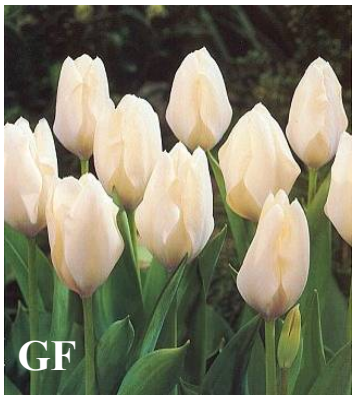
Introgression of *Tulipa fosteriana* into *Tulipa gesneriana*



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Credits thesis: 36 ECTS
Period: July 2008 –January 2009
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Preface

As the main tulip production country, tulip breeding is a continuous process in the Netherlands. Since 1963-1964, research on tulip breeding has been carried out at the DLO Centre for Plant Breeding and Reproduction Research (PRI; the former IVT) and with cooperation of the Dutch bulb industry. In 1974, seedlings of the PRI breeding programme were released to the Dutch tulip breeders. From 1999 to 2012, a project cofinanced by PT (Productschap Tuinbouw) and 10 tulip companies was set up, mainly focuses on selecting TBV resistant cultivars. In 2008, TTI-Green Genetics (Technological Top Institute Green Genetics) was also involved in this. Now it is the last step in the whole project which contains three parts: selection in GGF- population, introgression of TBV virus resistance gene from *T. fosteriana* to *T. gesneriana* and selection of virus resistant gene which could be used as marker to be probed on chromosome with FISH.

The present study is a part of the last step in the project.

Acknowledgements

I would like to express my gratitude to my supervisor Dr. Jaap Van Tuyl for giving me the chance to work in this group, and his guidance provided a good basis for this thesis.

I want to express my appreciation to my supervisor Dr. Agnieszka Marasek-Ciolakowska for her help in the laboratory work, and her constructive advices and encouragements have been very helpful.

I also want to thank Xie Songlin, whose kind support and guidance have been of great value to my study.

I wish to extend my warmest thanks to all those who have helped me with my work here, Dr Paul Arens, Nadeem Khan, Arwa Shahin, Maarten Holdinga and Alex van Silfhout.

Abstract

Pollen fertility was assessed in 113 tulip (2n = 24) genotypes by pollen staining and germination on media. 23 genotypes were selected based on high *2n* pollen percentage and will be used for crosses. Genomic *in situ* hybridization (GISH) was used to investigate the genome composition and the extent of intergenomic recombination in 2 F_1, 24 BC_1 and 2 BC_2 tulip progenies. F_1 comprised of 12 chromosomes of each parent without recombination and the percentage of introgressed *T. fosteriana* genome was 45.6%. In all BC_1 (GGF) and BC_2 (GGGF) hybrids recombinant chromosomes were observed and most of them had both G/F and F/G recombination. The number of *T. fosteriana* chromosomes as well as the number and type of recombinant chromosomes differed among progenies. In BC_1, the percentage of introgressed *T. fosteriana* genome ranged from 16.8% to 24.7%, while in BC_2 the percentage varied from 13.3% to 16.67%. The position of recombination distributed on both arms from distal to interstitial, but most of them were detected on terminal. The results of this could provide further information on possibility of introgression of the TBV resistance gene from *T. fosteriana* into *T. gesneriana*.

Keywords: *2n* pollen, chromosome recombination, GISH, karyotyping

Abbreviations:

F	*T. fosteriana*
G	*T. gesneriana*
GF	F_1 hybrid made by *T. gesneriana* (mother) and *T. fosteriana* (father)
GGF	BC_1 hybrid made by F_1 hybrid and *T. fosteriana* (father)
GGGF	BC_2 hybrid made by BC_1 hybrid and *T. fosteriana* (father)
F/G	a *T. fosteriana* centromere with *T. gesneriana* chromosome segment(s)
G/F	a *T. gesneriana* centromere with *T. fosteriana* chromosome segment(s)
FDR	First Division Restitution
SDR	Second Division Restitution
IMR	Indeterminate Meiotic Restitution
PMR	Post Meiotic Restitution

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Introgression of *Tulipa fosteriana* into *Tulipa gesneriana*

Chapter 1 General Introduction

1.1 The genus *Tulipa* L.

Of all the bulbous plants, the tulip is the most important one in The Netherlands. However, the primary gene centre of the genus *Tulipa* L. is located in the Pamir Alai and Tian Shan mountain ranges in Central Asia. Diversification occurred from this region, resulting in a distribution from Morocco to Western Europe and eastward to western China. A secondary gene centre has been found in the Caucasus (Van Tuyl and Van Creij, 2006).

Generally speaking, tulips are grown as fresh cut flowers, potted plants or used for landscaping. They have been extremely popular as cut flowers and garden plants for many centuries. In 2007, about 10,000 hectares were used for tulip bulb production in the Netherlands (http://www.flowerbulbs.co.uk/). Today the Netherlands produces three billion tulip bulbs each year, two billion of which are exported. The U.S. is the country where most of tulip bulbs are exported.

The number of cultivars being cultivated is also huge. There are more than 3000 varieties registered, while the number being cultivated on a commercial basis is not more than 750. The *T. gesneriana* (G) and Darwin hybrids consist of more than 1100 cultivars (Van Scheepen, 1996). Within these cultivars, 10 most popular ones occupy more than 35% of the planted acreage.

According to the taxonomic classification by Van Raamsdonk and De Vries (1995), the genus is divided into two subgenera: *Tulipa* and *Eriostemones* (Boissier). These subgenera are classified into eight sections**:**

Subgenus *Tulipa:* *Tulipa, Eichleres, Tulipanum, Kolpakowskianae, Clusianae*
Subgenus *Eriostemones:* *Australes, Saxatiles, Biflores*

T. gesneriana and *T. fosteriana* belong to the same subgenera Tulipa. Many crosses are made between them, and they are proved to be compatible with each other (Figure 1).

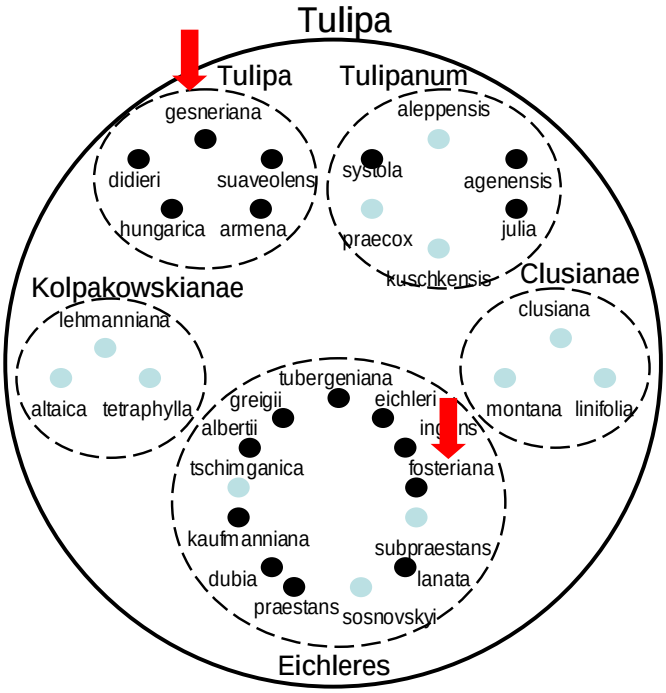


Figure 1 Tulipa subgenera and 5 sections within it. Black dot means the successful cross of *T. gesneriana* with other species of Tulipa section and species of Eichleres and Tulipanum. *T. gesneriana* and *T. fosteriana* are indicated by red arrow. (Van Raamsdonk et al. 1997).

T. gesneriana is one of the major cultivated group that originated from complex crossings of *Tulipa* species (Van Tuyl and Van Creij, 2006). Most *T. gesneriana* cultivars are diploid (2n = 2x = 24), a small number are triploid and few are tetraploid (Okazaki et al., 2005). Other cultivars derived from intraspecies crosses of *T. fosteriana* (F), *T. greigii*, and *T. kaufmanniana* have also been distributed to markets. Interspecific crossing of *T. gesneriana* and *T. fosteriana* has generated another commercial cultivar group, called Darwin Hybrid tulips, which are mostly diploid (2n = 2x = 24). And about 5% of Darwin Hybrids are triploid (2n = 36) which reveal large flower and good bulb yield (Van Tuyl and Van Creij, 2006).

1.2 Important Traits for Commercial Breeding

1.2.1 Flower colour, shape, longevity

The tulip is famous for its flower colour and flower shape. By exploring the diversity of its colour and shape, almost all the needs of the consumers could be satisfied. Biochemical analyses of flower pigments and crossing experiments were carried out to obtain more knowledge regarding to the inheritance of flower colour and flower shape (Straathof and Eikelboom, 1997).

The life of a cut flower or potted plant is one of the most important characteristics for the consumer. Research has shown that large genetic variation is available in the cultivar assortment. This research shows that more flower longevity is based on additive effects of several genes (Van Tuyl and Van Creij, 2006).

1.2.2 Disease resistance

Tulips can be affected by several diseases and the most important pathogens are *Fusarium oxysporum* (bulb-rot), *Botrytis tulipae* and Tulip Breaking Virus (TBV) Also other fungi (*Pythium* spp., *Rhizoctonia tuliparum*/*solani*), viruses (Tobacco Necrosis Virus (TNV) and Tobacco Rattle Virus (TRV)), mites and nematodes (*Trichodoridae*, *Pratylenchus penetrans* and *Ditylenchus dipsaci*) can cause economic losses. Host resistance is the best approach to prevent diseases. The use of resistant cultivars reduces the use of chemical control, increases bulb production, and requires less labor for sorting and selecting harvested bulbs. Neat, clean and pathogen free cultivars are also important for export.

