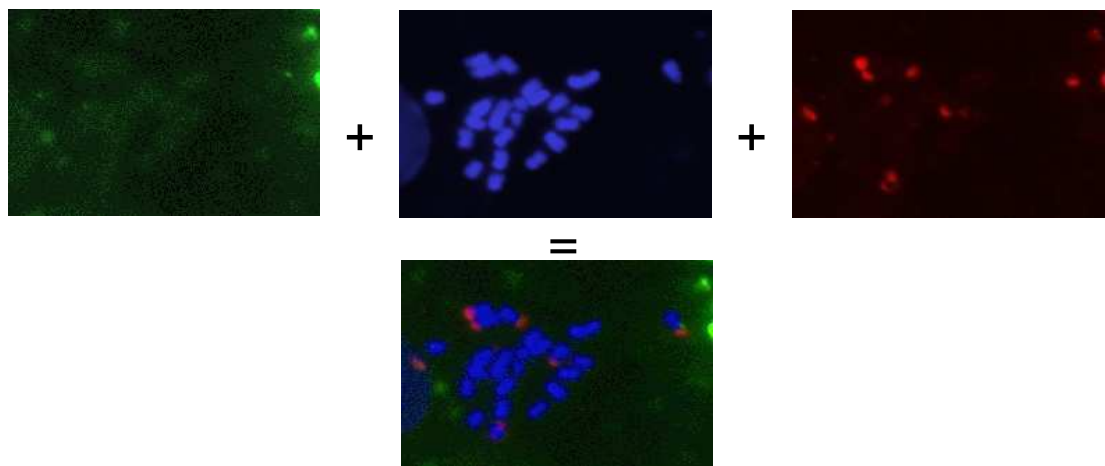**Universität für Bodenkultur**

University of Natural Resources and Applied Life Sciences

Localization of rDNA genes by Fluorescence *In Situ* Hybridization (FISH) in *Prunus* species

Diploma Thesis

Birgit Schmöllerl

Univ. Prof. Dr. Margit Laimer

Plant Biotechnology Unit (PBU)

Institute for Applied Microbiology (IAM)

Department of Biotechnology

Vienna, July 2009

Contents

Figures

Tables

Abstract

Prunus sp. is one of the most important European fruit species, thus rendering genetic studies particularly interesting. *Prunus* species with a very small nuclear genome have a basic chromosome number of 8 and range from diploid (2n = 2x = 16) to hexaploid (2n = 6x = 48).

In an attempt to provide scientific data answering the concerns about transgenic crops, the first step involves to characterize transgenic plants in detail at the genetic and molecular level.

By karyotyping, both in PIS (*P. incisa x serrula* (GM 9) Inmil[R]) and PAR (*Prunus subhirtella* autumnalis rosa), only chromosome number 1 could be clearly identified by its size. Therefore a FISH approach was chosen to determine the number and the physical position of rDNA in PIS (*P. incisa x serrula* (GM 9) Inmil[R]) and PAR (*Prunus subhirtella* autumnalis rosa).

To optimize the probe quality three different labelling methods were used. In this study the labelling of pTA71, containing the 45S rDNA, by Nick Translation was only successful if the plasmid was linearized. Although PCR labelling method showed a great improvement for probe labelling, it was limited by the size of the produced probe.

 A karyotype according to the size of the chromosomes was established. The simultaneous double colour hybridization with the 5S rDNA and the 45S rDNA of the PIS chromosomes allowed the identification of 4 further chromosomes of the tetraploid genome with 2n = 4x = 32 chromosomes, and of 3 further chromosomes of the diploid (2n = 2x = 16) genome of PAR. For PAR 6 signals for 45S rDNA were detected on chromosomes number 2, 3 and 6. The signal on chromosome 6 is weaker than the other signals for 45S rDNA. The 5S rDNA was detected close to the strong 45S rDNA signals on chromosome number 2 and 3. For PIS 12 signals for 45S rDNA and 4 signals

for 5S rDNA could be detected. The signals for 45S rDNA are on the terminal region of chromosomes number 2, 3 and 6. The signal for 5S rDNA is located on chromosome number 7.

This study is the first report of using FISH to determine the number and the physical position of rDNA on chromosomes of PIS and PAR.

Zusammenfassung

Prunus sp. umfasst die wichtigsten europäischen Obstarten, folglich sind genetische Studien besonders wichtig. *Prunus* Arten mit einem sehr kleinen Genom haben eine grundlegende Anzahl von 8 Chromosomen und sind diploid ($2n = 2x = 16$) bis hexaploid ($2n = 6x = 48$).

In dem Versuch mit wissenschaftlichen Daten Fragen an transgene Nutzpflanzen zu beantworten, beinhaltet der erste Schritt die detaillierte genetische und molekulare Charakterisierung der transgenen Pflanzen.

Bei der Erstellung des Karyotyps sowohl von PIS (*P. incisa x serrula* (GM 9) Inmil[R]) als auch von PAR (*Prunus subhirtella* autumnalis rosa) konnte nur Chromosom Nummer 1 eindeutig anhand der Größe identifiziert werden. Deshalb wurde eine FISH Methode ausgewählt, um die Anzahl und die physische Position der rDNS von PIS (*P. incisa x serrula* (GM 9) Inmil[R]) und PAR (*Prunus subhirtella* autumnalis rosa) festzustellen.

Um die Qualität der Sonde zu optimieren, wurden drei verschiedene Färbemethoden verglichen. In dieser Studie war die Färbung von pTA71, das die 45S rDNS beinhaltet, mittels Nick Translation nur erfolgreich, wenn das Plasmid linearisiert wurde. Obwohl die Färbung mittels PCR einen großen Fortschritt bedeutete, war sie durch die Größe der produzierten Sonde limitiert.

Entsprechend der Größe der Chromosomen wurde ein Karyotyp erstellt. Die gleichzeitige Hybridisierung der PIS Chromosomen mit 5S rDNS und 45S rDNS, die mit zwei Farbstoffen markiert waren, erlaubte die Identifikation von 4 weiteren Chromosomen des tetraploiden Genoms mit $2n = 4x = 32$ Chromosomen, und von 3 weiteren Chromosomen des diploiden Genoms ($2n = 2x = 16$) von PAR. Für PAR wurden 6 Signale für 45S rDNS auf den Chromosomen Nummer 2, 3 und 6 detektiert. Das Signal auf Chromosom

Nummer 6 ist schwächer als die anderen Signale für 45S rDNS. Die 5S rDNS wurde nahe an den starken 45S rDNS Signalen auf den Chromosomen Nummer 2 und 3 detektiert. Für PIS konnten 12 Signale für 45S rDNS und 4 Signale für 5S rDNS detektiert werden. Die Signale für 45S rDNS sind in der terminalen Region der Chromosomen Nummer 2, 3 und 6. Das Signal für 5S rDNS befindet sich auf Chromosom Nummer 7.

Diese Studie ist der erste Bericht über die Anwendung von FISH zur Bestimmung der Anzahl und Lokalisierung der rDNS auf Chromosomen von PIS und PAR.

Acknowledgements

I am deeply grateful to my advisor, Univ. Prof. Dr. Margit Laimer, for her encouragement and for giving me the chance to work on an interesting topic like this. She provided the lab at the IAM, all the material needed, constructive comments and suggestions to finish this study with success.

I also especially thank my co-advisor Dr. Fatemeh Maghuly for her practical support, constructive critics and helpful information. I also want to thank her for the effort she made to help me writing this thesis.

Finally, I would like to thank my family and friends for being always with me, never setting me under pressure and bringing me strength and patience.

Abbreviations

2n   sign of number of chromosome sets

AP   Alkaline Phosphatase

BAC   Bacterial Artificial Chromosome

bp   base pairs

c   concentration

CaMV   Cauliflower Mosaic Virus

CCD   Charge Coupled Device

CSPD   Disodium   3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)   tri-
            cycle [3.3.1.1]decan}-4- yl)phenyl

DIG   Digoxygenin

DKV   Driver and Kuniyuki Medium

dNTP   Deoxyribonucleotide Triphosphate

E. coli   *Escherichia coli*

EDTA   Ethylene-Diamin-Tetra-acetic Acid

EtBr   Ethidium Bromide

FACS   Fluorescence-Activated Chromosome Sorting

FISH   Fluorescence *In Situ* Hybridization

IAA   Indol-3-Acetic Acid

kb   kilo base pairs

LB	Luria Bertani Medium
PAC	Phage P1 based Artificial Chromosome
PAR	*Prunus subhirtella autumnalis rosa*
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIS	*Prunus incisa x serrula*
RAPD	Randomly Amplified Polymorphic DNA
rDNA	ribosomal DNA
RF	Recombination Frequency
RFLP	Restriction Fragment Length Polymorphism
RT	Room Temperature
SNP	single nucleotide polymorphism
sp.	Species
SSC	Sodium-Sodium Chloride
SSLP	Simple Sequence Length Polymorphism
TAE	Tris-Acetic acid-EDTA
T-DNA	Transgenic DNA
TE	Tris-EDTA
TST	Tris-Sodiumchloride-Tween
Tyr-FISH	Tyramide-Fluorescence *In Situ* Hybridization
v/v	volume to volume

VNTR   Variable Number Tandem Repeats

w/v   weight to volume

x   sign of ploidy-level

YAC   Yeast Artificial Chromosome

1. Introduction

1.1 *Prunus sp.* L. – General description

1.1.1 Botanical characteristics of *Prunus sp.*

The genus of *Prunus* within the subfamily *Prunoideae* belongs to the family *Rosaceae,* which are divided into three major subgenera: *Prunophora* (plums and apricots), *Amygdalus* (peaches and almonds) and *Cerasus* (sweet and sour cherries) (Esmenjaud and Dirlewanger, 2007)

Prunus includes approximately 400 species of trees and shrubs that grow throughout the Northern temperate regions worldwide. Many species and cultivars are grown for their edible fruits, while others are planted for their ornamental value. It is characterized by species that produce drupes known as "stone fruits" where the seed is encased in a hard, lignified endocarp referred to as the "stone", and the edible portion is a juicy mesocarp (Abbott et al, 2007).

1.1.2 Geographic origin and natural distribution of *Prunus sp.*

Watkins suggested in 1976 that the first diploid *Prunus* species arose in Central Asia, and that species in the section *Cerasus* (sweet, sour and ground cherry) were early derivatives of this ancestral *Prunus*. Hedrick (1915) described the geographic range of wild sweet cherry as the mainland of Europe far into the Southern U.S.S.R. and as far east as Northern India, with the greatest prevalence between the Casparian Sea and the Black Sea (Watkins, 1976).

1.1.3 Crossability

Interspecific (and intergeneric) relations in *Prunus* are not clear, perhaps due to the widespread presence of auto-incompatibility and the relative fertility of interspecific hybrids. The prolonged time period for flowering in the

Prunus species, influences the possibility of mutual pollination between different cultivars, as well as different species. The physiological or ecological diversity of the species caused sufficient isolation of their individual habitat. Interspecific barriers did not develop during natural evolution, but by the appearance of cultigenous hybrids, which started their development from the first steps of domestication up to the limits set by taxonomic divergence interspecific barriers appeared (OECD, 2002). Although cherries are perhaps the most distant from the rest of the species, bridging species between plums and cherries were found.

The introgression between cultivated and wild species of *Prunus* is scarcely documented. There is no doubt about the physical possibility. Escapes of cultivated varieties are frequently found in woods, pastures, abandoned orchards, ruderal, and other marginal areas. Intercrosses with really wild populations have very little chance, as blackthorn, hedge cherry and dwarf almond are extremely different in morphology and in adaptation, eventual hybrids could only survive in a very protected environment (OECD, 2002).

1.1.4 *Prunus* rootstocks

A rootstock provides a healthy root system, when used for grafting with a scionwood or budding from another plant. Rootstocks are most commonly used for fruit trees and grapevines. They are also selected for traits such as resistance to drought, soil composition, cold stress, root pests and diseases. The rootstock scion relationship is important for horticultural purposes, because it provides the basis for selecting the best graft combination for particular environmental conditions to yield high fruit quality (Gonçalves et al., 2005).

The use of *Prunus* rootstocks is one of the most effective ways to provide varying levels of control of vigor. The three most important traits for *Prunus* rootstocks are retaining the productivity over a long time period, reducing the tree size and imparting precocious flower bud induction. Further traits

are tolerances to problematic sites and resistance or tolerance to pests (Lang, 2000).

1.1.5 Breeding of *Prunus sp.*

The major breeding objectives for cherry are large, good flavoured fruits which are resistant to fruit cracking and large and constant yields. Further, breeding should improve tolerance and resistance to diseases and shorten the juvenile phase (Dirlewangler, 2007).

Compared to other temperate fruits breeding improvement for cherry was slow (Dirlewangler, 2007). Classical breeding programs are time consuming, especially for cherry that requires a minimum of 3 - 5 years of growth before flowering and fruit production (Merkle et al., 2000).

1.1.6 Genome contents

The phenomenon of polyploidy is widespread and of great importance in the evolution of new species or forms. Polyploidization is the increase in genome size initiated by the inheritance of an additional set of chromosomes. If the duplicated sets of chromosomes originate from the same or a closely related individual the progeny is considered as autopolyploid, if it originated from two different species it is called allopolyploid. Changes in genome structure usually have immediate effects on the phenotype and fitness of an individual. Changes in genome structure might allow evolutionary transitions that were impossible before polyploidization. For example, by introducing an additional set of chromosomes, polyploidization might release gene duplicates from the limitation of having to perform all of the functions of a gene, providing extra features upon which selection can favour new functions. Polyploidization can also stimulate further structural changes in the genome, providing genomic variation not available to diploid organisms (Otto, 2007).

All *Prunus* species have a basic chromosome number of eight and range from diploid (2n = 2x = 16) to hexaploid (2n = 6x = 48) (Arumuganathan and Earle, 1991). They have a small genome corresponding to about twice the size of *Arabidopsis thaliana* (Arumuganathan and Earle, 1991).

1.1.7 Botanic characterization of *Prunus* used in this study

PIS: *P. incisa x serrula* (GM 9) Inmil[R] is the most dwarfing cherry rootstock for cherry trees (Druart and Gruselle, 2007). PIS are rather unique in its capacity for regeneration of buds from *in vitro* grown roots. This trait of adventitious regeneration has been hardly reproducible in other *Prunus sp.* studied with the aim to genetically improve by transformation (Druart, 1981). PIS plants, due to their commercial value, relative small genome size (1C ~ 0.6 pg; tetraploid) and close relationship to other important *Rosaceae,* are the ideal model species for studying fruit trees (Maghuly et al, 2008).

Embryogenic calli of PIS were transformed following the procedure described by Druart (1981) using the *Agrobacterium tumefaciens* strain LBA4404 (Hoekema, 1983) containing pBinGUSint (Maghuly et al, 2008).

PAR: *Prunus subhirtella* autumnalis rosa is a deciduous, ornamental cherry tree which is up to 18 m tall, and most often encountered as a horticultural selection or botanical variety. It flowers from November to February and unlike the cultivar "autumnalis" its flowers have a conspicuously pink centre. It is used as rootstock for cherries due to its cold hardiness and has some potential for the genetic improvement of cherry (Maghuly et al, 2007), and has a small genome size (2*n* = 2x = 16, diploid, C-value = 0.3 pg) (Bennett and Leitch, 1995).

Transformed *P. subhirtella* (PAR) plants were regenerated from somatic embryos by inoculating an embryogenic callus with *Agrobacterium timefaciens* strain LBA 4404 (Hoekema, 1983) containing the pBinGUSint (da Câmara Machado et al, 1995).

Transgenic PAR and PIS lines were analyzed for stability and activity of transformed genes by PCR, Southern blotting and real time PCR by Maghuly et al. (2007, 2008).

1.2   Characterization of specific nucleic acid sequences

A variety of techniques are now available for genome-wide screening of alterations in copy number, structure, and expression of genes as well as DNA sequences. These include molecular cytogenetic techniques, e.g. comparative genomic hybridization, karyotyping, and multicolour Fluorescence *In Situ* Hybridization (mFISH), as well as molecular genetic techniques, e.g. restriction landmark genomic scanning, representational difference analysis, differential display, serial analysis of gene expression and microarray techniques.

1.2.1   Physical mapping

Several physical mapping techniques have been developed. The most important are: Restriction mapping, STS and FISH mapping (Brown, 2002).

1.2.2   Fluorescence *In Situ* Hybridization (FISH)

1.2.2.1 Historical background of FISH

The technique was first described by Gall and Pardue (1969) and John et al. (1969). Older methods that used radiolabel probes were soon replaced by fluorescence based techniques (Langer-Safer et al. 1982). In 1985 Rayburn and Gill introduced the *in situ* hybridization method in plant species by using biotin labelled probes which were detected by enzymatic reporter molecules. Later, various probes labelled with different fluorochromes were analyzed simultaneously and captured by special cameras or laser scanning microscopes and analyzed with digital imaging systems (Jiang and Gill 1994).

Developments of indirect detection allowed signal output to be increased artificially by the use of secondary reporters that bind to the hybridization probes. In the early 1980s, assays featuring nick-translated, biotinylated probes, and secondary detection by fluorescent streptavidin conjugates were used for detection of DNA (Manuelidis et al., 1982) and mRNA (Singer and Ward, 1982) targets. A decade later, improved labelling of synthetic, single-stranded DNA probes carrying enough fluorescent molecules allowed direct detection (Kislauskis et al., 1993). The development of bright fluorescent dyes such as Cy-3 (indocarbocyanine), Cy-5 (indodicarbocyanin) and Alexa series in combination with sensitive cooled charge coupled device (CCD) cameras made direct FISH the technique of choice for most applications in molecular cytogenetics. The use of CCD cameras enhanced the detection of targets as small as 1 – 3 kb (Trask, 1991; Jaing, 1996; De Jong, 1999). DNA targets smaller than 1 kb were successfully visualized in plant chromosomes using Tyr-FISH (Khrustaleva and Kik, 2001; Stephens et al., 2004). In Tyr-FISH a peroxidase conjugated antibody is used as the first layer of signal detection, and flourochrome-labelled tyramides are used as peroxidase substrates to generate and deposit many fluorochromes close to the *in situ* bound peroxidase (Raap et al., 1995). Another important factor for FISH is the spatial resolution. It is defined as the smallest physical distance between flanking target sequences that can be resolved using a conventional fluorescence microscope. The spatial, resolution for mitotic metaphase chromosomes remained limited, generally in the order of 2–5 Mbp (Jiang et al, 1996). Interphase nuclei provide better resolution, with values of as little as 50–100 kb, but lack recognizable chromosome structures and differ between cell types (De Jong, 1999).

1.2.2.2 The principle of FISH

The principle of FISH is the sequence specific hybridization of a DNA or RNA probe to the chromosomes of an organism. Therefore a clean

cytological preparation of full chromosome sets and good quality probes are the prerequisites.

There are several techniques to generate chromosome preparations. Two different methods for the preparation of metaphase chromosomes are described: the dropping and the squashing methods. For both methods the cell wall is digested by an enzyme mix. For the dropping method the digested cells are concentrated in a minimum of liquid by centrifugation, the cell suspension is dropped on a slide and the protoplasts burst by the upcoming forces. For the squashing method the digested meristems are put on a slide, the material is macerated by fine needles to separate the cells from each other and then a cover slip is applied. By tapping on the cover slip the protoplasts are destroyed to free the chromosomes (Fukui and Nakayama, 1996).

FISH is based on the use of non-radioactive labels that are incorporated into the probe DNA. Two basic methods of FISH, direct and indirect, are used depending on the requirements of specific experiments. In the direct method the label that has been incorporated into the probe can be examined by fluorescence microscopy immediately after the hybridization has been finished. Direct FISH is rapid, sensitive and exhibits very low background. In indirect FISH, biotin or digoxigenin (DIG)-conjugated nucleotides are incorporated into the hybridization probe. Hybridization signals are amplified and later visualized using one to three layers of fluorophore conjugated avidin, streptavidin and/or antibodies (Sergei, 2001).

