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REGULAR ARTICLE

ANALYSE OF RESTRICTION SITES OF AMARANTH *GBSSI* GENE IN MUTANT LINES

*Veronika Štefúnová[1], Mária Labajová[1]**

Address: [1]Ing. *Veronika Štefúnová*,PhD., Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 94976 Nitra.

[1]Ing. *Mária Labajová*, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 94976 Nitra.

*Corresponding author: e-mail: majka.labajova@gmail.com

ABSTRACT

The aim of the study was to identify and detect possible changes of *GBSSI* gene in 9 amaranth mutant lines and their 2 control samples. Polymerase chain reaction and restriction digest was used to identify *GBSSI* gene in *Amaranthus cruenthus* L. genotype Ficha and hybrid K-433 (*Amaranthus hypochondriacus* L. × *Amaranthus hybridus* L.). The presence of *GBSSI* gene was confirmes in all genotypes. Four parts of *GBSSI* gene was amplified in PCR. Detection of polymorphisms in *Pci*I restriction site was realized. The presence of the restriction site 5´ A*CATGT 3´ was confirmed in all samples. Restriction endonuclease *Pci*I digested the *GBSSI* gene in GBSSI 1 sequence in all samples equally. Two fragments with 1171 bp and 361 bp were obtained from restriction digest. Specific part GBSSI 3 of *GBSSI* gene was digested in all amaranth samples into two fragments with size 896 bp and 202 bp.

Keywords: amaranth, *GBSSI* gene, *Waxy* gene, restriction digestion.

INTRODUCTION

The genus *Amaranthus* L. (*Caryophyllales: Amaranthaceae*) is contained relatively by numerous group of about 60–70 species, which some of them are cultivated and some are weedy species **(Sauer, 1967)**. Plants of this genus have vast potential to be used as leaf vegetables (mainly in tropics) or forage, others for grains production, some are planted as ornamental plants and some are used in industry to produce of paper, dyes, cosmetics and etc. **(Moudrý *et al.*, 1996; Ofitserov, 2001; Kohaut, 2005; Neluheni, *et al.*, 2007; Čičová, 2008)**. Cultivation of amaranth grain has deep interest, considering**:** At the first, grain amaranth's balanced amino acid composition is close to the optimum protein reference pattern in the human diet according to FAO/WHO requirements **(Mlakar *et al.*, 2010)**. At the second, it is gluten-free pseudocereals, which are necessary for people with celiac disease **(Bressani *et al.*, 1992)**. Moreover, amaranth is suitable for planted in changing climatic conditions **(Ofitserov, 2001; Húska, 2011)**.

Mutagenesis is widely used method in plant breeding. Breeders use mutagenesis to improve characters and properties of plants and to increase genetic polymorphism within plant genomes. Mutagenesis experiments with amaranth were realized by **Gajdošová *et al.* (2002, 2005)** and **Kgang (2008)**.

Variously approaches and techniques of molecular analyzes provide methodological support for evaluation of biological materials through the monitoring and reporting of epidemiological changes in the human population **(Zeleňáková *et al.*, 2011, 2012)** evaluation of the authenticity and authentication of food **(Židek *et al.*, 2012; Ražná, Žiarovská, 2011; Revák *et al.* 2011**) and the diversity evaluation of the cultivated and wild species **(Hrubíková *et al.* 2007; Candráková *et al.*, 2008; Žiarovská *et al.*, 2009; Oslovičová *et al.* 2010; Vivodík *et al.* 2011).**

Complete CDS of *GBSSI* gene for *Amaranthus cruenthus* L. is available in NCBI database under access code AB456685. *GBSSI* gene with 3492 bp consist with 12 introne a 13 exones (Figure 1). **Park *et al.* (2009)** using BLAST analyse detected hight degree of similarites (based on aminoasid sequences analyses) between amaranthus species and other plants species.

Figure 1 Schematic representation of *GBSSI* gene

Legend: The rectangles represent exons and lines between them introns. CDS - coding region of the gene are follows (bp) 1-330, 503-423, 596-694, .873-784, 1238-1301, 1416-1516, 1598-1707, 1784-2027, 2116-2292, 2422-2613, 2707-2793, 2899-3027, 3121-3237. Modified by **(Park *et al.*, 2009)**.

