Genetic modification of tulip by means of particle bombardment

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Genetic modification of tulip by means of particle bombardment

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chapter 1

General introduction

Cultivation and breeding of tulips

Tulip: an important ornamental flower

Tulip (*Tulipa* L., Fig. 1) is the most important bulbous ornamental crop in the Netherlands. Figures from the Association of Dutch Flower Auctions (VBN) show that tulip cut flower turnover in 1994 was 269 million Dutch guilders which accounted for about one billion flowers. The number of bulbs produced in 1994 was about three billions, of which two billions were exported, representing an economic value of about 600 million Dutch guilders. The total area of tulip production was approximately 8500 ha. Other bulb producing countries are Japan, France, UK, USA and Denmark, but the Dutch production accounts for about 85% of the world bulb production (Le Nard and De Hertogh, 1993). The tulip originates from Central Asia and the Middle East, was introduced into Europe via Turkey in the second half of the 16th century and in 1994, the 400th anniversary of tulip's presence in Holland was celebrated. During these centuries it has been an object for breeding and hybridization. The genus *Tulipa* consists of about 50 species, roughly distributed over two sub-genera, *Eriostemones* and *Tulipa* (Van Raamsdonk and De Vries, 1995). The present cultivated tulips, bred for their ornamental value, belong to the sub-genus *Tulipa* and are members of the species *Tulipa gesneriana* L. A survey of the extensive literature on tulip research has been published by Le Nard and De Hertogh (1993). Some of the typical features of tulip culture will be discussed below. This survey will demonstrate, that investigations on propagation of tulip *in vitro* and on genetic modification are urgently wanted, and will have implications for a sustainable cultivation of tulips in the future.

The life cycle of tulip

Vegetative propagation is the general method applied for tulip multiplication and is based on the production of daughter bulbs. These develop from the buds which are located in the axils of the bulb scales. The average propagation rate of most tulip cultivars is between two and three bulbs each year.
For breeding purposes the generative cycle is utilized. After a cross, seeds have to be sown in autumn and be submitted to low temperatures, in order to induce germination. The seedling consists of only one cotyledonary leaf, one root and a dropper (Fig. 2), which produces a small bulb in the first year. Four to six additional years are needed to increase the size of the bulb until the first flower is produced.

Breeding Research

A long history of breeding has produced disease-resistant phenotypes (*Fusarium*), but in tulip, as in many other crops, there is a strong need for genetic sources of resistances against other pathogens. In addition to this, breeders need novel genetic variation to improve characteristics like forcing ability, flower longevity and flower quality, and to

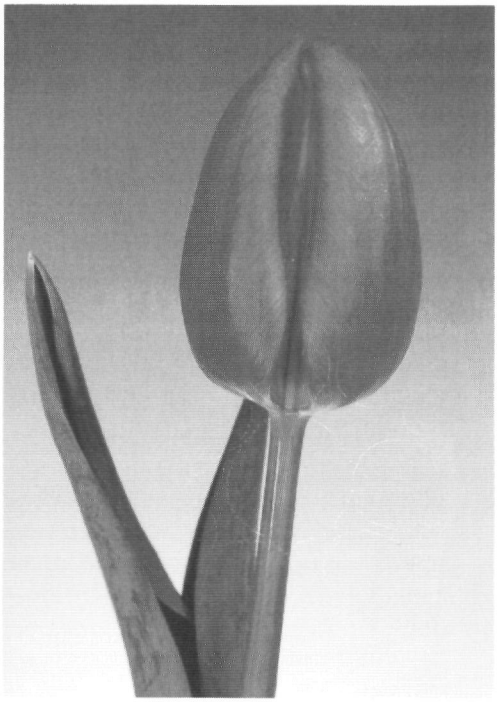

Fig. 1. *Tulipa gesneriana* (L.)

Fig. 2. Life cycle of tulip

broaden the assortment, for instance by introducing new flower colours.
Only recently, research has been started to enrich the cultivated tulip with foreign genetic material. This has been accomplished using interspecific hybridization with botanical species, which offers opportunities for the introduction of desired new traits in the cultivated tulip (Van Eijk *et al.*, 1991). However, further transfer of germplasm from interesting related species into the crop is hampered by fertility problems and strong crossing barriers, which can be overcome partly by special fertilization and embryo rescue techniques (Van Creij *et al.*, 1992).
The main drawback in breeding tulip by hybridization however, is the long juvenile period of the species. From seed till a flowering bulb takes four to six years (Fig. 2). Then it takes 10 years before all the characteristics of a promising hybrid are evaluated. Due to the low propagation rate an additional 10 years are necessary to produce enough bulbs to come to a commercial release of a new cultivar. In total, the entire process takes about 25 years (De Vroomen, 1995). The slow development and the long juvenile period delays the introduction of new cultivars.
This long juvenile period, the presence of crossing barriers and the lack of genetic sources of new traits require the introduction of new biotechnological methods for the introduction of new traits in tulip.

Environmental problems of tulip culture

Tulip is susceptible to many diseases. Serious threats to the tulip industry are fungi (*Fusarium, Botrytis, Pythium, Rhizoctonia*, and others), viruses (Tulip Breaking Virus, Tobacco Rattle Virus), insects and nematodes (*Trichodorus*) (Table 1) (Eikelboom *et al.*, 1992; Romanov *et al.*, 1991; Van Eijk *et al.*, 1978, 1979, 1983; Van Eijk and Eikelboom, 1990). Control of diseases and pests takes about 10% of the production costs of the crop. Especially the control of soil-born and other pathogens requires crop protecting chemicals which can cause severe environmental pollution.
Until now, there are only few alternatives for the use of disinfectants, insecticides, fungicides and herbicides in bulb culture to prevent diseases and to control weeds. In this section, we will discuss the problems and the alternatives for the use of chemicals, and indicate which investigations may result in the reduction of environmental stress.

Disease control

Control of diseases has long been done by using chemicals. However, insecticides and fungicides, like methylbromide (active against nematodes and fungi), etridiazol and fenaminosulf (against *Pythium*) have been banned since 1991 ("Meerjarenplan Gewasbescherming", Dutch Government). Resistances have developed against other agents which were active against *Pythium* (cyprofuram, furalaxyl, metalaxyl, propamocarb) or *Rhizoctonia* (tolclofosmethyl).
Alternatives for the use of chemicals have been searched in biological methods like inundation, soil-steaming, crop rotation or biological crop protection. A better genetic quality of the plant material is another factor to decrease the need for pesticides and fungicides. This can be reached through the introduction of resistance genes by crossing susceptible genotypes with resistant ones. Especially for tulip, this is a time consuming procedure, only after 20 to 25 years new resistant cultivars are available for commercial release (as described earlier).

Introduction of resistance genes

An alternative for genetic quality improvement has been found in the use of biotechnological techniques for introducing resistance genes. In these methods, isolated genes are introduced into plant cells, from which new plantlets are regenerated. These plantlets can be selected for the presence of the introduced genes and further propagated *in vitro*, which means a considerable gain of time with regard to the selection for the proper features. Since this way of molecular breeding bypasses recombination events in normal crossing, the new transgenic plants will be genetically identical to the original cultivar, except for the added resistance gene. Especially for tulip, with its long generation time, the introduction of such methods will have a great impact. Moreover, since new genetic sources become available, because isolated genes from various organisms can be used.

Table 1 **Most important diseases that hamper tulip culture**

Fungi
Fusarium oxysporum - *Botrytis* spp - *Pythium* spp - *Penicillium* spp - *Rhizoctonia tuliparum/solani*
Viruses
Tulip Breaking Virus (TBV) and other potyviruses - Tobacco Rattle Virus (TRV) - Tobacco Necrosis Virus (TNV)
Insects, mites
Aceria tulipae - aphids
Nematodes
Trichodoride nematodes, *Ditylenchus* spp.

Table 2 **Use of herbicides in tulip culture** (kg active agent used per year)

herbicide	kg.
glyphfosate	328
glufosinate-ammonium	220
paraquat	1466
diquat	746
paraquat/diquat	1769
chloorprofam	13461
chloorprofam/chloridazon	3576
chloridazon	2870
metamitron	8592
sethoxydim	192
aloxydim-natrium	600

Prospects for the introduction of resistance genes

Because diseases caused by viruses and fungi are important problems in tulip culture, especially resistance genes against these pathogens are of interest for introduction into tulip. Moreover, since the application of herbicides in tulip culture needs to be regulated more strictly, herbicide resistance genes might be important. Over the last years several strategies have been developed and will briefly be described.

Virus resistance
The introduction of viral coat protein genes is until now one of the most successful methods to obtain virus resistant plants. Coat protein genes mediated virus-resistance has been reported for the Alfalfa Mosaic Virus, Cucumber Mosaic Virus, Potato Virus X and Y, Tobacco rattle virus and Tobacco Mosaic Virus (Beachy *et al.*, 1990). Also for monocots it has been shown that coat protein genes can mediate virus resistance, transgenic rice plants have been shown to be resistant against the Rice Stripe Virus (Hayakawa *et al.*, 1992; Scholthof *et al.*, 1993). For lily virusses coat protein genes for the Lily virus X (LVX), the Lily Symptomless Virus (LSV) and the Tulip Breaking Virus (TBV-lily) have been isolated (Langeveld *et al* , 1991; Dekker *et al.*, 1993; Ohira *et al.*, 1994). If tranformation procedures are available, these genes can be introduced into Lily to confer virus resistance. For tulip a similar method can be applied for important pathogens like the Tulip Breaking Virus and the Tobacco Rattle Virus.

Fungal resistance

To achieve fungal resistance, genes coding for cell wall degrading enzymes like chitinases and glucanases have been isolated. Especially fungi are susceptible for these enzymes, because their cell walls mainly consist of chitine and ß-1,3-glucan. By introducing such genes into tobacco, plants resistant against *Rhizoctonia solani* have been obtained (Broglie *et al.*, 1991). Apart from isolating defense genes, another option would be to identify genes that confer natural resistance against certain pathogens from a variety of donors. This approach has led to the isolation of resistance genes against *Fusarium oxysporum* f. sp. *lycopersici* and against *Pseudomonas syringae* pv. *tomato*, which can now be introduced into susceptible crops (reviewed by Broglie and Broglie, 1993).

Genes for the introduction of virus and fungal resistance are available at this moment. For tulip, we consider the introduction of disease resistance genes as an important application for transformation that should have high priority as soon as a transformation procedure is available.

Herbicide resistance

In tulip culture, herbicides are used for weed control (Table 2). On a yearly basis, more than 33.000 kgs. of the active components of various herbicides are being used. Many of these agents are rather pollutive for environment. In the Netherlands, a reduction of the application of chemicals for weed control is necessary to reduce this pollution. Therefore the use of herbicides should be diminished in favor of ecologically sound agents, like glufosinate-ammonium (Basta, active compound phosphinothricin). One approach is the introduction of genes conferring resistance to Basta. If tulip could be protected by means of the Basta-resistance gene (*bar* gene), this would result in a more efficient use of this relatively easily degradable agent since preventive use of heavy agents could then be substituted by a minimized application of Basta only where needed.

The Dutch Urgency Programme for Research on Diseases and Breeding of Flower Bulbs; Aim of this research

In 1989, strong restraints were put on the use of chemicals for disease and weed control. According to the standards of the "Meerjarenplan Gewasbescherming", the use of these agents should be diminished, in order to reduce environmental pollution. The Dutch bulb industry was urged to find effective answers to these new standards, and decided to invest in research on environmental friendly alternatives. This resulted in an extensive research programme, financed by the Royal General Bulbgrowers' Association (KAVB), the Dutch Bulb Exporters Association (BVB), and the Commodity Board for Ornamental Plants (PVS) together with the Dutch government: the Dutch Urgency Programme for Research on Diseases and Breeding of Flower Bulbs.

For tulip, one of the options was, to introduce disease resistance, although existing breeding techniques were known to be inadequate to confer fast genetic improvement. Genetic modification in combination with *in vitro* propagation techniques however, offer

means to enable fast introduction of useful genes. Moreover, such techniques allow introduction of new genes into existing cultivars, which already have proven to be valuable in practice, whereas interspecific crosses will create new hybrids, of which all the characteristics need to be evaluated again.
To establish these techniques, a method for genetic modification of tulip had to be developed, and this thesis presents results on this research. The goals that were aimed can be subdivided in three parts:
- the development of a method for gene transfer for tulip
- the development of a regeneration method for tulip
- the introduction of foreign genes and the analysis of transgenic plants

Outline of this thesis

Several steps are necessary in order to develop a transformation procedure for tulip. At first, the effect of selective agents had to be evaluated, in order to select trangenic tissue by means of resistance. In chapter 2, the mode of action of various selective agents will be described, together with an evaluation of their use in monocots. The results on the use of antibiotics and herbicides in tulip are described in chapter 3. In this chapter we also describe the regeneration system we applied and adapted for floral stem segments. We choose to use this system of adventitious shoot formation after careful consideration of the possibilities that exist for tulip. For transformation and regeneration of monocots, in general, protoplasts, embryogenic cells or cells that are able to develop into embryogenic tissue are used. For tulip such an embryogenic system has not yet been established, and neither have protoplast cultures or cell suspension cultures. Callus formation has been reported in some cases, but callus growth is slow and embryogenesis from such callus is poorly reproducible (Famelaer, CPRO-DLO, personal communication). As an alternative, the use of zygotic embryos can be considered, but tulip plants are heterozygous and the sexual offspring shows strong genetic segregation. Hence, this approach contradicts with the need to introduce genes into existing cultivars. We concluded that the use of adventitious shoot formation offers more perspectives. This latter method generates leaf-like structures, which are in some cases able to develop again axillary meristems at their base. After a cold treatment such meristems can grow into a new bulb. The results with this system are described in chapter 3.
For the establishment of an effective transformation system, the activity of the promoter which has to direct the selection gene is of much importance. Therefore, the expression of various promoter constructs was tested on tissue of tulip and other *Liliaceae* in chapter 4, and we concluded that common monocot promoter constructs were not in all cases the best choice for *Liliaceae* tissues.
Additionally, in chapter 5, it was investigated which transformation method could best be used for tulip. In general, monocots are poorly susceptible for *Agrobacterium*, and indeed we concluded that the method of direct gene transfer by means of particle bombardment offered more perspectives, because it is genotype-independent.

The results of the experiments described above were used in two sets of transformation experiments, and are described in chapter 6. These experiments resulted in the formation of adventitious shoots, that were able to grow on selective medium, and they all were shown to be positive for the presence of the Basta resistance gene, as determined by PCR. However, the integration of the gene in the tulip genome was hard to demonstrate, because of the large size of the genome (25.10^6 kb). Final conclusions on this research and recommendations for future research are summarized in chapter 7.
Finally, in chapter 8, we evaluate the way in which genes integrate into genomes after direct gene transfer, and describe some ways to improve this process.