Labelled probes can be obtained in various ways (like PCR or enzymatic labelling systems, e.g. for DNA, Nick Translation, oligolabelling, endlabelling, and for RNA, *in vitro* transcription). PCR uses specific primers for a previously chosen DNA sequence of interest and by Random Primed Labelling the entire DNA available in the reaction can be amplified and further used as a probe. In Nick Translation an enzyme introduces nicks into the double strand of the DNA. The removed nucleotides are replaced by

another enzyme. By Nick Translation the DNA is not amplified (Schwarzacher & Heslop-Harrison, 2000; Fukui and Nakayama, 1996).

The chromosomes and the probe are denatured to produce single strands. Then the probe is added to the chromosome-preparation. Under conditions for renaturation the probe binds to the homologue DNA sequence on the chromosome. By different detection mechanisms (radioactivity, enzymatic reporter molecules, fluorochromes or antibodies) the localization of the probe can be visualized (see Figure 1-1).
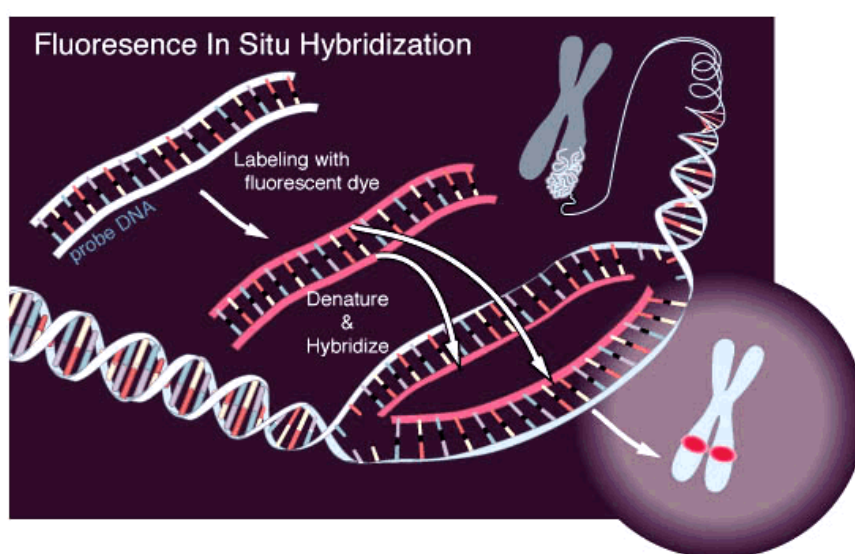
Figure 1-1: Principle of Fluorescence *In Situ* Hybridization (www.genome.gov/10000206)

1.2.2.3 Applications of FISH in plants

FISH can be used for the identification of different chromosomes within a genome. By the use of chromosome specific cytogenetic DNA markers the chromosome gets a distinctive feature to differentiate it from the other chromosomes (Jiang and Gill, 2006). The use of one or more probes generates specific hybridization patterns on chromosomes that can be used for karyotyping; further methods for the differentiation of chromosomes are C-banding or N-banding methods. In C-banding AT rich heterochromatin is visualized by AT-specific fluorescent dyes. In N-banding nucleolus

organizing regions are detected by the use of silver or Giemsa stains. The FISH karyotypes from some repetitive DNA probes are similar to karyotypes based on C- or N-banding analyses (Cuadrado et al., 1995; Pedersen and Langridge, 1997), it provides an evolutionary and phylogenetic view of related plant species (Lim et al., 2000; Hizume et al., 2002) and it is possible to visualize intergenomic chromosome translocations in polyploid species (Linc et al., 1999; Zhang et al., 2004). In 1986 Ambros et al. published a successful application of FISH for detection of a 17 kb T-DNA sequence in plant chromosomes The ability to localize transformation sequences was improved by the use of CCD cameras and the use of Tyr-FISH till even single copy T-DNA sequences smaller than 1 kb could be detected (Khrustaleva and Kik, 2001). In 1987 Meyerowitz adapted the methods for *in situ* hybridization to RNA, which were developed for animal tissue, to allow the detection of specific RNA in plant tissue. By the use of RNA specific probes the determination of the spatial and temporal expression of genes is possible.

One of the most important applications of the FISH techniques has been their use for physical mapping. For a long time only repetitive DNA sequences and multicopy gene families were physically mapped by FISH but several improvements of the techniques allow the detection of low and single copy genes (Jiang and Gill 1994).

1.2.2.4 Potential problems associated with FISH methods

One of the prerequisites for FISH is the production of high quality preparations of metaphase chromosomes. If the plant material is old or has a low ratio of cell division or small meristems it is difficult and time consuming to prepare slides with a sufficient amount of metaphase spreads which are complete, well separated and without disturbing cytoplasm. Furthermore, many woody species produce tannins, polyphenols and other secondary metabolites that hamper the production of high-quality preparations (Ma et al., 1996). To arrest cells in the metaphase stage of the

cell cycle different spindle inhibitors (colchicine, 8-hydroxychinoline, α-bromnaphtalene, taxanes and plant alkaloids) are used. The susceptibility of each plant to a specific spindle inhibitor is different.

Also, the amount of incorporated label can differ from reaction to reaction for all labelling methods. Too low amounts of labelled nucleotides will give a weak signal in FISH and too high amounts of labelled nucleotides can influence the structure of the probe hence the ability to hybridize to the chromosomes. The accessibility of the cytological targets and the size of the probes are important for efficient and sensitive FISH. Hybridization probes need to be long enough to hybridize stable to the target, but they should also be able to access the DNA within the chromatin configuration. A probe length of 100 – 300 bp is recommended (Schwarzacher, 2003).

Another important parameter in FISH technology is the spatial resolution of adjacent signals. It depends on the degree of chromatin compactness, and thus on the genome size and the phase of nuclear division. Well defined metaphase chromosomes are highly condensed and have a resolution of 5 – 10 Mb; the resolution of interphase nuclei can have values more than 100 kb. Microsporocytes at the pachytene stage are less condensed than chromosomes in the metaphase and single chromosomes are still distinguishable, which make this stage best suited for the mapping of genes (de Jong, 2003).

1.3 An overview of using FISH in *Prunus sp.*

So far there are only two reports about FISH application to *Prunus* species. In 1999 Yamamoto et al. studied the physical mapping of 18S rDNA of *P. persica* by FISH to prove the indicated existence of a relationship between rDNA sites and positive bands of chromomycin A_3 (a DNA binding fluorochrome specific to guanine-cytosine pairs). Six signal sites on six chromosomes were detected at the satellites, termini and proximal regions of the two respective

chromosomes. The working group already predicted FISH as a useful tool for the identification of chromosomes, which can not be distinguished by morphological characteristics. In 2004, Corredor et al. managed to karyotype almond *P. dulcis* by applying double colour FISH, using the 18S-5.8S-25S rDNA and the 5S gene as probes. Chromosomes with no signal, chromosomes with a signal for 18S-5.8S-25S rDNA and chromosomes with a signal for 5S rDNA could be identified on the basis of size and morphology.

2. Aim of the Work

Prunus sp. has become one of the most important European fruit species, rendering genetic studies particularly interesting.

FISH with labelled DNA probes provides additional valuable chromosomal landmarks that can be used for chromosome identification. Among such markers, the rDNA genes (18S–5.8S-25S and 5S rDNA) have been studied extensively in plant genomes. Because of the numerous copies (hundreds too many thousands) of these highly conserved families of repeated sequences their physical location on the chromosome can be visualized.

As there are a number of concerns about transgenic crops and, consequently, there is a need to characterize transgenic plants in further detail at the genetic and molecular levels, physical and genetic mapping of transgenes is an important part of this process (Salvo-Garrido et al, 2001).

In this work:

- Physical mapping of rDNA genes, i.e. 18S–5.8S–25S (45S) rDNA and 5S rDNA, and the karyotyping of non transgenic PAR and PIS plants should be carried out using double colour FISH. Additionally an attempt was made to localize the T-DNA on chromosomes of transformed PIS and PAR plants by FISH.

- Since the mitotic metaphase chromosomes of PAR and PIS are extremely small in size and lack morphological characteristics to be clearly specifiable without any kind of markers or labelling. Therefore the adaptation and optimization of the methods was the first challenge in this work.

- Good quality of the metaphase chromosome preparations is one of the major prerequisites for good results in FISH. Thus, different methods for chromosome preparation should be compared and evaluated.

- Different labelling methods should be compared for the preparation of the probes, including PCR, Nick-translation and high-prime.

- Since signal intensity is expected to be low, several different labelling methods should be applied to the best chromosome preparations. Signal intensity for each dye should be optimized for each filter to get a clear picture to produce chromosome preparations with lowest possible background signals.

- Karyotyping of PAR and PIS plants should be carried out.

3. Material and Methods

3.1 Plant material

Roots of transgenic *Prunus subhirtella* autumnalis rosa (PAR) and *Prunus incisa x serrula* (PIS) grown under in vitro and greenhouse conditions were used for this study. For in vitro cultures the rooting medium DKV containing macro- and microelements with 0.65% agar and 0.5 mg IAA was used. Cultures were kept at 23 ± 1 °C with a 16 h photoperiod provided by cool white fluorescent tubes with an irradiance of 60 μmol/m²s.

PAR and PIS are containing pBinGUSint (Vancanney et al., 1990) carrying the GUS gene with an intron (186 bp) under the control of the 35S or the apple CaM promoters besides the *nptII* gene (Maghuly et al, 2008, 2007) for selection. Further, flower buds of greenhouse plants (PAR GUS 40, PAR GUS 42, PAR GUS 20-1, PAR GUS 20-2 and PAR GUS 36) and a cherry tree from the field were used for meiotic chromosome preparations.

3.2 Plasmids

The clone pTa71, containing the 18S-5.8S-25S rDNA sequence of the bread wheat (Gerlach & Bedrook, 1979), the clone pBluescript containing the 18S rDNA and the clone pGEM-TEasy, containing a 121 bp 5S rDNA derived from *Melampodium divaricatum* were used as DNA probes. The T-DNA construct (Figure 3-1) of pBinGUSint (Vancanneyt et al., 1990) was used as DNA probe to localize the transgene in the plant-genome.
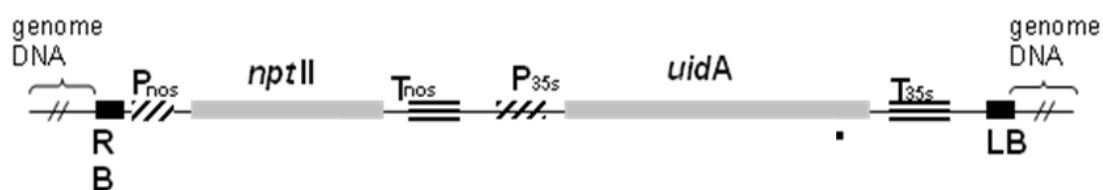
Figure 3-1: T-DNA construct of pBinGUSint

3.3 Chromosome preparation

DNA-DNA *in situ* hybridization technique is usually carried out on mitotic and meiotic cells where chromosomes and nuclei are released from cells and spread on a glass slide microscope. It is generally known that the quality of the chromosome preparations is very important for the further success of FISH.

3.3.1 Cleaning microscope slides

It is necessary that the slides used in fluorescence microscopy are extremely clean. Therefore, slides should routinely be washed with HCl. Omitting this step often leads to selective loss of nuclei and metaphase spreads and poor *in situ* hybridization results.

Protocol:

- put the slides in 6 M HCl for at least 4 h
- rinse the slides with tap water for 5 min
- rinse the slides with distilled water to remove the tap water
- air dry the slides
- put the slides in absolute ethanol until use
- air dry the slides right before use

Solutions:

- 6 M HCl
    - Dilute 37% HCl in distilled water
- absolute ethanol

3.3.2 Accumulation of mitotic chromosomes

To increase the number of cells in the metaphase stage of the cell cycle the material is treated with a spindle inhibitor. The spindle inhibitor prevents the formation of the spindle which separates the chromatides from each other

hence the cell division is stopped at metaphase. To avoid destruction of cell structures the material is fixed at this stage.

Protocol:

- Choose only young root tip meristems (lying immediately behind the root cap)
- Cut 0.4 – 1 cm long root tips with a razor blade
- Wash the root tips with water to get rid of media or soil

Note: Mitotic divisions are often synchronized; the best time to collect root tips is during the early morning.

Note: Prefer dry and aerated soil for rigorously growing root tips in pots.

- Put the root tips in 2 mM 8-Hydroxyquinoline for 2 h at RT followed by 2 h at 4 °C
- Discard the 8-Hydroxyquinoline

3.3.3 Accumulation of meiotic chromosomes

Meiotic chromosomes are generally studied in pollen mother cells. Finding the right stage (pachytene) is a matter of experience. Microsporogenesis proceed in most plants synchronously in the anthers of a flower bud so anthers from right stage will be fixed in fresh fixative to stop the cell cycle at the pachytene stage of meiosis I for further investigations.

Protocol:

- Choose young unopened flower buds (round shaped in comparison with leave buds)
- Cut them with a razor blade
- Free the flower buds from the surrounding leaves

3.3.4 Fixation and storage

Protocol:

- Fix the material in fresh fixative for 10 h (over night) at RT followed by one day in fresh fixative at 4 °C

Note: The material can be stored up to three month in fresh prepared fixative at – 20 °C

Solutions:

- 2 mM 8-Hydroxyquinoline
    - Dissolve 0.290 g of 8-Hydroxyquinoline in hot (80 °C) distilled water. Fill up to a final volume of 1000 ml.
    - Store solution at RT in the dark
- Fixative
    - 3 parts of absolute ethanol to 1 part of glacial acetic acid

Note: Fixative has to be prepared just before use to avoid formation of ester

3.3.5 Enzymatic treatment and chromosome spread preparation

The digestion of cell walls is an indispensable step for the production of high quality slides. The cells and the chromosomes should be well separated and free of cell debris and cytoplasm. A mixture of cellulase and pectinase suffices to digest the cell walls. Non-specific signals, subsequent detection and amplification steps are deposited on cytoplasm and cell debris and a thick layer of cytoplasm can mask the chromosome and hinder the access of probe and detection reagent.

Protocol:

- Wash the roots or flower buds 3 x 5 min in enzyme-buffer

Note: For meiotic chromosome preparation open the flower buds, cut the anthers and get rid of the surrounding material.

- Add 2 x enzyme-solution and incubate at 37 °C until the material is so soft that the root-tips fall off (1.5 to 2 h)

Note: Roots from the glasshouse plants need longer incubation time compared to *in vitro* plants

- Remove the enzyme solution carefully and wash material in enzyme buffer for 15 min at RT

Note: Within the 15 min select meristems or anthers and put each of them in a well of a multiwall plate. Incubate the material in 45 % acetic acid for 2–5 min and heat the material. This treatment reduces the amount of cell debris on the slide.

- Select meristems of anthers and put them on a cleaned object slide
- Remove all the material around the meristems or anthers
- Squash the tissue in 45% acetic acid over a well-cleaned slide using cover slips
- Tap gently over the cover slip with a razor blade or a needle to separate the cells
- Briefly heat the slide over a flame, without boiling the acetic acid. At this moment, cell wall should be broken and the cytoplasm surrounding the meiotic bivalent should have a very clear appearance, being almost invisible.

Note: Repeat heating two to three times, allowing cooling in between.

Note: If necessary, add some more 45% acetic acid by placing a small drop between the cover slip, do not try to get a better spreading by tapping again.

- Remove the excess liquid carefully with filter paper by gently pressing with fingers all around the cover slip, taking care not to move the cover slip
- Freeze the slide at -80 °C (10 min) then flick off the cover slip with a razor blade

- Allow the slide to air-dry

Note: To reduce background add a drop of ice cold fixative before the slide air dries

Solutions:

- 10 x enzyme-buffer pH 4.8
  - 7.70 g/l citric acid (40 mM)
  - 15.48 g/l sodium citrate (60 mM)
  - Dissolve chemicals in sterile distilled water and adjust pH = 4.8
- 1 x enzyme-buffer
  - Dilute 10 x enzyme buffer in distilled water
- 2 x enzyme-solution
  - 2 % (w/v) Cellulase Onzokura R-10 from *Trichoderma viride* (Serva Cat.Nr.: 16419)
  - 20 % (v/v) Pectinase solution from *Aspergillus niger* aqueous glycerol solution, ≥ 5 units/mg protein (Lowry) (Sigma Cat.Nr.: P4716)
  - Dissolve cellulase in 1 x enzyme buffer and add pectinase
  - Store in aliquots at -20 °C
- 45 % acetic acid

3.3.6 Counterstaining

Total DNA is usually stained independently of the labelled probes to show chromosome morphology and to provide contrast with colour used to visualize probe hybridization site. DAPI is a blue fluorescent dye which binds preferably to adenosine and tymidine.

Protocol:

- Add 35 µl of DAPI solution (5 µg/ml) on the slide in a dark room
- Incubate for 10 min at RT

- Wash the slides in 4 x SSC containing 0.05 % Tween 20

- Dry the slides for 4 – 5 minutes at RT

- Add 7 µl of Vectashield (Vector Laboratories, Inc.) on the place where the cell-squash is and apply a cover slip, avoid air blisters under the cover slip

- Store the slides over night in the dark before checking their quality using a fluorescence microscope

Solutions:

- DAPI solution 5 µg/ml
    - Prepare DAPI stock solution by dissolving 10 mg DAPI in 2 ml nuclease free water (c = 5 mg/ml)
    - Dilute DAPI stock solution in nuclease free water 1:1000 (c = 5 µg/ml)
    - Store in aliquots in the dark at -20 °C
- 20 x SSC
    - 175.33 g/l NaCl (3 M)
    - 88.23 g/l Tri-sodium citrate x 2 H_2O (300 mM)
    - Dissolve powder in 400 ml distilled water
    - Adjust pH = 7.0
    - Fill up with distilled water to 500 ml
    - Autoclave for 15 min at 121 °C
- 4 x SSC containing 0.2 % Tween-20
    - Dilute 20 x SSC 1:5 in sterile distilled water
    - Add 0.2 % Tween-20
- Vectashield mounting medium for fluorescence (Vector Laboratories, Inc.)

3.3.7 Checking slides quality

After making the preparation the slides should be screened and only top quality chromosome spread preparations selected.

Each slide was checked for the following quality criteria using a fluorescence microscope. The amount of overlying cytoplasm and cell debris around was checked by phase contrast. Selected slides were stored in the dark at 4 °C.

Quality criteria:

- Number of metaphase plates, more than 10
- Spreading of chromosomes, without underlying cytoplasm
- Complete chromosome sets
- Low to no background

3.4 Cloning, isolation and characterization of plasmid DNA

3.4.1 Transformation of plasmid DNA by Topo TA cloning Kit (Invitrogen)

The plasmids of interest are transformed to competent *E. coli* for further investigations. The transformed *E. coli* are cultured in selective medium containing kanamycin or ampicillin.

Protocol:

- Add 2 μl of the plasmid into a vial of One Shot® Chemically Competent *E. coli* and mix gently. Do not mix by pipetting up and down.
- Incubate on ice for 5 minutes.
- Heat-shock the cells for 30 seconds at 42 °C without shaking.
- Immediately transfer the tubes to ice.
- Add 250 μl of RT S.O.C. medium.

- Cap the tube tightly and shake the tube horizontally (200 rpm) at 37 °C for 1 h.
- Spread 10-50 μl from each transformation on a pre-warmed selective plate and incubate overnight at 37 °C. To ensure even spreading of small volumes, add 20 μl of S.O.C. medium
- Choose a single colony for culturing transformed cells for plasmid isolation

Solutions:

- SOC medium (TOPO TA cloning Kit))
    - 20.00 g/l trypton
    - 5.00 g/l yeast-extract
    - 0.60 g/l NaCl
    - 0.18 g/l KCl
    - 2.04 g/l $MgCl_2$
    - 2.46 g/l $MgSO_4$
    - Dissolve chemicals in water and adjust pH = 7.0
    - Add water to final volume
    - Autoclave for 15 min at 121 °C
    - Cool down the solution to RT
    - Add 20 ml 1 M sterile filtered glucose solution

Note: The glucose is added after sterilization to avoid Maillard reactions.

