



130
082
THS



7

7

This is to certify that the
thesis entitled
Localization of T-DNA Inserts of *Petunia*
hybrida Chromosomes by *in situ* Hybridization

presented by

JIMEI WANG

has been accepted towards fulfillment
of the requirements for

Master's degree in Plant Breeding and
Genetics-Horticulture


Major professor

Date 9/10/92

LIBRARY
Michigan State
University

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.

DATE DUE	DATE DUE	DATE DUE
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

MSU is An Affirmative Action/Equal Opportunity Institution

c:\crl\data\due.pm3-p.1

LOCALIZATION OF T-DNA INSERTS ON Petunia hybrida
CHROMOSOMES BY IN SITU HYBRIDIZATION

By

Jimei Wang

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture

1992

ABSTRACT

LOCALIZATION OF T-DNA INSERTS ON Petunia hybrida CHROMOSOMES BY IN SITU HYBRIDIZATION

By

Jimei Wang

The locations and distribution of the T-DNA region in plant chromosomes were detected by in situ hybridization. The T-DNA sequence was introduced into VR hybrid of Petunia hybrida genome by inoculation of leaf explants with Agrobacterium tumefaciens plasmid pMON200. The genes for kanamycin resistance and nopaline synthase are carried in T-DNA region. Putative transformed shoots were selected by their ability to grow in the presence of kanamycin. Transformed plants were confirmed by leaf-disc assay, nopaline synthase activity and slot blot assay.

Ten transgenic plants were used to determine the sites of T-DNA fragment in the host chromosomes. Nine plants showed one location of T-DNA sequence on chromosomes. One plant contained two T-DNA inserts in different chromosomes. The results indicated that T-DNA was integrated into different locations and distributed randomly among chromosomes in independent transformants. Each chromosome could be as a target for T-DNA insertion.

DEDICATION

To my parents
my husband
and
my daughter

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Kenneth Sink for his guidance and support throughout this research program. I would also like to thank Dr. Joanne Whallon for her guidance in the in situ hybridization and microscopic studies.

Appreciation also goes to the members of my guidance committee: Drs. Lowell Ewart and James Hancock for their direction and assistance during this research.

TABLE OF CONTENTS

	Page
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii

LITERATURE REVIEW

Introduction.....	1
Biology of <u>Agrobacterium</u> -mediated transformation.....	1
T-DNA as selectable markers.....	4
T-DNA as a selectable marker in protoplast fusion.....	7
Site of integration of T-DNA.....	8
T-DNA used as an insertion mutagen.....	13
<u>In situ</u> hybridization.....	14
T-DNA in use of plant breeding.....	17
Literature Cited.....	19

LOCALIZATION OF T-DNA INSERTS ON Petunia hybrida CHROMOSOMES BY IN SITU HYBRIDIZATION

Introduction.....	28
Materials and Methods.....	30
<u>Petunia</u> Karyotype.....	30
Transformation and Confirmation.....	31
<u>In Situ</u> Hybridization.....	32
Results and Discussion.....	34
Literature Cited.....	58
APPENDIX.....	62

LIST OF TABLES

Table	Page
1. Verification of kanamycin resistance, nopaline production, slot blot and T-DNA locations on chromosomes in transgenic VR hybrid <u>P. hybrida</u>	45

APPENDIX

I. Assay results of <u>P. hybrida</u> transformants for nopaline production, response to kanamycin and slot blot test.....	62
--	----

LIST OF FIGURES

Figure	Page
1. The karyotype of <u>P. hybrida</u>	46
2. Leaf explant transformation of <u>Petunia</u> VR hybrid co-cultivated with pMON200.....	47
3. Leaf explants from untransformed and transgenic <u>Petunia</u> plants on MS medium containing 300 ug/ml kanamycin.....	48
4. Transgenic <u>Petunia</u> plant 38 rooted in MS containing 300 ug/ml kanamycin.....	49
5. Nopaline synthase assay on non-transformed and transformed leaf tissue.....	50
6. Slot-blot analysis of <u>Petunia</u> transformants.....	51
7. <u>In situ</u> hybridization of T-DNA probe to metaphase chromosomes prepared from each transgenic <u>Petunia</u> plant.....	52
8. <u>In situ</u> hybridization with a T-DNA probe in transformed <u>P. hybrida</u>	55
9. Localizations of T-DNA inserts by <u>in situ</u> hybridization indicating on the karyogram of <u>Petunia</u> in 10 independent transgenic plants. The T-DNA locations labelled with arrows. Arabic numbers indicated plants 1-10.....	56
10. <u>In situ</u> hybridization with a ribosomal DNA probe in non-transformed <u>P. hybrida</u>	57

LITERATURE REVIEW

Plant cell and molecular genetic technologies provide opportunities to supplement traditional plant breeding. Foremost among these biotechnologies is gene isolation and subsequent transformation into crop species for improvement purposes (Perani et al. 1986; Gasswe et al. 1989). The transfer of foreign DNA into plants can be achieved in a variety of ways (Potrykus 1991). One prominent way is to engineer the T-DNA, transferred DNA, of Agrobacterium tumefaciens (Paul et al. 1988). The natural transformation capability of A. tumefaciens enables this organism to be used extensively as a vector of foreign genes into plants (Gheysen et al. 1985).

Biology of Agrobacterium-mediated transformation

A. tumefaciens infection causes tumorous plant growths, commonly called crown galls, by introducing T-DNA into the plant cells at a wound site. The ability of A. tumefaciens to transform plants is determined by the presence of two regions of the Ti, tumor-inducing, plasmid. These regions are the T-DNA and the virulence (vir) ones. The T-DNA has flanking sequences, each 25 bp long, termed the left border (LB) and right border (RB) that are essential for the transfer to occur. These borders are involved in excision

of the T-DNA fragment (Chilton et al. 1977; Koukolikova-Nicola et al. 1987). When Agrobacterium infects a plant, an interaction between the bacterium and the host plant cell stimulates excision of the T-DNA sequence. Some, but not all, steps in this process are known. Transfer begins with the nicks in the two borders producing the T-DNA molecule. Following activation of the vir genes, the T-DNA molecule is excised from the plasmid DNA and transferred into the nucleus of a plant cell where it eventually becomes integrated into one or more host plant chromosome(s) (Kerr et al. 1977; Matsumoto et al. 1990; Watson et al. 1992).

The vir genes, which consist of six loci (vir A, B, G, C, D and E), are required for transferring the T-DNA into plant cells (Klee et al. 1982; Stachel et al. 1986). They produce trans-acting proteins that are essential for plant cell transformation. These proteins must function within the agrobacterial cells for the T-DNA to be excised from the plasmid DNA (Zambryski et al. 1988; Watson et al. 1992). Vir D1/D2 endonucleases recognize the T-DNA border sequences (Albright et al. 1987; Wang et al. 1987) that generate the processed T-DNA molecules (Yanofsky et al. 1986; Yamamoto et al. 1987). Some proteins such as those encoded by Vir D2 and Vir E2 may also function later on in the plant for the conditioning of the plant cells during infection and for subsequent transfer of the T-DNA (Perani et al. 1986; Zambryski et al. 1988; Hohn et al. 1989). In

addition, another set of genes are indirectly involved in transformation (Watson et al. 1992). These genes are carried on the Agrobacterium chromosome and are responsible for proteins that bind the bacteria cells to the plant cells.

Native T-DNA also contains genes referred to as oncogenes which are responsible for tumor induction (Caplan et al. 1983; Perani et al. 1986). The oncogenes code for enzymes that lead to the production of phytohormones. Three of these genes are directly responsible for tumor formation (Caplan et al. 1983; Watson et al. 1992). Genes 1 and 2 code for enzymes that cause the production of an auxin. Gene 4 codes for an enzyme that leads to the production of cytokinin. Overproduction of these endogenous hormones results in tumorous growth of plant cells (Parani et al. 1986). Expression of the T-DNA genes in plant cells has been shown to be responsible for the observed hormone-independent cell growth (Lichtenstein et al. 1987).

The T-DNA also carries genes that code for the synthesis of unusual amino acids called opines. Depending on the opines that are synthesized, A. tumefaciens strains are classified as nopaline or octopine. The strains contain either the nopaline synthase gene or octopine synthase gene, respectively, in different types of Ti plasmids (Greve et al. 1984; Nester et al. 1984; Rogers et al. 1988). It has been demonstrated that the tumorous state is

characterized by the presence of an Agrobacterium plasmid fragment, T-DNA, which is transcribed into RNA. This makes it likely that plant cells are transformed by plasmid DNA, and are transferred during tumor induction. The enzymes mentioned above are encoded by one of the plasmid genes. Therefore, the presence of these enzymes can be considered as a marker for cell transformation. The opines offer a second marker in transformation studies (Otten et al. 1978).

A major breakthrough in the development of Ti plasmid vectors was to "disarm them" by deleting the oncogenes in the T-DNA sequence that elicit the typical crown gall tumors (Rogers et al. 1986; Misra 1990). This change in T-DNA sequences allowed shoot regeneration from transformed cells. Furthermore, the insertion of selectable marker genes, such as antibiotic resistance, aids in the selection of transformed tissues and in the confirmation of regenerated plants (Barton et al. 1983; Fraley et al. 1986; Zambryski et al. 1983). The selectable marker and the engineered genes of a disarmed T-DNA region of the Ti plasmid can insert and become stably integrated into the host nuclear DNA (Hooykaas et al. 1984; Horsch et al. 1988; Watson et al. 1988). Thus, various plant species have been transformed by disarmed vector strains of A. tumefaciens (De Block 1988; Ledger et al. 1991; Sitbon et al. 1991).

T-DNA as selectable markers

A very important factor in the development of T-DNA-based vectors is the availability of selectable markers which impose antibiotic resistance (Hauptmann et al. 1988; Misra 1990). Such genes give high levels of resistance in transformed cells to enable their selection from among wild-type cells. A variety of chimeric genes that express bacterial coding sequences under control of plant promoters have been used as selectable markers (Reynaerts et al. 1988). For example, the neomycin phosphotransferase gene (NPTII) confers resistance to kanamycin (Horsch et al. 1985). Other markers include: hygromycin phosphotransferase (HPT) giving resistance to hygromycin (Van Den Elzen et al. 1985), the bleomycin resistance gene confers resistance to bleomycin (Hille et al. 1986), the dihydrofolate reductase gene (DHFR) confers methotrexate resistance (Eichholtz et al. 1987), and the chloramphenicol acetyltransferase gene (CAT) confers resistance to chloramphenicol (Komari et al. 1990). These markers are included within the 25-bp repeats of the T-DNA, so that they too are transferred and integrated into the plant cells (Watson et al. 1992). The marker probably used most extensively is the NPTII gene (Bevan 1984; Fraley et al. 1983; Rogers et al. 1988). Thus, transformed plant cells carrying the NPTII gene are kanamycin resistant such that they grow in the presence of kanamycin (Klee et al. 1987; Misra 1990). Many engineered

co-integrated vectors also carry the nopaline synthase gene (NOS), which provides a second marker for confirming transformed cells and plants (Bevan et al. 1983; Horsch et al. 1985).

The chimeric NPTII gene has been very useful as a selectable marker in transformation of different plant species, including several Solanaceae such as Nicotiana spp. (Klee et al 1987), Petunia (Horsch et al. 1985; Shah et al. 1986), tomato (McCormick et al. 1986), potato (Shahin et al. 1986; Stiekema et al. 1988), and eggplant (Guri et al. 1988). Numerous other dicot plants including soybean, Brassica napus cv., and Arabidopsis have been transformed to date (Misra 1990; Potrykus et al. 1991).

