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Molecular Analysis of Stable Mutagenesis in Cucumber Induced by High Hydrostatic Pressure*

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Abstract: Two mutant cucumber lines are obtained from the Tianjin cucumber cultivar S9925-3 after treatment with high hydrostatic pressure. DNA fingerprint analyses are performed for the two mutants and their parental wild-type cucumber cultivars by AFLP (Amplified Fragments Length Polymorphism) analysis. A total of 30 randomly selected primer pairs are used for AFLP. Ten specific different bands between the mutants and the wild-type cucumber cultivars are excised from the polyacrylamide gel for DNA sequencing. Alignment of the obtained DNA sequences with NCBI (National Center for Biotechnology Information) database identifies the mutation sites in cucumber genome, which may be the reason of phenotypic changes of the mutant cucumbers. This is the first time that a study has showed at molecular level that treatment with high hydrostatic pressure results in stable mutations in cucumber cultivar. Our results indicate that high hydrostatic pressure-induced mutational breeding can be used as an effective approach for the crop improvement.

Key words: high hydrostatic pressure; DNA fingerprint; cucumber cultivar; amplified fragments length polymorphism (AFLP)

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1 Introduction

Plant mutagenesis was commonly used in crop breeding in order to obtain superior varieties in recent years. Development of high pressure bioscience allows people to use hydrostatic pressurization as a novel approach to breed new plant varieties. Similar to radiation, hydrostatic pressurization is a thermal-physical stress that affects various biological processes including chromatin structure, enzymatic activity, gene expression[1-2], etc. The inheritable mutants of rice induced by hydrostatic pressure were obtained and a distinct DNA polymorphism was observed in the mutant rice by RAPD (Random Am-

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plified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat) analysis[3]. This was the first attempt that people applied hydrostatic pressurization to mutagenize a plant species for the purpose of breeding. However, it is unclear with regard to the underlying molecular mechanisms of inheritable variations induced by high pressure.

Amplified fragments length polymorphism (AFLP) is a highly informative tool for detecting DNA polymorphisms. It is based on the selective PCR amplification of restriction fragments from the total digested genomic DNA and production of a large number of high quality markers without the need for prior sequence knowledge. Previous studies have shown that AFLP analysis has a greater discriminatory power than RAPD and other genomic fingerprinting methods. Thus, AFLP has become a reliable method for distinguishing small genomic difference[4].

Cucumber (*Cucumis sativus L.*) is one of the main vegetable crops widely grown in China, and much attention has been paid to its breeding. In this paper, the hydrostatic pressurization technique was used to induce the mutation of Tianjin cucumber cultivar s9925-3, and two cucumber strains with apparent morphological changes were obtained. AFLP coupled with DNA sequencing was applied to identify the genomic difference between two mutants and their original wild-type cucumber cultivars. To our knowledge, this is the first time to show that high hydrostatic pressure mutagenesis could result in stable genomic mutations in cucumber cultivar at molecular level. The results obtained in this paper provide possible molecular mechanisms for causing morphological variation after mutagenesis induced by treatment with hydrostatic pressure. These results also provide a theoretical foundation for plant breeding with hydrostatic pressurization.

2 Materials and Methods

2.1 Plant Material

Seeds of the cucumber cultivar (*Cucumis sativus*) s9925-3 cultivated by Tianjin Cucumber Research Institute were used in this research. The wild-type cucumber cultivar is three meters high with dark-green and palm-shaped leaves. The seeds of wild-type cucumber were introduced into a latex tube with appropriate amount of water and then put in the LECO reactor vessels for 4 h at 25 ℃ with a pressure range of 80 to 180 MPa. The high-pressure treated seeds were planted by Jilin Academy of Vegetables and Flowers (Jilin, China) to observe and select the mutant. Plants with the first expanded leaves were selected as experimental materials. At least three different plants were used for each of the following experiment.

