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GENETIC VARIABILITY OF ANTHER DONOR VERSUS SPONTANEOUS DOUBLED HAPLOID DESCENDENTS AND COLCHICINE INDUCED DOUBLED HAPLOID SWEET PEPPER (*Capsicum annuum* L.) LINES

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Abstract

Haploid (n) and doubled haploid (DH) plants were developed in anther culture of sweet pepper (*Capsicum annuum* L.). Regenerants were analyzed by flow cytometry for haploid (n = 12) and spontaneous doubled haploid (2n = 24) genomes. Haploid plants were forwarded to colchicine-treatment for induced doubled haploid (2n•) plant production. Molecular polymorphism of anther donor plants (2n), the haploid regenerants (n), the spontaneous (2n) and induced (2n•)-DH plants were analysed by RAPD-, SSR- and ISSR-PCR. The analysis of anther-donor plants compared to DH-descendents showed an unexpectedly wide range of molecular polymorphism. Our results suggest that genetic changes occurring during meiotic recombination is higher than those of occurring during colchicine-induced genomic duplication.

1.Introduction

In general, cultures of anther, pollen, ovary, and ovule produce haploid (n) regenerants as fixed gametoclonal variants resulted from meiosis. The two processes of meiotic recombination, the crossing over between homologous pairs of maternal and paternal chromosomes, and the random assortment of chromosomes generate genetically different haploid gametes. In the case of pepper (2n = 24) the number of possible gametes as a result of random assortment of chromosomes is equal to $2^n = 2^{12} = 4.096$. This number is higher considering the numbers of crossing over occurring during the first meiotic cell division. By the application of anther culture, as in the present study, male gametoclonal pepper variants could be selected with new genomic constitutions (Dumas de Vaulx *et al.,* 1981, Mitykó *et al*., 1995, Fári, 1986, Gémesné *et al*., 1998, Gyulai *et al*., 2000). Anther-culture-derived DH-lines are the most advanced inbreds in pepper breeding for hybrid development, new cultivar release with new fruit shape, size, colour etc., and for resistance especially to viruses such as *tobacco mosaic virus* (TMV) and to bacteria *Xantomonas vesicatoria.* The significance of the DH technique in breeding is, first, the ability to develop monoploid plants with one set of chromosomes, second, to produce homozygous, doubled haploid (DH) pure lines, and third, to reveal meiotic recombinants with unique genome constitutions (Caranta *et al*., 1996). The aim of the study presented was to select new meiotic recombinants of anther culture origin with a final aim of new cultivar release.

2. Materials and methods

2.1. Anther culture

Anthers were excised from flower buds of different pepper lines at the uninucleate microspore stage. Fifteen anthers per petridish, 6 cm in diameter, were excised and laid onto a nutritive medium of Dumas de Vaulx *et al.* (1981). Regenerated (DH-R_1) plants were potted and grown in greenhouses for R_2 - seed production prior to flow cytometric analysis.

2.2. Flow cytometry

The determination of ploidy levels was carried out by a PARTEC Ploidy Analyzer, PA-I. (Partec, Münster, Germany) equipped with a high-pressure-lamp (Osram HBO-100W/2) according to the method of Dolezel *et al.* (1989).

2.3. Chromosome doubling

For the development of induced doubled haploids, the haploid regenerants were placed 50-400 mg/l colchicine contained medium for 4-6 days for induced DH development.