Molecular methods and techniques allow analyzing specific parts – genes, places of interest of various organisms for *GBSSI* gene (granule bound starch synthetase I) – *Waxy* gene, too. *GBSSI* gene is responsible for the amylase synthesis one of the component of starch in plant storage organs. Two types of amaranth starch are known, waxy without amylose production seeds are glutinous and nonwaxy with amylose production seeds are nonglutinous. The characteristic of starch is controled by the one *Waxy* gene with nonglutinous starch dominant **(Okuno and Sakaguchi, 1982; Smith, 1997)**.

High degree of similarites, based on aminoacid sequences of *Waxy* gene was detected in *Amaranthus caudatus* L., *Amaranthus cruentus* L.and *Amaranthus hypochondriacus* L. (**Park *et al,* 2010)**.

Mutation of this gene eliminate or reduce the amilose content of starch throught disrupted expression or loss of function of *Waxy* gene. Understanding of the activites of *GBSSI* gene may by important to determine the functions of the food and nonfood industry. Analysis of genetic diversity is needed for effective utilization of genetic resources, creating new strains with different starch composition **(Park *et al.*, 2009, Park *et al.*, 2011)**.

The aim of the study was to identify and detect the *GBSSI* gene. The main goal was to amplify four parts of the *GBSSI* gene in polymerase chain reaction and analyse the presence or absence of restriction sites in *GBSSI* gene after the restriction digest with restriction enzyme *Pci*I in 9 amaranth mutant lines and their 2 control samples.

MATERIAL AND METHODS

Mutant lines of amaranth and their control plants without mutation affected were used in analysis. The characteristic of mutant lines and their control samples is listed in the Table

1. Individuals are characterized by statistically significant increase of weight of thousand seeds **(Gajdošová and Libiaková, 2002; Gajdošová *et al.*, 2005).** Biological material was provided by Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Nitra.

The amaranth seedlings were cultivated under *in vitro* conditions on Murashige, Skoog (1962) medium. DNA from fresh young leaves was isolated using the isolation kit Invisorb® Spin Plant Mini Kit (Invitek). DNA was quantified by fluorometer (Qubit TM). PCR technique was used to amplify *GBSSI* gene.

Table 1 Characteristics of amaranth biological material used in the work

Mutant lines	Origin of mutant lines
C 15/1, C 26/2, C 26/3,C 27/5, C 82/1, C 236/1, Control A	cultivar Ficha (*Amaranthus cruentus* L.)
D 54/1, D 279/1, D 282/1, Control B	hybrid K-433, result from interspecific hybridization of species (*A. hypochondriacus* L. × *A. hybridus* L.)

Primers for amplification of *GBSSI* gene (Table 2) with some diferences according to **Park *et al.* (2009)** were used. **Park *et al.* (2009)** designed primers on the base of sequence of 3492 bp *GBSSI* gene for *Amaranthus cruenthus* L. This sequence is available in NCBI database with access code AB456685.

Table 2 The sequences of primers and their annealing temperature

Name of primer	Sequences of primer	Anealing temperature of primer
GBSSI 1 - forward	CAGGCAGCTTTGGAGGCACCA	68°C
GBSSI 1 - reverse	TGGAGGCTACCACAGGCACCT	68°C
GBSSI 2 - forward	TTCAGGCCAGGGGACACCGT	65°C
GBSSI 2 - reverse	CGTGGGTAGTCCGCCAAGGC	65°C
GBSSI 3 - forward	ATGGAAACAGTAACATCTTCTCACT	61°C
GBSSI 3 - reverse	CATCTTTTCATAGAATAGCCAAGTCA	61°C
GBSSI 4 - forward	ATGTTAATTCCTAGCAGATTTGA	57°C
GBSSI 4 - reverse	CTTTGTGAATTTGTTGTTGAATA	57°C

Legend: Primer GBSSI 1 amplified part of amarnth *GBSSI* gene with size 1255 bp – 2725 bp, GBSSI 2 part of amarnth *GBSSI* gene with size 437 bp – 1659 bp, GBSSI 3 part of amarnth *GBSSI* gene with size 1 bp – 1098 bp and primer GBSSI 4 amplified part of amarnth *GBSSI* gene with size 2548bp – 3461 bp.