- Selective solid LB agar containing kanamycin or ampicillin (based on the used selection marker of the transformed *E. coli*)
    - 5 g/l yeast-extract
    - 10 g/l NaCl
    - 10 g/l trypton
    - 10 g/l agar
    - Dissolve chemicals in water, adjust pH = 7.5

- o Autoclave for 15 min at 121 °C
- o Cool down to 55 °C, add 100 µg/ml ampicillin or 50 µg/ml kanamycin
- o Pour agar in Petri dishes and wait until the agar solidifies

3.4.2 Culturing transformants

Transformed *E. coli* are cultured in selective medium to increase the amount of bacteria carrying the plasmid for mini- or maxiprep.

Protocol:

- Take a single colony with an inoculating loop and culture them overnight (16-21 hours) at 37 °C and 120 rpm in 5 ml LB medium containing kanamycin or ampicillin.

Notes: For increased culture volumes pre-culture the bacteria in 5 ml liquid selective LB medium for 8 h at 37 °C and 120 rpm and then use the whole culture volume to inoculate up to 500 ml of liquid selective LB medium.

Solutions:

- Selective LB medium containing kanamycin or ampicillin (see 3.4.1)

3.4.3 Extraction of plasmid DNA by Promega-Maxikit (Promega)

For low copy plasmids it is necessary to use increased culture volumes to get a sufficient amount of plasmid.

Protocol:

- Use 500 ml of *E. coli* culture (grown for 16 – 21 h)
- Pellet the cells by centrifugation at 5000 x g for 10 minutes and discard supernatant. Drain tubes on a paper towel to remove excess media.
- Resuspend the cell pellets in 12 ml of Cell Resuspension Solution.

- Add 12 ml of Cell Lysis Solution and mix by gently inverting the tube 3-5 times. Incubate for 3 minutes at RT.

- Add 12 ml of Neutralization Solution to the lysed cells, cap the tube and mix by gently inverting the tube 10-15 times.

- Centrifuge the lysate at 14000 x g for 20 minutes at RT in a fixed-angle rotor. This centrifugation will pellet the bulk of the cellular debris.

- When the spin is complete, examine the cleared lysate. It should be clear, with small amounts of cell debris.

- Assemble a column stack by placing a blue PureYield. Clearing Column on the top of a white PureYield. Maxi Binding Column. Place the assembled column stack onto the vacuum manifold.

- Pour approximately one-half of the lysate into the blue PureYield Clearing Column.

- Apply maximum vacuum. The lysate will pass through the clearing membrane in the PureYield Clearing Column. The DNA will bind to the binding membrane in the PureYield. Maxi Binding Column.

- Add the remainder of the lysate to the appropriate clearing column and allow it to pass through both columns as described above.

- Slowly release the vacuum from the filtration device before proceeding.

- Add 5 ml of Endotoxin Removal Wash to the PureYield. Maxi Binding Column, turn on the vacuum and allow the vacuum to pull the solution through the column.

- Add 20 ml of Column Wash to the binding column and allow the vacuum to draw the wash through.

- Dry the membrane by applying a vacuum for 5 minutes.

- Remove the PureYield. Maxi Binding Column from the vacuum manifold. Tap the tip of the column on a paper towel to remove excess ethanol. Wipe any excess ethanol from the outside of the tube.

- To eluate the DNA place the PureYield. Maxi Binding Column into a new 50 ml disposable plastic centrifuge tube. Add 1.5 ml of Nuclease-Free Water to the DNA binding membrane in the binding column.

- Centrifuge the PureYield. Maxi Binding Column in a swinging bucket rotor at 2000 x g for 5 minutes.

- Collect the eluate from the 50 ml tube and transfer to a 1.5 ml tube if desired.

- Check the purity and quality of the isolated plasmid via agarose gel electrophoresis

Solutions:

- Cell Resuspension Solution
- Cell Lysis Solution
- Neutralization Solution
- Endotoxin Removal Wash
- Column Wash
- Nuclease-Free Water

3.4.4 Extraction of Plasmid DNA by Alkaline Lysis with SDS (Miniprep)

For plasmids with a high copy number the use of 2 – 4 ml of bacteria culture are sufficient to extract enough plasmid for most applications.

Protocol

- Add 2 ml of the culture into an Eppendorf tube and centrifuge and centrifuge at 14 000 g for 1 min at RT
- Remove the supernatant
- Resuspend the bacterial pellet in 100 µl of ice cold solution 1 by vigorous vortexing
- Incubate for 5 min at RT

- Add 200 µl of fresh prepared solution 2 and mix carefully by inverting the tube 10 times.
- Incubate on ice for 10 min
- Add 150 µl of solution 3, mix it carefully and incubate the tube on ice for another 10 min
- Centrifuge at 14 000 g for 15 min at 4 °C
- Transfer supernatant into a fresh tube
- Precipitate the DNA with 0.6 volumes of isopropanol and incubate at RT for 15 min
- Centrifuge at 14 000 g for 5 min at RT and discard supernatant
- Rinse the pellet with 1 ml of 70 % ethanol cooled to -20°C
- Centrifuge at 14 000 g for 2 min at 4 °C
- Discard the supernatant and dry the pellet under vacuum
- Dissolve the pellet in 30 µl of TE buffer
- Add 2 µl RNAse A (10 mg/ml) and incubate at 37 °C for 30 min
- Store the plasmid at -20 °C

Solutions:

- Solution 1
    - 9.01 g/l glucose (50 mM)
    - 2.42 g/l Tris (20 mM)
    - 2.92g/l EDTA (10 mM)
    - Dissolve solutions and set pH = 8.0
    - Autoclave for 15 min at 121 °C
    - Store at 4 °C
- Solution 2
    - 8.8 ml sterile water
    - 0.2 ml 10 mol/l NaOH (399.97 g/l NaOH)
    - 1.0 ml 10 % SDS

Note: Solution 2 needs to be prepared freshly

- Solution 3
    - 60.0 ml 5 mol/l KOH (275.55 g/l KOH)
    - 11.5 ml glacial acetic acid
    - 28.5 ml sterile water
    - Set pH = 4.8

3.4.5 Linearization of plasmid DNA

To determine the amount of plasmid via agarose gel electrophoresis as well as for improved susceptibility of enzymes in the Nick Translation mix the plasmid needs to be linearized. Three different plasmids were used (pTa71, pBluescript – 18S rDNA and pBinGUSint).

pTa71:

- 1 µg pTa71
- 0.1 x volume 10 x Buffer B (Roche)
- 1 U restriction enzyme *Hind* III (Roche)
- Add nuclease free water to the final reaction volume
- Mix well and digest for 1 h at 37 °C
- Load an aliquot on an agarose gel to check if the digestion is complete and to check the amount of plasmid DNA in comparison with the DNA molecular weight marker III (Roche).
- Clean the linearized plasmid, using the QIAquick PCR Purification Kit Protocol

pBluescript – 18S rDNA

- 1 µg pBluescript containing the 18S rDNA
- 0.1 x volume 10 x Buffer B (Roche)
- 1 U restriction enzyme *EcoR*V (Roche)

- Add nuclease free water to the final reaction volume

- Mix well and digest for 1 h at 37 °C

- Load an aliquot on an agarose gel to check if the digestion is complete and to check the amount of plasmid DNA in comparison with the DNA molecular weight marker III (Roche).

pBinGUSint

- 1 µg pBinGUSint

- 0.1 x volume 10 x Buffer O$^+$ (Fermentas)

- 1 U restriction enzyme *EcoR*I (Fermentas)

- Add nuclease free water to the final reaction volume

- Mix well and digest for 1 h at 37 °C

- Load an aliquot on an agarose gel to check if the digestion is complete and to check the amount of plasmid DNA in comparison with the DNA molecular weight marker III (Roche).

3.4.6 Isolation of insert DNA

The plasmid was digested with restriction enzymes to isolate the insert DNA and to get rid of the plasmid backbone.

Double digestion with restriction enzymes *Mss*I and *Sac*I:

- 1 µg pBinGUSint

- 0.1 x volume 10 x buffer B

- 2 U restriction enzyme *Mss*I (Fermentas)

- 4 U restriction enzyme *Sac*I (Fermentas)

- Add nuclease free water to the final reaction volume

- Mix well and digest for 1 h at 37 °C

- Load an aliquot on an agarose gel to check if the digestion is complete

- After complete digestion, load the rest of the DNA on a gel, until the bands are well separated

- Cut out the band of interest and extract the DNA using QIAquick Gel Extraction Kit (see 3.4.7.1)

3.4.7 Purification of DNA

The cleaning of the DNA before labelling is required to produce only specific probes and to remove enzymes and salts which might disturb the labelling reaction.

3.4.7.1 Gel extraction using QIAquick Gel Extraction Kit (Qiagen)
Protocol:

- Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

- Weigh the gel slice in a colourless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

- Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min.

- After the gel slice has dissolved completely, check that the colour of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the colour of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The colour of the mixture will turn to yellow.

- Add 1 gel volume of isopropanol to the sample and mix. This step increases the yield of DNA fragments < 500 bp and > 4 kb.

- Place a QIAquick spin column in a provided 2 ml collection tube.

- Apply the sample to the QIAquick column, and centrifuge for 1 min.

- Discard flow-through and place QIAquick column back in a collection tube.

- Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

- Add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

- Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17900 x g.

- Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

- Add 50 µl water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min.

- Store DNA at –20 °C

- Check the cleaned DNA via agarose gel electrophoresis

Solutions:

- Buffer QG
- Isopropanol
- Buffer PE

3.4.7.2 QIAquick PCR Purification Kit Protocol (Qiagen)

Protocol:

- Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and mix.

- Check that the colour of the mixture is yellow (similar to Buffer PBI without the PCR sample). If the colour of the mixture is orange or violet, add 10 µl of 3 M sodium acetate (pH 5.0) and mix. The colour of the mixture will turn to yellow.

- Place a QIAquick spin column in a provided 2 ml collection tube.

- Apply the sample to the QIAquick column and centrifuge for 30–60 s.

- Place the QIAquick column back into the same tube.

- Add 0.75 ml Buffer PE to the column and centrifuge for 30–60 s.

- Discard flow-through and place the QIAquick column back in the same tube.

- Centrifuge the column for an additional 1 min.

- Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

- Add 30 μl of nuclease free water to the centre of the QIAquick membrane, let the column stand for 1 min, and centrifuge the column for 1 min.

- Store DNA at -20 °C

Solutions:

- Buffer PBI, PE, EB

3.4.8 Agarose gel electrophoresis

Via agarose gel electrophoresis DNA is separated by size in an electric field by the use of an agarose gel matrix. The higher the concentration of agarose the compacter is the gel matrix hence the resolution of the gel electrophoresis experiment. The negative charged DNA migrates to the anode of the electric field. Short DNA fragments move faster through the gel than long DNA molecules. By comparing the position of the band of the sample to the positions of the bands of a DNA molecular weight marker the size of the sample can be determined. By comparison of the intensity of the bands is also possible to estimate the amount of DNA in the sample.

Protocol:

- Add 3 μl loading dye to the samples (2 – 5 μl, total sample volume if gel extraction will be done) and to the DNA molecular weight marker (2 μl)
- Put the gel into the tray
- Fill the tray with 1 x TAE buffer
- Load the samples and the DNA molecular weight marker
- Place lid over tray, plug in the power supply
- Run the gel at 100 V to 120 V for 30 – 60 min

- The gel is viewed under UV and photographed using a digital camera UVP (ultraviolet products) from BIO TRADE

Solutions:

- TAE-Buffer 50 x
    - 242.0 g/l Tris-base
    - 57.1 ml/l acetic acid
    - 100 ml/l of 0.5 mM EDTA-solution (0.146 g/l EDTA)
    - Adjust pH = 8.5
    - Fill up to final volume with distilled water
    - Autoclave for 15 min at 121 °C
- 1 x TAE buffer
    - Dilute 50 x TAE buffer in distilled water
- EtBr stock solution 10 mg/ml
    - Dissolve 200 mg EtBr in 20 ml distilled water
    - Store in the dark at 4 °C

Note: EtBr has to be collected and discarded according to safety instructions (www.retrologistik.de)

- Agarose gel
    - Dissolve 1-2 % agarose (depending on the demanded resolution; for high resolution take more agarose) to 100 ml 1 x TAE buffer by heating the mixture in the microwave.
    - Cool down the gel to 55 °C
    - Add 5 µl EtBr stock solution (10 mg/ml)
    - Mix well and pour gel in a casting platform with well former sideways. Avoid air blisters
    - Wait until the gel solidifies
    - Take gel out of the platform; store in 1 x TAE containing EtBr at 4 °C

3.4.9 DNA quantification

The isolated plasmids were quantified by using a UV/VIS BioPhotometer (Eppendorf) or by comparison of band intensity of samples and DNA molecular weight marker in agarose gel (see 3.4.8).

3.5 Probe preparation

3.5.1 DNA labelling

3.5.1.1 PCR labelling

PCR labelling works like a normal PCR with two primers, which hybridize to the target sequence and the thermo stable enzyme Taq Polymerase elongates the primers. By repeating the temperature program for denaturation, annealing of the primer and elongation the target will be accumulated. The difference to a normal PCR is that a part of the dTTP is replaced by DIG or biotin conjugated dUTP which is incorporated by chance into the synthesized probe. Therefore each probe contains another amount of labelled nucleotides even if the DNA sequence is the same.

Protocol using a HotStarTaq DNA polymerase (Qiagen):

PCR reaction mix:

Place a sterile microcentrifuge tube on ice and, for each PCR, add to the tube

- 5 µl of 10x concentrated PCR buffer (Qiagen)
- 2 µl of 50 mM $MgCl_2$ (Qiagen)
- 5 µl of 2 mM dNTPs containing 2 mM each of dATP, dCTP, and dGTP;
- 5 µl of 1.3 mM dTTP; 0.7 mM DIG-dUTP or biotin-dUTP (2:1 ratio of dTTP:DIG-dUTP or biotin-dUTP) (Roche)
- 10 pmol of each primer
- 0.1-0.2 ng template DNA
- 1.2 U HotStarTaq (Qiagen).

- Add sterile double distilled water to a final reaction volume of 50 µl.
- Mix solutions on ice and centrifuge briefly to collect the sample at the bottom of the tube.
- Place samples in a thermal cycler and start PCR.

PCR Programme:

- Initial denaturation: 5 min   95 °C
- 35 cycles of:
  - Denaturation: 50 s   95 °C
  - Annealing: 50 s   according to Table 3-2
  - Elongation: 60 s   72 °C
- Final elongation step: 7 min   72 °C
- Hold   4 °C
- Check PCR labelled probe via agarose gel (see 3.4.8) and compare the size of the band to unlabelled PCR product (see 3.5.4.2).
- Storage of the labelled probe: Short term, at 2 - 8 °C long terms, at -20 °C, stable for at least one year.

Table 3-1: PCR primers and their annealing temperature

primer	sequence
M13fwd	5´-GTAAAACGACGGCCAGT-3´
M13rev	5´-GGAAACAGCTATGACCATG-3´
T3	5´-AATTAACCCTCACTAAAGGG-3´
UP46	5´-GTGCGATCATACCAGCAGGTTAATGCACCGG-3´
UP47	5´-GACGTGCAACACGAGGACTTCCCAGGAGG-3´
GUSfwd	5´-ATGTTACGTCCTGTAGAACCC-3´
GUSrev	5´-TCATTGTTTGCCTCCCTGCTG-3´
Kmfwd	5´-AGAGGCTATTCGGCTATGAC-3´
Kmrev	5´-ACTCGTCAAGAAGGCGATAG-3´

Table 3-2: DNA-products of primer-pairs and templates

DNA-product	forward primer	reverse primer	template	annealing temperature
18 S rDNA	M13fwd	T3	pBluescript	55 °C
5 S rDNA	M13fwd	M13rev	pGEM-Teasy	55 °C
5 S rDNA	UPF46	UP47	genomic DNA	55 °C
uidA	GUSfwd	GUSrev	pBinGUSint	60 °C
nptII	Kmfwd	Kmrev	pBinGUSint	59 °C

3.5.1.2 Random Primed Labelling with DIG- or Biotin-High Prime reaction mix

In Random Primed Labelling reaction, the template DNA is linearized, denatured, and annealed to a primer. Starting from the 3´-OH of the random primer which annealed to the template the Klenow enzyme synthesizes new DNA along the single stranded substrate and will also incorporate the labelled nucleotides. The size of the labelled DNA depends on the amount and size of the template DNA.

Protocol:

- Add 1 µg template DNA (linear or supercoiled) to a 1.5 ml microcentrifuge tube, and sterile, double dist. water to a final volume of 16 µl.

- Denature the DNA by heating the tube in a boiling water bath for 10 min.

Notes: Complete denaturation is essential for efficient labelling.

- Chill the reaction tube quickly on ice.

- Mix DIG-High Prime or Biotin-High Prime thoroughly and add 4 µl to the denatured DNA.

- Mix the Solutions, and centrifuge briefly to collect the reaction mixture at the bottom of the tube.

- Incubate the tube at least 1 h at 37 °C.

Note: Longer incubations (up to 20 h) increase the yield of labelled DNA.

- Add 2 µl 0.2 M EDTA (pH 8.0) to the reaction tube and/or heat the tube to 65 °C for 10 min, to stop the reaction.

Solutions:

High Prime reaction mix (Roche): Contains 5x concentrated reaction buffer in50% glycerol; 1 U/µl Klenow enzyme, labelling grade; 5x concentrated random primer mix; 1 mM each of dATP, dCTP, and dGTP; 0.65 mM dTTP; and 0.35 mM X-dUTP (X = DIG or biotin).

3.5.1.3 Nick Translation labelling

In this labelling method the DNA is nicked with DNAse I, the exonuclease activity of DNA Polymerase I extends the nicks to gaps. These gaps will be filled by the DNA Polymerase I again with new and labelled nucleotides. The recommended size of the probe after the reaction should range from 200 – 500 bp.

Protocol:

- Add 16 µl sterile, double dist. water containing 1 µg template DNA to a 1.5 ml microcentrifuge tube on ice.
- Add 4 µl of 5 x concentrated Nick Translation mix
- Mix ingredients and centrifuge tube briefly.
- Incubate at 15 °C for 2 h.
- Chill the reaction tube on ice.
- Take an aliquot from the tube and run the sample on an agarose gel with DNA Molecular Weight Marker VIII (Roche).

Depending on the average size of the probe, do one of the following:

- If the probe is between 200 and 500 bp long, stop the reaction by adding 2 µl EDTA-solution and heating to 65 °C for 10 min.
- If the probe is longer than 500 bp, incubate the reaction tube further at 15 °C until the fragments have the proper size.

Solutions:

Nick Translation mix (Roche): contains 5x concentrated reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM X-dUTP (X = DIG or Biotin)

3.5.2 Purification of the probe by QIAquick Nuclotide Removal kit (Qiagen)

After labelling the probe has to be cleaned to remove all the enzymes, nucleotides, very small DNA fragments and salts. The QIAquick Nucleotide Removal Kit removes nucleotides and short fragments resulting from the different labelling methods. This step is required to reduce the unspecific signals, and for adequate quantification via dot blot.

Protocol:

- Add 10 volumes of Buffer PN to 1 volume of the reaction sample and mix. For DNA fragments <100 bp, only 5 volumes of Buffer PN are required.
- Place a QIAquick spin column in a provided 2 ml collection tube.
- Apply the sample to the QIAquick column and centrifuge for 30–60 s.
- Discard flow-through. Place the QIAquick column back into the same tube.
- Add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
- Discard flow-through. Place the QIAquick column back in the same tube.