Kanamycin resistance has been used to optimize different transformation systems: co-cultivation of cultured plant cells with A. tumefaciens (Marton et al. 1979; De Black et al. 1984), infection of leaf discs (Horsch et al. 1985), callus transformation (An 1985), direct gene transfer to protoplasts (Paszkowski et al. 1984; Saul et al. 1987), and symmetric and asymmetric protoplast fusions (Toki et al. 1990; Bates et al. 1987). Kanamycin resistance has provided a stringent selection means both at the cell and plant levels (Herrera-Estrella et al. 1983a). The production of transgenic plants can be divided into the following main steps: introduction of genes into modified A. tumefaciens strains; transformation of T-DNA into plant cells or

tissues; selection and regeneration of transformants; and verification and analyses of gene expression in transformed plants and the offspring (Rogers et al. 1986; Power et al. 1986). Due to the efficiency of this selection procedure, stable transformants can be routinely obtained in many dicot species (Hauptmann et al 1988).

T-DNA as a selectable marker in protoplast fusion

The T-DNA sequences can also be used as a marker for selecting somatic hybrids. For example, the general utility of T-DNA has already been demonstrated in both symmetric and asymmetric nuclear genome transfers via protoplast fusion (Bates et al. 1987; Ichikawa et al. 1990). The transfer of the T-DNA of A. tumefaciens, previously introduced into plant cells, via protoplast fusion from T-DNA donor Nicotiana paniculata; to recipient N. tabacum has been reported (Muller-Gensert et al. 1987). The hybrid cell lines survived the kanamycin selection treatment by exhibiting sustained growth. Dot-blot analysis showed that besides the T-DNA other nuclear genomic DNA of the donor species had also been transferred in various amounts. Moreover, asymmetric somatic hybridization in Nicotiana by fusion of irradiated and T-DNA inserted donor protoplasts of N. plumbaginifolia with N. tabacum was reported by Bates et al. (1987). Selection on kanamycin medium allowed the recovery of asymmetric somatic hybrids retaining the

chromosome, or chromosome fragment(s), with the gene encoding kanamycin resistance. A single chromosome can also be transferred by donor-recipient fusion as long as the donor contains a nuclear encoded marker gene (Dudits et al. 1980; Gupta et al. 1984; Bates et al. 1987). T-DNA was detected in asymmetric somatic hybrids between Brassica nigra and B. napus, and cytological analysis showed the addition of donor chromosomes (Sacristan et al. 1989). The T-DNA marker may also be used in the transfer of clustered nuclear genes in the form of isolated chromosomes (Dudits et al. 1985).

Since the T-DNA becomes a marker for the tagged genes or chromosomes, a system of selecting those genes and chromosomes is available for somatic hybridization. Such a system is possible by the use of T-DNA in a dual role as a marker for plant chromosome or gene(s) of interest and as cell level selectable trait (Herrera-Estrella et al. 1983b; Misra 1990). Therefore, it might be possible to use the T-DNA as a chromosome and/or gene marker in transformation experiments with crop plants.

Site of integration of T-DNA

It is of practical and theoretical value to know whether the T-DNA integrates only at a limited number of specific sites or random throughout the host genome. It has been reported that the T-DNA insertions integrate randomly into

the plant genome (Wallroth et al. 1986). Wallroth et al. confirmed the presence of the expected T-DNA fragments in six transformed Petunia plants. Southern hybridization and genetic analyses showed that the size of the plant/T-DNA junction fragments are different, thus, suggesting the integrations occurred in different sites (Wallroth et al. 1986). Agrobacterium-mediated T-DNA insertions in the Lycopersicon, tomato, genome were at random locations based on the genetic analysis (Chyi et al. 1986). Based on genetic analysis, 10 independent T-DNA insertions were located on 10 different chromosome loci. Herman et al. (1990) also found that the T-DNA sequences integrated into plant chromosomes at random positions. They measured the frequency with which a promoterless reporter gene (NPTII) is activated after T-DNA insertion into the Nicotiana tabacum SR1 genome. When adjacent to the left or right T-DNA border sequences, about 35% of the transformants expressed the marker gene. Therefore, T-DNA integration is random in respect to sequence specificity. Another evidence for random insertion is that the integration of foreign genes in the transgenic plant genome commonly occurs at random sites. For example, Ambros et al. (1986b) detected the locations of the T-DNA of the Ri plasmid in the host chromosomes of Crepis by in situ hybridization. The results indicated that the T-DNA region of A. rhizogenes Ri plasmid also integrated randomly in the four chromosomes of Crepis.

On the other hand, Paszkowski et al. (1988) presented evidence for directed T-DNA integration into a predicted location in the host plant genome. They constructed five strains of transgenic tobacco plants containing chromosomal copies of a non-functional, partially deleted APH(3')II gene. Protoplasts of these plants were used for DNA-mediated direct transformation with the missing part of the gene in order to form an active gene. Molecular and genetic data confirmed the integration of foreign DNA directed to specific loci through homologous recombination.

The integration of previously missing sequences resulted in the formation of an active gene in the host chromosome. This suggested the possibility of integration of the T-DNA into an homologous location in the plant genome. The integration of Agrobacterium T-DNA into a plant chromosome was also investigated in tobacco tumor cell lines (Matsumoto et al. 1990). Nucleotide sequence analysis showed significant homology between the region adjacent to the integration target site and both external regions of the T-DNA breakpoints. In addition, a short stretch of plant DNA near the integration site was deleted. This deletion seems to have been promoted by homologous recombination. Analyses of T-DNA/plant DNA junctions have shown that in some cases plant sequences on both sides of the integration site are homologous to the plasmid sequences outside of the T-DNA breakpoints. These results

indicated that the T-DNA integrated by a mechanism similar to that for homologous recombination. Therefore, sequence homology between the incoming T-DNA and the plant chromosomal DNA may have an important function in T-DNA integration. The homology may promote close association of both termini of T-DNA molecule on a target sequence; then T-DNA may in some case be integrated by a mechanism at least in part analogous to homologous recombination (Matsumoto et al. 1990).

Genetic analysis has shown the deletion of the right border abolishes tumorigenicity. This result indicates the polarity of T-DNA transfer (Wang et al. 1984; Zambryski 1988). IN addition, Mouras and Negroid (1989) located the T-DNA insertion site in a tobacco crown-gall line by in situ hybridization: the hybridization signal was found on a small metacentric chromosome. Chromosome and molecular analyses showed there were specific chromosome rearrangements in the transformed cells. The chromosome may translocate onto other chromosomes to make marker chromosomes through specific rearrangements. The insertion of T-DNA is random within the plant genome and the activity of the introduced genes may be affected by adjacent plant DNA (Hobbs et al. 1990). The expression of the T-DNA introduced genes was studied in ten tobacco transformants. They found the T-DNA was apparently integrated at different sites in the plant genome. The insertion site of the

foreign DNA (Km^r) in two independent transformed tobacco plants was localized on different homologous chromosomal pairs by in situ hybridization (Mouras et al. 1987).

When the genes of interest are introduced into the plant genome in relatively high copy number, in order to have a significant effect, this presents no technical problem. Usually, multiple copies of T-DNA integrate at a single random site in the plant chromosome (Watson et al. 1992). The T-DNA can integrate in the form of tandem repeats (Watson et al. 1988). Transformed tobacco plants containing 3-5 multiple copies of the T-DNA sequence were analyzed at the molecular level (Paszkowski et al. 1984). The genetic (Potrykus et al. 1985) and in situ hybridization data (Mouras et al 1987) of the same plants confirmed that the T-DNA was integrated at one chromosomal location in multiple copies. Combined molecular and genetic analysis of transformed SR₁ tobacco plants have shown that the integration of the foreign DNA usually takes place at one physical location within the genome as a multiple of both functional and unfunctional copies (Negrutiu et al. 1987). One transgenic Petunia plant was reported to have two possible tandemly repeated T-DNAs (Fraley et al. 1985).

On the other hand, Wallroth et al. (1986) reported that another transformant Petunia possibly had two T-DNA inserts. The integrations were in different sites of the Petunia genome. In transformed Crepis plants, Southern

hybridization showed that each root line was the result of one or more independent Ri T-DNA insertion events (Ambros et al. 1986b). The results of in situ hybridization indicated that the transformed root line contained one or two A. rhizogenes Ri T-DNA insert(s) on the host chromosomes (Ambros et al. 1986b).

T-DNA used as an insertion mutagen

The T-DNA is used as a gene/chromosome marker since the T-DNA inserts can integrate into the host genome. T-DNA-mediated gene tagging in plants was investigated on T-DNA-linked mutation (Koncz et al. 1989). T-DNA is proving to be a very efficient insertion mutagen that causes inactivation of plant genes. The potential of T-DNA as an insertional mutagen was studied in Arabidopsis (Lijsebettens et al. 1991). Arabidopsis lines transformed with T-DNA vector were generated by selection on kanamycin medium. One mutation, named pf1, was found to affect mainly morphology of the first seedling leaves and was mapped to chromosome 1. The detection of recombination between pf1 and T-DNA, and analysis of genetic distance suggested that the pf1 mutation was induced by insertion of the T-DNA.

Insertional mutagenesis may be used to tag genes (Marks et al. 1989). Marks et al. found the mutation causing via T-DNA insertion is tightly linked to a T-DNA insert. Complementation analysis with genetically characterized

trichome mutant revealed that the new mutation in an allele of the GL1 locus. Thus, the T-DNA tagged with the GL1 gene in Arabidopsis thaliana. Those T-DNA-linked mutations have been selected and isolated. Furthermore, the use of T-DNA as gene/chromosome marker was demonstrated in another experiment. Transgenic tobacco plants carried copies of a partial, non-functional kanamycin-resistance gene in the nuclear DNA (Mouras et al. 1987). Such plants were used as recipients for DNA molecules containing the missing part of the gene. Molecular and genetic data confirmed that the kanamycin resistant gene integrated into the protein coding region, and resulted in the formation of an active gene. The selection for kanamycin resistance conferred by the marker gene made the detection of target gene possible (Paszkowski et al. 1988).

In situ hybridization

When T-DNA inserts are used for tagging genes of interest, it is necessary to establish the location of the T-DNA inserts among the chromosomes of a particular genome. In situ hybridization provides the means to detect and localize the position of specific gene sequences and makes it possible to visualize DNA fragments on metaphase chromosomes (Huang et al. 1989). Since the first in situ hybridization of nucleic acid probes was used by Gall et al. (1969) to detect an RNA-DNA hybrid molecule on

chromosomes, this technique has undergone continuous refinement. Some refinements have enhanced the efficiency and sensitivity of hybrid detection (Shen et al. 1987). Thus, it is possible to localize single gene sequences or low copy numbers. The use of biotin-labeled probes produced higher resolution and lower background interference than did conventional in situ hybridization using isotope labeling and autoradiography (Manuelidis et al. 1982; Singer et al. 1982). The technique can indicate exactly which chromosome(s) and the particular site(s) of the chromosome(s) where specific DNA sequences are located (Simpson et al. 1990).

The repetitive DNA sequences such as tandem repeats of the pea 5S rDNA in pea (Simpson et al. 1990), 18S.26S rDNA gene repeat unit in wheat (Mulai et al. 1991), and a TGRI satellite repeat in tomato (Lapitan et al. 1989) have been detected. Low copy number and single-copy endogenous plant genes have also been detected by in situ hybridization. A 6.6 kb of the chalcone synthase gene was observed on chromosomes in parsley by in situ hybridization (Huang et al. 1988). A legumin gene DNA fragment of 13.5 kb was localized in pea (Simpson et al. 1988). To test the sensitivity of the method, a cloned 10.8 kb genomic DNA fragment encoding the waxy locus mRNA and the same DNA fragment with insert sizes of 6.6, 4.7, 3.5, 2.3, 1.9 and 0.8 kb were used for in situ hybridization on maize

pachytene chromosomes. The data showed that single copy sequences longer than 1.9 kb could be detected at the correct position (Shen et al. 1987). A 900 bp sequence from a cDNA clone of the rye endosperm-storage-protein gene Sec-1 was visualized on the chromosome in rye (Gustafson et al. 1990).

