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ORIGINAL PAPER

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GENETIC DIVERSITY OF CHRYSANTHEMUM PLANTS DERIVED VIA SOMATIC EMBRYOGENESIS USING RAPD MARKERS

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ABSTRACT

The genetic diversity was investigated among two chrysanthemum (*Chrysanthemum* × *grandiflorum* Ramat./Kitam.) cultivars 'Lady Salmon' and 'Lady Vitroflora' and its 15 lines of plants derived from somatic embryos in using ten random amplified polymorphic DNA (RAPD) markers. All primers gave 108 bands with 1218 products from 148.65 to 4391.20 bp in size. The average number of bands per primer was 10.8. Most fragments (54; 50%) were polymorphic, 9 (0.8%) specific and others (45; 49.2%) were monomorphic. Cluster analysis grouped all the cultivars and their lines into two main clusters and two subclusters. Most genetic diversity was characteristic for LS2 lines of plants derived via somatic embryogenesis from cultivar 'Lady Salmon'. All lines were different from each other and from the original cultivar propagated by meristematic explants. RAPD markers are a helpful tool to detect the genetic diversity of chrysanthemum plants derived via somatic embryogenesis (SE). Our result will provide useful information for production laboratory and for breeding programmes.

Key words: *Chrysanthemum* × *grandiflorum*, molecular markers, somatic embryos, genetic distance

INTRODUCTION

Chrysanthemum (*Chrysanthemum* × *grandiflorum* Ramat./Kitam.) is one of the most important ornamental plants in horticultural production. It is grown both for cut flowers or as potted plant. It is one of the leading species on the global market. Its story began in the Far East, Japan and China but today it is present on all the continents [Teixeira da Silva 2004]. It is characterized by enormous wealth of colours, shapes and types of inflorescences as well as the plant habit. Due to its long flowering period, it is often used in the compositions and arrangements of space, both in the gardens of the countryside and in the cities. In addition to its decorative qualities, it also has numerous health-promoting qualities [Lin and Harnly 2010]. In order to increase the production, it is more and more frequently propagated in *in vitro* culture laboratories. The laboratory production is extremely important to maintain the stability of the genetic variations. It is not easy with chrysanthemums, given the fact that about 50% of the cultivars are periclinal chimeras with the outer layer of cells altered only [Stewart and Dermen 1970, Bush et al. 1976, Van Harten 1998, Zalewska et al. 2007]. Such plants retain the properties characteristic of reproduction only by using meristematic explants and only on media without growth regulators added. The remain-

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ing 50% of the cultivars are homogeneous tissue. They can be propagated through the non-meristematic explants since, regardless of which layer regenerates new shoots or embryo, chimera separation of components does not occur. This is possible by applying two methods: adventitious shoots [Broertjes et al. 1976, Jerzy and Zalewska 1997, Song et al. 2011, Teixeira da Silva et al. 2015] and somatic embryogenesis [Lema-Rumińska and Śliwińska 2015]. These methods involve growth regulators; in addition, the regeneration often occurs indirectly via callus [Zalewska et al. 2011, Xu et al. 2012, Naing et al. 2013a, b, Lema-Rumińska and Niedojadło 2014]. As a consequence, the resulting plants can be unstable [Miler and Zalewska 2014]. Until now it is the flow cytometry (FCM) which has been a widely used method to study the genetic stability of plants produced by micropropagation [Pinto et al. 2010, Naing et al. 2013b, Prado et al. 2010, Konieczny et al. 2012, Currais et al. 2013, Lema-Rumińska and Śliwińska 2015]. However, changes in the genetic material may be not only quantitative but also qualitative. The latter, even the smallest change point, can be detected using molecular markers. RAPD markers have been still used for the study of genetic differentiation between cultivars [Wolff and Peters-Van Rijn 1993, Wolff et al. 1995, Wolff 1996, Myśków et al. 2001, Mukherjee et al. 2013], or a group of the cultivars produced by mutagenesis [Lema-Rumińska et al. 2004, Chattarjee et al. 2006, Miňano et al. 2009].

To understand the molecular systematics, genetic differences and the population structure of germplasms in chrysanthemums, except for RAPD markers, as well as other types of molecular markers: amplified fragment length polymorphism (AFLP) [Roein et al. 2014], inter simple sequence repeats (ISSR) [Mukherjee et al. 2013, Baliyan et al. 2014], the simple sequence repeats (SSR) genomic polymorphism [Zhang et al. 2014], conserved DNA-derived polymorphism (CDDP) markers are used [Li et al. 2013]. The start codon targeted (SCoT) analysis was performed to investigate genetic diversity and relationships in medicinal *Chrysanthemum morifolium* cultivars [Feng et al. 2016].

