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Somaclonal Variation in Long-term Micropropagated Tulips (*Tulipa gesneriana* L.) Determined by FISH Analysis

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ABSTRACT

A new protocol for tulip micropropagation by cyclic multiplication of adventitious shoots, in the presence of thidiazuron, was developed. True-to-type plants and somaclonal variants selected from *Tulipa gesneriana* cv. 'Prominence' plants derived from long-term *in vitro* cultures were analysed for karyotype rearrangements and stability of ribosomal DNA (rDNA) using fluorescence *in situ* hybridization (FISH). The study focused on the polymorphism of number, appearance and chromosomal localization of rDNA sites. The chromosome number in the plants of the original cultivar propagated conventionally (standard) and the somaclones equalled 2n = 2x = 24 chromosomes. The karyotypes of the standard and the micropropagated plants consisted of median, submedian and subterminal chromosomes. The difference in number of each type of chromosomes was observed in somaclones but not in the standard. In all analysed plants FISH with 5S and 45S rDNA probes identified many rDNA sites on each chromosome which provided markers for individual chromosome identification. Loci of 45S rDNA were located at telomeric positions on the long arm of the chromosomes. 5S rDNA sites were predominately located in intercalative positions on the long arms in close proximity to the centromere and in the telomeric position on the short arms. In addition, 5S rDNA loci were located intercalary on the short arm on the first pair of median chromosomes. Karyotype comparison exhibited variation in the number of 5S and 45S rDNA loci and in the size of hybridization signals both between standard 'Prominence' and somaclones as well as among somaclones. In conclusion, we demonstrated the usefulness of FISH karyotyping with cloned 5S and 45S rDNAs in analysis of genome re-structuring in long-term micropropagated tulips.

Keywords: 5S rDNA, 45S rDNA, *in vitro* propagation, karyotype instability

INTRODUCTION

The genus *Tulipa* includes approximately 100 species (Botchantzeva 1982; Bryan 2002). *T. gesneriana* is the most cultivated tulip group for cut flowers that resulted from complex crossing of *Tulipa* species (Killingback 1990). The constant need for new, enriched varieties, e.g. with new flower colours and shapes, pathogen resistance or a short forcing period, is hampered by the slow propagation rate of tulip *via* traditional methods. A new method for tulip (*Tulipa gesneriana* L.) propagation using *in vitro* techniques was developed to speed up breeding, and to provide new genotypes on the market (Podwyszynska and Marasek 2003). The method is based on cyclic multiplication of adventitious shoots in the presence of thidiazuron (TDZ). The new protocol was generally elaborated using two model cultivars of *T. gesneriana*, 'Blue Parrot' and 'Prominence'. Shoots were propagated *in vitro* for one and a half to four years for 'Prominence' and for one and a half to six years for 'Blue Parrot' before being cultured *ex vitro* (Podwyszynska 2005). Plants regenerated from tissue culture can exhibit substantial changes (Lee and Phillips 1988). The nature and causes, as well as positive and negative aspects of such phenomenon, termed somaclonal variation (SV) occurring in tissue culture (TC)-derived plants has been widely reviewed, e.g. by Jain (2001). The occurrence of somaclonal variation in mass *in vitro* production is undesirable. Such variation can be caused by epigenetic or genetic changes. Several methods such as molecular markers based on DNA amplification *via* polymerase chain reaction (PCR) as well as cytological analyses were used for evaluation of SV (Jain 2001; Gostimsky *et al*. 2005). Within the PCR-based markers, random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLP) were used most frequently in the last decade.

In previous studies, SV occurring in tulip plants propagated *in vitro* with our methods, was evaluated by phenotypic observation and DNA markers such as RAPD, ISSR and AFLP (Podwyszynska 2005; Podwyszynska *et al*. 2006, 2007). Thus several somaclonal variants with minor or major morphological changes were selected in both studied cultivars, 'Blue Parrot' and 'Prominence', however the later showed higher diversity and stability of somaclonal variants. Molecular analyses revealed that phenotypic variation resulted from altered DNA. We supposed that such genetic changes could also be detected by cytological analysis. Simple staining techniques permitted observation of variation in chromosome number (Nakano *et al*. 2006) and their morphology such as chromosome breaks and deletions (McCoy *et al*. 1982; Lepitan *et al*. 1984; Lee and Phillips 1988; Creissen and Karp 1985; Kharabian and Darabi 2006). Giemsa C-banding technique, which enables the linear differentiation of chromosomes, has allowed for the detection of deletions and translocations in somaclones, e.g. in *Medicago sativa* L. (Masoud *et al*. 1991) and cereal species (Armstrong *et al*. 1983; Lepitan *et al*. 1984; Wang and Marshall 1996). Until now, there is no information on the chromosome fidelity in tissue culture derived tulips.

