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Restriction Analyze of Starch Synthesis Genes in Amaranth Mutant Lines

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Abstract: The increase interest and study of underutilized and neglected crops is actual for the last years. In the study, the restriction polymorphisms of *GBSSI* and *SSSI* gene in gamma-irradiated mutant lines of amaranth was performed. To evaluate polymorphisms of restriction sites endonucleases *Aci*I, *Bsa*JI, *Fat*I *Eco*RI, *Bam*HI, *Pst*I, *Hpa*II and *Pci*I were used. *GBSSI* gene responsible to amylose synthesis was digested with *Hpa*II and *Pci*I restriction endonucleases. Two *Pci*I restriction sites and three *Hpa*II restriction sites were evaluated and any changes in restriction sites were recorded. Profile changes of the SSSI gene in mutant lines C 15/1 and C 236/1 were recorded after the restriction digest by *Bsa*JI. Restriction cleavage polymorphism was recorded after the restriction digest of segment 5250-6854 bp with restriction endonuclease *Fat*I, too.

Key words: Amaranth, mutagenesis, GBSSI gene, SSSI gene, restriction digestion

INTRODUCTION

A lot of questions about healthful and nutritionally rich foodstuffs accompany human race from time immemorial. In this nutritional respect, the group of rediscovered plants play an interest role. The alternative crops such as *Amaranthus* and quinoa (Tomoskozi *et al*., 2011), yacon (Milella *et al*., 2005, 2011) or buckwheat (Baumgertel *et al*., 2010) are intensively studied. Nowadays, up to the 70 species of the genus Amaranth are known, but not all of them are cultivated, weedy species are known, too (Sauer, 1967). A big potential of amaranth is in its ability to grow in less favorable conditions and so, it is one of the most important leafy vegetables widely grown in the tropics with a potential to broaden man's food base in Africa (Neluheni *et al*., 2007). Another advantage of amaranth is its positive linkage to the consumption when celiac disease. For dietary purposes it is very suitable because of the optimum protein pattern (Mlakar *et al*., 2009, 2010).

Improvement of plant traits is the main role of breeding programs for ensures sufficient quantity and quality of agricultural crops. The stimulus for the research of amaranth was initiated by the Rodale Foundation and the Rodale Research Center in the mid-1970s, where they started with comprehensive research into amaranth (Stallknecht and Schulz-Schaeffer, 1993). Goals in improving cultivars of grain crops (the same case is amaranth) are usually similar- raising yield, increasing pest resistance and improving harvest stability (Brenner *et al*., 2010). One of the tools of breeding is mutagenesis. It is widely used. In FAO/IAEA database is registered more than 3218 mutant varieties of crops. Amaranth genotype Ficha and hybrid K-433 are reported as the objects for the treatment by γ-radiation in the literature. As a result of the treatment, increased weight of thousand seeds is reported for their putative mutant lines. Across the positive selection in 2nd to the 8th generation, this characteristics seems to have the tendency to be stabilized (Galova *et al*., 2008; Gajdosova *et al*., 2008).

One of planted crops characteristics with the influence on germination, seed vigor or yield is the thousand seeds weight (Moshatati and Gharineh, 2012). The yield is important economic indicator, so the study of this trait is very important. On the genetic level thousand grain weights is encodes by many genes. Quantitative Trait Loci (QTLs) for thousand grain weights have been intensively studied in rice (Yu *et al*., 2008) and oilseed rape (Fan *et al*., 2010). For breeders and for marker assisted improvement purposes, actually QTLs that are environmentally stable are interesting (Wu *et al*., 2012).

Biochemical analysis of amaranth seeds has showed the content of the proteins at the level of 15% and the content of the starch at the level of 60% (Becker *et al*., 1981; Pedersen *et al*., 1987). Amaranth grains contain 50-60% starch that consists of two major components:

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amylose and amylopectin such as endosperm starch. Amylose is synthesized by a Granule-bound Starch Synthase (GBSS) encoded by the *Waxy* gene, whereas amylopectin biosynthesis is catalyzed by 'Soluble' Starch Synthase (SSS), starch branching enzyme, and starch debranching enzyme (Fujita *et al*., 2008).

The sequence of SSSI gene is listed in NCBI database under the accession number AB626804. *GBSSI* gene with 9500 bp consist 15 introns and 16 exones. The sequence of GBSSI gene (*Waxy* gene) is listed in NCBI database under the accession number AB456685. As is referred in the database, the length of the *GBSSI* gene is about 3492 bp with a total of 12 introns. Mutation of this gene eliminate or reduce the amylose content of starch through disrupted expression or loss of function of *Waxy* gene. For a good amaranth utilization and genetic resources management, knowledge about its genetic diversity and breeding of varieties with different starch composition are actual (Park *et al*., 2009; Park *et al*., 2010).