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chapter 2

Selective agents and marker genes for use in transformation of monocotyledonous plants

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Plant Molecular Biology Reporter 11: 165-185 (1993)

Abstract

Even the most successful transformation systems for monocotyledonous plants have a very low efficiency. To overcome this, the selection system is of utmost importance. Established selection systems based on kanamycin resistance are not generally applicable for monocotyledonous plants, because monocot cells and tissues are relatively insensitive to kanamycin. Selection can be improved by using other antibiotics or herbicides and their respective resistance genes, which can lead to a substantial reduction in the number of untransformed regenerants. In chosing a selection system the resistance mechanism, either detoxification of the selective agent or modification of the target enzyme, should also be taken into account. Detoxification of the selective agent by expression of a modifying enzyme in transformed cells can enable untransformed cells to escape. This does not happen when resistance is based on the production of a modified target enzyme. By use of vectors specifically designed for high expression in monocots, the level of resistance in transformed cells and tissues can be enhanced, resulting in an increased efficiency of selection. This paper presents an overview of the various antibiotics and herbicides available and the application of the related resistance genes to improve transformation of monocots.

Keywords: monocot transformation, selection, antibiotics, herbicides, monocot expression vectors

Abbreviations: AAC(3'), gentamycin 3-N-acetyltransferase; ALS, acetolactate synthase; APH(3')II, aminoglycoside 3'-phosphotransferase II, also called NPTII, neomycin phosphotransferase II; APH(4') or HPT, hygromycin phosphotransferase; CS, chlorsulfuron; EPSP, 5-enolpyruvyl-shikimate-3-phosphate; EPSPS, 5-enolpyruvyl-shikimate-3-phosphate synthase; G418, eneticin; GS, glutamine synthase; PAT, phosphinothricin-N-acetyltransferase; PPT, phosphinothricin.

Gene names and corresponding products: *Act1*, actin; *Adh1*, alcohol dehydrogenase; *aphA2*, aminoglycoside 3'-phosphotransferase II; *aroA*, 5-enolpyruvyl-shikimate-3-phosphate synthase; *bar*, phosphinothricin-N-acetyltransferase; *bxn*, bromoxynil-nitrilase; *csr1-1*, mutant acetolactate synthase; *dhfr*, dihydrofolate reductase; *Epsp*, 5-enolpyruvyl-shikimate-3-phosphate synthase; *GST1*, gluthathione-S-transferase I; *hpt*, hygromycin phosphotransferase; *pat*, phosphinothricin-N-acetyltransferase; *psbA*, Q_B; *RbcS*, small subunit of riblose-biphosphate carboxylase; *tfdA* 2,4-dichlorophenoxyacetate monoxygenase.

Introduction

Since monocotyledonous plant species generally are insensitive to *Agrobacterium*-mediated transformation, alternative procedures are being developed for genetic modification of such plants. Gene transfer by particle bombardment is presently one of the most successful methods. In this procedure, the efficiency of DNA delivery and especially the efficiency of stable integration of the introduced genes is rather low compared to transformation by *Agrobacterium*. For instance, in tobacco, only 1.9 % of the cells that initially expressed the *ß-glucuronidase* gene after particle bombardment became transformed by integration of the gene (Klein *et al.*, 1988). Although Spencer *et al.* (1991) obtained rather high values, from 5 to 10%, after particle bombarding maize cells, others obtained very low frequencies of about 0.1% (Gordon-Kamm *et al.*, 1990). Such a low efficiency of integration stresses the importance of a good selection of the small number of stably transformed cells.

The antibiotic kanamycin, which is most widely used as a selective agent for transformation of dicotyledonous tissues, cannot easily be applied to monocot tissues (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989). Monocots are often insensitive to relatively high levels of kanamycin, which allows regeneration of untransformed plant cells on kanamycin-containing medium. For instance, a concentration of 100 mg/l kanamycin allowed growth of 70% of untransformed rice calli (Dekeyser *et al.*, 1989), and protoplasts isolated from suspension cells of *Lolium perenne* were even able to divide in the presence of 800 mg/l kanamycin (Potrykus *et al.*, 1985). In our own research on tulip, adventitious shoot formation on flower stem explants still continued in the presence of 500 mg/l kanamycin in the medium. At these concentrations growth of untransformed cells of dicotyledonous plants such as petunia and tobacco (Fraley *et al.* 1983), and soybean (Christou *et al.*, 1988) is fully inhibited.

Therefore, many attempts have been made to replace the selective agent kanamycin by other antibiotics or herbicides. This seems a reasonable approach because the various substances differ in their mode of action and in the way they are taken up and transported in plant tissues. Moreover, it should be realised that the corresponding resistance genes act differently, either by inhibiting the selective agent by detoxification or by modification of the target enzyme (Table 1). Also the level of expression of the resistance gene is important, to ensure a sufficient level of resistance in the transformed cells. In this way transformed cells will be allowed to divide and further develop amidst the majority of untransformed highly susceptible cells. High expression levels can be obtained by the use of specific expression cassettes, specifically constructed for monocotyledonous plants.

In this paper the effects of the various selective agents (Table 1) will be summarized, and their suitability for monocot transformation will be evaluated. In addition, the use of various expression vectors will be discussed.

Table 1.

Summary of the effects of various selective agents and the corresponding resistance genes

	effect	resistance gene	resistance enzyme	resistance mechanism
Antibiotics:				
Aminoglycosides				
kanamycin	protein synthesis	*aphA2*	APH(3')II, (NPTII)	phosphorylation of selective agent
G418	protein synthesis	*aphA2*	APH(3')II, (NPTII)	phosphorylation of selective agent
hygromycin	protein synthesis	*hpt*	APH(4')	phosphorylation of selective agent
Other antibiotics				
bleomycin	DNA lesions	-	-	-
Herbicides:				
Basta, PPT	aminoacid synthesis	*bar, pat*	PAT	acetylation of selective agent
roundup, glyphosate	aminoacid synthesis	*aroA, epsps*	EPSPS	modification or amplification of the target enzyme
sulfonylureas	aminoacid synthesis	*csr1*	ALS	modification of the target enzyme
bromoxynil	photosynthesis	*bxn*	nitrilase	degradation of the selective agent
atrazine	photosynthesis	*pbsA*	Qb	modification of the target enzyme
Other selective agents:				
methotrexate	nucleotide synthesis	*dhfr*	DHFR	modification of the target enzyme
2,4 D	auxin	*tfdA*	DPAM	degradation of the selective agent

Antibiotics

Antibiotic resistance genes of bacterial origin, expressed under the direction of plant promoters, have been shown to be effective in plant tissue. Expression of such a gene in plant cells results in transformed cells which show acquired resistance to the corresponding antibiotic. Representatives of a major class of antibiotics, the aminoglycosides, and the corresponding resistance genes are widely used for plant transformation studies.

Aminoglycoside antibiotics and resistance genes

The best known member of the aminoglycoside antibiotics is kanamycin, which is widely applied as a selective agent in transformation experiments. Other agents are gentamycin, G418, neomycin, paromomycin and hygromycin. The properties of kanamycin and the inactivating enzymes of various aminoglycoside antibiotics have been reviewed by Nap *et al.* (1992), and will be briefly mentioned here. The application in tissue culture will be discussed for kanamycin; G418 and hygromycin will be described as main alternatives.

All known aminoglycoside antibiotics inhibit protein synthesis in prokaryotic cells. Kanamycin, gentamycin and its derivative geneticin (G418), neomycin and paromomycin bind to the 30S ribosomal subunit thus inhibiting the initiation of translation, and consequently protein synthesis. Hygromycin occupies the ribosomal binding site of the elongation factor EF-2, inhibiting peptide chain elongation (Kors, 1991). Ribosomes of mitochondria and chloroplasts of higher plants are related to bacterial ribosomes, and are also susceptible to antibiotics. The most dramatic visible effect on plants is chlorosis, bleaching of the leaves, caused by lack of chlorophyll synthesis.
Resistance to kanamycin, G418, neomycin and paromomycin is obtained by the *aphA2* gene from Tn5 of *E. coli* (Bevan *et al.*, 1983). This gene codes for the enzyme APH(3')II (aminoglycoside 3'-phosphotransferase II), also called NPTII (neomycin phosphotransferase II), which phosphorylates a specific hydroxyl group of these antibiotics (Fig. 1), using the γ-phosphate group of ATP. Due to this phosphorylation, binding of the antibiotic to the ribosome is inhibited. Kanamycin can also be detoxified by AAC(3') enzymes, which acetylate the 3' aminogroup of ring II. Also gentamycin can be detoxified by AAC(3') enzymes (gentamycin 3-N-acetyltransferase) (Fig. 1), but does not contain a hydroxyl group at the 3' position of ring I. Hence it cannot be phosphorylated by APH(3'). However, gentamycin's derivative G418 contains an extra hydroxyl group at the 4' position of ring I, which enables phosphorylation by the NPTII enzyme. Genes encoding AAC(3') enzymes have been used for transformation of *Petunia hybrida* and *Arabidopsis thaliana* (Hayford *et al.*, 1988) and tobacco (Carrer *et al.*, 1991). Hygromycin can be inactivated by phosphorylation of a hydroxyl group (Fig. 1) by the enzyme APH(4'), also called HPT (hygromycin phosphotransferase). The *hpt* gene which codes for this enzyme was also isolated from *E. coli* and rendered suitable for expression in plant cells (Van den Elzen *et al.*, 1985, Waldron *et al.*, 1985).

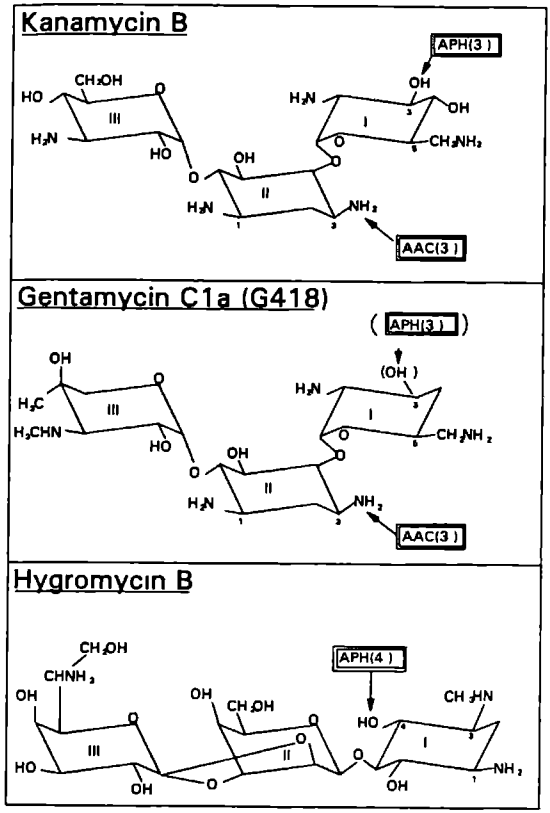

Fig 1 Chemical structures of kanamycin, gentamycin (geneticin), and hygromycin The arrows indicate the sites of action of the detoxifying enzymes G418, derivative of gentamycin, and the corresponding change in the chemical stucture is given between brackets

Application of kanamycin selection

Generally, kanamycin is added to the growth medium in a concentration that previously showed to be inhibitory for regeneration of untransformed cells. Little is known about the transport in plant tissue. Kanamycin does not seem to be mobile in the vascular tissue, it rather seems to diffuse through the plant tissue via intercellular spaces. It has been observed that this diffusion only occurs over rather short distances (Weide *et al* , 1989), which means that in large explants not all parts are reached by the antibiotic. In a study on the transformation of adventitious shoots in tulip, we observed that kanamycin selection was much more effective when small explants were used (personal observation).

From prokaryotic studies, it is known that the strongly positive molecule binds to the negatively charged cell membrane, followed by an active transport into the cell, mediated by carriers. This last step renders the process pH dependent; if the pH drops below 6, binding is strongly inhibited. Moreover, bivalent ions in the medium (Ca^{2+} and Mg^{2+}), compete with kanamycin for the carrier binding sites (Nap *et al.*, 1992) Additionally, other components in tissue culture media can influence the efficacy of kanamycin. for

instance, if the synthetic gelling agent gelrite is used instead of agar, the kanamycin concentration has to be increased because kanamycin seems to bind to gelrite (personal observation). These aspects should be taken into consideration when kanamycin is applied in tissue culture: preferably the pH should not drop below 6, whereas the normal tissue culture pH is 5.8 or even lower, and the presence of bivalent ions in the medium should be reduced to a minimum level.

Application of antibiotics in monocot transformation

The use of kanamycin proved to be very effective for the development of transformation procedures for dicot species. However, research in monocot transformation demonstrated that in these species kanamycin was less effective as a selective agent. Many monocots, especially *Gramineae*, show a high level of natural tolerance to the antibiotic. When using kanamycin for selection of protoplasts of *Gramineae*, it was shown that, for an optimal effect, the antibiotic had to be applied as soon as possible after protoplast isolation, before restoration of the cell wall (Hauptmann *et al.*, 1988). This suggests that transport over the cell wall is the limiting factor. Additional problems have been reported for rice: regeneration of plants from transformed calli proved to be impossible if kanamycin was used as the selective agent (Peng *et al.*, 1992). Only when G418 was used for selection, calli expressing the *aphA2* gene could be regenerated into plants. It was concluded that kanamycin somehow impairs the regeneration potential of rice calli. Similar phenomena were reported by Toriyama *et al.* (1988), Zhang *et al.* (1988) and Battraw and Hall (1992).
G418 (geneticin) has been tested as an alternative selectable marker in various monocots, e.g. rice, *Lolium* and other *Gramineae*, which were highly resistant to kanamycin (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989). In all cases G418 was shown to be more effective. This might be due to a more effective binding to ribosomes.
Another useful antibiotic is hygromycin, for which most tissues, including monocot tissues, show a higher sensitivity compared to kanamycin (Meijer *et al.*, 1991). Hygromycin has been applied in establishing transformation procedures for maize (Walters *et al.*, 1992), rice (Shimamoto *et al.*, 1989; Li *et al.* 1992[a]; Datta *et al.* 1992), and orchardgrass (Horn *et al.*, 1988).

Other antibiotics

Bleomycin is an antibiotic that acts in a completely different way compared to the aminoglycosides in that it causes lesions in DNA. Cells do not die but DNA synthesis and mitosis are blocked. Especially actively dividing tissue is very susceptible and apical meristems are lost. The resistance gene has been isolated from Tn5 of *E. coli*, but the mechanism by which the gene confers resistance to the plant cells is unknown (Hille *et al.*, 1986). After its description as selective agent, there have been no reports about its use in transformation studies.

Herbicides

In addition to antibiotics, herbicides are being used very frequently and with success for plant transformation. Resistance genes originate from various organisms, such as

bacteria and plants. The mechanisms by which these genes act have been reviewed by Comai and Stalker (1986) and will only be briefly described here.

L-Phosphinothricin

L-Phosphinothricin (PPT), also known as glufosinate, is an analog of glutamate and acts as a competitive inhibitor of the enzyme glutamine synthetase (GS) (Fig 2A). This enzyme is involved in assimilation of ammonia and plays a key role in the regulation of nitrogen metabolism (De Block *et al* , 1987). Inhibition of the enzyme causes accumulation of NH_4^+ in the cell. This ammonia accumulation rather than the lack of glutamine causes the death of plant cells (Tachibana *et al.*, 1986[a,b]). PPT is synthesised chemically (Basta; Hoechst AG), or by fermentation of *Streptomyces hygroscopicus* (Herbiace, produced by Meiji Seika Ltd). The latter product, also called Bialaphos, is a tri-peptide compound, which consists of PPT and two alanine residues, which are removed in the plant cell by peptidases (De Block *et al* , 1987).