PCR reactions were performed in a buffer solution 1 × PCR containing 100 mmol.dm^{-3} Tris-HCl (pH 8.8), 500 mmol.dm^{-3} KCl a 1.5 mmol.dm^{-3} $MgCl_2$ (Applichem), together with 0.08 mmol.dm^{-3} d NTP (Invitrogen^{TM}) 450 nmol.dm^{-3} primer (Microsynth), 1 U Taq polymerase (Applichem) and 50ηg of template DNA. PCR water was added into 15 µl final volume of reaction. The condition listed in the Table 3 was used to amplify of *GBSSI* gene.

Amplified fragments were electrophoretically separated in 1,5 % agarose gel (Applichem). Electrophoreograms were used to visual determine the profile changes of the mutant lines in the comparison with control plants. Electrophoresis was conducted at a voltage of 60 V at 3 h. Electrophoreograms were processed with documentation system G:Box in GeneSnap program – Product version: 7.09 (Syngene) and GeneTools – Product version: 4.01 (Syngene). Profiles were evaluated for the presence or absence of amplification GBSSI products.

Table 3 Time and temperature profile of PCR for amplification of *GBSSI* gene

Steps of GBSSI reaction	Time profile	Temperature profile	Number of cycle
Initial denaturation	3 minute	95 °C	-
Denaturation	15 second	95 °C	32
Annealing	40 second	57–68 °C Depending on the primers*	
Extension	2 minute	72 °C	
Final extension	7 minute	72 °C	-
Cooling	10 minute	4 °C	-

Legend: * Temperatures in Tablele 2.

PCR products were digested with restriction endonuclease *Pci*I. Restriction digest was performed in a buffer solution Buffer Tango 1 × containing 33 mmol.dm^{-3} Tris-acetate (pH 7.9), 10 mmol.dm^{-3} magnesium acetate, 66 nmol.dm^{-3}potassium acetate, 0.1 mg.ml^{-1} BSA (Fermentas), together with 10 u.µl^{-1} *Pci*I, 10 mmol.dm^{-3} Tris-HCl (pH 7.4 25°C), 100 mmol.dm^{-3} KCl, 1 mmol.dm^{-3} DTT, EDTA, 0.2 mg.ml^{-1} BSA a 50 % glycerol and DNA nuclease-free water.

Restriction digest was performed in termobox at 37°C for 14 hours. Restriction fragments were electrophoretically separated in 2 % agarose gel (Applichem) after restriction digest. Electrophoreograms were used to determine the profile changes of the mutant lines and the control plants. Electrophoresis was conducted at a voltage of 60V at 3 h. Electrophoreograms were processed with documentation system G:Box in GeneSnap program – Product version: 7.09 (Syngene) and GeneTools – Product version: 4.01 (Syngene). Profiles were evaluated for the presence or absence of restriction products.

RESULTS AND DISCUSSION

Knowledge about genetic diversity and relationships between cultivated speciec and their relative wild species are necesary for study, formation and effective utilisation of individual collection of genetic resources **(Chan and Sun, 1997).**

Diversity among organisms is a result of variations in DNA sequences and effect of environmental conditions **(FAO, 2007)**. Molecular tools provide to detect genetic diversity of species at the DNA level. **(Somasundaram and Kalaiselvam, 2011).** Varied molecular analyses was done to study variability in amaranth genome. RAPD molecular markers and isoensymes was used to analyse by **(Chan and Sun, 1997)**, AFLP and ISSR **(Xu and Sun, 2001),** AFLP **(Costea, *et al.*, 2006)**. Park *et al.* **(2009, 2010 and 2011)** analysed variability of *GBSSI* gene.

GBSSI gene for *Amaranthus cruenthus* L. with size 3492 bp is available in NCBI database under the access code AB456685. *GBSSI* gene is one of the genes with extensive interest, which is responsiable to the synthesys of storage substance – starch. The change in nucleotide sequene in *GBSSI* gene can by the main cause of increase of higher weight of thousand seeds in our amaranth mutant lines samples. Higher weight of thousand seeds in samples can by depend on changes in quantity of starch production.