Karyology of tulips is well-documented with respect to chromosome number (Sayama *et al*. 1982; Van Raamsdonk and De Vries 1995) and C-banding (Filion 1974; Van Raamsdonk and De Vries 1995). *Tulipa* chromosomes show similar sizes, and are rather uniform in morphology except for median chromosomes (Marasek *et al*. 2006). Such characteristics make the identification of individual chromosomes difficult by conventional cytological means, thus limiting the possibility for investigations of chromosome variation

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following *in vitro* culture and regeneration. Development of fluorescence *in situ* hybridization (FISH) has opened the possibility of cytogenetic studies in species with morphologically little differentiated chromosomes. FISH is a technique that enables the physical location of genes or other DNA sequences on chromosomes. The two types of ribosomal RNA genes, 45S rDNA encoding for 18S-5.8-25S ribosomal RNAs and 5S rDNA have been commonly used as probes for FISH experiments in various plant species (Maluszynska 2002). In all higher eukaryotes the 45S rRNA genes (45S rDNAs) are clustered in tandem arrays (Appels and Honeycutt 1986) and can be located at one or several chromosomal loci providing reliable landmarks for chromosome identification. Simultaneous use of these probes has provided effective markers for analysis of genome evolution in *Clivia* (Ran *et al*. 2001) and chromosome identification and karyotyping, e.g. in *Brachypodium* (Hasterok *et al*. 2004), *Brassica* (Hasterok *et al*. 2001, 2005), *Lilium* (Marasek *et al*. 2004) and *Hordeum* (Taketa *et al*. 1999). FISH has been successfully applied to detect somaclonal variation in *Triticum aestivum* L. derived from long-term suspension culture (Leitch *et al*. 1993) and in a regenerated variant of *Allium tuberosum* (Do *et al*. 1999). In *Tulipa*, the number and position of the ribosomal genes have been used for evaluating the cytological diversity between *T. gesneriana* and *T. fosteriana* cultivars (Mizuochi *et al*. 2007) and for identification of chromosomes in hybrids (Marasek and Okazaki 2008).

The objective of the present study was to analyze the genome variability in long-term propagated tulips. The investigation focuses on the polymorphism of the number, appearance and chromosomal location of ribosomal DNA (rDNA) sites in *T. gesneriana* cv. 'Prominence' propagated conventionally and plants derived from *in vitro* culture. This cultivar was chosen due to high diversity of somaclonal variants which different phenotypes were stable in a few growing seasons.

MATERIALS AND METHODS

Plant material

In this study, shoots of tulip (*Tulipa gesneriana* L.) cv. 'Prominence' were used which were propagated *in vitro* for one and a half and four years. Plant material was multiplied *in vitro* by the means of adventitious shoot regeneration in the presence of thidiazuron (TDZ) (Podwyszynska and Marasek 2003). Bulbs were formed *in vitro* and then grown outdoors in insect-proof-tunnel (Podwyszynska 2005, 2006). First tissue culture (TC)-derived plants, including true-to-type Prt 3, were planted *ex vitro* in 1999, and subsequent plants in 2000 and 2001. During subsequent years of cultivation, 2003-2005 SV was determined based on phenotype observation (Podwyszynska 2005). In the present study, morphology of micropropagated plants was evaluated in 2006 and 2007 and one true-to-plant Prt 3 and four somaclonal variants with malformed flowers and leaves were selected for karyotype analysis (**Table 1, Fig. 1B-E**). Morphology was evaluated when plants were in full bloom, which occurred at the beginning of May. The morphological traits recorded were determined as in the previous study (Podwyszynska 2005). Plants of the original cultivar which had been propagated by conventional means served as the standard (**Fig. 1A**). Bulbs of selected somaclonal variants were collected in mid June and stored at 20°C till October then cooled for three months and rooted in soil. Two different plants for standard and one plant per mutant were taken for karyotype analysis.

Preparation of chromosomes

For karyotype analysis, root tips were pre-treated with 0.05% colchicine for 2.5 h, fixed in 3:1 ethanol: glacial acetic acid, then washed in enzyme buffer (0.01 M sodium citrate, pH 4.8) and digested in an enzyme mixture comprising 2% (w/v) cellulase Onozuka-RS (Yakult Honsha Co., LTD. Japan) and 1% (w/v) pectolyase Y-23 (Kyowa Chemical Products Co., LTD. Japan) for 2.5 h at 37°C. Squashing was performed by the dry ice method.

Table 1 Phenotypes of plants used for karyotype analysis.

Code	Years *in vitro*	Phenotype
Standard	-	'Prominence' – propagated conventionally: plants height 45.5 cm, flower dark red, flower length 6.5 cm, base ivy green edged with yellow, pollen blush black.
Prt 3	1.5	Plant height 45.9 cm, flower length 6.0 cm, all other characteristics as in standard.
Prs 54	4	Plant height 33 cm, flower length 5.5 cm, five tepals with highly irregular edges and white stripes, no stamens, pistil malformed.
Prs 72	4	Plant height 48 cm, flower length 6.1 cm, tepals with irregular edges and white stripes, the least malformed flower.
Prs 74	4	Plant height 42.8 cm, flower length 4.8 cm, narrow tepals with irregular edges, white stripes, shorter flower stem.
Prs 75	4	Plant height 36 cm, flower length 5 cm, tepals with irregular edges and white stripes.