- Centrifuge the column for an additional 1 min. Residual ethanol from Buffer PE by additional centrifugation.

- Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

- Add 30 µl of nuclease free water to the centre of the QIAquick membrane, let the column stand for 1 min, and centrifuge the column for 1 min.

- Store DNA at –20 °C

- Check the cleaned DNA via agarose gel electrophoresis

Solutions:

- Buffer PN, PE, EB

3.5.3 Purification of the probe by precipitation of DNA with Ethanol

Protocol:

- Add one-tenth of the labelling reaction volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes 96% ethanol.

- Mix well and store at -20 over night.

- Pellet the DNA at 14000 g for 20 minutes at 4 °C. Discard the supernatant.

- Wash the pellet with 100 µl 70% ethanol, mix well and centrifuge at 14000 g for 10 minutes.

- Discard the ethanol, dry the pellet, and resuspend the DNA in 20 µl nuclease free water

- Store DNA at –20 °C

Solutions:

- 3 M sodium acetate pH = 5.2
    - Dissolve 246.09 g/l sodium acetate (3 M) in distilled water and adjust pH = 5.2

- o Fill up with water to the final volume
- o Autoclave for 15 min at 121 °C
- 70 % and 96 % ethanol

3.5.4 Checking incorporation of label

After labelling it is advisable to check the incorporation of the labelled nucleotide. Biotin or DIG label incorporation can be measured using agarose gel electrophoresis or dot blot assay.

3.5.4.1 Dot blot

In dot blot the incorporated label is detected via enzyme linked antibodies. The antibodies bind to the label and then a substrate for the linked enzyme is added to produce a chemiluminescent signal. The stronger the signal the more label is incorporated. The intensities of the signals of the different dilutions are compared to the intensities of the signals of the labelled standard DNA; same intensities indicate the same amount of incorporated label.

Protocol:

Dilution of probes and DIG-labelled control DNA

- Pre-dilute the probes and the DIG-labelled control DNA to 1 ng/µl (A) with nuclease-free water and mix well
- Dilute A 1:10 with nuclease-free water (B) and mix well, c = 100 pg/µl
- Dilute B 1:10 with nuclease-free water (C) and mix well, c = 10 pg/µl
- Dilute C 1:10 with nuclease-free water (D) and mix well, c = 1 pg/µl
- Dilute D 1:10 with nuclease-free water (E) and mix well, c = 0.1 pg/µl

Dot blotting

- Cut a piece of positively charged membrane
- Cut one corner to avoid exchanging upside and downside

- Using a pencil, mark the membrane lightly with a grid (x-axis: dilutions; y-axis: probes and DIG-labelled control DNA)

- Denature the diluted probes and the DIG-labelled control DNA at 95 °C for 10 min using the thermomixer

- To avoid renaturation chill the diluted probes and the DIG-labelled control DNA on ice for at least 5 min

- Centrifuge briefly to collect the probe on the bottom of the tube

- Apply 1µl of each dilution to the marked membrane. Avoid touching the membrane with the pipette-tip

- Air dry the membrane for about 2 min

- Fix the labelled DNA to the membrane by baking at 120 °C for 30 min or 80 °C for 2 h

Chemilumeinescent detection

- Equilibrate the membrane in buffer 1 for 1 min

- Incubate the membrane in 20 ml of 1% blocking reagent (diluted in 1 x buffer 1) for 5 min

- Centrifuge Anti-DIG-AP1 for 5 min at full speed

- Dilute Anti-DIG-AP1 1:20000 in 1% blocking reagent (diluted in 1 x buffer 1)

- Incubate the membrane in antibody solution for 10 min

- Discard antibody solution

- Wash the membrane two times 5 min in buffer 1

- Discard buffer 1

- Equilibrate the membrane in buffer 3 for 2 min

- Place membrane on a piece of plastic foil and apply some drops of CSPD ready to use

- Put the surface of the membrane on the substrate, cover the membrane with another piece of plastic foil and scatter the substrate carefully until there are no air-bubbles visible

- Incubate for 1 min at RT

- Squeeze out excess liquid and seal the development folder completely

- Incubate the membrane at 37 °C for 10 min

- Detect signal by using a Lumi-Imager F1 working station (Boehringer Mannheim)

Solutions:

- 1 x washing buffer (buffer 1)
    - 12.11 g/l maleic acid (100 mM)
    - 8.77 g/l NaCl (150 mM)
    - Dissolve the salts in about 800 ml distilled water and adjust the pH 7.5 by adding 10 M NaOH
    - Fill up to final volume with distilled water
    - Autoclave for 15 min at 121 °C
- 10 % (w/v) Blocking reagent stock solution (buffer 2)
    - Dissolve 10 % (w/v) Blocking reagent granules in buffer 1 by heating in the microwave
- 1 x Detection buffer (buffer 3)
    - 12.11 g/l Tris base (100 mM)
    - 5.84 g/l NaCl (100 mM)
    - Dissolve the salts in about 800 ml distilled water and adjust the pH 9.5 by adding concentrated HCl
    - Fill up to final volume (1 l) with distilled water
    - Autoclave for 20 min at 121 °C

3.5.4.2 Agarose gel electrophoresis

In PCR the incorporated label is detected by comparing the sizes of a labelled and an unlabelled DNA fragment. Depending on the amount of incorporated label the DNA fragment has a higher molecular weight and runs slower on the gel than the unlabelled DNA fragment. If the labelled probe seems to be of much higher molecular weight on the gel the amount of incorporated label

is high, if there is no difference between the labelled and the unlabelled fragment there are no or not many labelled nucleotides incorporated.

3.6 Fluorescence *In Situ* Hybridization

3.6.1 Pretreatment of metaphase spreads on slides

Pretreatment of chromosome preparations is required to: a) remove RNA and proteins which bind to probe and detection reagents, increasing background. b) Fix the preparation of chromosomes and nuclei to avoid loss of DNA from the slide during the procedure. c) Stabilize the structure of the metaphase chromosomes by the use of formaldehyde.

Protocol:

- To get rid of the cover slip put the slides in 2 x SSC until it falls off.
- Re-fix the material in fresh prepared fixative for 20 min
- Dehydrate the slide through a series of ethanol (70%, 90%, absolute ethanol) by incubating for 2 min in each ethanol concentration

Note: Series of ethanol can be used 3 – 4 times

- Dry the slides at RT for at least 20 min to be sure that all the ethanol residue is gone
- Pipet 200 µl RNAse A (100 µg/ml) in 2 x SSC on the slide
- Cover the slides with a plastic cover slip and put them in a pre-warmed humid chamber
- Incubate for 1 h at 37 °C in a pre-warmed humid chamber (put a tissue in the chamber pour sterile 2 x SSC on the tissue, close the chamber and put it in the hybridization oven)
- Wash the slides 3 times for 5 min in 2 x SSC
- Dehydrate the slide through a series of ethanol (70%, 90%, absolute ethanol) by incubating for 2 min in each ethanol concentration
- Dry the slides at RT for at least 20 min to be sure that all the ethanol residue is gone

- Incubate the slides in 0.01 % (w/v) pepsin in 0.1 M HCl (pH= 2.0) for 20 min at 37 °C

- Wash the slides two times in 1 x PBS for 5 min

- Wash the slides in 1 x PBS containing 50 mM $MgCl_2$ for 5 min

- Wash the slides in 1 x PBS containing 50 mM $MgCl_2$ and 4 % formaldehyde for 10 min

- Wash the slides in 1 x PBS for 5 min

- Dehydrate the slide through a series of ethanol (70%, 90%, absolute ethanol) by incubating for 2 min in each ethanol concentration

- Dry the slides at RT for at least 20 min to be sure that all the ethanol residue is gone

Solutions:

- 2 x SSC
  - Dilute 20 x SSC (see 3.3.6) in sterile distilled water
- Fixative (see 3.3.4)
- RNAse A stock solution (10 mg/ml)
  - Prepare buffer 1: dissolve 1.21g/l Tris base and 0.87 g/l NaCl, in sterile distilled water and adjust the pH = 7.5
  - Dissolve 100 mg RNAse A in 10 ml of buffer 1 and mix well
  - Prepare 500 μl aliquots in 1.5 ml Eppendorf tubes
  - Heat the 1.5 ml Eppendorf tubes to 99 °C for 10 min
  - Store the aliquots at -20 °C
- RNAse A (100 μg/ml)
  - Dilute RNAse A stock solution in 2 x SSC
- 70% ethanol, 90% ethanol, absolute ethanol
- Pepsin stock solution (2.5 mg/ml)
  - Dissolve 100 mg Pepsin (Sigma) in 40 ml 0.01 M HCl
  - Prepare 1 ml aliquots and store at 4 °C
- Pepsin solution (25 μg/ml)

- o Dilute pepsin stock solution 1: 100 in 0.01 M HCl
- 10 x PBS
    - o 80.0 g/l NaCl
    - o 14.4 g/l Na$_2$HPO$_4$
    - o 2.0 g/l KCl
    - o 2.4 g/l KH$_2$PO$_4$
    - o Dissolve salts in distilled water and adjust pH = 7.4 by adding HCl
    - o Autoclave for 15 min at 121 °C
    - o Store at RT
- 1 x PBS
    - o Dilute 10 x PBS in sterile distilled water
- 1 x PBS containing 50 mM MgCl$_2$ x 6 H$_2$O
    - o Add 2.033 g/200 ml MgCl$_2$ x 6 H$_2$O (50 mM) to 1 x PBS just before use and mix well
- 1 x PBS containing 50 mM MgCl$_2$ x 6 H$_2$O and 4 % (v/v) formaldehyde
    - o Add 2.033 g/200ml MgCl$_2$ x 6 H$_2$O (50 mM) and 4 % (v/v) formaldehyde to 20 ml of 10 x PBS and fill up to a final volume of 200 ml just before use and mix well

3.6.2 Denaturation and hybridization

To allow hybridization of the labelled probe and chromosomal DNA, DNA single strands are required and the chromosomal structure should not be lost. Therefore the denaturation of the cytological preparations is performed in a hybridization solution which should help to overcome this problem. The formamide in the hybridization solution decreases the melting point of the DNA and dextran sulphate is used to increase the density of the solution. The probe is denaturated independently from the chromosomes and chilled to prevent renaturation.

Protocol:

- Add probe DNA to a microcentrifuge tube, and sterile, double dist. water to a final volume of 20 µl.

- Dilute probe in 80 µl 1.25 x hybridization solution

- The final concentration of the probe:

    o 40 ng pTa71

    o 100 ng 5S rDNA

    o 2 - 400 ng pBinGUSint T-DNA

- Denature the probe at 95 °C for 5 min using the thermomixer

- Chill the probe on ice for 5 min to avoid renaturation

- Add 10 µl of hybridization mixture to each slide

- Apply a cover slip. Avoid air bubbles under the cover slip

- Seal the cover slip with Fixogum Rubber Cement (Fa. Marabu GmbH&Co)

- Wait until the Fixogum solidifies; cover the slides to avoid bleaching of the fluorescent-labelled antibodies

- To hybridize, place the slide in a PCR-hybridization- block using the following program:

    o 80 °C   3 min

    o 65 °C   2 min

    o 55 °C   2 min

    o 45 °C   2 min

    o 37 °C   Hold

- Put the slides in a prewarmed dark humid chamber and hybridize over night at 37 °C in a hybridization oven

Solutions:

- 1.25 x Hybridization solution (0.8 ml)

    o 400 µl deionized formamide

    o 200 µl of 50 % (w/v) dextran sulphate

- o 100 μl of 20 x SSC (see 3.3.6)
- o 100 μl of 10 x blocking reagent
- o Mix all solutions well and store at -20 °C

3.6.3 Detection

Detection of hybridization sites is an important part of *in situ* hybridization allowing visualization of the probe-target hybrids formed.

3.6.3.1 Stringency washes

Within stringency washes all the probes which are not hybridized or do not bind well to the chromosomes are washed away. By changing the parameters of the stringency the ratio of mismatches can be controlled.

Protocol:

- Remove the Fixogum with a needle without moving the cover slip
- Wash the slides 5 min in 2 x SSC at 42 °C
- Wash the slides 3 times 5 min in 0.1 x SSC at 42 °C
- Wash the slides 5 min in 2 x SSC at 42 °C

Note: The cover slip should fall off during the first washing step. If it does not fall off, move the slide up and down until it descends.

- Wash the slides 5 min in 2 x SSC at RT

Solutions:

- 0.1 x SSC, 2 x SSC (see 3.3.6)

3.6.3.2 Antibody application

To detect DIG and biotin labelled probes the application of fluorescence labelled antibodies is necessary. By the application of a series of antibodies the signal can be amplified but the background signals are amplified as well. For direct labelled probes the application of antibodies is not needed.

Protocol:

- Wash the slides 5 times in 2 x SSC for 2 min
- Wash the slides in TST buffer for 5 min
- Apply 100 µl of 1 x blocking reagent on each slide
- Cover the slides with a plastic cover slip
- Incubate in a humid chamber at 37 °C for 30 min
- Wash the slides 3 times for 5 min in TST buffer at RT

Note: If the amplification of signal is not needed skip the application of antibody-solution 1 and antibody-solution 2 and continue with the application of antibody solution 3

- Apply 100 µl antibody-solution 1 on each slide
- Cover the slides with a plastic cover slip
- Incubate in a humid chamber at 37 °C for 30 min
- Wash the slides 3 times for 5 min in TST buffer at RT

Note: The cover slip should fall off during the first washing step. If it does not fall off move the slide up and down until it descends.

- Apply 100 µl antibody-solution 2 on each slide
- Cover the slides with a plastic cover slip
- Incubate in a humid chamber at 37 °C for 30 min
- Wash the slides 3 times for 5 min in TST buffer at RT

Note: The cover slip should fall off during the first washing step. If it does not fall off, move the slide up and down until it descends.

- Apply 100 µl antibody-solution 3 on each slide
- Cover the slides with a plastic cover slip
- Incubate in a humid chamber at 37 °C for 30 min
- Wash the slides 3 times for 5 min in TST buffer at RT

Note: The cover slip should fall off during the first washing step. If it does not fall off, move the slide up and down until it descends.

- Dehydrate the slide through a series of ethanol (70%, 90%, absolute ethanol) by incubating for 2 min in each ethanol concentration
- Dry the slides at RT for at least 20 min to be sure that all ethanol is gone
- Counter stain with DAPI as described (see 3.3.6). Use larger cover slips than you used for squashing to be sure to cover all the chromosome-sets.
- Seal the cover slip with nail-polish to avoid that the slide dries out and to avoid movement of the cover slip.
- Store the slides over night in the dark
- Check the slides, using a fluorescence microscope.

Solutions:

- 2 x SSC (see 3.3.6)
- 1 x PBS (see 3.6.1)
- 1 x Blocking solution
    - Dilute 10 x Blocking solution in 1 x PBS
- Antibody-solution 1
    - 40 µl of Anti-DIG monoclonal antibody against DIG (Roche, Fluorescent Antibody Enhancer Set for DIG Detection)
    - 1.1 µl of Cy™3-conjugated Streptavidin (1.8 mg/ml) (Jackson ImmunoResearch Laboratories, Inc.)
    - Fill up to 1 ml with 1 x Blocking solution and mix well
- Antibody-solution 2
    - 40 µl of Anti-mouse-Ig-DIG (Roche, Fluorescent Antibody Enhancer Set for DIG Detection)
    - 5 µl of Biotinylated-Anti-Streptavidin (1 mg/ml) (Vector Laboratories)

- o Fill up to 1 ml with 1 x Blocking solution and mix well
- Antibody-solution 3
  - o 40 µl of Anti-DIG Fluorescein (Roche, Fluorescent Antibody Enhancer Set for DIG Detection)
  - o 1.1 µl of Cy™3-conjugated Streptavidin (1.8 mg/ml) (Jackson ImmunoResearch Laboratories, Inc.)
  - o Fill up to 1 ml with 1 x Blocking solution and mix well
- TST buffer
  - o 12.11 g/l Tris base (100 mM)
  - o 8.77 g/l NaCl (150 mM)
  - o 0.05 % (v/v) Tween 20
  - o Dissolve salts in water and adjust pH to 7.5
  - o Autoclave for 15 min at 121 °C
  - o Add 0.05% (v/v) Tween 20 and mix by inverting several times
- 70 % ethanol, 90 % ethanol, absolute ethanol
- DAPI solution c = 5 µg/ml (see 3.3.6)
- 4 x SSC containing 0.2 % Tween-20 (see 3.3.6)
- Vectashield (see 3.3.6)

3.7 Analysis of signal

An Olympus BX61 fluorescence microscope using standard filters and objectives was used for visualizing *in situ* hybridization sites. The F-View II camera was used for acquisition of digital black and white pictures which were automatically pseudo coloured by a software called cell^F. The exposure time was optimized for each filter and each chromosome set to get a clear picture with low background signals. By the software the pictures are stored as multidimensional data formats. To export the pictures to another program the multidimensional picture were separated to each colour channel and then each channel was stored as tiff or jpg. If there is no need for further optimization of the picture the multidimensional picture can also be reduced

to a one-dimensional picture including all colours, which can be stored as tiff or jpg as well.

The pictures were optimized for brightness, contrast and the level of the colour channel using the Adobe Photoshop CS2. The Mode was changed to RGB Colour the right colour channel (green for FITC, red for Cy3 and blue for DAPI) was chosen and then the picture was optimized. To overlap the different colour channels the whole picture of each colour channel was marked and copied to a new picture with white background. The DAPI picture of the chromosomes with the white background was chosen and then the other pictures of this set were added by the menu command "Apply Image…". For Blending "Add…" was chosen.

3.7.1 Karyotyping

The DAPI pictures of countable and full chromosome sets were opened with the Adobe Photoshop CS2 and the Mode was changed to Grayscale. To do karyotyping it was necessary to store the picture as bmp (8 bit). Chromosome measurements were made using the freeware MicroMeasure 3.3 (http://www.colostate.edu/Depts/Biology/MicroMeasure). The results of measuring were stored as Excel sheets and compared using Microsoft Office Excel 2007. The mean length of each chromosome and the standard deviation were calculated for the total chromosome length, the long arm, the short arm and the arm ratio of each chromosome. From the generated data pictures have been made using CATIA V5R16 and Adobe Photoshop Elements 4.0.

4. Results

4.1 Slide preparation

PAR and PIS lines collected from greenhouse or *in vitro* cultures at the PBU (Plant Biotechnology Unit) showed the expected chromosome number of 2n = 16 (diploid) and 2n = 32 (tetraploid) respectively.

4.1.1 Mitotic chromosome preparation

After some initial attempts with traditional cell squashes which gave unsatisfactory results (high background and poor signal), mitotic chromosome preparations from root tip meristematic cells from *in vitro* and glasshouse material were made for karyotyping and FISH analysis. Well digested cells from root tip meristems were used in order to reduce penetrability problems of probes and antibodies and their non-specific binding.

Spread preparation slides from glasshouse material, which was always harvested early in the morning, had higher quality than the roots from *in vitro* culture.

The modified chromosome spread preparation was used for 13 different PAR and PIS lines (Table 4-1). The total ratio of slides with a high quality of chromosome preparations was 10.72%.

Table 4-1: Comparison of yield of good quality slides of different plant lines

Line	% +	% -
PAR cont	30.95	69.05
PAR GUS 9 (2n)	15.00	85.00
PAR GUS 14 (2n)	4.65	95.35
PAR GUS 40 (2n)	2.65	97.35
PAR GUS 43 (2n)	12.90	87.10
PAR GUS 65 (2n)	5.32	94.68
PAR nt (2n)	4.58	95.42
PIS GUS 2 (4n)	10.92	89.08
PIS GUS 12 (4n)	25.00	75.00
PIS CAM 30 (4n)	19.78	80.22
PIS CAM 31 (4n)	7.50	92.50
PIS nt (4n)	2.35	97.65
IxS nt (2n)	19.05	80.95
total	10.72	89.28

4.1.2 Meiotic chromosomes preparation

Pachytene chromosome preparations were made from flower buds of *Prunus* lines (PAR GUS 40, PAR GUS 42, PAR GUS 20-1, PAR GUS 20-2 and PAR GUS 36) grown in the glasshouse and a field sample. The flower buds from the field were harvested in the middle of March. The preparations from this material had a high ratio of good quality; 46% of the produced slides were with low background and more than 10 sets of meiotic pachytene chromosomes.

