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USING OF AFLP TO EVALUATE GAMMA-IRRADIATED AMARANTH MUTANTS

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To determine which of several gamma-irradiated mutants of amaranth Ficha cultivar and K-433 hybrid are most genetically similar to their non-irradiated control genotypes, we performed amplified fragment length polymorphism (AFLP) based analysis. A total of 40 selective primer combinations were used in reported analyses. First analyses of gamma-irradiated amaranth mutant lines were done used the AFLP. In the study, primers with the differentiation ability for all analysed mutant lines are reported. The very specific changes in the mutant lines´ non-coding regions based on AFLP length polymorphism were analysed. Mutant lines of the Ficha cultivar (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0,11 and their ISSR profiles are more similar to the Ficha than those of K-433 hybrid mutant lines. The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0,534 but are more distinct to their control plant as a whole, as those of the Ficha mutant lines. Different AFLP fingerprints patters of the mutant lines when compared to the Ficha cultivar and K-433 hybrid AFLP profiles may be a consequence of the complex response of the intergenic space of mutant lines to the gamma-radiance. Although a genetic polymorphism was detected within accessions, the AFLP markers successfully identified all the accessions. The AFLP results are discussed by a combination of biochemical characteristics of mutant lines and their control genotypes.

Key words: AFLP, gamma-radiance, amaranth mutant lines, Ficha, K-433

INTRODUCTION

Nowadays, people interested of the planted and consumption of healthful and nutritionally rich crops as well as the cultivation of plants for changing climate conditions.

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Therefore, (1) the crops once grown by our ancestors, whose lost significance for some time, are getting at the last years to the awareness, again), (2) different breeding methods are used to improving the character and qualities of crops (NAC, 1985; BERGHOFER, SCHOENLECHNER, 2002). Amaranth is one of such crops, obviously. The genus *Amaranthus* L. (*Caryophyllales*: *Amaranthaceae*) consists of about 60–70 species and is growing in many parts of the world (PANDEY, SINGH 2011; RIVELLI *et al*. 2008). Three ancient cereal Amaranthus species, *A. caudatus* L., *A. cruentus* L. and *A. hypochondriacus* L., are nowadays studied and cultivated worldwide because of their exceptional nutritional value of both seeds and leaves (BRENNER *et al.* 2000). Amaranth plant is extensively studied: cultivation in diverse condition (FEJÉR 2011), nutrient value (GÁLOVÁ *et al*. 2008; HRICOVÁ *et al*. 2011), industrial applications (TELI *et al*. 2009), and utilization as source of bioenergy (VIGĽASKÝ *et al.* 2009).

Treatment by γ-radiation was used also for enhancing quality and quantity of amaranth grain of two selected genotypes: *Amaranthus cruentus* genotype 'Ficha' and hybrid K-433. Thanks to positive selection which was performed from 2^{nd} to 8^{th} mutant generation several putative mutant lines of *Amaranthus cruentus* and hybrid K-433 were selected characterized by highly significantly increased weight of thousand seeds with an obvious tendency to stabilization of this trait (GAJDOŠOVÁ *et al*. 2008). Induced mutagenesis, i.e. changes in the genetic basis of the plant using chemical compounds or radioactivity, was employed after World War Two. So-called "Green Revolution" in the 50-ies involved the simultaneous development of new varieties of crop plants and altered agricultural practices that greatly increased crop yields (OVESNÁ *et al.* 2002). Biochemical analyses of *Amaranthus cruentus* genotype 'Ficha' and hybrid K-433 samples realised (HRICOVÁ *et al*. 2011; MÚDRY *et al.* 2011, KEČKEŠOVÁ *et al.* 2012). They states that nutritional value of selected mutant lines in comparison with untreated controls remain unchanged. The highest result for nutritional value was observed in mutant line C82/1.

In this study, we present the molecular characterization of mutant amarant samples by using AFLP markers. The objectives of this work were: evaluate ability of AFLP marker technique to detect genetic diversity of amaranth samples, estimate the genetic relationships between the *A. cruenthus* and hybrid K-433 (*A. hypochondriacus* × *A. hybridus*) and genetic relationships between mutant lines and their control samples in each group, behalf detection of changes in genome of amaranth mutant lines.