DNA probes and fluorescence *in situ* hybridization

The 5S rDNA probe was amplified and labelled by PCR with digoxigenin-11-dUTP (Roche) of the cloned *T. fosteriana* 5S rDNA sequence (Mizuochi *et al*. 2007) using universal primers T3 and T7. The thermal cycle conditions were as follows: 95°C for 4 min, 32 cycles of 95°C for 20 sec, 53°C for 1 min, and 72°C for 1 min, followed by 72°C for 7 min. The 45S rDNA probe was labelled by standard nick translation with tetramethyl-rhodamine-5-dUTP (Roche) of the cloned *T. fosteriana* 45S rDNA (Mizuochi *et al*. 2007).

FISH was performed as described by Hasterok *et al*. (2001) with minor modifications. Slides were pre-treated with RNase A (100 µg/ml) in 2× SSC for 1 h at 37°C, then washed in 2× SSC, treated with 10 mM HCl at 37°C for 2 min followed by incubation in pepsin solution (5 µg/ml) for 10 min (Wako Pure Chemical Industries, Ltd.), washed in 2× SSC and post-fixed in 1% formaldehyde in PBS buffer for 10 min, washed again in 2× SSC and dehydrated in absolute ethanol. The hybridization mixture consisted of target DNA (150 ng of each per slide), 50% deionised formamide, 10% dextran sulphate, 2× SSC, 1% SDS, and salmon sperm blocking DNA in 80- to 100-fold excess of labelled probes. The hybridization mixture was pre-denatured at 75°C for 10 min and applied to the chromosome preparations followed by denaturing at 70°C for 10 min and allowed to hybridize overnight in a humid chamber at 37°C. The post-hybridization washes were carried out for 15 min in 2× SSC at room temperature, followed by washes in 0.1× SSC at 42°C for 30 min. The digoxigenin probe was immunodetected using a standard protocol with FITC-conjugated anti-digoxigenin (Roche). The chromosomes were counterstained with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) in Vectashield (Vector Laboratories, Inc. Burlingame. CA). Images of chromosomes spreads were captured with a digital camera (DP70, Olympus, Japan) attached to a microscope (BX60, Olympus, Japan) with an appropriate filter and then processed using software (DP Manager, Olympus, Japan).

Karyological study

Ten metaphase plates of 'Prominence' and each of TC-derived plants were used for chromosome measurements. Data were collected from two different plants for standard and one plant per mutant. The short arm, long arm and total chromosome length and centromeric index (percentage of short arm length to the total length of chromosome) and arm index (ratio of long arm to short arm lengths) were determined using freeware application MicroMeasure available on the Internet at the http://www.colostate.edu/Depts/Biology/MicroMeasure. Standard deviations were calculated for mean values. Chromosomes were positioned within the karyotype based on the decreasing length of the short arm, centromeric index and signal patterns observed after fluorescence *in situ* hybridization. Nomenclature for the centromeric position on the chromosome was according to Levan *et al*. (1964) based on

Fig. 1 Standard 'Prominence' propagated conventionally (**A**) and *in vitro* produced tulip plants at the full flowering coded Prs 54 (**B**), Prs 72 (**C**), Prs 74 (**D**), Prs 75 (**E**).

arm index (long to short arm ratio) (1.0–1.7: median chromosomes; 1.7–3.0: submedian; 3.0–7.0: subterminal; 7.0–∞: terminal).

RESULTS

Somaclonal variation in long–term micropropagated tulip plants and phenotype observation

The somaclonal variants analysed in this study were considered as plants with major changes. They revealed three recurrent phenotypes: six plants of Prs 72, three plants of Prs 74 and two plants of Prs 75, while Prs 54 was the single plant (**Table 1, Fig. 1B-E**). Such phenotypes were also observed in 2007 (data not presented). The basic colour of tepals dark red was unchanged in all of the somaclonal variants. Flowers of these plants were malformed with white stripes, less or more irregular notched tepals, malformed pistils and less number of stamens or partly malformed stamens. Leaves had thicken, vitreous venation which subsequently got necrotic. Prs 74 had the most malformed flowers and slightly shorter flower stem and tepals. The flower of Prs 72 were the least malformed. With the exception of this phenotype, the average plant heights and flower lengths of the remaining somaclonal variants were lower compared to standard. TC-plant considered as true-to-type Prt 3 had the same flower colour and size as standard (**Table 1**).

Karyotype analysis in standard 'Prominence' and TC-derived plants

Both standard 'Prominence' and TC-derived plants were diploids and they contained 24 chromosomes (2n = 2x = 24). In general, their karyotypes were very similar in shape, consisting of median, submedian and subterminal chromosomes; however the difference in number of each type of chromosomes has been recorded in somaclones as compared to the standard (**Table 2**). Detailed morphometric analysis revealed a small difference in the total length of all metaphase chromosomes. In standard 'Prominence' the total length of chromosome set was 288.35 ± 13.46 μm, whereas in TC-derived plants it ranged from 244.24 ± 18.79 μm in Prs 54 to 284.48 ± 15.64 μm in Prs 75.