Flower buds from the glasshouse material were harvested in the middle of May. None of the produced chromosome preparations showed enough pachytene chromosomes or had a good quality. Although different sizes of the buds were used for the preparations, spores were already ripened.

4.2 Probe preparation

For *in situ* hybridization, 4 DNA probes 45S rDNA, 18S rDNA, 5S rDNA and T-DNA of pBinGUSint were used. Biotin-16-dUTP or DIG-11-dUTP (Roche) probes were produced by Random DNA Priming or Nick Translation or PCR.

4.2.1 45S rDNA probe

The 45S rDNA probe was obtained by Nick Translation labelling with biotin-16-dUTP or DIG-11-dUTP (Roche) of the clone plasmid pTA71 containing the wheat derived 18S-5.8S-25S gene repeat sequence. *E. coli* were transformed with pTA71. To produce a high amount of the contained ribosomal genes *E. coli* strain DH5α were grown under optimal conditions over night at 37 ℃ in 20 ml LB medium containing 100 µg/ml ampicillin. The plasmid was isolated from 4 ml of culture using miniprep. The mean amount of plasmid DNA was 830 ng/µl.

For Nick Translation the plasmid was linearized using the restriction enzyme *Hind* III (Roche), and cleaned by PCR-Purification spin protocol (Qiagen). The end concentration was 200 ng/µl. Nick Translation labelling was not successful without linearization of the plasmid. The linearized plasmid was incubated with the Nick Translation mix (Roche) until the size of the fragments ranged from 200 to 500 bp. The size of the fragments was checked by gel electrophoresis using a 2 % agarose gel and the molecular weight marker VIII (Roche). If the labelled fragments had right size the reaction was stopped by adding 1 µl/20µl EDTA (c = 5.0 mol/l) and by incubation at 65 ℃ for 10 min. The labelled DNA fragments were cleaned using the nucleotide removal kit (Qiagen) to get rid of the not incorporated nucleotides as well as enzymes and salts.

4.2.2 5S rDNA probe

The 5S rDNA probe was obtained by PCR labelling with biotin-16-dUTP or DIG-11-dUTP (Roche) of genomic DNA or the clone plasmid pGEM-Teasy containing 121 bp fragment of 5S rDNA gene. *E. coli* were transformed with pGEM-Teasy using the TOPO TA cloning kit. The transformed *E. coli* strain DH5α was cultured over night at 37 °C in 20 ml LB medium with ampicillin for selection. The plasmid pGEM-Teasy was isolated by miniprep. The mean concentration of the isolated plasmid was 1 µg/µl.

The 5S rDNA probe was obtained by PCR labelling of PIS genomic DNA using primers UP 46 and UP 47 (Table 3-1). The expected fragment size was 100 bp. After PCR the primer pairs turned out to produce more than one fragment. These probes were not used for FISH.

The M13 forward and reverse primers (Table 3-1) were used for amplification of the 121 bp insert of 5S rDNA in pGemTEasy plasmid. The expected fragment size was about 400 bp because the M13 primers binding sites were not directly at the border of the insertion. However, two different PCR products (~800 and ~400 bp) were obtained. The ~400 bp band was extracted using the QIAquick gel extraction protocol (Qiagen).

PCR were always run with a positive and a negative control. The positive control was used to check if the PCR conditions used can successfully amplify the target sequence. The negative control containing no template DNA was included to ensure that the solutions used for PCR were not contaminated with the template DNA.

4.2.3 18S rDNA

The 18S rDNA probe was obtained by Nick Translation labelling and by PCR labelling with biotin-16-dUTP or DIG-11-dUTP (Roche) of the pBluescript plasmid containing the 18S rDNA gene. Competent *E. coli*, Strain Top 10 F´ were transformed with pBluescript plasmid containing the 18S rDNA gene

using the Topo TA cloning kit. Ampicillin was used for selection of transformed *E. coli*. Bacteria were cultured over night in 5 ml LB medium containing 100 μg/ml ampicillin at 37 °C and 120 rpm. The plasmid was isolated by miniprep from 2 ml culture. The concentration of plasmid ranged from 0.8 μg/μl to 2.1 μg/μl. For Nick Translation the plasmid was linearized using the *Eco*RV restriction enzyme and for PCR labelling the plasmid was diluted to 0.2 ng/μl.

For PCR the M13fwd and the T3 primers (Table 3-1) were used. The obtained PCR product had 3 kb, but the intron was only 2 kb. Because of the size of the amplicon it was not sure if the backbone of the plasmid was amplified or if the amplified fragment was the 18S rDNA intron including a part of the backbone. The fragments were not used for further experiments.

Therefore, Nick Translation was used for labelling of pBluescript containing the 18S rDNA gene. Experiments with a linearized and a not linearized plasmid showed that there was no need to linearize the pBluescript plasmid to produce labelled fragments by Nick Translation. However the labelling was better with the linearized plasmid.

4.2.4 T-DNA probe

For the pBinGUSint plasmid it was not possible to isolate an appropriate amount for Nick Translation by miniprep (max 35 μmol/μl). However, mean concentration of the isolated plasmid using Promega Maxikit was 140 ng/μl.

The pBinGUSint plasmid was used for Nick Translation and for Random Primed Labelling as well as PCR labelling method. For PCR labelling the whole plasmid was used as template. By the use of the GUS primer set the expected fragment size was 2 kb. The amplicon was checked by gel electrophoresis and the size of labelled fragment was compared to a not labelled control. For random primed and Nick Translation labelling the 15 kb pBinGUSint plasmid was digested by the *Mss*I and *Sac*I restriction enzymes

to cut out the 5 kb T-DNA construct (Figure 3-1). The ~5 kb band was extracted from agarose gel using the QIAquick gel extraction kit (Qiagen). The concentration after gel extraction was 50 ng/µl. Positive PCR results for *uidA* and the *nptII* genes (Table 3-1) proved that the extracted fragment was the T-DNA.

The labelled probe was cleaned using the QIAquick nucleotide removal kit.

4.3 Checking incorporation of label

The incorporation of label was checked by performance of a dot blot. The probes and a labelled standard DNA were diluted, denatured and the put on ice to avoid renaturation. A dilution series was made, and 1 µl of each dilution was loaded on a positively charged membrane and baked. After equilibration and application of blocking reagent an Anti-DIG-AP or Streptavidin-AP binding to DIG or Biotin was applied. The unbound antibodies were washed out, substrate CSPD ready to use was added to the Alkaline Phosphatase linked antibody, and the resulting chemiluminescence of the reaction was visualized using the Lumi-Imager F1 working station.

The resulting spots of the different probes were compared to the spots of the standard DNA to estimate the concentration of incorporated label. For biotin labelled probes such a standard DNA did not exist. The results of biotin labelled probes were compared to the results of DIG labelled probes; if the spots of the biotin labelled probe were visible in the same dilutions as on the membrane of the DIG labelled probe the concentration of incorporated label was assumed to be the same.

The comparison of the different labelling methods is complex: a) the amount of tymidin bases which are partly replaced by labelled dUTPs is different, b) the standard DNA (Roche) and the probes had different DNA sequences and size. The comparison of the spot intensity gave no linear result but a good estimation of the amount of incorporated label is still possible.

The dot blot of the three different labelling methods showed that the labelling by PCR is most variable in the amount of incorporated labelled nucleotides. Nick Translation was the least efficient labelling method (Figure 8-1 and Figure 8-2).

4.4 Fluorescence *In Situ* Hybridization

For FISH the best slides were chosen, and Pepsin as well as RNAse A were used to get rid of RNA and proteins. The hybridization buffer, the timing and temperature of the probe and target denaturation were optimized.

Different signal detection systems were tested: a) detection with one antibody for DIG or Cy3 conjugated Streptavidin for biotin, b) detection with three antibodies for the amplification of the signal for DIG labelled probes or a series of Cy3 conjugated Streptavidin followed by Biotinylated-Anti-Streptavidin and Cy3 conjugated Streptavidin again for biotin labelled probes. The best results were obtained without signal amplification. Detection of biotin labelled probes showed stronger signals but with more background compare to DIG labelled probes.

A mixture of two probes, one labelled with DIG and one with biotin, was applied. The amount of labelled probe ranged from 2 - 400 ng, but a clear signal was visible for a probe with concentration more than 40 ng for the 45S rDNA and more than 100 ng for the 5S rDNA.

4.5 Analysis of signal

Hybridized chromosome sets showed six (PAR) or twelve (PIS) signals for 45S rDNA, which could be detected on up to three different chromosomes of PAR (Figure 4-1, green arrows) and PIS (Figure 4-2, green arrows). For PAR chromosomes four out of 6 signals were clearly stronger. While for PIS chromosomes there is no obvious difference in the signal intensity for the 45S rDNA probe. In Figure 4-2 two 45S rDNA signals of PIS (green arrows) seemed to be over a longer distance of the chromosomes, but these were

DNA-traces of the satellites which were separated from the chromosomes. The signals were at the terminal region of the chromosomes, but because of the intensity of the signal it was not possible to localize them more exactly within the chromosome.

For 5S rDNA it was possible to get 4 clear signals for PAR chromosomes using 100 ng probes. The signals are located on two different chromosomes and both are close to the strong 45S rDNA signals (Figure 4-1, red arrows). For PIS it was possible to detect a signal of 5S rDNA on one of the chromosomes (Figure 4-2, red arrows) using 100 ng probe.
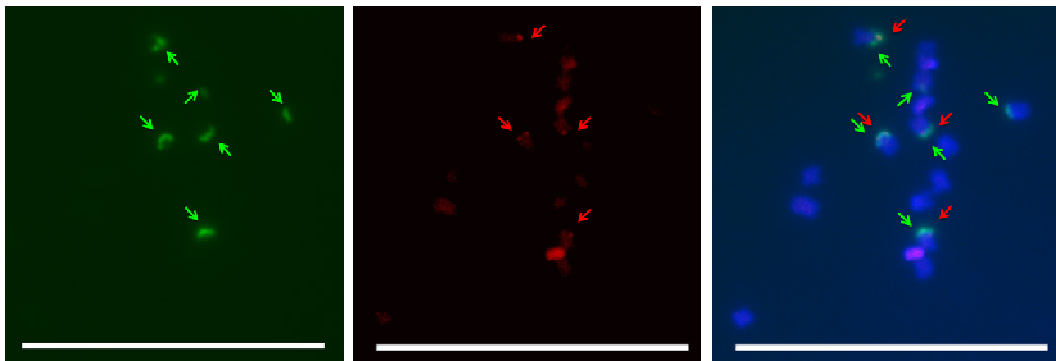
Figure 4-1: PAR GUS 11 chromosomes hybridized with 45S rDNA (DIG, green arrows) and 5S rDNA (biotin, red arrows) (bar = 20 μm)
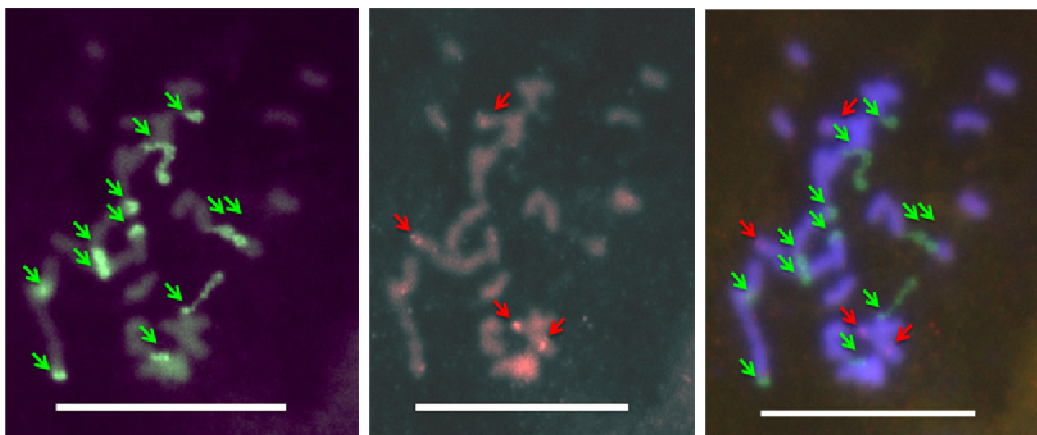
Figure 4-2: PIS GUS 12 chromosomes hybridized with 45S rDNA (DIG, green arrow) and 5S rDNA (biotin, red arrow) (bar = 10 μm)

FISH of meiotic chromosomes with the 45S rDNA probe (red arrow) and the 18S rDNA probe (green arrows) was also successful and showed the overlapping signals at the same positions of the chromosomes (Figure 4-3).
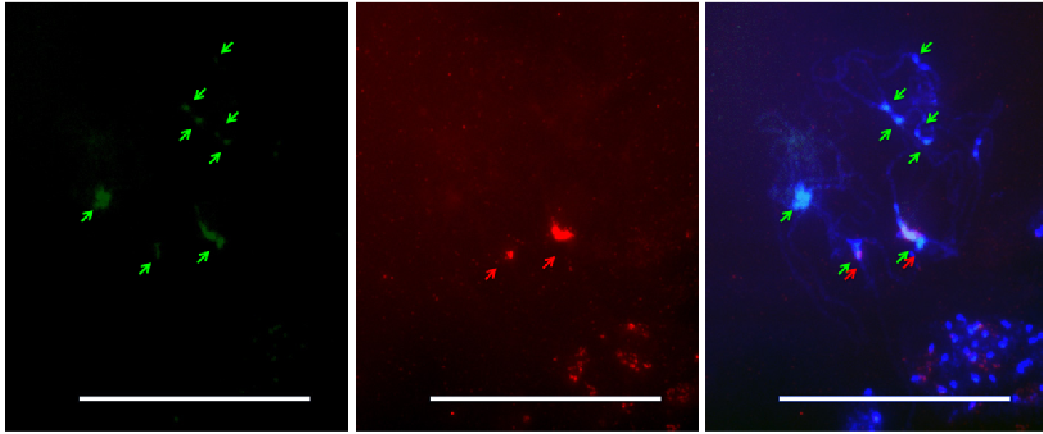
Figure 4-3: Pachytene chromosomes of a field tree hybridized with 45S rDNA (biotin, red arrows) and 18S rDNA (DIG, green arrows) (bar = 20 µm)

For T-DNA it was not possible to get a clear hybridization signal.

4.6 Karyotyping

To establish the karyotypes of non transgenic PAR and PIS plants pictures of countable and full chromosome sets were used (Figure 4-6 and Figure 4-9). From the generated data schematic pictures have been made (Figure 4-4 and Figure 4-7). For high statistical relevance 29 full chromosome sets of PAR and 11 full chromosome sets of PIS were used for the calculation of the mean length of the chromosomes, the mean length of the short and the long arm and the arm ratio as well as for the standard deviation.

All chromosomes of PAR and PIS except for chromosome 1 are hardly distinguishable by size (Figure 4-5 and Figure 4-8). It was not possible to define, which of the chromosomes are metacentric or submetacentric. The high standard deviation for the arm ratios could result from a wrong categorization of the chromosomes by length only. For PAR chromosomes no

secondary constriction was found. For PIS, chromosome 2 could be distinguished from the other chromosomes because it carries a secondary constriction.

Measuring of PAR chromosomes which were hybridized with the 45S rDNA probe showed that chromosomes 2, 3 and 6 carry the 45S rDNA. The signal on chromosome 6 is the weaker signal. For 5S rDNA it was possible to detect 4 signals located close to the strong 45S rDNA signals on chromosomes number 2 and 3.

Measuring of PIS chromosomes which were hybridized with 45S rDNA and 5S rDNA showed that chromosome number 2, 3 and 6 carried the signals for 45S rDNA and chromosome number 7 carried the signal for 5S rDNA.
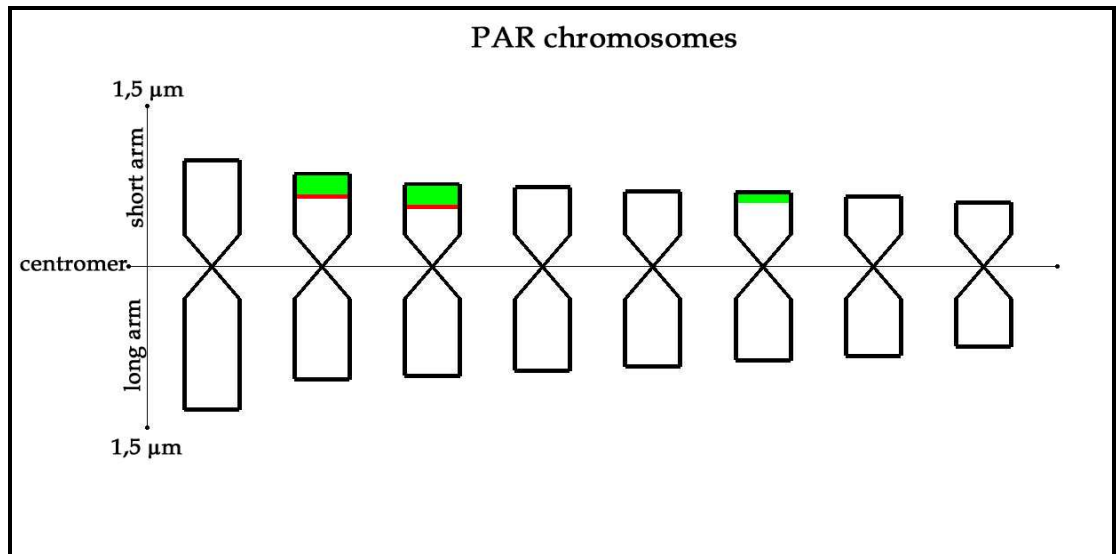
Figure 4-4: Schematic figure of PAR chromosomes; the green bands represent the 45S rDNA signals; the red band represents the signal for 5S rDNA

Figure 4-5: Mitotic metaphase chromosomes of PAR hybridized with 45S rDNA (green signal) and 5S rDNA (red signal) sorted by size (bar = 20 μm)
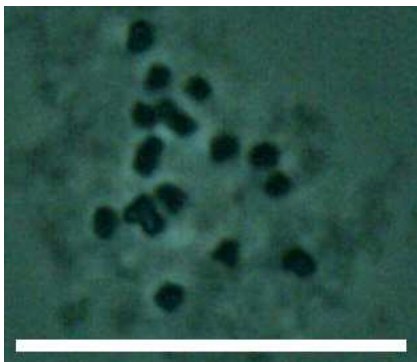
Figure 4-6: Phase contrast picture of metaphase chromosomes of PAR (bar = 20 μm)
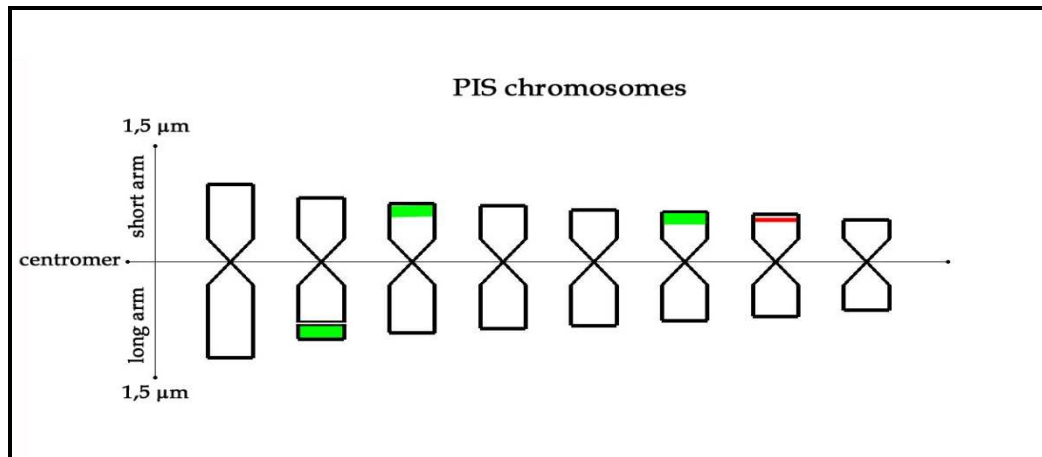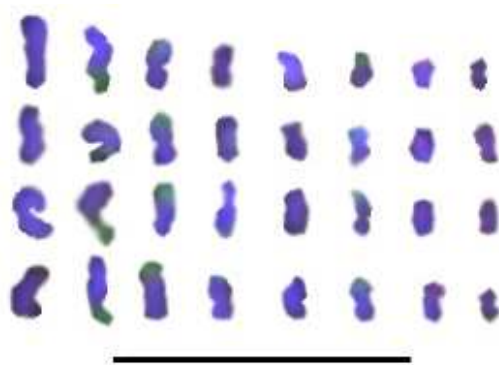
Figure 4-7: Schematic figure of PIS chromosomes the green bands represent the 45S rDNA signals; the red band represents the signal for 5S rDNA