In the future, resistance to the three main diseases of tulip should be combined to get multi-resistant tulips. PRI has been conducting research to accomplish that task in co-operation with a group of tulip growers. The most suitable method for the transmission of disease resistance is interspecific hybridization and therefore work is being done in the introgression of virus resistance from *T. fosteriana* into *T. gesneriana.*

1.2.3 Forcing qualities

For cut flower and potted plant production, forcing is an essential process. During forcing, flowering is controlled by simulating the temperature and light conditions required in nature. The cold period can be given partially by storing the bulbs in temperature controlled and highly ventilated rooms, prior to planting the bulbs. This called “pre-cooling”. The optimal length of the total cold treatment varies with the genotype and bulb size.

1.3 Methods of Overcoming Interspecific Hybridization Barriers

Interspecific hybridization proved to be the most important tool for developing complete new hybrid groups. Many crosses between *T. gesneriana* and other tulip species have been carried out to enrich the commercial assortment with desirable traits from these species. However, incongruity barriers hinder sexual reproduction in interspecific hybridization.

1.3.1 Overcoming pre-fertilization barriers

Pre-fertilization barriers have been overcome in several different interspecific crosses after bud-pollination, cut-style method or the grafted-style method (Van Tuyl et al., 1991), placental pollination and pollination of isolated ovules. In tulip, the cut-style method (CSM) and placental pollination have been studied (Van Creij et al., 2000).

1.3.2 Overcoming post-fertilization barriers

Embryo culture, ovule culture, ovary-slice culture have been developed to enable hybrid embryos to survive *in vitro*. The application of embryo rescue techniques in tulip breeding has been reported by Van Tuyl et al. (1991), Custers et al. (1995) and Van Creij et al. (2000). Compared to embryo culture, with ovule culture, more embryos could be rescued from an earlier developmental stage (4 weeks. post-pollination), and also more embryos could be rescued at each culture date. For ovary-slice culture, ovaries were cut transversely in eight sections and placed on medium. The results of ovary-slice culture, started at various dates after pollination, were comparable to or better than the results of direct ovule culture.

1.3.3 Overcoming F_1 sterility

Post-fertilization barriers may cause sterility of F_1-hybrids caused by the lack of chromosome pairing during meiosis. In many crops, e.g., lily (Barba-Gonzalez et al., 2006) chromosome doubling with oryzalin or colchicine has restored fertility. Similarly, tetraploid tulip cultivars have been produced after treating *in vitro* grown tulip bulbs with oryzalin or colchicine (Van Tuyl et al., 1992).

In the past, diploid pollen has been used to overcome interploidy crossing barriers between 4*x* and 2*n* species and to transmit the important agronomic trait from diploid wild alleles to tetraploid cultivars. And this has been proven useful in many species such as potato (Mendiburu et al., 1974), alfalfa (Veronesi et al., 1986), and red clover (Smith et al., 1985). In addition, Van Tuyl et al. (1989) reported that *2n* pollen is useful to overcome pollen sterility in interspecific hybrids of lilies. In tulip, N_2O treatment was used to yield *2n* pollen (Okazaki et al., 2005; Barba-Gonzalez et al., 2006).

1.4 Scope of the thesis

The aims of this thesis were

1) To check the pollen fertility from a number of genotypes, and select desirable genotypes with relatively high *2n* pollen percentage (>10%). The results could be used for the selection of crossing parents for the coming year.

2) To use GISH to analyze genome composition of F_1, BC_1 and BC_2 of *T. gesneriana* and *T. fosteriana* (GF) population of Tulip and establish the chromosome karyotypes, with some information for introducing Tulip Breaking Virus resistance into *T. gesneriana* cultivars.

With GISH the following information were collected:
I. Discrimination of parental chromosomes
II. Determination of the genome composition in BC_1 and BC_2 progenies
III. Identification of the recombinant chromosome(s) or chromosome segment (s)

Chapter 2 Pollen fertility analysis of *T. fosteriana* and *T. gesneriana* hybrids

2.1 Introduction

Triploid tulips are in great demand because they display agronomically important traits such as attractive flower colour, large flower size and vigorous growth Moreover, they are suitable for various forcing methods (Kroon and Van Eijk, 1977; Okazaki, 2005; Marasek et al., 2006). However, as most of cultivated tulip (*T. gesneriana*) cultivars are diploid (2n = 2x = 24) (Okazaki et al., 2005), there is a great demand for polyploidization.

Generally speaking, there are two ways for polyploidization, which can be categorized into mitotic polyploidization and meiotic polyploidization (Lim and Van Tuyl, 2006). Mitotic polyploidization is obtained by artificial chromosome doubling via treating vegetative tissue with spindle inhibitors such as colchicine (Blakeslee and Avery 1937) or oryzalin (Van Tuyl et al., 1992). However, due to preferential pairing between homologous chromosomes at metaphase I of the meiosis, there is a lack of homoeologous recombination between parental genomes. Thus, decrease the opportunity to introduce required traits to other progeny plants. Another alternative and preferred method is meiotic polyploidization.

Meiotic polyploidization, also called sexual polyploidization, involves the use of *2n* gametes that occur occasionally in interspecific hybrids. *2n* gametes are directly formed in diploid plants rather than mitotic chromosome doubling, recombination can occur between the parental chromosomes. In this way, *2n* gametes can transmit broad heterozygosity to the polyploid offspring. (Lim et al., 2001a; Ramanna et al., 2003).
2n pollen has been used to transmit important agronomic traits from diploid wild-type alleles to teraploid cultivars in alfalfa (Veronesi et al., 1986), orchard grass (Van Santen et al., 1991), and white clover (Bullitta et al., 1995). In addition, *2n* pollen are useful to overcome F_1 sterility in interspecific hybrids in lilies (Van Tuyl et al., 1989) and *Alstroemeria* (Ramanna et al., 2003).

The aim of this research was to check the percentage of *2n* pollen for a number of genotypes, and to compare them with pollen germination ratio. The selected genotypes with relatively high *2n* pollen percentage (>10%) could be used for the selection of crossing parents for the coming year.

2.2 Materials and methods

Materials:

In the second and third week of April 2008 the anthers were collected from the field of Plant Research International, Wageningen UR, the Netherlands. Pollen was stored at 18℃ till being used for crosses. In total, 113 genotypes were involved in this test: 77 genotypes of F_1, 29 genotypes of BC_1 and 7 genotypes of parents of BC_1

Methods:

Pollen fertility was estimated first by germination on media and then by acetocarmine staining.

Pollen germination
Pollen germination media was consisted of ½ strength of MS medium supplemented with 10% sucrose (w/v), 0.5% bacteriological agar (w/v), 0.002% boric acid (w/v) and 0.02% calcium nitrate (w/v). Pollen grains were spread evenly on the surface of the medium. Petri dishes were placed upside down in dark at around 20℃ for 24h. Pollen grains were scored as germinated when pollen tube length was at least twice to pollen grain diameter (Tyagi et al., 1995).

Pollen staining
A small amount of pollen grains were collected from 3 - 6 anthers of each genotype and were mixed. Then, applied one or two drop(s) of acetocarmine and dispersed evenly before the coverslip was placed. For each genotype, three slides were prepared, and each slide 5 randomly selected fields were observed under 100 × magnification. Pink stained pollen was counted as fertile and pollen without stain or very lightly stained ones were considered as sterile (Rigamoto and Tyagi, 2002).

2.3 Results

Pollen viability was determined by germination of pollen on pollen germination medium and their staining with acetocarmine. Pollen staining helped to differentiate *n* and *2n* gametes. *2n* gametes can be distinguished by their larger size compared to *n* gametes (Figure 2-1). Pollen grain size was checked for four 'Purissima' hybrids (Table 2-1) and it was found that *2n* pollen was lager than *n* pollen from 106.27% to 140.71%. The results of pollen analysis were present in Table 2-2.

All 23 genotypes listed in Table 2-2 had relatively higher *2n* pollen percentage, from 10.2% (99342-22) to 79.9% (98689-1), which was useful information for choosing parents for the next cross. At the same time, variations for stainable pollen were observed, ranged from 23.1% (99345-174) to 89.3% (Pax mix). There was no correlation between the percentage of stainable pollen and *2n* pollen.

Pollen germination results also had a lot of variations, from 0% to 80% (Table 2-2). In 21 genotypes of F_1 and BC_1 listed in Table 2-2, up to 29% of them have as high as 80% pollen germination rate. And 19% of them have 60% germination rate. This result showed that higher *2n* pollen percentage could lead to higher germination rate.

