

Received 9 December 2002
Accepted 14 April 2003

Short Communication

AFLP MARKER POLYMORPHISM IN CUCUMBER (*Cucumis sativus* L.) NEAR ISOGENIC LINES DIFFERING IN SEX EXPRESSION

JUSTYNA WITKOWICZ, EWA URBAŃCZYK-WOCHNIAK
and ZBIGNIEW PRZYBECKI

Department of Plant Genetics Breeding and Biotechnology, Warsaw
Agricultural University, Nowoursynowska 166, 02-787 Warsaw, Poland

Abstract: The AFLP technique was used to evaluate the level of polymorphism between two pairs of isogenic cucumber (*Cucumis sativus* L.) lines (NIL) differing in flower sex expression. The BSA techniques were also applied to find molecular markers linked to sex determination genes (dominant alleles) in those cucumber lines. Sex determination in cucumber is controlled by three main bands: *F*, *M* and *Gy*. The interaction of these bands is responsible for the formation of the various flower phenotypes with respect to sex in the analyzed lines: a female line 2gg with a ff/MM/gygy genotype, isogenic to a monoecious line B10 (genotype ff/MM/GyGy), and a female line Gy3 with a FF/MM/GyGy genotype, isogenic to a hermaphroditic line HGy3 (genotype FF/mm/GyGy). Using 56 combinations of AFLP primers, used for the analysis of lines 2gg and B10, gave 3794 bands, of which 155 (4.1%) were polymorphic. Ten bands distinguished gynoeious and monoecious bulks appearing at the same time in the appropriate parent; they are believed to be linked to the *Gy* locus. The isogenic lines Gy3 and HGy3 showed a higher level of polymorphism (14.2%). In this case, 55 combinations of primers gave 2996 reaction products, of which 430 showed variation. Twenty bands occurred in one bulk and in one parent, so they are probably associated with the *M* locus. Using the AFLP technique, the isogenicity of the lines was evaluated. The level of polymorphism (per pair of primer) between lines 2gg and B10 is 0.072% and is four times lower than that between the Gy3 and HGy3 lines (0.27%). The differences in the isogenicity of the lines can result from the degree of their relatedness, which may reflect the way they were derived.

Key Words: AFLP, NIL, BSA, Sex Gene Markers, Polymorphism, Cucumber

Abbreviations used: AFLP - amplified fragment length polymorphism; NIL - near isogenic lines; BSA - bulked segregant analysis.

INTRODUCTION

AFLP (amplified fragment length polymorphism) is an effective method for the analysis of genomic DNA. The specificity of the technique allows the identification of genotypes, the construction of a high saturation genetic map and gene cloning [11, 13]. Amplified fragment length polymorphism analysis is a PCR-based technique allowing the detection of more than 50 independent bands in a single PCR reaction. These markers can also reveal polymorphisms between unrelated and related genotypes (mutants, NIL, RIL, DH). The advantage of the system is also its high reproducibility. In this study, this method was used to detect polymorphism between two pairs of isogenic cucumber lines: female 2gg and monoecious B10; and female Gy3 and hermaphroditic HGy3. The above-mentioned lines are characterized by a differentiated expression of flower sex, as they differ in the alleles responsible for this trait; i.e. *M* and *Gy*. The presence of a recessive allele is responsible for the recessively-caused femaleness of flowers from the 2gg line in the absence of a dominant allele in *locus F*. On the other hand, the recessive allele of the *M* gene increases the maleness of flowers [5]. The main aim of the presented work was to find the polymorphic AFLP products in the above-mentioned lines, and to detect AFLP products linked to sex in both NIL pairs.

MATERIALS AND METHODS

The plant material consisted of two pairs of isogenic cucumber lines characterized by a differentiated expression of flower sex. The female line 2gg (ff/MM/gygy) is isogenic to the monoecious line B10 (ff/MM/GyGy), and the female line Gy3 (FF/MM/GyGy) is isogenic to the hermaphroditic line HGy3 (FF/mm/GyGy). The plants were cultivated in a plastic tunnel under long day conditions – an external factor responsible for the proper expression of flower sex.