2.4. PCR study and DNA-isolation

DH-R_1 seeds were germinated in petridishes for molecular analysis. For genomic DNA extraction, two young fresh leaves were collected from each of DH-R_2 plants and pooled in bulks. For control, a bulk of DNAs of five individuals of anther donor pepper plants (DH-R_0) was applied. Leaves were subjected to leaf squeezing (Ravenel Spec. Inc.) using 750 µl of CTAB-buffer, cethyltrimethylammonium bromide, followed by the method used by Gyulai *et al.* (2000), utilizing the modifications of Gyulai *et al.* (1999). DNA samples were treated with 5 µl RNAse-A (from bovine pancreas), for 30 min at 37°C. *PCR analysis.* Amplification reactions were run in a volume of 25 µl (Williams *et al*., 1990) by a PE9700 thermocycler. The primers were the sets OP/A, B, AB, C, J, E, T for RAPD-analysis, SSR: $(GACA)_4$, $(GATA)_4$, $(ACTG)_4$, and ISSR: $CA(GACA)_4$, $AC(GACA)_4$, $(GACA)_4AC$, and $(GACA)_4CA$.

3. Results

Anthers excised at the uninucleate microspore stage showed direct embryo development by the fourth to eight week of incubation time. The frequency of plant development was 1-11 %. On average, 100 responsive anthers produced 18 ± 2.5 pollen embryos at the globular developmental stage. Regenerants were subjected to flow cytometric analysis to determine DNA C-level. The flow cytometric analysis of individuals of DH-R_1 regenerants revealed on average 68.5 % haploid plants (1C = n = 12); 29.8 % spontaneous DH plants (2C = 2n = 24); 0.7 % tetraploid plants (4C = 4n = 48) and 1 % aneuploid plants consisting one chimera with genetic mosaicism of haploid and diploid genomes. All the spontaneous and colchicine DH plants were grown in glasshouses for seed production of DH-R_2 generation for PCR analysis.

Haploid plants treated with 400 mg/l colchicine for six days produced the most of induced doubled haploids. 50-95 % of colchicine treated haploid plants became diploid. The PCR analysis, especially RAPD revealed a high level of genetic diversity among of doubled haploid DH-R_2 plants compared to the control of anther-donor DH-R_0 plants. Of the primers of Operon sets OP/C-15 (GAC GGA TCA G), OP/D-12 (CAC CGT ATC C), and OP/AB-09 (GGG CGA CTA C) produced polymorphic PCR-banding patterns with additional new PCR-fragments in DH-plants compared to anther-donor plants.

4. Discussion

In the case of adult plants, it is easy to differentiate haploids from diploids based on morphological characters. However, flow cytometry has provided a rapid and precise tool to detect ploidy levels since its first application in plant genome study (Heller, 1973). It is also very important to distinguish the regenerants in the juvenile stage to subject the haploids to colchicine treatment. The flow cytometric analysis revealed 29.8 % of the regenerants at doubled haploid (2n) genome level as a result of obvious chromosome doubling, which is common in pepper anther culture (Vagera, 1990). The 68.5 % haploid (1C = n) regenerants were subjected to colchicine treatment for chromosome doubling which provided optimal material for a further experiment to compare genetic diversity between spontaneous- and induced DH plants. The use

of PCR-based techniques has also become increasingly important, not only for genetic analysis but also for the cultivar discrimination of plants of DH origin (Paran *et al*., 1998, Prince *et al*., 1995). Our results facilitate the direct selection of DH-descendants by molecular markers in the molecular discrimination of DH-plants. Further PCR analysis of the individual plants of polymorphic bulks, and the linkage analysis of the polymorphic bands observed as putative genetic markers of meiotic recombinants is in progress.

In conclusion, spontaneous doubled haploid and colchicine-induced doubled haploid fertile pepper plants (DH-R_1) were developed and distinguished by flow cytometric analysis. PCR analysis revealed genetic diversity among DH-R_2 plants with additional new PCR-fragments in DH-R_2 plants compared to anther-donor plants. The DH-plants as meiotic recombinants with new genomic constitutions have been directly involved in breeding programs. The results also proved to be useful for PCR analysis in the recognition and selection of meiotic recombinants with a final aim of new cultivar release in sweet pepper. Our results prove a certain change between plants of anther-donor and DH-descendents, and among DH-descendents as well, due to the results of meiotic recombination. The experiences also suggest that genetic changes occurring during colchicine-induced genomic duplication do not seem to be higher than the changes caused of meiotic recombination.

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