The T-DNA sequences have also been localized in the host genome by in situ hybridization. A 17 kb T-DNA unique sequence was detected on chromosomes of transformed Crepis capillaris by Ambrose et al. (1986a). The results indicated that T-DNA was present in a different location in each root line, and that each chromosome had been a target for T-DNA insertion at least once (Ambrose et al. 1986b). A 5.4 kb kanamycin resistance gene (Km'), with 3-5 copies, was localized on different homologous chromosome pairs in transgenic tobacco (Mouras et al. 1987). And, a 6 kb T-DNA was detected in a tobacco crown-gall line by Mouras and Negroid (1989). The hybridization signal was found on a small metacentric chromosome, which could translocate onto other chromosomes. The smallest target T-DNA sequence detected to date was a 1-2 kb fragment (Mouras et al. 1989).

T-DNA in use of plant breeding

In spite of rapid developments in genetic engineering of plants which have allowed many genes to be cloned and

successfully introduced into transgenic plants, most genes of agronomic importance have not yet been isolated and characterized. The genes that have been successfully engineered to date are single-copy and well-defined, whereas, agronomically important traits such as yield, disease resistance and flower color may be controlled by polygenes. Since the biochemical products of these genes are not known, it will be some time before their isolation for gene transfer is possible (Le et al. 1991). An alternative method is to tag the gene(s) of interest. Thus, the T-DNA inserts may be used as a selective marker on the chromosome(s) mapping the gene of interest. Subsequently, the tagged gene, chromosome or chromosome fragment may be transferred by protoplast fusion. This approach provides an efficient means for chromosome engineering of desired traits in crops (Dudits et al. 1985). The system is potentially applicable to crop species where Agrobacterium based transformation and plant regeneration are available.

Many transgenic plants including Petunia have been produced by the leaf-disc transformation method (Horsch et al. 1985; Rogers et al. 1988). The fate of the foreign genes in such transformants has been studied. Since regenerates are often fertile and transmit the newly inserted gene in a Mendelian fashion to the progeny (Horsch et al. 1984; Rogers et al. 1986; Uchimiya et al. 1986), transgenic plants can be readily used in genetic studies.

The objective of the current study was to use the in situ hybridization technique to directly visualize the location of T-DNA on the host chromosomes of transformed P. hybrida plants. The distribution of T-DNAs among the chromosomes was an necessary step for their further use in chromosome tagging in cell fusion. P. hybrida was chosen as the material since it is one of the most-studied dicotyledons in terms of both genetics and cytology and as an experimental subject to investigate molecular aspects of biological phenomena (Cornu et al. 1983). Hence, it has become an ideal model system for molecular biological studies. It has a diploid chromosome number of $2n=2x=14$. Flower color genetics has been well established and some of these genes are mapped (Cornu 1984). Moreover, plant transformation and protoplast regeneration have been successful (Horsch et al. 1985; Frearson et al. 1973). But the distribution of T-DNA in chromosomes by in situ hybridization has not been reported. Analysis of several different transformed plants should allow a valid comparison of independent T-DNA insertion events. In addition, the growing root tips are an abundant source of metaphase chromosomes, a feature which is essential for in situ hybridization.

LITERATURE CITED

- Albright L, Yanofsky M, Leroux B, Ma D and Nester E. 1987. Processing of the T-DNA of Agrobacterium tumefaciens generates borders and linear, single-stranded T-DNA. J Bacteriol 169:1046-1055.
- Ambros P, Matzke M and Matzke A. 1986a. Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by in situ hybridization. Chromosoma 94:11-18.
- Ambros P, Matzke A and Matzke M. 1986b. Localization of A. rhizogenes T-DNA in plant chromosome by in situ hybridization. EMBO 5:2073-2077.
- An G. 1985. High efficiency transformation of cultured tobacco cells. Plant Physiol. 79:568-570.
- Barton K, Binns A, Matzke A and Chilton M. 1983. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA and transmission of T-DNA to R1 progeny. Cell 32:1033-1043
- Bates G, Hasenkampf C, Contolini C and Piastuch W. 1987. Asymmetric hybridization in Nicotiana by fusion of irradiated protoplasts. Theor. Appl. Genet. 74:718-726.
- Beven M, Barnes W and Chilton M. 1983. Structure and transcription of the nopaline synthase gene region of T-DNA. Nuc. Acids Res. 11:369-389.
- Caplan A, Herrera-Estrella L, Inze D, Van Haute E, Van Montagu M, Schell J and Zambryski P. 1983. Introduction of genetic material into plant cells. Science 222:815-821.
- Chilton M, Drummond M, Merlo D, Sciaky D, Montoya A, Gordon M and Nester E. 1977. Stable incorporation of plasmid DNA into higher plant cells: The molecular basis of crown gall tumorigenesis. Cell 11:263-271.
- Chyi Y-S, Jorgensen R, Goldstein D, Tanksley S and Loaiza-Figueroa F. 1986. Locations and stability of Agrobacterium mediated T-DNA insertions in the Lycopersion genome. Molec. Gen. Genet. 204:46-49.
- Cornu A and Maizonnier D. 1983. The genetics of Petunia. Plant Breeding Reviews 2:11-57.
- Cornu A. 1984. Genetics. In: Sink K. (Ed) Petunia 9:34-48. Springer-verlag, Berlin.

De Black M, Herrera-Estrella L, Van Montagu M, Schell J and Zambryski P. 1984. Expression of foreign genes in regenerated plants and in their progeny. EMBO J 3:1681-1689.

De Block M. 1988. Genotype-independent leaf disc transformation of potato using Agrobacterium tumefaciens. Theor. Appl. Genet. 76:767-774.

Dudits D, Fejer O, Hadlaczky G, Koncz C, Lazar G and Horvath G. 1980. Intergeneric gene transfer mediated by plant protoplast fusion. Molec. Gen. Genet. 179:283-288.

Dudits D and Praznoysky T. 1985. Intergeneric gene transfer by protoplast fusion and uptake of isolated chromosomes. In: Zaitlin M, Day P and Hollaender A. (Eds). Biotechnology in Plant Science: relevance to agriculture in the eighties. Academic Press. Orlando. p115-127.

Eichholtz D, Rogers S, Horsch R, Klee H, Hayford M, Hoffmann N, Bradford S, Fink C, Flick J, O'Connell K and Fraley R. 1987. Expression of mouse dihydrofolate reductase gene confers methotrexate resistance in transgenic Petunia plants. Somatic Cell Molec. Gen. 13:67-76.

Fraley R, Rogers S, Horsch R, Sanders P, Flick J Adams S, Bittner M, Brand L, Fink C, Fry J, Galluppi G, Goldberg S Hoffmann N and Woo S. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80:4803-4807.

Fraley R, Rogers S, Horsch R, Eichholtz D, Flick J, Fink C, Hoffmann N and Sanders P. 1985. The SEV system: a new disarmed Ti plasmid vector for plant transformation. Bio/Technology 3:629-635.

Fraley R, Rogers S and Horsch R. 1986. Genetic transformation in high plants. CRC Critical Rev. Plant Sci.4:1-46.

Frearson E, Power J and Cocking E. 1973. The isolation, culture and regeneration of Petunia leaf protoplasts. Dev. Biol. 33:130-137.

Gall J and Pardue M. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. Natl. Acad. Sci. USA 63:378-383.

Gasswe C and Fraley R. 1989. Genetically engineering plants for crop improvement. Science 244:1293-1299.

Gheysen G, Dhaese P, Van Montagu M and Schell J. 1985. B.Hohn and E.S. Dennis, Genetic flux in plants, Advances in

plant gene research. 2:11-17 Springer-Verlag, Wien.

Greve H, Dhaese P, Seuinck J, Lemmers M, Van Montagu M and Schell J. 1983. Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti-plasmid encoded octopine synthase gene. *Molec. Appl. Genet. J.* 1:499-511.

Gupta P, Schieder O and Gupta M. 1984. Intergeneric nuclear gene transfer between somatically and sexually incompatible plants through asymmetric protoplast fusion. *Molec. Gen. Genet.* 197:30-35.

Guri A and Sink C. 1988. *Agrobacterium* transformation of eggplant. *J. Plant Physiol.* 133:52-55.

Gustafson J, Butler E and McIntyre C. 1989. Physical mapping of a low-copy DNA sequence in rye (*Secale cereale* L.). *Proc. Natl. Acad. Sci. USA.* 87:1899-1902.

Hauptmann R, Vasil V, Ozias-Akins P, Tabaeizdeh Z, Rogers S, Fraley R, Horsch R and Vasil I. 1988. Evaluation of selection markers for obtaining stable transformants in the gramineae. *Plant Physiol.* 86:602-606.

Herman L, Jacobs A, Van Montagu M and Depicker A. 1990. Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. *Molec. Gen. Genet.* 224:248-256.

Herrera-Eatrella L, De Block M, Messens E, Hernalsteens J, Van Montagu M and Schell J. 1983a. Chimeric genes as dominant selectable markers in plant cells. *EMBO J.* 2:987-995.

Herrera-Estrella L, Depicker A, Van Montagu M and Schell J. 1983b. Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature.* 303:209-213.

Hille J, Verheggen F, Roelvink P, Franssen H, Kammen A and Zabel P. 1986. Bleomycin resistance: a new dominant selectable marker for plant cell transformation. *Plant Molec. Biol.* 7:171-176.

Hobbs S, Kpodar P and DeLong C. 1990. The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. *Plant Molec. Biol.* 15:851-864.

Hohn B, Koukolikova-Nicola Z, Bakkeren G and Grimsley N. 1989. *Agrobacterium*-mediated gene transfer to monocots and dicots. *Genome* 31:987-993.

- Hooykaas P and Schilperoort R. 1984. The molecular genetics of crown gall tumorigenesis. *Adv. Genet.* 22:209-230.
- Horsch R, Fraley R, Rogers S, Sanders P, Lloyd A and Hoffmann N. 1984. Inheritance of functional foreign genes in plants. *Science* 223:496-498.
- Horsch R, Fry J, Hoffmann N, Eichholtz D, Rogers S and Fraley R. 1985. A simple and general method for transferring gene into plants. *Science* 227:1229-1231.
- Horsch R, Fry J, Hoffmann N, Neidermeyer J, Rogers S and Fraley R. 1988. Leaf disc transformation. *Plant molec. biology. Manual.* 5:1-9.
- Huang P-L, Hahlbrock K and Somssich E. 1988. Detection of a single-copy gene on plant chromosomes by in situ hybridization. *Molec. Gen. Genet.* 211:143-147.
- Huang P-L, Hahlbrock K and Somssich E. 1989. Chromosomal localization of parsley 4-coumarate: CoA ligase genes by in situ hybridization with a complementary DNA. *Plant Cell Reports* 8:59-62.
- Ichikawa H and Imamura J. 1990. A highly efficient selection method for somatic hybrids which used an introduced dominant selectable marker combined with iodoacetamide treatment. *Plant Sci.* 67:227-235.
- Kerr A, Manigault P and Tampe J. 1977. Transfer of virulence in vivo and in vitro in Agrobacterium. *Nature* 265:560-561.
- Klee H, Gordon M and Nester E. 1982. Complementation analysis of Agrobacterium tumefaciens Ti plasmid mutations affecting oncogenicity. *J. Bacteriol.* 150:327-331.
- Klee H, Horsch R and Rogers S. 1987. Agrobacterium-mediated plant transformation and its further application to plant biology. *Ann. Rev. Plant Physiol.* 38:467-486.
- Komari T, Saito Y, Nikaido F and Kumashiro T. 1990. Efficient selection of somatic hybrids in Nicotiana tabacum L. using a combination of drug-resistance markers introduced by transformation. *Theor. Appl. Genet.* 77:547-552.
- Koncz C, Martini N, Mayerhofer R, Koncz-Kalman Z, Korber H, Redei G and Schell J. 1989. High-frequency T-DNA-mediated gene tagging in plants. *Proc. Natl. Sci. USA* 86:8467-8471.
- Koukolikova-Nicola Z, Albright L and Hohn B. 1987. The