Genomic DNA was isolated from young leaves using the standard protocol of Scott [9] permitting the removal of polysaccharides from cucumber DNA. AFLP analysis was performed using a commercially available kit from LifeTechnologies, the AFLP Analysis System Kit I. Restriction, adaptor ligation, preamplification and selective amplification were performed according to the manufacturer's protocol with minor modifications. These modifications were connected with an about five-fold reduction of reaction amounts. 250ng of total genomic DNA was completely digested by *EcoRI* and *MseI* restriction enzymes. Next, *EcoRI* and *MseI* adaptors were ligated to the restriction fragments and primers with one additional nucleotide were used for the first amplification, named pre-amplification. Primers with three selective nucleotides were utilized for the last step, named selective amplification. The products of selective amplification were detected using a radiolabelling method. 50.04ng *EcoRI* primers for the last PCR reaction were labelled with 1ul of γ -³³P-ATP (ICN) using 0.2 U of T4 polynucleotide kinase for 2 hours at 37°C. 10ul of stop

solution (95% deionized formamide, 0.02M EDTA, pH 8.0, 1% bromophenol blue xylene cyanol dye solution, 10mM NaOH) were added to the mix after the reaction. Selective amplification products were loaded on 6% polyacrylamide denaturing sequencing gels. Electrophoresis was run at 55 W constant power, transferred to 3mm Whatman paper, dried and exposed to BIO-RAD intensifying screen for 10-12 hours. Semi-automated AFLP fragment scanning was performed with Quantitation Software, Quantity One[®].

To identify the molecular markers linked with the *Gy* or *M* gene, two DNA pools were formed for the 2gg and B10 F₂ segregants. Three DNA pools were constructed for the Gy3 and HGy3 F₂ progeny. Each pool consisted of 20 F₂ plants, displaying either monoecious or gynoeious sex expression in the first case, and monoecious, gynoeious and hermaphroditic in the second pair. The bulks were made by pooling equal amounts of 20 isolated DNAs (10ng) obtained from 2gg and B10 progeny and Gy3 and HGy3 progeny.

In order to evaluate polymorphisms between the above-mentioned pairs of isogenic cucumber lines, 64 combinations of AFLP starters were used (8 EcoRI primers and 8 MseI primers). The primers used are part of a standard AFLP kit (Life Technologies) and contain three selective nucleotides. Polymorphism between the lines was determined on the basis of the presence or absence of an amplification product of a given *locus*, while the variation was expressed as a percentage. Polymorphism per primer pair was estimated, as we had used a different number of AFLP primer combinations for each NIL pair.

RESULTS

AFLP polymorphism

In order to detect the level of variation between isogenic cucumber lines 2gg and B10, and Gy3 and HGy3 56, 55 combinations of AFLP primers were used (in each case). In the case of lines 2gg and B10, a total of 3794 amplification products were obtained, of which 155, i.e. 4.1%, were defined as variable. Each pair of primers allowed 67 bands to be obtained on average. The used pairs of starters for lines Gy3 and HGy3 allowed 2996 amplification products to be obtained, of which 430, i.e. 14.2%, were polymorphic. On average, 54 bands were obtained (per primer combination). The results of the polymorphism analysis are presented in Tab. 1. We used a different number of AFLP primer combinations for each NIL pair analysis. For comparing detected polymorphism and estimating the level of similarity (isogenicity), we found a common ratio, polymorphism per primer pair, as a relationship between the percentage of polymorphism in each NIL and the number of primer pairs used. This data allowed us to estimate that the similarity between lines 2gg and B10 is 0.072%, and that this is four times higher than that between Gy3 and HGy3 (0.27%) (Tab. 1).

Identification of AFLP bands linked to the *Gy* or *M* locus

We used applied BSA techniques to find the AFLP bands linked to the *Gy* or *M* locus.

Tab. 1. AFLP polymorphism of near-isogenic cucumber (*Cucumis sativus* L.) lines

Near-isogenic cucumber lines	Number of primer combinations	Number of analyzed bands	Average number of bands per starter pair	Number of variable bands	Variable products present in 2gg/Gy3	Variable products present in B10/HGy3	Percentage of polymorphic bands	Polymorphism per primer pair [%]	Polymorphic product per primer combination
2gg, B10	56	3794	67	155	67	88	4.085	0.072	2.7
Gy3, HGy3	55	2996	54	430	217	212	14.18	0.27	7.8

Gy locus markers

Among the 56 EcoRI and MseI primer combinations, eleven pairs produced AFLP bands, present in the suitable parent and in bulk, simultaneously in the 2gg and B10 isogenic pair. Ten bands were found to be associated with monoecious (determined by the dominant allele – Gy) sex expression. The bands corresponded with the monoecious pool E-AAC/M-CAC₁₂₀, E-ACA/M-CTA₁₈₀, E-ACA/M-CTT₁₉₀, E-ACT/M-CTT₁₂₀, E-AGG/M-CAG₇₀, E-AGG/M-CAG₁₄₀, E-ACG/M-CAG₄₀₀, E-ACG/M-CAT₂₈₀, E-ACG/M-CTT₁₆₀, E-ACG/M-CTT₁₉₀