MATERIALS AND METHODS

DNA was extracted from young leaves of amaranth plants with DNeasy® Plant Mini kit (Quiagen). Amplified fragment length polymorphism analyses were done according to the AFLP Plant Mapping Protocol (Applied Biosystems, Foster City, USA). AFLP technique was performed using AFLP Kit for Regular Plant Genomes (Applied Biosystems): (1) AFLP^{TM} Ligation and Preselective Amplification Module (Applied Biosystems) was used to restriction of DNA and a ligation of oligonucletide adapters. DNA digestion was carried out using the restriction enzymes EcoRI (Fermentas) and MseI (Biolabs) and ligated with T4 DNA ligase (Fermentas) 2 hours by 37°C. 9 µl of the ligation mixture was diluted with 91 µl $TE_{0.1}$. (2) The preselective amplification was prepared using diluted DNA product after the first step with the preselective primer pairs and AFLP Core mix (AFLP Amplification Core Mix Module – Applied Biosystems) according to the thermal cycler parameters for preselective amplification listed in AFLP Plant Mapping Protocol. 5 µl of the ligation mixture was diluted with 75 µl $TE_{0.1}$. (3) The selective amplifications with MseI primers and fluorescently labelled EcoRI primers (Table 1)

were performed as a multiplex PCR in a 10 µl reaction mixture (0.2 mM dNTP, 1 µM MseI primer, 3x 0.1 µM fluorescently-marked EcoRI primers (Applied Biosystems), 0.5 U Taq polymerase (Qiagen), 1x buffer with 15 mM $MgCl_2$ (Quiagen) and 1 µl diluted preselective amplification product) according to the thermal cycler parameters for selective amplification listed in AFLP Plant Mapping Protocol. Samples were prepared for electrophoresis using 4.8 µl of formamide, 0.4 µl of GENESCAN^{TM}-500 ROX size standard and 1.5 µl of selective amplification product. The amplification products were separated by capillary electrophoresis in ABI PRISM 310 and analysed using GeneScan™ and Genotyper™ software (Applied Biosystems).

Table 1. Selective primer combinations used for AFLP reactions

MseI	EcoRI		
	FAM (blue)	JOE (green)	NED (yellow)
CAC	ACT, ACA	AAC, ACC, AGC	AAG, AGG, ACG
CTA	ACT, ACA	AAC, ACC, AGC	AAG, AGG, ACG
CTC	ACT, ACA	AAC, ACC, AGC	AAG, AGG, ACG
CTT	ACT, ACA	AAC, ACC, AGC	AAG, AGG, ACG
CGA	ACT, ACA	AAC, ACC, AGC	AAG, AGG, ACG

AFLP electrophoreograms 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 (1908) coefficient (GD_{JC}) were calculated from binary matrix.

Similarity indices were calculated by the relationship: $(GD_{JC}) = 1- [N_{11} / (N_{11} + N_{10} + N_{01})]$, where N_{11} is the number of bands present in both individuals; N_{10} is the number of bands present only in the individual a; N_{01} is the number of bands present only in the individual b. GD_{JC} takes into consideration only matches between bands–alleles that are present and ignores pairs in which a band is absent in both individuals. Unweighted pair group method with arithmetic means (UPGMA) based on genetic similarity by the Jaccard similarity coefficients was used for cluster analysis to show genetic similarities among amaranth samples.