Resistance to the herbicide is conferred by the enzyme phosphinothricin-N-acetyltransferase (PAT) This enzyme inactivates PPT by acetylation, using acetyl coenzyme A as a cofactor The gene that codes for the PAT enzyme, the Basta resistance gene or *bar* gene, has been isolated from *S. hygroscopicus* (Murakami *et al.*, 1986, Thompson *et al* , 1987). Another resistance gene, the *pat* gene, has been isolated from *S. viridochromogenes*, and has been shown to have significant homology to the *bar* gene (Wohlleben *et al.*, 1988).
In the field, Basta is used as weed control. It is a biologically metabolizable herbicide, which is less harmful for the environment than other herbicides In transformation studies Basta or PPT have been used to select for PPT resistant plants, by spraying full-grown plants or by adding it to selective medium in earlier stages In the medium, one to three mg/l PPT is often adequate to select for transformed cells (De Block *et al* , 1987; Gordon-Kamm *et al.*, 1990) Dekeyser *et al* (1989) found a concentration of 10 mg/l optimal to discriminate between transformed and untransformed rice calli They also showed that, for an effective selection, it was important to omit amino acids from the selective medium, because several amino acids (glutamic acid, proline, arginine) allowed growth of untransformed cells in the presence of PPT This seems in contradiction with data indicating that ammonia accumulation leads to cell death (Tachibana *et al* ,

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A Glutamine synthetase (GS)

$$\text{glutamate} + NH_4^+ + ATP \xrightarrow{GS^*} \text{glutamine} + ADP + P_i + H^+$$

B Enolpyruvylshikimate-phosphate synthase (EPSPS)

$$\text{phosphoenolpyruvate} + \text{shikimate 3P} \xrightarrow{EPSPS^*} \text{5 enolpyruvylshikimate 3 P} \rightarrow\rightarrow\rightarrow \text{aromatic aminoacids}$$

Fig 2 Biochemical pathways which are affected by the use of A Basta, B Roundup The target sites of the herbicides are indicated with an asterisk

1986[a,b]). However, it shows that both the composition of the selective medium and the concentration of PPT are important factors in selection for transformed plant cells.
As a selective agent, PPT has successfully been applied in *Brassica napus* (De Block *et al.*, 1987; De Greef *et al.*, 1989), *Helianthus annuus* (Escandón and Hahne, 1991), and others. It has proven to be effective in monocots such as maize (Gordon-Kamm *et al.*, 1990; Spencer *et al.*, 1991; Spencer *et al.*, 1992), oat (Somers *et al.*, 1992) and rice (Dekeyser *et al.*, 1989, Datta *et al.*, 1992, Cao *et al.* 1992), which have a high tolerance for antibiotics. In contrast to aminoglycosides, PPT has been shown to be mobile in both xylem and phloem (Shelp *et al.*, 1992).

Glyphosate

Glyphosate or N-phosphonomethylglycine inhibits the enzyme 5-enolpyruvate shikimate 3-phosphate synthase (EPSPS). The enzyme is involved in the shikimate pathway (Fig. 2B). Inhibition of this enzyme results in an accumulation of shikimate, inhibition of synthesis of aromatic amino acids and secondary metabolites, and results in cell death (Comai *et al.*, 1985). Glyphosate is mobile in the phloem and tends to accumulate in the apex of stem and root, so it affects especially meristematic and apical cells (Comai *et al.*, 1989). Glyphosate is the active compound in the well known herbicide Roundup, produced by Monsanto.

Resistance to glyphosate is not based on inactivation of the herbicide, but is achieved by the introduction into the plant of a gene coding for an EPSPS enzyme with a reduced affinity to glyphosate. There are several genes encoding such a modified enzyme, for instance the *aroA* gene coding for 5-enolpyruvyl-3-phosphoshikimate synthetase from *Salmonella typhimurium*. This modified EPSPS enzyme has one amino acid substitution (Comai *et al.* 1985). Another modified enzyme is encoded by a mutated *epsps* gene isolated from *Petunia* (Hinchee *et al.*, 1988).
A different approach to glyphosate resistance is based on overproduction of the unmodified enzyme. From a glyphosate tolerant *Petunia* MP4-G cell line, a gene has been isolated as a result of overproduction of the enzyme. This overproduction was due to amplification of the gene. When this gene was introduced into *Petunia* cells under control of the CaMV 35S promoter to obtain a high level of expression, transformed plants were tolerant to glyphosate (Shah *et al.*, 1986[a]).

Glyphosate has successfully been applied as a selective agent in transformation of soybean (Hinchee *et al.*, 1988), wheat (Vasil *et al.*, 1991) and others. In these studies, the modified *Petunia epsps* gene was used, under control of the CaMV 35S promoter.
A lot of attention has been paid to improve the selection system based on glyphosate resistance. This included isolation of promoters that are effective in apices and meristems, where glyphosate accumulates (Comai *et al.*, 1989). A higher transformation efficiency is also expected to be achieved by targeting the enzyme to the chloroplast, where the shikimate pathway is located. To achieve this, the *aroA* gene from *S.*

typhimurium was fused to the transit peptide coding region of ribulose biphosphate carboxylase small subunit (Comai *et al.*, 1988), but so far this chimeric gene has not been used in transformation experiments. However, a fusion between the transit peptide of the *Petunia epsps* gene and the resistance gene *aroA* from *S. typhimurium* improved the selection of transgenic plants (Della-Cioppa *et al.*, 1987; Botterman and Leemans, 1988).

Other herbicides

Several other herbicides have occasionally been used as selective agents in transformation studies, but only few data are ava8ilable for use in monocots.

Chlorsulfuron, other sulfonylureas and imidazolinones are herbicides that affect amino acid biosynthesis by inhibiting the enzyme acetolactate synthase (ALS). This enzyme is involved in the synthesis of branched chain amino acids. The resistance gene is a mutated *csr1* gene (chlorsulfuron resistance) encoding an ALS enzyme with a reduced affinity for the herbicide, isolated from *Arabidopsis thaliana* (Haughn *et al.*, 1988). Other mutations that confer resistance against chlorsulfuron (CS) have been found in *E. coli*, *Salmonella typhimurium*, *Saccharomyces cerevisae* and in various plant species, such as tobacco and sugarbeet.
Chlorsulfuron has been applied as selective agent in transformation of tobacco (Haughn *et al.*, 1988), maize (Fromm *et al.*, 1990) and sugarbeet (D'Halluin *et al.*, 1992). D'Halluin *et al.* (1992) compared the use of CS and other sulfonylurea compounds, PPT and kanamycin. They concluded that, after the selection procedure, 90% of the shoots that survived the application of CS were actually transformed, while for PPT and kanamycin these figures were 30% and 50% respectively. These results indicated that CS is an efficient selectable agent for transformation of sugarbeet. Also transgenic poplars (Brasileiro *et al.*, 1992) and fertile transgenic rice plants (Li *et al.*, 1992[b]) have been obtained by using the *crs1* gene in combination with the CaMV 35S promoter.
Bromoxynil is a herbicide that inhibits photosynthesis in plants, by binding to electron transport components of photosystem II in the thylakoïd membrane. The *bxn* gene (bromoxynil nitrilase gene) from *Klebsiella ozaenae* codes for a specific nitrilase that degrades bromoxynil. Transgenic tobacco plants harboring this gene have been obtained (Stalker *et al.*, 1988).
Atrazine also inhibits photosynthesis by binding to the Qb protein in the thylakoïd membrane, thus blocking electron transport. The resistance gene has been isolated from an atrazine resistant *Amaranthus hybridus*. The gene is a mutated *psbA* gene (photosystem II thylakoid membrane protein gene), that codes for a modified Qb. It has been fused to a transit peptide sequence for chloroplast targeting. Transgenic tobacco plants indeed appeared to be tolerant to 100 μM atrazine (Cheung *et al.*, 1988), but no other reports concerning the use of atrazine as a selective agent in transformation studies are known. There also exists an atrazine-detoxifying system based on the

glutathione-S-transferase *gst* gene (Shah *et al.*, 1986[b]), but this has not been applied in transformation studies.
2,4-D, 2,4-dichlorophenoxyacetic acid, is an auxin analog that is competitive to indol acetic acid (IAA), by occupying binding sites of auxin receptors. The resistance gene *tfdA* has been cloned from the soil bacterium *Alcaligenes eutrophus*. This *tfdA* gene codes for the enzyme 2,4-dichlorophenoxyacetate monooxygenase that is involved in the degradation of 2,4-D. The gene has been introduced into tobacco (Streber and Willmitzer, 1989), resulting in transgenic plants with an enhanced tolerance to 2,4-D. However, in this study, kanamycin was used to select for transformed tissue and selection by 2,4-D alone did not yield any transformed shoots, possibly because of the overgrowth of untransformed tissue. Thus the use of 2,4-D as selective agent is doubtful, also because 2,4-D is very toxic for dicotyledonous plants, whereas monocotyledonous plants have shown to be tolerant (Streber and Willmitzer, 1989).

Other selective agents: methotrexate

Methotrexate causes a lack of thymidylate by inhibiting the enzyme dihydrofolate-reductase. Because thymidylate is a precursor of one of the components of DNA, nucleotide biosynthesis is blocked, and cell death results. Resistance can be conferred by the mouse dihydrofolate reductase gene (*dhfr*). This gene codes for an enzyme with a reduced affinity for methotrexate. It has been applied in transformation of *Petunia hybrida* (Eichholtz *et al.*, 1987), *Brassica napus* (Pua *et al.*, 1987), and transformation of monocots like rice (Meijer *et al.*, 1991). Also for grass species such as *Panicum maximum* it has shown to be a useful selective agent (Hauptmann *et al.*, 1988). In all cases, transgenic plants or transgenic calli have been obtained. However, Meijer *et al.* (1991) question the suitability of methotrexate as a selective agent for rice because of an inhibition of other enzymes involved in the synthesis of nucleotides.

Resistance mechanisms and the selection of transgenic plants

In the previous sections various selective agents and selectable marker genes have been described. Their applicability for plant transformation is related to factors like transport of the chemicals through the plant tissues, but is also dependent on the mechanism by which resistance is conferred to the plant cells.
Resistance of plant cells to the aminoglycoside antibiotics mentioned above, is obtained by introduction of a gene encoding a detoxifying enzyme into the plant genome. This novel enzyme detoxifies the selective agent and allows growth of transformed cells, whereas untransformed cells are hampered in growth. One of the major problems encountered when using aminoglycoside antibiotics, is protection of untransformed tissue by surrounding transformed cells (Christou *et al.*, 1991, Escandón and Hahne, 1991, Hinchee *et al.*, 1991). By detoxification, the effective concentration of the antibiotic in the vicinity of transformed cells is decreased. Such a "cross-protection" allows regeneration of untransformed cells, leading to many escapes. Hinchee *et al.*

(1991) investigated the effect of the expression level of the resistance genes on the number of escapes by using various promoters. The use of a less effective promoter upstream of the kanamycin resistance gene decreased the number of escapes, possibly due to a slower detoxification of the antibiotic and hence less cross protection.
There are few data concerning cross protection when G418 or hygromycin were used as selective agents. Christou *et al.* (1991) mentioned the problem when using hygromycin for transformation of rice. Considering the analogous mode of action and because of the very similar detoxifying resistance mechanism, the problem of cross protection certainly has to be taken into account.
Resistance to PPT is also obtained by a detoxifying enzyme, so the same problems concerning cross protection might be expected. Indeed, Christou *et al.* (1991) found that non-transformed tissue of rice had the same regeneration capacity compared to transformed tissue, as a result of detoxification of the selective agent by transformed cells. Other approaches, not based on detoxification, can be used to obtain PPT-resistant plants as summarized by De Block *et al.* (1987). The level of GS expression can be increased by gene amplification, or by overexpressing a GS encoding gene. Another possibility is the introduction of a mutant form of GS not inhibited by PPT. Irrespective these alternatives, the detoxifying system such as the combination of the selective agent Basta and the resistance gene *bar* has been applied successfully for many plant species.
Resistance to methotrexate and to herbicides like glyphosate, sulfonylureas and atrazin is not obtained by detoxification, but the target enzyme is rendered less sensitive, either by introducing into the plant genome a gene encoding a modified target enzyme, or by overproducing the target enzyme. In potato the use of glyphosate in combination with the modified *epsps* gene led to reduced numbers of escapes compared to the use of kanamycin in combination with the *aphA2* gene (Hinchee *et al.*, 1991). This might be explained by the different resistance mechanisms. The modified EPSPS enzyme does not detoxify the herbicide, so there is no cross protection of untransformed cells. Moreover, the mobility of glyphosate in the phloem of the vascular bundles ensures a better distribution of the herbicide in the plant tissue.

Vectors specifically designed for monocot transformation.

To obtain a sufficient selection efficiency the level of expression of the marker gene is very important. Especially in experiments where the number of stable transformation events is low, as in particle bombardment of monocot tissues, it is important to achieve an adequate expression level of the resistance gene in the transformed cells. However, care must be taken to control the number of escapes (Hinchee *et al.*, 1991) as discussed in the previous section.
Expression of the transgene depends on several factors, for instance on its position in the plant genome and its copy number. At present, no well established procedures exist to regulate the site and mode of integration of genes. However, the expression of the resistance gene can be influenced by changing the promoter sequences fused to it.

The CaMV 35S promoter is a strong, constitutive promoter used in many transformation studies (Benfey and Chua, 1990), including those for the monocotyledons rice (Christou *et al.*, 1991; Datta *et al.*, 1990), wheat (Vasil *et al.*, 1992) and maize (Gordon Kamm *et al.*, 1990). However, Peterhans *et al.* (1990) showed that, although this promoter was active in rice, transcript accumulation was three times less than in tobacco. Also, the results of Mc Elroy *et al.* (1991) indicate that the activity of the CaMV 35S promoter in transformed monocot (rice and maize) cells is relatively low compared to tobacco. However, it was shown that the 35S promoter was much more active in rice than the promoter of the *csr1* gene of *Arabidopsis*, while this latter promoter was very active in the dicots tobacco and *Brassica napus* (Li *et al.*, 1992[b]). This indicates that the activity of dicot promoters in monocot tissues might even be lower than the activity of the CaMV 35S promoter.

Major improvements in transformation of monocotyledonous plants have been obtained by using promoters from monocot genes, like the pEmu promoter, a modified promoter of the maize *adh1* gene (Last *et al.*, 1991). Also the Act1 promoter of the rice *act1* actin gene (McElroy *et al.*, 1990) has been used in monocot transformation. In transgenic rice plants, the homologous Act1 promoter was more active than the heterologous CaMV 35S and maize Adh1 promoters (Zhang *et al.*, 1991). Transgenic sugarcane has been obtained by using the selectable marker gene *nptII* controlled by the pEmu promoter in combination with G418 as the selective agent. This promoter appeared to be sixty times stronger than the CaMV 35S promoter in sugarcane tissue (Bower and Birch, 1992).