- mechanism of T-DNA transfer from Agrobacterium tumefaciens to plant cell. In Hohn T and Schell J. Plant gene research. 4:109-148. Springer, Wien.
- Lapitan N, Ganai M and Tanksley S. 1989. Somatic chromosome karyotype of tomato based on in situ hybridization of the TGR1 satellite repeat. Genome 32:992-998.
- Le H and Armstrong K. 1991. In situ hybridization as a rapid means to assess meiotic pairing and detection of alien DNA transfer in interphase cells of wide crosses involving wheat and rye. Molec. Gen. Genet. 225:33-37.
- Ledger S, Deroles S and Given N. 1991. Regeneration and Agrobacterium-mediated transformation of chrysanthemum. Plant Cell Reports. 10:195-199.
- Lichtenstein C and Fuller S. 1987. Vectors for the genetic engineering of plants. Genetic engineering 6:104-171.
- Lijsebettens M van, Vanderhaeghen R and van Montagu M. 1991. Insertional mutagenesis in Arabidopsis thaliana: isolation of a T-DNA-linked mutation that alters leaf morphology. Theor. Appl. Genet. 81:277-284.
- Manuelidis L, Langer-Safer P and Ward D. 1982. High-resolution mapping of satellite DNA using biotin-labeled DNA probes. J. Cell. Biol. 95:619-625.
- Marks M and Feldmann K. 1989. Trichome development in Arabidopsis thaliana. 1. T-DNA tagging of the GLABROUS1 gene. Plant Cell. 1:1043-1050.
- Marton L, Wullems G, Molendijk L and Schiperoort R. 1979. In vitro transformation of cultured cells from Nicotiana tabacum by Agrobacterium tumefaciens. Nature 227:129-130.
- Matsumoto S, Ito Y, Hosoi T, Takahashi Y and Machida Y. 1990. Integration of Agrobacterium T-DNA into a tobacco chromosome: Possible involvement of DNA homology between T-DNA and plant DNA. Molec. Gen. Genet. 224:309-316.
- McCormick M, Niedermeyer J, Fry J, Baranson A, Horsch R and Fraley R. 1986. Leaf disc transformation of cultivated tomato using A. tumefaciens. Plant cell Rep. 5:81-84.
- Misra, S. 1990. Transformation of Brassica napus L. with a disarmed octopine plasmid of Agrobacterium tumefaciens: Molecular analysis and inheritance of the transformation phenotype. J. Exp. Bot. 41:269-275.

- Mouras A, Saul M, Essad S and Potrykus I. 1987. Localization by in situ hybridization of a low copy chimeric resistance gene introduced into plants by direct gene transfer. *Molec. Gen. Genet.* 207:204-209.
- Mouras A, Negroid I, Orth M and Jacobs M. 1989. From repetitive DNA sequences to single-copy gene mapping in plant chromosomes by in situ hybridization. *Plant Physiol. Biochem.* 27:161-168.
- Mouras A and Negroid I. 1989. Localization of the T-DNA on marker chromosomes in transformed tobacco cells by in situ hybridization. *Theor. Appl. Genet.* 78:715-720.
- Mukai Y, Endo T and Gill B. 1991. Physical mapping of the 18S.28S rRNA multigene family in common wheat: Identification of a new locus. *Chromosoma* 100:71-78.
- Muller-Gensert E and Schieder O. 1987. Interspecific T-DNA transfer through plant protoplast fusion. *Molec. Gen. Genet.* 208:235-241.
- Negroid I, Shillito R, Potrykus I, Biasini G and Sala F. 1987. Hybrid genes in the analysis of transformation condition. *Plant Molec. Biol.* 8:363-373.
- Nester E, Gordon M, Amasino R and Yamada Y. 1984. Crown gall: a molecular and physiological analysis. *Ann. Rev. Plant Physiol.* 35:387-413.
- Otten L and Schiperoort R. 1978. A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta.* 527:497-500.
- Paszkowski J, Shillito R, Saul M, Mandak V, Hohn T, Hohn B and Potrykus I. 1984. Direct gene transfer to plants. *EMBO J* 3:2717-2722.
- Paszkowski J, Baur M, Bogucki A and Potrykus I. 1988. Gene targeting in plants. *EMBO J.* 7:4021-4026.
- Paul J and Hooykaas J. 1988. Agrobacterium molecular genetics. *Plant Molec. Biol. Manual.* 4:1-13.
- Perani L, Radke S, Wilke-Douglas M and Bossert M. 1986. Gene transfer methods for crop improvement: Introduction of foreign DNA into plants. *Physiol. Plant.* 68:566-570.
- Potrykus I, Paszkowski J, Saul M, Petruska J and Shillito R. 1985. Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. *Molec.*

Gen. Genet. 199:169-177.

Potrykus I. 1991. Gene transfer to plants: Assessment of published approaches and results. Ann. Rev. Plant Physiol. 42:205-225.

Power J, Davey M, Freeman J, Mulligan B and Cocking E. 1986. Fusion and transformation of plant protoplasts. Methods in Enzymol. 118:578-594.

Reynaerts A, De Block A, Hernalsteens J and Montagu M. 1988. Selectable and screenable markers. Plant Molec. Bio. Manual. 9:1-16.

Rogers S, Horsch R and Fraley R. 1986. Gene transfer in plants. Methods in Enzymol. 118:627-640.

Rogers S, Klee H, Horsch R and Fraley R. 1988. Use of cointegrating Ti plasmid vectors. Plant Molec. Biol. Manual 2:1-12.

Sacristan M, Gerdemann M and Schieder O. 1989. Incorporation of hygromycin resistance in Brassica nigra and its transfer to B. napus through asymmetric protoplast fusion. Theor. Appl. Genet. 78:194-200.

Saul M, Paszkowski J, Shillito R and Potrykus I. 1987. Methods for direct gene transfer to plant. Plant Physiol. Biochem. 25:361-364.

Shah D, Horsch R, Klee H, Kishore G, Winter J, Turner N, Hironaka C, Sanders P, Gasser S, Aybent N, Siegel S, Rogers S and Fraley R. 1986. Engineering herbicide tolerance in transgenic plants. Science 233:478-481.

Shahin E and Simpson R. 1986. Gene transfer system. HortScience 21:1199-1201.

Shen D-L, Wang Z-F and Wu M. 1987. Gene mapping on maize pachytene chromosomes by in situ hybridization. Chromosoma 95:311-314.

Simpson P, Newman M-A and Davies R. 1988. Detection of legumin gene DNA sequences in pea by in situ hybridization. Chromosoma 96:454-458.

Simpson P, Newman M-A, Davies R, Ellis N and Matthews P. 1990. Identification of translocations in pea by in situ hybridization with chromosome-specific DNA probes. Genome 33:745-749.

Singer R and Ward D. 1982. Actin gene expression visualized

in chicken muscle tissue culture by using in situ hybridization with a biotinated analog. Proc. Natl. Acad. Sci. 79:7331-7335.

Sitbon F, Sundberg B, Olsson O and Sandberg G. 1991. Free and conjugated indoleacetic acid (IAA) contents in transgenic tobacco plants expressing the iaaM and iaaH IAA biosynthesis genes from Agrobacterium tumefaciens. Plant Physiol. 95:480-485.

Stachel S and Zambryski A. 1986. Agrobacterium tumefaciens and the susceptible plant cell: a novel adaption of extracellular recognition and DNA conjugation. Cell. 47:155-157.

Stiekema W, Heidekamp F, Louwerse J, Verhoeven H and Dijkhuis P. 1988. Introduction of foreign genes into potato cultivars Bintje and Desiree using an Agrobacterium tumefaciens binary vector. Plant Cell Rep. 7:47-50.

Toki S, Kameya T and Abe T. 1990. Production of a triple mutant, chlorophyll-deficient, streptomycin-, and kanamycin-resistant Nicotiana tabacum, and its use in intergeneric somatic hybrid formation with Solanum melongena. Theor. Appl. Genet. 80:588-592.

Uchimiya H, Hirochika H, Hashimoto H, Hara A, Masuda T, Kasumimoto T, Harada H, Ikeda J.-E and Yoshioka M. 1986. Co-expression and inheritance of foreign genes in transformants obtained by direct DNA transformation of tobacco protoplasts. Molec. Gen. Genet. 205:1-8.

Van Den Elzen P, Townsend J, Lee K and Bedbrook J. 1985. A chimeric hygromycin resistance gene as a selectable marker in plant cells. Plant Molec. Biol. 5:299-302.

Wallroth M, Gerats A, Rogers S, Fraley R and Horsch R. 1986. Chromosomal localization of foreign genes in Petunia hybrida. Molec. Gen. Genet. 202:6-15.

Wang K, Herrera-Estrella L, Van Montagu M and Zambryski P. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from Agrobacterium to the plant genome. Cell 38:455-462.

Wang K, Stachel S, Timmerman B, Van Montagu M, Zambryski P. 1987. Site-specific nick in the T-DNA border sequence as a result of Agrobacterium vir gene expression. Science 235:587-591.

Watson J, Hopkins N, Roberts J, Steitz J and Weiner A. 1988. Molecular biology of the gene. p817-831. The

Benjamin/Cummings Publishing Company, Inc.

Watson J, Gilman M, Witkowski J and Zoller M. 1992. Genetic engineering of plants. Recombinant DNA. W.H. Freeman and Company.

Yamamoto A, Iwahashi M, Yanofsky M, Nester E, Takebe I and Machida Y. 1987. The promoter proximal region in the virD locus of Agrobacterium tumefaciens is necessary for the plant-inducible circularization of T-DNA. Molec. Gen. Genet. 206:174-177.

Yanofsky M, Porter S, Young C, Albright L, Gordon M and Nester E. 1986. The virD operon of Agrobacterium tumefaciens encodes a site-specific endonuclease. Cell 47:471-477.

Zambryski P, Joos H, Genetello C, Leemmans J, Van Montagu M and Schell J. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2:2143-2150.

Zambryski P. 1988. Basic processes underlying Agrobacterium-mediated DNA transfer to plant cells. Ann. Rev. Genet 22:130.

Localization of T-DNA Inserts on Petunia hybrida
Chromosomes by in situ Hybridization

INTRODUCTION

The production of transgenic plants using Agrobacterium tumefaciens as a vector is well established for many species (Klee et al. 1987; Potrykus 1991). The introduced T-DNA sequences are known to integrate stably into plant chromosomes (Perani et al. 1986; Matsumoto et al. 1990). As well, the T-DNA loci have been genetically mapped and found to be transmitted in a Mendelian manner in two solanaceous genera (Chyi et al. 1986; Wallroth et al. 1986). These genetic analyses indicated that the T-DNA inserts were distributed randomly in chromosomes of the Lycopersicon and Petunia genomes, respectively. Although the T-DNA inserts have been used principally as a vector of genes to date, they also show promise as a tag of genes (Andre et al. 1986), or of a chromosome or chromosome fragment(s) (Wallroth et al. 1986) and as a cell selectable trait via an antibiotic resistance. Such a role may be particularly useful for the introduction or identification of economically important genes where the molecular biology is not established. Thus, an efficient means to localize the T-DNA insert sites and determine their distribution among chromosomes of transgenic plants is needed. In situ

hybridization is such a technique that has potential for this purpose.