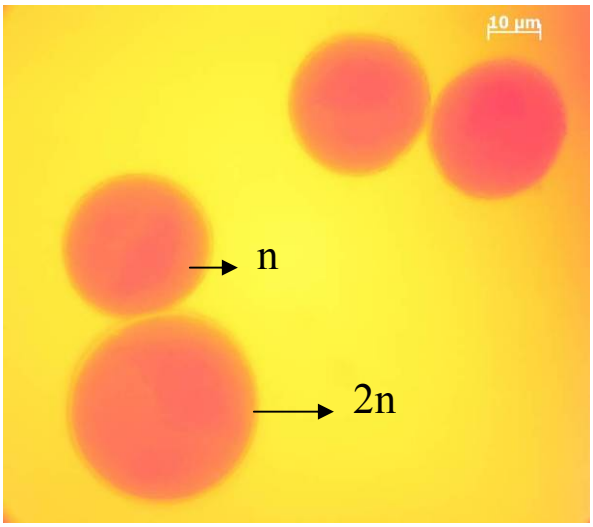


Figure 2-1 Picture of *n* pollen and *2n* pollen of 'Purissima' hybrids 99346-9

Table 2-1 Sizes of *n* pollen and *2n* pollen of four 'Purissima' hybrids (µm)

Accession Number	Size(µm)		
	n	***2n***	***2n/n%***
99346-1	23.21	28.86	124.34
98689-1	29.84	31.71	106.27
99342-22	23.85	33.56	140.71
99346-9	29.12	40.33	138.50

Table 2-2 Pollen staining results compared with pollen germination results

Generation	Accession Number	Pollen Staining				%Pollen germination
		% stainable	% unstainable	% n pollen*	% *2n* pollen*	
F_1	20185-1	63.7	36.3	44.2	55.8	80
	20192-11	49.5	50.5	86.6	13.4	60
	20206-2	85.5	14.5	65.2	34.8	80
	20230-1	59.4	40.6	52.5	47.5	80
	20230-3	82.1	17.9	88.8	11.2	20
	20231-19	69.3	30.7	88.9	11.1	60
	20259-8	37.7	62.3	74.1	25.9	10
	1563	68.2	31.8	89.7	10.3	60
	Pax mix	89.3	10.7	84.9	15.1	N/A
	Pax wit mix	38.8	61.2	69.9	30.1	N/A
	Bellona mix	62.2	37.8	84.9	15.1	N/A
BC_1	99342-22	72	28	89.8	10.2	0
	99344-13	46.5	53.5	60.7	39.3	80
	99345-37	51.2	48.8	61.3	38.7	0
	99345-64	42.5	57.5	76.4	23.6	80
	99345-174	23.1	76.9	89.1	10.9	0
	99346-1	79.8	20.2	45.6	54.4	40
	99346-8	33.4	66.6	88.9	11.1	60
	99346-9	54.6	45.4	52.9	47.1	40
	98689-1	80.3	19.7	20.1	79.9	80
	98690-1	23.6	76.4	76.4	23.6	50
Parent	Barbedos 090065 070029	60.2	39.8	80.3	19.7	N/A
	'Purissima' T	25.9	74.1	89.2	10.8	N/A

*% *n* pollen and % *2n* pollen were checked for stainable pollen

2.4 Discussion

In tulip, a large number of interspecific hybrids are fertile. For example, 'Purissima' is an interspecific hybrid from crossing *T. gesneriana* and *T. fosteriana.* It is reported that most of these hybrids are diploid (2n = 2x = 24) and can be used in backcrossing with other genotypes (Marasek and Okazaki, 2008). In this report, variations for stainable *n* pollen were observed (Table 2-2), which is ranged from 20.1% (98689-1) to 89.8% (99342-22). This showed that tulip could produce considerable numbers of functional *n* gametes. This situation is in opposite with *Lilium* (Barba-Gonzalez et al., 2004) and *Alstroemeria* (Ramanna et al., 2003) where most of the F_1 hybrids are highly sterile due to the failure of chromosome pairing and very few could produce *2n* gametes with considerable frequencies.

T. gesneriana and *T. fosteriana* belong to the same subgenera Tulipa. Many crosses are made between them, and they are proved to be compatible with each other (Figure 1). They can produce diploid, triploid or tetraploid hybrid by normal crossing (Kroon and Van Eijk, 1977). In the hybrids of species that possess very closely related genomes, it is not a problem to have diploid forms that can produce functional *n* gametes due to normal segregation of chromosomes during meiosis (Khan et al., 2008; Weiss and Maluszynska, 2000; Gu et al., 2005). However, because triploid tulips have excellent traits such as large flower, attractive colour (Marasek et al., 2006), they are popular in the market. For the sake of producing more triploid tulip hybrids, choosing parents which have relatively higher *2n* pollen fertility is important. The genotypes listed in Table 1 could be used for this purpose to get triploid BC_1 progenies.

2n gametes are used to produce polyploids and have several advantages compared with mitotic polyploidization. Research on *Lilium* (Barba-Gonzalez et al, 2004) and *Alstroemeria* (Ramanna et al., 2003) proved that *2n* gametes could be most useful for the introgression of alien genes and chromosomes into cultivars (Ramanna and Jacobsen, 2003) due to recombination during meiosis. Another advantage is the polyploids heterozygosity is not fixed so that they are more promising for breeding (Barba-Gonzalez, 2005). As *2n* gametes have different modes of origin such as first division restitution (FDR), second division restitution (SDR), indeterminate meiotic restitution (IMR) and post meiotic restitution (PMR), therefore their genetic consequences are also varied from each other (Barba-Gonzalez, 2005). In many crops, such as *Lilium* (Lim et al., 2001a; Barba-Gonzalez et al., 2004; Zhou, 2007) and *Alstroemeria* (Ramanna et al., 2003), extensive research have been done on to study meiotic nuclear restitution mechanism. However, so far, in tulip, there is limited investigation on defining the type of meiotic abnormalities or various types of *2n* gametes occurrence. May be it is possible to do further study to clarify which type is dominant and stabilized for the possible tulip polyploidy formation.

Results from the pollen fertility test (data not shown) showed that the variation of the frequencies of *2n* pollen were very large, from 0.0% to 79.9%. Considerable variations of *2n* pollen frequencies have been reported in the interspecific hybrids of other monocotyledonous genera such as *Lilium* (Barba-Gonzalez et al., 2005a), *Triticum* and *Aegilops* (Fukuda and Sakamoto, 1992). It was found that the frequencies of *2n* pollen varied depending on genotypes and different cross combination. In 113 genotypes tested, 20% (23 genotypes) of them produced higher rate of *2n* gametes (>10%), which is relatively higher as compared with lily (Barba-Gonzalez et al., 2004). Consequently, from this aspect, triploid tulips are easier to

produce. Pollen staining is the simplest way to detect *2n* gametes; nevertheless, it is not a reliable technique to determine pollen fertility. In some experiments, the pollen from the same genotypes could be stained but there was no indication of pollen tube formation (data not shown). Pollen germination test should be done immediately after anthesis, as after storage, germination rate would decrease. But pollen staining could be done one or two months later after collecting. That's the reason some pollen staining results are not always correspond with pollen germination test (data not shown).

Chapter 3 GISH analysis of *T. fosteriana* and *T. gesneriana* hybrids

3.1 Introduction

3.1.1 Tulip Breaking Virus and disease resistance

Tulip is very susceptible to virus diseases; 22 viruses have been reported to affect this crop (Mowat, 1995). The most prevalent and damaging disease is tulip breaking, caused by Tulip Breaking Virus (TBV). TBV has been reported in many countries of both hemispheres and is likely to appear wherever tulips are grown (Juodkaitë et al., 2005). TBV causes colour 'breaking' or streaking of the flower in pink, purple and red flowered tulips, but white and yellow flowered cultivars are not affected. Foliar symptom is chlorotic mosaic (Hammond and Chastagner, 1989). At the end, this virus leads to reduction in bulb number, their weight and ultimately the quality of the bulbs (Romanow et al., 1991). Tulip Breaking Virus is transmitted by virus in the field during growing season and possibly during bulb storage (Hammond and Chastagner, 1989). Cultural control by rouging and application of mineral oil or insecticides is done frequently but it is time-consuming, expensive and not always effective. An alternative and more efficient approach is by using TBV resistant cultivars.

A high degree of TBV resistance was found in *T. fosteriana* cultivars, such as 'Cantata' and 'Princeps'. However, as the main cultivated species, *T. gesneriana* cultivars, such as 'Madame Lefeber' and 'Juan' were susceptible to TBV (Eikelboom et al., 1992). Thus, from breeding point of view, it is essential to combine these two species and transmit resistant trait from *T. fosteriana* to *T. gesneriana*.

3.1.2 Progress of Genomic *in situ* Hybridization (GISH) Technique

Genomic *in situ* hybridization has applications in distinguishing the parental genomes and their segments in distant hybrid polyploids, polyhaploids and recombinant breeding lines; and in detecting and localizing the amount of introgressed alien chromatin (Crasta et al., 2000; Friebe et al., 1996). This technique has also given new insights into somaclonal variation (Molnár-Láng et al., 2000), the origin of B chromosomes (Stark et al., 1996; Poggio et al., 1999), control of chromosome pairing (Benavente et al., 1996) and other aspects of chromosome evolution. It is an effective technique in molecular cytogenetics. In wheat (*Triticum aestivum*), GISH was proved as a useful tool to precisely characterize genetic stocks derived from crosses with *Thinopyrum intermedium* and *Thinopyrum elongatum* (Han et al., 2003). GISH is proved to be a powerful technique in many crops with intergeneric and interspecific hybrid origin, such as 'Gasteria' × 'Aloe' (Takahashi et al., 1997), Brassica (Wang et al., 2005) and so on. Applying GISH with probe pre-annealing, Belyayev and Raskina (1998) detected and physically mapped the location of conserved and variable intergenomic repetitive sequences in the chromosomes of the cross-pollinated species *Aegilops spelotoides* Tausch.