RESULTS AND DISSCUSSION

Characterization of germplasm implies the use of DNA fingerprinting technique for the precise establishing, identification and quantitative determination of genetic diversity (MILOSEVIČ *et al.,* 2010). For more accurate study of genetic diversity and phylogenetic relationships between *Amaranthus* species, different molecular markers including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (ALFP) have been used (XU and SUN 2001; WASSOM and TRANEL 2005; LEE *et al.* 2008; RAY *et al.* 2008, POPA *et al.* 2010). The genetic backgrounds of amaranth on a DNA level have been studied extensively and many difference molecular markers techniques are

used to study of amaranth genome: RAPD markers (RANADE *et al*. 1997; ŠTEFÚNOVÁ and BEŽO 2003; POPA *et al.* 2006; RAY and ROY 2009), SSR markers (LEE *et al*. 2008; MALLORY *et al*. 2008), ISSR markers (RAY and ROY 2007; NOLAN *et al.* 2010; RAŽNÁ *et al.* 2012) SCAR markers (RAY and ROY 2009), AFLP markers (XU and SUN 2001; WASSOM and TRANEL 2005; COSTEA *et al.* 2006; CHANDI *et al.* 2013).

Initially, 40 AFLP primer combinations were tested on mutant lines C15 and D 279 and their control lines and of these, only those that generated reproducible and polymorphic profiles were chosen for further analyses. Primer combinations reported in this study produced 1027 different AFLP fragments of which 76,92 % were polymorphic. This is in concordance with the reported power of AFLP to identify also inter-clonal variation (BELAJ *et al.* 2003, GEUNA *et al*. 2003, OVESNÁ *et al.* 2011), so the effective discrimination of individual mutant lines of amarath was expected. The 40 MseI and EcoRI primer combination for AFLP reaction produced 1027 fragments for *A. cruenthus* and hybrid K-433, together (Table 2). 539 fragments (52.48%) from total 1027 were polymorphic. The most productive were primer combinations CTA/AAC and CGA/ACC with 42 fragments, respectivelly. Percentual, the primer combination CTT/AAC with 76.92% of polymorphic fragments, was the most polymorphic. Across *A. cruentus* accessions, we detected 754 fragments, 78 (10.34%) of witch, were polymorphic. The most polymorphic fragments were detected by CGA/AGC primer combination with 7 polymorphic fragments. Percentual, the primer combination CAC/ACC with 37.50% of polymorphic fragments, was the most polymorphic. Hybrid K-433 accessions were more polymorphic than *A. cruentus* accessions. The percentage range of polymorphic fragments in *A. cruenthus* accessions was (0%–37.50%), whereas in hybrid K-433 accessions was (5.71%–51.85%). Across hybrid K-433 accessions, we detected 939 fragments, 291 (30.99%) of witch, were polymorphic. The most polymorphic fragments were detected by CAC/AGC primer combination with 15 polymorphic fragments. Percentual, the primer combination CTA/AGC with 51.85% of polymorphic fragments, was the most polymorphic

The genetic structure of the analysed grain amaraths and their putative mutant lines was visualised by applying a hierarchical method (Neighbour-Joining method) based on a Jaccard coefficient (Table 3) and UPGMA. The resultative dendrogram (Figure 1) shows the collection divided into 2 clearly separated clusters. As AFLP fingerprints results visualized in dendrogram show, all the mutant lines of the Ficha genotype (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0,106 and their AFLP profiles are more similar to the Ficha than those of K-433 mutant lines. The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0,576 and as the dendrogram (Figure 1) shows, are more distinct to their control genotype (their average Jaccard coefficient is 0,492) as a whole, as those of the Ficha mutant lines (0,114). *Amaranthus cruentus* mutant lines were grouped in the first major cluster and the average Jaccard dissimmilarity coefficient for all of the Ficha mutant lines was 0,11 (Table 3). Similar, the second cluster is compounded only from K-433 mutant lines with the average dissimilarity coefficient of 0,534. As the table 3 shows, no genetic uniformity was observed among the Ficha mutant lines, nor among the K-433 mutant lines, but low average Jaccard dissimilarity coefficient indicate that their AFLP profiles were similar, but not the same completelly.

