PLANT RESEARCH INTERNATIONAL

Eindrapportage PT-13242

Veredelingsonderzoek naar de ontwikkeling van virusresistente broei tulpen

Application of GISH-techniques in breeding research of virus resistant forcing tulips

Productschap Tuinbouw

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Summary

The application of genomic in situ hybridisation (GISH), a chromosome painting technique in breeding research of virus resistant forcing tulips proved an important breakthrough in this field. It is known that *Tulipa fosteriana* transmits TBV-resistance to the Darwin hybrids (*T. gesneriana x T. fosteriana*). Until now these Darwin hybrids were sterile and introgression of virus-resistance to the forcing tulip was impossible. In a project focussed on resistance breeding in tulip however GF-hybrids were found with pollen-fertility. By backcrossing to *T. gesneriana* various BC1 populaties were produced. By using GISH of 99 GF-hybrids the genome composition was analysed. 92 of them proved to be real GF-hybrids. By analysing GGF-hybrids a high percentage of intergenomic recombination (recombination between G- and F-chromosomes) was found. This proves that through introgression of *T. fosteriana* chromosome segments into the genome of *T. gesneriana* introgression of virus resistance is possible. In a second backcross population (BC2) of 'Purissima' (which appeared to be a GF-hybrid) further introgression was found. In these hybrids only about 10% of the genome originated from F. fosteriana. On this moment in TTI- research molecular markers are developed which will be used to trace the virus resistance in these GGF-hybrids.

Samenvatting

Door toepassing van Genomische in situ hybridisatie (GISH), een chromosoomkleuringstechniek bij de veredeling van virusresistente broeitulpen hebben aangetoond dat er een belangrijke doorbraak is bereikt. Het is bekend dat *Tulipa fosteriana* TBV resistentie bezit en doorgeeft aan de Darwin (GF) hybriden (*T. gesneriana x T. fosteriana*). Tot nu toe waren deze Darwin hybriden steriel en was introgressie van virus resistentie in het broeisortiment onmogelijk. In een project gericht op gestapelde resistenties bij tulp zijn echter GF hybriden gevonden met fertiliteit. Hieruit zijn diverse terugkruisingspopulaties (GGF) verkregen. Met behulp van GISH is van 99 GF-hybriden de genoomsamenstelling vastgesteld. 92 bleken inderdaad echte GF-hybriden te zijn. In GGF hybriden is aanzienlijke intergenomische recombinatie aangetoond. Dit toont aan dat door introgressie van *T. fosteriana* chromosoomsegmenten in het *T. gesneriana* genoom introgressie van virusresistentie mogelijk is. In een tweede terugkruisingsgeneratie van 'Purissima', een GF-hybride werd voortgaande introgressie aangetoond. In deze hybriden (G x GGF) bleek nog slechts 10% van het genoom afkomstig te zijn van *T. fosteriana.* In dit materiaal wordt in voortgaand TTI-onderzoek met behulp van moleculaire merkers de virus resistentie aangetoond.

1. Introduction

According to taxonomic classification by Van Raamsdonk and De Vries (1995) and Van Raamsdonk *et al*. (1997), the genus *Tulipa* is divided into two subgenera *Tulipa* and *Eriostemones*. Subgenus *Tulipa* comprises of about 55 species which are arranged into five sections, including the cultivated *T. gesneriana*. Subgenus *Eriostemones* comprises about 20 species arranged in three sections (Van Raamsdonk and De Vries 1992). Many tulip varieties have been developed mainly in the Netherlands and more than 8,000 of them are included in the list of 'tulips names' (Van Scheepen 1996). Of the primary cultivars distributed to the commercial markets consisting of more than 1100 cultivars (Van Scheepen 1996), the majority of them belong to *T. gesneriana* L from the section *Tulipa* which is the collective name given to a large number of varieties of unknown origin (Killingback 1990). The second commercial group is Darwin hybrid tulips, which have been obtained from interspecific crosses between cultivars of *T. gesneriana* and *T. fosteriana* Hoog ex W. Irving genotypes of the section *Eichleres* (Van Tuyl and Van Creij 2007).

Crossability in genus *Tulipa*

In order to enrich the commercial assortment with desirable traits interspecific crosses are usually made between genotypes of *T. gesneriana* and other *Tulipa* species. *T. gesneriana* has been crossed successfully with only 12 out of the approximately 55 tulip species by using conventional breeding methods (Van Eijk *et al*. 1991, Van Raamsdonk *et al*. 1995). Several hybrids have been obtained from crosses between *T. gesneriana* and species of the section *Eichleres*; like the hybrids obtained between *T. gesneriana* and *T. fosteriana* Hoog, *T. kaufmanniana* Regel, *T. greigii* Regel, *T. eichleri* Regel, *T. ingens* Hoog, *T. albertii* Regel (formerly *T. vvedenskyi*) and *T. didieri* Jord (**Fig 1**). In many other interspecific crosses hybrid development was prevented by crossing barriers. Crosses between *T. gesneriana* and species from the *Eriostemones*, like *T. tarda* Stapf, *T. pulchella* Fenzl and *T. turkestanica* Regel have never been successful (Van Eijk *et al*. 1991; Van Raamsdonk *et al*. 1995).

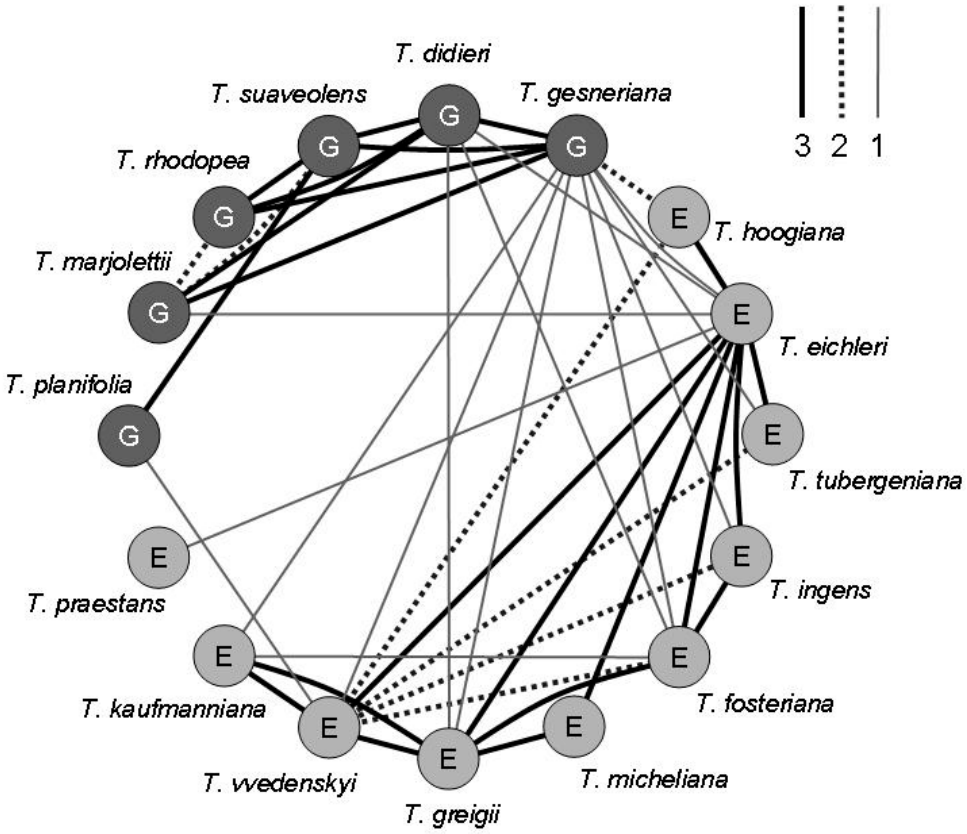

Fig. 1. Crossing polygon of species of section *Eichleres* ('Ei') and *Tulipa* ('Ge'). Meaning of lines: 1: several successful attempts, effectivity low; 2: one successful attempt, effectivity high; 3: several successful attempts, effectivity high. Low effectivity: less than $5F_1$ bulbs per seed pod; high effectivity: more than 15 F_1 bulbs per seed pod. The data shown are pooled results of all crosses carried out per combination. Modified from Van Raamsdonk *et al*. (1995).

Tulip breaking virus resistance (TBV) in genus *Tulipa*

One of the most important pathogen in Tulip is Tulip breaking Virus, the causal agent of flower breaking. Although producing beautiful flames in pigmented flowers TBV is a serious problem in the production of tulip bulbs and flowers. The virus causes a reduction in bulb number, weight and quality. The virus spread in the field is difficult to control. The virus is transmitted by aphids and can be therefore spread through the field in a short period of time. Host resistance is the best approach to prevent such diseases. The use of resistant cultivars reduce the use of chemical control, increase bulb production and require less labor for sorting and selecting harvested bulbs.