The different performance of promoters in dicotyledonous or monocotyledonous cells might be due to differences in the respective transcription factors, it appears that dicot promoters are better recognised in heterologous dicots than in monocots. Furthermore, the CaMV 35S promoter is more active in dicot tissue than in monocot tissue. It can be concluded, that monocot promoters are the best choice for monocot transformation when a high level of expression is needed.

The activity of promoters in monocots can further be increased by the addition of an intron between promoter and coding region. The presence of the Adh1 intron strongly promoted expression in maize cells (Callis *et al.*, 1987; Luehrsen and Walbot, 1991). Such an enhancement of expression was also obtained in rice and maize cells with constructs containing the rice *act1* intron, whereas in tobacco the presence of the intron decreased gene expression (McElroy *et al.*, 1991). Also with the maize *shrunken1* intron an enhancement of gene expression has been found for various grass species (Vasil *et al.*, 1989). Stronger gene expression as a result of insertion of exon 1 and intron 1 of the *Shrunken-1* gene was shown in barley cells (Maas *et al.*, 1992), and in rice and maize protoplasts, while gene expression was impaired in tobacco protoplasts (Maas *et al.*, 1991).

These data show, that promoters isolated from monocot genes, such as pEmu or pAct1, in combination with a monocot intron between promoter and gene, can greatly enhance expression of the resistance gene and can be used to improve transformation efficiency in monocots.

Conclusions and practical use

In this paper, an overview is given of the effects of various selective agents and respective resistance genes (Table 1). Emphasis was put on their suitability for monocot transformation and on the use of specific vectors that cause a high expression level of the selectable marker gene.

Most selective agents used are inhibitors of protein synthesis, the aminoglycosides on the level of translation, the herbicides on the level of amino acid biosynthesis. Less frequently used agents with a different mode of action are methotrexate and bleomycin that inhibit DNA synthesis and cause DNA breakdown respectively. Bromoxynil and atrazine are photosynthesis inhibitors and 2,4-D affects hormone action in plants.

In transformation studies, kanamycin is still one of the most frequently used selective agents. However, especially in monocot tissues, a major problem is the relatively high resistance to this antibiotic, resulting in a high number of untransformed regenerants (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989). For plants showing such a high tolerance, G418 or hygromycin (Walters *et al.*, 1992) can offer an effective alternative, because many tissues generally exhibit a higher sensitivity for these antibiotics. Another possibility is the use of a herbicide as selective agent. PPT and glyphosate are more and more used in monocot transformation. The number of escapes is then greatly reduced, especially when the resistance mechanism is based on modification of the target enzyme, as in the *epsps* - glyphosate system. Moreover, introduction of the gene is sometimes a goal on its own, because for some crops herbicide resistance is a commercially interesting trait.

The effective concentration of the selective agent to be applied in tissue culture has to be determined for each crop and for each type of explant. Usually, a concentration is chosen that stops growth of untransformed tissue, but allows proliferation of transformed cells, without causing too much cell death. Dying cells release products which are toxic and inhibit the regeneration of transformed cells (Klee *et al.*, 1987). Moreover, especially in an untransformed environment, single transformed cells can only proliferate when the surrounding cells are not killed, but just hampered in growth.

Success of a selection procedure is also based on the resistance mechanism involved: detoxification of the selective agent by means of an enzyme encoded by the transgene or production of a modified target enzyme of the selective agent in transgenic plants. In the first approach, there is a chance for cross protection by detoxification of the selective agent mediated through the expression of the resistance gene in transformed cells (Christou *et al.*, 1991; Escandón *et al.*, 1991). The number of escapes can be reduced by controlling the level of expression of the resistance gene.

In general, selection is applied rather soon after transformation. In *Agrobacterium*-mediated transformation, the selective agent is added to the medium after a cocultivation period of one to five days. In direct gene transfer protocols, selection is applied immediately or within a few days. There are no reliable studies on the effect of the timing of the selection but immediate selection will give transformed cells a rapid

selective advantage over untransformed cells. On the other hand, the resistance gene also needs some time to become expressed, and cells need some time to recover from the transformation event. Delayed selection can account for this, but then preferential division of untransformed cells might result in the formation of chimaeras and escapes which is a problem in transformation studies (Fromm *et al.*, 1990). So, the moment at which the selection pressure should be applied, has to be established carefully for each transformation protocol.

In summary, there are several ways in which an effective selective regime for monocot transformation can be established. Kanamycin is not the most appropriate agent to use for selection of transformed cells, but G418 and hygromycin provide good alternatives. The use of herbicides however, has an even better potential, especially if these are transported in the vascular tissue and if the resistance mechanism is based on modification of the target enzyme, as is the case of glyphosate resistance. Also herbicides like PPT, that are detoxified by a resistance enzyme expressed in transgenic cells, have a very good potential for use in monocot transformation. The expression can be regulated by the use of appropriate promoter and intron combinations.

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Chapter 3

Adventitious shoot formation in tulip: Histological analysis and response to selective agents

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Abstract

On explants taken from young floral stems of tulip (*Tulipa gesneriana* L.) adventitious shoot formation was induced. The shoots were formed directly from the explants without an intermediate callus phase. More shoots developed on explants taken from dry-stored bulbs than from bulbs stored in ice. At the basis of the shoots a meristem was formed that developed into a new bulb. The ability to form this bulb meristem was compared in eight cultivars, and was highest in 'Lucky Strike' and 'Monte Carlo'. Adventitious shoot formation was initiated in the first two subepidermal cell layers and a number of cells of the original explant contributed to the shoot formation. To avoid the formation of chimeric transformed shoots in future transformation, an optimal selection system was established. For selection purposes, the aminoglycoside antibiotics were not very effective in inhibiting shoot formation, since tulip tissue showed a high tolerance for kanamycin, while G418 and hygromycin induced severe necrosis of the explants at low concentrations. In contrast, the herbicides phosphinothricin and glyphosate were very effective and offer good perspectives to be used for selection of transformed shoots.

Introduction

Over the last years for many monocotyledonous crops transformation procedures have been established. Transgenic plants have been obtained from cereals like rice [1], maize [2,3], wheat [4], oat [5], sorghum [6] and barley [7]. Also for sugarcane [8] and tall fescue [9] successful transformation procedures have been developed. In most cases, selectable marker genes were introduced into cells or protoplasts derived from embryogenic tissues.

For the establishment of a transformation system for tulip, a monocotyledonous ornamental crop which can only be regenerated via adventitious shoot formation, clonal material should be applied as target tissue. In this species, adventitious shoot formation has been described for bulb segments by Nishiuchi [10] and for floral stem segments by Le Nard *et al.* [11]. Because bulb segments suffered from reduced vitality and browning [12], the floral stem system seems to be more appropriate to be used in a transformation procedure. This system, however, also deals with a number of problems that have to be solved. Firstly, the floral stems can only be used during a limited period of time, when they start developing at the end of dry storage (December till February). Le Nard [13] adapted the post-harvest treatment of bulbs, and by using more elongated floral stems the regeneration season was extended. In our system we chose to store bulbs with developing floral stems in frozen soil (-2°C; ice-stored bulbs), by which they maintained their regenerative capacity for several months (January till April). Secondly, regenerated shoots from floral stem segments are mostly leaf-like structures. Only a small percentage (5-20%) is having functional meristems and is capable of developing bulbs [14-16] which can be transplanted into soil.

Here we report on research on a number of aspects of the tulip adventitious shoot regeneration system that are important for establishment of a genetic transformation system for this crop. First, we compared the regeneration capacity of floral stems from dry- and ice- stored tulip bulbs. Secondly, we investigated whether the ability of adventitious shoots to form a functional meristem was cultivar-dependent, in order to find a cultivar with good meristem formation to be used for transformation experiments. Thirdly, the regeneration process was analyzed histologically, in order to determine the single or multicellular origin of the adventitious shoots. In this part of our study, we demonstrated that the adventitious shoots had a multicellular origin. After transformation, this might raise problems concerning the efficiency of selection and the occurrence of chimerism. Transformed cells need a selective advantage, but the surrounding cells should not be killed [17]. Selective agents commonly used in dicot transformation procedures proved to be inefficient for monocots [18]. At last, the effect of various selective agents (antibiotics and herbicides) on shoot regeneration from floral stem segments was evaluated and an appropriate selective regime for tulip was established.

Material and methods

Plant material
Tulip bulbs of eight cultivars (Table 1), all belonging to *Tulipa gesneriana* L. were obtained from commercial growers in September. They were stored at 20°C until October, followed by 17°C storage until the beginning of December. At this stage, part of the material was used for regeneration (dry-stored bulbs), the remaining bulbs were planted in pots. After a four weeks period rooting at 9°C, these bulbs were frozen at -2°C. These so called ice-stored bulbs provided the floral stem material for regeneration experiments. At various times they were thawed at 17°C and floral stems were excised 2-3 days after thawing, when they were 2-3 cm long.

Regeneration from floral stem explants
Ten segments of 1-2 mm thick were excised from a single floral stem and incubated in 6 cm petri dishes containing MS medium [19] supplemented with sucrose 40 g/l, casein hydrolysate 1.5 g/l, 1-naphthalene acetic acid (NAA) 1 mg/l, 2-isopentenyl adenine (IPA) 1.5 mg/l and Daichin agar 7 g/l ("regeneration medium"). The pH was adjusted at 6.0 before autoclaving. Cultures were kept at 17°C in continuous darkness for 10 weeks. Floral stem segments from dry bulbs were incubated in December and the beginning of January, segments from ice-stored bulbs were incubated from January until August.

Development of functional meristems in various cultivars
Adventitious shoots were cultured to develop functional bulb meristems. Ten weeks after incubation on regeneration medium, shoots were subcultured in jars with tulip growth medium and kept at 17°C and 16 h light (Philips TL 85 fluorescent lamps, 36 μmol/m^{2}s). The growth medium consisted of MS macro elements at half strength, MS micro elements at full strength, adeninesulphate 80 mg/l, thiamine 10 mg/l, sucrose 30 g/l, benzyladenine (BA) 1 mg/l, indolebutyric acid (IBA) 0.5 mg/l and Daichin agar 7 g/l. The pH was adjusted at 6.0. This medium was refreshed every 7 weeks. Ten segments

of 8 bulbs of 8 cultivars (Table 1) were examined. After 24 weeks the number of adventitious shoots that had developed a functional meristem were scored.

Histological analysis
The origin of the adventitious shoots was studied with the cultivar Lucky Strike. At various times after incubation on regeneration medium, floral stem segments were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer pH 7, rinsed in 0.1 M phosphate buffer pH 7 and dehydrated through an ethanol series of 50-100%. They were embedded in a series of 25-100% Technovit 7100 (Kulzer) according to manufacturer's instructions. Sections of eight micron prepared with a microtome were stained with toluidine blue (1 g toluidine blue and 1 g Na tetracarbonate in 100 ml H_2O), covered with glycerine and analysed microscopically.

Selection experiments
Antibiotics (kanamycin, G418 and hygromycin) and herbicides (phosphinothricin (PPT), commercial Basta and glyphosate) were tested at various concentrations. Kanamycin, G418, hygromycin and PPT were purchased from Duchefa, glyphosate was provided by Monsanto Co. and Basta is a commercial herbicide, produced by Hoechst.
Ice-stored bulbs of cv. Prominence were used for the experiments, which were carried out from January until April. Ten floral stem segments of 10 bulbs were incubated on selective agent-containing regeneration medium. The medium was refreshed every 4 weeks and after 16 weeks the number of shoots (larger than 2 mm) was determined and expressed as a percentage of control values (*percentage of regeneration*). The *percentage of survival* was determined from the number of explants that remained vital and did not show necrosis relative to the control value. The effect of selective agents was also studied histologically, in order to determine the effect on cell division and proliferation.

Results

Shoot regeneration
Incubation of floral stem segments from dry-stored bulbs or ice-stored bulbs on regeneration medium resulted in the direct formation of adventitious shoots at the base of the explants (Fig. 1A). After 10 weeks of culture, adventitious shoots were transferred to growth medium and light, resulting in strong elongation and greening (Fig. 1B). Explants of some genotypes (e.g. Lucky Strike and Monte Carlo) started to form new adventitious shoots at the base of the primary ones, resulting in an increase of the regeneration frequency. Shoots that survived showed a thickened, yellow coloured shoot base (Fig. 1B), indicative for the presence of a functional meristem. This could be visualized by a longitudinal section through the shoot base (Fig. 1C). Shoots that did not contain such a meristem, deceased 20-30 weeks after start of the cultures.
Table 1 shows the regeneration capacity of eight tulip cultivars. Dry-stored bulbs were highly regenerative from December till February. Ice-stored bulbs were equally regenerative in January, the regeneration capacity declined until March, after that they could not be used any longer. By using both types of bulbs, the experimental period was doubled. All tested cultivars formed adventitious shoots. Comparison of cultivars with

respect to their regeneration capacity showed a large variation, but it was clear that for all cultivars dry-stored bulbs regenerated better than ice-stored bulbs.
The genotypic variation observed with respect to the number of shoots per explant was consistent for dry- and ice-stored bulbs. Highly regenerative cultivars were Lustige Witwe and Prominence, they produced significantly more shoots than e.g. Apeldoorn and Monte Carlo. With respect to the ability of adventitious shoots to form a functional meristem, Monte Carlo and Lucky Strike appeared to be outstanding, as demonstrated by a MS/S ratio of more than 100% (Table 1). This high MS/S ratio is due to secondary adventitious shoot formation during the growth period as described above. These newly formed shoots were all able to develop a functional meristem.

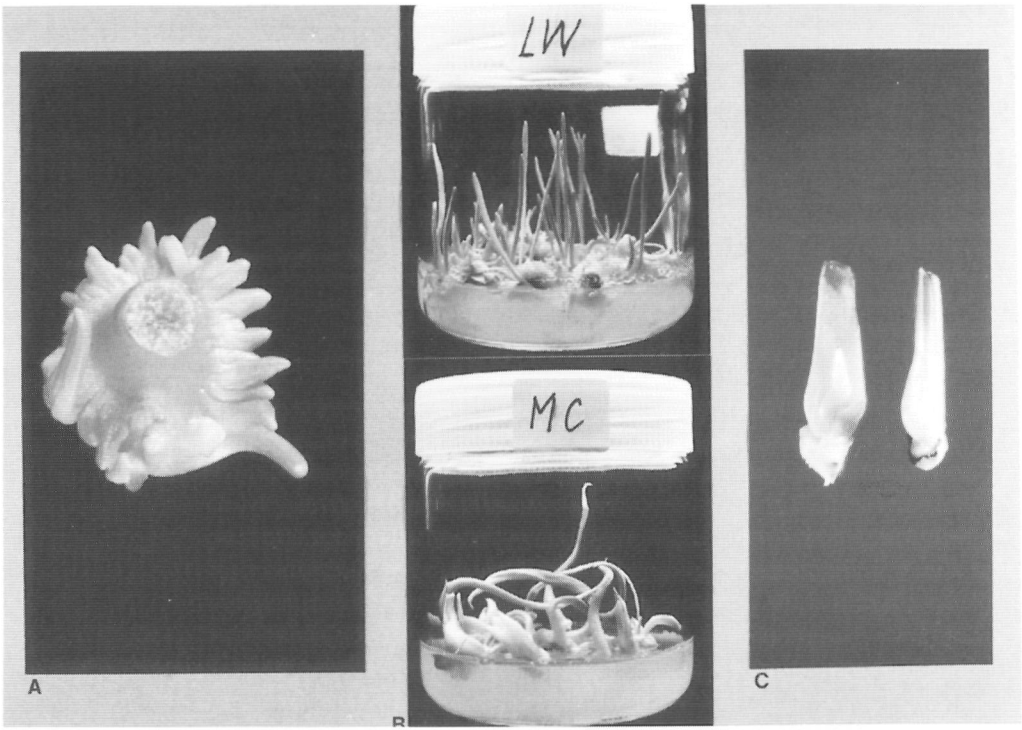

Fig. 1. Adventitious shoot formation of tulip.
A. Regeneration of a whorl of young shoots from a floral stem explant of cv. Prominence, 8 weeks after incubation on regeneration medium. B. Development of adventitious shoots in cv. Lustige Witwe (LW) and Monte Carlo (MC), after 24 weeks of culture. C. Presence of a meristem in an adventitious shoot of cv. Monte Carlo shown after longitudinal sectioning of the shoot base.