Primers marked as: GBSSI 1, GBSSI 2, GBSSI 3 and GBSSI 4 was used to amplify of entire *GBSSI* gene (Figures 2-5). Sequences of GBSSI primers are listed in the Table 2.

Polymerase chain reaction and restriction digest of PCR products with restriction endonuclease *Pci*I was used to the analyze of potential changes in *GBSSI* gene of amaranth Ficha genotype – *Amaranthus cruentus* L. and hybrid K-433 (*Amaranthus hybridus* L. × *Amaranthus hypochondriacus* L.).

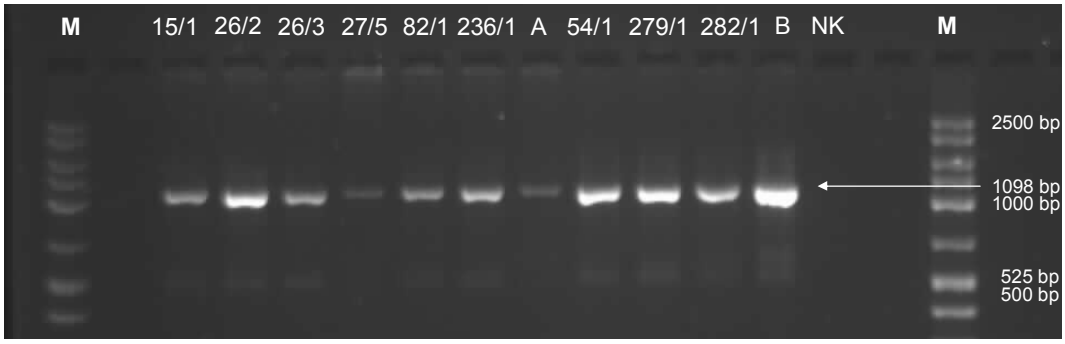

Figure 2 Amplification of part *GBSSI* gene 1 bp – 1098 bp with primer GBSSI 3

Legend: M – Marker Lonza 50–2500 bp, NK – negative control, samples of *Amaranthus cruenthus* L. 15/1, 26/2, 26/3, 27/5, 82/1, 236/1, A – control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B – control.

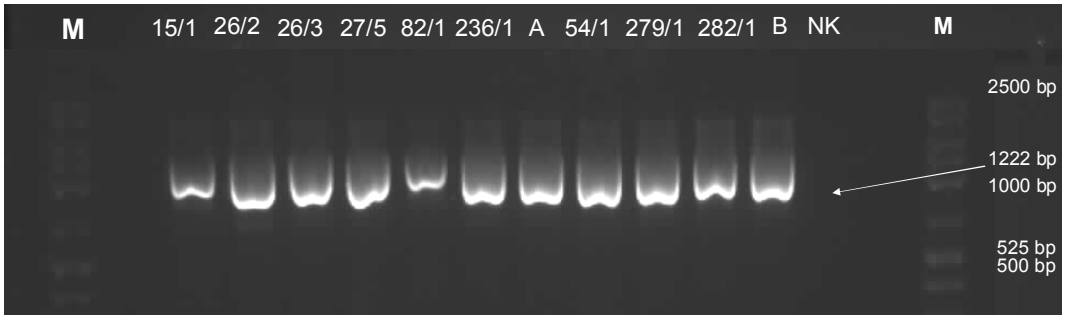

Figure 3 Amplification of part *GBSSI* gene 437 bp – 1659 bp with primer GBSSI 2

Legend: M - Marker Lonza 50-2500 bp, NK - negative control, samples of *Amaranthus cruenthus* L. 15/1, 26/2, 26/3, 27/5, 82/1, 236/1, A - control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B – control