T. gesneriana cultivars are characterized by various flower colors, good forcing quality, resistance to *Fusarium oxysporum* (bulb-rot) and susceptibility for Tulip Breaking Virus (TBV). The high levels of resistance for this virus are found in some cultivars of *T. fosteriana* (Romanow *et al*. 1991; Eikelboom *et al*. 1992; Straathof and Eikelboom 1997). For instance, *T. fosteriana* cultivars 'Cantata' and 'Princepss' are characterized by a high degree of resistance, while the level of resistance in 'Juan' and 'Madame Lefeber' varied in the experiments.

An important goal in tulip breeding is to combine the desirable horticultural traits from these two sections into new cultivars. Many interspecific crosses have been made between resistant to TBV *T. fosteriana* cultivars and *T. gesneriana* cultivars (Van Tuyl and Van Creij 2007), which generated highly TBV resistant genotypes called Darwin Hybrid tulips (Eikelboom *et al*. 1992; Van Tuyl and Van Creij 2007). F_1 tulip hybrids resulted from crosses between *T. gesneriana* and *T. fosteriana* genotypes are usually sterile or show low fertility. However through large scale screening it is possible to select genotypes of GF hybrids with reasonable high frequencies of fertile pollen that could be used for backcrossing.

Meiotic polyploidization

The majority of tulip species and cultivars is diploid (2n = 2x = 24) but also triploids (2n = 3x = 36), tetraploids (2n = 4x = 48) and even some pentaploids (2n = 5x = 60) have been found (Holitscher 1968; Kroon 1975: Zeilinga and Schouten 1968a, b; Kroon and Jongerius 1986; Van Scheepen, 1996). According to Kroon and Van Eijk (1977) triploid and tetraploid tulips are likely to have arisen as a result of the occurrence of diploid gametes in diploid cultivars. An important feature of diploid Darwin Hybrid tulips hybrids is that they can produce functional n gametes but also 2n gametes. This provides the opportunity to generate diploid and polyploidy BC1 progenies from backcrossing FG hybrids to *T. gesneriana* parents. Among Darwin hybrid tulips resulting from interspecific crosses between *T. gesneriana* and *T. fosteriana*, diploid (2n = 2x = 24), triploid (3x = 36) e.g., 'Apeldoorn', 'Ad Rem', 'Pink Impression' and some tetraploid (2n = 4x = 48) hybrids such as 'Tender Beauty' can be found, in spite of the fact that both of the parental cultivars are diploid (2n = 2x = 24) (Van Scheepen 1996). By studying karyotypes, Marasek *et al*. (2006) demonstrated that the triploid Darwin Hybrid tulip 'Yellow Dover' has two copies of the *T. gesneriana* genome and one copy of the *T. fosteriana* genome which suggest that *T. gesneriana* has supplied the diploid gamete. The most important advantage of meiotic polyploidization is that homoeologous recombination occurs between parental chromosomes during meiosis.

Polyploid tulip may have also resulted from interploidy crosses (). Crosses between diploid (2n = 2x = 24), triploid (2n = 3x = 36) and tetraploid (2n = 4x = 48) varieties were conducted. By making crosses between tetraploids, new tetraploids were obtained of which the best known is 'Judith Leyster' (Straathof and Eikelboom 1997). Crossing tetraploids with diploids (4x X 2x) can result in vigorously growing triploids e.g., 'World's Favourite' originating from a tetraploid seedling 'Denbola' x 'Lustige Witwe' crossed with a diploid *T. fosteriana* seedling (Straathof and Eikelboom 1997). Triploid varieties such as 'Lady Margot', 'Benny Neyman' and 'Sun Child' have been obtained by crossing diploid varieties with those that are tetraploid (2x X 4x) (e.g., 'Mrs. John T. Scheepers') (Van Scheepen, 1996). Upcott and Philip (1939) in diploid-triploid crosses observed progenies with chromosomes numbers from 24 to 48 while aneuploids having 25 chromosomes were most common (37%). According to Bamford *et al.* (1939) 50% of progenies resulting from the 2x X 3x crosses had 25 chromosomes while the chromosome number in other genotypes ranged from 24 to 31. In contrast, Okazaki and Nishimura (2000) reported that, in the 2x X 3x crosses 92.6% were diploids and 7.4% were aneuploids, while in the 3x X 2x crosses 60.0% were diploids and 40% were aneuploids.

3. Description of the project

Existing knowledge

GISH

Genomic *in sit*u hybridization (GISH) is a cytogenetic technique which utilizes genomic DNA of one parental genotype as a probe and excessive fragmented DNA of another parent as blocking DNA. GISH will enable the discrimination of parental genomes in hybrids and polyploid forms of tulips. This technique also detects chromosome recombination between chromosomes from different genomes and can be used to visualize the level of introgression in backcrossed progenies.

4. Results

4.1 Cytogenetic analysis of F1 hybrids

The genome constitution of 99 F1 genotypes has been analysed by GISH technique. Simultaneous application of differentially labelled total genomic DNA of *T. gesneriana* cultivar 'Ile de France' and *T. fosteriana* 'Princeps' enabled the discrimination of the parental genomes in Darwin Hybrid genotypes. The results are presented in Table 1 and Figure 1. The hybrid status has been confirmed for 92 genotypes. All F1 hybrid tested were diploids (2n = 2x = 24). Diploid F1hybrids consisted of 12 chromosomes of *T. gesneriana* and 12 chromosomes of *T. fosteriana* (Fig. 1a) whereas triploid 035083-3 comprised 24 T. gesneriana chromosomes and 12 *T. fosteriana* (Fig. 1b).

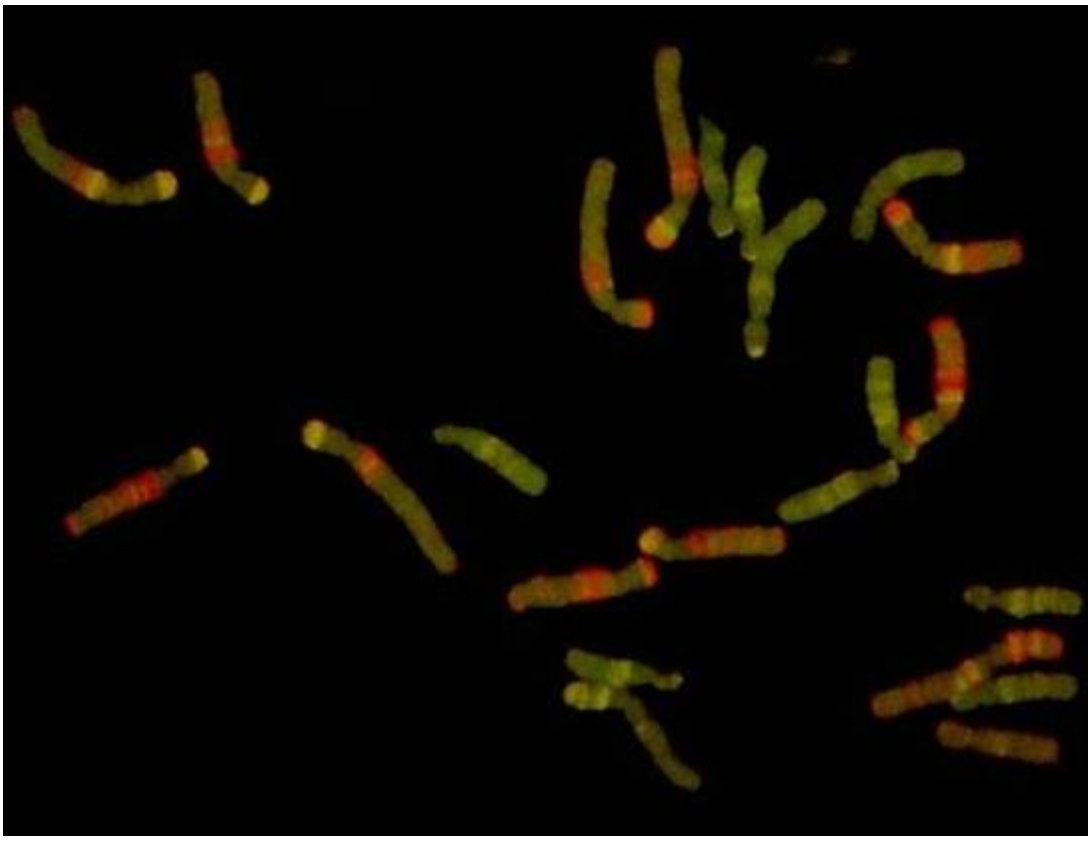

Fig. 1 GISH picture of diploid F1 hybrids 20622-36. Red fluorescence represents *T. gesneriana* genome and green fluorescence *T. fosteriana* genome, respectively.