M locus markers

Bands which distinguished gynoeious or hermaphroditic bulks appearing at the same time in the appropriate parent were obtained using 55 EcoRI and MseI primer combinations. Twenty of them were associated with a pool carrying the M allele allowing female sex expression in Gy3 lines: E-AAC/E-CAA₇₀, E-AAC/M-CAT₈₀, E-AAC/M-CTA₁₀₀, E-AAG/M-CAC₈₀, E-AAG/M-CAC₁₂₀, E-AAG/M-CAT₈₀, E-ACA/M-CTA₈₀, E-ACA/M-CTA₉₀, E-ACC/M-CAG₈₀, E-ACC/M-CAT₉₀, E-ACC/M-CTA₂₀₀, E-ACC/M-CTC₇₅, E-ACG/M-CTG₈₀, E-ACT/M-CAG₄₀, E-ACT/M-CAG₆₀, E-ACT/M-CAT₅₀, E-AGC/M-CAG₈₅, E-AGC/M-CAT₇₅, E-AGC/M-CAT₇₅, E-AGG/M-CTA₈₀.

DISCUSSION

Cucumber sex determination is controlled by two main genes, namely m and F. The expression of these genes is modified by other genes and environmental factors. Gene F induces a higher degree of femaleness and controls male flower formation by suppression. Plants with FF and Ff only produce female flowers, while ff plants are monoecious [3]. Gene F, which is strongly modified by the environmental and genetic background, is from a Japanese variety. The recessive allele m of the M gene controls andromonoecism and comes from the Lemon variety [5]. The observed strong interactions between F and m allow for the production of four basic sexual types: monoecious (MMff), andromonoecious

(mmff), female (FFMM) and hermaphroditic (mmFF) [3]. The analyzed Gy3 gynoeceious lines contain two dominant *F* alleles and two dominant *M* alleles. The HGy3 hermaphroditic line is dominant homozygous for *F* and recessive homozygous for *m*. The HGy3 line's origin is not well known; it was obtained by Kubicki using cross-fertilization, and it is claimed to be isogenic to Gy3. Gynoeceious 2gg lines contain another *gy* allele of the *Gy* gene. This recessive allele is for high-degree female sex expression [4] Gene *gy* controls recessive femaleness in cucumber and induces mutation in the Borszczagowski variety [3], so this line is near-isogenic line to Borszczagowski. Line 2gg was obtained through chemical mutagenesis (using ethyleneimine) of the inbred Borszczagowski line at the very beginning of seventies [3].

Both the 2gg and HGy3 lines obtained in our department by Prof. Kubicki, as well as two other lines, have been self-pollinated over the last 30 years (oral information from breeders).

The level of variation detected using the AFLP techniques depends on the combination of restriction enzymes selected for the first stage, on the number of combinations of primers used, and on the genetic distance between the analyzed genotypes. The AFLP technique permits the detection of the variation of many loci simultaneously, allowing the analysis of over 50 independent amplification products in a single reaction. AFLP markers are widely used for the evaluation of genetic variation between forms with a differentiated degree of relatedness, especially between genotypes with a small genetic distance, such as between a variety and the mutants derived from it, between recombinant lines and between isogenic lines [1, 11].

The detection of a polymorphism between a variety and a mutant derived from it requires the use of a sensitive marker system, due to the fact that an unusually low frequency of mutations occurs when agents and methods of mutation are used which do not lower plant viability and induce the desired changes in the genetic material. In order to detect variation between a mutant variety of grape (*Vitis vinifera*), Flame Seedless, and the initial variety, 64 combinations of AFLP primers were used. Each pair permits about 100 reaction products to be obtained. The mutants differed from the initial forms in their developmental characteristics. Among the 6400 obtained reaction products, 0.03% were polymorphic and (at the same time) unique fragments differentiating the mutant and its parental variety [11]. In order to distinguish AFLP markers linked with a trait using a generation of inbred lines, a much higher number of AFLP bands must be analyzed [9].

The use of the AFLP technique for the detection of differentiation between isogenic lines allows the evaluation of the degree of isogenicity of lines to be expressed as the level of polymorphism. The level of polymorphism calculated per pair of primers for lines 2gg and B10 was 0.072%, and for lines Gy3 and HGy3 was 0.27%. For comparison, the polymorphism defined for tomato lines differing in the gene responsible for carotene content was 0.0013% [15] (our own calculations on basis of data from the literature). However, for closely

isogenic barley lines differing in the alleles at the *Mla* locus, the polymorphism calculated for one primer pair was 0.00011% [9] (our own calculations). As can be seen the isogenicity of the pairs of lines, expressed as the degree of polymorphism of AFLP markers, is very variable and may even differ by several orders of magnitude. Of course, the higher the value of polymorphism, the lower the isogenicity of the lines. Differences in the isogenicity of the lines may, in the case of cucumber, be due to the degree of their relatedness, which may reflect the way they were derived. A four-fold lower polymorphism was observed for line 2gg and B10 than for line Gy3 and HGy3. This seems to confirm the assumption that these differences are probably due to the way the isogenic lines were derived.

