide the appropriate amount of fragments. The optimal amount of G/C bases is less than 50% for suitable production of *Mse*I fragments. *EcoR*I also tends to produce more fragments in G/C poor genomes. In cases where the G/C base content is unknown for the assessed organism, the efficiency for restriction enzymes must be determined empirically. It was reported [31] that a greater number of fragments in the profile increases the probability of detecting genetic polymorphism, but also increases the likelihood of poorly separated fragments. The amplification of the fragments was performed in variants after gamma irradiation, as well as in the controls of both amaranth species in the case of 93.75 % when using 80 primers based on one selective nucleotide *Mse*I primer and one *EcoR*I primer.

Only 30 primer combinations were used from 64 primer combinations tested to evaluate the variability of two common cabbage cultivars, because of providing the appropriate level of DNA polymorphism [43]. The level of detected polymorphism depends on individual primer combinations [35]. The interspecific polymorphism between the seven species of amaranth reached the values from 94% to 99%. Based on the evaluation of intraspecific variability, they found that even intraspecific variability is relatively high, although it is significantly lower compared to interspecific variability [35]. A polymorphism of 44% was recorded among the species of *Amaranthus cruentus*, 34% among *A. gangeticus*, 49% among *A. caudatus*, 55% among *A. viridis*, 50% of *A. hypochondriacus*, and 62% among *A.hybridus*. Here, amplification of unique fragments characteristic for controls only provided the differentiation of gamma-irradiated variants from the control. A total of 11 fragments characteristic of the Ficha control and 21 fragments characteristic of the K-433 control were amplified. Separating of gamma-irradiated variants of the amaranth from the control ensured the amplification pattern of the fragments that is a very specific only for the breeding lines with bands that absent for the controls. In the Ficha variety, three primer combinations have resulted in the unique fragment characteristic for gamma irradiated variants. In the K-433 line of the interspecific hybrid, two gamma-only fragments were detected.

4. Conclusions

The UPGMA and PcoA of ISSR and AFLP markers data produced a very similar results in the polymorphism analysis of control and gamma treated amaranth species accessions. It shows that both marker systems have similar properties in genome polymorphism evaluation and therefore can be used simultaneously of can be choosing only one of them to get the relevant genomic information based on the locus specific genotyping.

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