Table 1.
Regeneration capacity of eight tulip cultivars. Floral stem explants from dry- and ice-stored bulbs were incubated in December and March respectively. At both of the two dates, per cultivar eight floral stems were used and 10 explants were taken from each stem. Numbers of adventitious shoots initiated (> 2 mm, S) were counted after 10 weeks of culture, and numbers of shoots having a meristem (MS) after 24 weeks of culture. Numbers represent mean numbers of shoots per floral stem (that is per 10 explants).

	Dry-stored bulbs				Ice-stored bulbs			
Cultivar	S 10 wks		MS 24 wks	MS/S *100%	S 10 wks		MS 24 wks	MS/S *100%
Apeldoorn	19,3	ab	1,5	8	9,8	a	0,4	4,1
Christmas Marvel	46,8	c	nd	nd	31,5	bc	5,1	16,1
Kees Nelis	41,6	bc	nd	nd	18,1	abc	6,1	33,7
Leen vd Mark	28,1	bc	nd	nd	11,2	a	0,7	6,3
Lucky Strike	nd		nd	nd	17,8	b	21,1	118,5
Lustige Witwe	97,3	cd	3,6	4	38,1	c	0,8	2,1
Monte Carlo	17,1	a	nd	nd	16,2	ab	22,1	136,4
Prominence	96,3	d	4,9	5	40,8	c	1,2	2,9

a,b,c,d: Values followed by the same letter are not significantly different, based on LSD determined by Students' t-test ($P \leq 0.05$).
nd: not determined

Histological analysis

Floral stem segments of the cultivar Lucky Strike (ice-stored bulbs), incubated on regeneration medium for various periods, were embedded and sectioned. Transversal sections representing different developmental stages are presented in Fig. 2. During the first days after the start of incubation only enlargement of cells was observed, but after about 13 days first cell divisions were observed in the cortex (Fig. 2A). They were found all over the explant and division planes were randomly orientated. A few days later, after 17 days of culture, cells in the first subepidermal cell layer underwent periclinal divisions (Fig. 2B), and gave rise to the formation of elongated cell clusters. Subsequently, anticlinal divisions occurred in these newly formed cells, and clusters of eight to ten cells wereformed out of the original subepidermal cells. At the same time, periclinal cell divisions were visible in the second subepidermal cell layer and gave rise to the formation of elongated clusters of cells as well (Fig. 2C). Fig. 2D shows that the clusters originating from both subepidermal cell layers enlarge by means of periclinal and anticlinal cell divisions. In contrast to this, cells of the epidermis only occasionally underwent periclinal divisions. In most pictures, the dark stained cell walls that indicate the original subepidermal cell layer boundaries remained visible (e.g. arrows in Fig. 2B,C,D,F). Fig. 2E shows the onset of the regeneration process after 20 days. In the cell clusters that originated in the two subepidermal cell layers, meristematic regions had appeared. This

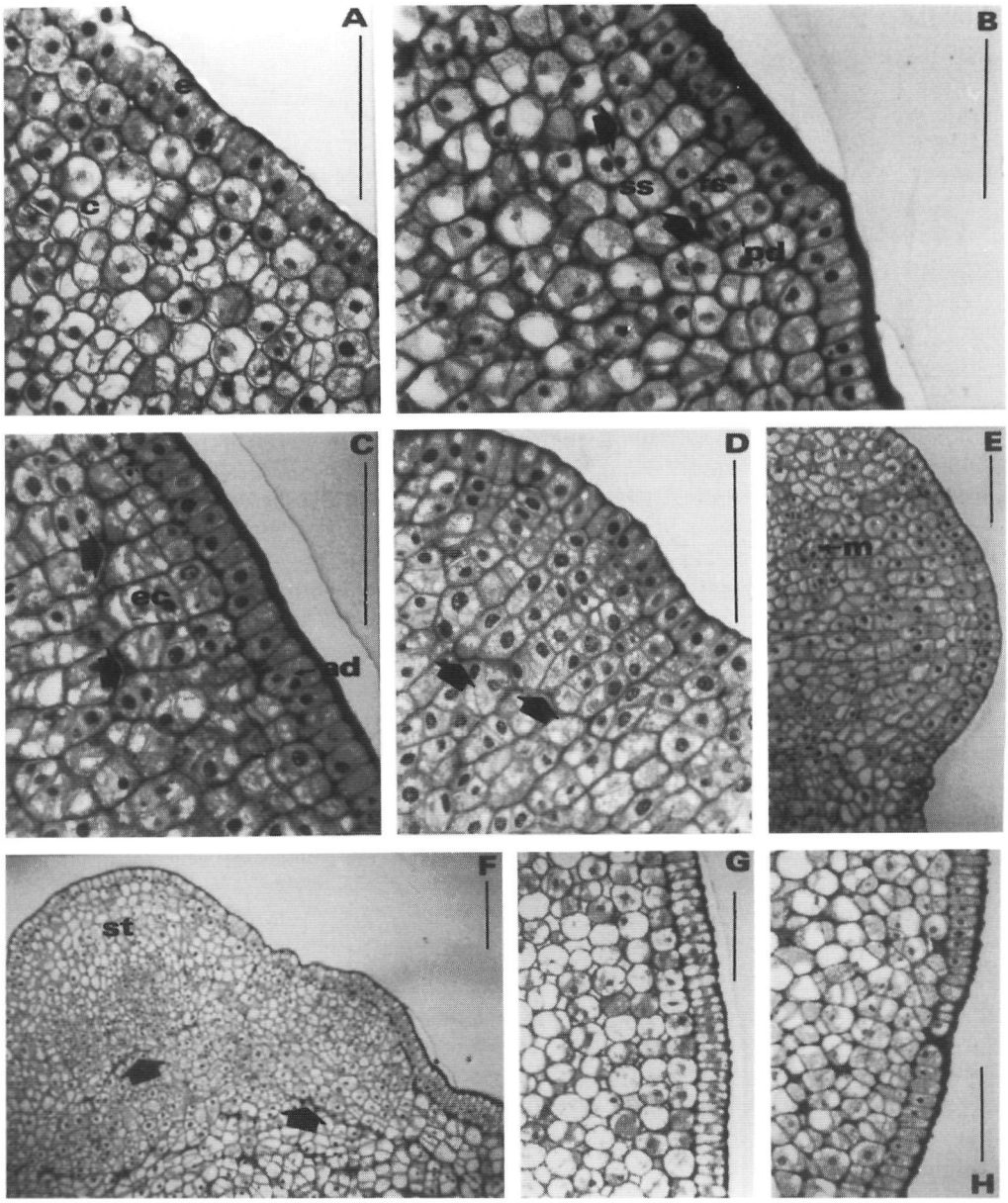

Fig. 2. Transversal sections representing the regeneration process at various days after incubation on regeneration medium: A: 13 days; B/D: 17 days; E: 20 days; F: 27 days; G: The regeneration process under selective conditions: 1 mg/l Basta, 23 days on selective medium; H: 50 days on selection medium. Bars represent 200 μM.

e = epidermal layer; c = cortex; fs = first subepidermal cell layer; ss = second subepidermal cell layer; pd = periclinal divisions; arrow(dw) = darker stained older cell wall; ec = elongated cell cluster that originates from one subepidermal cell; ad = anticlinal divisions; m = meristematic region; st = tip of the future shoot, originating from the first subepidermal cell layer.

is also evident in Fig. 2F, that illustrates the further development of the regeneration process 27 days after incubation. The origin of these meristematic regions from the first and second subepidermal cell layers is still visible. Fig. 2F is a typical illustration of young primordia that will grow out into new adventitious shoots.
The pictures clearly show that meristems originate from several cells, two subepidermal cell layers are involved in adventitious shoot formation. The original epidermal cells only undergo anticlinal and transversal divisions, and as such form the epidermis of the new adventitious shoots.

Selection experiments
The effects of various selective agents on regeneration of tulip tissue were tested by incubating floral stem segments on regeneration medium containing various concentrations of antibiotics or herbicides. For these studies we used ice-stored bulbs from the cultivar Prominence, that showed a high regeneration capacity. The results are shown in Fig. 3. Tulip tissue had a high tolerance for kanamycin (Fig. 3A,B). All explants survived when this antibiotic was present in the regeneration medium, there was no necrosis at all, not even at the highest concentration (Fig. 3A). The percentage of regeneration was reduced to about 30% in the presence of 50 mg/l kanamycin, but even at a high concentration of 500 mg/l a low level of 6% regeneration was still observed. The use of more extreme concentrations was not considered. Instead, the antibiotics G418 (Fig. 3C,D) and hygromycin (Fig. 3E,F) were tested at various concentrations, and showed a stronger effect. Necrosis was observed in most explants at concentrations of 25 and 10 mg/l respectively (Fig. 3C,E), inhibition of shoot regeneration occurred at even lower concentrations. Only at the lowest concentrations of G418 and hygromycin tested (5 mg/l), a regeneration level of 15-25% was observed compared to the control treatment (Fig 3D,F). Obviously, G418 and hygromycin performed better than kanamycin as a selective agent, however, fast progression of necrosis was a problem. In order to search for agents that inhibit regeneration, whithout too much tissue damage, the use of herbicides was investigated.
At all concentrations of the herbicides phosphinothricin and glyphosate tested the explants remained relatively healthy (Fig. 3G,I). On the other hand the herbicides had a dramatic effect on adventitious shoot formation. Regeneration was almost completely inhibited at concentrations PPT of 5 mg/l and higher (Fig. 3H). For glyphosate a concentration of 0.1 mM (17 mg/l), although commonly used for transformant selection [20, 4], completely blocked regeneration of tulip tissue (Fig. 3K). The prospectives of the use of PPT were further investigated. The effect of the active compound (PPT) was compared with the effect of the herbicide itself (Basta). Addition of the herbicide had a stronger effect, both the percentage of survival and the percentage of regeneration were reduced compared to addition of PPT (Table 2). If Basta was applied 1 week after the start of the incubation, tissue death was reduced, but regeneration was strongly inhibited. The effect of Basta could also be enhanced by omitting casein hydrolysate from the medium. Under these conditions, the vitality of the explants was reduced to 18 % or less when 5 mg/l Basta or more was applied, and formation of adventitious shoots was fully inhibited, even at the lowest Basta concentrations used (1 mg/l) (Table 2).In order to visualize the effect of selective pressure on the initiation of shoots, we transferred explants one week after incubation from regeneration medium to medium

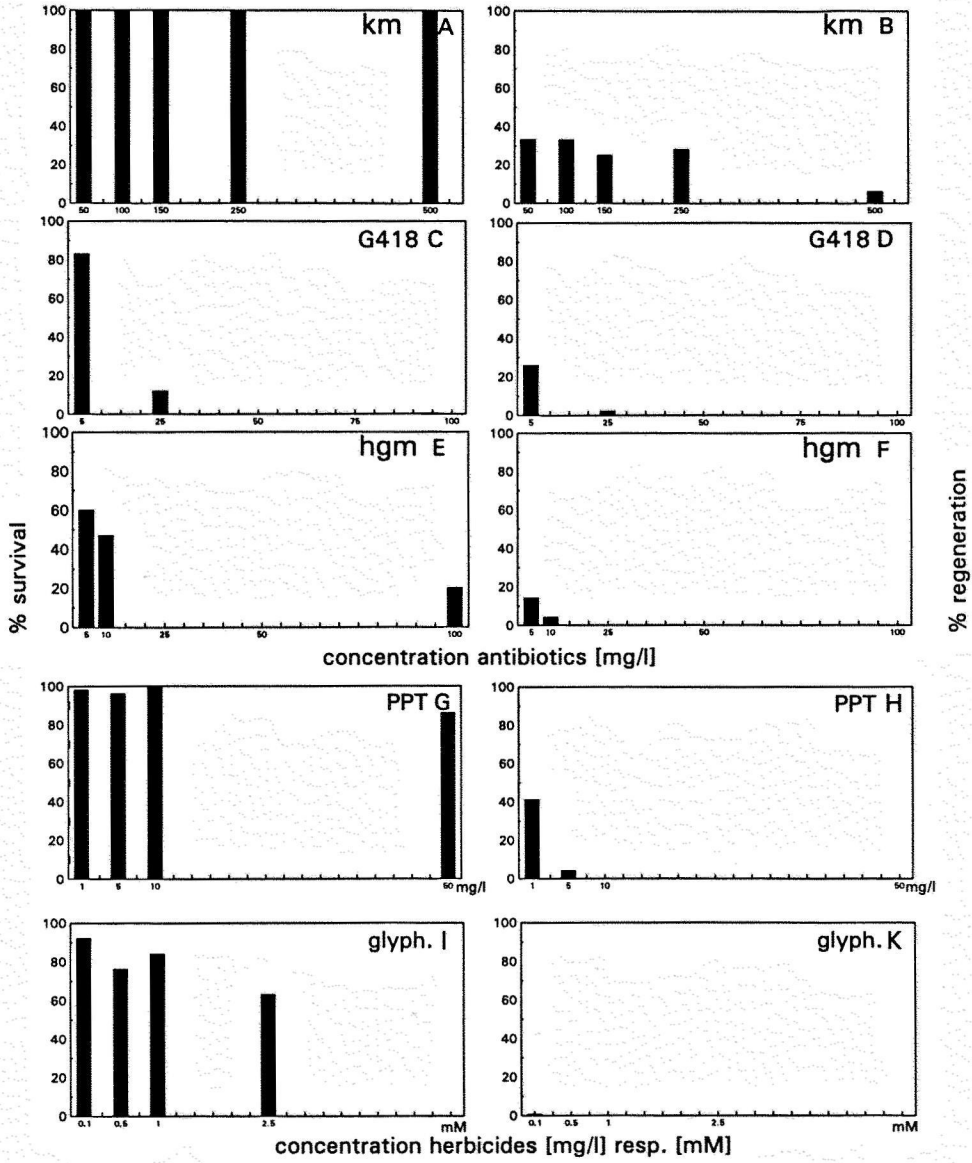

Fig. 3. Regeneration and survival of tulip floral stem explants after incubation on medium containing various concentrations of antibiotics or herbicides. The % regeneration and the % survival represent respectively the number of regenerating shoots and the number of vital shoots as a percentage of the value of the control treatment (no selective agent). All concentrations that were tested are given on the X-axis.