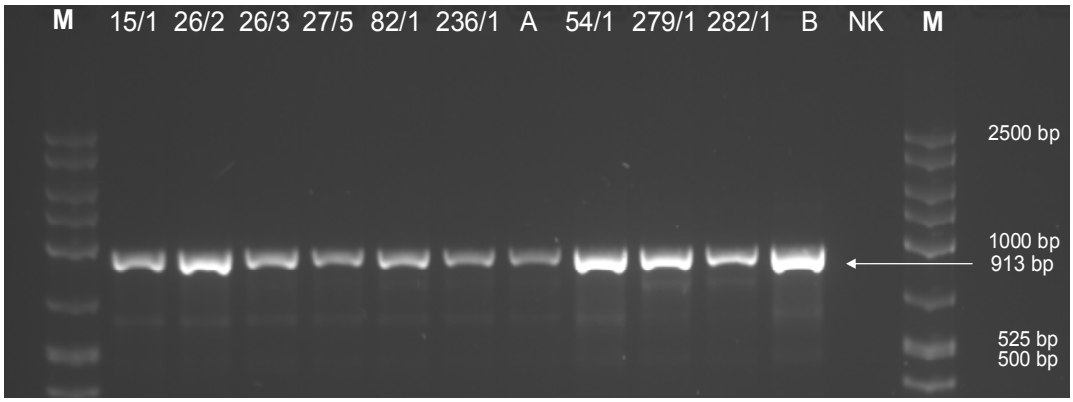

Figure 4 Amplification of part *GBSSI* gene 1255 bp – 2725 bp with primer GBSSI 1

Legend: M – Marker Lonza 50–2500 bp, NK – negative control, samples of *Amaranthus cruenthus* L. 15/1, 26/2, 26/3, 27/5, 82/1, 236/1, A – control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B – control.

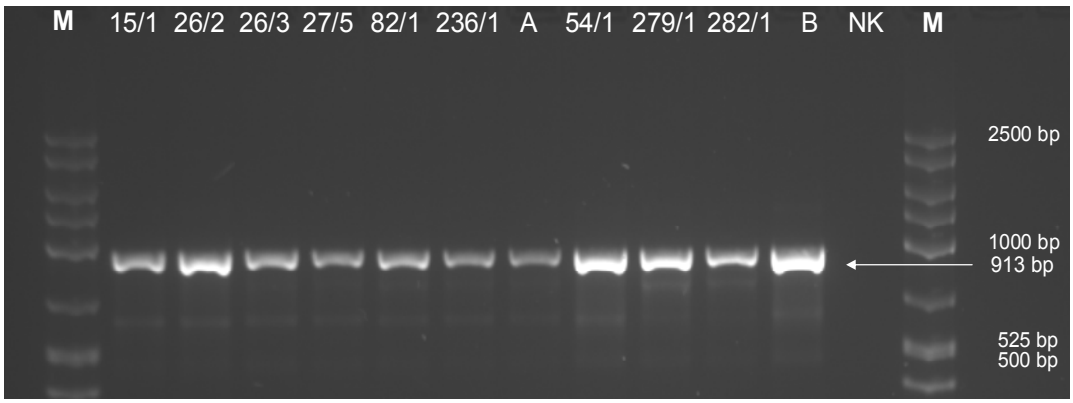

Figure 5 Amplification of part *GBSSI* gene 2792 bp – 3461 bp with primer GBSSI 4

Legend: M – Marker Lonza 50–2500 bp, NK – negative control, samples of *Amaranthus cruenthus* L. 15/1, 26/2, 26/3, 27/5, 82/1, 236/1, A – control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B – control.

Restriction enzyme *Pci*I was used to the restrition digest of *GBSSI* gene. Program NEBcutter2 (http://tools.neb.com/NEBcutter2/) was used to analyse of sequence of *GBSSI* gene before the restrition digest of *GBSSI* gene. Map of restriction sites (Figure 6) with used endonuclease *Pci*I for amaranth *GBSSI* gene was created.

