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CHEMICAL MUTAGENESIS IN HEAD CABBAGE (*Brassica oleracea var. capitata* L.). IV. DNA POLYMORPHISM

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ABSTRACT: ISSR method was applied for a detection of induced variability on a DNA level in M_1 generation after Ethylmethan Sulphonate (EMS) chemical treatment in concentrations 0.5, 0.6 and 0.7% of seeds from head cabbage, Brassica oleraceae L., var. Capitata, cv. Ditmarsko.

Different patterns of mutant plants obtained from the studied species were established by randomly selected primer sequences among tandem repeats. Polymorphisms were revealed among M_1 and control plants. Diverse DNA patterns of mutant plants were identified. As a result of the pre-breeding screening done in M_1, altered plants were selected and seeds for growing the next M_2 generation were gathered.

Key words: *head cabbage, Brassica oleraceae L., ISSR-PCR, induced mutagenesis, EMS, DNA polymorphism*

INTRODUCTION

Using of induced mutagenesis for creating of new genotypes with useful characters is a potentially valuable source for the plant breeding (Kahl et al., 2001; Lavi U., 2001). Cultivated cabbage problems boil down to restricted genetic basis (Joshi and Khalatkar, 1998). In last time new methods for selection of valuable mutants are applied in order to reduce the delay to develop new genetic resources (Charters et al., 1996; Chopra, 2005). However applied in *Brassica oleraceae* the treatments are ineffective. Most mutations appeared are recessive and altered characters could not be manifested. The variety is biannual and it takes more than two years to establish them. The assembling of recessive alleles is long and difficult process in species like *B. oleraceae* due to their pollination peculiarities (Daskalov, 1987). The detection of the variability in cabbage population after treatments with mutagens based on traditional screening procedures, at times might be not correct. Applying of induced mutagenesis and new DNA based methods for mutation detections both together could create valuable tools for plant breeding work (Nielen, 2001).

Therefore the present study was designed to evaluate the genetic potential variability of *B. oleraceae* cv. Ditmarsko individual plants after EMS treatment on DNA level and to introduce ISSR technique, inexpensive and suitable for routine screening application.

Material and methods

The experimental work was carried out in 2004-2005 in the Molecular Biology Laboratory of Maritza Vegetable Crops Research Institute on the wide-spread Bulgarian cv. Ditmarsko (*Brassica oleraceae* L.). Genetically pure seeds of the head cabbage variety with uniform size and shape were exposed to chemical mutagenesis. Seeds were soaked for 18 hrs with different concentrations of Ethylmethan Sulphonate (EMS) as follow: 100 seeds with 0.5% E S, 100 seeds with 0.6% E S, 100 seeds with 0.7% E S.All treated and 10 untreated seeds for control were planted and grown in 1.All the survived control plants (9) and 9 plants of M_1 generation after EMS treat-

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ments that showed morphological, physiological or cytological differences from the controls were selected for DNA analyses.

DNA was isolated from the 18 plant samples using 100 mg fresh or 33 mg lyophilized leaf material from individual plants by CTAB protocol (Murray et al., 1980), with additional modification for increasing it effectiveness. It was applied an additional purification of DNA to improve it quality. In some cases DNA was additionally isolated by PhytoPure Kit protocol purchased from LKB-Amersham Biotechnology. The DNA quantity was evaluated visually through Lambda DNA (Fermentas №SD0011) in different concentrations on Agarose gels - 0.6-0.7%.

Inter Simple Sequence Repeats - Polymerase Chain Reaction (ISSR-PCR) method was performed with different primers (Table 1). The primers were selected in NCBI gene bank on the basis of information for *Brassica spp*. complete gene sequences and MICAS web-based server. Bibliography data for tandem repeats of genome nucleotide sequence of related species were also used (Love et al., 2004). ISSR 3'-anchored and 5'- anchored and unanchored primers, 14-21-oligomers as priming sites, were used to amplify regions between two SSRs (Table 1). ISSR-PCR was performed through different programs for each primer justified empirically in the process of the analyses (Table 2) in an Applied Biosystems 2720 Thermal Cycler. Genomic DNA 25-30 ng was used as template for each ISSR reaction. For the PCR mix the chemicals were ordered from Amersham Pharmacia Biotech - Ready to Go PCR beads, containing stabilizers, BSA, dATP, dCTP, dGTP, dTTP. The concentration of each dNTP is 200 μM in 10 mM Tris HCl(pH 9.0), 50 mM KCl and 1.5 mM $MgCl_2$, ~ 2.5 U Pure*Taq* polymerase enzyme and reaction buffer, primer (5-10 pmol) in 15 ml reaction volume. The electrophoresis after PCR reactions were performed on 1,5% gels from Agarose SPI (purchased from Duchefa Biochemie).