Table 1 Hybrid status of F1 generation evaluated by GISH analyses (G- *T. gesneriana* chromosomes; F – *T. fosteriana* chromosomes)

No.	F1	Mather	Father	GISH	Hybrid status
1	20161-5	Bellona	103 Juan x Cantata	12G+12F	hybrid
2	20161-3	Bellona	103 Juan x Cantata	12G+12F	hybrid
3	20160-5	Bellona	102 Juan x Cantata	12G+12F	hybrid
4	20183-3	Bellona	127 Mad. Lef. x Ca	12G+12F	hybrid
5	20185-2	Bellona	135 Cantata x Mad. Lef.	24G	Not hybrid
6	20179-1	Bellona	121 Cantata x Juan	12G+12F	hybrid
7	20176-2	Bellona	118 Cantata x Juan	12G+12F	hybrid
8	20179-2	Bellona	121 Cantata x Juan	12G+12F	hybrid
9	20168-3	Bellona	110 Juan x Cantata	12G+12F	hybrid
10	20170-4	Bellona	112 Juan x Cantata	12G+12F	hybrid
11	20171-8	Bellona	113 Juan x Cantata	12G+12F	hybrid
12	20176-1	Bellona	118 Cantata x Juan	12G+12F	hybrid
13	20164-1	Bellona	106 Juan x Cantata	24G	Not hybrid
14	20164-2	Bellona	106 Juan x Cantata	12G+12F	hybrid
15	20164-4	Bellona	106 Juan x Cantata	12G+12F	hybrid
16	20164-5	Bellona	106 Juan x Cantata	12G+12F	hybrid

17	20170-7	Bellona	112 Juan x Cantata	12G+12F	hybrid
18	20171-4	Bellona	113 Juan x Cantata	12G+12F	hybrid
19	20179-4	Bellona	121 Cantata x Juan	12G+12F	hybrid
20	20180-3	Bellona	122 Cantata x Juan	12G+12F	hybrid
21	20165-2	Bellona	107 Juan x Cantata	12G+12F	hybrid
22	20165-4	Bellona	107 Juan x Cantata	24G	Not hybrid
23	20166-1	Bellona	108 Juan x Cantata	12G+12F	hybrid
24	20166-2	Bellona	108 Juan x Cantata	12G+12F	hybrid
25	20241-3	Pax	102 Juan x Cantata	12G+12F	hybrid
26	20243-3	Pax	113 Cantata x Juan	12G+12F	hybrid
27	20230-9	Ile de France	155 Princeps x Cantata	12G+12F	hybrid
28	20231-1	Generaal de Wet	102 Juan x Cantata	12G+12F	hybrid
29	20231-8	Generaal de Wet	102 Juan x Cantata	12G+12F	hybrid
30	20232-2	Generaal de Wet	104 Juan x Cantata	12G+12F	hybrid
31	20249-1	Pax	123 Cantata x Juan	12G+12F	hybrid
32	20249-2	Pax	123 Cantata x Juan	12G+12F	hybrid
33	20160-1	Bellona	102 Juan x Cantata	12G+12F	hybrid
34	20160-4	Bellona	102 Juan x Cantata	12G+12F	hybrid
35	20233-1	Generaal de Wet	104 Juan x Cantata	12G+12F	hybrid
36	20241-2	Pax	102 Juan x Cantata	12G+12F	hybrid
37	20233-10	Generaal de Wet	104 Juan x Cantata	12G+12F	hybrid
38	20242-1	Pax	104 Juan x Cantata	24G	Not hybrid
39	20242-4	Pax	104 Juan x Cantata	12G+12F	hybrid
40	20233-7	Generaal de Wet	104 Juan x Cantata	24G	Not hybrid
41	20221-3	Ile de France	137 Canta x Mad. Lef.	24G	Not hybrid
42	20214-2	Ile de France	121 Cantata x Juan	12G+12F	hybrid
43	20230-4	Ile de France	155 Princeps x Cantata	12G+12F	hybrid
44	20222-4	Ile de France	138 Cantata x Mad. Lef.	12G+12F	hybrid
45	S-20253-1	Pax	137 Cantata x Mad. Lef.	12G+12F	hybrid
46	20193-2	Bellona	148 Cantata x Princeps	12G+12F	hybrid
47	20214-1	Ile de France	121 Cantata x Juan	24G	Not hybrid
48	S-20250-2	Pax	126 Mad. Lef. x Cantata	12G+12F	hybrid
49	S-20254-1	Pax	141 Mad. Lef. x Princeps	12G+12F	hybrid
50	20193-7	Bellona	148 Cantata x Princeps	12G+12F	hybrid
51	20190-3	Bellona	143 Princeps x Mad. Lef.	12G+12F	hybrid
52	S-20248-1	Pax	121 Cantata x Juan	12G+12F	hybrid
53	S-20229-1	Ile de France	154 Princeps x Cantata	12G+12F	hybrid
54	S-20248-2	Pax	121 Cantata x Juan	12G+12F	hybrid
55	20189-8	Bellona	141 Mad. Lef. x Princeps	12G+12F	hybrid
56	20192-2	Bellona	147 Cantata x Princeps	12G+12F	hybrid
57	20190-4	Bellona	143 Princeps x Mad. Lef.	12G+12F	hybrid
58	S-20171-2	Bellona	113 Juan x Cantata	12G+12F	hybrid
59	S-20171-1	Bellona	113 Juan x Cantata	12G+12F	hybrid
60	S-20186-2	Bellona	136 Cantata x Mad. Lef.	12G+12F	hybrid
61	20186-3	Bellona	136 Cantata x Mad. Lef.	12G+12F	hybrid
62	20189-1	Bellona	141 Mad. Lef. x Princeps	12G+12F	hybrid
63	20187-13	Bellona	137 Cantata x Mad. Lef.	12G+12F	hybrid
64	S-20170-1	Bellona	112 Juan x Cantata	12G+12F	hybrid
65	S-20165-5	Bellona	107 Juan x Cantata	12G+12F	hybrid
66	S-20170-6	Bellona	112 Juan x Cantata	12G+12F	hybrid
67	20255-2	Pax	147 Cantata x Princeps	12G+12F	hybrid
68	20254-6	Pax	141 Mad. Lef. x Princeps	12G+12F	hybrid
69	20181-1	Bellona	123 Cantata x Juan	12G+12F	hybrid

70	20259-23	Pax	155 Princeps x Cantata	12G+12F	hybrid
71	20259-12	Pax	155 Princeps x Cantata	12G+12F	hybrid
72	20259-13	Pax	155 Princeps x Cantata	12G+12F	hybrid
73	20251-1	Pax	135 Cantata x Mad. Lef.	12G+12F	hybrid
74	20250-6	Pax	126 Mad. Lef. x Cantata	12G+12F	hybrid
75	20259-11	Pax	155 Princeps x Cantata	12G+12F	hybrid
76	20259-1	Pax	155 Princeps x Cantata	12G+12F	hybrid
77	20256-3	Pax	149 Cantata x Princeps	12G+12F	hybrid
78	20258-1	Pax	154 Princeps x Cantata	12G+12F	hybrid
79	20252-1	Pax	136 Cantata x Mad. Lef.	12G+12F	hybrid
80	20254-4	Pax	141 Mad. Lef. x Princeps	12G+12F	hybrid
81	20185-1	Bellona	135 Cantata x Mad. Lef.	12G+12F	hybrid
82	20176-3	Bellona	118 Cantata x Juan	12G+12F	hybrid
83	20185-4	Bellona	135 Cantata x Mad. Lef.	12G+12F	hybrid
84	20251-3	Pax	135 Cantata x Mad. Lef.	12G+12F	hybrid
85	20251-2	Pax	135 Cantata x Mad. Lef.	12G+12F	hybrid
86	20256-2	Pax	149 Cantata x Princeps	12G+12F	hybrid
87	20255-4	Pax	147 Cantata x Princeps	12G+12F	hybrid
88	20185-5	Bellona	135 Cantata x Mad Lef.	12G+12F	hybrid
89	20191-4	Bellona	Princeps x Mad Lef.	12G+12F	hybrid
90	20208-2	Ile de France	Juan x Cantata	12G+12F	hybrid
91	20239-20	Gen. de Wet	Cantata x Juan	12G+12F	hybrid
92	20622 –12?	Pax	Unknown	12G+12F	Hybrid
93	20167-31	Bellona	109 Juan x Cantata	12G+12F	Hybrid
94	20172-32	Bellona	114 Juan x Cantata	12G+12F	Hybrid
95	20180-3	Bellona	122 Juan x Cantata	24G	No hybrid
95	20180-32	Bellona	122 Juan x Cantata	12G+12F	Hybrid
97	20190-31	Bellona	143 Princeps x Mad. Lef.	12G+12F	Hybrid
98	20622-36	Bellona	129 Mad Lef x Cantata	12G+12F	Hybrid
99	20622-38	Pax	117 Cantata x Juan	12G+12F	Hybrid

4.2 Chromosome characteristic in Darwin hybrids

Morphometric analysis in 23 F1 hybrids revealed a difference in the total length of chromosomes representing genomes of *T. gesneriana* and *T. fosteriana*. The percentage of *T. gesneriana* and *T. fosteriana* genomes in these hybrids equaled 55.18±0.78% and 44.92 ± 0.6% respectively.