Figure 4-8: Mitotic metaphase chromosomes of PIS hybridized with 45S rDNA (green signal) and 5S rDNA (red signal) sorted by size (bar = 5μm)
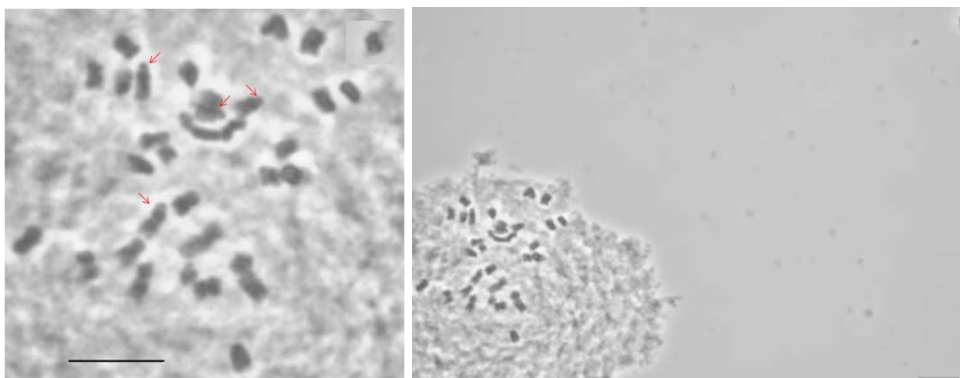

Figure 4-9: Metaphase-chromosomes of PIS; the secondary constriction of chromosome 2 is marked by red arrows; phase contrast (bar = 5 μm)

Table 4-2: Results of measuring for 29 full sets of PAR chromosomes

PAR	total length				long arm				short arm				arm ratio			
	Mean	Std. dev	Min	Max	Mean	Std. dev	Min	Max	Mean	Std. dev	Min	Max	Mean	Std. dev	Min	Max
1	2.329	0.325	1.693	3.056	1.336	0.190	0.918	1.698	0.993	0.207	0.602	1.411	1.395	0.336	1.000	2.736
2	1.912	0.162	1.415	2.191	1.049	0.127	0.728	1.320	0.864	0.107	0.559	1.056	1.236	0.246	1.004	1.899
3	1.793	0.150	1.371	2.003	1.023	0.131	0.732	1.280	0.770	0.105	0.528	0.955	1.362	0.316	1.011	2.340
4	1.712	0.146	1.308	1.948	0.971	0.123	0.713	1.323	0.741	0.106	0.490	0.923	1.345	0.318	1.005	2.491
5	1.635	0.135	1.282	1.847	0.932	0.122	0.654	1.191	0.702	0.098	0.443	0.884	1.362	0.315	1.000	2.267
6	1.568	0.132	1.238	1.833	0.875	0.107	0.664	1.129	0.693	0.090	0.448	0.834	1.291	0.267	1.000	1.985
7	1.484	0.119	1.183	1.721	0.831	0.097	0.651	1.107	0.653	0.069	0.443	0.787	1.286	0.212	1.017	1.840
8	1.343	0.121	1.067	1.563	0.747	0.085	0.534	0.982	0.596	0.079	0.389	0.751	1.275	0.235	1.003	2.031

Table 4-3: Results of measuring for 11 full sets of PIS chromosomes

PIS	length each				long arm				short arm				arm ratio			
	Mean	Std. dev	Min	Max	Mean	Std. dev	Min	Max	Mean	Std. dev	Min	Max	Mean	Std. dev	Min	Max
1	2.228	0.445	1.132	3.225	1.244	0.303	0.658	1.843	1.007	0.538	0.461	1.398	1.300	0.236	1.988	1.017
2	1.832	0.378	0.855	2.502	1.001	0.256	0.490	1.490	0.831	1.516	0.343	1.179	1.255	0.223	1.885	1.004
3	1.672	0.350	0.782	2.243	0.920	0.246	0.438	1.368	0.756	2.556	0.343	1.119	1.256	0.220	1.882	1.005
4	1.577	0.332	0.772	2.086	0.863	0.221	0.408	1.217	0.726	3.597	0.279	1.018	1.292	0.336	2.937	1.005
5	1.487	0.316	0.712	1.984	0.824	0.217	0.384	1.167	0.672	4.646	0.294	0.929	1.315	0.336	3.118	1.008
6	1.403	0.314	0.659	1.912	0.761	0.200	0.364	1.030	0.650	5.688	0.279	0.926	1.234	0.173	1.890	1.013
7	1.310	0.291	0.640	1.793	0.707	0.191	0.339	1.063	0.617	6.735	0.289	0.904	1.200	0.169	1.696	1.005
8	1.117	0.342	0.068	1.553	0.627	0.186	0.249	1.062	0.544	7.793	0.230	0.741	1.204	0.236	2.329	1.005

5. Discussion

5.1 Slide preparation

Good quality of the metaphase chromosome preparations is one of the major prerequisites for good results in FISH (Schwarzacher and Haslop-Harrison, 2000). In this study the quality of metaphase spreads was improved by maceration of the meristems, longer incubation in 45 % acetic acid and by the application of fixative directly after flicking off the cover slip. This procedure seems to reduce the amount of disturbing cytoplasm and leads to well separated cells. Although the number of slides which had enough sets of mitotic chromosomes could not be increased, the modified protocol gave high quality slides. Transgenic and non transgenic lines with different quality of slides (Table 4-1) showed that the transformation of the plants did not influence the susceptibility for the spindle inhibitor 8-hydroxyquinoline. It is therefore not the possible reason for the low metaphase index occurring in the preparations. Andras et al. (1999) described 8-hydroxychinoline to be the optimal spindle inhibitor for preparation of small chromosomes and it was also successfully used by Yamamoto et al. (1999) for *Prunus* species. In general the use of 8-hydroxychinoline is most common, but in some cases another spindle inhibitor might deliver better results. Corredor et al (2003) used ice water to accumulate metaphase chromosomes and Ohmido et al. (1998) used no accumulation of metaphase chromosomes for the preparation of rice chromosomes which are also very small. An increased yield of metaphase plates is necessary, because so far it is very time consuming to produce enough good-quality metaphase chromosome preparations for any kind of hybridization experiment.

5.2 Probe preparation

Three different methods were used for the preparation of the probes. Although PCR labelling method shows a great improvement for probe labelling, it is limited by the size of the produced probe. The entire T-DNA fragment and the 45S rDNA template are too large for PCR labelling, so it was performed only for the 5S rDNA. The dot blot control showed variable results for the efficiency to incorporate DIG or biotin linked dUTPs. In comparison, the PCR labelling method is less time consuming. No previous digestion and cleaning of the plasmid are needed and the labelling is sequence specific. A part of the T-DNA construct was also labelled by PCR but the chance to get a strong hybridization signal was even lower than for the whole T-DNA construct because the target is smaller.

The pBinGUSint plasmid was used for the production of the T-DNA probe. It is a very big plasmid (15 kb). It has to be isolated by the use of a maxikit to get an efficient amount and concentration of the plasmid. The isolation of the T-DNA and the cleaning which are required for labelling reduced the amount of DNA dramatically (75 % of isolated DNA were lost). So the only labelling method applicable for the T-DNA construct was the Random Primed Labelling because the whole construct is too long for PCR labelling.

In this study the labelling of pTA71 by Nick Translation without linearization of the plasmid was not successful. Although, the smaller plasmid pBluescript could be labelled sufficiently without linearization, the Nick Translation seems to be more efficient if the plasmid is linearized or digested into small (1 – 3 kb) pieces as described by Salvo-Garrido et al. (2001). In the same work is described that the use of the released insert helps to map the gene more accurate because there is no network formation and a higher homology to the target.

A method which compares the advantages of the two other labelling methods (PCR labelling and Nick Translation) – amplification of DNA and independence

of the template size is the Random Primed Labelling. In this study it was used for T-DNA labelling of the pBinGUSint. Random Primed Labelling was the only method which produced a high amount of sufficiently labelled T-DNA probe. The T-DNA insert was released from the plasmid to avoid the formation of networks and to achieve accurate physical mapping.

The dot blot showed that the PCR labelling method is most variable for efficient incorporation of labelled nucleotides. The dot blots of two different labelling reactions with the same conditions showed one with high amount of label incorporated and the other with the low amount of incorporated label. These results show that the quality of the probes should be checked before hybridization. As the incorporation of labelled nucleotides is always random; the results for FISH can be very different. Probes produced by Nick Translation and Random Primed Labelling showed less variable results for the incorporation of label. Another important fact is that probes and a standard DNA of different sequence are compared, so it is only possible to compare the total amount of incorporated label, but not the ratio of labelled dUTPs to the unlabelled dTTPs.

5.3 Fluorescence *In Situ* Hybridization

This study is the first report of using FISH to determine the number and the physical position of rDNA in PIS and PAR.

The simultaneous double colour hybridization with the 5S rDNA and the 45S rDNA of the PIS chromosomes allowed the identification of four chromosomes of the tetraploid genome with 2n = 4x = 32 chromosomes. Yamamoto et al. (1999) and Corredor et al. (2003) also described three loci for 45S rDNA on three different chromosomes by the use of FISH in *Prunus persica* and *amygdalus* respectively. Corredor et al. (2003) also found only one locus for 5S rDNA and were able to identify all the chromosomes of almond by the total size of the chromosomes, the hybridization signals and arm ratios. For PAR, 6 signals for

45s rDNA could be detected on three different chromosomes. Two of the signals were weaker than the other four signals. The weak signals were located on chromosome number 6. The difference in the intensity of the hybridization signal could be caused by a difference in the copy number of the rDNA. Different signal intensities were also described by Corredor et al. (2003). In that study it was also described that only two of three chromosomes carrying the 45S rDNA participate in the organization of the nucleolus. As the 5S rDNA probe was suitable for the detection of 5S rDNA of PIS it was also possible to detect 4 signals for 5S rDNA in PAR. They were located close to the 45S rDNA on chromosomes number 2 and 3.

The use of different labels (DIG and biotin) and the detection system for each of the labels resulted in different signal to noise ratios. The DIG detection system produced less background signals than the biotin detection system, while the biotin detection system resulted in stronger signals. A biotin detection system was also the first nonradioactive detection system which was used successfully for the detection of a single copy gene (Ambros et al, 1986).

So far several studies are reported of successful application of FISH for the detection of small probes and single copy genes (Ohmido et al., 1998; Desel et al., 2001; Salvo-Garrido, 2001, Khrustaleva and Kik, 2001). Anyway it is still reviewed (de Jong, Fransz and Zabel, 1999; Svintashev and Somers, 2002; Jiang and Gill, 2006) that FISH with small targets is still a challenge and that even under optimal hybridization and detection conditions, and with the use of the best CCD cameras the sensitivity remains dependent principally on the DNA condensation and consequently the accessibility of the target. Further the frequencies of the detected small probes are low and in many cases not reproducible in other labs using the same technique (Jiang and Gill, 2006). The low metaphase index of the *Prunus* plants used in this study, as the propable loss of chromosomes during pretreatment and denaturation (Rapp et al., 1986) and the low detection frequency of small targets as well as the possible

inaccessibility of the target are reasons for the failure of the detection of the T-DNA in this report. Although different probe concentrations, labelling techniques and detection systems were used this hurdles could not be overcome yet. Anyway the results of PCR, Southern blotting and real time PCR confirm the transgenicity of all transgenic lines. Southern blots further indicated that the transgenic PAR carry an average of 1.6 T-DNA copies (Maghuly et al, 2007). For PIS all individuals from 35S-GUS lines carried 5 or 6 copies of the *uid*A and all individuals from CaM-GUS only one copy of *uid*A gene (Maghuly et al, 2008).

5.4 Karyotyping

PIS has a very small genome size (1C ~ 0.6 pg; tetraploid). Like other small chromosomes (Andras et al., 1999), PIS chromosomes also lack morphological markers for karyotryping. Only chromosome number 1 could be clearly identified by its size. By FISH the identification of 4 further chromosomes was possible. The measurement of the chromosomes delivered different mean length of the nonhomologues chromosomes but according to the high standard deviation and the impossibility to identify weather a chromosome is metacentric or submetacentric are these results of low reliability. The preparation method (dropping or squashing) of the chromosomes (Schwarzacher and Heslop-Harrison, 2000) and the exact cell cycle stage could be reasons for the high standard deviation as they influence the condensation, and therefore the size of the chromosomes. Another factor influencing the high standard deviation is for sure the manual measurement of the chromosome length and especially for PIS the low amount of full chromosome sets available.

For the karyotype of PAR a higher amount of full chromosome sets was used. The standard deviation for the calculated mean length of the chromosomes is lower than for the PIS chromosomes but still too high to identify all the PAR chromosomes exactly as the sizes overlap when the standard deviation is included. The high standard deviation and especially the varying arm ratios

point out that the simple distinction by size may lead to wrong assigns. Yamamoto et al. (1999) also failed in the identification of all chromosomes of *Prunus persica*. Anyway Corredor et al. established (2004) a karyotype of almond with standard deviations for the total length of the chromosomes similar to the standard deviation obtained in this report, but they were also possible to distinguish metacentric and submetacentric chromosomes.

6. Perspectives

For further investigations of the PIS and PAR chromosomes there is a need to increase the yield of metaphase chromosomes within slide preparation. Several strategies like the synchronization of the cell cycle (Doležel et al., 1999) or the use of other spindle inhibitors could be useful. For less condensed metaphase chromosomes (Schwarzacher and Heslop-Harrison, 2000) a dropping method for small chromosomes (Andras et al., 1999) could deliver a clearer chromosome structure and therefore more accurate results, especially for the arm ratio of the karyotyping. Another approach to identify all the chromosomes of the two different *Prunus* species is the FISH method with other telomere or centromere specific probes. The application of traditional banding techniques could also be used for the identification of the chromosomes. The successful karyotyping of *P. avium* by C-banding was described by Schuster and Ahne (1999).

The establishment of the karyotype for PIS and PAR will allow the assignment of linkage groups to chromosomes in *Prunus* by physical mapping of the linkage groups by FISH (Yamamoto et al., 1999).

The characterization of the transgene loci in the different lines of PIS and PAR will also deliver important information as the copy number of the transgene, the site of integration and the structural organization of the transgene loci (Svintashev and Somers, 2002). One approach to circumvent the difficulty for detecting small DNA probes is the use of large-insert genomic DNA probes that are anchored by the targeted small DNA probes (Jiang and Gill, 2006).The use of pachytene chromosomes of PIS and PAR with a higher spatial resolution and a better accessibility could also help to increase the chances for single copy detection on metaphase chromosomes.

7. Literature

Abbott, A. G., Arús, P., & Scoza, R. (2007). Peach. In C. Kole, *Genome Mapping and Molecular Breeding in Plants* (Volume 4 Fruits and Nuts , pp. 137 - 156). New York: Springer-Verlag Berlin Heidelberg.

Ambros, P. F., Matzke, M. A., & Matzke, A. J. (1986). Detection of a 17 kb unique aequence (T-DNA) in plant chromosomes by in situ hybridization. *Chromosoma (Berlin), 94*, 11 - 18.

Andras, S. C., Hartman, T. P., Marshall, J. A., Power, J. B., Cocking, E. C., & Davey, M. R. (1999). A drop-spreading technique to produce cytoplasm-free mitotic preparations from plants with small chromosomes. *Chromosome Research, 7*, 641 - 647.

Arumuganathan, K., & Earle, E. D. (1991). Nuclear DNA Content of some important plant species. *Plant Molecular Biology, 9*, 208 - 219.

Bennett, M. D., & Leitch, I. J. (1995). Nuclear DNA Amounts in Angiosperms. *Annals of Botany, 76*, 113 - 176.

Brown, T. A. (2002). *Genome 2.* New York and London: Garland Science.

Corredor, E., Román, M., García, E., Perera, E., Arús, P., & Naranjo, T. (2004). Physical mapping of rDNA genes establishes the karyotype of almond. *Annals of applied Biology, 144*, 219 - 222.

Cuadrado, A., Ceolony, C., & Jouve, N. (1995). Variation in highly repetitive DNA composition of heterochromatin in rye studied by fluorescence in situ hybridization. *Genome, 38*, 1061 - 1069.

da Câmara Machado, A., Puschmann, M., Pühringer, H., Kremen, R., Katinger, H., & Laimer da Câmara Machado, M. (1995). Somatic embryogenesis of *Prunus*

subhirtella autumnalis rosa and regeneration of transgenic plants after *Agrobacterium*-mediated transformation. *Plant Cell Reports, 14*, 335 - 340.

de Jong, H. (2003). Visualizing DNA domains and sequences by microscopy: a fifty-year history of molecular cytogenetics. *Genome*, *46*, 943 - 946.

de Jong, J. H., Fransz, P., & Zabel, P. (1999). High resolution FISH in plants - techniques and applications. *Trends in plant science, 7*, 258 - 263.

Desel, C., Jung, C., Cai, D., Kleine, M., & Schmidt, T. (2001). High-resolution mapping of YACs and the single-copy gene *Hs1pro-1* on *Beta vulgaris* chromosomes by multi-colour fluorescence in situ hybridization. *Plant Molecular Biology*, *45*, 113 - 122.

Dirlewanger, E., Claverie, J., Wünsch, A., & Iezzoni, E. (2007). Cherry. In C. Kole, *Genome Mapping and Molecular Breeding in Plants* (Volume 4 Fruits and Nuts, 103 - 118). Springer-Verlag Berlin Heidelberg.

Dolezel, J., Cihalikova, J., Weiserova, J., & Lucretti, S. (1999). Cell cycle synchronization in plant root meristems. *Methods in Cell Science, 21*, 95 - 107.

Druart, P. (1981). Embryogenèse somatique et obtenion de plantules chez *P. incisa x serrula* (GM9) cultivé in vitro. *Bull. Rech Argon , 3*, 205 - 220.

Druart, P., & Gruselle, R. (2007). Behaviour of *Agrobacterium rhizogenes-*Mediated Transformants of "Inmil" (*P. incisa x serrula*) Cherry Rootstock. *Acta Horticulturae, 738*, 589 - 592.

Esmenjaud, D., & Dirlewanger, E. (2007). Plum. In C. Kole, *Genome Mapping and Molecular Breeding in Plants* (119 - 135). Springer-Verlag Berlin Heidelberg.

Fukui, K., & Nakayama, S. (1996). *Plant Chromosomes: Laboratory Methods*. Boca Raton: CRC Press.

Gall, J. G., & Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Genetics*, 378 - 383.

Gerlach, W. L., & Bedrook, J. R. (1979). Cloning and characterization of robosomal RNA genes from wheat and barley. *Necleic Acids Research*, *7*, 1869 - 1885.