Table 2 Total length of chromosomes in metaphase plate and karyotype formula in 'Prominence' standard and its progeny micropropagated *in vitro*.

Code	Karyotype length (μm) ± SD[a]	Type of chromosomes		
		Median	Submedian	Subterminal
Standard	288.35 ± 13.46	2	14	8
Prt 3	281.36 ± 17.12	5	12	7
Prs 54	244.24 ± 18.70	2	12	10
Prs 72	273.04 ± 12.10	3	13	8
Prs 74	270.24 ± 18.26	4	12	8
Prs 75	284.48 ± 15.64	2	14	8

a – mean was calculated for 10 metaphase plate (abbreviation: SD – standard deviation)

Number and distribution of ribosomal DNA in standard 'Prominence'

Fig. 2A illustrates chromosomal distributions of the 45S and 5S rDNA sites revealed by FISH using tetramethyl-rhodamine-5-dUTP labelled 45S rDNA (red fluorescence) and digoxigenin-11-dUTP labelled 5S rDNA (green fluorescence) as probes in standard 'Prominence'. Idiogramic representation for this metaphase is presented in **Fig. 3A**. The most notable feature of standard 'Prominence' is the large number of rDNA sites which effectively mark an individual chromosome or group of chromosomes. Each chromosome was characterized by the presence of two or three loci of 5S genes (53 sites). 45S rDNA loci were carried on 14 chromosomes (8 large discrete and 6 minor sites). 45S rDNA were located at telomeric positions on the long arm of the chromosomes. 5S rDNA sites were predominately located in intercalative positions on the long arms, in close proximity to the centromere and in the telomeric position on the short arms. In addition, the first pair of median chromosomes possessed two 5S rDNA loci intercalary located on the short arm and the chromosomes of the twelfth pair had pericentromeric 5S rDNA loci on the short arms (**Fig. 3A**). The labeling pattern of 45S and 5S rRNA gene probes was identical on homologous chromosomes with an exceptions of tenth pair of chromosomes where the signal of 5S rDNA was exhibited on the long arm only on one of chromosome (**Fig. 3A**).

Cytological diversity with respect to number and size of rDNA loci in TC-derived plants

The results of the FISH experiment for TC-derived plants are shown on **Fig. 2B-F** and **Fig. 3B-F**. Distribution of 5S rDNA loci in plants derived *in vitro* is summarised in **Table 3**. Most of the chromosomes showed the hybridization pattern of standard 'Prominence' regarding the position of rDNA loci. The common feature is the telomeric position of 45S rDNA loci on the long arm whereas 5S rDNA loci oc-

Table 3 Distribution of 5S rDNA loci in 'Prominence' standard and TC-derived plants.

	Labelled chromosomes				Position of 5S rDNA loci		
	One arm	Two arms	Two bands on one arm	Three bands on one arm	Telomeric	Pericentromeric	Intersticial
Standard	3	21	4	-	22	2	29
Prt 3	3	21	5	-	22	1	27
Prs 54	4	20	5	1	20	4	26
Prs 72	2	22	5	1	22	2	27
Prs 74	7	17	4	-	19	-	26
Prs 75	6	18	5	-	22	-	24

