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ISSR MARKERS IN THE GENETIC DIVERSITY OF CHRYSANTHEMUM PLANTS DERIVED VIA SOMATIC EMBRYOGENESIS

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Summary. The objective of the study has been to determine the usefulness of six different Inter Simple Sequence Repeat (ISSR) primers for the genetic stability in the chrysanthemum plants produced through somatic embryogenesis (SE). The research material consisted of the lines of two cultivars: 'Lady Salmon' (LS1–LS5) and 'Lady Vitroflora' (LV1–LV5). The plants derived from meristems constituted the standard. The primers used in the PCR reaction have shown a notable polymorphism between the genotypes analyzed. All the primers (S1–S6) demonstrated a polymorphism in the inter-microsatellite region; the highest polymorphism in 'Lady Salmon' and 'Lady Vitroflora' lines with primer S3, whereas the lowest – in 'Lady Salmon' cultivar with primers S5 and S6, and in 'Lady Vitroflora' – with primer S1. UPGMA dendrogram clearly separated all the cultivars and its lines into two main clusters and two subclusters. ISSR markers can be applied to assess the genetic stability of SE-derived chrysanthemum.

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

INTRODUCTION

Chrysanthemum ×grandiflorum Ramat./Kitam. is the second, immediately after the rose, ornamental plant with the greatest economic importance [Teixeira da Silva et al. 2013]. A significant economic importance results from a large number of cultivars used as cut flowers, potted plants or garden perennials [Nencheva 2013]. In addition to the rich range of cultivars, chrysanthemums reveal amazing colours, shapes, types of

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inflorescence and a varied growth [Carvalho-Zanão et al. 2012]. In addition to decorative qualities, chrysanthemums have many health-promoting properties used in medicine [Liang-Yu et al. 2010]. The market requirements call for an ongoing search for new breeding and propagation methods to produce a large number of plants in a relatively short time [Kulpa 2012]. Using *in vitro* cultures, in addition to a larger number of plants, the material is rejuvenated, of higher quality and it demonstrates a significantly higher regenerative potential. Chrysanthemum micropropagation involves *in vitro* cuttings with existing meristems (axillary or apex), while breeding – adventitious organogenesis or somatic embryogenesis. The latter is a biological process with somatic embryos being formed from vegetative cells [Haccius 1978]. It is commonly regarded as one of the most effective micropropagation methods, facilitating the generation of a large number of plants in a very short time [Kulpa 2012]. Plant regeneration with chrysanthemum via somatic embryogenesis gives higher efficiency than adventitious organogenesis [Zalewska et al. 2007]. Somatic embryogenesis in chrysanthemum is induced on MS medium [Murashige and Skoog 1962] with addition of auxin – 2,4-dichlorophenoxyacetic acid (2,4-D) or α-naphthylacetic acid (NAA) as well as cytokinins: 6-benzylaminopurine (BAP) or kinetin (KIN), and generally this process occurs indirectly, via callus [Naing et al. 2013, Lema-Rumińska et al. 2015, Lema-Rumińska and Śliwińska 2015]. The presence of growth regulators can threatens the genetic stability and can lead to somaclonal variation [Miler and Zalewska 2014]. One of the methods used to study genetic stability is flow cytometry (FCM) [Śliwińska and Thiem 2007, Kulpa 2012]. Cytometric analysis in chrysanthemum, 'Lady Vitroflora' and 'Lady Salmon', did not show any differences in the ploidy level, however, significant biochemical differences were found [Lema-Rumińska and Śliwińska 2015]. For that reason, we decided to investigate the lines of two cultivars of chrysanthemum derived via somatic embryogenesis using more advanced molecular methods. The methods more and more commonly used to determine the level of polymorphism are the Amplified Fragment Length Polymorphisms (AFLP) molecular markers [Garcia et al 2004], Simple Sequence Repeat (SSR) [Yu et al. 2000], Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) [Kalia et al. 2011, Bhagyawant 2016]. The method of ISSR very fast and easy to perform, it is cheap in contrast to ALFP and it does not require knowledge of flanking sequences, as for SSR markers [Kalia et al. 2011, Ng and Tan 2015]. ISSR markers were used to determine the genetic diversity of *Camellia sinesis*, *Canarium album*, Indian *Impatiens* or *Tulipa gesneriana* [Podwyszyńska et al. 2010, Mei et al. 2017, Tamboli et al. 2018]. The preliminary studies of the genetic map of chrysanthemum cultivars involved various molecular markers (RAPD, ISSR, SSR, AFLP) [Zhang et al. 2010, Chang et al. 2018], however studies on genetic diversity evaluation in chrysanthemum plants produced via somatic embryogenesis with genetic markers are missing. A genetic diversity in plants derived as a result of somatic embryogenesis in chrysanthemum has been studied previously with RAPD markers [Lema-Rumińska and Mellem 2017].