GISH was applied to many plant species to establish the parentage of the

chromosomes of the F_1-hybrids and the BC_1 and BC_2 progenies, for example in *Triticum* (Anamthawat-Jonsson et al., 1990; Han et al., 2003), *Alstroemeria* (Kuipers et al., 1997), *Allium* (Khrustaleva et al., 1998), etc. This technique has also been used successfully in lily cytogenetic research. After three genomes of *Lilium*, viz., Longiflorum (L), Asiatic (A) and Oriental (O) genome were recognized with GISH (Karlov et al., 1999), some F_1 LA and OA hybrids, BC_1 and BC_2 progenies have been analyzed continuously (Lim et al., 2001a, 2003; Barba-Gonzalez et al., 2004, 2005a, 2005b, etc). Many of these triploid BC_1 progenies were analysed to explain the genetic variation in BC_1 plant obtained after sexual polyploidization (Barba-Gonzalez et al., 2005b). The mechanism of *2n* gametes formation (Lim et al. 2001a; Barba-Gonzales et al., 2004, 2005a) as well as homoeologous recombination is also studied using this technique (Lim et al., 2003b; Barba-Gonzales et al., 2005b)

3.1.3 Progress of GISH in tulip interspecific hybridization

GISH was used to study the process of interspecific tulip hybrid diploid (2n = 2x = 24), triploid (2n = 3x = 36) and tetraploid (2n = 4x = 48) cultivar formation (Marasek and Okazaki (2007). Results showed that diploid cultivar 'Shirayukihime' contained one set of chromosomes from *T. gesneriana* and one set from *T. fosteriana*. In triploid cultivars 'Come Back', 'Diplomate', 'Pink Impression' and 'Oxford' comprised 24 chromosomes from *T. gesneriana* and 12 chromosomes from *T. fosteriana*. And for tetraploid cultivar 'Ollioules' 36 chromosomes were derived from *T. gesneriana* and 12 chromosomes from *T. fosteriana*. GISH and FISH has been done to analyse the introgression of the *Tulipa fosteriana* genome into *Tulipa gesneriana* (Marasek and Okazaki, 2008). GISH demonstrated that 'Purissima' (2n = 2x = 24) was an interspecific hybrid comprised of one genome of *Tulipa* (*T.*) *gesneriana* and one genome of *T. fosteriana*. Progenies of backcrossed cultivar from *T. gesneriana* with 'Purissima' were studied by GISH and FISH. In different cultivars, the number and type of recombinant chromosomes are different. The number of recombinant *T. fosteriana* chromosomes in 'Judith Leyster' (2n = 4*x* = 48) is four. And in 'Purissima' progeny, the number of recombinant *T. fosteriana* chromosomes differed from two in 'Hatsuzakura' to six in 'Kikomachi' and 'Momotaro'. In 'Kikomachi' only one translocation was observed compared with six translocations in 'Hatsuzakura'. It was found that these translocations resulted from single crossover event (Marasek and Okazaki, 2008)

3.2 Materials and Methods

3.2.1 Plant material

The original parental materials belong to two tulip species. These two species are from the same subgenus (Tulipa), but different sections. One parent is *T. gesneriana,* which belongs to Tulipa section, susceptible to Tulip Breaking Virus (TBV). Another parent is *T. fosteriana,* which is in Eichleres section, proved to be TBV resistant species. Crossings were made in the field of Plant Research International, Wageningen UR, the Netherlands. Crossing scheme was depicted in Figure 3-1

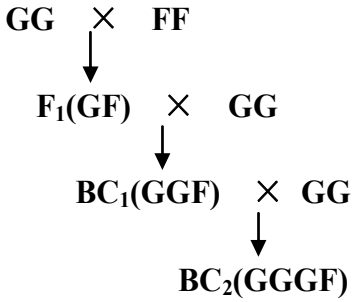


Figure 3-1 Crossing Scheme

In this experiment, we investigated three types of progenies i.e., F_1, BC_1 and BC_2, which were shown in Table 3. In BC_1 progenies, some of them were generated by the same parental combination.

For the reason that not all the genotypes had bulbs or seeds, we obtained roots either from bulbs or from tissue cultured seeds (Table 3-1)

Table 3-1 Materials used for analysis

Generation	Number	Mother	Father	Roots origin
F_1	20185-5	Bellona	135	Bulb
	20191-4	Bellona	144	Bulb
	20208-2	Ile de France	114	Bulb
	20239-20	Generaal de Wet	118	Bulb
BC_1	061150	Kees Nelis	Mix white F_1	Tissue Culture
	061161	Yellow flight	Mix white F_1	Tissue Culture
	061178	Lustige Witwe	Mix white F_1	Tissue Culture
	071363	060058	ZW 00-2	Tissue Culture
	081153	Ile de France	20185-5	Tissue Culture
	081439	060055	20191-4	Tissue Culture
	081148	060055	20208-2	Tissue Culture
	081154	Ile de France	20239-20	Tissue Culture
	081418	070006	20239-20	Tissue Culture
	99342-2	Bellona	Purissima	Bulb
	99342-15	Bellona	Purissima	Bulb
	99342-22	Bellona	Purissima	Bulb
	99342-47	Bellona	Purissima	Bulb
	99342-60	Bellona	Purissima	Bulb
	99343-6	Chr. Marvel	Purissima	Bulb
	99344-15	Debutante	Purissima	Bulb
	99345-25	Golden Melody	Purissima	Bulb
	99345-37	Golden Melody	Purissima	Bulb
	99345-102	Golden Melody	Purissima	Bulb
	99345-108	Golden Melody	Purissima	Bulb
	99345-123	Golden Melody	Purissima	Bulb
	99346-7	Ile de France	Purissima	Bulb
	99346-9	Ile de France	Purissima	Bulb
	99347-2	Pax	Purissima	Bulb
BC_2	083275	070018	99343-6	Tissue Culture
	083276	070018	99345-16	Tissue Culture

Roots from bulbs

After cold treatment at 4℃ for 2 weeks, bulbs were put in plates containing water. The plates were then placed at 4℃ chamber. After 4 days, the roots in the basal plate become very prominent. After one week, the roots of some genotypes reached 0.5-1cm, and were collected (Figure 3-2). Different genotypes had different growth rate. Roots were collected continuously for more than 1 month (10, Nov - 19, Dec).

Figure 3-2 Roots growing from bulb

Roots from tissue cultured seeds

Seeds germination:
7 genotypes were selected for tissue culture to obtain roots (081153, 081154, 081419, 081439, 081148, 083275, and 083276). Seeds were rinsed in H_2SO_4 for 1-2 min and washed in tap water for 1-2 min to remove remaining H_2SO_4. Then after washing in 80% ethanol for 2 min, seeds were sterilised in 6% sodium hypochlorite (NaOCl) for 10 min followed by three times washing in sterile water.

Seed were placed on germination medium in Petri dishes. Medium comprised by half strength MS (Murashige and Skoog, 1962), 3% (w/v) sucrose and 0.7% microagar. pH was adjusted to 5.6. Media were autoclaved at 120℃ for 20 min. Then fungicides Nystatin 20mg/l, antibiotics Cefotaxime 50 mg/l and Vancomycin 50 mg/l were added to the media. Seeds were placed at 4℃ in darkness.

After 1.5 to 2 month, germinated seeds (Figure 3-3) were transferred to propagation media. The time of germination depended on the genotype. Propagation media contained full strength MS (including Vitamins), 6% (w/v) sucrose, pH was adjusted to 6.0 and then added 0.7% Daichin agar.

Figure 3-3 Germinated seeds from tissue culture

Figure 3-4 in vitro cultured root

After 1 – 3 weeks in propagation medium, when roots reached 1-2cm (Figure 3-4), they could be collected.

3.2.2 Methods

Root treatments:
To accumulate anthesis metaphases, excised root tips (5-20 mm long) were treated with metaphase arresting agents. For roots from bulbs, the following 3 agents were tested:

1) 0.05% (w/v) colchicine for 3-6 h at room temperature
2) 0.1% (w/v) colchicine for 3-5 h at room temperature
3) 0.07 mM cyclohaximide for 4- 6 h at 4 ℃.

For roots from tissue culture, they were normally weaker than roots from bulbs, so 0.05% (w/v) colchicine for 2.5-3 h at 4℃ was enough for metaphases arresting. After these agents, transferred root tips to Carnoy's solution (fixative: alcohol-acetic acid 3:1) for at least 2 h at room temperature and stored at -20℃ until they are used.

Slides preparation

- Spread method (roots from bulbs)

Root tips were washed in citerate buffer (0.01M sodium citrate, pH 4.8) for 20 min and digested in enzyme solution (1% (w/v) cellulose RS and 1% (w/v) pectolyase Y23 in citrate buffer, pH 4.8) at 37℃ for 1.5 h. Root meristem was dissected and spread evenly on the slide in one drop of 60% acetic acid followed by putting on slide heater for 2-3 min and then washed with Carnoy's solution, and air dried.

- Squash method (*in vitro* cultured roots)

Meristem tissue was dissected under dissecting microscope in 60% acetic acid and squashed with a coverslip. Then slide was frozen in liquid nitrogen. Coverslip was removed followed by washing with Carnoy's solution and rinsing in 100% ethanol for 15 min. Air dried.

DNA isolation

Total genomic DNA of 'Princeps' (*T. fosteriana*), 'Ile de France' (*T. gesneriana*) and *T. tarda* (belongs to subgenus *Eriostemones*, section *Biflores*) was extracted according to Fulton et al. (1995). In general, the tulip bud from bulb was ground in liquid nitrogen and the powder was stored at -80℃. 750 µl microprep buffer (2.5 parts extraction buffer, 2.5 parts lysis buffer, 1.0 part 5% (w/v) Sarkosyl, and 0.38 g sodium bisulfite/100ml buffer immediately before use) were added to the powder, then incubated in 60℃ water bath for 30 - 60 min. After this, mixture was extracted with 800 µl chloroform and centrifuged for 5 min at 13,000. 400 - 600 µl aqueous phase was pipetted off then equal volume cold isopropanol was added. Tubes were inverted repeatedly until DNA precipitates. After centrifugation for 5 min, the pellet was washed with 500 µl 70% ethanol and spun 5 min. Pellet was dried followed by resuspension of DNA in 90 µl TE buffer. 10 µl of RNase was added and then incubated at 37℃ for 1 h (Figure 3-5).