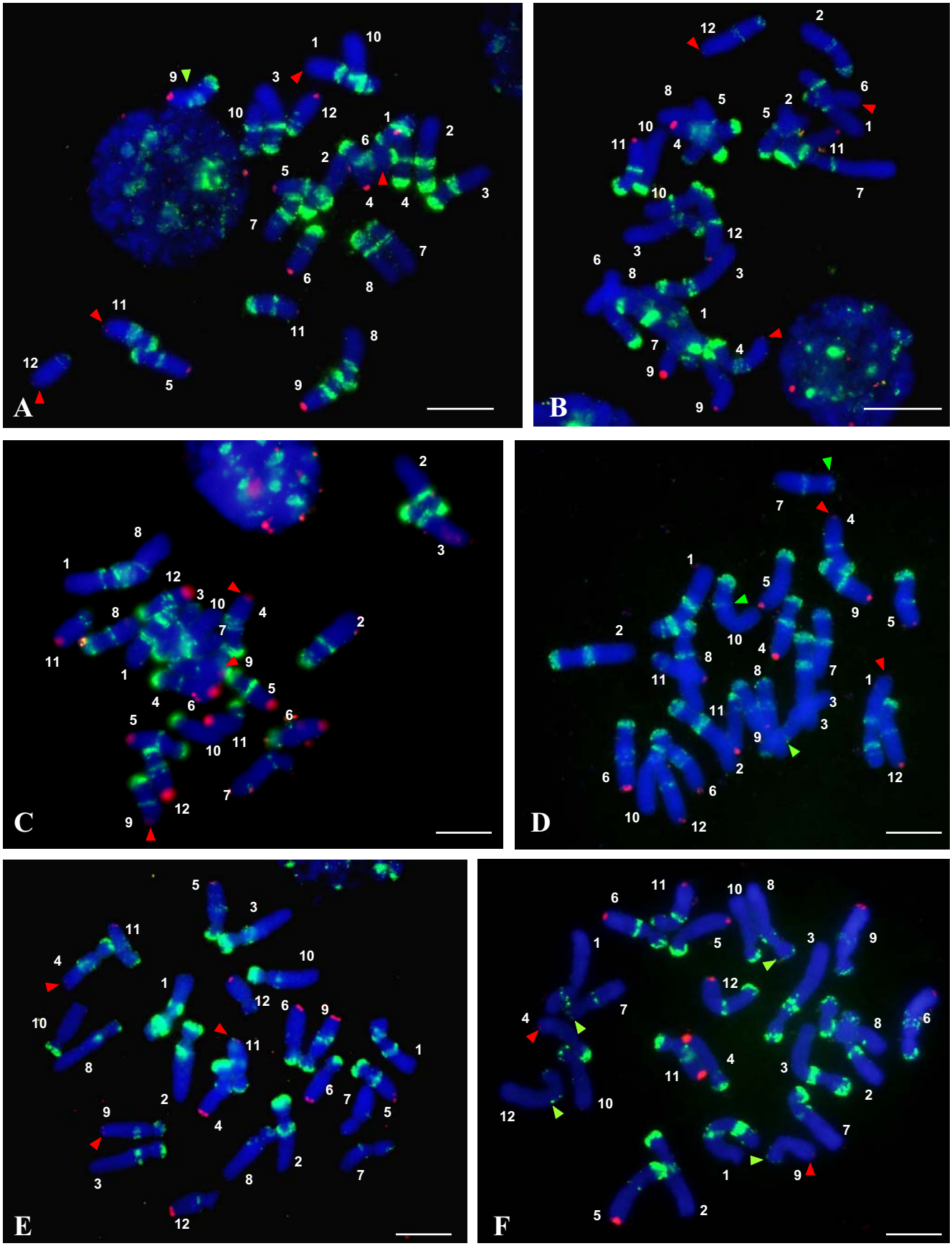

Fig. 2 Double-target FISH of 5S rDNA (green fluorescence) and 45S rDNA (red) probes to chromosomes of 'Prominence' (**A**), and *in vitro* produced tulip plants coded Prt 3 (**B**), Prs 54 (**C**), Prs 72 (**D**), Prs 74 (**E**), Prs 75 (**F**). Arrowheads indicate the faint 45S rDNA (red) and 5S rDNA (green) signals, which are detected but are invisible or poorly visible in this figure. Chromosomes are stained with DAPI. The bars represent 10 µm. Numbers from 1 to 12 refer to position of chromosomes on idiograms in **Fig. 3**.

cupied a predominately interstitial position on the long arm and terminal positions on the short arm.

Detailed analysis revealed that the size of hybridization signals, as well as the number of sites of 45S and 5S genes significantly differed between standard 'Prominence' and TC-derived plants (**Table 4**). A loss of rDNA loci as well as

the reduction of the loci size occurred in all regenerated plants (**Fig. 2, Fig. 3**). A few of the chromosomes exhibited a gain of 5S rDNA loci, e.g. in Prt 3 one of the chromosomes of the sixth pair gained a minor site in the pericentromeric position on the short arm (**Fig. 3B**). The percent of length of rDNA signals in entire chromosome set of the standard 'Prominence' equalled 29.26 whereas in the TC-derived plants it ranged from 27.57% in Prs 54 to 20.6% in Prs 75. The lowest number of 45S rDNA sites was observed in accession Prt 3 (9), while the highest was found in Prs 72 (13). The number of 5S rDNA genes was determined to range from 46 in Prs 75 to 52 in Prs 54. In three genotypes (Prs 54, Prs 72 and Prs 74) the difference was observed in the number of 5S rDNA loci depending on metaphases analysed (**Table 4**).

A noticeable feature is the higher polymorphism of rDNA loci on homologous chromosomes as compared to standard (**Table 4**). In Prt 3 six pairs of polymorphic chromosomes were recorded regarding 5S rDNA loci and one pair for 45S rDNA loci (**Fig. 3B**). In Prs 54 the lack of the

Table 4 Characteristics of rDNA loci in 'Prominence' standard and TC-derived plants.