The objective of our research has been to develop the knowledge on genetic diversity in 12 lines (LS1–LS7 and LV1–LV5) of 'Lady Salmon' and 'Lady Vitroflora' (LS0, LV0) produced as a result of somatic embryogenesis with ISSR markers.

MATERIAL AND METHODS

Plant material and DNA isolation

The research material consisted of two cultivars and their 12 lines of *Chrysanthemum ×grandiflorum* Ramat./Kitam., derived as a result of some earlier studies on somatic embryogenesis in 'Lady Salmon' (lines LS1–LS7) and 'Lady Vitroflora' (lines LV1–LV5) [Lema-Rumińska and Śliwińska 2015, Lema-Rumińska and Mellem 2017]. The controls (standards) were plants from explants containing meristems, marked as LS0 and LV0, because propagation by meristematic explants give the maximum genetic stability of plants. All the somatic embryos were obtained by indirect somatic embryogenesis in 10 weeks. The DNA was isolated from fresh chrysanthemum leaves growing in a greenhouse (100 mg of each) using the Genomic Mini AX Plant (A&A Biotechnology, Poland). The DNA content was measured using Quantus Fluorometer DNA (Promega, Poland).

ISSR analysis

Molecular analysis was performed using six different ISSR primers (Genomed S.A., Poland). Six PCR reactions were performed for 14 genotypes (in two repetitions) as follows:

- S1 – sequence 5'→ 3' GAGGGTGGAGGATCT [Palai and Rout 2011];
- S2 – sequence 5'→ 3' CGAGAGAGAGAGAGAGA [Palai and Rout 2011];
- S3 – sequence 5'→ 3' GTGAGAGAGAGAGAGAGA [Mukherjee et al. 2013];
- S4 – sequence 5'→ 3' GACAGACAGACAGACA [Mukherjee et al. 2013];
- S5 – sequence 5'→ 3' GAGAGAGAGAGAGAGAGAT [Mukherjee et al. 2013];
- S6 – sequence 5'→ 3' CAGAGAGAGAGAGAGAG [Palai and Rout 2011].

The 25 µl reaction mixture contained: 12.5 µl PCR Mix Plus, 2.5 µl of primer, DNA template and nuclease-free sterile water (A&A Biotechnology, Poland).

Amplification was performed using the C1000 Touch^{TM} thermocycler (BIO-RAD, USA). The PCR conditions were as follows: initial denaturation at 94°C for 4 min, followed by 45 cycles of 1 min at 94°C, 1 min at 53°C, 2 min at 72°C, and final extension during 4 min at 72°C. The products were separated on a 1.5% agarose gel in the presence of ethidium bromide in 1× TBE buffer. Identification, visualization and analysis of the products separated were made using the Molecular Imager® Gel Doc^{TM} XR and transilluminator (BIO-RAD, USA) and GelAnalyzer 2010 programme.

Statistical methods

The banding patterns obtained with the PCR reaction using ISSR primers were the binary matrix as present (1) or absent (0). Only clear and reproducible bands which do not differ between replications were used. Cluster analysis was performed using the unweighted pair group method (UPGMA) applying Statistica 13.1 software with hierarchical, agglomerative grouping (StatSoft, Poland).

RESULTS AND DISCUSSION

ISSR primers showed a high polymorphism across the genotypes tested. They resulted in 457 products in the range of 314–3,107 bp (average 76.2 per primer) for both cultivars and their lines derived via somatic embryogenesis. ISSR band profiles for all the genotypes studied with an exemplary primer are given in Figure 1.