2.2 Hydrostatic Pressurization

Experiments with different pressures were conducted by using Leco-HR-1B-2 High Pressure-High Temperature Hydrothermal Research System (USA), which is equipped for operation with 60 000 psi (400 MPa) pressure and 1 000 ℃.

2.3 DNA Extraction

Genomic DNA was isolated from the expanded leaves of wild-type (s9925-3) and the two mutant lines of cucumber by CTAB procedure with slight modifications[5]. DNA was subsequently purified by phenol extractions.

2.4 Maintenance of Plant Material

The two mutants used in this paper had been maintained along with their exact parental lines by strict selfing for two generations to develop into mutant lines. These two stabilized mutant lines were further planted for one more generation in Academy of Vegetable and Flower Sciences of Jilin Province, which still maintained the same morphological changes.

2.5 AFLP Analysis

DNA (250 ng) was digested with restriction enzymes EcoRI/MseI (NEB, Ipswich, MA) for 2 h at 37 ℃ and then ligated to the double-stranded EcoRI/MseI adapters as described in the AFLP Manual[6]. After ligation, the pre-amplification reactions containing the diluted DNA were carried out for 20 cycles (94 ℃ for 30 s, 56 ℃ for 60 s, and 72 ℃ for 60 s). The product of the pre-amplification was diluted for 50 times for selective amplification including 14 touchdown cycles with a reduction of annealing temperature of 0.7 ℃ for each cycle and 26 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s and 72 ℃ for 60 s. The AFLP products were denatured for 2—5 min at 95 ℃ and then separated on 6% (w/v) polyacrylamide gels (1 300 V, 55 ℃) in 1× TBE buffer (0.09 mol/L Tris-borate, 2 mmol/L EDTA) using a Model S2001 gel electrophoresis system. After electrophoresis, the gel was silver stained and dried as described previously[7].

2.6 Isolation of the Polymorphic AFLP Fragments

DNA bands of interest in the silver-stained AFLP gels were excised, re-amplified with a given combination of primers and then cloned into TA cloning vector. The cloned DNA fragments were sequenced. The obtained DNA sequences were analyzed by using the advanced BLASTN program at the NCBI (National Center for Biotechnology Information) website. Clustal X 1.83 was used for alignment analysis and editing.

3 Results

3.1 Morphological Changes

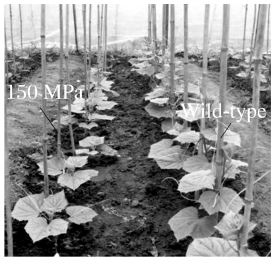
Two mutant lines of cucumber with visible phenotypic variations at the first generation were selected among the survived plants. The edge of leaves in the mutant line 1 (No. 184), which was treated with a pressure of 130 MPa, turned into an obvious lighter shade of color, as shown in Fig. 1(a) and Fig. 1(b). Mutant line 2 (No. 197) treated with the maximum pressure (150 MPa) showed a significantly shorter plant height ((162.33±63.01)cm) than the wild-type plant ((211.56±31.32)cm, $P<0.01$) measured at end of eight weeks after field planting, as shown in Fig. 1(c) and Fig. 1(d). The original wild-type cultivar and two mutant lines were further planted for one more generation in Academy of Vegetable and Flower Sciences of Jilin Province.



(a) Mutant line 1 (strain No.184)



(b) Original wild-type of strain No.184



(c) Strain No.197 at end of one week



(d) Strain No.197 at end of eight weeks

Fig. 1　Morphological changes of two genetically stable mutants of cucumber cultivars

3.2 Different AFLP Bands

A total of 30 combinations of primers were used for selective amplification, and 10 of these combinations generated polymorphic DNA bands. These 10 combinations of primers and the numbers of the resulting polymorphic DNA bands were listed in Table 1. Amplification with the 10 selected AFLP primer combinations resulted in a total of 609 AFLP fragments, of which 36 (5.91%) fragments were differentially displayed between the wild-type and mutant lines of cucumber.