Fig. 1a-b shows GISH painted chromosomes complement of diploid GF hybrid 20208-2 whereas detailed morphometric data of its chromosomes are shown in Table 2. In this hybrid the difference of 28.2μm in the total length of all metaphase chromosomes between *T. gesneriana* and *T. fosteriana* genomes was observed. The differences in chromosome size of particular chromosomes were also observed (Fig. 1a.b; Table 2). For instance, the difference in the length of the longest matching chromosomes between *T. gesneriana* and *T. fosteriana* genomes was 4μm and the difference in the length of the shortest matching chromosomes was 1.2μm. According to Levan et al. (1964) the chromosomes within each genome could be classified to median, submedian and subterminal chromosomes. F1 hybrids comprised of one pair of median chromosomes and variable number of submedian and subterminal chromosomes which ranged from 3-9 submedian and 2-8 subterminal chromosomes in *T. fosteriana* genome and from 5-8 and 2-6 subterminal in *T. gesneriana* genome.

An interesting aspect of *in situ* hybridization in Darwin hybrids tulips is the lack of uniform chromosome painting along entire somatic chromosome arms where telomeric and certain blocks of intercalary regions of chromosomes showed stronger fluorescence intensity (Fig 2). In situ hybridization with 5S rDNA and 45S rDNA probes to metaphase chromosomes of F1 hybrids showed that these regions are rich in repetitive DNA. Figure 2 shows the chromosome complement of F1 Darwin hybrid 20208-2 (Bellona x (Princeps x Cantata)) with enlarged median chromosomes (inset). 45S rDNA loci were localized exclusively in the telomeric position of the long arm of chromosomes (green fluorescence), whereas strong 5S rDNA signals were localized in the telomeric position on the short arm of chromosomes and in intercalary positions on the long arms (red fluorescence) with the exception of median chromosomes having additional strong intercalary positions of 5S rDNA locus on the short arm. Thus, the banding pattern after GISH painting revealed additional information, which allowed identification of a few individual chromosomes.

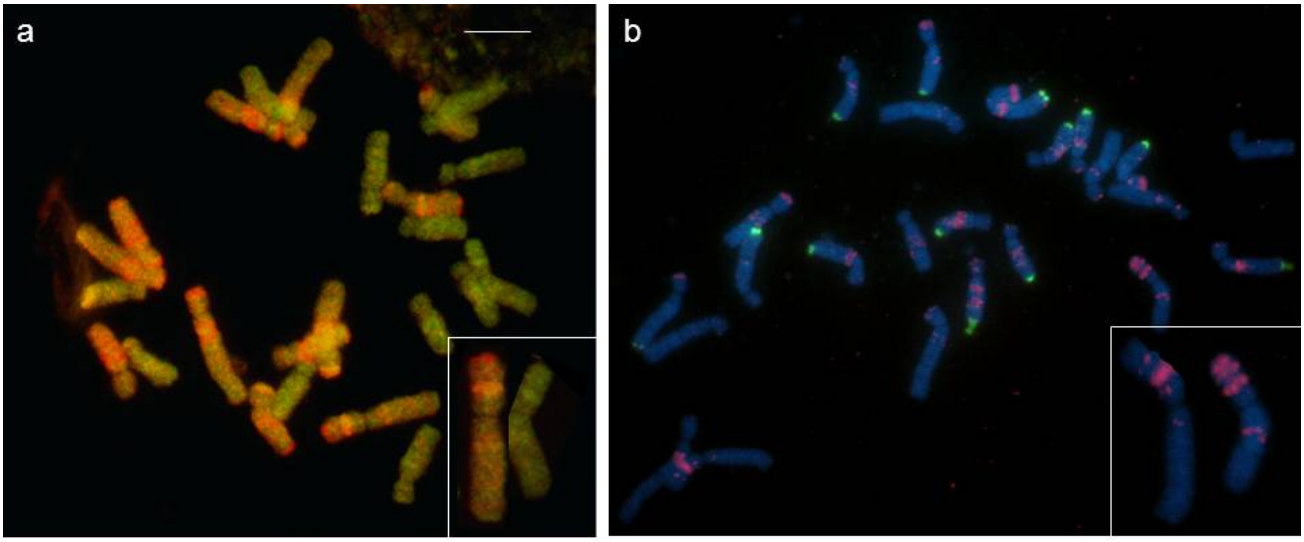

Figure 2 Chromosome painting in diploid F1 hybrids 20208-2 (2n = 2x = 24). a Genomic in situ hybridization to somatic metaphase cchromosome compliment showing 12 F and 12 G chromosomes. *T. gesneriana* DNA is detected with Cy3-streptavidin system (red) and *T. fosteriana* with FITC (green); b Double target fluorescence in situ hybridization of 45S r DNA (green) and 5S rDNA (red) to somatic metaphase cchromosome compliment. Insets show enlarged median chromosomes. Bar = 10 µm

Table 2 Chromosome characteristics in F1 Darwin Hybrid tulip 20208-2

Genome	Chr. No	p[a] (μm)	q[b] (μm)	p+q (μm)	R L[c] (%)	Cen. index (%)	p/q	Type
T. gesneriana	1	7.0	11.3	18.3	11.1	38.2	1.6	m
	2	4.1	13.3	17.4	10.6	23.6	3.2	st
	3	3.8	12.2	16.0	9.7	23.8	3.2	st
	4	3.6	11.7	15.3	9.3	23.5	3.2	st
	5	3.3	11.5	14.8	9.0	22.1	3.5	st
	6	3.6	9.9	13.5	8.2	26.9	2.7	sm
	7	3.2	8.7	11.9	7.3	27.0	2.7	sm
	8	3.3	8.4	11.7	7,1	28.1	2.5	sm
	9	3.3	8.5	11.8	7.2	27.8	2.6	sm
	10	3.6	8.0	11.6	7.1	31.1	2.2	sm
	11	3.5	7.4	10.9	6.7	32.1	2.1	sm
	12	3.2	7.2	10.4	6.4	31.2	2.2	sm
	Total			163.7				
T. fosteriana	1	5.2	9.1	14.3	10.5	36.5	1.7	m
	2	2.7	11.0	13.7	10.1	19.9	4.0	st
	3	3.0	10.4	13.4	9.9	22.8	3.4	st
	4	2.4	10.4	12.8	9.4	18.8	4.3	st
	5	3.1	8.7	11.8	8.7	26.4	2.8	sm
	6	3.4	8.1	11.5	8.5	29.7	2.3	sm
	7	2.0	8.1	10.1	7.5	20.0	4.0	st
	8	2.5	7.5	10.0	7.4	25.3	2.9	sm
	9	2.8	6.8	9.6	7.1	29.1	2.4	sm
	10	2.2	7.5	9.7	7.1	22.4	3.4	st
	11	2.4	7.0	9.4	6.9	25.6	2.9	sm
	12	2.5	6.7	9.2	6.8	27.6	2.6	sm
	Total			135.5				

[a]Short arm. [b]Long arm. [c]Relative length. [d]Median chromosomes. [e]Submedian chromosomes. [f]Subterminal chromosomes.

4.3 Intergenomic recombination in the genus *Tulipa* based on GISH analysis

4.3.1 BC1 progenies

The genome composition was assessed in diploid BC1 plants (2n = 2x = 24) resulted from crossing *T. gesneriana* cultivar with GF hybrids (Table 3). Figure 3 shows an example GISH picture of the diploid GGF BC_1 hybrids 061161-14 resulted from 'Yellow flight' x Eco F1 cross. By GISH it was possible to distinguish chromosomes from both parental genomes. The number of G genome chromosomes (chromosomes of which centromere was from *T. gesneriana* genome) predominated in the BC_1 progenies and varied from 14 to 20 whereas the total number of *T. fosteriana* chromosomes in hybrids ranged from 4 to 10. In all BC1 plants the recombinant chromosomes were observed. The number of recombinant chromosomes differed among hybrids from 5 to 10 (Table 3). Such diploid

BC1 plants with recombinant chromosomes indicate that normal meiosis had occurred in F1 GF hybrids. These results mean that in tulip introgression breeding is possible at diploid level.

Table 3. Genotypic information on number of *T. gesneriana* (G), *T. fosteriana* (F) and recombinant chromosomes of BC_1 population.