Code	Number of 5S rDNA loci	Number of 45S rDNA loci	% rDNA [b]	Polymorphic 5S rDNA sites[c]	Polymorphic 45S rDNA sites[c]
Standard	53	14	29.26	10^{th} (1q)	-
Prt 3	50	9	21.33	1^{st} (1p); 2^{nd} (1q); 6^{th} (1p); 9^{th} (1q); 10^{th} (1q); 11^{th} (1q)	6^{th} (1q)
Prs 54	46-50[a]	11-12[a]	27.57	1^{st} (1p); 2^{nd} (1p); 6^{th} (1q); 7^{th} (1p); 10^{th} (1q); 11^{th} (1p); 12^{th} (1p,1q)	9^{th} (1q)
Prs 72	48-51[a]	13	23.13	5^{th} (1q); 6^{th} (2q); 8^{th} (1q); 10^{th} (1q)	1^{st} (1q)
Prs 74	44-45[a]	12	23.16	10^{th} (1q); 12^{th} (1p, 1q)	-
Prs 75	46	11	20.60	1^{st} (1p, 1q); 4^{th} (1p); 5^{th} (1q); 6^{th} (1q); 9^{th} (1q); 11^{th} (1q); 12^{th} (1q)	12^{th} (1q)

[a] The number of rDNA loci differs depending on metaphase plates
[b] Percent of rDNA signals in entire chromosome set
[c] Polymorphic chromosome pairs with respect to rDNA sites, in brackets the number and position of polymorphic loci (abbreviation: p – short arm; q – long arm)

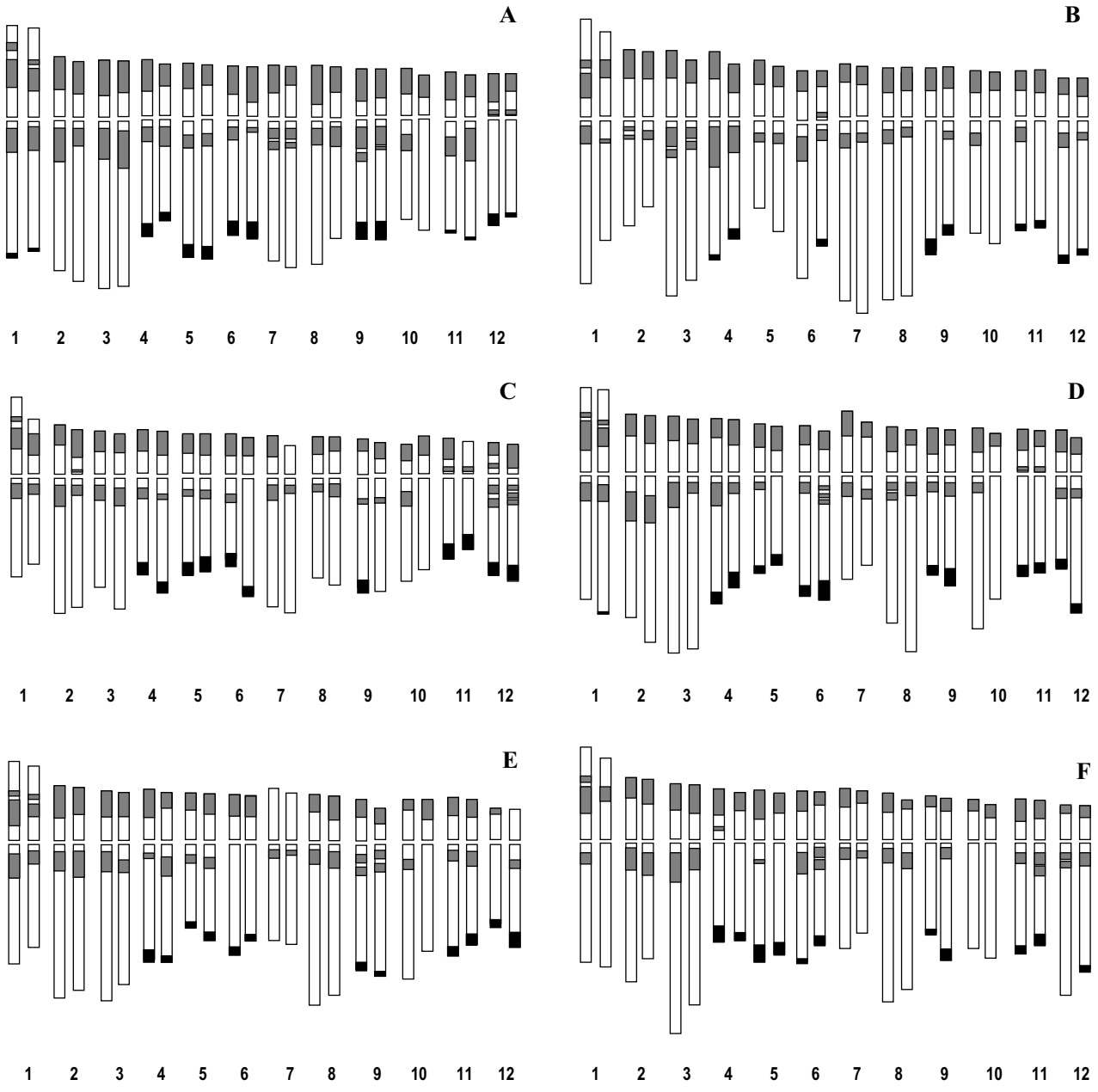

Fig. 3 Idiograms of metaphase chromosomes depicting the position of 5S rDNA (grey bands) and 45S rDNA loci (black bands) in standard 'Prominence' (**A**), and *in vitro* produced tulip plants coded Prt 3 (**B**), Prs 54 (**C**), Prs 72 (**D**), Prs 74 (**E**), Prs 75 (**F**).