DNA probe and block preparation

DNA of 'Princeps' and 'Ile de France' were used as probes and DNA of *T. tarda* was used as block. 'Princeps' and 'Ile de France' DNA was sonicated for 5 sec in order to get 1-10 kb fragments and used as probes. 'Princeps' and 'Ile de France' DNA fragments were labeled by digoxigenin 11-dUTP and biotin- 16 dUTP with standard nick translation (Roche, Germany) respectively. *T. tarda* DNA was autoclaved at 120℃ for 5 min to get 100-500bp fragments and was used as block (Figure 3-6).

***In situ* Hybridisation**

The method was described based on Marasek and Okazaki (2008) with some minor modifications. Slides were left overnight at 37℃. Then treated with RNase A (100 µg/ml) at 37℃ for 1 h followed by 3 times washing in 2× SSC for 5 min. After incubating in 10 mM HCL for 2 min, slide was treated in pepsin (5 µg/ml) for 15 min. Then slides were incubated in 4% paroformaldehyde at room temperature for 10 min,

washed another three times in 2× SSC and dehydrated through a graded ethanol series (70%, 90% and 100% for 3 min each) and air dried. Hybridisation mix was composed of 60% (v/v) formamide, 8% dextran sulphate, 2× SSC, 0.2% (w/v) SDS, 100ng probe DNA each, haring sperm blocking DNA (80-100-fold excess over the probe concentration). *T. Tarda* blocking DNA (20-40-fold excess over the probe concentration) to reduce cross-hybridization of the two labeled probe DNA. The mixture was pre-denatured at 75℃ for 10 min and directly placed on ice for at least 10 min. 40 μl of mixture was added to each slide and denatured at 70℃ for 5 min and incubated 16-18 h in a humid chamber at 37℃. Stringency washing was carried out by washing in 2× SSC for 15 min at room temperature followed by washing in 0.1× SSC for 30 min at 42℃, and 2× SSC at room temperature for 15 min. The probe labeled with dig-11-dUTP and biotin-16-dUTP was detected with the digoxigenin and biotin detection system respectively. The chromosomes were counterstained with 1 μg/ml DAPI (4, 6-diamidino-2-phenylindole) in Vectashield (Vector Laboratories, Burlingame, CA). Photos of fluorescently stained chromosomes were acquired using Zeiss Axiophot eqifluorescence microscope. During the experiments, another method was also carried out to reduce cross-hybridisation. This pre-annealing method was according to Anamthawat-Jonsson and Reader (1995) and Belyayev et al. (2001) (Appendix).

Karyotype analysis

For each genotype, the karyotype was drawn based on at least three metaphases. Chromosome measurements were made using the freeware computer application MicroMeasure version 3.3, available on the Internet at http://www.colostate.edu/Depts/Biology/MicroMeasure.

Chromosomes were paired based on the length of both arms and arm ratio. Nomenclature for the centromeric position on the chromosomes was according to Levan et al. (1964) based on arm ratio (1.0-1.7 median chromosomes; 1.7-3.0 submedian; 3.0-7.0 subterminal; 7.0−∞ terminal). And paired chromosomes were arranged in order of decreasing short arm length.

3.3 Results

3.3.1 Germination rate

Two months after putting seeds on germination media, germination rate was calculated (Table 3-2). The rates were variable, ranged from 29.2% to 100.0%. Both of the two BC_2 progenies (083275 and 083276) had higher germination rates.

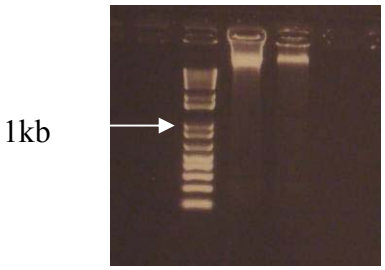
Table 3-2 Germination rate of tissue cultured seeds:

Accession Number	Germinated seeds	Total No. of seeds	Germination rate %
081154	7	24	29
081153	16	25	64
081419	10	20	50
081439	26	26	100
083275	46	50	92
083276	36	40	90

3.3.2 DNA isolation

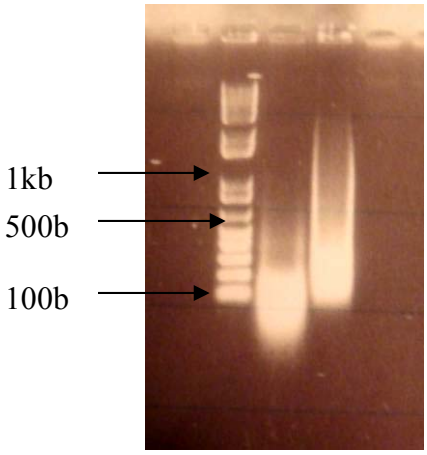
The method described by Fulton et al. (1995) was proved to be efficient on isolating total genomic DNA of tulip (Figure 3-5).

Concentration and quality of a sample DNA was measured with a NanoDrop machine.



Lane 1: Molecular mass markers
Lane 2: Total genomic DNA of *T. tarda*
Lane 3: Total genomic DNA of *T. gesneriana* cultivar: 'Ile de France'

Figure 3-5 Gel electrophoresis of isolated total genomic DNA



Lane 1: Molecular mass markers
Lane 2: autoclaved blocking DNA of *T. tarda*
Lane 3: sonicated probe DNA of *T. gesneriana* cultivar: 'Ile de France'

Figure 3-6 Gel electrophoresis of autoclaved blocking DNA and sonicated probe DNA

In order to get proper size of DNA as block and probe, we applied some treatments on total genomic DNA and proved our results by gel electrophoresis (Figure 3-6)

After autoclaving, blocking DNA of *T. tarda* was smeared from less than 100bp to 500bp on the gel, and sonicated probe DNA was ranged from 100bp to 8kb, which was smaller size than the optimal size.

3.3.3 GISH analysis

GISH analysis determines the genomic composition of the parents and identifies the number of recombinant chromosomes in the subsequent progenies. Four F_1, twenty-four BC_1 and two BC_1 progenies were analysed, and some results were shown below (Figure 3-7).

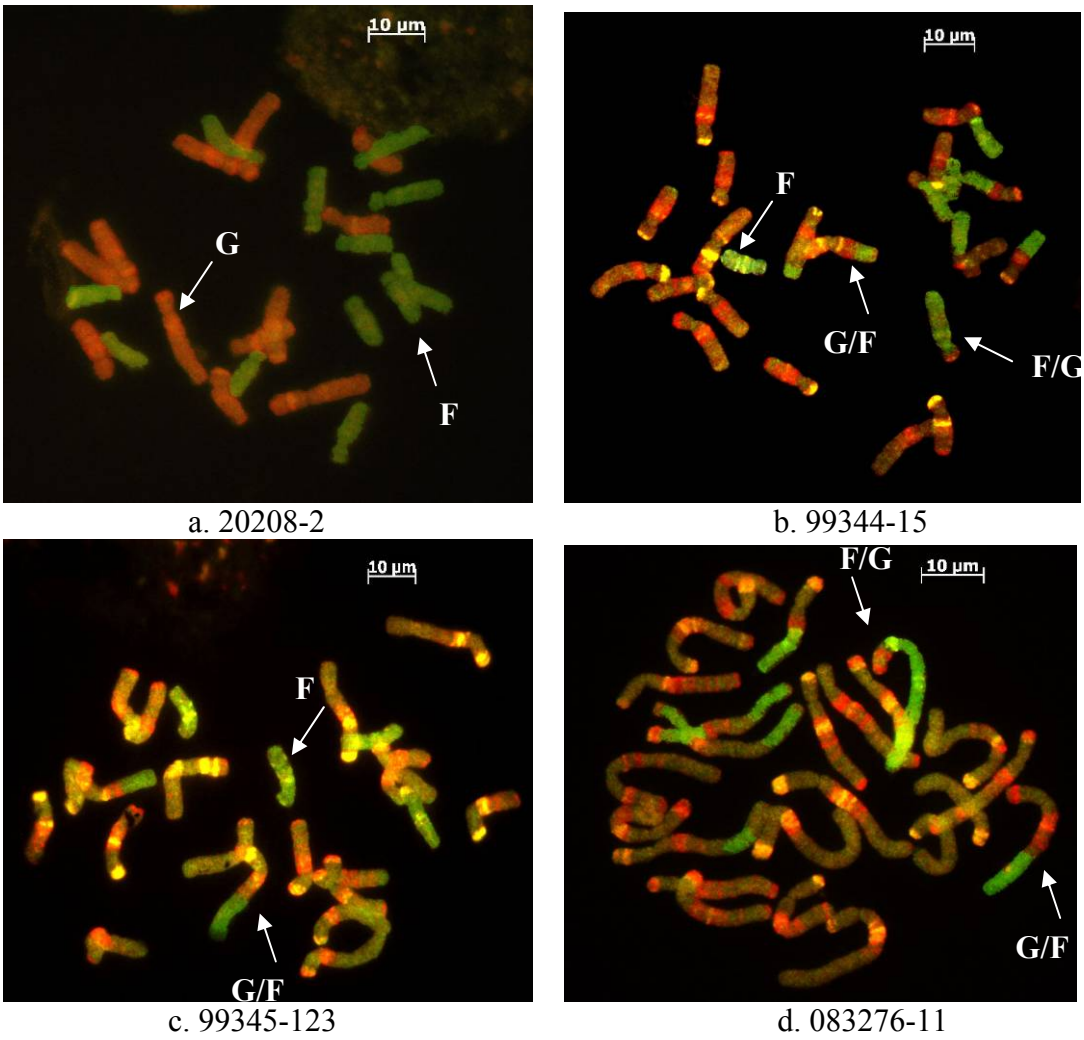
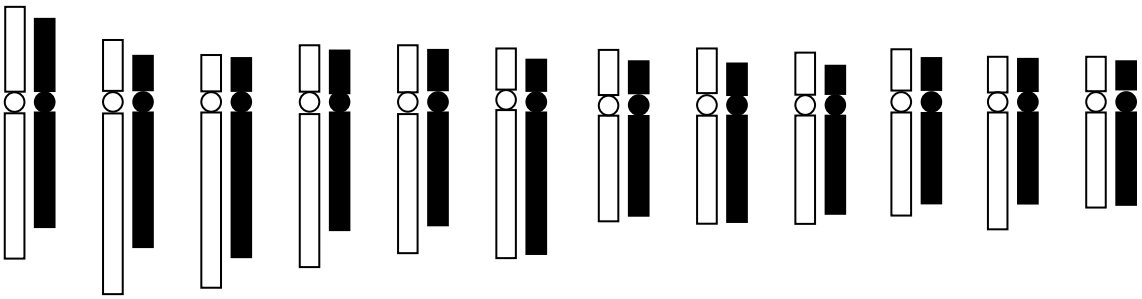
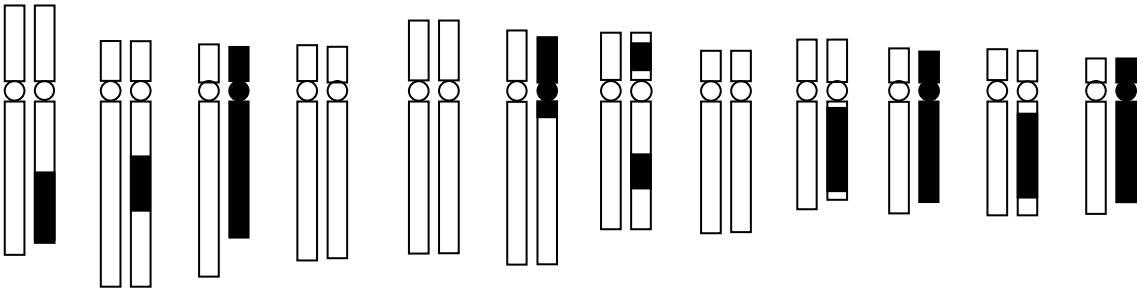