Cross no.	Parents		Genome composition		No of recombinant chromosomes	% of F-genome
	Female	Male	G(G/F)	F(F/G)		
061150-1	Kees Nelis	Eco F1 wit	18 (2)	6 (3)	5	24.56
061150-3	Kees Nelis	Eco F1 wit	18 (2)	6 (4)	6	23.54
061161-1	Yellow flight	Eco F1 wg	14 (2)	10 (7)	9	24.66
061161-2	Yellow flight	Eco F1 wg	15 (-)	9 (6)	6	21.15
061161-3	Yellow flight	Eco F1 wg	20 (4)	4 (2)	6	21.88
061161-13	Yellow flight	Eco F1 wg	18 (3)	6 (5)	8	19.71
061161-14	Yellow flight	Eco F1 wg	20 (6)	4 (4)	9	21.91
061161-24	Yellow flight	Eco F1 wg	17 (4)	7 (6)	10	21.88
061178-1	Lustige Witwe	Eco F1 wg	14 (-)	10 (8)	8	23.18
061178-12	Lustige Witwe	Eco F1 wg	17 (1)	7 (5)	5	20.48
061178-13	Lustige Witwe	Eco F1 wg	16 (2)	8 (6)	8	22.24
061178-20	Lustige Witwe	Eco F1 wg	18 (2)	6 (4)	6	22.28
061178-21	Lustige Witwe	Eco F1 wg	16 (2)	8 (5)	7	21.11

F/G and G/F recombinant chromosomes with *T. fosteriana* centromere with *T. gesneriana* chromosome segment(s) and *T. gesneriana* centromere with *T. fosteriana* chromosome segment(s), respectively

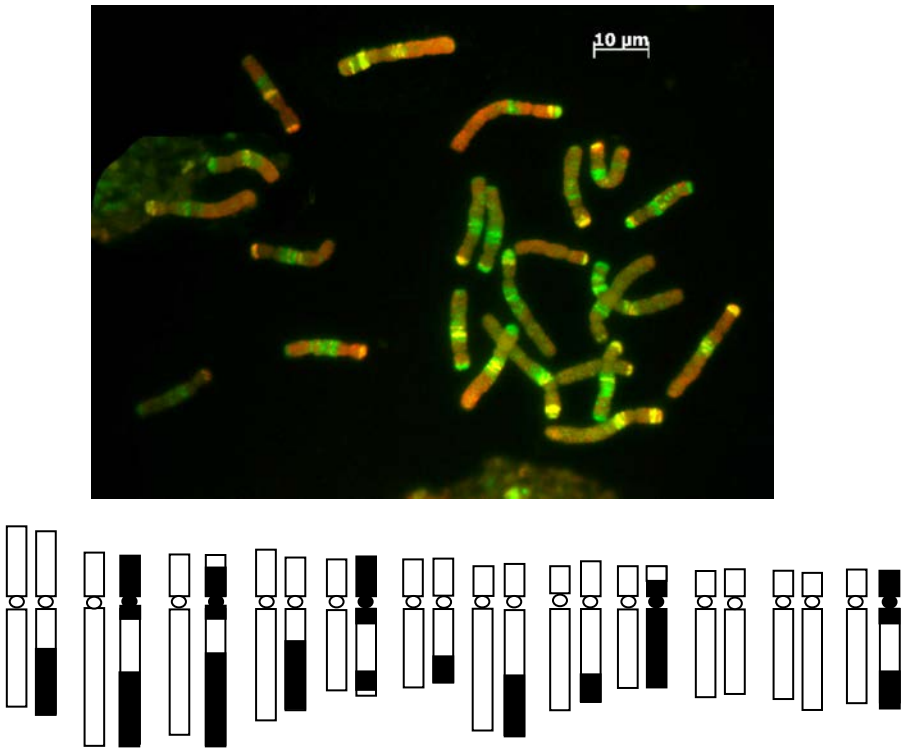

Fig. 3 (A). Diploid BC_1 GGF hybrid 061161-14, *T. gesneriana* (green) and *T. fosteriana* (red). (B). Diagrammatic representation of metaphase chromosomes of diploid BC_1 hybrid. The black represents the chromatin of *T. fosteriana*.

4.3.2 BC1 – progenies of Darwin Hybrid 'Purissima'

GISH has been applied to analyse BC1 hybrids resulted from crosses between *T. gesneriana* cultivars (G) and **'**Purissima' (GF) (Fig.4) for their ploidy level, the number of *T. gesneriana* (G) end *T. fosteriana* (F) chromosomes and the number of recombinant chromosomes. The results for 21BC1 progenies are summarised in Table 4. All BC1 plants were diploids (2n=2x=24) with the exception of a tetraploid (2n=4x=48) genotype, 99345-37 (Fig.4b). By GISH it was possible to distinguish chromosomes from both parental genomes as well as the recombinant chromosomes. In diploid BC1 progenies the number of G genome chromosomes (chromosomes with centromere of *T. gesneriana* genome) predominated and their number varied from 18 to 21 per complement whereas the total number of *T. fosteriana* chromosomes in hybrids ranged from 3 to 6. GISH clearly distinguished the presence of recombinant chromosomes in all BC1 hybrids tested. In all genotypes, with the exception of 99343-6 and 99345-123, there were two distinct types of recombinant chromosomes. Chromosomes with a *T. gesneriana* centromere possessing *T. fosteriana* recombinant segment, indicated as G/F, whereas chromosomes with a *T. fosteriana* centromere possessing *T. gesneriana* recombinant segment were indicated as F/G. The numbers of these two types of

recombinant chromosomes varied in different BC1 genotypes and the total ranged from 3 to 10 (Table 4). The number of recombination sites was counted for individual chromosomes and they varied from 1 to 3 per chromosome. The total number of recombination sites per BC1 genotype varied from 3 to 12 (Fig.5). Of the total number of 84 recombinant chromosomes that were found in 14 BC1 plants, 57 (67.85%) were the results of single crossover events. The recombination sites were distributed along the entire length of the chromosomes and their positions ranged from highly proximal to distal. However, only 18 recombination sites were found on the short arm of *T. gesneriana* and *T. fosteriana* genomes.

GISH analysis of the tetraploid progeny, 99345-37 (2*n* = 4*x* = 48) resulted from a cross between 'Golden Melody' and 'Purissima'revealed that its karyotype consists of 42 chromosomes of *T. gesneriana* (2G/F) and 6 chromosomes of *T. fosteriana* (4F/G) (Fig. 4b; Table 4), where the amount of introgressed *T. fosteriana* genome was 11.54%. The chromosome composition of the exceptional tetraploid has obviously resulted from the functioning of 2n gametes from both parents.

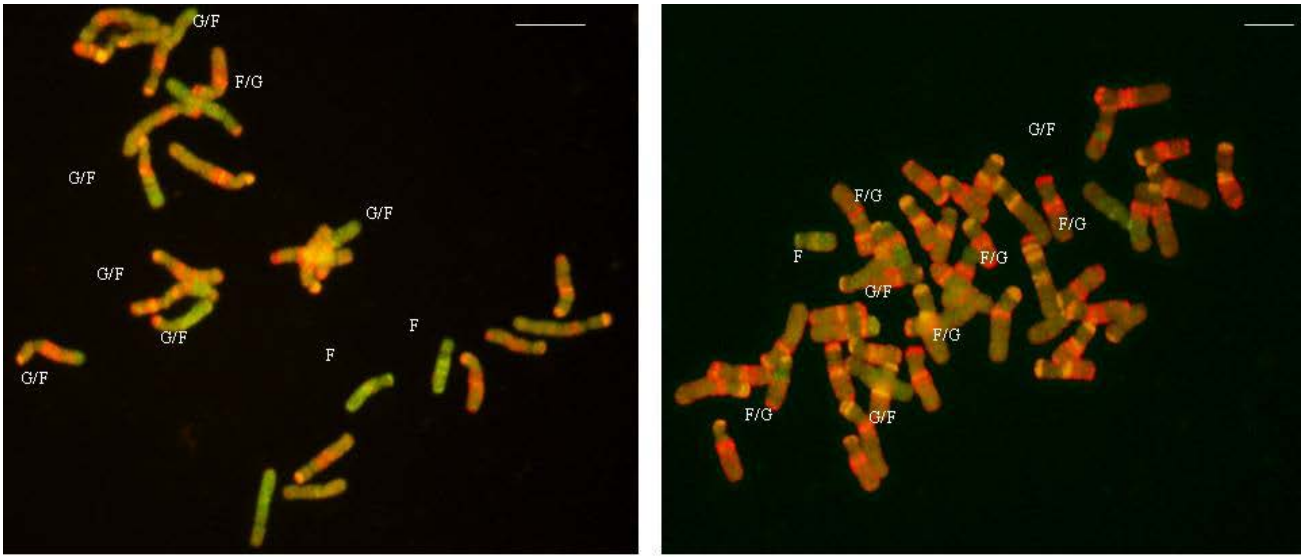

Figure 4 The representative GISH results for BC1 progenies. **a** Diploid BC1 hybrid 99344-15 (2n = 2x = 24) with 20 G chromosomes (6 G/F) and 4 F chromosomes (2F/G); **b** Chromosome compliment of tetraploid BC1 hybrids 99345-37 (2n = 4x = 48) with 42 G chromosomes (2 G/F) and 6 F chromosomes (5F/G). *T. gesneriana* DNA is detected with Cy3-streptavidin system (red) and *T. fosteriana* with FITC (green). Recombinant chromosomes are defined as F/G and G/F indicating a *T. fosteriana* centromere with *T. gesneriana* chromosome segment(s) and a *T. gesneriana* centromere with *T. fosteriana* chromosome segment(s), respectively. The arrows indicate the recombinant segment. Bar = 10 μm.