Goncalves, B., Moutinho-Pereira, J., Santos, A., Silva, A. P., Bacelar, E., Correia, C.,Rosa, E. (2005). Scion-rootstock interaction affects the physiology and fruit quality of sweet cherry. *Tree Physiology*, *26*, 93 - 104.

Griffiths, A. J., Gelbart, W. M., Miller, J. H., & Lewontin, R. C. (1999). Genomics. In *Modern Genetic Analysis.* New York: W. H. Freeman and Company.

Hedrick, U. P. (1915). The history of cultivated cherries. In U. P. Hedrick, *The Cherries of New York* (39-64). Ithaca, USA: Albany: J. B. Lyon Company.

Hizume, M., Shibata, F., Matsusaki, Y., & Garajova, Z. (2002). Chromosome identification and comparative karyotypic analysis of four *Pinus* species. *Theoretical and Applied Genetics*, *105*, 491 - 497.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J., & Schilperoort, R. A. (1983). A binary plant vector strategy based on separation of vir- and T-Region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature*, *303*, 179 - 180.

Jiang, J., & Gill, B. S. (2006). Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. *Genome, 49*, 1057 - 1068.

Jiang, J., & Gill, B. S. (1994). Nonisotopic in situ hybridization and plant genome mapping: the first 10 years. *Genome*, *37*, 717 - 725.

Jiang, J., Hulbert, S. H., Gill, B. S., & Ward, D. C. (1996). Interphase fluorescence in situ hybridization mapping: a physical mapping strategy for plant species with large complex genomes. *Molecular and General Genetics*, *252*, 497 - 502.

John, H. A., Birnstiel, M. L., & Jones, K. W. (1969). RNA-DNA hybrids at the cytological level. *Nature*, *223*, 582 - 587.

Kato, A., Veger, J. M., Han, F., Lamb, J. C., & Birchler, J. A. (2005). Advances in plant chromosome identification and cytogenetic techniques. *Curent Opinion in Plant Biology, 8,* 148 - 154.

Khrustaleva, L. I., & Kik, C. (2001). Localization of single-copy T-DNA insertion in transgenic shallots (*Allium cepa*) by using ultra-sensitive FISH with tyramide signal amplification. *The Plant Journal, 6,* 699 - 707.

Kislauskis, E. H., Li, Z., Singer, R. H. and Taneja, K. L. (1993). Isoform-specific 3'-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *Journal of Cell Biology, 123,* 165 - 172

Langer-Safer, P. R., Levine, M., & Ward, D. C. (1982). Immunological method for mapping genes on *Drosophila* polytene chromosomes. *Proc. NatL Acad. Sci. USA , 14,* 4381 - 4385.

Lim, K. Y., Matyasek, R., Lichtenstein, C. P., & Leitch, A. R. (2000). Molecular cytogenetic analysis and phylogenetic studies in the *Nicotiana* section *tomentosae. Charomosoma , 109,* 245 - 258.

Linc, G., Friebe, B., Kynast, R. G., Molnar-Lang, M., Koszegi, B., Sutka, J., et al. (1999). Molecular cytogenetic analysis of *Aegilops cylindrica* host. *Genome, 42,* 497 - 503.

Ma, Y., Islam-Faridi, M. N., Crane, C. F., Stelly, D. M., Price, H. J., & Byrne, D. H. (1996). A new procedure to prepare slides of metaphase chromosomes of roses. *Horticultural Science, 31,* 855 - 857.

Maghuly, F., Khan, M. A., Fernandez, E. B., Druart, P., Watillon, B., & Laimer, M. (2008). Stress regulated expression of the GUS-marker gene (*uid*A) under the control of plant calmodulin and viral 35S promoters in a model fruit tree rootstock: *Prunus incisa x serrula. Journal of biotechnology, 135,* 105 - 116.

Maghuly, M., da Câmara Machado, A., Leopold, S., Khan, M. A., Katinger, H., & Laimer, M. (2007). Long-term stability of marker gene expression in *Prunus subhirtella* a model fruit tree species. *Journal of biotechnology*, *127*, 310 - 321.

Manuelidis, L., Langer-Safer, P. R. and Ward, D. C. (1982). High-resolution mapping of satellite DNA using biotin-labelled DNA probes. *Journal of Cell Biology, 95*, 619 -625.

Meyerowitz, E. M. (1987). In Situ Hybridization to RNA in Plant Tisue. *Plant Molecular Biology Reporter, 5*, 242-250.

Ohmido, N., Akiyama, Y., & Fukui, K. (1998). Physical mapping of unique nucleotide sequences on identified rice chromosomes. *Plant Molecular Biology*, *38*, 1043 - 1052.

Olden, E., & Nybom, N. (1968). On the origin of *Prunus cerasus* L. *Hereditas , 70*, 3321 - 3323.

Otto, S. P. (2007). The Evolutionary Consequences of Polyploidy. *The Cell , 131*, 452- 462.

Pardue, M. L., & Gall, J. G. (1969). Molecular hybridization of radioactive DNA to the DNA of cytological preparations. *Genetics, 64*, 600 - 604.

Pedersen, C., & Langridge, P. (1997). Identification of the entire chromosome complement of bread wheat by two-color FISH. *Genome*, 40, 589 - 593.

Raap, A., van de Corput, M., Vervenne, R., van Gijlswijk, R., Tanke, H., & Wiegant, J. (1995). Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome tyramides. *Human Molecular Genetics*, *4*, 529 - 534.

Rayburn, A. L., & Gill, B. S. (1985). Use of biotin-labelled probes to map specific DNA sequences on wheat chromosomes. *The Journal of Heredity*, *76*, 78 - 81.

Salvo-Garrido, H., Travella, S., Schwarzacher, T., Harwood, W. A., & Snape, J. W. (2001). An efficient method for the physical mapping of transgenes in barley using in situ hybridization. *Genome*, *44*, 104 - 110.

Schuster, M., & Ahne, R. (1998). Karyotype Analysis of *Prunus avium* L. *Acta Horticulturae , 484*, 387 - 390.

Schuster, M., & Schreiber, H. (2000). Genome investigation in sour cherry *P. cerasus* L. *Acta Horticulture*, *538*, 375 - 379.

Schwarzacher, T. (2003). DNA, chromosomes, and in situ hybridization. *Genome*, *46*, 953 - 962.

Schwarzacher, T., & Heslop-Harrison, J. S. (2000). *In situ hybridization.* Oxford U.K.: BIOS Scientific Publishers.

Singer, R. H. and Ward, D. C. (1982). Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinylated nucleotide analog. *Proc. Natl. Acad. Sci. USA, 79,*7331 -7335.

Stephens, J. L., Brown, S. E., Lapitan, N. L., & Knudson, D. L. (2004). Physical mapping of barley genes using an ultrasensitive fluorescence in situ hybridization technique. *Genome*, *47*, 179 - 189.

Svitashev, S. K., & Somers, D. A. (2002). Characterization of transgene loci in plants using FISH: A picture is worth a thousand words. *Plant Cell, Tissue and Organ Culture, 69*, 205 - 214.

Tessadori, F., van Dril, R., & Fransz, P. (2004). Cytogenetics as a tool to study the gene regulation. *Trends in Plant Science*, *9*, 147 - 153.

Trask, B. J. (1991). Fluorescence in situ hybridization: Applications in cytogenetics and gene mapping. *Trends in Genetics*, *7*, 149 - 154.

Vancanneyt, G., Schmidt, R., O´Connor Sanchez, A., Willmitzer, L., & Rocha Sosa, M. (1990). Constuction of an intron-containing marker gene. *Molecular Genetics and Genomics*, *220*, 245 - 250.

Watkins, R. (1976). Cherry, plum, peach, apricot and almond. In N. W. Simmonds, *Evolution of Crop Plants* (pp.242 - 247). New York: Longman.

Wegel, E., Vallejos, R. H., Christou, P., Stoger, E., & Shaw, P. (2005). Large-scale chromatine decondensation induced in a developmentally transgene locus. *Journal of Cell Science, 118*, 1021 - 1031.

Yamamoto, M., Shimada, T., Haji, T., Mase, N., & Sato, Y. (1999). Physical Mapping of the 18S Robosomal RNA Gene of Peach (*Prunus persica* (L.) Batsch) Chromosomes by Fluorescence *in situ* Hybridization. *Breeding Science*, *49*, 49 - 51.

Zhang, P., Li, W. L., Friebe, B., & Gill, B. S. (2004). Simultaneous painting of three genomes in hexaploid wheat by BAC-FISH. *Genome, 47*, 979 - 987.