In 'Lady Salmon' and its lines 236, from which 23 products were polymorphic (Table 1). The highest number of products was recorded for primer S2 (55 products in the 443–1,761 bp range), and the lowest number of bands was obtained as a result of reaction with primer S5 (30 products in the size ranging from 585 to 1,323 bp). 'Lady Vitroflora' and its lines gave rise to a total of 221 products, 19 of which were polymorphic (Table 2). Most generated bands were produced for primer S3, in the size range of 528–1,188 bp (49 products), while the lowest number – by primers S1 and S5 (each of 30 products). The earlier studies on genetic diversity in chrysanthemum by Lema-Rumińska and Mellem [2017] using RAPD markers have shown more polymorphic bands than found in the present studies with ISSR markers. The number of polymorphic bands was up to

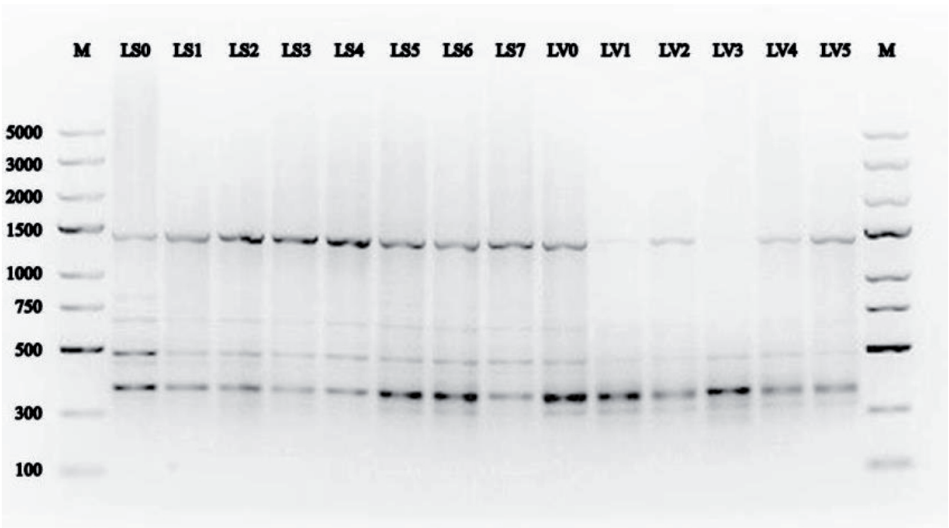

Fig. 1. Profiles of amplification products obtained with ISSR primer S1 for two cultivars of chrysanthemum and their lines – a negative picture: M – marker, Gene Ruler^{TM} Express DNA Ladder (Thermo Scientific), LS0 – 'Lady Salmon' standard plants derived from meristematic explants, LS1–LS7 lines of 'Lady Salmon' plant after somatic embryogenesis, LV0 – control plants of 'Lady Vitroflora' from meristematic explants, LV1–LV5 lines of 'Lady Vitroflora' plant after somatic embryogenesis

Rys. 1. Profile produktów amplifikacji otrzymane przy pomocy startera ISSR S1 dla dwóch odmian chryzantemy i ich linii – obraz w negatywie: M – marker, Gene Ruler^{TM} Express DNA Ladder (Thermo Scientific), LS0 – 'Lady Salmon' rośliny kontrolne z eksplantatów merystematycznych, LS1–LS7 linie roślin 'Lady Salmon' po embriogenezie somatycznej, LV0 – rośliny kontrolne 'Lady Vitroflora' z eksplantatów merystematycznych, LV1–LV5 linie roślin 'Lady Vitroflora' po embriogenezie somatycznej

12 (for primer H) applying RAPD markers, and only eight for primer S3 ISSR. Biswas et al. [2010] investigated the use of various markers: RAPD, ISSR, IRAP (Inter Retrotransposon Amplified Polymorphism) and REMAP (Retrotransposon Microsatellite Amplified Polymorphism), for the assessment of genetic diversity and relationships among *Citrus* spp.