Results and discussion

Conditions to perform each ISSR reaction were optimized till obtaining patterns with good resolution. Data on the table 2 summarize the best parameters for the steps of the amplification with the 7 primers (Table 1) used in different ISSR reactions.

Tab. 1. Sequences of applied primers for ISSR reaction

Primers	Abbreviations	Sequences			Primer length
		5'- anchor	Core sequence	3'-anchor	
ISSR1	i1		(CA)6	(AG) (CT)	16
ISSR3	i3		(AGC)4	(CT)	14
ISSR4	i4		(AGAC)6		24
ISSR5	i5	(AC)	(GACA)4		16
ISSR6	i6		(GACA)4	(AC)	18
ISSR8	i8	(CAG)	(CT)8		19
ISSR11	i11	(CGC) (ACA)	(AGA)5		21

Each one primer amplified successfully genomic DNA fragments of the treated cabbage population and generated different number of bands on the agarose gel. Five to eight bands showed a high resolution patterns and were interpreted. The fragments selected for analyses were reproductive in the repeated ISSR reactions and different DNA extractions from the same samples.

In result of the amplification reaction of the genomic DNA fragments some of the obtained patterns were monomorphic and resembled the controls. In each tested primers male-sterile mutants showed polymorphic electrophoretic phenotypes that differed from the controls and among them, too.

The control plants among them were monomorphic, except the case of the reaction with i1 primer (Table 1, Table 2). Patterns of the control plants (wild type), obtained after amplification with i1, differed among them and were marked as 1(sample 1, Figure 1) and 2(sample 2, Figure 1). Eight plants possessed p1 (8 fragments) which prevailed over p2 (6 fragments) - in one of the 9 plants. Two fragments, with length 350 and 500 bp, were polymorphic and presented on p1 and absent on p2. This fact proved the ability of the se-

Tab. 2. PCR programmes for ampliphication of DNA fragments

i. PCR programme for i1:

Initial denaturation		5 min	at	94°C
35 cycles of	Denaturation	1 min		94°C
	Annealing	2 min		45°C
	Extension	30 sec		72°C
Final extension		5 min		72°C
Sustaining		∞		6°C

ii. PCR programme for i3:

Initial denaturation		5 min	at	94°C
35 cycles of	Denaturation	30 sec		94°C
	Annealing	30 sec		46°C
	Extension	1 min 30 sec		72°C
Final extension		7 min		72°C
Sustaining		∞		6°C

iii. PCR programme for i4, i5:

Initial denaturation		5 min	at	94°C
35 cycles of	Denaturation	30 sec		94°C
	Annealing	30 sec		50°C
	Extension	1 min 30 sec		72°C
Final extension		7 min		72°C
Sustaining		∞		6°C

iv. PCR programme for i6:

Initial denaturation		5 min	at	94°C
35 cycles of	Denaturation	30 sec		94°C
	Annealing	30 sec		54°C
	Extension	1 min 30 sec		72°C
Final extension		7 min		72°C
Sustaining		∞		6°C

v. PCR programme for i8:

Initial denaturation		5 min	at	94°C
35 cycles of	Denaturation	30 sec		94°C
	Annealing	30 sec		45°C
	Extension	30 min		72°C
Final extension		1 min 30 sec		72°C
Sustaining		∞		4°C

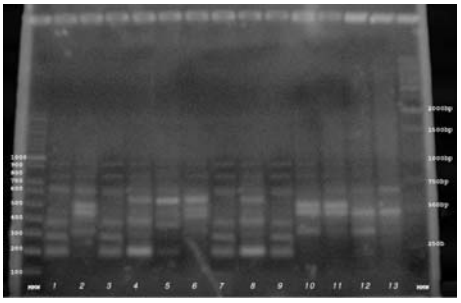
vi. PCR programme for i11:

Initial denaturation		5 min	at	94°C
35 cycles of	Denaturation	30 s		94°C
	Annealing	45 s		50°C
	Extension	2 min		72°C
Final extension		5 min		72°C
Sustaining		∞		6°C

lected method to distinguish genetically closely related forms. One of the male sterile mutants possessed p2 pattern (sample 6, Figure 1), the same like the pattern of one of the control plants. The revealed variability was not due to a mutation event. Each variety represents a population and it is composed from different lines. DNA polymorphism among lines (control plants) could be detected. In the presented study ISSR method with i1 primer was able to detect it.