5S rDNA locus was observed in 7 chromosome pairs and one pair was polymorphic regarding 45S rDNA loci (**Fig. 3C**). In Prs 72 four chromosome pairs were polymorphic with respect to the 5S rDNA loci and first pair of median chromosomes with respect to the 45S rDNA signals (**Fig. 3D**). In Prs 74 polymorphism was exhibited in two chromosome pairs (**Fig. 3E**) while in Prs 75 the total number of polymorphic chromosome pairs was seven (**Fig. 3F**).

DISCUSSION

Somaclonal variation in TC-derived plants – phenotype observation

There have been several reports of somaclonal changes in various TC-derived plants, such as bushy growth in rhubarb (Zhao *et al*. 2008), and malformed flowers in orchid (Chen *et al*. 1998), oil palm (Rival *et al*. 2000), rhubarb (Zhao *et al*. 2008) and rice (Lee *et al*. 1999). In the case of tulips, micropropagated according to our new protocol, the first reliable information on phenotypic SV was obtained in 2005, when more than 30% of the plants had flowered that occurred as early as in fourth or fifth growing season (Podwyszynska 2005). Those results showed that the variation was not detected or was less than 3.3% in progeny lines derived from cultures maintained *in vitro* for less than three years. However, within the plants derived from four-year-old cultures, the half of plants of 'Prominence' was changed. The variation concerned minor or major changes in flower and leaf morphology. All of the off-types had normal coloured flowers and most of them had minor changes. They represented two repeated phenotypes, in the first one, tepals had acute tips and the outer ones curved back as in lily-flowered tulips, and in the second one, tepals were slightly notched. Some plants were extremely abnormal with significantly shorter flower stems, malformed tepals, stamens and pistils, their leaves had thicken, glassy venation. Subsequent studies showed that molecular analysis with RAPD, ISSR and AFLP markers revealed DNA polymorphism within practically all of the tested somaclonal variants and standard plants (Podwyszynska *et al*. 2006, 2007). Some of the true-to-type TC-derived plants, analyzed with ISSR or AFLP markers had also altered DNA pattern compared to standard plant (Podwyszynska *et al*. 2007). That corresponds with our present results concerning the changed karyotype of true-to-type plant Prt3. Results of the present study, where phenotype was evaluated in 2006 and 2007, showed that the changes such several types of flower malformation and leaf thicken venation have been stable and transmitted through vegetative propagation, similarly as it was observed in the earlier studies mentioned above. Thus the described tulip phenotypic variation, induced by *in vitro* culturing had the nature of genetic changes that was detected both on DNA and chromosomal levels, and proved by the cytological and molecular analyses.

Karyotype analysis

A wide range of numerical variation of chromosome among regenerated plants has been reported (Orton 1980; Creissen and Karp 1985; Menéndez-Yuffá *et al*. 2000; Nakano *et al*. 2006). In our study, however all the tulips derived from long-term *in vitro* culture had normal chromosome number (2n = 2x = 24). The karyotypes of the standard and the micropropagated plants were very similar in shape consisting of median, submedian and subterminal chromosomes. The differences in number of each type of chromosomes and in the total length of all metaphase chromosomes may be either explained by structural changes in the chromosomes or they are an artefact of preparation by squashing, which may increase total chromosome length and change the arm indices (Bosemark and Bormotov 1962). These deformations are not evenly distributed over the chromosome arms; on average, long arms are stretched more than short arms (Sybenga 1959), they may also depend on the chromosome's position in relation to the centre of the squash.