The aim of the study was to identify restriction pattern of *SSSI* and *GBSSI* genes. The main goal was to analyse the presence or absence of selected restriction sites in *SSSI* and *GBSSI* gene in amaranth mutant lines and their 2 control samples.

MATERIALS AND METHODS

Mutant lines of amaranth and their control plants without mutation affected were used in analysis. The characteristic of used accessions is listed in the Table 1. Individuals are characterized by statistically significant increase of weight of thousand seeds (Gajdosova and Libiakova, 2002; Gajdosova *et al*., 2005). The amaranth seedlings were cultivated under *in vitro* conditions on Murashige and Skoog, 1962) medium. DNA from fresh young leaves was isolated using the isolation kit Invisorb® Spin Plant Mini Kit (Invitek).

Table 1: Species and accessions of Amaranthus studied

Species	Accession	Type	WTS (g)*
Amaranthus cruentus	Ficha cultivar	Control A	0.86**
	C 15/1		0.969*
	C 26/2		1.000*
	C 26/3		1.014*
	C 27/5		0.978*
	C 82/1		0.975*
	C 236/1		1.086*
A. hypochondriacus x *A. hybridus*	K-433 hybrid	Control B	0.73 **
	D 54/1		0.896*
	D 279/1		0.804*
	D 282/1		0.811*

*Weight of thousand seeds in M5 positively selected generation (based on data from Gajdosovs and Libiakova, 2002; Gajdosova *et al*., 2005), **Weight of thousand seeds (personaly communication Janovská Dagmar, VURV-Praha Rruzyne)

The sequences of *GBSSI* gene and *SSSI* gene are listed in Table 2. *GBSSI* gene primers were created with some differences according to Park and collaborators (Park *et al*., 2010). *SSSI* gene primers were designed on the base of sequence of 9500 bp *SSSI* gene for *Amaranthus cruenthus* L. This sequence is available in NCBI database with access code AB626804.

PCR for amplification of *SSSI* gene were performed in Termo Scientific Dream *Taq* PCR Master Mix (2x); Dream *Taq* DNA polymerase is supplied in 2x Dream *Taq* buffer, dATP, dCTP, dGTP and dTTP (0,4 mmol dm^{-3} each) and 4 mmol. dm^{-3} $MgCl_2$, together with 260 nmol dm^{-3} of SSSI 2, SSSI 5 and SSSI 6 primer or 200 nmol. dm^{-3} of SSSI 3 and SSSI 4 primer (Microsynth) and 50 ng of the genomic DNA in a volume of 15 μL and PCR for amplification of *GBSSI* 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} dNTP (Invitrogen™) 450 nmol dm^{-3} primer (Microsynth), 1 U Taq polymerase (Applichem) and 50 ήg of template DNA. The amplification of *GBSSI* and *SSSI* genes was carried by the condition listed in Table 3 and 4.

PCR products were digested with *Aci*I, *Bsa*JI, *Fat*I (BioLabs), *Eco*RI, *Bam*HI, *Pst*I (Gibco), *Hpa*II, *Pci*I (Fermentas) restriction endonucleases. Restriction digest was performed in buffer solution (determined by manufactures for each restriction endonuclease), 1U restriction endonuclease and DNAnuclease-free water.

Restriction digest was performed at 37°C for 14 h *Hpa*II, *Pci*I (Fermentas), at 37°C for 2 hours *Aci*I (BioLabs), *Eco*RI, *Bam*HI, PstI (GibcoBRL), at 55°C for 2 hours *Fat*I (BioLabs) and at 60°C for 2 h *Bsa*JI (BioLabs).

Table 2: The primers sequences and their annealing temperatures

Name of primer	Sequences of primer	Anealing temperature of primer (°C)
GBSSI 1-F	CAGGCAGCTTTGGAGGCACCA	68
GBSSI 1-R	TGGAGGCTACCACAGGCACCT	68
GBSSI 2-F	TTCAGGCCAGGGGACACCGT	65
GBSSI 2-R	CGTGGGTAGTCCGCCAAGGC	65
GBSSI 3-F	ATGGAAACAGTAACATCTTCTCACT	61
GBSSI 3-R	CATCTTTTCATAGAATAGCCAAGTCA	61
GBSSI 4-F	ATGTTAATTCCTAGCAGATTTGA	57
GBSSI 4-R	CTTTGTGAATTTGTTGTTGAATA	57
SSSI_F2	CTGGTTCCATTGTTGGGTTTGA	62
SSSI_R2	ACCTGATTGTCCCCAAAAGCA	62
SSSI_F3	GGTGTTGATTGGGTAGGGGA	65
SSSI_R3	GCGACATTACCTGGCTGACT	65
SSSI_F4	GTATTTCCCGAGTGGGCCAG	63
SSSI_R4	TAGAGGTGTTCAACGTGCCG	63
SSSI_F5	GGCGCCTGTAGGACAAAGAA	62
SSSI_R5	TGGTGGGATTTCATTGAGACCA	62
SSSI_F6	GCAAGTCCTGTTGCATATCG	62
SSSI_R6	ACTACAAGCATTCAGTTCCCCT	62
SSSI_F7	CACAAGCGAATTACCTAGCGA	63
SSSI_R7	ACCATTAACCAAACATCCCGT	63