Figure 3-7 GISH results from different genotypes of hybrids. *T. gesneriana* (G) genome was labeled by biotin-16-dUTP (red) and *T. fosteriana* (F) genome was labeled by dig-11-dUTP (green) (a) F_1 hybrid 20208-2; (b) BC_1 'Purissima' hybrid 99344-15; (c) BC_1 'Purissima' hybrid 99345-123; (d) BC_2 hybrid 083276-11 from tissue culture. 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. Arrows indicted types of genome or recombination.

For each genotype at least three metaphases were selected for analysis. Based on the images of GISH experiment (Figure 3-7), total length of each chromosome from *T. fosteriana* and *T. gesneriana,* and the length of recombinant segments represented by red and green signal were measured separately. Based on these measurements the karyotypes were drawn for each genotype (Figure 3-8).

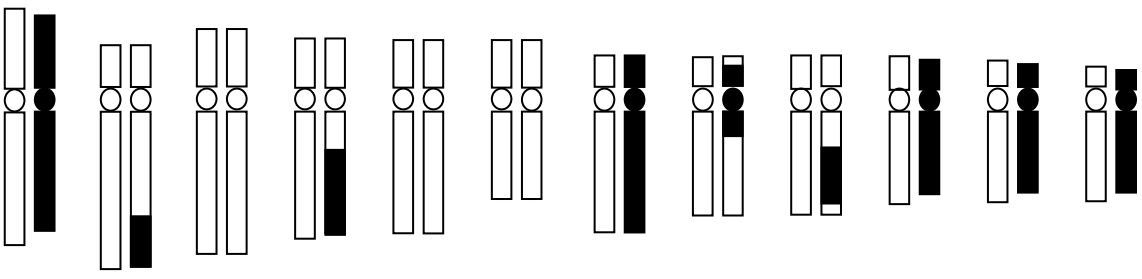


a. 20208-2

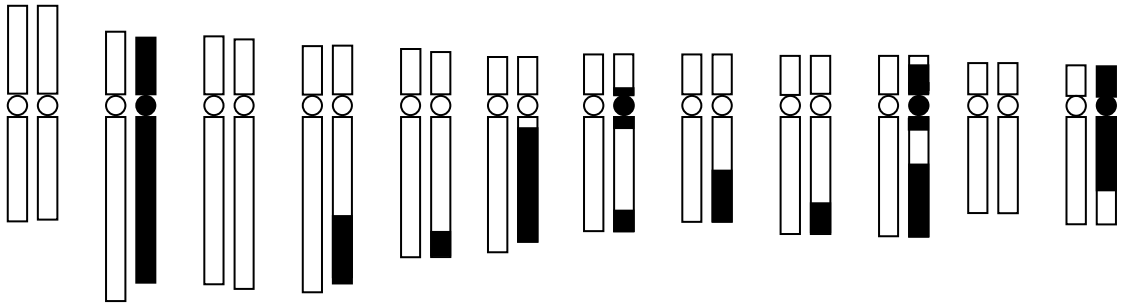


b. 99342-60

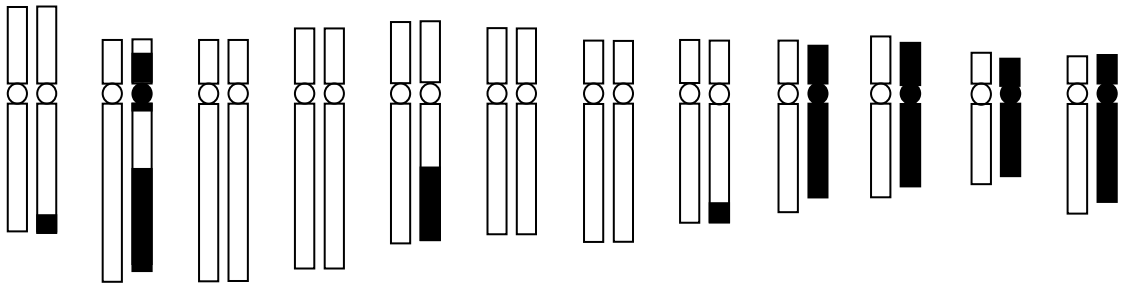
c. 99344-15



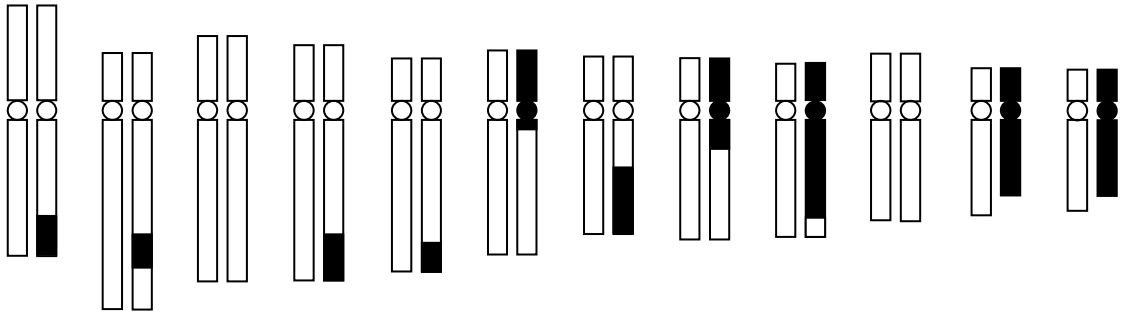
d. 99345-102



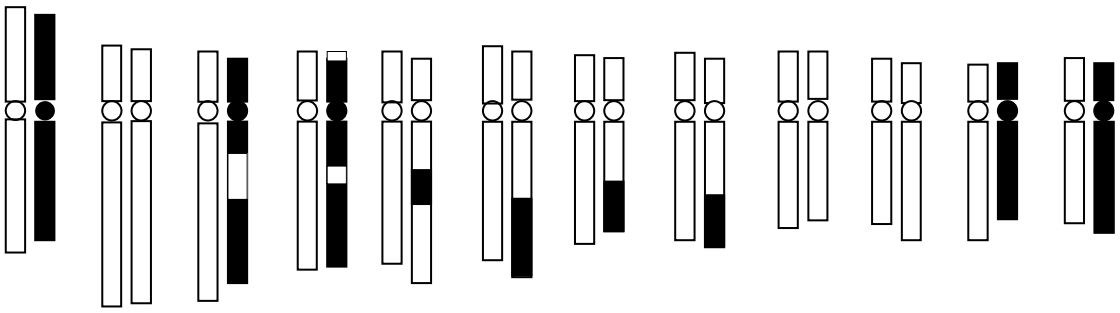
e. 99345-108



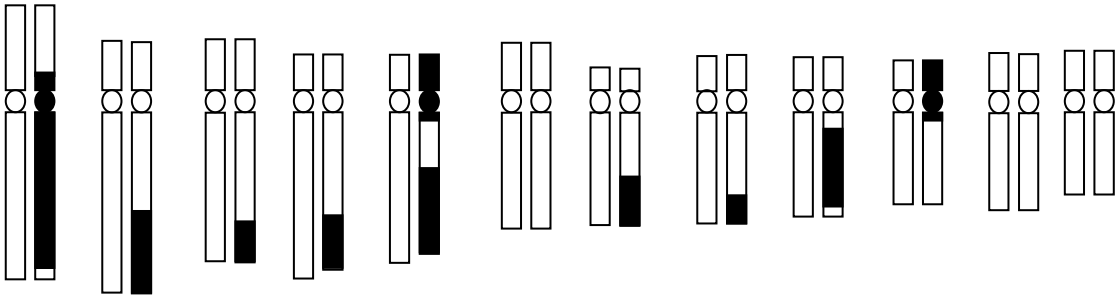
f. 99345-123



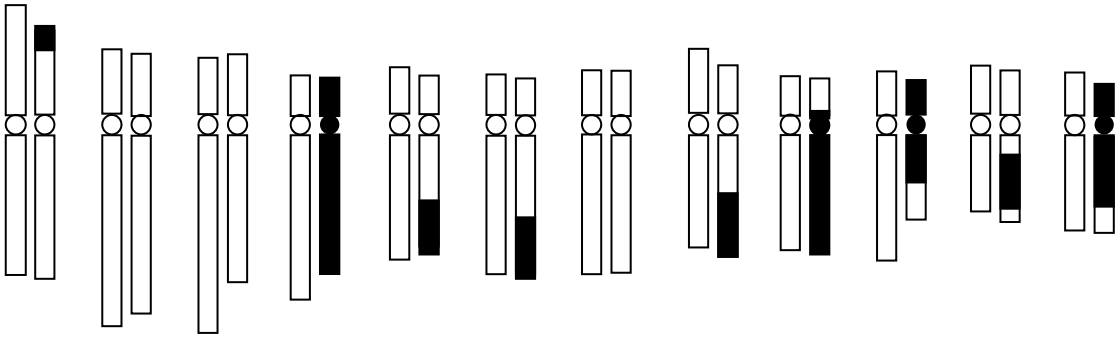
g. 99346-9



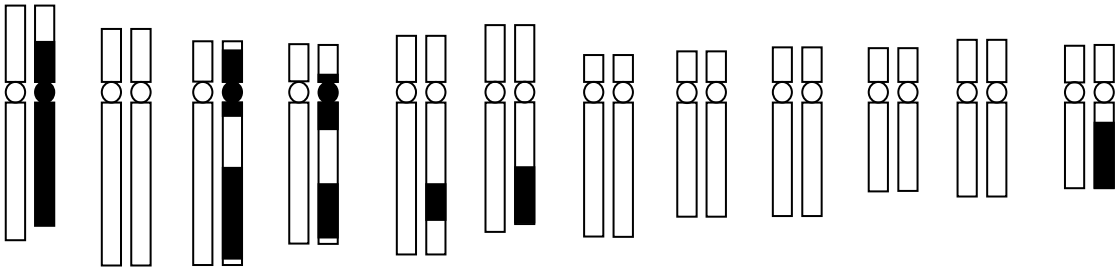
h. 99343-6



i. 083275-10



j. 99345-16



k. 083276-11

Figure 3-8 Karyotypes of *T. gesneriana* and *T. fosteriana* hybrids. *T. fosteriana* fragments were marked as black. (a) 20208-2, (b) 99342-60, (c) 99344-15, (d) 99345-102, (e) 99345-108, (f) 99345-123, (g) 99346-9, (h) 99345-16 (Marasek, data not published), (i) 083275-10, (j) 99343-6 (Marasek, data not published), (k) 083276-11

The genomic constitutions of the hybrids investigated are summarized in Table 3-3, according to karyotype analysis (Figure 3-8).

Table 3-3 Genomic information of GF hybrids and number of recombinant chromosomes

Accession No.		No. of chromosomes contributed by		No (type) of recombinant chromosomes			% of introgressed F-genome
		G-genome	F-genome	total	G/F	F/G	
F_1	20208-2	12	12	0	0	0	45.6
BC_1	99342-60	20	4	6	5	1	21.3
BC_1	99344-15	20	4	7	6	1	24.1
BC_1	99345-102	18	6	4	3	1	24.7
BC_1	99345-108	20	4	8	5	3	18.6
BC_1	99345-123	19	5	4	3	1	17.7
BC_1	99346-9	19	5	8	5	3	16.8
BC_2	083275-10	21	3	9	6	3	16.7
BC_2	083276-11	21	3	6	3	3	13.3

F_1 hybrid (20208-2) comprised of 12 chromosomes of *T. gesneriana* (green fluorescence) and 12 chromosomes of *T. fosteriana* (red fluorescence) and displayed no recombination (Figure 3-8 a) as expected. The percentage of introgressed *T. fosteriana* genome into F_1 hybrid 20208-2 equaled 45.6%.

All the BC_1 and BC_2 progenies investigated had recombinant chromosomes and most of them had both G/F and F/G recombination (Figure 3-7 b-d; Figure 3-8; Table 3-3). G/F type of recombination ranged from 3 - 6, while F/G type ranged from 0 - 3. It was found that G/F type was dominating. In all the BC_1 progenies tested, there was at least one whole chromosome from *T. fosteriana* while it was not the case in BC_2 hybrids (Figure 3-8). The maximum recombinant segments on one chromosome were three. The percentage of introgressed *T. fosteriana* genome into BC_1 hybrids ranged from 16.8% to 24.7%, and introgressed *T. fosteriana* genome into BC_2 hybrids ranged from 13.3% to 16.7%. The position of recombination distributed on both arms from distal to interstitial, but most of them were detected on terminal position of chromosomes (Figure 3-8).