Markers for tulip chromosomes

The genes for 45S rRNA and 5S rRNA have provided useful markers for chromosome identification and karyotyping in diverse plant species such as *Brassica* (Hasterok *et al*. 2005), *Medicago* (Falistocco and Falicinelli 2003), *Hordeum* (Leitch and Heslop-Harrison 1993) and *Lilium* (Marasek *et al*. 2004). In *Tulipa*, *in situ* hybridization with 5S rDNA and 45S rDNA probes has been used previously for evaluating the cytological diversity within *T. gesneriana* and between *T. gesneriana* and *T. fosteriana* cultivars (Mizuochi *et al*. 2007), and for chromosome identification in 'Purissima' (*T. fosteriana*), and its progenies (Marasek and Okazaki 2008). In our study, fluorescence *in situ* hybridization with rDNA probes established markers, which enabled accurate identification of all of individual chromosomes in metaphase plate. The noticeable feature of analysed genotypes is the large number of rDNA loci. In standard, 53 loci of 5S rDNA were distributed on each chromosome whereas 45 S rDNA loci were carried on 14 chromosomes in metaphase plate. This is with agreement with previous study on *Tulipa* genome where the number of 5S rDNA sites for *T. gesneriana* cultivars ranged from 45 to 56 and they were located on each chromosome, the number of 45S rDNA sites differed from 11 to 13 (Mizuochi *et al*. 2007). The distribution of rDNA sites in 'Prominence' karyotype agrees with data of Mizuochi *et al*. (2007) for other *T. gesneriana* cultivars. In both studies, the 45S rDNA sites were located exclusively in the telomeric position on the long chromosome arms whereas 5S rDNA predominately occurred on the short arm at telomeric region and at interstitial position on both chromosome arms. Similar distribution of 45S and 5S ribosomal genes were also recorded in other plants, e.g. telomere mapping of 45S rDNA loci was also recorded in *Paeonia* (Zhang and Sang 1999) and *Brassica* (Hasterok *et al*. 2005) whereas the variable differential position of 5S rDNA sites along chromosome was also observed, e.g. in *Lilium* (Marasek *et al*. 2004) and *Brassica* (Hasterok *et al*. 2005). A few pair of 'Prominence' standard chromosomes, e.g. the longest median chromosomes shared the same pattern of rDNA sites with other *T. gesneriana* cultivars analysed by Mizuochi *et al*. (2007), which may suggest that those chromosomes are highly conserved in all *T. gesneriana* cultivars.

Chromosome rearrangements in regenerated plants

Chromosome rearrangements have been implicated as closely associated with abnormal morphology of regenerated plants (Sacristan and Wendt-Gallitelli 1971; Brettell *et al*. 1986). In our study, fluorescence *in situ* hybridization with ribosomal gene (rDNA) probes provided the clear evidence that the chromosome structural variation occurred in TC- derived tulips exhibiting variation in plant morphology. A few of the chromosomes had undergone several rearrangements involving rDNA loci lost and gain, e.g. in Prs 54 5S rDNA loci were lost for example from telomeric region on the short arm of one of the homologous chromosomes of the seventh and eleventh pairs whereas one of the chromosomes of the second pair gained 5S rDNA locus in pericen-tromeric position on the short arm (**Fig. 3C**). There are many possible mechanisms which generate variation in size, number and position of rDNA sites, e.g. events leading to direct exchange of chromosome or chromatid fragments in mitotic and meiotic cell divisions (homologous and non-homologous unequal crossing–over) and events generating chromosome rearrangement (translocation, deletion, duplication and inversion) (Lee and Phillips 1988; Leitch and Heslop-Harrison 1993; Moscone *et al*. 1999).

In long term micorpropagated tulips, it seems that there is a tendency to reduce the number of rDNA loci. The lost of the loci has occurred both in interstitial and telomeric re-

gion of chromosomes and the total number of lost 45S rDNA loci differed form one in Prs 72 to five in Prt 3 and 5S rDNA loci ranged from three in Prt 3 to seven in Prs 75 (**Fig. 3**). These results are consistent with earlier reports on the lost of the rRNA genes in a long-term wheat suspension culture where most sequences have been lost by terminal deletion (Leitch *et al*. 1993). In triticale derived from tissue culture in general rRNA genes were stable except for one family where there was a marked reduction of the gene number and most of deficiencies occurred in one of homologous chromosome (Brettell *et al*. 1986). This is in contrast with the observation of wild *Allium tuberosum* and its regenerated variant which exhibited the same chromosomal location of both rDNAs (Do *et al*. 1999).

The significant difference in the intensity of the hybridization signal was observed which may suggest that there is a difference in the copy number of repeat unit between loci. The strength of hybridization signal is generally considered related to the copy number of genes (Maluszynska and Heslop-Harrison 1993). The small size of the loci may be either explained by the partial deletion of a major locus or the retention of a minor locus.

In conclusion, the micropropagation of tulip based on multiplication of adventitious shoots can increase the risk of mutation, especially when the shoots are maintained *in vitro* for four years or more. Assessing the stability of plant materials would reduce the risk of mutated plant production. Molecular techniques such as RAPD, ISSR or AFLP have been most frequently used for routine monitoring the genotype stability (Aravanopoulos 2003; Renau-Morata *et al*. 2005; Saker *et al*. 2006; Podwyszynska *et al*. 2007). In our study we proved the ribosomal genes are also important tools for assessing the stability of TC-derived plants.

In spite of somaclonal variation occurring within tulip TC-plants, the new micropropagation method can be used for production of the virus-free stock plants of new genotypes. However, the time of shoot multiplication *in vitro* should be limited to two or three years and plant material ought to be controlled for genetic stability by phenotypic evaluation or molecular as well as the advanced cytological analyses. Application of the micropropagated method markedly reduces the time needed for introduction of the new cultivars on the market from 25 years to a half. On the other hand, the high frequency of genetic variation associated with long-term-cultures can be further enhanced by the use of chemical or radial mutagens and may be used for a mutation breeding of tulips.

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