A,B: Kanamycin (km); C,D: G418; E,F: Hygromycin (hgm); G,H: Phosphinothricin (PPT); I,K: glyphosate (glyph.); A,C,E,G,I: % survival; B,D,F,H,K: % regeneration.

Table 2
Effects of PPT and Basta on survival (%surv) and adventitious shoot formation (%reg) of floral stem segments of tulip Basta was applied in medium with casein hydrolysate immediately after incubation (Basta), one week after incubation (Basta delay) and in medium without casein hydrolysate (Basta-cas) PPT was applied immediately Analysis was done after a culture period of 15 weeks. Data represent the percentage of the control value 0 mg/l (100%)

conc	PPT		Basta		Basta delay		Basta-cas	
mg/l	%surv	%reg	%surv	%reg	%surv	%reg	%surv	%reg
1	98	41	100	12	104	0 4	88	0
5	96	0	66	0	100	0	18	0
10	100	0	73	0	100	0	4	0
50	86	0	73	0	105	0	0	0

containing 1 mg/l Basta and no casein hydrolysate, and embedded them at various times for histological analysis. Generally speaking, the process of cell divisions in the subepidermal cell layers was delayed in the presence of Basta. After 23 days of incubation, in most explants cell division was arrested completely (Fig. 2G), or occasionally periclinal and random divisions were formed in the subepidermal layer (Fig 2H). Under non-selective conditions adventitious meristems had already been formed at that time, as shown in Fig. 2E.

Discussion

In this paper we described the application of a procedure for adventitious shoot formation on explants from floral stems of tulip. One of the main problems in tulip tissue culture is the lack of a continuous propagation system. New cultures have to be started from fresh bulbs, on developing floral stem explants. Explants taken from dry-stored bulbs are only available during a rather short season, but cold storage of bulbs extended the period in which material for regeneration studies was available. The number of adventitious shoots formed on explants of ice-stored bulbs was lower than the number obtained by using as starting material bulbs kept under normal practical conditions (Table 1) [21, 11]. Another way to extend the regeneration season was described by Le Nard [13], who used early-lifted, cooled bulbs from September till November. By combining these various methods, and by using bulbs grown on the Southern hemisphere, it is now possible to produce adventitious shoots of tulips in various seasons The quality of the formed shoots is strongly dependent on the formation of a meristem that produces the first new bulb [14]. Most cultivars tested produced only few meristem-containing shoots, and for these cultivars the regeneration procedure still awaits further improvement [15, 16]. It is intriguing to notice that especially cultivars which are able to develop secondary adventitious shoots have a high MS/S ratio (Monte Carlo and Lucky Strike) These secondary adventitious shoots all developed a functional meristem, and further improvement of the regeneration procedure should perhaps be focussed on this phenomenon.

The cellular origin of the adventitious shoots was investigated by a histological characterization of the regeneration process. The results clearly showed that shoots did not originate from single cells, but from multi-cellular, subepidermal regions, while the epidermal layer participated by means of anticlinal and transversal divisions, and developed into the epidermis of the new shoots. This is in contrast with the results from histological studies on tulip as performed by Chanteloube *et al.* [22], who concluded that adventitious shoots developed from epidermal cells only. Despite extensive histological studies, we have not been able to find periclinal epidermal cell divisions. From our studies, we have to conclude that adventitious shoots emerged from the outer two subepidermal cell layers, while the epidermal layer formed the new epidermis.

The multi-cellular origin of the adventitious shoots might cause problems in the application of this regeneration system for transformation. To prevent chimerism, transgenic cells need to have a selective advantage. Surrounding cells should be inhibited, but not killed, because dying cells might inhibit growth of potentially transformed cells [17]. Moreover, the percentage of regeneration of untransformed tissue in the presence of a selective agent should be as low as possible, to prevent the formation of escapes after transformation. Like reported before for other monocots [18], we found a high kanamycin tolerance in tulip tissue. The use of herbicides appeared to be much more advantageous, which agrees well with studies on other monocotyledonous species [3, 5, 23, 24, 25]. The higher efficiency of Basta compared to its active compound PPT might be due to the presence of effluents in the herbicide, that might cause a better uptake of the herbicide in the explant. Moreover, Dennehey *et al.* [26] have shown that use of the herbicide instead of the active compound did not have negative effects on the growth and health of transgenic maize callus. The selection system could be further improved by omitting casein hydrolysate from the regeneration medium, because the high concentration of amino acids in casein hydrolysate overcomes the inhibiting effect of the herbicide [25]. A one week delay in the application of Basta appeared to be beneficial for the condition of the explants, probably because explants are permitted to overcome wounding due to excision in the absence of selective pressure. Because first cell divisions only occur after two weeks (Fig 2A), this delay in selection is not expected to favour the proliferation of non-transformed cells In our opinion, this selection system (i e. 1 mg/l Basta, to be applied one week after transformation, in a medium without casein hydrolysate) would give the best chances for resistant cells to proliferate, while non-resistant cells are inhibited in growth without dying The system will now be used in transformation experiments with tulip In preliminary experiments it has already been shown that particle bombardment can be used for the introduction of genes in floral stem segments [27]. We will now focus on obtaining Basta-resistant adventitious shoots, and bulblets developed from these shoots.

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chapter 4

Activity of constitutive promoters in various species from the *Liliaceae*

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Abstract

In this paper we first review literature on the performance of various promoters in monocotyledonous species. In general, promoters isolated from monocots show a higher activity in monocot species. Moreover, the presence of an intron between the promoter and reporter gene increases transcription levels. We used the same approach to study gene expression in *Liliaceae*.
The activities of the CaMV 35S, maize Adh1-based pEmu, rice Act1 and maize Ubi promoters, coupled to the *ß-glucuronidase (gus)* reporter gene were evaluated for transient gene expression following particle bombardment of tissues of tobacco, rice, tulip, lily and leek. Although monocot promoters performed very well in rice tissues, the results of this study show that this cannot be generalized for other monocot species. The transcription inducing effects of monocot promoters were less pronounced or even absent in tissues of *Liliaceae*, while the presence of an intron between promoter and *gus* gene reduced promoter activity.

Abbreviations:
CaMV: Cauliflower Mosaic Virus; Adh: Alcohol Dehydrogenase; Act: Actin; Ubi: Ubiquitin

Activity of various promoters in monocot and dicot species.

Expression cassettes specific for monocots may contribute to the development of efficient transformation procedures for such monocots, as was reviewed by McElroy and Brettell [16]. In general, the CaMV 35S promoter is active in dicot tissues, but in monocots a much lower activity is obtained [9, 15, 18]. A number of investigations has been devoted to compare monocot promoters and the CaMV 35S promoter in both monocots and dicots. We will shortly summarize the quantified data, before discussing our own results. Data are presented in Table 1, in which the increase in gene expression using monocot promoters is normalized relative to the activity of the CaMV 35S promoter. The variation between experiments was very large and the expression increasing effect in some cases might be influenced by the low absolute activity of 35S in monocots. However, it can be concluded that monocot promoters are more active in monocot tissues than in dicot tissues (i.e. tobacco and carrot). The maize Adh1 promoter was active at a relatively low level in the monocots tested. However, the addition of multiple enhancer elements to a truncated part of the Adh1 promoter (Emu) resulted in high constitutive expression [9]. Other constitutive monocot promoters used in these studies were the rice actin promoter Act1 [15, 24] and the maize ubiquitin promoter Ubi [2], which achieved a far better expression compared to 35S in all monocots tested. The increase in expression sometimes amounted to several tenfolds. Dicot tissues showed a different response: the activities of monocot promoters were comparable to or less than the activity of the CaMV 35S promoter (Table 1).

Table 1 Summary of literature on the activity of various promoters in monocot and dicot crops. 35S is the intronless CaMV promoter, 35Si is the CaMV 35S promoter in combination with the maize adh1 intron, unless shown otherwise. The maize promoters Adh1 and Emu are used in combination with the adh1 intron as well, while the rice Act1 and the maize Ubi promoters each are combined with their original intron. Also see Fig. 1. Data were calculated relative to the activity of the CaMV 35S promoter (activity = 1)

	35S	35Si	Adh1	Emu	Act	Ubi	ref
tobacco	1	0,3[1]			0,0		[15]
	1	1,2		0,8			[9]
	1					0,1	[2]
	1			0,0	0,0	0,1	[18]
carrot	1	2,1		1,2			[17]
maize	1	5,6	2,2		29		[15]
	1	1,6		41			[9]
	1					12,5	[2]
	1	8,0[2]	2,5			13	[4]
	nd		1			2,5	[20]
	1			6,7	11,1	13,2	[18]
wheat	1	0,6		40			[9]
	nd		1			34	[20]
rice	1	4,2	4		23		[15]
	1	1,3		25			[9]
	nd	1	1,5		1,5	10	[3]
barley	1			15,6	6,4	11,9	[18]
lolium	1	0,5		8,8			[9]
sugarcane	1	0,5		400			[17]
	1			10	10	50	[5]

[1]: measured with *act1* intron, see Table 2
[2] measured with *adh1* intron 6

Promoter activity increases with introns of monocot genes

A number of experiments have been described in the literature showing enhanced gene expression in monocots by introducing a monocot intron between the promoter and reporter gene. Table 2 summarizes results of studies on the effect of various monocot introns (act1, adh1 and sh1) on the activity of the CaMV 35S promoter. Although the

relative activities vary between the experiments, it is clear that the presence of an intron increased *gus* expression in most monocots. The shrunken1 (sh1) intron proved especially effective, and even higher activities were obtained by using the shrunken first exon in combination with the first intron [11]. A negative effect of the adh1 intron between CaMV 35S and *gus* gene on promoter activity in monocots was only observed by Last *et al.* for wheat and *Lolium* [9] and by Rathus *et al.* for sugarcane [17]. For tobacco, a dicot, a similar decrease in expression was reported [11, 15].
Because of the promoting effect of the first intron, it might be questioned whether the activating effect of monocot promoters as shown in Table 1 was due to the promoter only or partly due to the presence of a monocot intron in these constructs. Comparison of the activity of the 35Si, Adh1 and Emu promoter (all containing the adh1 intron) shows the expression-activating effect of the intron, relative to the activity of the intron-less CaMV 35S promoter. But the high activity of the Emu promoter compared to the Adh1 is not due to the presence of the intron, since both constructs contain the same adh1 intron, but is the result of the addition of enhancer elements (Fig. 1). For a true comparison of promoter- versus intron-effects however, also the monocot promoters should be tested with and without addition of an intron.

Activity of monocot promoters in tulip, lily and leek

Tulip (*Tulipa gesneriana* cv Lucky Strike), lily (*Lilium longiflorum* cv Snowqueen) and leek (*Allium ampeloprasum* L.) belong to the *Liliaceae*, and for these crops transformation procedures are being developed [21, 23]. The objective of this study was to determine the activity of monocot promoters in tissues of the non-cereal monocots tulip, lily and leek. For this we evaluated transient expression levels, which we assume to be indicative for stable expression levels when constitutive promoters are used.
The experiments were performed by particle bombardment of leaf explants or cell suspensions using particles coated with DNA constructs of four promoters, all driving the *gus* gene (Fig. 1). Expression was determined histochemically by counting the number of GUS expressing cells per bombardment of a 6 cm petri disk, or fluorimetrically, and data are given in rate, relative to the activity of the CaMV 35S promoter.
The activity of the chimeric gene constructs was tested in leaf explants (tulip and lily) or cell suspensions (leek). For comparison we used leaf explants from *Nicotiana tabacum* SR1 and cell suspensions of *N. plumbaginifolia* and rice (*Oryza sativa* cv. Indica rice 52). The results are presented in Table 3, absolute values for GUS expression are in Table 3A, while expression is normalized relative to 35S-directed expression in Table 3B.

N. tabacum and N. plumbaginifolia. The CaMV 35S promoter directed high expression levels in *N. plumbaginifolia*. The number of GUS positive cells observed in cell suspensions after bombardment with 35S-*gus* was approximately five-fold higher than the number found in the rice cell suspensions (Table 3A). The activity of all tested monocot promoters was decreased relative to the CaMV 35S promoter in cell suspensions of *N. plumbaginifolia*. The activity of Act was hardly detectable, in

Table 2. Summary of literature on the effect of various monocot introns on the activity of the 35S promoter. All values are relative to the activity of the intronless 35S promoter (activity = 1). *Act1* is the 35S promoter in combination with the rice *actin1* intron, *adh1* is the 35S promoter in combination with the maize *adh1* intron, unless otherwise indicated, *sh1* is the 35S promoter with the maize *shrunken1* intron.

	act1	*adh1*	*sh1*	ref.
tobacco			0,1	[11]
			1,3[4]	[11]
	0,3			[15]
maize		8,4		[1]
		4	35-50	[22]
		12-20[2]		[13]
	6,7[1]	3,7		[10]
			136	[11]
			875[4]	[11]
	60	5,6		[15]
		1,6		[9]
		8,0[3]		[4]
rice			110	[11]
			910[4]	[11]
	40	4,2		[15]
		1,3		[9]
barley			100-250[4]	[12]
wheat		0,6		[9]
panicum			25	[22]
pennisetum			11	[22]
lolium		0,5		[9]
sugarcane		0,5		[17]

[1] measured with *act3* intron
[2] adh1 introns 2 resp. 6
[3] adh1 intron 6
[4] *shrunken exon1/intron1*

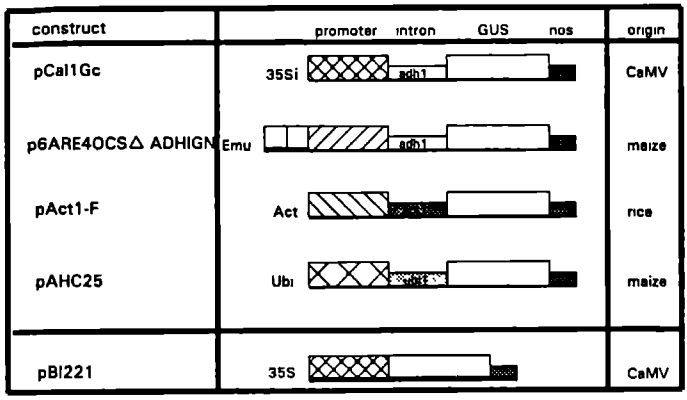

Fig. 1
Schematical representation of the constructs used for determination of promoter activity, as described in Table 3.

agreement with observations of McElroy *et al.* [15] and Schledzewski and Mendel [18]. The promoters Emu and Ubi performed better, but did not reach the expression level of the CaMV 35S promoter. These results, obtained with cell suspensions of *N. plumbaginifolia* were also found with leaf explants of *Nicotiana tabacum* (Table 3B). By combining these results and the results presented in Table 1, the conclusion can be drawn that the 35S promoter is more active than monocot promoters in *Nicotiana* species. It has been suggested that the molecular mechanisms underlying gene expression are not the same for monocots and dicots, there may be differences with respect to transcription factors and the recognition of promoter sequences [9]. However, a more simple explanation might be that monocot promoter activity is inhibited in dicots by the presence of the intron between promoter and gene, present in all constructs tested. This will be discussed further in the next section.