3.4 Discussion

Introgression breeding

The percentage of introgressed F-genome in F_1 progeny was only 45.6% (Table 3-3) and less than expected 50%. After drawing karyotype of this progeny (Figure 3-8 a), it is clear that the genome of *T. fosteriana* was shorter than that of *T. gesneriana.* This result is in agreement with the result shown by Marasek (Marasek et al., 2006). The expected genomic composition of BC_1 diploids is 25% from *T. fosteriana* and the rest from *T. gesneriana*. The percentage of all the *T. fosteriana* genomes in BC_1 was less than 25% (Table 3-3) and varied among individuals from 16.8% (99346-9) to 24.7% (99345-102). One reason could be the genome of *T. fosteriana* (F) is shorter than that of *T. gesneriana* (G) or the introgression of F genome is slower than expected. Therefore, further analysis of BC_1 progenies is needed.

Here the BC_2 progeny from GGF backcross with *T. gesneriana* has been reported for the first time. Compared with BC_1 progeny, BC_2 is expected to contain 12.5% of the donor parent—*T. fosteriana* rather than 25% in BC_1. Analysis of two BC_2 progenies (Table 5) displayed that 083275-10 and 083276-11 contained 16.7% and 13.3% F-genome respectively. Both of them were exceeded the expectation (12.5%). So the introgression process is more than expected. If these progenies could show desirable traits, such as virus resistance, vigorous growth, probably no need to do further back crosses.

Karyotype analysis

In Figure 3-8, chromosomes were paired based on the length of both arms and arm ratio. The paired chromosomes were arranged generally based on chromosome length. Genome information of BC_1 (Marasek et. al. in press) and BC_2 were summarized in Table 3-4 in order to find a relationship between father and progeny. The percentage of introgressed F-genome is supposed to decrease from 25% (BC_1) to 12.5% (BC_2). But in fact, the decreased percentage was only 3% to 4% (Table 3-4), far less than the expected percentage. To prove this, more progenies should be taken into account.

Table 3-4 Comparison of the genome information between BC_1 and BC_2

Accession No.		Father	Break point	No. of chromosomes contributed by		No (type) of recombinant chromosomes			% of introgressed F-genome
				G-genome	F-genome	total	G/F	F/G	
BC_1	99343-6		10	19	5	6	3	3	21.4
BC_2	083275-10	99343-6	12	21	3	9	6	3	16.7
BC_1	99345-16		9	21	3	7	5	2	16.3
BC_2	083276-11	99345-16	13	21	3	6	3	3	13.3

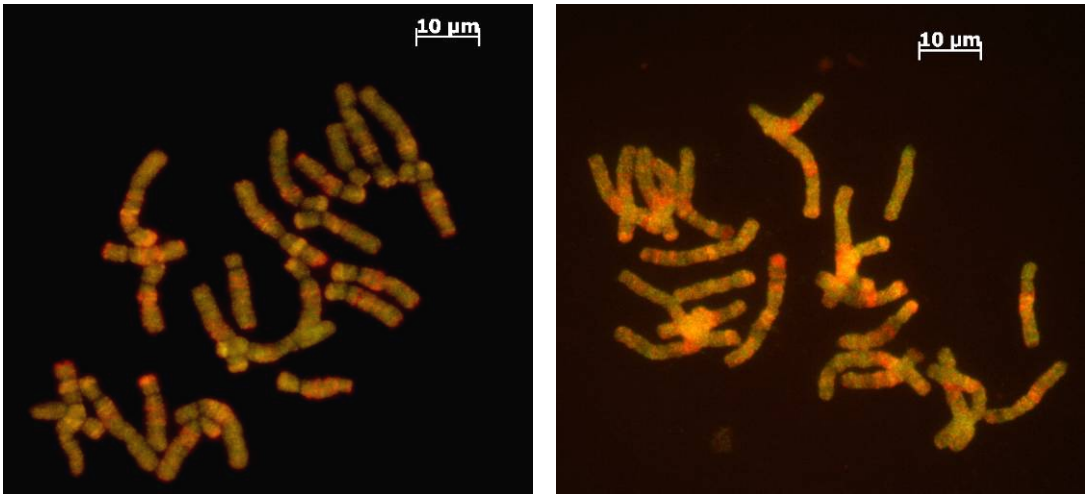
In the comparison of 083275-10 and its male parent 99343-6, the number of recombinant chromosome and breakpoints were increased (Table 3-4). In 083276-11, one recombinant chromosome was less than its farther (99345-16). However, with

respect to breakpoint in chromosomes, the number had increased. 99345-16 had 9 breakpoints (Figure 3-8 j) while 083276-11 had 13 breakpoints (Figure 3-8 k, Table 3-4). This means that during segregation of chromosomes in meiosis, some new recombination events occured. In addition, more interstitial recombinations were present in BC_2 as compared to BC_1. It was found that there is more potential of introgressions from BC1 to BC2 progenies which ultimately results into the introgression of desirable traits from the donor parents to their progenies with minimum linkage drag (Hospital 2001).