In situ hybridization allows for visualization of labelled DNA fragments on the chromosomes, and has already been successfully used on plants (Rayburn et al. 1985; Huang et al. 1989). In situ hybridization provides an efficient means for mapping and physical identification of chromosomes, chromosomal segments, and transformed genes (Bergey et al. 1989). To date, in situ hybridization has already allowed both T-DNA and genomic DNA sequences as small as 1-2 kb to be detected (Mouras et al. 1989; Shen et al. 1987). For example, a legumin gene DNA sequence and a multiple copy rDNA gene were identified in pea (Simpson et al. 1988; 1990). The chalcone synthase gene was localized on parsley chromosomes (Huang et al. 1988). Ambros et al. (1986a) identified the site of a 17 kb A. rhizogenes Ri T-DNA on the chromosomes of a transformed Crepis root line. The detection of T-DNA inserts containing the Km' gene on the chromosomes of transformed tobacco cells was reported by Mouras et al. (1987), the Km' insertion sites were found to be localized on different homologous chromosome pairs in two independent transformed tobacco plants. Recently, a 0.9 kb DNA fragment was detected on the chromosomes of rye (Gustafson et al. 1990). In addition, in situ hybridization has been used for detecting species-specific repetitive DNA in order to identify recombined donor chromosome fragments

in asymmetric somatic hybrid plants (Piastuch et al. 1990).

While most studying in situ hybridization indicates that the detection of foreign DNAs in the plant genomes, the distribution of physical location for the insertion sites has only been reported for A. rhizogenes Ri T-DNA in transformed Crepis capillaris plants (Ambros et al. 1986b). The Ri T-DNA inserts in four separate transformed C. capillaris ($2n=2x=6$) were distributed randomly among all three chromosomes.

In this study, in situ hybridization was used to visualize the location and distribution of T-DNA inserts on chromosomes of ten independent transgenic plants of P. hybrida.

MATERIALS AND METHODS

Petunia karyotype

The VR (R51 X V23) hybrid of Petunia hybrida, a gift from T. Gerads, Vrije University, was examined cytologically to establish the karyotype. Root tips were collected in dH₂O and pretreated at 0°C for 24 h. They were fixed in 3:1 ethanol:acetic acid (v/v) solution at 4°C for 24 h. After rinsing with distilled water, the root tips were incubated in 1.0 M HCl at 60°C for 10 min, rinsed again in distilled water and placed on a microscope slide. A drop of 1% acetocarmine in 45% acetic acid was added and the

meristematic region which was cut off from the root tip was gently squashed. Chromosomes were observed on a Carl Zeiss microscope (CARL ZEISS, Oberkochen/West Germany) using 40X objective and phase contrast. Photographs were taken on Kodak Technical Pan film (ASA 125) (Eastman Kodak Company).

Transformation and Confirmation

The VR hybrid was transformed with plasmid pMON200 (9.5 kb) which contains chimeric NOS:NTPII:NOS genes. The Agrobacterium tumefaciens containing pMON200 was cultured as described by Guri et al. (1988). The transformation was carried out by the leaf-disc method of Horsch et al. (1985), with some modifications. The leaves were sliced into 1.0 x 0.6 cm sections and soaked in the bacterial culture about 5 min. to ensure that all leaf edges were infected by the bacteria. The inoculated explants were placed on solidified MS salts and vitamins medium (Murashige et al. 1962) plus benzyladenine 1.0 ug/ml, naphthaleneacetic acid 0.1 ug/ml, and 100 ug/ml kanamycin without a nurse culture. Uninoculated VR hybrid leaf explants were cultured identically as the control.

To confirm transgenic status, 50 regenerated plants, the original shoots taken from separate sites of origin, were tested for the presence of kanamycin resistance by leaf-disc and rooting assays as described by Rogers et al.

(1986). Analysis of nopaline synthase activity in leaf tissue was done according to Otten et al. (1978) except that the samples were run at 300 V for 80 min. For slot blot hybridization, total genomic DNA was extracted from young leaf tissue of putative transformed and non-transformed plants using the procedure of Dellaporta et al. (1985). An isolated 900 bp PstI fragment of plasmid pB10S, encoding the NPTII sequence, was used as the probe (gift from Dr. W. Kopachik, Michigan State Univ.). The probe was ³²P-dCTP labelled (approx. 1×10^8 cpm) by a random primed DNA labeling system according to the supplier's instruction (Boehringer Mannheim Inc.). Slot blot hybridization was performed as described by Rivin (1986).

In Situ Hybridization

Ten independent transgenic plants that were confirmed by the previous four assays were used for the detection of T-DNA inserts by in situ hybridization. The root tips of transformed and non-transformed plants grown in the greenhouse were collected 10 to 14 days after the plants had been repotted. The plants were watered 18-24 h before the root tips were taken, and the root tips were harvested at 10:30 am. Samples for in situ hybridization were prepared according to the method described by Mukai et al. (1990) with several minor modifications. The root tips were pretreated in water at 0°C for 18-20 h and fixed in 3:1

ethanol:glacial acetic acid (v/v) at 4°C for 24 h. After washing with distilled water, the root tips were incubated in 1.0 M HCl for 15 min at 37°C and rinsed in distilled water. They were soaked in 0.7% acetocarmine in 45% acetic acid solution for 30 to 60 min and placed in 45% acetic acid on acid-cleaned slides. The meristematic region cut off from a single root tip was gently squashed under a acid-cleaned coverslip, placed on dry ice for 10 to 15 min followed by removal of the coverslip. The slides were dehydrated in an ethanol series (70-95-100%, 5 min each) and then allowed to air dry.

The 900 bp NPTII gene fragment described previously was used as the probe to visualize the T-DNA inserts. The probe was labelled by nick translation with biotin-16-dUTP according to the instructions of the supplier (Borhringer Mannheim GmbH, West Germany).

The protocol of hybridization and post-hybridization washes followed that of Rayburn et al. (1985) with a few changes. The chromosomal DNA on the slides was denatured by incubation in 70% formamide, 2X SSC at 70°C for 2 min. The slides were immediately dehydrated through prechilled (-20°C) 70, 95 and 100% ethanol for 5 min each and then air dried. The hybridization solution consisted of 50% deionized formamide, 10% dextran sulfate, 2X SSC, 500 ug/ml salmon sperm DNA, 500 ug/ml yeast tRNA, and 2.5 ng/ul biotinylated probe DNA. This mixture was denatured in

boiling water for 10 min and quenched on ice. The hybridization solution, 15 μ l, was applied per slide and covered with an acid-cleaned coverslip. Slides were incubated at 37°C in a humidified chamber for 12-16 h. Following hybridization, the samples were washed twice in 2X SSC at 25°C, each for 15 min, 2X SSC at 37°C for 10 min, 2X SSC at 25°C for 10 min, 0.1% Triton in phosphate-buffer saline (PBS) at 25°C for 2 min and finally in phosphate-buffer saline (PBS) at 25°C for 5 min; twice.

After PBS post-hybridization washing, the slides were drained, but not allowed to dry during the detection process. The DETEK I-hrp signal generating system (ENZO Diagnostics, Inc.) was used for locating hybridization sites. The supplier's instructions were followed except that the DETEK I-hrp complex was diluted 1:150 with 1X complex dilution buffer. To reduce background hybridization, slides were additionally washed at 25°C in 4X SSC, 0.1% Triton in 4X SSC, 4X SSC, 10 min for each solution. The chromosomes were stained with 2% Giemsa for 5 min and a coverslip positioned using double distilled water as the mounting medium.

To test the procedure of in situ hybridization working well, a ribosomal DNA of wheat (gift of Dr. H.J. Price and Mr. Charles Crane, Texas A&M University) was used as a probe on Petunia.

The chromosomes and hybridization regions were observed

on a Zeiss 10 Laser Scanning Confocal Microscope (Carl Zeiss, West Germany) by using 100X objective and brightfield. Black-and-white photographs were taken with Kodak T-max (ASA 100) (Eastman Kodak Company).

RESULTS AND DISCUSSION

Both transgenic and non-transformed VR hybrid plants had $2n=2x=14$ chromosomes which is consistent with previous reports for P. hybrida (Maizonnier 1984). In order to assign the T-DNA inserts to specific chromosomes, it was necessary to distinguish all 7 pairs. Maizonnier et al. (1979) and Smith et al. (1973) previously described the Petunia karyotype and the VR hybrid is consistent with their description (Fig. 1). Briefly, chromosome I is the longest; with a median centromere. Chromosome II is nearly the same length as chromosome I, but has a sub-telocentric centromere and satellites of varying size. Chromosome III is similar to chromosome II with respect to length and centromere position but without satellites. Chromosome IV is slightly shorter than the three preceding ones and has a sub-median centromere. The centromere of chromosome V is intermediate between sub-telocentric and sub-median. Since chromosome VI is very similar to chromosome V, they can not be distinguished by morphology. Maizonnier (1984) also reported that chromosomes V and VI of Petunia were indistinguishable although they were discerned by Smith et

al. (1972; 1973) through fluorescence. Chromosome VII is the shortest and has a median centromere.

Calluses appeared on pMON200 inoculated leaf explants in 2 weeks; between 1 to 2 weeks later about 5 to 10 shoots arose per explant (Fig. 2). When the shoots were subcultured on root-inducing medium, the shoots grew roots in the presence of 100 ug/ml kanamycin (Fig. 3). Non-transformed leaf explants did not produce callus or shoots on MS with kanamycin at 100 ug/ml and eventually turned yellow and died.

Leaf explants taken from 49/50 independent putative transformed plants formed callus on MS medium containing 300 ug/ml kanamycin (Fig. 4B, Table I). Conversely, leaves from non-transformed plants did not produce callus (Fig. 4A). Nopaline was detected in leaf extracts of 48/50 transformed plants, but not in a non-transformed plant (Fig. 5, Table I). Horsch et al. (1985) reported that 20/25 transformed VR hybrid Petunia plants were resistant to kanamycin, and nopaline was detected in 17/25 of those plants. The loss of expression of kanamycin resistance and nopaline synthase genes in certain transformants may be due to influences from surrounding DNA or chromatin structure at the site of insertion of the foreign genes into a plant chromosome (Horsch et al. 1985). Of 50 regenerated plants, 37 (74%) had a positive slot blot assay (Fig. 6, Table I). Some putative transformants that were negative in the slot

blot assay may be due to incomplete transformation or the T-DNA may be lost (Klee et al. 1987). The slot blot assay also revealed various T-DNA intensities. Such plants may contain variable numbers of T-DNA inserts. The 37 verified transgenic plants were eventually grown in the greenhouse. They were pollen fertile and had leaf and flower morphology identical to control plants. The results of plant transformation and confirmation were shown in the Appendix.

Ten of 37 plants which were positive in all three transformation assays were used for in situ hybridization. The signals obtained with the T-DNA probe by in situ hybridization were visible as twin spots, one on each chromatid of a single chromosome (Fig. 7). The T-DNA locations on the chromosomes were detected in three independent cells of every plant.

For each plant, a signal was clearly present at a particular chromosomal location. In plants 1 and 6, the signals were present at the satellite of chromosome II (Fig. 7A & 7F). The T-DNA insert of plant 2 integrated into the middle of an arm of chromosome I (Fig. 7B). For plant 4, the signal was found near the telomere of chromosome I (Fig. 7D). The signal observed in plant 5 was at a centromere position on chromosome V or VI (Fig. 7E). In plants 3, 7 and 8, the T-DNA inserts were localized at the telomere of the short arm of chromosomes V or VI (Fig. 7C), chromosome VII (Fig. 7G) and chromosome IV (Fig. 7H),

respectively. The T-DNA insert in plant 10 was integrated into a proximal position of the short arm of chromosome III (Fig. 7J). In plant 9, two T-DNA inserts were observed on two different chromosomes: One insert was near the end of chromosome IV, and the other integrated in the long arm of chromosome III (Fig. 7I). As expected, no signal of T-DNA was observed in the non-transformed plant (Fig. 8). The summary of verifying assays for transformation and detection of the T-DNA inserts by in situ hybridization were shown in Figure 9 and Table 1.