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

Steps of GBSSI reaction	Time profile	Temperature profile (°C)	No. of cycle
Initial denaturation	3 min	95	-
Denaturation	15 sec	95	32
Annealing	40 sec	57-68	
Depending on the primers*			
Extension	2 min	72	
Final extension	7 min	72	-
Cooling	10 min	4	-

*Temperatures in Table 2

Table 4: Time and temperature profile of PCR for amplification of *SSSI* gene

Steps of SSSI reaction	Time profile min	Temperature profile (°C)	Number of cycle
Initial denaturation	3	95	-
Denaturation	1	95	40
Annealing	1	62-65	
Depending on the primers*			
Extension	2	72	
Final extension	7	72	-

*Temperatures in Table 2

Restriction fragments were electrophoretically separated in 2% agarose gel (Applichem). 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) andGeneTools -Product version: 4.01 (Syngene). Profiles were evaluated for the presence or absence of restriction products.

RESULTS

Genetic mapping of natural or induced genomic variation remains a powerful approach to understand the function of genes in a variety of biological processes (Baird *et al*., 2008).

We identified and evaluated the polymorphisms of *GBSSI* gene and *SSSI* genes in mutant lines of amaranth samples with increase of weight of thousand seeds.

At the first, presence of *GBSSI* and *SSSI* gene was detected. The genes were not amplified in whole length but in segments. Concretely, *GBSSI* gene was amplified in three and *SSSI* gene in seven segments. We identified both genes in all of the mutant and control samples.

The second part of the analysis was the detection of restriction sites of *GBSSI* and *SSSI* genes. Restriction endonucleases *Aci*I, *Bsa*JI, *Fat*I, *Eco*RI, *Bam*HI, *Pst*I, *Hpa*II, *Pci*I, were used as a tool for evaluating polymorphisms of restriction sites. Changes in restriction sites will be analysed further in the research of genetic background of the increasement of weight of thousand seeds. *GBSSI* gene responsible to amylose synthesis was digested with *Hpa*II and *Pci*I restriction endonucleases.

Fig. 1: Cleavage of *GBSSI* gene segment 1255 bp-2725 bp with RE-*Hpa*II Lane: M: Marker Lonza 50-2500 bp, NK-negative control, samples of *Amaranthus cruenthus* Lane 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

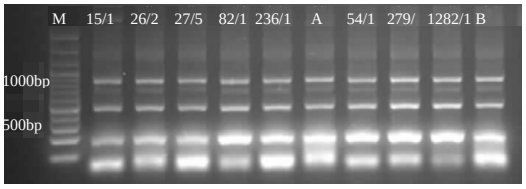

Fig. 2: Cleavage of *SSSI* gene segment 6772 bp-8372 bp with RE- *Eco*RII Lane: M: Marker 100-3000bp, NK-negative control, samples of *Amaranthus cruenthus* Lane 15/1, 26/2, , 27/5, 82/1, 236/1, A-control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B-control

Two *Pci*I restriction sites and three *Hpa*II restriction sites were evaluated and any changes in restriction sites were recorded. Restriction endonucleases *Pci*I and *Hpa*II digested the *GBSSI* gene in all samples equally (Fig. 1).

Restriction analysis of *SSSI* gene in amaranth mutant lines and they control shown some another results. Six restriction endonucleases were used to evaluate amylopectin synthesis responsible SSSI gene. Restriction endonuclease *Aci*I was used to evaluate *SSSI* gene segment 3740-5370 bp, *Bsa*JI and *Fat*I was used to evaluate *SSSI* gene segment 5250- 6854 bp, *Eco*RI was used to evaluate *SSSI* gene segment 6772-8372 bp, *Bam*HI was used to evaluate *SSSI* gene segment 3740-5370 bp and *Pst*I was used to evaluate *SSSI* gene segment 1994-3854 bp. The analysis of AciI, BamHI, EcoRI, PstI restriction sites didn't show any polymorphisms. The profiles of restriction fragments in all samples were monomorphic (Fig. 2).