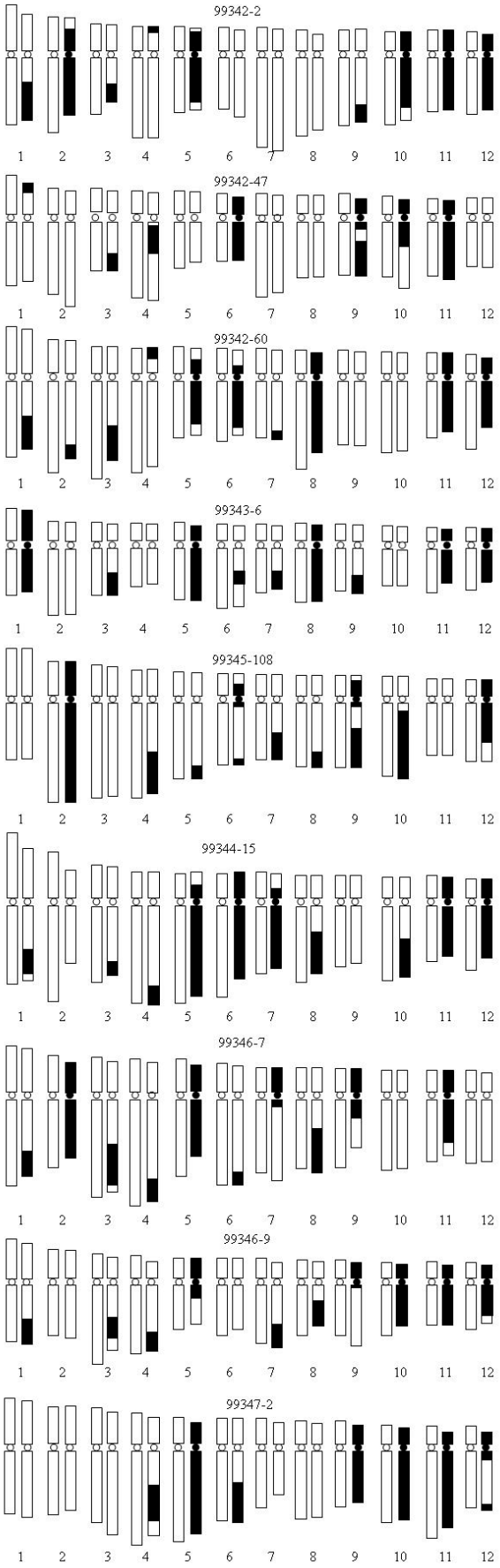

Figure 5. A diagrammatic representation of chromosomes in BC1 hybrids. In this figure the black color represents the *T. fosteriana* genome while white represents *T. gesneriana* one.

Table 4 The genome composition of BC1 hybrids derived from backcrossing 'Purissima' (GF) to *T. gesneriana* cultivars (the number of recombinant chromosomes are in brackets)

Generation	Cross no.	Parents		Ploidy level	Genome composition		No. of recombination sites	% of F-genome
		Female	Male		G(G/F)	F(F/G)		
BC1	99342-2	Bellona	Purissima	2*x*	19 (4)	5 (3)	8	18.9
	99342-47	Bellona	Purissima	2*x*	20 (3)	4 (2)	7	20.4
	99342-12	Bellona	Purissima	2*x*	20 (5)	4 (2)	7	20.5
	99342-40	Bellona	Purissima	2*x*	23 (9)	1 (1)	10	20.1
	99342-60	Bellona	Purissima	2*x*	19 (4)	5 (2)	8	21.3
	99343-6	Chr. Marvel	Purissima	2*x*	19 (4)	5 (0)	5	21.4
	99344-5	Debutante	Purissima	2*x*	19 (3)	5 (5)	11	20.0
	99344-15	Debutante	Purissima	2*x*	19 (6)	5 (2)	8	24.4
	99345-1	Golden Melody	Purissima	2*x*	18 (4)	6 (3)	7	19.3
	99345-16	Golden Melody	Purissima	2*x*	21 (5)	3 (2)	7	20.6
	99345-25	Golden Melody	Purissima	2*x*	18 (3)	6 (2)	8	22.1
	99345-37	Golden Melody	Purissima	4*x*	42 (2)	6 (4)	9	11.5
	99345-47	Golden Melody	Purissima	2*x*	21 (3)	3 (2)	5	11.5
	99345-102	Golden Melody	Purissima	2*x*	18 (3)	6 (1)	5	24.7
	99345-108	Golden Melody	Purissima	2*x*	20 (5)	4 (3)	12	18.5
	99345-123	Golden Melody	Purissima	2*x*	20 (3)	4 (0)	3	17.7
	99346-7	Ile de France	Purissima	2*x*	19 (4)	5 (3)	7	18.1
	99346-9	Ile de France	Purissima	2*x*	19 (5)	5 (3)	9	17.8
	99346-12	Ile de France	Purissima	2*x*	21 (4)	3 (1)	5	21.6
	99347-2	Pax	Purissima	2*x*	19 (3)	5 (2)	6	22.3
	99347-20	Pax	Purissima	2*x*	20 (3)	4 (3)	6	20.2

4.4 Genome composition of BC2 progenies and transmission of recombinant chromosomes

The genome composition determined through GISH in 5 BC1 parents and 32 BC2 progenies is given in Table 5, and some are illustrated in Figs 6 and 7. With the exception of one BC2 plant 083275-4, which was an aneuploid, all others BC2 genotypes were diploids. The total number of recombination sites per BC1 genotype varied from 2 to 11. A maximum of 6 recombinant chromosomes were, for example, found in one BC2 plant, 083569-4, of which one was the same as in the BC1 parent whereas three were new recombinant chromosomes. In this genotype two original recombinant chromosomes were involved in the second cycle of homoeologous recombination.

Table 5 The genome composition of 5 BC1 hybrids and their BC2 derivatives analyzed by GISH (the number of recombinant chromosomes are in brackets)

Generation	Cross no.	Parents		Ploidy level	Genome composition		No. of recombi-nation sites	% of F-genome
		Female	Male		G (G/F)	F (F/G)		
BC1	99342-2	Bellona	Purissima	2*x*	19 (4)	5 (3)	8	18.9
BC2	083508-1	Target	99342-2	2*x*	22 (0)	2 (2)	3	3.9
	083508-2	Target	99342-2	2*x*	22 (1)	2 (2)	3	3.8
	083508-4	Target	99342-2	2*x*	23 (1)	1 (1)	3	4.6
	083508-5	Target	99342-2	2*x*	22 (0)	2 (2)	3	5.3
BC1	99342-47	Bellona	Purissima	2*x*	20 (3)	4 (2)	7	20.4
BC2	083568-1	Target	99344-47	2*x*	23 (3)	1 (1)	5	7.1
	083568-3	Target	99344-47	2*x*	22 (4)	3 (3)	10	12.7
	083568-4	Target	99344-47	2*x*	23 (5)	1 (1)	6	10.5
	083568-5	Target	99344-47	2*x*	21 (2)	3 (3)	5	10.7
	083568-8	Target	99344-47	2*x*	23 (3)	1 (1)	6	6.3
	083568-10	Target	99344-47	2*x*	23 (4)	1 (1)	7	8.6
BC1	99343-6	Chr. Marvel	Purissima	2*x*	19 (4)	5 (0)	5	21.4
BC2	083275-4	Snowboard	99343-6	2*x* +1	25 (4)	0	5	4.5
	083275-5	Snowboard	99343-6	2*x*	23 (3)	1 (1)	4	5.4
	083275-6	Snowboard	99343-6	2*x*	23 (3)	1 (1)	5	7.3
	083275-7	Snowboard	99343-6	2*x*	22 (3)	2 (2)	5	9.3
	083275-8	Snowboard	99343-6	2*x*	23 (4)	1(1)	5	7.0
	083275-9	Snowboard	99343-6	2*x*	24 (5)	0	5	7.3
BC1	99345-25	Golden Melody	Purissima	2*x*	19 (3)	6 (2)	8	22.1
BC2	083569-1	Target	99345-25	2*x*	21 (2)	3 (2)	5	12.3
	083569-2	Target	99345-25	2*x*	23 (3)	1 (0)	3	7.8
	083569-3	Target	99345-25	2*x*	22 (3)	2 (2)	7	6.9
	083569-4	Target	99345-25	2*x*	21 (3)	3 (3)	11	8.2
	083569-5	Target	99345-25	2*x*	23 (3)	1 (1)	8	3.6
	083569-6	Target	99345-25	2*x*	23 (3)	1 (1)	4	6.3
	083569-7	Target	99345-25	2*x*	22 (1)	2 (2)	4	6.9
	083569-9	Target	99345-25	2*x*	23 (3)	1 (1)	4	6.8
	083569-10	Target	99345-25	2*x*	24 (2)	0	4	2.4
BC1	99346-9	Ile de France	Purissima	2*x*	19 (5)	5 (3)	9	17.8
BC2	083272-1	Freeman	99346-9	2*x*	23 (3)	1 (1)	5	4.9
	083272-3	Freeman	99346-9	2*x*	22 (2)	2 (2)	6	6.2
	083272-5	Freeman	99346-9	2*x*	23 (3)	1 (1)	6	5.5
	083272-6	Freeman	99346-9	2*x*	24 (1)	0	2	1.1
	083272-7	Freeman	99346-9	2*x*	22 (2)	2 (2)	6	8.0
	083272-8	Freeman	99346-9	2*x*	23 (2)	1 (1)	5	5.7
	083272-9	Freeman	99346-9	2*x*	22 (2)	2 (2)	5	6.3