The previous cytometry studies by Lema-Rumińska and Śliwińska [2015] with the chrysanthemums SE line revealed no changes in ploidy plants despite morphological and biochemical differences, which gave rise to more detailed research on the genetic diversity of the lines applying molecular markers. To our knowledge, our study is the first attempt to analyze genetic stability of somatic embryo-derived plants of chrysanthemum.

The aim of this study has been to analyze the genetic diversity of somatic-embryo derived plants of chrysanthemum cultivars and their lines by the use of RAPD markers.

MATERIALS AND METHODS

Plant material

The two cultivars of *Chrysanthemum* × *grandiflorum* Ramat./Kitam., 'Lady Salmon' and 'Lady Vitroflora' by Jerzy and Zalewska [1996] and their 15 lines of plants obtained via somatic embryogenesis by Lema-Rumińska and Śliwińska [2015] were studied. In chrysanthemum cultivars 'Lady Salmon' and 'Lady Vitroflora' the number of chromosomes is 2n = 6x = 54 [Lema-Rumińska and Zalewska 2002]. The 'Lady Salmon' cultivar has a chimera structure, while 'Lady Vitroflora' – a non-chimera structure (homohistont form). Both cultivars were selected due the highest somatic embryogenesis ability.

Eight lines of 'Lady Salmon' (LS1-LS8) and seven lines of 'Lady Vitroflora' (LV1-LV7) were derived from somatic embryos and control plants LS0 and LV0 – from *in vitro* microcuttings shoots apexes (with meristems). For the purpose of DNA extraction, 100 mg of young fresh leaves from each line and cultivar grown in greenhouse was collected.

DNA extraction

Leaves were homogenized using liquid nitrogen. DNA was extracted with the Genomic Mini AX Plant (A&A Biotechnology, Poland) following the manufacturer's instructions. In addition to the kit, RNA-se was used. The DNA concentration was quantified using a UV-VIS Spectrophotometer (UV-VIS 1601 SHIMADZU, Japan).

RAPD analysis

RAPD loci were generated with ten arbitrary primers (DNA Gdańsk, Poland) selected based on the references and sequences shown in Table 1. PCR reactions were performed twice for all plant materials in Eppendorf tubes in 25µl containing 1 µl of template DNA (20 ng/µl), 12.5 µl 2 × PCR MIX PLUS (A&A Biotechnology, Poland), 2.5 µl of each primer (1 µM) and ultrapure water. Amplification was performed in Bio-Rad Thermal Cycler C1000 Touch™ (Hercules, CA, USA) programmed according to Lema-Rumińska et al. [2004]. PCR products were separated on 1.5% agarose gels stained with ethidium bromide in 1 × TBE buffer. The DNA bands size was identified with the use of 'Gel Doc' gel documentation system (Bio-Rad, Hercules, CA, USA) and GelAnalyzer 2010 software (Copyright 2010 by Istvan Lazar and Dr. Istvan Lazar).

Data analysis

The band patterns received with each RAPD primer scored as present (1) or absent (0). Only distinct, reproducible bands which did not vary between replications were used for the calculations. Cluster analysis was carried out and a dendrogram was constructed with the STATISTICA'12 software package (StatSoft). The dendrogram generated using unweighted pair group method (UPGMA) with arithmetic averages and the agglomerative, hierarchical clustering was made. Additionally, to evaluate the genetic similarity, Ney's coefficient [Nei and Li 1979] was calculated.