A high level of polymorphism was observed in all of the four types of marker systems. Interestingly, RAPD markers generated the highest number of polymorphic bands. In addition, there was a high correlation in *Citrus* spp. between the genetic similarity due to the application of RAPD and ISSR markers. Also Mei et al. [2017] suggest, based on the research of *Canarium album* classification in different regions of China, that both RAPD and ISSR markers are useful for studying the genetic diversity. In our studies, the highest average percentage of polymorphism for both cultivars and their lines was demonstrated by primer S3 (64.58%) and the lowest – primer S1 (33.33%). The genetic analysis showed that in 'Lady Salmon' polymorphic *loci* accounted for 51.11%, monomorphic – for 44.44%, while specific – for 4.44%, whereas 'Lady Vitroflora' polymorphic *loci* – for 44.19%, monomorphic – for 39.53%, and specific – for 16.28%.

The cluster analysis demonstrated that the cultivars studied as well as their lines were separated into two main clusters, one of which formed line LV3 itself as a cluster separate from other cultivars. In addition, the lines were grouped in two subclusters in which LS0 got clearly separated forming a subcluster separate from the other lines (except for LV3) – Figure 2.

The cluster analysis also identified the highest similarity between LS2 and LS3 lines as well as LV0 and LV1. The RAPD markers research reported by Lema-Rumińska and Mellem [2017] observed some different grouping within the lines produced via somatic embryogenesis in chrysanthemums. Also a pilot study investigated by Baliyan et al. [2014] based on morpho-agronomic and ISSR markers in chrysanthemum has shown to be highly useful for asserting genetic diversity in cultivars of chrysanthemum and

Table 1. Number of amplification products derived using individual primers (S1–S6) for 'Lady Salmon'

Tabela 1. Liczba produktów amplifikacji otrzymanych dla poszczególnych starterów (S1–S6) dla 'Lady Salmon'

Specification Wyszczególnienie	Number of products Liczba produktów	*Loci*				Polymorphism Polimorfizm [%]
		polimorphic polimorficzne	monomorphic monomorficzne	specific specjalne	total ogółem	
S1	42	4	3	1	8	50.00
S2	55	3	3	0	6	50.00
S3	32	5	3	0	8	62.50
S4	40	5	4	0	9	55.56
S5	30	3	3	1	7	42.86
S6	37	3	4	0	7	42.86
Total – Ogółem	236	23	20	2	45	NA*
Share *loci* Udział *loci* [%]	NA	51.11	44.44	4.44	NA	NA

*NA – not applicable – nie dotyczy.

Table 2. Number of amplification products derived using individual primers (S1–S6) for 'Lady Vitroflora'

Tabela 2. Liczba produktów amplifikacji otrzymanych dla poszczególnych starterów (S1–S6) dla 'Lady Vitroflora'

Specification Wyszczególnienie	Number of products Liczba produktów	*Loci*				Polymorphism Polimorfizm [%]
		polimorphic polimorficzne	monomorphic monomorficzne	specific specjalne	total ogółem	
S1	30	1	4	1	6	16.67
S2	34	2	4	1	7	28.57
S3	49	8	1	3	12	66.67
S4	35	3	2	1	6	50.00
S5	30	2	2	1	5	40.00
S6	43	3	4	0	7	42.86
Total Ogółem	221	19	17	7	43	NA*
Share *loci* Udział w *loci* [%]	NA	44.19	39.53	16.28	NA	NA

*NA – not applicable – nie dotyczy.

UPGMA dendrogram clearly separated the genotypes studied into distinct groups. Our research has confirmed the effectiveness of the ISSR markers application for the study of genetic diversity in the chrysanthemum line produced via somatic embryogenesis in chrysanthemums.