The length of chromosome was varied among genotypes. In tulip, it was not easy to distinguish each chromosome and establish karyotype for *T. fosteriana* and *T. gesneriana* as Lim et al. (2001b) did in *Lilium Longiflorum* and *Lilium rubellum*, although a lot of efforts had put on this. Mizuochi et al. (2007) reported that 5S and 45S rDNA were not successful to discriminate chromosomes of F and G, as there were so many rDNA sites located on each chromosome. Marasek et al. (2006) observed no remarkable chromosome landmarks in these two species, such as secondary construction. In addition, morphological analysis, such as length of chromosomes and arm ratio, were not able to discriminate chromosomes except median chromosomes. Because of this, it is difficult to compare each chromosome in the karyotype of BC_1 and its corresponding progeny, thus we could not find out which chromosomes had new recombination event. So therefore, we could only compare them as a whole, concerning to breakpoint and interstitial recombination obtained after GISH analysis (Table 3-4). Moreover, karyotype analysis of BC_1 (Figure 3-8 b-g) showed that each genotype contained 1 (99345-108) to 5 (99345-102) entire chromosome from *T. fosteriana*. However, only a part of *T. fosteriana* chromosome was present in BC_2 population (Figure 3-8 h, i). It illustrated the possibility of using introgression breeding to insert small fragments of the donor genome and reduce linkage drag. The comparison of genome information from BC1 progenies with same parental combination (Table 3-1) showed that there were a lot of variations in number and type of recombinant chromosomes as well as percentage of introgressed F-genome (Figure 3-8 d-f). This information strengthens the application of introgression breeding to introgressed various traits of interest in a breeding program.

Problem concerning cross-hybridization

During the experiment, many genotypes other than ‘Purissima’ hybrids were used. However, the results were not promising. Although we used the same hybridisation mixture and all the other conditions were the same as ‘Purissima’ hybrids, we can not distinguish red and green signal (Figure 3-9). We were unable to know the genome composition of these genotypes. This cross-hybridization depends on genotypes and it was noticed in Table 3-1 that all the other genotypes except ‘Purissima’ were from tissue culture, maybe this kind of cross-hybridization is because of tissue cultured seeds.



a. 081153-1 b. 081448-1

Figure 3-9 GISH results from different genotypes of hybrids. *T. gesneriana* genome was labeled by biotin-16-dUTP (red) and *T. fosteriana* genome was labeled by dig-11-dUTP (green) (a) BC_1 hybrid 081153-1; (b) BC_1 hybrid 081448-1

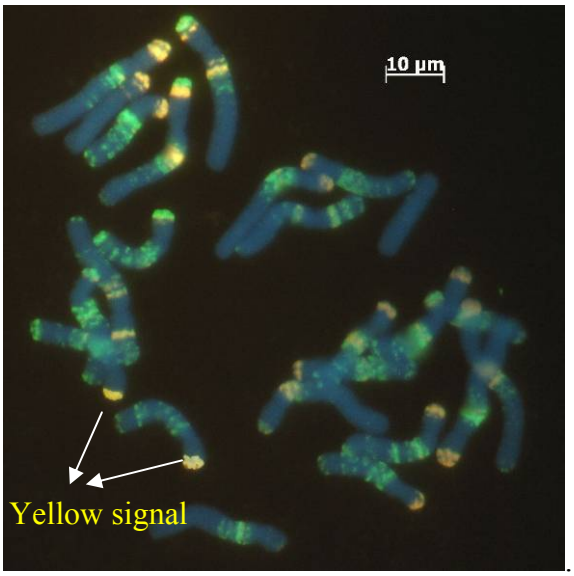


Figure 3-10 GISH results from BC_1 (061178-17) of hybrids by pre-annealing method. *T. gesneriana* genome was labeled by dig-11-dUTP (green) and *T. fosteriana* genome was labeled by biotin-16-dUTP (red)

In order to overcome this problem, we tried another modified method of GISH, which was pre-annealing method (Appendix). The idea was that before applying the hybridization mix to the slide, pre-annealing the two probes, and blocked out common DNA sequences between the different genomes, could increase species specificity of the probes. Unfortunately, we still didn't get good results. It seems that there were many cross-hybridizations giving out yellow or orange signals at telomeric and intercalary locations (Figure 3-10) (red and greed signal overlapped gives out yellow or orange signal). This kind of cross-hybridization can be explained by the high level of conservation among rDNA genes. The cross-hybridization of rDNA genes has also been reported in other species, such as *Brassica* (Hasterok et al, 2001), *Hordeum* (Taketa et al., 2001) and *Beta* (Desel et al., 2002)

In order to differentiate genomes in BC_1 hybrids (e.g., genotype 081153, 081448), further modification of GISH protocol is needed.

Usefulness of GISH in introgression breeding

In this investigation, identification of different recombinant chromosomes transmitted to the progenies (Figure 3-8) demonstrated the usefulness of GISH in introgression breeding in tulip. In the absence of this technique, accurate detection of introgressed chromosome segments would be impossible. GISH was also successfully used in detecting introgressed genome in *Lilium* (Lim et al., 2001a), *Astroemeria* (Kamstra et al, 1999). Van Eijk et al., (1991) and Custers et al., (1995) suggested the possibility of transmitting important agricultural traits (e.g., virus resistance) from *T. fosteriana* to *T. gesneriana* genome by interspecific breeding and these traits could be monitored in the subsequent progenies using GISH technique.

Future work

The introgression of *T. fosteriana* chromatin to *T. gesneriana* was recorded before (Marasek and Okazaki, 2008). However, the presence of TBV resistance in the plant was not checked. Similarly, present work has not been confirmed yet. And this can be done by visually scoring the resistant genotypes in the fields for several years or could be checked by an Elisa test. Nowadays, molecular Marker Assisted Selection (MAS) plays a very important role in breeding. Molecular markers linked to resistant traits, such as *Fusarium oxysporum,* Tulip Breaking Virus, can speed up breeding and selection processes rapidly (Van Heusden et al., 2002). However, because of the huge genome size of tulip, there are many problems in using RAPD and AFLP marker, so more attention is needed on this research area to construct a genetic map, a prerequisite for MAS. As we know that TBV resistant gene is a monogenic trait, it is much easier than polygenic traits such as *Fusarium oxysporum* resistance. The future work for the whole project, as I state before, could be to build up markers for TBV resistant gene and subsequently, to locate these markers on the chromosome by Fluoresce *in situ* Hybridization (FISH) technique.

Appendix

Protocol of Pre-annealing method of GISH

- ✓ Probe labeling: the same as we did before
- ✓ Probe denaturation and pre-annealing:

Probe mix

	1 ×
50% DS	8μl
20 SSC	4μl
10 SDS	1μl
Probe cantata-biotin	2μl
Probe Ile-digo	2μl
MQ	23μl
	40μl

Boil for 7 min, centrifuge briefly and transfer immediately to an incubator for pre-annealing either at 37℃ overnight (16-18h), or at 58℃ for 30 min

- ✓ Chromosome denaturation
  1. The slides are denatured in a preheated formamide solution (70% in 2 x SSC) at 70℃ for 2 min
  2. Dehydrate in an ice cold ethanol series (70, 90 and 100%, 2 min each) and then allow air-dry
- ✓ Hybridization
  1. Probe mixture are applied to the slides and covered with glass coverslips
  2. Slides are incubated in a preheated humid chamber at 63℃ for 2 h of hybridization.
- ✓ Washing
  1. Gently remove the coverslips
  2. Slides are washed for 2 × 5 min in 2 × SSC at 63℃
  3. Transfer to an incubator at 37℃ for 20 min, and allowed to cool to RT.
- ✓ Detection
  1. Wash the slides in buffer 1 for 5 min
  2. Add 200μl blockbuffer to each slide. Cover the slides with a glass/plastic coverslip and incubate for 5 min
  3. Dilute 2μl anti-dig-FITC and 0.4μl streptavidin-CY3 anti-bodies in 100μl blockbuffer per slide
  4. Drain the slides and add the diluted antibodies to the slide. Cover the slides with coverslips, place the slides in a humid chamber for 1 h at 37℃
  5. Wash in buffer 1 for 5 min at 37℃
  6. Wash in Tween/4xSSC 2 times for 8 min at 37℃
- ✓ Counterstaining and mounting
  1. Dehydrate in an ethanol series (70, 90 and 100%, 1 min each) and then allow air-dry, all these steps should be done in dark
  2. Use vectashield(0.5ml)+DAPI(1μl) 20μl each slide and cover the slides with coverslips
  3. Store the slides in +4℃

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