The segment of 5250-6854 bp of SSSI gene was digested using two different restriction endonuclease, *Bsa*JI and *Fat*I. In both cases, changes in restriction

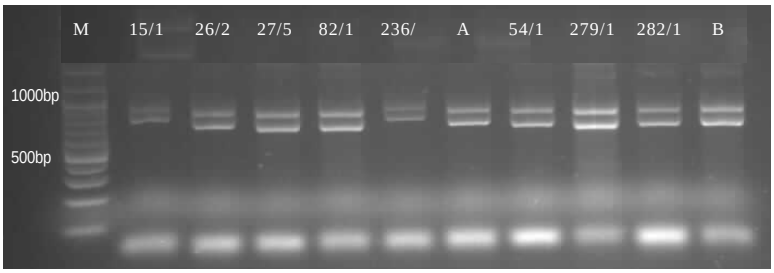

Fig. 3: Cleavage of *SSSI* gene segment 5250-6854 bp with RE -*Bsa*JI, Lane: M: Marker 100-3000bp, NK-negative control, samples of *Amaranthus cruenthus* Lane 15/1, 26/2, , 27/5, 82/1, 236/1, A-control. Samples of K-433 hybrid 54/1, 279/1, 282/1, B-control

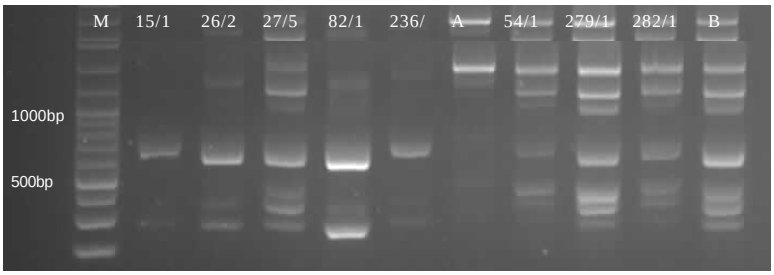

Fig. 4: Cleavage of *SSSI* gene segment 5250 bp-6854 bp with RE-*Fat*I, Lane: M: Marker 100-3000bp, NK-negative control, samples of *Amaranthus cruenthus* Lane 15/1, 26/2, , 27/5, 82/1, 236/1, A-control, Samples of K-433 hybrid 54/1, 279/1, 282/1, B-control

polymorphism of this segment were recorded (Fig. 3 and 4). Profile changes in samples C 15/1 and C 236/1 were recorded on the electrophorteogram of restriction digest when restriction endonuclease *Bsa*JI was used. Restriction cleavage polymorphism was recorded after the restriction digest of segment 5250-6854 bp with restriction endonuclease FatI, too and mainly in *A. cruenthus* samples (Fig. 4).

DISCUSSION

Amaranth plant is extensively studied: cultivation in diverse condition (Rivelli *et al*. 2008; Fejer *et al*., 2011), nutrient value (Galova *et al*., 2008; Hricova *et al*. 2011), industrial applications (Teli *et al*., 2009), utilization as source of bioenergy and genetic background (Stefunova and Bezo, 2003; Ray and Roy 2009) is studied actually.

Mutation induction and some other means of genetic modification, such as genetic transformation, are tools that provide variation in some of the plant characters (Javed *et al*., 2000). The increase of weight of seeds after the mutagenesis was recorded by authors (Gajdosova and Libiakova, 2002; Dwimahyani and Ishak, 2004; Basu *et al*., 2008).

Genetic mapping of natural or induced genomic variation remains a powerful approach to understand the function of genes in a variety of biological processes (Baird *et al*., 2008).

The variability of amaranth *GBSSI* and *SSSI* gene evaluated Park and co-laborants (Park *et al*., 2009; 2010).

It was identified and evaluated the polymorphisms of *GBSSI* and *SSSI* genes in mutant lines of amaranth samples with increase of weight of thousand seeds. We used restriction digest with endonucleases *Aci*I, *Bsa*JI, *Fat*I, *Eco*RI, *Bam*HI, *Pst*I, *Hpa*II, *Pci*I restriction endonucleases, to evaluate polymorphisms of restriction sites.

GBSSI gene responsible to amylose synthesis was digested with *Hpa*II and *Pci*I restriction endonuclese but any changes in profiles of restriction sites was recorded. Mutations of this gene eliminate or reduce the amylose content of starch through disrupted expression or loss of function of *Waxy* gene (Park *et al*., 2009, 2012).

The cleavage of *SSSI* gene detected some polymorphisms. The profiles of all samples after the cleavage with restriction endonucleases AciI, BamHI, EcoRI, PstI didn't shown any changes of restriction sites.

The highest level polymorphism was observed in a *SSSI* gene segment 5250-6854 bp.

Mutations can cause substitution of single nucleotides, insertion or deletion of DNA fragments of various or duplication or inversion of DNA fragments. 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 may lose their functionality completely, or even gain a novel function (FAO, 2007). Determination of changes responsible to increase of thousand weights may be used for MAS purposes in the future.

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