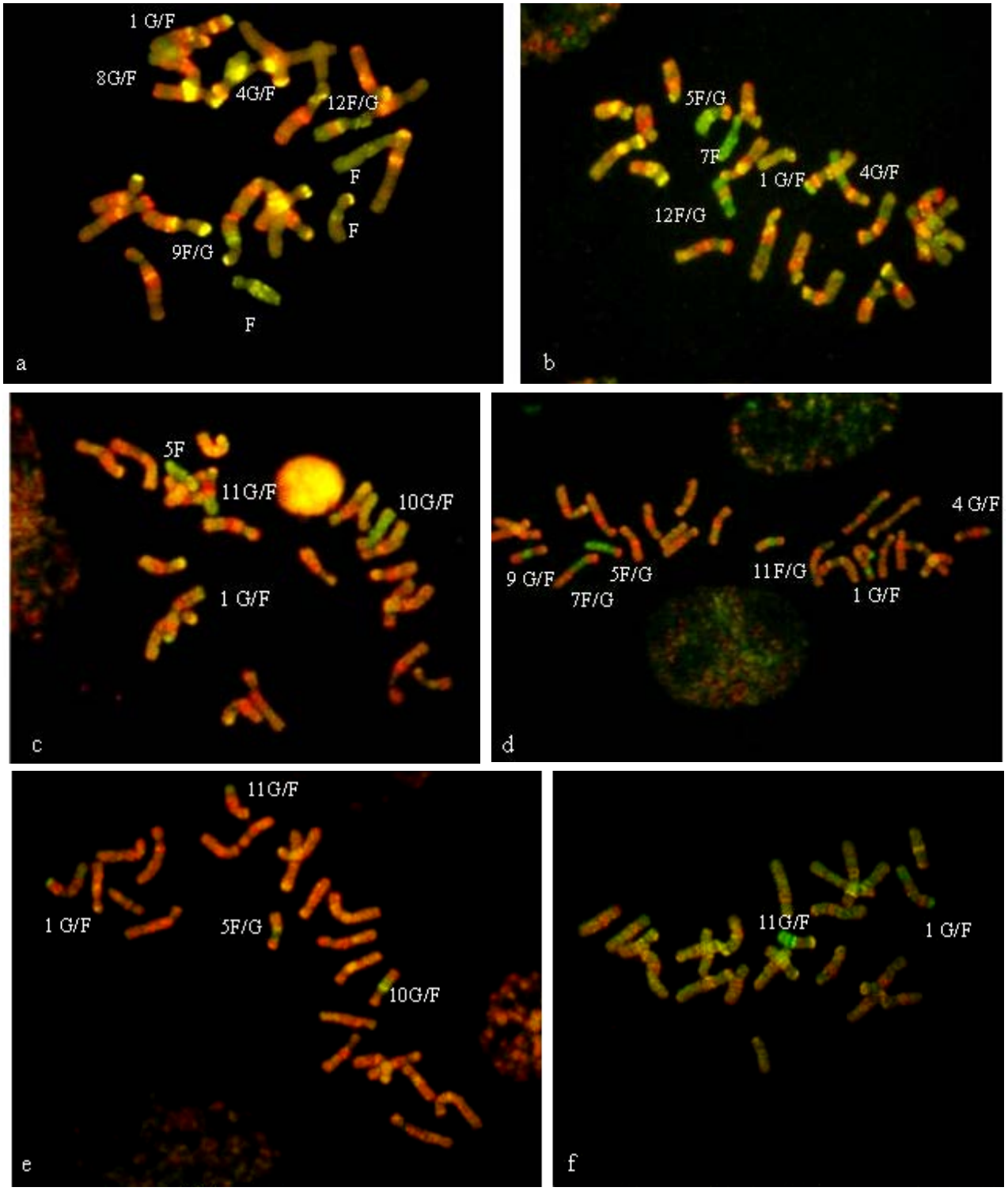

Fig. 6 GISH results for BC1 diploid GGF hybrid (2n = 2x = 24) and its representative BC2 progenies. **a** Chromosome compliment of BC1 hybrids 99345-25 showing 5 F chromosomes (2F/G) and 19 G chromosomes (3G/F); **b** BC2 progeny 083569-1 (2n = 2x = 24) with 3F chromosomes (2F/G) and 21G chromosomes (2G/F); **c** BC2 progeny 083569-2 (2n = 2x = 24) with 1F chromosomes and 23G chromosomes (3G/F); **d** BC2 progeny 083569-4 (2n = 2x = 24) with 3F chromosomes (3F/G) and 21G chromosomes (3G/F); **e** BC2 progeny 083569-5 (2n = 2x = 24) with 1F chromosomes (1F/G) and 23G chromosomes (3G/F); **f** BC2 progeny 083569-10 (2n = 2x = 24)

with 0F chromosomes and 24G chromosomes (2G/F). *T. gesneriana* DNA is detected with Cy3-streptavidin system (red) and *T. fosteriana* with FITC (green). Recombinant chromosomes are defined as F/G and G/F indicating a *T. fosteriana* centromere with *T. gesneriana* chromosome segment(s) and a *T. gesneriana* centromere with *T. fosteriana* chromosome segment(s), respectively.

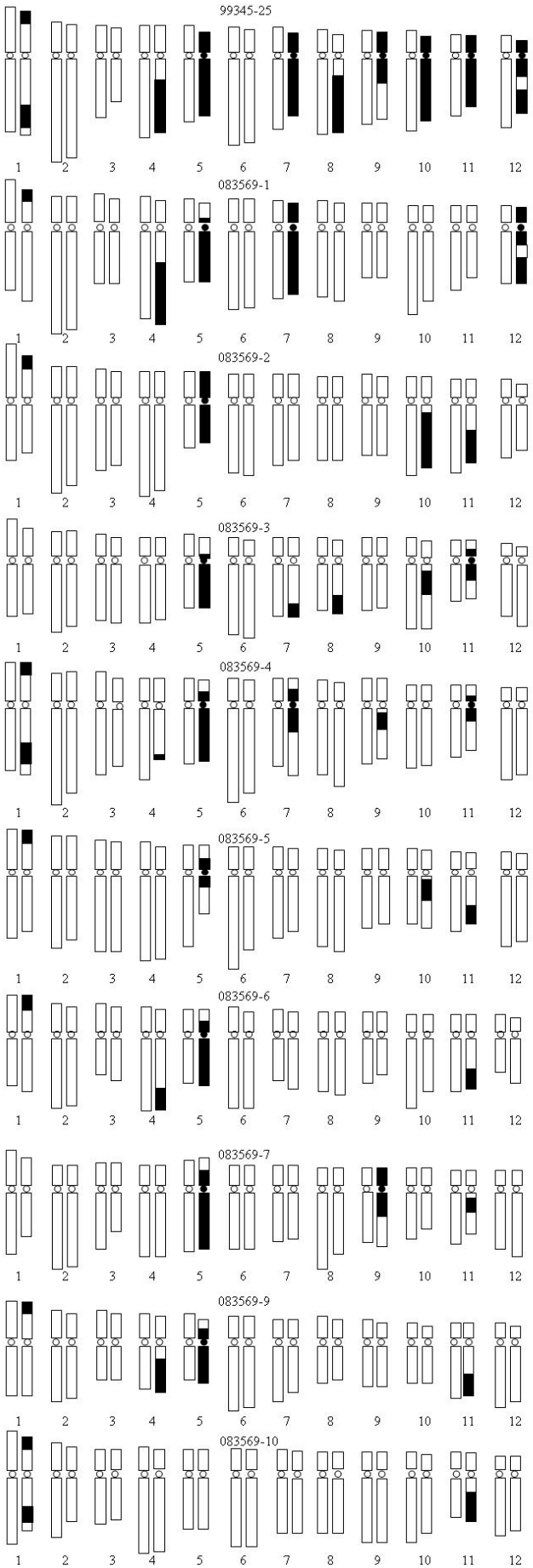

Figure 7. A diagrammatic representation of chromosomes in 99345-25 BC1 hybrids and its BC2 progenies. In this figure the black color represents the *T. fosteriana* genome while white represents *T. gesneriana* one.