AFLP technique was used to evaluate of amaranth mutant samples AFLP, because AFLP assays require no previous sequence knowledge, variability can be assessed at a large number of independent loci (20–100 loci per assay) data are obtained quickly and are reproducible (MAJER *et al.* 1996; MAUGHAN *et al.* 1996; POWELL *et al*. 1996; ZHONG and

STEFFENSON 2001). The technique has previously been used successfully in diversity studies for: durum wheat (MARTOS 2005), potato (ESFAHANI *et al.* 2009), wheat and *Aegilops* (KHALIGHI 2008), garlic (OVESNÁ *et al.* 2011), common bean (MARAS *et al*. 2008), cabbage (FALTUSOVÁ *et al*. 2011), tobacco (ZHANG *et al.* 2008) pathogens of crops (LEISOVÁ-SVOBODOVÁ *et al.* 2012a; 2012b), too.

Table 2. Summary of obtained AFLP fragments in analysed irradiated mutant lines and their control genotypes.

PRIMER COMBINATIONS		AMARANTHUS CRUENTHUS			HYBRID K-433			AMARANTHUS CRUENTHUS and HYBRID K-433		
MseI	EcoRI	no. of fragment	no. of polymorphic fragment	percentage of polymorphic fragment	no. of fragment	no. of polymorphic fragment	percentage of polymorphic fragment	no. of fragment	no. of polymorphic fragment	percentage of polymorphic fragment
CAC	ACT	14	2	**14,29**	24	11	**45,83**	25	15	**60,00**
CAC	AAG	16	3	**18,75**	20	9	**45,00**	21	15	**71,43**
CAC	AAC	26	5	**19,23**	29	14	**48,28**	32	19	**59,38**
CAC	ACA	18	5	**27,78**	23	8	**34,78**	26	14	**53,85**
CAC	ACG	14	3	**21,43**	12	6	**50,00**	16	10	**62,50**
CAC	ACC	8	3	**37,50**	12	6	**50,00**	13	9	**69,23**
CAC	AGG	16	3	**18,75**	20	7	**35,00**	22	12	**54,55**
CAC	AGC	26	5	**19,23**	32	15	**46,88**	35	22	**62,86**
CTA	ACT	19	0	**0,00**	24	8	**33,33**	26	13	**50,00**
CTA	AAG	14	0	**0,00**	18	4	**22,22**	20	11	**55,00**
CTA	AAC	29	1	**3,45**	37	5	**13,51**	42	19	**45,24**
CTA	ACA	21	0	**0,00**	31	11	**35,48**	34	23	**67,65**
CTA	ACG	13	1	**7,69**	18	5	**27,78**	18	7	**38,89**
CTA	ACC	15	0	**0,00**	19	5	**26,32**	20	9	**45,00**
CTA	AGG	28	3	**10,71**	34	7	**20,59**	35	13	**37,14**
CTA	AGC	18	1	**5,56**	27	14	**51,85**	29	22	**75,86**
CTC	ACT	5	0	**0,00**	8	3	**37,50**	10	7	**70,00**
CTC	AAG	15	1	**6,67**	22	4	**18,18**	23	10	**43,48**
CTC	AAC	13	1	**7,69**	15	6	**40,00**	17	9	**52,94**
CTC	ACA	23	3	**13,04**	30	12	**40,00**	34	19	**55,88**
CTC	ACG	14	2	**14,29**	21	6	**28,57**	22	14	**63,64**
CTC	ACC	9	2	**22,22**	12	3	**25,00**	14	9	**64,29**
CTC	AGG	11	1	**9,09**	16	4	**25,00**	17	7	**41,18**
CTC	AGC	11	0	**0,00**	16	8	**50,00**	17	12	**70,59**
CTT	ACT	17	2	**11,76**	21	9	**42,86**	23	13	**56,52**
CTT	AAG	24	2	**8,33**	28	5	**17,86**	33	16	**48,48**
CTT	AAC	26	3	**11,54**	32	13	**40,63**	33	16	**48,48**
CTT	ACA	27	1	**3,70**	33	7	**21,21**	37	17	**45,95**
CTT	ACG	10	0	**0,00**	12	3	**25,00**	12	3	**25,00**
CTT	ACC	15	2	**13,33**	23	10	**43,48**	26	20	**76,92**
CTT	AGG	22	2	**9,09**	27	9	**33,33**	29	15	**51,72**
CTT	AGC	24	3	**12,50**	37	12	**32,43**	38	22	**57,89**
CGA	ACT	10	0	**0,00**	10	2	**20,00**	12	6	**50,00**
CGA	AAG	21	5	**23,81**	20	7	**35,00**	22	11	**50,00**
CGA	AAC	11	0	**0,00**	13	1	**7,69**	14	4	**28,57**
CGA	ACA	28	1	**3,57**	30	6	**20,00**	33	13	**39,39**
CGA	ACG	24	4	**16,67**	23	4	**17,39**	27	13	**48,15**
CGA	ACC	34	1	**2,94**	39	10	**25,64**	42	19	**45,24**
CGA	AGG	33	0	**0,00**	35	2	**5,71**	39	11	**28,21**
CGA	AGC	32	7	**21,88**	36	10	**27,78**	39	20	**51,28**