RESULTS AND DISCUSSION

All 10 primers gave reproducible profiles of amplification products used to study the genetic diversity of cultivars and cultivar lines derived via somatic embryogenesis. All the primers generated a total of 108 bands with 1,218 products ranging in size from 148.65 to 4391.20 bp. The number of fragments per primer ranged from 8 to 15 bands. On average, one primer generated 10.8 bands. Most, 54 bands (50%), were polymorphic, and only 9 were specific bands (0.8%), the rest (45 bands) were monomorphic. The amplification profiles of the genotypes investigated with representative primer are shown on Figure 1. Percentage of polymorphic to monomorphic bands for all the primers ranged from 4.35% (LS1) to

Table 1. Sequences of the primers selected and the number of amplification-generated products in RAPD analysis

Primers code	Sequence	Total numer of bands	Number of bands per primer	No of polymorphic bands per primer	No of specific bands per primer	References
A	5'-GGG AAT TCG G-3'	119	13	9	1	Lema-Rumińska et al. [2004]
B	5'-GAC CGC TTG T-3'	131	9	4	0	
C	5'-GGA CTG GAG T-3'	100	11	6	1	
D	5'-GCT GCC TCA GG-3'	128	9	3	1	Shibata et al. [1998]
E	5'-TAC CCA GGA GCG-3'	85	8	5	1	
F	5'-CAA TCG CCG T-3'	76	8	3	2	Wolf [1996]
G	5'-GGT GACGCA G-3'	145	11	4	1	
H	5'-CCC AGT CAC T-3'	112	15	12	1	Martin and Gonzales-Benito [2005]
I	5'-TGG CGT CCT T-3'	191	15	6	1	
J	5'-AGC GTG TCT G-3'	131	9	2	0	Chattarjee et al. [2006]
Total		1218	108	54	9	

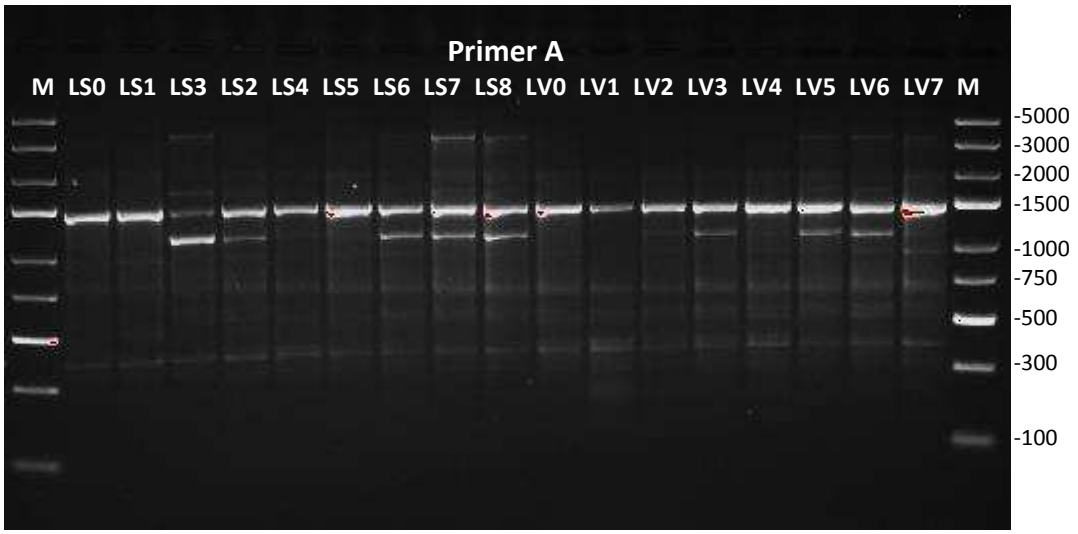

Fig. 1. RAPD profiles of bands of chrysanthemum cultivars and their lines obtained via SE: M, Gene--Ruler™ DNA Ladder (Thermo Scientific, Poland), LS0 – control plants of 'Lady Salmon' from meristematic explants, LS1-LS8 somatic embryo-derived plants of 'Lady Salmon', LV0 – control plants of 'Lady Vitroflora' from meristematic explants, LV1-LV7 somatic embryo-derived plants of 'Lady Vitroflora'