DNA polymorphism among mutants was also observed. Sample 4 and 6, sample 5, samples 12 and 13 did not resemble to the wild type patterns (Figure 1).

Fig. 1. ISSR patterns (i1) of EMS treated population of head cabbage - control and mutant 1 plants



Two polymorphic DNA fragments among 6 amplified were detected using i3 primer (Table 1, Table 2). Bands with length 180 bp and 580 bp differed among male-sterile mutants.

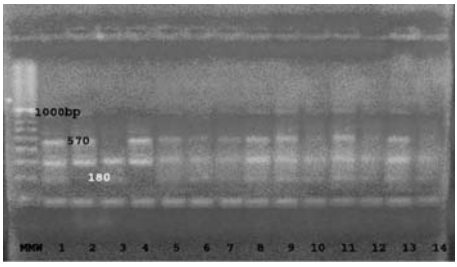
Polymorphic DNA patterns were detected using i4, i5 and i6 primer for the amplification (Table 1, Table 2). The three primers were sequenced to be unanchored (i4), 5'- anchored (i5) and 3'-anchored (i6) (Table1). The three different reactions produced the same electrophoretic patterns with 7 and 8 fragments. In the ISSR patterns one of the fragments (460 bp, 600 bp, 700 bp) of each male sterile cabbage samples was not presented on the gels.

Five reproducible bands were revealed using i8 in the ISSR reaction (Table 1, Table 2). In one of the male sterile mutants (sample 3), a fragment with length 570 bp was not produced (Figure 2).

ISSRs were also polymorphic when amplification was performed using primer i11 (Table 1, Table 2). Male sterile plants differed from the 9 controls. Pattern variability was detected in band with length 400 bp.

The suggestion for mutations occurred using ISSRs was proved in other plant species, such as wheat (Bennetzen et al., 2001), pepper (Daskalov, 1986; Tomlekova, unpublished data). Albani et al. (1998) confirmed the genetic changes occurred in S. tuberosum regenerants in result of somaclonal variation in a potato variety.

Fig. 2. ISSR patterns (i8) of EMS treated population of head cabbage - control and mutant 1 plants



Different primers annealed to different loci of the cabbage genome and amplified diverse fragments. Male sterile mutants were produced with all the three concentrations - 0.5%, 0.6% and 0.7% EMS. In all reactions polymorphisms among male sterile mutants and wild type plants were presented. Bands with different length were not been amplified in mutants. Mutations caused severe genome changes related to losses of ISSR loci. According FAO/IAEA data in most of the cases, treatments with EMS produce 50 bp deletions. When these alterations affect coding regions controlling important functions of the plant they could have a lethal effect. In fact, all the male sterile mutants, except one, included in this study did not survive and dead in a later stage of plant development. This fact demonstrates that the routine and cheap techniques like ISSR for DNA analysis could be explored to study the chemo-sensibility of the genotypes in the chemical mutagenesis. They also could be explored to make prognosis for the plant vitality and safe time and all costs necessary to grow plants which do not carry a new potential. Lethality due to the applied doses of the EMS mutagen could be suggested in early stage of plant development with ISSR method.

For a criterion of the chemo-sensibility are widely used the plant growth reducing, the survived plants, sterility of M_1 plants, chromosomal aberrations in the first mitosis. On the

basis of the obtained results in this study we could suggest the DNA based markers to be used for the same purpose because of their reliability and infinitive nature.

Conclusions

The sequenced primers selected in the NCBI gene database were applied for amplification reactions in cabbage successfully. The DNA patterns were reproducible.

The application of the method to study mutant populations in M_1 was approved.

Polymorphism among mutants and wild type plants was established.

In some cases ISSRs (reaction with primer i1) were able to distinguish variability in individual plants of the same variety.

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SUMMARY

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The application of the method to study mutant populations in M_1 was approved.

Polymorphism among mutants and wild type plants was established (Figure 1, Figure 2).

In some cases ISSRs (reaction with primer i1) were able to distinguish variability in individual plants of the same variety (Figure 1).