The signal of ribosomal DNA was observed on chromosome II proximal to the satellite in a non-transformed plant (Fig. 10). As a rule, the ribosomal DNA is located in the nucleolus organizing region (NOR) (Mukai et al. 1991). This was the case herein where the NOR region in Petunia is cytologically discerned as a secondary constriction associated with a nucleolus and a distal satellite on chromosome II. Therefore, the signals of rDNA were obtained at expected positions.

Sometimes, the results were interfered by the background hybridization. Based on the followings, we may distinguish the signals of T-DNA from the background. The signal was twin spots (Fig. 7C, black arrow indicated), but the interference was not (Fig. 7C, white arrow indicated). The color of the signal visualized on microscope was black-brown twin spots, and the dirt was seen to be black single

spot. Also, the signal was observed at the same position in the repeated experiment, however, the site of background hybridization was not repeatable.

The important factors contributing to the success of in situ hybridization in this study mainly involved in the following aspects. First one was the preparation of clean metaphase chromosome spreads from root tip cells. The signals observed by in situ hybridization only occurred on those chromosomes and nuclei which were free of cellular contaminants such as cell wall and cytoplasmic debris. Similarly, Ambros et al. (1986a), using plant protoplasts to prepare chromosome spreads for in situ hybridization, found that those preparations without the cell wall were a prerequisite for visualizing the Ri T-DNA signal. In addition, vigorous cell division in root-tip cells was found at 10:30 am and good cell division phases were also found at 12:30 am and 4:00 pm. Watering the plants 18-24 hours before collection at 10:30 am helped to obtain more dividing cells.

Secondly, the degree of background hybridization influenced the efficacy of detecting the T-DNA probe signals. Thus, an additional post-hybridization wash of slides in 4X SSC, 0.1% Triton in 4X SSC, 4X SSC before Giemsa staining helped to eliminate such interference.

Since the procedure of in situ hybridization were enzymatic reaction, the third important factor was giving

longer reacting time and supplying enough substrate for each reaction. Thus, more products were formed and the size of the signal were enhanced.

In situ hybridization allowed the clear identification and localization of T-DNA sequences in the chromosomes of transgenic P. hybrida plants. Since this technique allowed direct visualization of T-DNA sequences in the host chromosomes, it provides direct information not otherwise obtained from slot blot or Southern blot analyses. Likewise, in situ hybridization eliminates the lengthy process of location by genetic mapping such as the work of Chyi et al. (1986). A non-radioactive probe detection system was used in in situ hybridization because it also permitted rapid detection of hybridized probe sequences at different chromosomal locations (Rayburn et al. 1985).

The T-DNA sequence which is integrated into the host plant chromosomes from pMON200 is about 7.8 kb (Rogers et al. 1986; 1988). The in situ hybridization procedure allowed to detect clearly and localize the sites of T-DNA, present in the chromosomes of transgenic plants, with a target size of around 7.8 kb with 900 bp NPTII fragment as probe. T-DNA inserts have already been detected by in situ hybridization with the length of target from 17 kb (Ambros et al. 1986a) to 1-2 kb (Mouras et al. 1989). Single-copy target sequences as short as 0.9 kb in plant (Gustafson et al. 1990) and as 0.5 kb have been localized on human

chromosomes by in situ hybridization (Jhanwar et al. 1983).

The results in the study indicate that T-DNA can integrate into numerous locations in Petunia chromosomes. In addition, two T-DNA inserts were found in the genome of plant 9. Previous results obtained from Southern blot experiments have shown that T-DNA inserts can integrate into either unique or repetitive plant DNA sequences (Fraley et al. 1985; Wallroth et al. 1986). Wallroth et al. (1986) mapped the genetic locations of the T-DNA inserts in nine independent transgenic VR hybrid Petunia, and reported that all of which are in unique sites distributed over four of the seven chromosomes. Of those, 7/9 plants had one T-DNA insert, one plant had two T-DNA inserts, and another plant had two possible tandemly repeated T-DNAs. Their data on Southern hybridization and genetic analysis showed that the T-DNA inserts were distributed among chromosomes I, III, IV and V, and suggested that the integration was at random, but not distributed equally over the different Petunia chromosomes. However, the locations of T-DNA inserts obtained by our in situ hybridization study indicated not only that the T-DNA integrates randomly into Petunia chromosomes but also that each chromosome appears equally likely to be a target. Similar results were obtained in a study of the Ri T-DNA integrations in transgenic Crepis plants ($2n=6$) by in situ hybridization (Ambros et al. 1986b), whose results indicated that the T-

DNA inserted into all 3/3 chromosomes in four independent transformed root lines. For the T-DNA position, some signals were found near the telomere of chromosomes (three of five), and the T-DNA also integrated into chromosomes (two of five).

The hybridization intensity of slot blot analysis showed that independent transgenic plants contained different T-DNA copy numbers. The 10 plants which had a high intensity hybridization (+++ or ++) with the T-DNA probe in slot blot assay were chosen to localize the sites of T-DNA inserts by in situ hybridization. However, the signal of T-DNA was not observed in the plants which had low intensity hybridization (+) of slot blot. Furthermore, most of transgenic plants (9/10) revealed only one T-DNA insert except plant 9 that had two T-DNA inserts present on different chromosomes. These results suggested that usually T-DNA integrates into a single site, 9 plants. The possibility that two or more tandemly repeated T-DNAs inserted in Petunia as has been reported (Fraley et al. 1985). Our results do not excluded the possibility that two or more tandemly repeated copies are present in a single site. Tandemly repeat T-DNA sequences were also found at a single position in chromosomes of transgenic tobacco plants (Mouras et al. 1987). On the other hand, occasionally two T-DNA inserts were present on separate chromosomes, in one Petunia plant (plant 9). Ambros et al. (1986b) reported

that a transgenic Crepis plant contained two T-DNA inserts on separate chromosomes. Thus, genetic analyses support both insertion patterns which were found in Petunia (Fraley et al. 1985; Wallroth et al. 1986).

The signals of T-DNA inserts were detected and visualized by in situ hybridization procedure used in this study not only on metaphase chromosomes but also in interphase chromosomes. This result indicates that the visualization of the T-DNA sequences is possible in structurally well-preserved plant nuclei. These detections give further confirmation of T-DNA fragment integrating into the plant genome. In plants 1 and 2, while one signal of T-DNA insert was detected on the metaphase chromosomes, one hybridization signal was obtained in the interphase nuclei. Furthermore, two separate signals of T-DNAs were observed in a nucleus of transgenic plant 9 in which two T-DNA inserts were present on different chromosomes. Le et al. (1991) reported that detection of alien DNA of interphase chromosomes may permit rapid decision-making and efficiency in plant breeding.

The study of localization and distribution of T-DNA sequences on plant chromosomes is only an initial step for their use in plant breeding. The long term goal is to improve crop species by transferring genes of agronomic importance such as disease resistance and flower color which are hard to isolate but have been genetically mapped.

The approach presented in this study has such potential applications. Since T-DNA inserts were present in different chromosomal location in each transgenic plant, and each chromosome has been a target for T-DNA insertion at least once, T-DNA inserts could be used as a selectable marker at the chromosome level. The method described would make it possible to identify individual chromosomes or chromosome fragments containing the genes of interest with T-DNA as a probe. Using a T-DNA marked donor species, genes of interest could be transferred by asymmetric hybridization with T-DNA as a selectable marker as already demonstrated by Bates et al. (1987) and Toki et al. (1990).

Table 1. Verification of kanamycin resistance, nopaline production, slot blot and T-DNA locations on chromosomes in transgenic VR hybrid Petunia.

Plant No.	Leaf-disc assay	Nopaline	Slot blot intensity	Chromosome number	T-DNA position on chromosome(s)
1	+	+	++	II	satellite
2	+	+	+++	I	middle of the arm
3	+	+	+++	V/VI	telomere
4	+	+	+++	I	near telomere
5	+	+	+++	V/VI	centromere
6	+	+	+++	II	satellite
7	+	+	++	VII	telomere
8	+	+	++	IV	telomere
9	+	+	+++	III & IV	middle of long arm near telomere
10	+	+	++	III	middle of short arm

Slot blot:
 +: Low intensity
 ++: High intensity

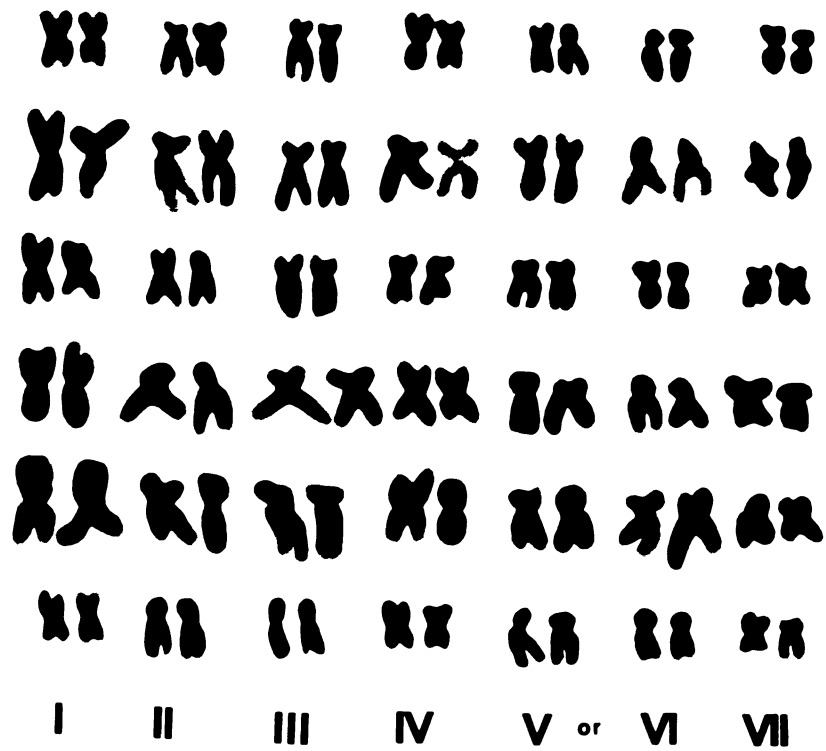


Figure 1. The karyotype of P. hybrida, the genotype VR hybrid.

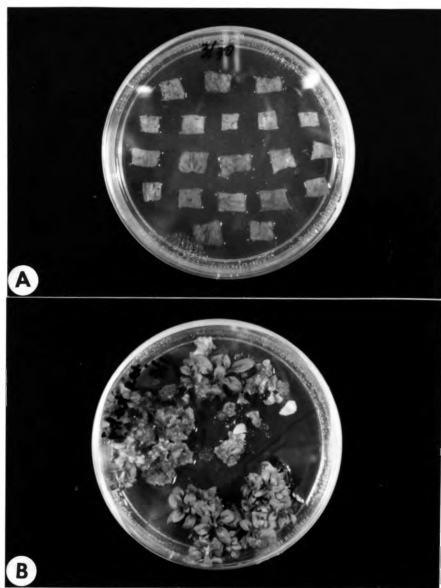


Figure 2. Leaf explant transformation of *Petunia* VR hybrid co-cultivated with pMON200. (A) Leaf explants placed on MS medium containing kanamycin 100 ug/ml, (B) Callus and regenerated shoots, after three weeks in culture.



Figure 3. Transgenic Petunia plant 2 rooted in MS containing 100 ug/ml kanamycin.