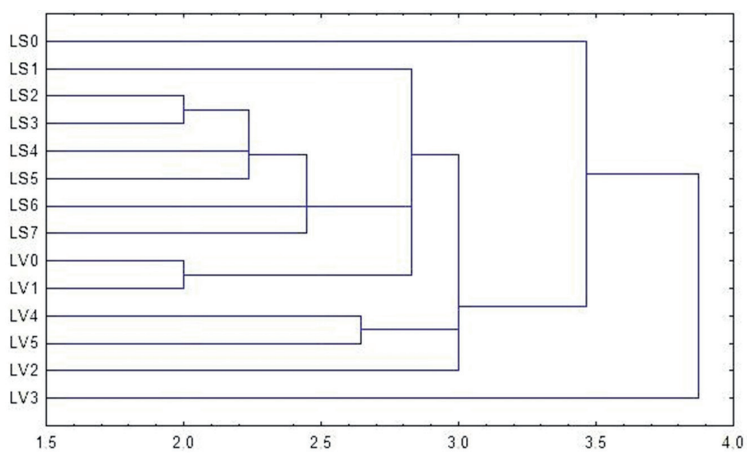

Fig. 2. Dendrogram of chrysanthemum 'Lady Salmon' and 'Lady Vitroflora' and their lines based on the matrix data of ISSR markers using the unweighted pair group method (UPGMA). Abbreviations as in Figure 1

Rys. 2. Dendrogram chryzantemy 'Lady Salmon' and 'Lady Vitroflora' i ich linii na podstawie danych matrycowych markerów ISSR przy użyciu metody nieważonej grupy par (UPGMA). Oznaczenia jak na rysunku 1

CONCLUSIONS

In the *Chrysanthemum ×grandiflorum* Ramat./Kitam. lines derived applying somatic embryogenesis in 'Lady Vitroflora' and 'Lady Salmon', a high genetic diversity was found both across the lines tested and within the lines as well as in relation to control plants propagated from meristems. All that makes it possible to consider SE as a method useful in breeding programmes for chrysanthemums.

It was found that all the primers (S1–S6) used for the ISSR analysis allowed to detect polymorphism between studied cultivars and their lines. The highest polymorphism was found using primer S3. The lowest polymorphism was recorded using primers S5 and S6 for the lines of 'Lady Salmon', and primer S1 for the lines of 'Lady Vitroflora'.

The UPGMA dendrogram clearly separated all the cultivars and their lines into two main clusters and two subclusters. All the cultivars were different from one another and from the standard plants.

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MARKERY ISSR W ZRÓŻNICOWANIU GENETYCZNYM CHRYZANTEM UZYSKANYCH W WYNIKU EMBRIOGENEZY SOMATYCZNEJ

Streszczenie. Chryzantema wielkokwiatowa (*Chrysanthemum ×grandiflorum* Ramat./Kitam.) zajmuje wysoką pozycję na rynku ogrodniczym, a jej produkcja z każdym rokiem wzrasta. W celu zaspokojenia rosnących potrzeb rynku poszukuje się nowych metod rozmnażania roślin oraz ich hodowli. Jedną z najbardziej wydajnych metod regeneracyjnych jest embriogeneza somatyczna, która może być pomocna w hodowli nowych odmian roślin na etapie regeneracji z pojedynczych komórek somatycznych. Celem pracy było określenie przydatności sześciu różnych starterów sekwencji międzymikrosatelitarnych (ISSR) do badań stabilności genetycznej roślin chryzantemy wielkokwiatowej, uzyskanych w wyniku embriogenezy somatycznej. Materiał badawczy stanowiły linie dwóch odmian 'Lady Salmon' (LS1–LS5) oraz 'Lady Vitroflora' (LV1–LV5) uzyskane w wyniku embriogenezy somatycznej. Kontrolą były rośliny uzyskane z merystemów (LS0 i LV0). Analizę otrzymanych produktów reakcji wykonano z użyciem programu GelAnalyzer 2010. Startery wykorzystane w reakcjach PCR wykazały znaczny polimorfizm między analizowanymi genotypami. U odmiany 'Lady Salmon' otrzymano 236 produktów, z których 23 prążki były polimorficzne, a u 'Lady Vitroflora' powstało 221 produktów, z czego 19 wykazało zmienność. Z tego względu metoda oparta na embriogenezie somatycznej może być szczególnie przydatna w programach hodowlanych chryzantemy, gdyż proces ten może być traktowany jako dodatkowe źródło zmienności.

Słowa kluczowe: *Chrysanthemum ×grandiflorum*, markery molekularne, zarodki somatyczne