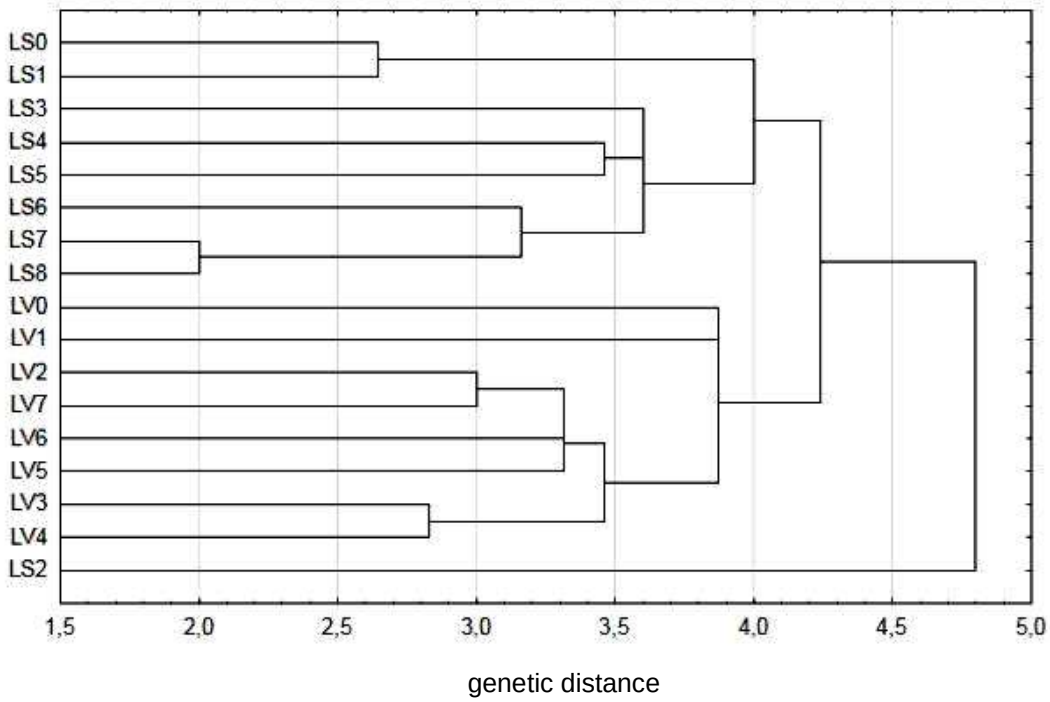

Fig. 2. Dendrogram of the cultivars and their chrysanthemum lines obtained via somatic embryogenesis revealed by cluster analysis (UPGMA). Abbreviations see Fig. 1

27.54% (LS2) in 'Lady Salmon' and its cultivar line, while in 'Lady Vitroflora' (LV0-LV7) it ranged from 8.57% (LV1 and LV4) to 17.14% (LV6).

The greatest number of polymorphic bands, reaching 30%, was found in line LS2, moreover, we have shown the most specific bands in this line. Our studies have shown that all the lines derived from 'Lady Salmon' and 'Lady Vitroflora' via SE differ in the number and position of the bands of the control cultivar (propagated by meristematic explants) and on the other lines.

All the products obtained with 10 primers were used to construct the dendrogram and cluster analysis using UPGMA method (fig. 2). Cluster analysis readly separated the cultivars and lines into two main groups, with one of the major group being further divided into two subgroups. Cluster analysis confirmed line LS2 greatest genetic distance, which formed a separate cluster distant from the other two cultivar subgroups LS0-LS8 (without LS2) and LV0-LV7. Within the subgroup of 'Lady Salmon' the greatest genetic similarity occurred in line LS7 and LS8, while the greatest similarity to the control plants derived from meristematic explants occurred in line LS1. Cluster analysis confirmed that the cultivars and lines tested generated by somatic embryogenesis are different.

A high level of polymorphisms provided, in the cluster analysis, a dendrogram in which there clearly separated 2 major groups, divided into two subgroups. Line LS2 has formed a distant cluster being a group separate from the other lines, LS and LV. Genetic diversity evaluation with RAPD markers for the line resulting from somatic embryogenesis has shown a surprisingly high genetic variation between individual lines LS1-LS8 and LV1-LV7 and control plants (derived from meristematic explants) in chrysanthemums. Genetic variability in plants with Nei

Table 2. Similarity coefficients among cultivars and their lines obtained via SE in chrysanthemum using RAPD markers