Table 1 Numbers of differentially displayed AFLP bands in two mutants comparing with wild-type lines of cucumber

Primers	Mutant 1	Mutant 2	Primers	Mutant 1	Mutant 2
M-CAA/E-AAC	1	6	M-CTC/E-AAC	1	1
M-CAA/E-AAG	2	3	M-CTC/E-ACG	2	5
M-CAA/E-ACA	0	1	M-CTG/E-AAC	1	0
M-CAA/E-ACC	0	1	M-CTG/E-AAG	0	1
M-CAG/E-AAG	1	1	M-CTT/E-AAG	3	6

Note: Primer sequences correspond to M-5'-GACGATGAGTCCTGAGTAA-3' and E-5'-CTCGTAGACTGCGTACCAATTC-3'.

3.3 Sequencing and BLAST Analysis for the Polymorphic AFLP Fragments

Six of the 36 AFLP polymorphic DNA fragments were sequenced and analyzed by BLASTN in NCBI Genebank (Fig. 2). BLAST analysis show that the DNA sequences of only one polymorphic band bare significant similarities to a gene with known functions, NADH dehydrogenase. Clustal X sequence alignment analysis show that the obtained DNA sequences are homologous to the *nad*7 gene, encoding subunit 7 of NADH dehydrogenase. DNA sequence of another AFLP band has low similarity with Populus EST sequences from severe drought-stressed leaves. As seen in Table 2, DNA sequences

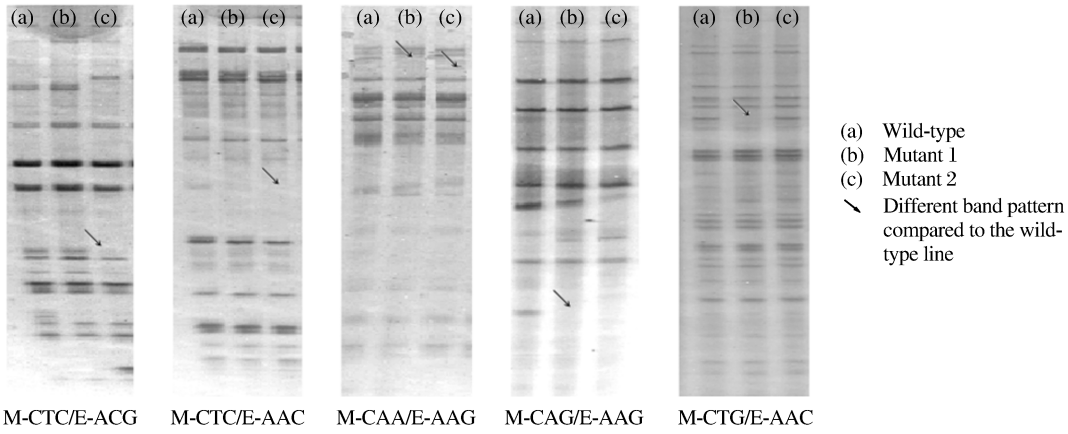


Fig. 2 Molecular analysis of two genetically stable mutants and wild-type lines of cucumber cultivars

Table 2 BLAST analysis for the sequences obtained from the differentially displayed AFLP fragments between two mutants and wild-type cucumber lines

Band No.	NCBI Genbank Acc. No.	Similar protein	*E* value	Max identities/(%)
2	FJ007643.1	Cucumis sativus NADH dehydrogenase subunit 7 gene	10^{-9}	100
3		No match		
5		No match		
7	CU228719.1	Populus EST from severe drought-stressed leaves	2.4	92
B		No match		
C		No match		

of the remaining four bands do not exhibit significant matches to any known sequences in the database. These sequences may present uncharacterized genes.