8. Appendix
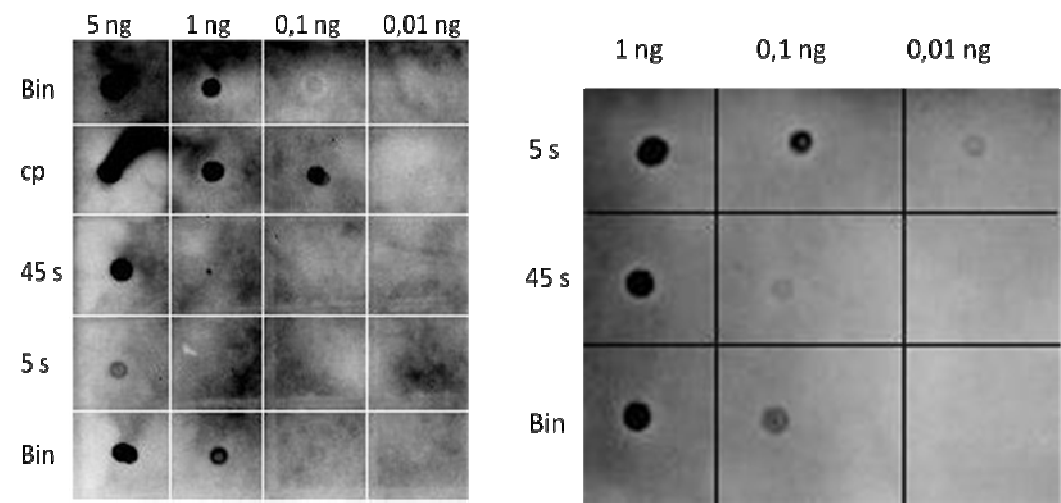
Figure 8-1: Dot blots of biotin labelled probes
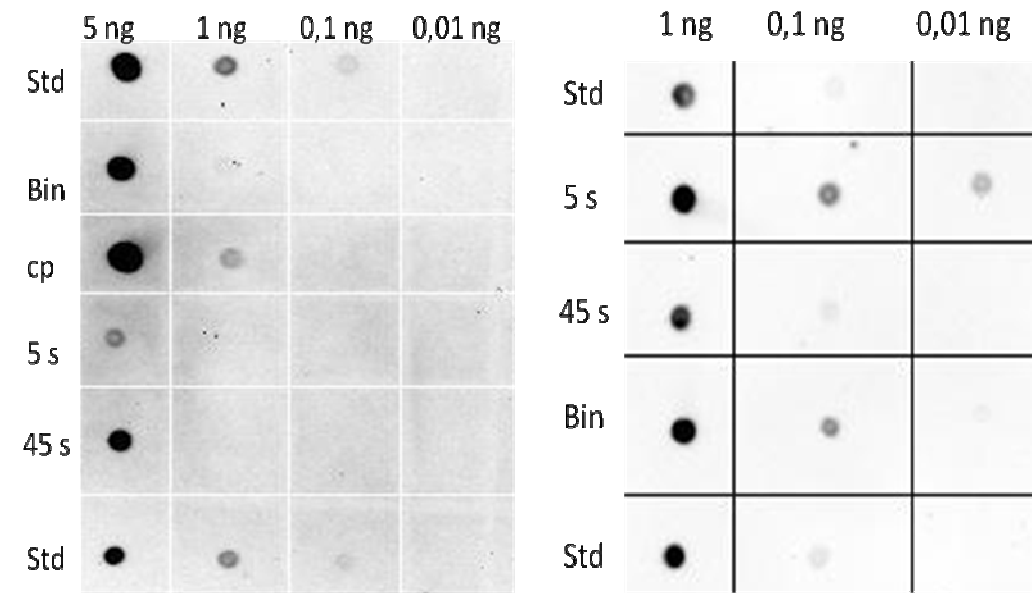
Figure 8-2: Dot blots of DIG labelled probes
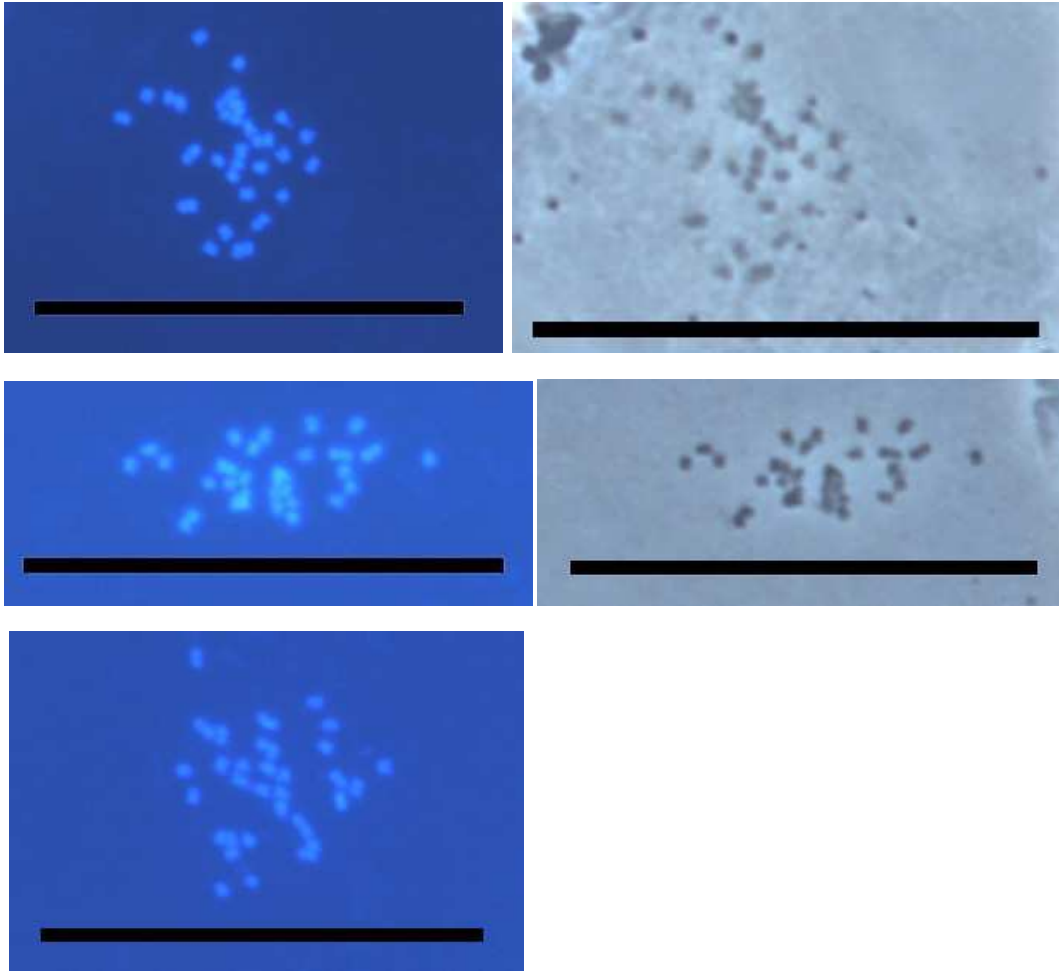
Figure 8-3: Pictures of PIS GUS 12 chromosomes used for karyotyping (bar = 50 μm)
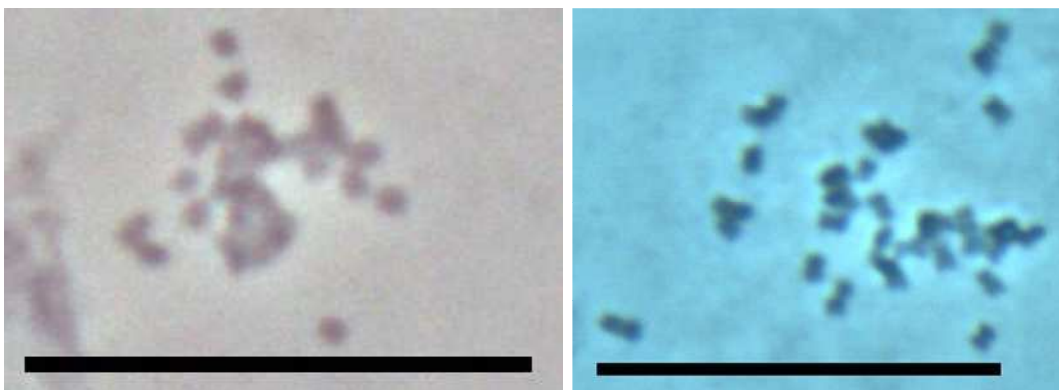
Figure 8-4: Pictures of PIS nt chromosomes used for karyotyping (bar = 20 μm)
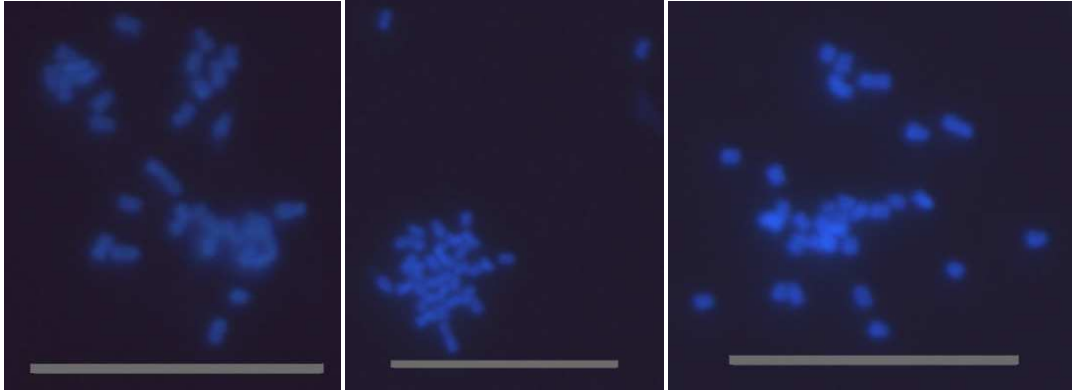
Figure 8-5: Pictures of PIS CAM 31 chromosomes used for karyotyping (bar = 20 μm)
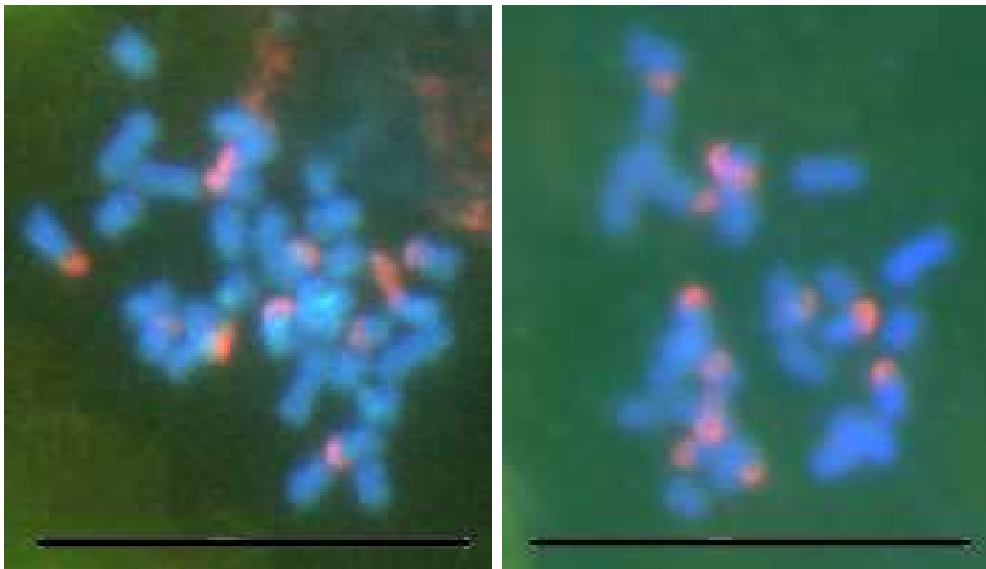
Figure 8-6: FISH of PIS GUS 12 chromosomes with 45S rDNA (red signal) and 5S rDNA (green signal) (bar = 10 μm)
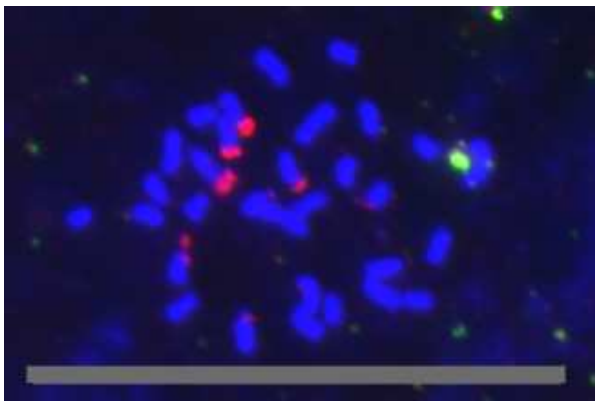
Figure 8-7: FISH of PIS GUS 2 chromosomes with 45S rDNA (red signal) and the T-DNA construct (green signal) (bar = 10 μm)
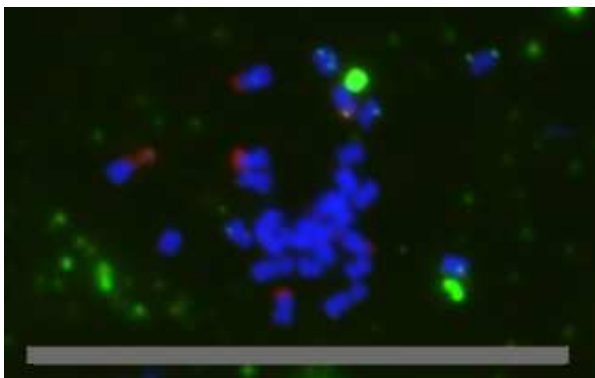
Figure 8-8: FISH of PIS GUS 2 chromosomes with 45S rDNA (red signal) and the T-DNA construct (green signal) (bar = 20 μm)
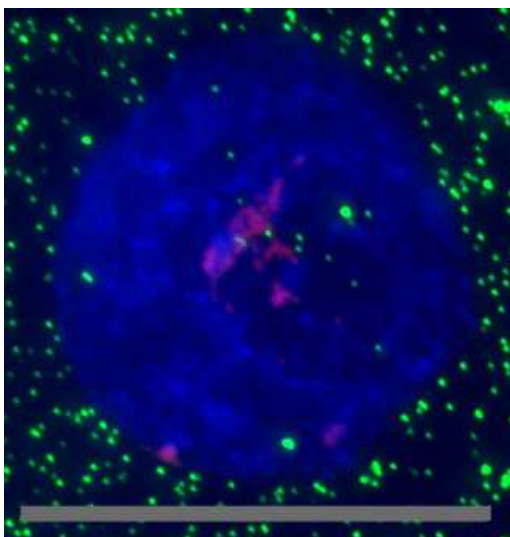
Figure 8-9: Interphase nucleus of PIS GUS 2 hybridized with 45S rDNA (red signal) and T-DNA construct (green signal) (bar = 10 μm)
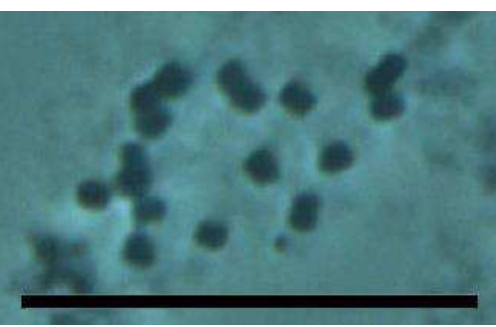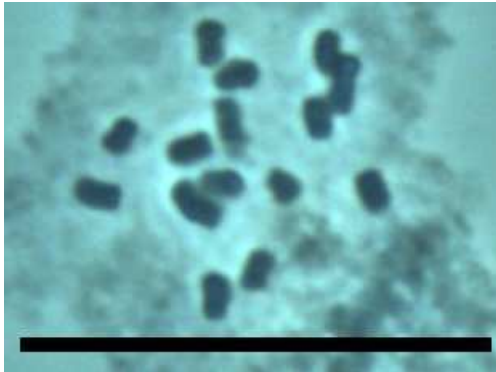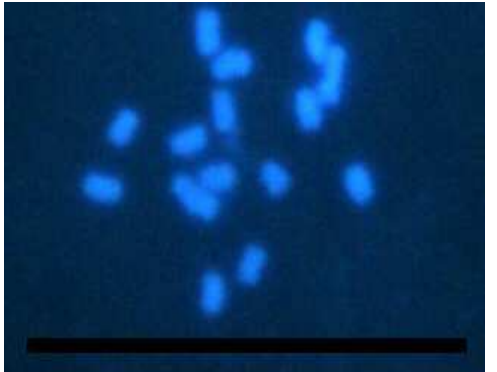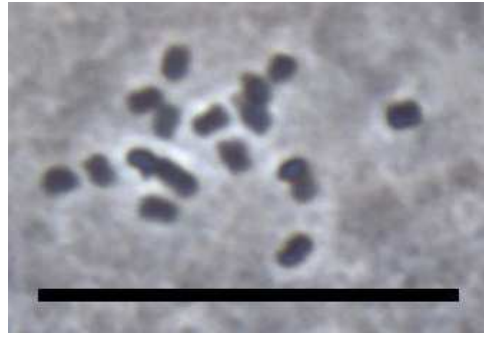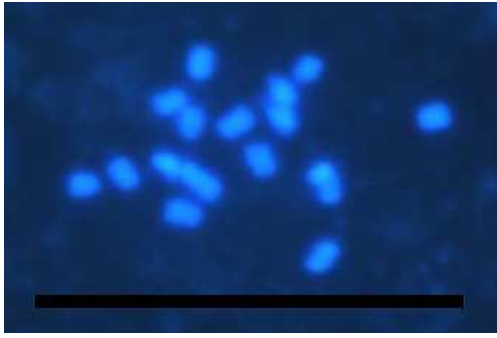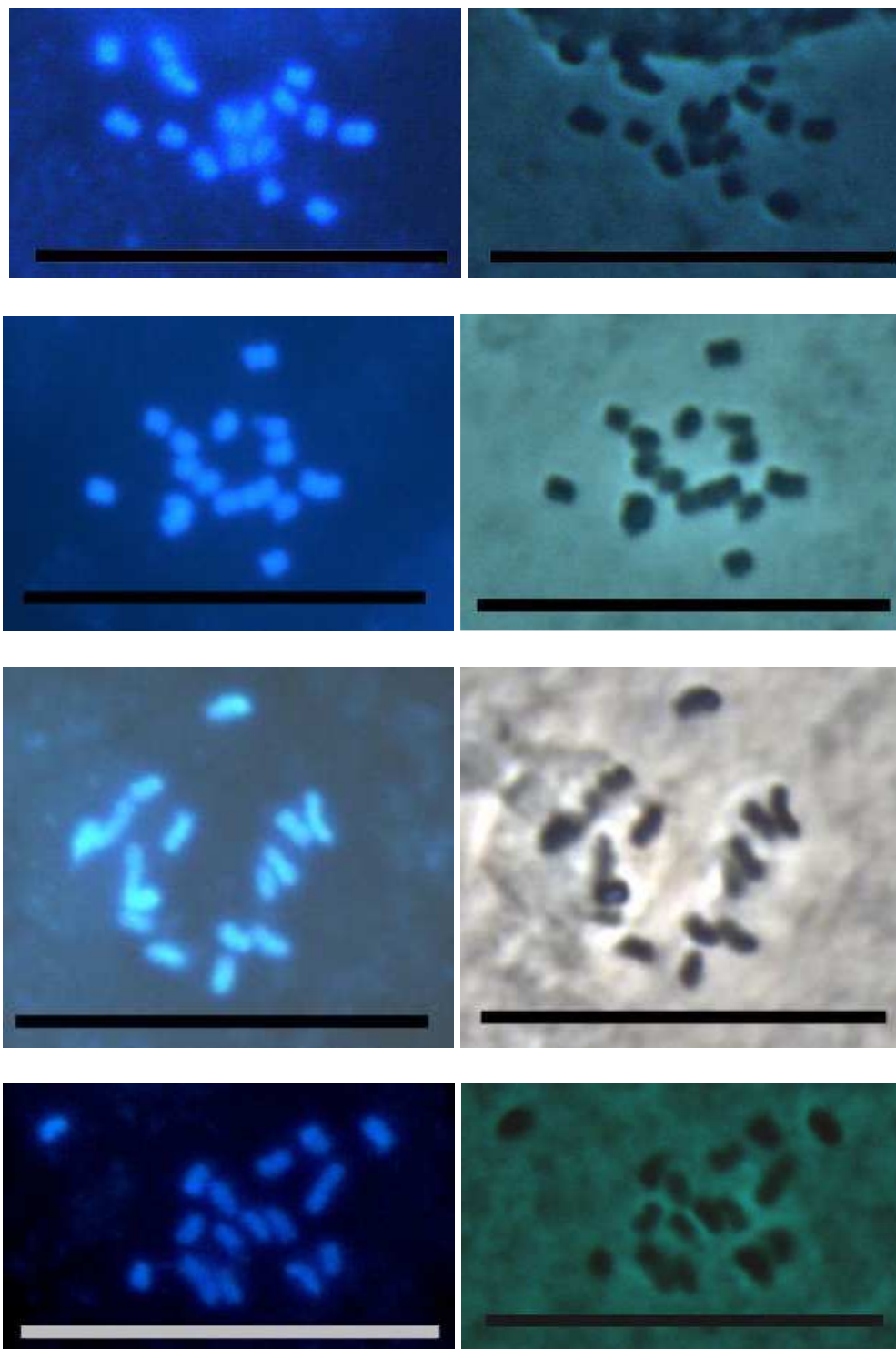
Figure 8-10: Fluorescence (left) and phasecontrast (right) pictures of DAPI stained PAR nt chromosomes (bar = 20 μm)
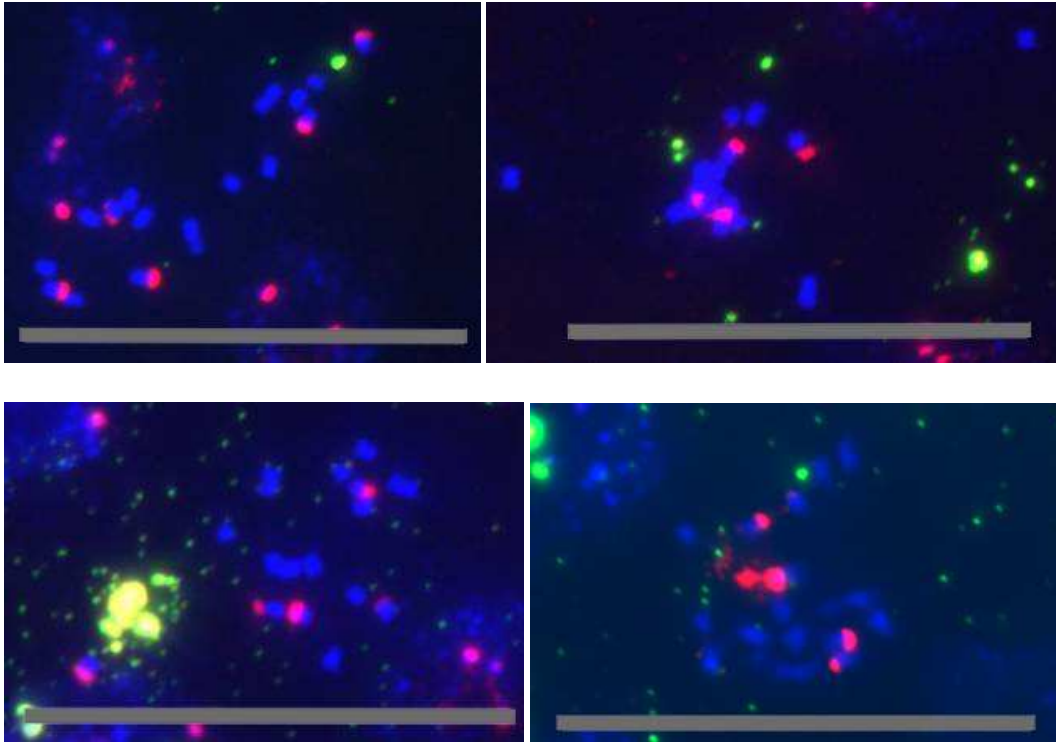
Figure 8-11: PAR GUS 40 chromosomes hybridized with 45S rDNA (red signal) and T-DNA construct (green signal) (bar = 20 μm)
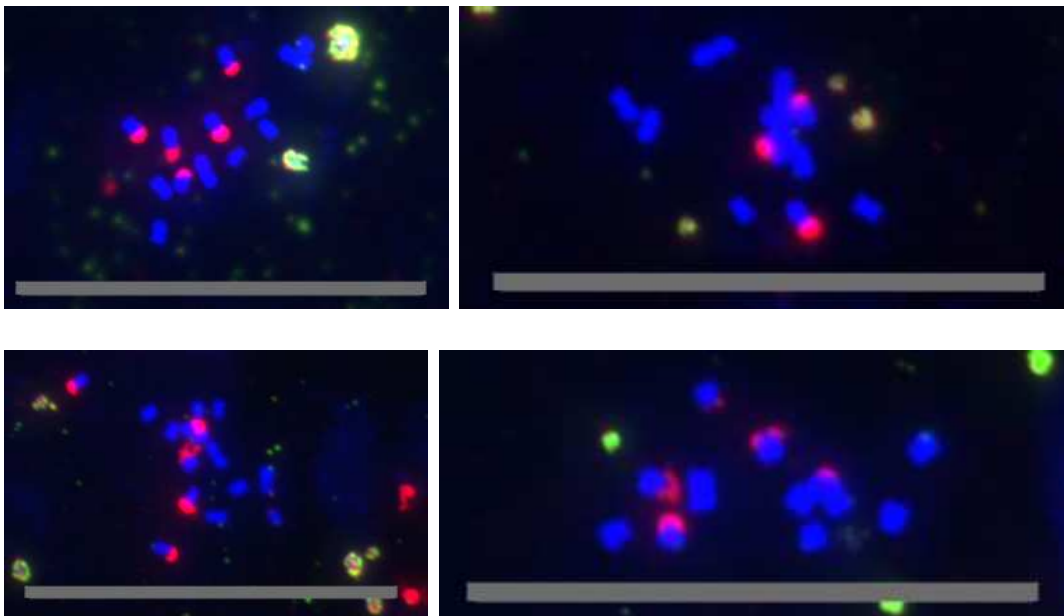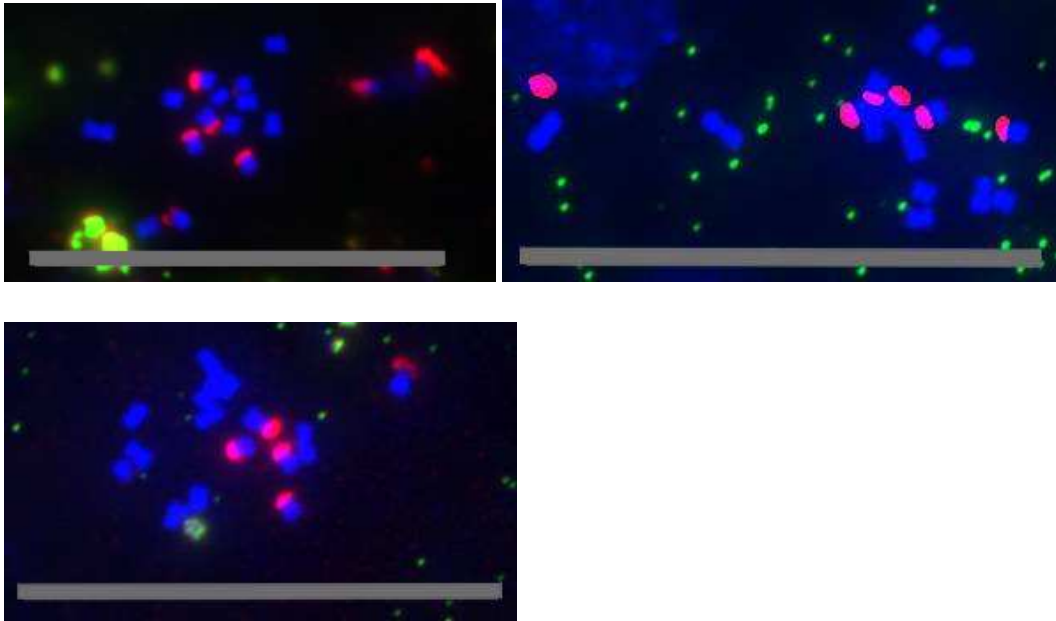
Figure 8-12: PAR GUS 43 chromosomes hybridized with 45S rDNA (red signal) and T-DNA construct (green signal) (bar = 20 μm)
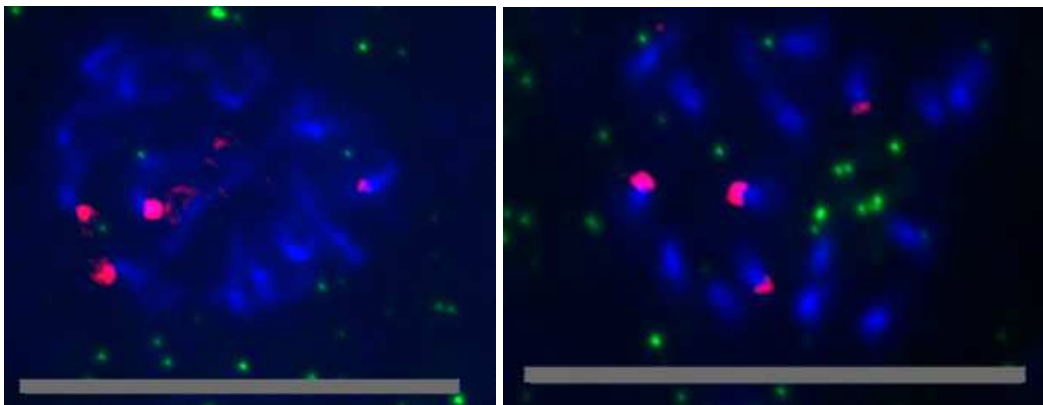

Figure 8-13: Less condensed PAR GUS 43 chromosomes hybridized with 45S rDNA (red signal) and T-DNA construct (green signal) (bar = 20 μm)
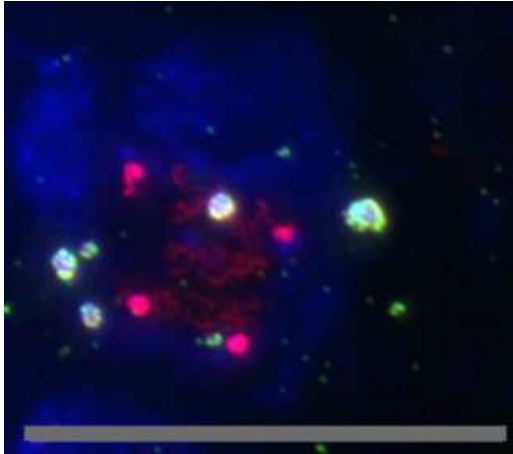
Figure 8-14: Interphase nucleus of PAR GUS 43 hybridized with 45S rDNA (red signal) and T-DNA construct (green signal) (bar = 20 μm)