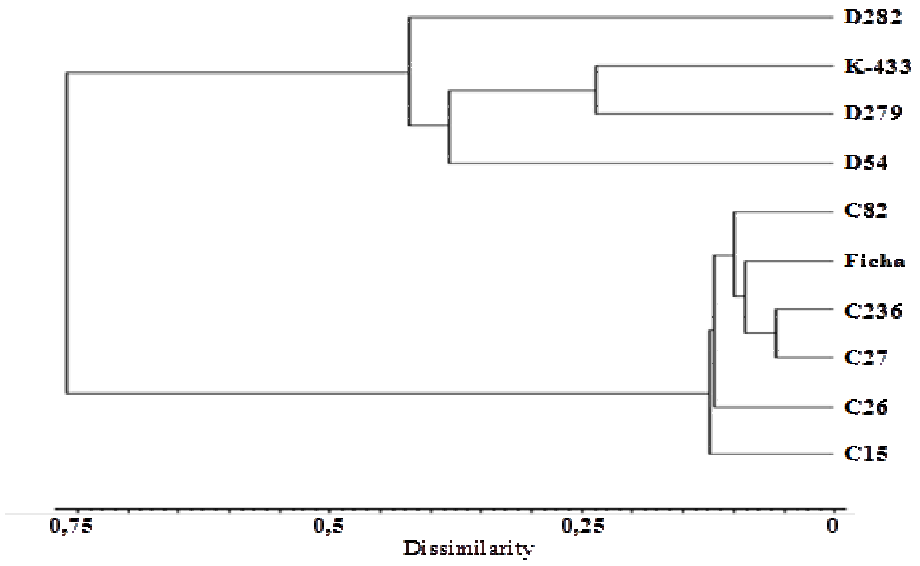

Figure 1. Dendrogram of Ficha and K-433 mutant lines as analysed by AFLP.

Table 3. Jaccard dissimilarity indices of analysed mutant lines and their control genotypes.

	C15	C26	C27	D54	D82	C236	D279	D282	Ficha	K-
C15	0.000									
C26	0.125	0.000								
C27	0.125	0.106	0.000							
D54	0.607	0.613	0.599	0.000						
D82	0.110	0.118	0.084	0.605	0.000					
C236	0.118	0.119	0.058	0.600	0.105	0.000				
D279	0.825	0.816	0.813	0.366	0.827	0.814	0.000			
D282	0.787	0.791	0.776	0.461	0.793	0.777	0.406	0.000		
Ficha	0.145	0.132	0.086	0.608	0.112	0.093	0.821	0.790	0.000	
K-	0.833	0.837	0.829	0.397	0.842	0.824	0.237	0.400	0.819	0.000

AFLP data were tested in a some of studies demonstrating their use for detecting relationships among cultivars and species in amaranth. XU and SUN (2001) reported that, AFLP has a great potential for generating a large number of informative characters for phylogenetic analysis of closely related species. COSTEA *et al.* (2006) reported AFLP technique as one with a wide range of applications, such as usable to estimate genetic diversity within and among intraspecific accessions, landraces, or wild species; to evaluate taxonomic status, evolutionary relationships, and geographic provenance of germplasm collection; to study interspecific hybridization and intogression; and to manage germplasm collections using marker-assisted reduction of redundancy.