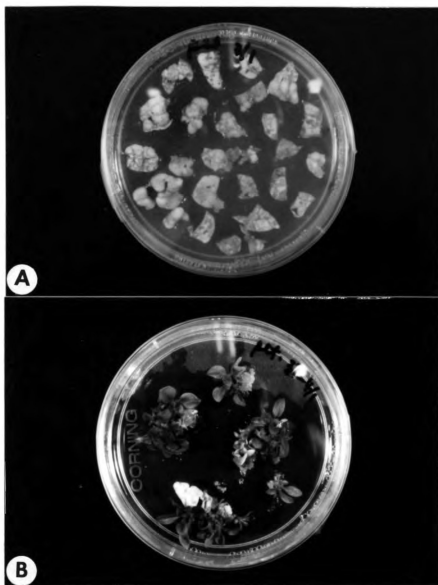


Figure 4. Leaf explants after three weeks in culture from
A) non-transformed and B) transgenic Petunia VR hybrid
plants on MS medium containing 300 ug/ml kanamycin.

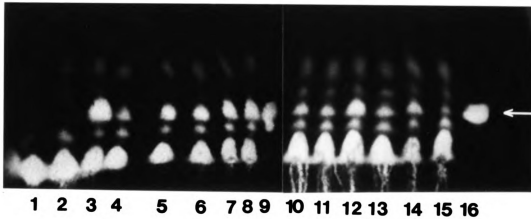


Figure 5. Nopaline assay on non-transformed and transformed leaf tissue. Arrow indicates spot representing nopaline. Lane 1: Arginine marker. Lane 2: Untransformed plant. Lane 3-8, 10-13: Independent transgenic plants 9, 8, 2, 3, 5, 6, 1, 4, 7 and 10 in order. Lane 14-15: Another two transgenic Petunia plant. Lane 9 and 16: Nopaline marker.

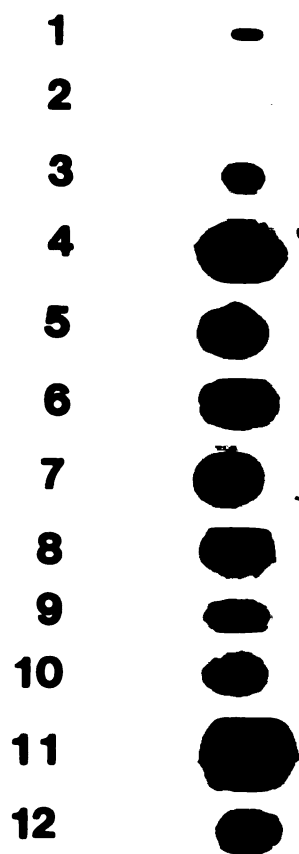


Figure 6. Slot-blot analysis of Petunia transformants. The blots were probed with NPTII gene fragment labeled with dCTP ³²P. Lane 1: Positive control. Lane 2: Negative control. Lanes 3-12: independent transgenic plants 1 to 10.

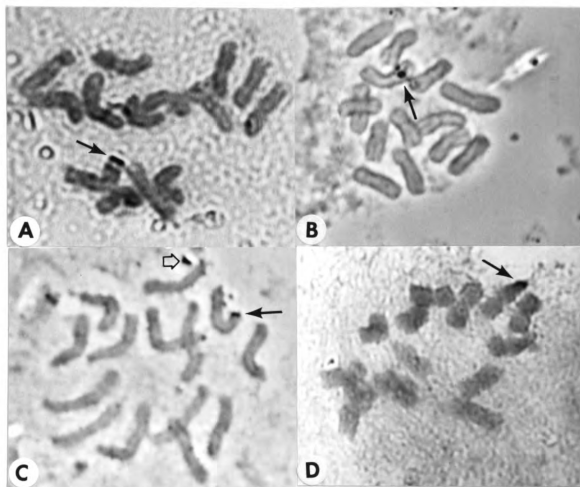


Figure 7. *In situ* hybridization of NPTII fragment as probe to metaphase chromosomes of *Petunia* plants. The signals obtained in plants 1 to 10 are shown in photographs A to J. Black arrows indicate the locations of T-DNA insert. White arrow indicates background hybridization

Figure 7. cont'd

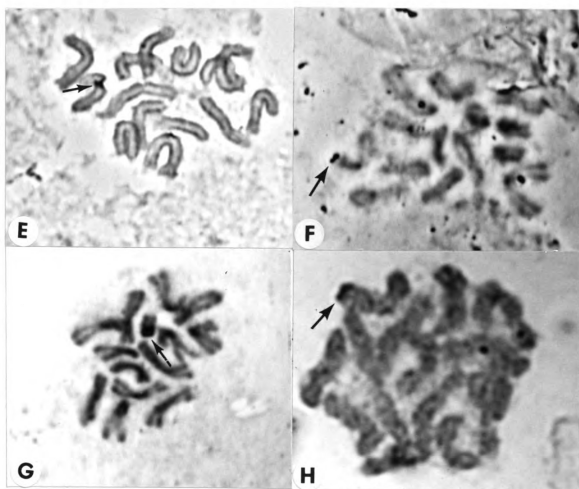
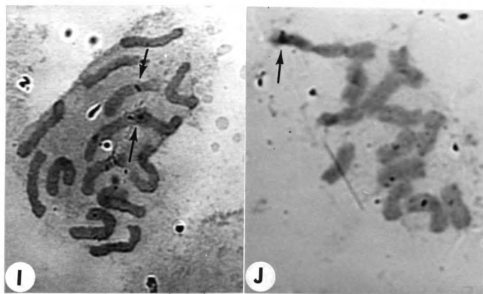


Figure 7. cont'd



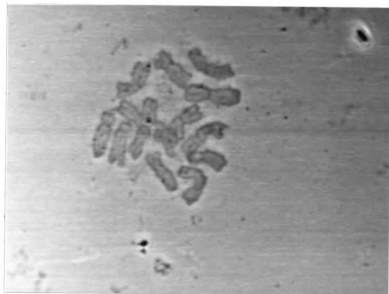


Figure 8. In situ hybridization with NPTII probe in untransformed P. hybrida. No twin spots indicating on chromosomes.

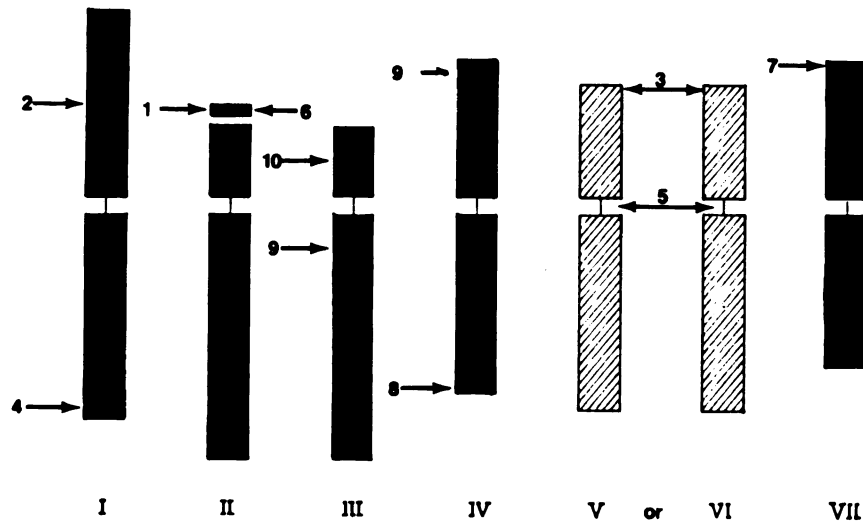
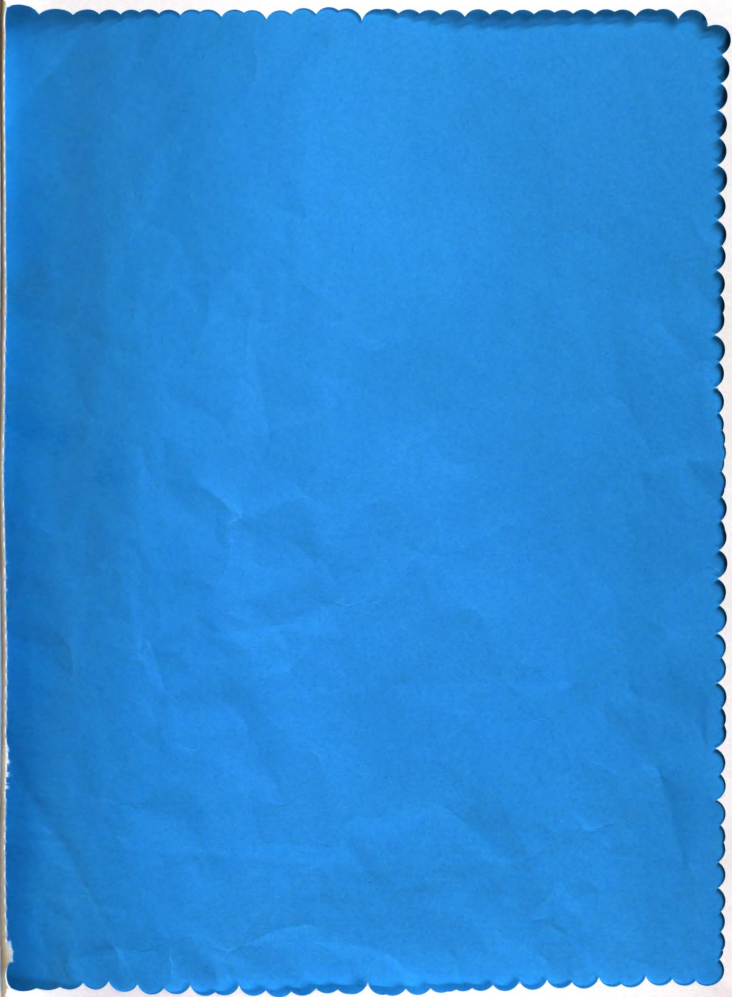
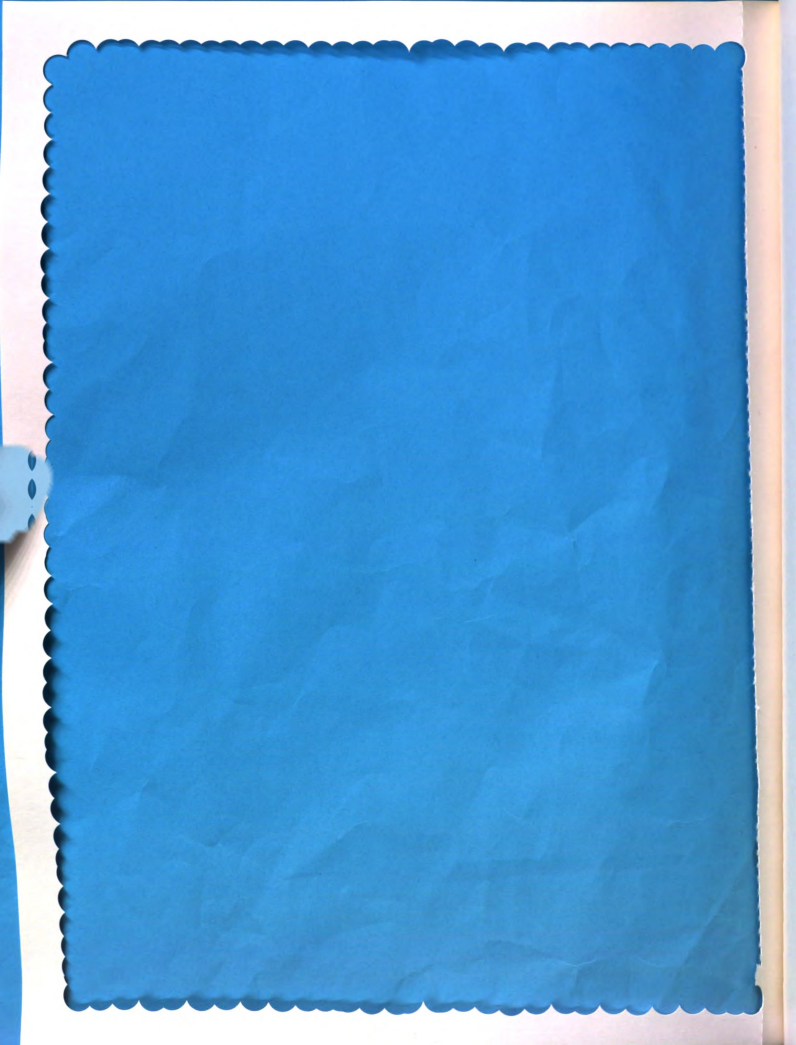


Figure 9. Localizations of T-DNA inserts by in situ hybridization indicating on the karyogram of Petunia in 10 independent transgenic plants. The T-DNA locations labelled with arrows. Arabic numbers indicated plants 1-10.