The AFLP and BSA techniques used with NIL are good techniques for finding molecular markers closely linked to the desired trait. The same tools were used for finding the AFLP markers linked to the M_{OB} locus, influencing the B gene, in lycopersicon. The B locus increases fruit b-carotene content at the expense of lycopene [16]. 64 AFLP primer combinations were used for the analysis, and two AFLP products were identified, co-segregated with the M_{OB} locus in NIL differing in the B locus.

Existing maps of cucumber establishing linkages between molecular markers and important morphological traits include only the *F* sex gene [2, 6, 12]. Genes *m* and *Gy* have been placed on the map published by Pierce and Wehrner [8]. The *Gy* gene was mapped by Kubicki [3] in the first linkage group, with a 4 % cross-over to the *F* gene; *m* belongs to the second linkage group. So far, no molecular markers linked to them are known. Using the above segregating population size (bulks), 30 markers were found which appear to be fully linked to the dominant alleles of the analyzed genes (no traces of bands in the recessive bulks). Markers linked with recessive alleles were not taken into consideration because of the impossibility of isolating heterozygous bulks. Virtual map distances between the markers and genes were not evaluated. This is the subject of further investigation using much larger segregating populations.

Acknowledgements. This work was supported by grant No. PBZ/KBN/029/P06/2000 from the Polish State Committee For Scientific Research.

REFERENCES

1. Agrama, H.A., Houssin, S.F. and Track, M.A. Cloning of AFLP markers linked to resistance to *Peronosclerospora sorghi* in maize. **Mol. Gen. Genet.** 267 (2002) 814-819.
2. Bardeen, J.M., Staub, J.E., Wye, C., Antonise, R. and Peleman, J. Towards an expanded and integrated linkage map of cucumber (*Cucumis sativus* L.) **Genome** 44 (2001) 111-119.

3. Kubicki, B. New sex types in cucumber and their uses in breeding work. In: **XIXth International Horticultural Congress**. Warszawa 11-18, September, 1974, 475-485.
4. Kubicki, B. New possibilities of applying different sex types in cucumber breeding. **Genetica Polon.** 6 (1965) 241-250.
5. Malepszy, S. and Niemirowicz-Szczytt, K. Sex determination in cucumber (*Cucumis sativus*) as a model system for molecular biology. **Plant Sci.** 80 (1991) 39-47.
6. Micheltore, R.W., Paran, I. and Kesseli, R.V. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. **Proc. Natl. Acad. Sci. USA** 88 (1991) 9828-9832.
7. Park, Y.H., Sensoy, S., Wye, C., Antonise, R., Peleman, J. and Havey, M.J. A genetic map of cucumber composed of RAPDs, RFLPs, AFLPs, and loci conditioning resistance to papaya ringspot and zucchini yellow mosaic viruses. **Genome** 43 (2000) 1003-1010.
8. Pierce, L.K. and Wehner, T.C. Review of genes and linkage groups in cucumber. **HortScience** 25 (1990) 605-615.
9. Schwarz, G., Michalek, W., Mohler, V., Wenzel, G. and Jahoor, A. Chromosome landing at the *Mla* locus in barley (*Hordeum vulgare* L.) by means of high-resolution mapping with AFLP markers. **Theor. Appl. Genet.** 98 (1999) 521-530.
10. Scott, D.M., Manorama, C.J and Richard, M.A. Removal of polysaccharides from plant DNA by ethanol precipitation. **BioTechniques** 17 (1994) 274-276.
11. Scott, K.D., Ablett, E.M., Lee, L.S. and Henry, R.J. AFLP markers distinguishing an early mutant of Flame Seedless grape. **Euphytica** 113 (2000) 245-249.
12. Staub, J.E., Serquen, F.C., and Gupta, M. Genetic markers, map construction and their application in plant breeding. **HortScience** 31 (1996a) 729-741.
13. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. AFLP: A new technique for DNA fingerprinting. **Nucl. Acid Res.** 23 (1995) 4407-4414.
14. Wehner, T.C. Gene list for cucumber. **Cucurbit Genetics Cooperative Report** 20 (1997) 66-88.
15. Zabeau, M. and Vos, P. Selective restriction fragment amplification: A general method for DNA fingerprinting. **European Patent Application** 92402629,7 (publication No. 0 534 858 A1). (1993).
16. Zhang, Y. and Stommel, J.R. RAPD and AFLP tagging and mapping of Beta (B) and Beta modifier (*Mo_B*), two genes which influence β -carotene accumulation in fruit of tomato (*Lycopersicon esculentum* Mill.). **Theor. Appl. Genet.** 100 (2000) 368-375.