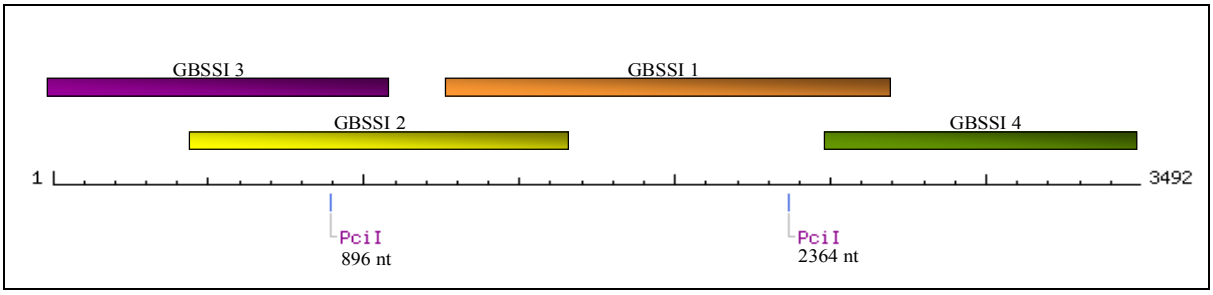

Figure 6 Map of restriction sites with used endonuclease *Pci*I for amaranth *GBSSI* gene

Specifically restriction sites of *GBSSI* gene for restriction endonuclease *Pci*I are shown at the Figure 7. Program NEBcutter2 has enabled virtual view to the electrophoreogram with restriction fragments after the restriction digest of amaranth *GBSSI* gene (Figure 8).

PciI sites

5'...A▼C A T G _T... 3,
3'...T _G T A C▲A... 5'

	Cut position	Site with flanks
1	896/900	886 TTGATTTCAG A▼CATG_T ACCACATTCG
2	2364/2368	2354 ATCGTGGTCT A▼CATG_T ATGGTGTACC

Figure 7 Restriction site for cleavage *GBSSI* gene RE *Pci*I

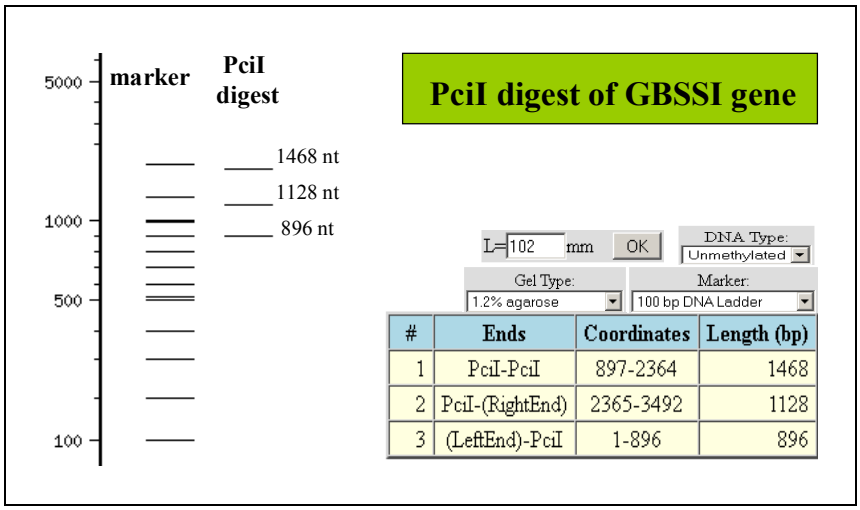

Figure 8 Restriction digest of *GBSSI* gene restriction endonuclease *Pci*I by NEBcutter2

Restriction endonuclease *Pci*I digested the *GBSSI* gene in GBSSI 1 sequence in all samples equally. Two fragments with 1171 bp and 361 bp were obtained from restriction digest (Figure 9). Specific part GBSSI 3 of *GBSSI* gene was digested in all amaranth samples into two fragments with size 896 bp and 202 bp.