When performing AFLP based UPGMA analyse of gamma-irradiated mutans, two major clusters were observed at a dissimilarity coefficient of a 0,75, what means, that the used primers have generated two relativelly different sets of fingerprints, one for the Ficha and its mutant lines and one for the K-433 and its mutant lines. When comparing results of molecular assessment of putative mutant lines based on AFLP and those obtained by KEČKÉŠOVÁ *et al.* (2013), interesting findings can be summarized. Relationships showed in dendrogram (Figure 1) are not only a simple fingerprints of AFLP length polymorphism within a genome but links to the fractional composition of proteins can be found when comparing our data to those reported by KEČKEŠOVÁ *et al*. (2013). Within K-433 mutant lines, both, D54 and D282 are located in the same cluster as their control line, but above it, what declare different AFPL pattern for the lines. Similary in nutritional quality analysis done by KEČKÉŠOVÁ *et al.* (2013), both of the lines mentioned above were determined as significantly different when D54 has the highest total protein content together with the lowest albumin and globulins content. Mutation-derived line is reported as indicating the highest content of prolamins and glutelins same authors.

Among Ficha mutant lines (C15, C26, C27, C82, C236), AFLP based relationships also share similar pattern as in biochemical analysis was found. C15 line is reported as significantly different to the control Ficha in the content of albumin and globulin (KEČKÉŠOVÁ et al. 2013) and here, it is among all other C-lines most distinct to the Ficha, too (Table 3).

As the dendrogram (Figure 1) figures, mutant lines C15 and C27 share the specific positions to the Ficha control genotype - they are the most distinct to the Ficha. The result correspond to the results of the biochemical analyses of the same mutant lines as in this study, where both of them are reported as statistically different to the Ficha in the content of prolamins plus glutenins and C26 is reported as to having the lowest content of total proteins when compared to the Ficha (HRICOVÁ *et al.* 2011, KEČKÉŠOVÁ *et al.* 2013). In the present study, although genetic polymorphism was detected within accessions, the AFLP markers successfully identified all the accessions. The AFLP results were further discussed by a combination of biochemical characteristics of mutant lines and their control genotypes. Based on the clear separation illustrated by the topologies of the dendrogram produced in this study, the variability within the mutant lines based on AFLP data is needed to be proved for possible correlation to the biochemical characteristics in the future. Our data indicates, the use of AFLP molecular marker systems in amaranth gamma-rays mutant lines should be advanced, because of the possible linkage to the changes of the non-coding regions of their genomes after the gamma-rays treatment and the positive selection for the thousand seeds weight (GAJDOŠOVÁ *et al.* 2005).

The first analyses of gamma-rays amaranth mutant lines developed by GAJDOŠOVÁ *et al*. (2007) were done used the AFLP molecular markers approach. We have shown that AFLP profiling produces a high level of genetic discrimination in amarath gamma-irradiadiated mutant

lines. We have documented the genetic structure present in a collection of mutant lines, where a weight of thousand seed is decreased and statistically confirmed (HRICOVÁ *et al.* 2011).

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PRIMENA METODA AFLP U VREDNOVANJU MUTANATA AMARANTUSA DOBIJENIH GAMA ZRAČENJEM

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Izvod

Korišćena je metoda AFLP u cilju utvrđivanja koji su najviše genetički slični kontrolnim genotipovimaod mutanata amarantisa, kultivar Ficha i hibrida K-433 Korišćeno je 40 selektivnih prajmer sekvenci. Utvrđene su prajmer sekvence koje imaju sposobnost diferencijacije svih ispitivanih mutanata. Vršena je analiza mutanata sa specifičnim promenama u nekodirajućim sekvencama koje su utvrđene primenom AFLP. Različit izgled AFLP fingerprinta mutanata linija kada se uporede genotip Ficha i K-433 hibrid je verovatno posledica kompleksnog odgovora intergenskog prostora u mutant – linija dobijenih gama zračenjem. Rezultati dobijeni analizom korišćenjem AFLP su diskutovani i kombinaciji sa biohemijskim karakteristikama linija mutanata i kontrolnih genotipova.

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