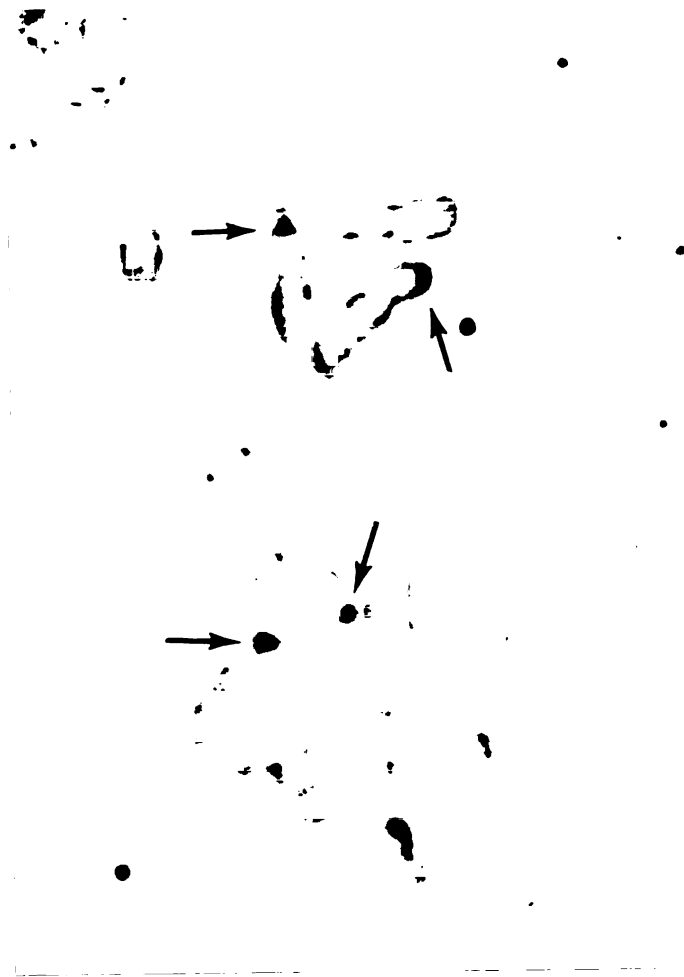


Figure 10. In situ hybridization with ribosomal DNA probe on non-transformed *P. hybrida* chromosomes. The rDNA fragment was detected in the nucleolar organizer region associated with the satellites. Arrows indicate the signals.

LIST OF REFERENCES

LITERATURE CITED

- Ambros P, Matzke M and Matzke A. 1986a. Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by in situ hybridization. Chromosoma 94:11-18.
- Ambros P, Matzke A and Matzke M. 1986b. Localization of A. rhizogenes T-DNA in plant chromosome by in situ hybridization. EMBO 5:2073-2077.
- Andre D, Colau D, Schell J, Van Montagu M and Hernalsteens J-P. 1986. Gene tagging in plants by a T-DNA insertion mutagen that generates APH(3')II-plant gene fusions. Molec. Gen. Genet. 204:512-518.
- Bates G, Hasenkampf C, Contolini C and Piastuch W. 1987. Asymmetric hybridization in Nicotiana by fusion of irradiated protoplasts. Theor. Appl. Genet. 74:718-726.
- Bergey D, Stelly D, Price J and McKnight T. 1989. In situ hybridization of biotinylated DNA probes to cotton meiotic chromosomes. Stain Technol. 64:25-37.
- Chyi Y-S, Jorgensen R, Goldstein D, Tanksley S and Loaiza-Figueroa F. 1986. Locations and stability of Agrobacterium mediated T-DNA insertions in the Lycopersion genome. Molec. Gen. Genet. 204:46-49.
- Dellaporta S, Wood J and Hicks J. 1985. Maize DNA miniprep. In: Molecular biology of plants. p.36-37. Cold Spring Harbor. New York.
- Fraley R, Rogers S, Horsch R, Eichholtz D, Flick J, Fink C, Hoffmann N and Sadlers P. 1985. The SEV system: a new disarmed Ti plasmid vector for plant transformation. Bio/Technology 3:629-635.
- Guri A and Sink K. C. 1988. Agrobacterium transformation of eggplant. J. Plant Physiol. 133:52-55.
- Gustafson J, Butler E and McIntyre C. 1989. Physical mapping of a low-copy DNA sequence in rye (Secale cereale L.). Proc. Natl. Acad. Sci. USA. 87:1899-1902.
- Horsch R, Fry J, Hoffmann N, Eichholtz D, Rogers S and Fraley R. 1985. A simple and general method for transferring gene into plants. Science 227:1229-1231.

Huang P-L, Hahlbrock K and Somssich E. 1988. Detection of a single-copy gene on plant chromosomes by in situ hybridization. Molec. Gen. Genet. 211:143-147.

Huang P-L, Hahlbrock K and Somssich E. 1989. Chromosomal localization of parsley 4-coumarate: CoA ligase genes by in situ hybridization with a complementary DNA. Plant Cell Reports 8:59-62.

Jhanwar S, Neel B, Hayward W and Chaganti R. 1983. Localization of c-ras oncogene family on human germ line chromosomes. Proc. Natl. Acad. Sci. USA 80:4794-4797.

Klee H, Horsch R and Rogers S. 1987. Agrobacterium-mediated plant transformation and its further application to plant biology. Ann. Rev. Plant Physiol. 38:467-486.

Le H and Armstrong K. 1991. In situ hybridization as a rapid means to assess meiotic pairing and detection of alien DNA transfer in interphase cells of wide crosses involving wheat and rye. Molec. Gen. Genet. 225:33-37.

Maizonnier D and Moessner A. 1979. Localization of the linkage groups on the seven chromosomes of the *Petunia hybrida* genome. Genetica 51,2:143-148.

Maizonnier D. 1984. Cytology. In: Sink K (Ed) *Petunia*. Springer-Verlag Berlin Heidelberg. p21-33.

Matsumoto S, Ito Y, Hosoi T, Takahashi Y and Machida Y. 1990. Integration of Agrobacterium T-DNA into a tobacco chromosome: Possible involvement of DNA homology between T-DNA and plant DNA. Molec. Gen. Genet. 224:309-316.

Mouras A, Saul M, Essad S and Potrykus I. 1987. Localization by in situ hybridization of a low copy chimeric resistance gene introduced into plants by direct gene transfer. Molec. Gen. Genet. 207:204-209.

Mouras A, Negrutiu I, Horth M and Jacobs M. 1989. From repetitive DNA sequences to single-copy gene mapping in plant chromosomes by in situ hybridization. Plant Physiol. Biochem. 27:161-168.

Mukai Y, Endo T and Gill B. 1990. Physical mapping of the 5S rRNA multigene family in common wheat. J Hered. 81:290-295.

Mukai Y, Endo T and Gill B. 1991. Physical mapping of

the 18S.26S rRNA multigene family in common wheat: Identification of a new locus. *Chromosoma* 100:71-78.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. plant.* 15:473-479. 373.

Otten L and Schiperoort R. 1978. A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta.* 527:497-500.

Perani L, Radke S, Wilke-Douglas M and Bossert M. 1986. Gene transfer methods for crop improvement: Introduction of foreign DNA into plants. *Physiol. Plant.* 68:566-570.

Piastuch W and Bates G. 1990. Chromosomal analysis of Nicotiana asymmetric somatic hybrids by dot blotting and in situ hybridization. *Molec. Gen. Genet.* 222:97-103.

Potrykus I. 1991. Gene transfer to plants: Assessment of published approaches and results. *Ann. Rev. Plant Physiol.* 42:205-225.

Rayburn A and Gill B. 1985. Use of biotin-labeled probes to map specific DNA sequences on wheat chromosomes. *J Hered.* 76:78-81.

Rivin, C. 1986. Analyzing genome variation in plants. *Methods Enzymol.* 118:75-86.

Rogers S, Horsch R and Fraley R. 1986. Gene transfer in plants. *Methods Enzymol.* 118:627-640.

Rogers S, Klee H, Horsch R and Fraley R. 1988. Use of cointegrating Ti plasmid vectors. *Plant Molec. Biol. Manu.* 2:1-12.

Shen D-L, Wang Z-F and Wu M. 1987. Gene mapping on maize pachytene chromosomes by in situ hybridization. *Chromosoma.* 95:311-314.

Simpson P, Newman M-A and Davies R. 1988. Detection of legumin gene DNA sequences in pea by in situ hybridization. *Chromosoma* 96:454-458.

Simpson P, Newman M-A, Davies R, Ellis N and Matthews P. 1990. Identification of translocations in pea by in situ hybridization with chromosome-specific DNA probes. *Genome* 33:745-749.

Smith F and Oud J. 1972. The possibility to distinguish chromosomes of Petunia hybrida by quinacrine fluorescence. *Genetica* 43:589-596.

Smith F, Oud J and Jong J. 1973. A standard karyogram of Petunia hybrida. *Hort. Genetica* 44:474-484.

Toki S, Kameya T and Abe T. 1990. Production of a triple mutant, chlorophyll-deficient, streptomycin-, and kanamycin-resistant Nicotiana tabacum, and its use in intergeneric somatic hybrid formation with Solanum melongena. *Theor. Appl. Genet.* 80:588-592.

Wallroth M, Gerats A, Rogers S, Fralay R and Horsch R. 1986. Chromosomal localization of foreign genes in Petunia hybrida. *Molec. Gen. Genet.* 202:6-15.

APPENDIX

Table I. Assay results of *P. hybrida* transformants for nopaline production, response to kanamycin and slot blot test.

Plant	Leaf-disc assay to kanamycin	Nopaline	Slot blot intensity	Plant	Leaf-disc assay to kanamycin	Nopaline	Slot blot intensity
1	R ¹	+	- ²	21	R	+	+
2	R	+	+ ³	22	R	+	+
3	R	+	+	23	R	+	+
4	R	+	+	24	R	-	-
5	R	+	+	25	R	+	-
6	R	+	+	26	R	+	+
7	R	+	++ ⁴	27	R	+	++
8	R	+	-	28	R	+	-
9	R	+	++	29	R	+	-
10	R	+	-	30	R	+	+
11	R	+	+	31	R	+	-
12	R	+	-	32	R	+	++
13	R	+	+	33	R	+	-
14	R	+	+	34	R	+	-
15	R	+	-	35	R	+	+
16	R	+	++	36	R	+	-
17	R	+	+	37	R	+	++
18	R	+	+	38	R	+	-
19	S ¹	-	-	39	R	+	-
20	R	+	-	40	R	+	+

1- R: resistance; S: sensitive

2- -: negative

3- +: low intensity

4- ++: middle intensity

Note: 6 plants which showed high intensity (+++) were used for in situ hybridization. Thus, they (+++) did not show in this table.

MICHIGAN STATE UNIV. LIBRARIES



31293008978706