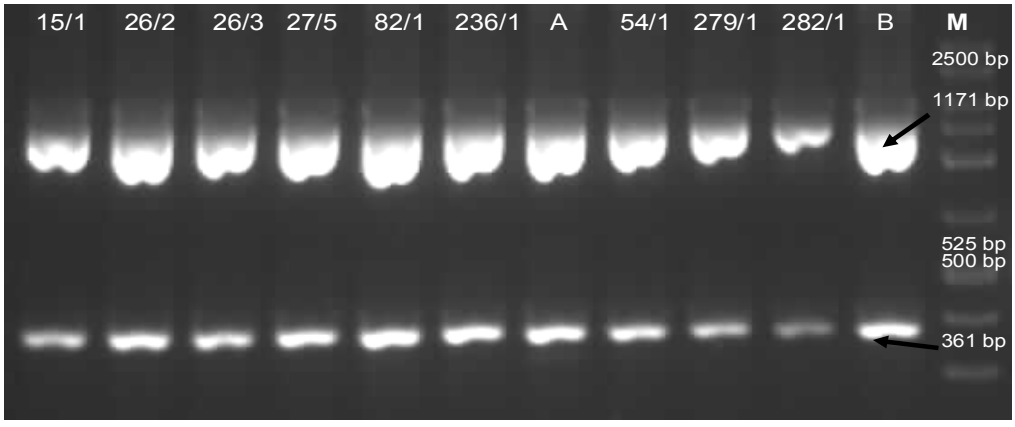

Figure 9 Cleavage of *GBSSI* gene segment 1255 bp – 2725 bp with RE – *Pci*I

Legend: M – Marker Lonza 50–2500 bp, NK – negative control, samples of *Amaranthus. cruenthus* L. 15/1, 26/2, 26/3, 27/5, 82/1, 236/1, A – control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B – control

Any changes were detected in restriction sites of mutant lines samples compare to no mutant effected control samples. Gamma radiation has no effect to change of nucleotide in restriction sites of restriction endonuclease *Pci*I in amaranth *GBSSI* gene.

DNA variations are mutations resulting from substitution of single nucleotides (single nucleotide polymorphisms – SNPs), insertion or deletion of DNA fragments of various lengths (from a single to several thousand nucleotides), or duplication or inversion of DNA fragments **(FAO, 2007)**.

Any polymorphism of *GBSSI* gene was detected in 9 amaranth mutant lines and 2 control samples after the restriction digest with restriction endonuclease *Psi*I.

Optimization of simple enzyme polymorphism analyses for genotype Ficha and mutant line of K-433 hybrid was described in **Múdry *et al.* (2011)** research. The best result was obtained in the case of phosphoglucomutase, where both genotypes were distinguished.

Hricová *et al.* (2011) realized biochemical research of mutant lines *Amaranthus cruentus* L. genotype 'Ficha' and hybrid K-433. 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.

Mutations in key nucleotides of a coding sequence may change the amino acid composition of a protein, and lead to new functional variants. Such variants may have an increased or decreased metabolic efficiency compared to the original "wild type", may lose their functionality completely, or even gain a novel function **(FAO, 2007)**.

We detected presence or absence and restriction sites of *GBSSI* gene with RE *PciI* in amaranth samples. *GBSSI* gene was presence in all samples and any changes was detected in restriction sites of mutant lines samples compare to no mutant effected control samples.

Park *et al.* (2010) analysed presence of polymorphic sites in GBSSI nucleotide sequence of three amaranth species. The result was the detection of 16 polymorphic sites in introns and exons, too. SNP in coding regions was found in exon 10 and 13, but this changes effeced any changes in aminoacid composition or in enzymatic activity.

It is distinguished two type of amaranth starch, waxy without amylose production-seeds are glutinous and nonwaxy with amylose production - seeds are nonglutinous. The characteristic of starch is controled by the one *Waxy* gene with nonglutinous starch dominant **(Okuno and Sakaguchi, 1982; Smith, 1997)**.

Various mutations of nucleotide sequences have been recorded in Waxy gene. For example in wheat, there are major deletions of the protein in coding region **(Vrinten *et al.* 1999)**, and in barley there are the waxy phenotype originated by deletion of the 5´-terminal part of the gene **(Domon *et al.* 2002)**.

Testing of genomic diversity at the molecular level using molecular markers in amaranth is effective and it is highly used, too **(Park, Lee, Kim, 2009).**

CONCLUSION

Restriction digest of PCR fragments is an effective tool for species-specific analyzes. It allows effectively and in a short time identificate of the presence and/or changes in the specific sequence of nucleotides in plant genomes. The presence of restriction site in the amaranth *GBSSI* gene in control samples so in mutant lines showed, that this part of nucleotide sequence was not changed by radiation mutagenesis. It is a precondition to designate of primers for further molecular analysis of *GBSSI* gene.

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