4.5 General information on ploidy levels of two different types of crossings based on flow cytometry analysis.

4.5.1. Ploidy testing in one year old seedlings of tulips through flow cytometry

In total 308 one year old F1 seedlings resulted from crosses between diploid and triploid *T. gesneriana* cultivars and diploid 2n gametes producers (20168-3, 20170-4, 20230-9, S-20253-1, 20190-4, 20168-3, 20241-2) have been tested by flow cytometry analysis (Table 6). According to the flow cytometry results, 193 out of 202 progenies resulted from crosses at diploid level (2x X 2x) were diploids, whereas 9 seedlings were triploids (Table 6). The 2*n* pollen grains seem to be functional in crosses 2x X 2x but the amount of triploid progenies were low, approximately 5%. In crosses 3x X 2x, 81 genotypes were tetraploids and 25 seedlings were pentaploids (Table 6).

Table 6 General information of ploidy levels of two different types of crossings

Cross	No. of progeny analyzed	Ploidy levels of the progeny			
		2X	3X	4X	5X
2x X 2x	202	193	9	0	0
3x X 2x	106	0	0	81	25

4.5.2 GISH analysis in the progeny of diploids crossed with diploids producing 2n gametes

25 BC1 hybrids resulted from 2x X 2x cross have been used for GISH analysis (**Table 7**). All hybrids were diploids except for triploid 0913062-1 (2n = 3x = 36) resulted from cross Michail x 20253-1 (**Fig 8**), where male genotype produce 2n pollen at 18.1%. Although some male genotypes could produce 2n pollen at 82.78% e.g., 20168-3, their BC1 progenies tested by GISH analysis were diploids (e.g. 0912189-1 and 0912189-2).

Table 7 Chromosome numbers in the progeny of diploids crossed with diploid fathers producing of 2n gametes

Genotype	Cross	Parents	Genome	Ploidy	Chromosome number
0911467-1	2x x 2x	WM x 20180-3	GGF	2x	24
0911467-2	2x x 2x	WM x 20180-3	GGF	2x	24
0911467-3	2x x 2x	WM x 20180-3	GGF	2x	24
0911467-7	2x x 2x	WM x 20180-3	GGF	2x	24
0912189-1	2x x 2x	WM x 20168-3	GGF	2x	24
0912189-2	2x x 2x	WM x 20168-3	GGF	2x	24
0913062-1	2x x 2x	Michail x 20253-1	GGF	3x	36
0913062-2	2x x 2x	Michail x 20253-1	GGF	2x	24
0913062-4	2x x 2x	Michail x 20253-1	GGF	2x	24

0913062-5	2x x 2x	Michail x 20253-1	GGF	2x	24
0913062-7	2x x 2x	Michail x 20253-1	GGF	2x	24
0913062-8	2x x 2x	Michail x 20253-1	GGF	2x	24
0913062-10	2x x 2x	Michail x 20253-1	GGF	2x	24
912517-5	2x x 2x	L v/d Mark x 20190-4	GGF	2x	24
912517-7	2x x 2x	L v/d Mark x 20190-4	GGF	2x	24
912517-9	2x x 2x	L v/d Mark x 20190-4	GGF	2x	24
912517-12	2x x 2x	L v/d Mark x 20190-4	GGF	2x	24
9121151-3	2x x 2x	Ile de France x 20253-1	GGF	2x	24
9121151-4	2x x 2x	Ile de France x 20253-1	GGF	2x	24
9121151-5	2x x 2x	Ile de France x 20253-1	GGF	2x	24
9121151-6	2x x 2x	Ile de France x 20253-1	GGF	2x	24
9121151-8	2x x 2x	Ile de France x 20253-1	GGF	2x	24
912645-11	2x x 2x	AC12 x 20168-3	GGF	2x	24
0911471-1	2x x 2x	White Marvel x 20259-23	GGF	2x	24
0911471-6	2x x 2x	White Marvel x 20259-23	GGF	2x	24

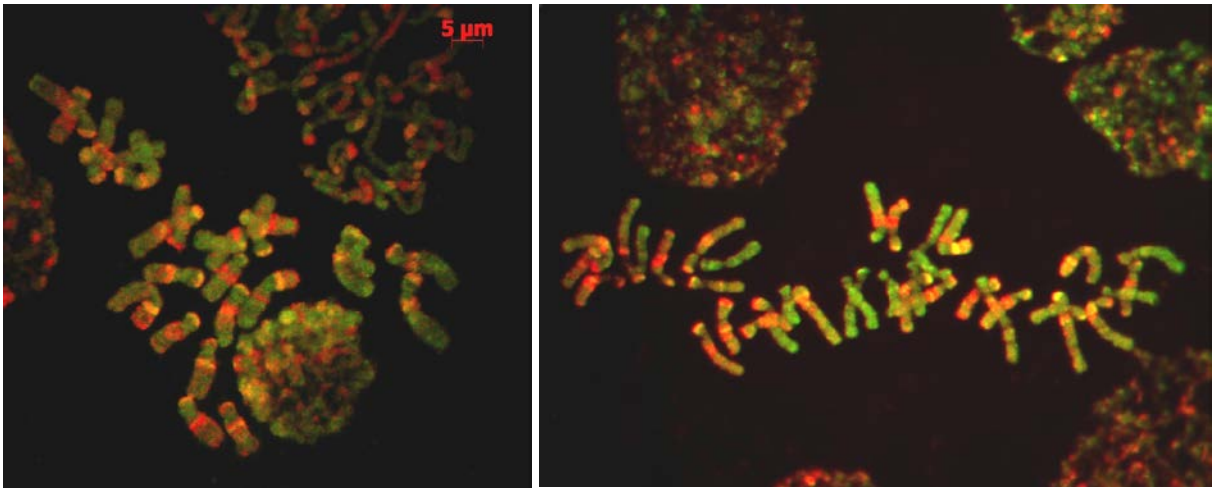

Fig 8 GISH pictures of BC1 hybrids resulted form 2x x 2x cross **a** diploid hybrid 0913062-7(2n = 2x = 24) **b** triploid hybrid 0913062-1 (2n = 3x = 36) both resulted form cross Michail x 20253-1. Red fluorescence represents *T. gesneriana* genome and green fluorescence *T. fosteriana* genome, respectively.

4.6 The list of publication resulted form the project

The following publication resulted from cytogenetic study on introgression in the genus *Tulipa* have been published so far:

A. Marasek-Ciolakowska, M.S. Ramanna, J.M. Van Tuyl 2008. Introgression of virus resistance of *Tulipa fosteriana* into *T. gesneriana* cultivars analyzed by GISH. Lecture Bulb symposium Lisse, book of abstr page 26.

A. Marasek-Ciolakowska, M.S. Ramanna, J.M. Van Tuyl 2009. Introgression breeding in genus *Tulipa* Analysed by GISH. Acta Hort. 836: 105-110.

A. Marasek-Ciolakowska, M.S. Ramanna, J.M. Van Tuyl. Introgression breeding in genus *Tulipa* Analysed by GISH. Poster Eucarpia Leiden 2009, book of abstr page 37.

A. Marasek-Ciolakowska, M.S. Ramanna, J.M. Van Tuyl 2011. Introgression of Chromosome Segments of *Tulipa fosteriana* into *T. gesneriana* Detected through GISH and Its Implications for Breeding Virus Resistant Tulips. Acta Hort. 886: 175- 182

A. Marasek-Ciolakowska, M.S. Ramanna, P .Arens, J.M. Van Tuyl 2011 Breeding and cytogenetics in the genus *Tulipa*. Global Science Books (in press).

A. Marasek-Ciolakowska, H. He, M.S. Ramanna, P. Bijman P. Arens, J.M. Van Tuyl Species differentiation in the two parents of Darwin Hybrid tulips, *Tulipa gesneriana* and *T. fosteriana*: an assessment of intergenomic recombination through GISH analysis of F1 hybrids and progenies. Plant Syst. Evol. (2012) 298:887-899.

The following manuscripts have been submitted for publishing:

A. Marasek-Ciolakowska, S. Xie, M.S. Ramanna, P. Arens, J.M. Van Tuyl. Sexual polyploidization in Darwin Hybrid tulips. To be submitted to Euphytica.

5. Conclusions

GISH and FISH analysis

Introgression of important agricultural traits is one of the main goals in interspecific hybridization. Many crosses have been made to introgress the resistance to TBV present in *T. fosteriana* germplam into *T. gesneriana* cultivars. The Darwin hybrids resulting from these crosses turned out to be very useful intermediate parents for introgressing the *T. fosteriana* germplasm into the *T. gesneriana* assortment. In genus *Tulipa*, GISH enables not only the monitoring of the hybridity of progenies resulting from interspecific hybridization, but also the analysis of the introgression of chromosomes and chromosome segments into hybrids. Through GISH it is also possible to trace the mode of origin of polyploid tulips and the role of 2*n* gametes in polyploidization. It was found that some tulip F1 hybrids not only produced *n* gametes but also 2*n* gametes. This provided unique opportunities to generate polyploid as well as diploid BC1 progenies from backcrossing GF hybrids (Darwin hybrids) to *T. gesneriana* parents. The identification of individual chromosomes of tulip has been improved by the application of FISH with repetitive DNA probes. In future the FISH method can be applied for the physical mapping of resistance genes or molecular markers of virus resistance on tulip chromosomes and to trace their inheritance in progenies.

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