Rice. In rice cells the monocot promoters Emu, Act1 and Ubi were very efficient and respectively 5, 9 and 12 times more active than the 35S promoter (Table 3A and 3B). The activity of the 35S promoter was lower than in any other species tested. This low value positively influences the relative effect of the monocot promoters, but also the absolute numbers of spots found in rice cells after bombardment with Emu-, Act- and Ubi-*gus* were considerably higher than in *N. plumbaginifolia* cells bombarded with the same construct (Table 3A).

Few other reports described such a comparison of three promoters in one study. Gallo-Meagher *et al.* [5] reported a ten-fold enhancement of expression levels compared to a 35S construct using Emu and Act1, while Ubi performed 50 times better in sugarcane leaf explants. Schledzewski and Mendel [18] reported a 6 to 16 fold expression enhancement of theEmu, Ubi and Act promoters in maize and barley. The activity of the CaMV 35S promoter was relatively low in all tested monocot tissues, while the expression of the monocot promoters in tobacco was extremely low (Table 1).

Table 3A. Absolute values representing activity of promoters in *N. plumbaginifolia*, rice and leek cells, determined after particle bombardment of cell suspension material. Values for GUS activity were determined as numbers of blue cells per disk, using the histochemical assay, as described by Jefferson *et al.* [7]. Results are means (with SEM) from various experiments (n = 7 for *N. plumbaginifolia*, 10 for rice and leek). 0.5 ml of packed cell volume (PCV) was bombarded per disk.

	35S	35Si	Emu	Act	Ubi
N.plumbaginifolia cells	591 (84)	195 (18)	313 (18)	3 (1)	177 (19)
rice cells	122 (17)	149 (16)	627 (47)	1053 (89)	1461 (320)
leek cells	627 (69)	249 (33)	151 (25)	13 (2)	641 (53)

Table 3B. Relative activity of promoters in *Liliaceae*, compared to the activity in tobacco and rice, determined after particle bombardment of plant material. All results are given relative to the activity obtained by using the plasmid pBI221 (35S). GUS activity was determined as described in Table 3A, only for tulip leaves, activity was too high to discern discrete spots and activity was measured using the quantitative, fluorimetric assay [7]. Results are means (with SEM) from various experiments (n = 10 for rice, tulip, lily and leek, 12 for *N. tabacum* leaf and 7 for *N. plumbaginifolia* cells). For cell suspensions, 0.5 ml of PCV was used per disk.

	35S	35Si	Emu	Act	Ubi
N.tabacum leaf	1 (0.2)	0.7 (0.1)	0.4 (0.0)	0.0 (0 0)	0.5 (0.1)
N.plumbaginifolia cells	1 (0.1)	0 3 (0 0)	0.5 (0.0)	0.0 (0.0)	0.3 (0.0)
rice cells	1 (0.1)	1.2 (0.1)	5.1 (0 4)	8.6 (0 7)	12.0 (2.6)
tulip leaf	1 (0.1)	0.6 (0.1)	0.6 (0.1)	1.0 (0.1)	0.5 (0.0)
lily leaf	1 (0.2)	0.5 (0 1)	0.6 (0.1)	1.5 (0.3)	1.4 (0.4)
leek cells	1 (1.1)	0.4 (0.1)	0.2 (0 0)	0.0 (0.0)	1.0 (0.1)

Constructs: pCal1Gc, carrying the CaMV 35S promoter in combination with the maize adh1 intron (35Si) was a gift from V. Walbot; pBI221, carrying the 35S promoter without intron (35S), was described by Jefferson *et al.* [7], and purchased from Clontech; the pEmu construct was described and made available by D. Last *et al.* [9] (Emu) and pAct-F was described and provided by R. Wu and D. McElroy *et al.* [14, 15] (Act). The Ubiquitin promoter (plasmid pAHC25, [2]) was made available by P. Quail (Ubi) (also see Fig. 1).

Liliaceae. In contrast to rice, in the monocots tulip, lily and leek the activities of the monocot promoters were much lower, and even did not significantly exceed the activity of the CaMV 35S promoter. For leek cells, a very low level of Emu promoter activity was found, and also the activity of Act was hardly detectable (Table 3A, 3B). If absolute numbers of GUS positive spots are compared in the bombarded cell suspensions, for 35S similar numbers of spots were found in *N. plumbaginifolia* and leek, while Emu activity in leek was only half of that in *N. plumbaginifolia* (Table 3A). The absolute numbers of

spots found in *Liliaceae* cell suspensions were comparable to those found in *N. plumbaginifolia*, and much lower than for rice. For tulip leaf, overall expression of all promoters was very high, even too high to count discrete spots, so expression was measured using the fluorimetric assay. This indicates that certain tissue accessibility differences do exist when evaluating transient expression after particle bombardment. However, relative activities compared to 35S were very clear, and consistent with results found for lily and leek.

These observations with *Liliaceae* contrast the results obtained with other monocots, where higher gene expression was observed in most cases (Table 1). Similar differences in expression between monocot species have been observed before by Last *et al.* [9], who noticed a deviating promoter activity in *Lolium* compared to other monocots. A construct with the octopine synthase enhancer elements fused to the modified Adh1 promoter gave the highest expression in tobacco, and was also highly expressed in *Lolium*, while the expression was extremely low in wheat. They concluded that these differences probably rely on different sets of transcription factors in various cell lines, and that the *Lolium* cell line may resemble tobacco more than wheat in this respect.

In our experiments, the Act and Ubi promoters directed higher gene expression only in lily leaves, but not to the same extent as in rice cells. Surprisingly, extremely low levels of expression were found with the Act promoter in leek, and a good explanation for this is lacking so far. Taking all results together leads to the conclusion that promoter activity in *Liliaceae* resembles the activity in *Nicotiana* species more than activity in cereal monocots. The performance of Act in leek demonstrates that results concerning the activity of certain promoters cannot be extrapolated to members of related species. The activity of a promoter should be investigated for each species.

Effect of introns on expression in Liliaceae

In our experiments the inhibitory effect of the presence of the adh1 intron between CaMV 35S promoter and *gus* gene in *Nicotiana* species and the enhancing effect in rice were both in good agreement with results found by Last *et al.* [9] and McElroy *et al.* [15] (Table 2).

However, the presence of this intron decreased *gus* gene expression in all *Liliaceae* tested. The activity of the CaMV 35S promoter in combination with the adh1 intron was reduced to 40-60% of the activity of the intron-less 35S promoter. This is in contrast with results found for most *Gramineae* species, but in agreement with results found for sugarcane and *Lolium* (Table 2).

To confirm the inhibitory effect of monocot introns in *Liliaceae* we analyzed the effect of placing the shrunken-1 intron between the CaMV 35S promoter and *gus* gene. Replacement of 35S-shrunken-1 intron-*gus* by 35S-*gus* resulted in a 250-300 times enhancement of GUS-positive leek cells, showing that the presence of a monocot intron between promoter and reporter gene can negatively affect expression in leek.

The mechanism by which introns between promoter and gene influence gene expression in monocots is not yet fully elucidated. Luehrsen and Walbot [10] suggested a

posttranscriptional effect, the splicing event being responsible for the increase of steady state RNA and expression level. From studies on the processing of monocot and dicot pre-mRNAs in tobacco Keith and Chua [8] concluded, that there may exist differences in sequences required for RNA processing between monocot and dicot plants. Monocot introns were spliced at lower rates in dicots compared to monocots, while dicot introns were spliced at high efficiencies in monocots. Goodall and Filipowicz [6] confirmed these suggestions by showing that dicot plant introns are characterized by AU-rich sequences, while these sequences were not essential in monocots. They showed that monocot splicing seems to be more "permissive" than dicot splicing, the monocot maize was shown to be able to recognize and splice many introns that were spliced poorly or not at all in tobacco. This indicates that monocots differ from dicots in their mechanism of intron recognition.
From our results and from literature data on sugarcane and lolium, it appears that the effect of a monocot intron cannot be generalized for all monocots. In *Liliaceae* and in tobacco, a similar effect of the presence of the adh1 intron was found on the expression of the reporter gene, contrary to the effect in cereal monocots. These conclusions are based on results using the 35S promoter and a monocot intron. A dicot intron will probably have similar effects, because Tanaka *et al.* [19] described the expression enhancing effect of a dicot intron in rice, but not in tobacco. An interesting topic for further research will be to test the activity of monocot promoters without a monocot intron.

In summary, we conclude that expression levels of monocot promoter and intron combinations in the tested *Liliaceae* species resemble those found in tobacco more than those found in rice. Moreover, we conclude that unexpected expression levels can occur, as we found in tulip with respect to Ubi expression and in leek with respect to Act expression. Therefore, we feel that it is not possible to extrapolate results on expression levels to related species, and suggest that each crop should be tested with a set of promoters.

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chapter 5

Expression of the GUS-gene in the monocot Tulip after introduction by Particle Bombardment and Agrobacterium

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Abstract

Gene transfer to the monocotyledon tulip (*Tulipa sp.* L.) was obtained both by particle bombardment and *Agrobacterium* transformation. Using a Particle Delivery System, transient expression of the reporter gene for ß-glucuronidase was demonstrated. It was shown that the CAMV 35S as well as the TR2' promoter were active in flower stem explants. Various wildtype and disarmed *Agrobacterium* strains, harbouring the 35S GUSintron gene on a binary plasmid, were used for infection of flower stem explants of 7 cultivars and 7 botanical *Tulipa* species. In nine genotypes the GUSintron gene was expressed, despite the fact that tulip tissue did not produce detectable amounts of virulence-inducing substances. *Agrobacterium rhizogenes* appeared to be most effective in gene transfer to tulip tissue.

Introduction

Tulip (*Tulipa gesneriana* L.) is the most important flower bulb in the Netherlands. It is a monocot species belonging to the Liliaceae with a long generation cycle, due to a juvenile period of 5 years. This strongly hampers breeding of the crop. To accelerate genetic improvement it is important to establish transformation procedures for the introduction of interesting genes e.g. disease resistance. Although transformation of plants is most efficiently achieved by using the natural *Agrobacterium* gene transfer system, many monocotyledonous species seem to be insensitive to *Agrobacterium* infection and subsequent transformation. However, the detection of opines in *Chlorophytum capense, Narcissus* cv Paperwhite (Hooykaas-Van Slogteren *et al.* 1984), *Asparagus officinale* (Hernalsteens *et al.* 1984), *Zea mays* (Graves and Goldman 1986), *Gladiolus* (Graves and Goldman 1987) and *Allium cepa* (Dommisse *et al.* 1990), indicated that at least some monocots are susceptible to *Agrobacterium* infection. Moreover, *Agrobacterium* T-DNA integration into the plant genome has been demonstrated for *Asparagus officinales* (Bytebier *et al.* 1987), *Dioscorea bulifera* (Schäfer *et al.* 1987), *Oryza* (Raineri *et al.* 1990), and *Zea mays* (Gould *et al.* 1991). Some cereals have been proven to belong to the *Agrobacterium* host-range by using the method of "agroinfection" (Grimsley *et al.* 1988).

It has been suggested that the inefficiency of transformation of monocotyledonous species is caused by the lack of production of virulence inducing substances (Usami *et al.* 1987; Sahi *et al.* 1990). In some monocotyledonous plants virulence inducing compounds have been demonstrated (Usami *et al.* 1988), but their presence does not necessarily imply successful transformation (Messens *et al.* 1990).

The recalcitrance of several plants to *Agrobacterium*-mediated transformation has led to the development of direct DNA delivery methods. From the various methods described, especially particle bombardment has successfully been used for monocotyledonous plants (Klein *et al.* 1989, Wang *et al.* 1988).

In this paper we report on the introduction of the reporter gene for ß-glucuronidase (GUS) in tulip. Young floral stem segments of tulip are able to form adventitious shoots

(Alderson *et al.* 1986; Le Nard 1989). These were used for transformation. First, the expression of the GUS-gene driven by the CAMV 35S promoter or the TR2' promoter was tested by means of particle bombardment. The ability of tulip tissue to produce virulence inducing compounds was investigated using an *Agrobacterium* strain containing a virB-lacZ gene fusion. Various subsections of the genus Tulipa, were tested for their susceptibility to various wildtype *Agrobacterium tumefaciens* strains and their disarmed derivatives, and to a wildtype *Agrobacterium rhizogenes* strain.

Material and Methods

Plant material

Seven cultivars and seven botanical species of tulip (*Tulipa sp.* L.) were used (listed in Table 3). Bulbs of the cultivars were obtained from commercial growers, whereas botanical species were obtained from the CPRO collection. All bulbs were harvested in summer and stored dry at 20°C for 4 months, transferred to 17°C for 2 weeks and then to 9°C for 4 weeks, before being frozen at -2°C. These so called ice-tulips were thawed at 17°C and floral stems were excised when they were approximately 4 cm long. One mm segments taken from the nodal part were incubated on MS medium (Murashige and Skoog 1962) supplemented with 1.5 g/l caseinhydrolysate, 40 g/l sucrose, 7 g/l daichin agar (Brunschwig), 1 mg/l 1-naphtyl acetic acid (NAA) and 1.5 mg/l 2-isopentenyl adenine (2iP). The pH of the medium was adjusted at 6.0. After 2 to 3 months in culture (17°C, darkness), when regeneration had been initiated (Le Nard 1989, Alderson *et al.* 1986), the explants were used for transformation experiments. *Nicotiana tabacum* shoot cultures were grown *in vitro* on MS medium at 24 °C.

Bacterial strains and plasmids

The *Agrobacterium* strains used are listed in Table 1. To cover a wide host range, various *A.tumefaciens* strains were tested: the octopine strain Ach5 and its disarmed derivative LBA4404, the disarmed nopaline strain C58C1, the supervirulent A281 and its disarmed derivative EHA101; and the wildtype *A. rhizogenes* strain LBA9402. All strains were provided with the binary plasmid p35SGUSintron, harbouring the *NPTII* gene regulated by the NOS promoter, and the intron containing ß-glucuronidase gene, controlled by the CaMV 35S promoter (Vancanneyt *et al.* 1990). In strain EHA101 the binary 35S GUSintron plasmid was provided with a spectinomycin resistance gene (pCPO201; J. vd Berg, CPRO). C58C1 (pMP90) was used as a negative control.
For particle delivery mediated DNA transfer, two plasmids were used. The 5.8 kB plasmid pCAL1Gc, which was a gift from V. Walbot, harboured the ß-glucuronidase gene driven by the CaMV 35S promoter in combination with the maize adh1 intron and with NOS termination signals. The 11.8 kB plasmid pCPO1.2'GUS was constructed at CPRO (B. Visser, personal communication) and harboured the GUS gene under control of the dual TR2' promoter from *Agrobacterium* (Saito *et al.* 1991), and the APH (3')II kanamycin resistance gene regulated by the NOS promoter (Van der Leede-Plegt *et al.* 1991). Plasmids were present in *E.coli* strains HB101 (pCAL1Gc) or XL blue DH5α (pCPO1.2'GUS). Bacteria were lysed with alkali according to Maniatis *et al.* (1982), and plasmid DNA was purified using phenol-chloroform, precipitated and dissolved in 10 mM Tris, 1mM EDTA (pH8).