	LS0	LS1	LS2	LS3	LS4	LS5	LS6	LS7	LS8	LV0	LV1	LV2	LV3	LV4	LV5	LV6	LV7
LS0	1																
LS1	0.93	1															
LS2	0.70	**0.69**	1														
LS3	0.80	0.83	0.82	1													
LS4	0.87	0.81	0.76	0.89	1												
LS5	0.85	0.84	0.78	0.87	0.91	1											
LS6	0.80	0.77	0.78	0.91	0.88	0.89	1										
LS7	0.79	0.77	0.79	0.88	0.82	0.84	0.93	1									
LS8	0.79	0.77	0.78	0.87	0.81	0.82	0.93	**0.98**	1								
LV0	0.79	0.79	0.73	0.79	0.82	0.87	0.81	0.80	0.80	1							
LV1	0.80	0.82	0.81	0.83	0.83	0.85	0.82	0.79	0.81	0.89	1						
LV2	0.81	0.80	0.75	0.81	0.84	0.86	0.83	0.81	0.82	0.88	0.84	1					
LV3	0.82	0.79	0.80	0.82	0.80	0.80	0.80	0.80	0.80	0.84	0.83	0.90	1				
LV4	0.81	0.85	0.81	0.78	0.83	0.79	0.79	0.79	0.83	0.84	0.73	0.94	0.95	1			
LV5	0.79	0.82	0.83	0.77	0.82	0.82	0.83	0.83	0.81	0.81	0.76	0.85	0.89	0.89	1		
LV6	0.85	0.81	0.80	0.79	0.82	0.81	0.85	0.82	0.85	0.77	0.76	0.88	0.84	0.89	0.83	1	
LV7	0.82	0.83	0.79	0.80	0.80	0.80	0.82	0.81	0.83	0.81	0.77	0.94	0.90	0.91	0.88	0.89	1

and Li [1979] similarity coefficient was from 0.69 to 0.98, respectively, in LS2/LS1 and in LS7/LS8 (tab. 2). As many as 50% were polymorphic bands and 0.8% of the bands were specific. Differences in colour and the content of pigments in inflorescences (particularly evident in line LS) have been identified by Lema-Rumińska and Śliwińska [2015]. The authors also investigated the genetic stability of those cultivars and lines applying flow cytometry, however, they did not show any changes in the ploidy of the test plant material.

The reports by Lema-Rumińska et al. [2004] point to a variation in the genetic level (from 0.50 to 0.93) using RAPD markers in mutants in the 'Lady' cultivar group, including control cultivars 'Lady Salmon' (LS0) and 'Lady Vitroflora' (LV0), which was similar as in the study of the plants derived from somatic embryos. Similarly Chattarjee et al. [2006], investigating 10 original chrysanthemum cultivars and 11 mutants, found a variation at a similar genetic level in the range from 0.17 to 0.90 using RAPD analysis. Similar results were reported also by Mukherjee et al. [2013] in RAPD and ISSR studies of 40 non-related chrysanthemum cultivars; the cultivars showed a high level of polymorphism (from 0.23 to 0.68), which makes them distinguishable on the basis of banding pattern. In our study on somatic embryogenesis derived plants genetic variability was lower (from 0.69 to 0.98) than in non-related chrysanthemums, but similar to mutants derived in mutation breeding.

The greatest genetic similarity (of up to 0.99) among the somaclons studied with a different inflorescence colour obtained by regeneration of adventitious shoots in 'Albugo' Group and 'Alchimist' Group was received by Miler and Zalewska [2014]. A genetic diversity in mutants gave rise to distinguishing related mutant cultivars by Lema-Rumińska et al. [2004]. Our studies also show that the genetic diversity of plants derived via somatic embryogenesis is similar as in the mutants obtained by induced mutagenesis. Such changes can be due to the growth regulators added to the medium at induction stage SE in *in vitro* cultures (especially 2.4-D) and /or instability resulting from the indirect regeneration via callus.

The studies by Viehmannova et al. [2014], using ISSR markers and flow cytometry in yacon on somatic embryo-derived plants also showed polymorphisms evidence of somaclonal variation, however a cytometric study showed no differences. No differences in ploidy, which could be detected by FCM, demonstrates that during SE, the number of chromosomes or the ploidy of the test plants does not change. The changes detected by the use of molecular markers is usually sowing small changes occur in both the coding and non-coding sequences distributed over the entire huge genome contained in 54 chromosomes of chrysanthemums. The variability detected by RAPD markers shows that regardless of whether the chrysanthemum cultivar is a chimera ('Lady Salmon') or not-chimera ('Lady Vitroflora'), the variability is at a similar level.

CONCLUSIONS

Cluster analysis of chrysanthemum plants resulting from somatic embryogenesis grouped all the cultivars and their lines into two main clusters and two subclusters. All lines LS1-LS8 and LV1-LV7 were different from each other and from the original cultivar LS0 or LV0 respectively.

The large variability (from 0.69 to 0.98) at genetic level of chrysanthemums plant derived via somatic embryogenesis can be considered as a new source of variation in breeding programmes.

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