4 Discussion

Similar to temperature, pressure is another key physical parameter, which has been shown to induce multiple cellular activities in eukaryotic cells. Studies have demonstrated that hydrostatic pressurization plays an important role in the formation of advanced protein structure and interaction between protein and DNA. Shen and coworkers[3] demonstrate that the DNA molecules are changed in the genome of rice when conducted under high hydrostatic pressure by RAPD and ISSR analysis. However, other studies[8] show that no variation is observed in the genome after the plant is treated with high pressure. Our AFLP analysis presents the changes in the whole genome of the pressurized cucumber cultivar. The DNA loci changed at the genome level are relatively low because only 36 variant DNA bands are observed among a total of 609 bands. Generally, double-stranded DNA molecules are quite stable compared to the proteins under high pressures. Radiation treatment can induce various types of DNA damages and produce gene mutations. In contrast, hydrostatic pressurization rarely acts directly on DNA, but protein is particularly vulnerable to pressure. High pressure has been shown to aggregate proteins and induce denaturation of monomeric proteins in *Escherichia coli* and *Listeria monocytogenes*[9], which diminishes enzyme activity. Changes in intracellular enzymes activity, especially repairs of antioxidant enzymes can increase the genomic mutation rate[10]. Recent studies have documented that the DNA methylation state alters frequently in response to various stresses, which may explain why high hydrostatic pressurization causes phenotypic changes in the plants. Either through methylation or activity changes of intracellular enzymes, the mutation alleles induced by high hydrostatic pressurization occur randomly in the whole genome. Our data show that different novel phenotypes are gotten in the first generation after high hydrostatic pressure treated and no mutation hotspots in the genome are found.

In our study, we sequence 6 DNA fragments that are polymorphic between two mutants and wild-type cucumber lines. BLAST analysis shows that one of the sequences is homologous to the gene encoding NADH dehydrogenase subunit 7 (*nad*7). NADH dehydrogenase, the first enzyme of the mitochondrial electron transport chain, plays an important role in the energy metabolism by transferring a high energy electron to ubiquinone. It has been demonstrated that the mutation in the mitochondrial gene, *nad*7, results in the absence of functional complex Ⅰ, leading to partial male sterility and slower shoot growth[11]. This might be one reason for the shorter plant height of the mutant line 2. For the bands isolated from the mutant with fade leaves (Fig. 1), the sequences that match the databases are not identified. Large scale screen by AFLP and cDNA-AFLP may provide more clues on the changes of gene expression induced by high pressure.

5 Conclusions

Although the molecular mechanism of mutagenesis induced by pressure is still largely unclear, our experimental data have demonstrated unequivocally that the DNA mutations in cucumber arise as a result of high pressure treatment. Through sequencing and BLAST analysis for the polymorphic AFLP fragments, it is confirmed that the morphological variations of the cucumber strain can be caused by DNA sequences. Moreover, high hydrostatic pressure-induced mutation breeding can be used as an effective approach for crop improvement.

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高静水压诱导黄瓜稳定遗传变异系的分子分析

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摘要: 采用高静水压法诱变了黄瓜品种 S9925-3, 产生了两个稳定的可遗传变异株。采用 30 对随机配对引物, 对该品种的原种及两个变异株进行了扩增片段长度多态性(AFLP)分析。结果表明原种与两个变异植株之间存在明显的多态性。从聚丙烯酰胺凝胶上切下 6 个差异明显的条带进行 DNA 测序, 并将获得的 DNA 序列与 NCBI(National Center for Biotechnology Information)数据库中的黄瓜 DNA 数据进行比对分析, 确定了变异位点, 找到了变异黄瓜系表型变化的可能原因。首次完成了对高静水压处理引起的黄瓜稳定遗传变异系的分子水平分析。研究结果表明, 高静水压技术是一种有效的改良作物品系的新诱变工具。

关键词: 高静水压; DNA 指纹图谱; 黄瓜品系; 扩增片段长度多态性

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