Particle Gun-mediated transformation

Plasmid DNA was precipitated onto tungsten particles (average diameter 1.1 μm) using a procedure modified from Klein *et al.* (1987). The precipitation mixture included 1.5 mg tungsten particles, 2.5 μg DNA, 1 M $CaCl_2$ and 16 mM spermidine in a total volume of 62.5 μl. After adding the components in this sequence, the mixture was vortexed for a few seconds, incubated for 10 min and centrifuged for 3 min (10000 g), and 45 μl of the supernatant was decanted. From the remaining 17.5 μl of suspension, 2-μl aliquots were pipetted onto the macroprojectiles for bombardment. Floral stem segments, approximately 40 per petri dish, were bombarded three times using a DuPont Biolistic Particle Delivery System 1000, with a vacuum pressure of 30 inch Hg. Tobacco leaf explants were used as a control. GUS assays were performed two days after bombardment.

Table 1. *Agrobacterium* strains used in transformation experiments

name	chrom	virulence plasmid	binary plasmid
A. rhizogenes			
LBA9402(pGUSint)	1855	pRi1855 (T)	p35SGUSintron
A. tumefaciens			
Ach5(pGUSint)	Ach5	pTiAch5 (T)	p35SGUSintron
LBA4404(pGUSint)	Ach5	pAL4404 (NT)	p35SGUSintron
A281(pGUSint)	C58	pTiBo542 (T)	p35SGUSintron
EHA101(pCPO201)	C58	pEHA101 (NT)	pCPO201
C58C1(pMP90,pGUSint)	C58	pMP90 (NT)	p35SGUSintron
C58C1(pMP90)	C58	pMP90 (NT)	-

T: wildtype plasmid with oncogenes (Tumorigenic); NT: vir-plasmid without oncogenes (Not Tumorigenic); references for strains: p35SGUSintron, Vancanneyt *et al.* (1990); LBA9402, Petit *et al.* (1983); Ach5, Depicker *et al.* (1983); LBA4404, Hoekema *et al.* (1983); A281, Komari *et al.* (1986); EHA101, Hood *et al.* (1987); CPO201, p35SGUSintron harbouring spectinomycin-resistance gene; C58C1(pMP90), Koncz and Schell (1986).

Determination of GUS-activity

ß-Glucuronidase activity was determined using a histochemical assay modified from Jefferson *et al.* (1987). Explants were vacuum infiltrated for 10 min with 0.1 M $NaPO_4$ (pH 7.5), 0.02% (w/v) NaN_3, 0.1% (v/v) Triton, 0.01 M EDTA,0.5 μM ferrocyanide, 0.5 μM ferricyanide solution containing 0.5 mg/ml X-gluc, and incubated overnight at 37°C. After staining, explants were rinsed in 50% and 75% (v/v) ethanol for 30 min, and kept in 96% (v/v) ethanol for microscopical examination.

Assay for virulence-inducing activity

The activation of virulence genes by wounded plant tissue was determined using the octopine strain LBA 2516 harbouring a virB-lacZ gene fusion (Melchers *et al.* 1989), which was a gift of dr. P.J.J. Hooykaas, University of Leiden. LBA 2516 was grown in

minimal medium (Hooykaas *et al.* 1979). For virulence induction, 50 µl of an overnight culture (OD550 of 0.8) was inoculated in 4 ml induction medium (Melchers *et al.* 1989) containing 1 g of sliced immature floral stem segments of tulip or sliced tobacco leaves. Samples were tested with and without the addition of 1 mM acetosyringone (As). As a control 250 µl of the bacterium culture was grown in 4 ml induction medium in the absence of plant material. For tulip tissue, the pH was adjusted at 7.0 (tulip tissue lowers the pH of the medium to an optimum pH of 5.3 to 5.5). After overnight incubation, 1 ml samples were taken for ß-galactosidase assay (Miller 1972). ß-Galactosidase activity was expressed as units produced per bacterium per minute.

***Agrobacterium*-mediated transformation**

A. tumefaciens strains were grown in LB (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, 1 g/l glucose); *A. rhizogenes* strains in YMB (0.4 g/l yeast extract, 10 g/l mannitol, 0.5 g/l K_2HPO_4, 0.1 g/l NaCl, 0.2 g/l $MgSO_4$). The media were supplemented with 1 mM acetosyringone (As) and the appropriate antibiotics. Prior to inoculation, bacteria were pelleted and resuspended to an OD550 of 1.0 in MS medium containing 1 mM acetosyringone. Hundred µl of this suspension was applied to each floral stem segment with a sterile syringe. For each bacterium strain / tulip genotype combination, three explants were used and the experiment was repeated two times. After three days of cocultivation, explants were transferred to fresh medium containing 250 mg/l cefotaxime and 250 mg/l vancomycin. GUS assays were performed 12 days after transformation. As a control, tobacco leaf explants were transformed according to the leaf-disk transformation method reported by Horsch *et al.* (1985).

Results

Transient expression of the GUS gene in tulip tissue after Particle Bombardment

Expression of the ß-glucuronidase gene was monitored after introduction of plasmid DNA into floral stem segments by particle bombardment. The stem segments had been cultured for 8 weeks. At that time the formation of adventitious shoots had just started. Blue spots were found on and at the bases of regenerating shoots (Fig. 1A). When testing the plasmids pCPO1.2'GUS and pCAL1Gc, both 35S and TR2' promoters appeared to be effective in tulip tissue.

All seven cultivars tested were responsive, and no differences in susceptibility among cultivars were found. Explants of the botanical species showed very low numbers of blue spots compared to the cultivars. It was noticed that these explants were softer and formed more callus than explants of the cultivars (results not shown). The number of blue spots varied between shots and between explants. Most of the positive explants showed numbers between 1 and 10, but higher numbers, up to 145 were found (Fig. 2).

Induction of virulence by wounded plant tissue

Tulip tissue was tested for its ability to induce virulence genes of *Agrobacterium*; the results are listed in Table 2.

Addition of an excess of 1 mM acetosyringone (As) to a culture of LBA 2516 resulted in a high expression of the lacZ gene under control of the virB promoter. Sliced tobacco

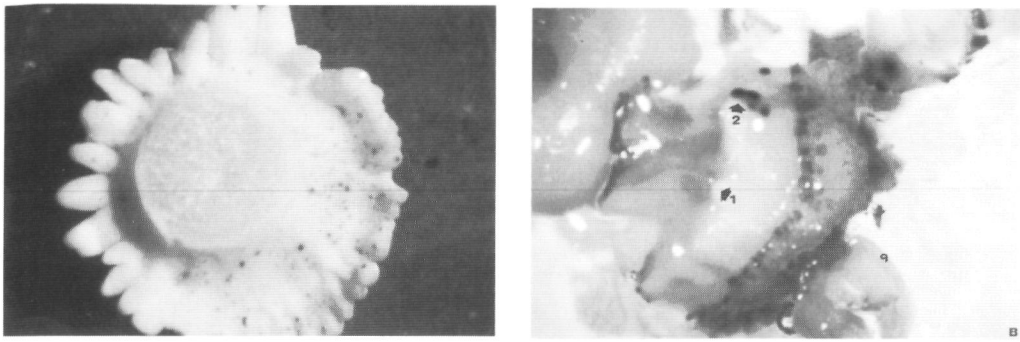

Fig. 1. Floral stem segment of cv Prominence developing adventitious shoots and showing blue spots after particle bombardment with the plasmid pCal1Gc and subsequent histochemical reaction with X-Gluc (A); Part of a floral stem segment of cv Lustige Witwe developing adventitious shoots (1) and showing blue spots (2) after infection with strain LBA 9402 and subsequent histochemical reaction with X-gluc(B).

Table 2. Virulence induction, expressed as units ß-galactosidase produced per bacterium per minute by *Agrobacterium* strain LBA 2516 harbouring a VirB-LacZ gene fusion, as affected by addition of wounded plant tissue (1 g) and acetosyringone (As, 1mM).

	As	units ß-gal	±	SD
No plant material	-	57	±	16
No plant material	+	5313	±	549
Tobacco	-	2550	±	757
Tobacco	+	4383	±	1462
Tulip	-	57	±	22
Tulip	+	1847	±	630

leaf material was also able to induce virulence, and it appeared that the presence of actively metabolizing plant tissue was necessary for this induction. If purified tobacco exudate was used, after removing the tissue parts by centrifugation or filtration, induction of ß-galactosidase did not occur. Induction of virulence by tobacco tissue did not reach the level of induction caused by 1 mM As alone and addition of As indeed enhanced the activity. In contrast to tobacco, tulip tissue did not induce galactosidase activity, indicating that it does not produce detectable amounts of virulence inducing substances. Moreover, it inhibited the induction of virulence by As.

Transient expression of the GUS gene in tulip tissue after infection with *Agrobacterium*

Various *Agrobacterium* strains harbouring the 35S GUSintron gene (Table 1) were used to infect flowerstem explants. The results of experiments with 7 cultivars and 7 botanical *Tulipa* species are presented in Table 3. The number of transformation events

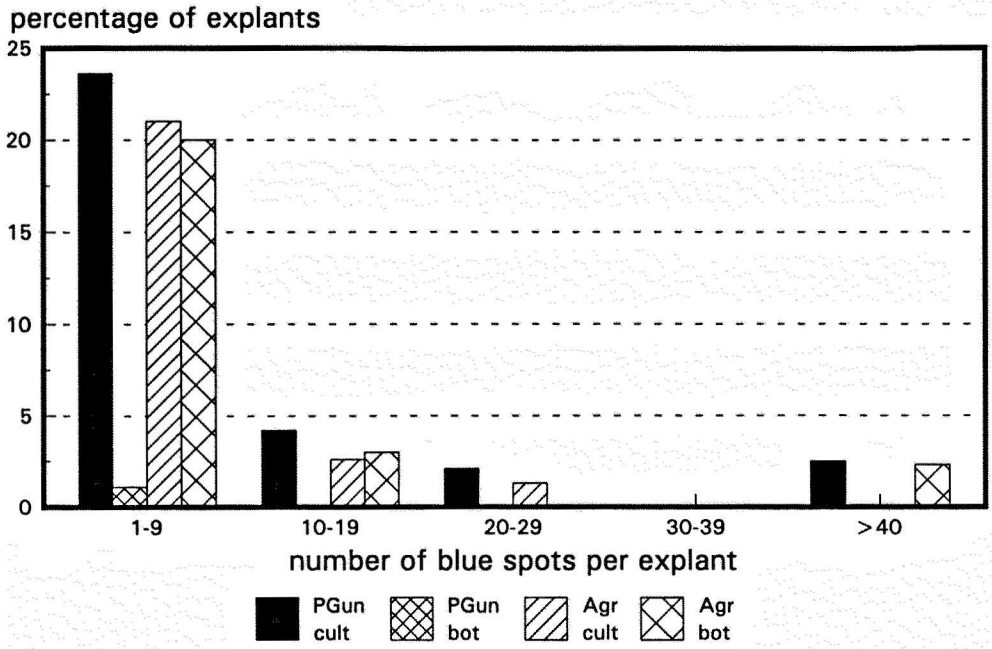

Fig. 2 Percentage of explants showing blue spots after transformation by particle bombardment (PGun) with pCal1Gc, compared to transformation by *Agrobacterium* rhizogenes LBA9402 (Agr). Distinction was made between the reaction of cultivars (cult) and botanical species (bot). PGun experiments were performed once for the 7 cultivars and twice for the 7 botanical species, each time with 30 explants per genotype. Agr experiments were performed three times (total 70 explants for cultivars and 70 for botanical species). Standard deviations were very high and ranged up to 50% of the ultimate value.

varied considerably between experiments and between genotypes. The spots were spread over the surface of the explant, including those regions where adventitious shoot formation occurred (Fig. 1B). Explants of botanical species developed more callus on the surface, where many blue spots appeared.

After infection with the agropine *A. rhizogenes* strain LBA9402, blue spots were found on segments of five cultivars and four botanical species (Table 3). In contrast to *A. rhizogenes*, most *A. tumefaciens* strains were less efficient in transferring the ß-glucuronidase gene. Occasionally the wild type strains A281 and Ach5, and the disarmed strain LBA4404 gave rise to blue spots on four genotypes. All *Agrobacterium* strains were used successfully in control experiments with tobacco leaf disks. Additional control experiments with the wild type strain LBA9402(pRi1855) next to the regular control C58C1(pMP90) always were negative.

The results of the gene-delivery by *Agrobacterium* and particle bombardment were compared (Fig. 2). No differences were found in the percentage of explants expressing the GUS gene. Explants transformed by particle bombardment show some more scatte-

Table 3. Susceptibility of 7 cultivars and 7 botanical species of Tulipa L to 6 *Agrobacterium* strains.

Genotype	Strain:				
	LBA 9402	Ach5	LBA4404	A281	C58C1/ EHA101*
Section *Leiostemones*					
subsection *Gesnerianae*·					
T. gesneriana:					
Prominence	+	-	-	-	-
Apeldoorn	+	-	-	-	-
Lustige Witwe	+ +	+	-	-	-
Leen van de Mark	-	-	-	-	-
Monte Carlo	+	-	-	-	-
Kees Nelis	-	-	-	-	-
Christmas Marvel	+	-	-	-	-
subsection *Eichleres:*					
T. eichleri	+ +	-	-	+	-
T. fosteriana	+ +	-	-	-	-
T vvedenskyi	-	-	-	-	-
subsection *Spiranthera:*					
T. kaufmanniana	-	-	-	-	-
Section *Eriostemones.*					
subsection *Saxatiles:*					
T. pulchella	-	-	-	-	-
subsection *Biflores:*					
T biflora	+ +	+	+	-	-
T. turkestanica	+	-	-	+	-

Notes:
+ : blue spots found in one out of repeated experiments
+ + : blue spots found repeatedly, in independent experiments
- : no blue spots found, in repeated experiments
* : experiment performed once
Agrobacterium strains are presented in Table 1.

ring of blue spots over the surface of the explant (Fig. 1A). These results agreed verywell with former experiments in which construct efficiencies were compared (results not shown).

Discussion

By using the Particle Delivery System, it was shown that the ß-glucuronidase gene is expressed in flower stem explants of tulip. Two gene constructs were used, which were equally effective. This means that both the CAMV 35S promoter and the TR2' promoter are operational in this monocot.
The results also are the first demonstration that the monocotyledonous species tulip is susceptible to *Agrobacterium* transformation. In nine out of 14 genotypes the blue spots of glucuronidase activity show that the GUS gene is introduced, correctly spliced and transiently expressed.
Tulip is a member of the Liliaceae, a family of the order Liliales. De Cleene (1985) showed that six families of this order reacted upon *Agrobacterium* infection by producing swellings. *Chlorophytum* (Hooykaas-van Slogteren *et al.* 1984), *Asparagus* (Hernalsteens *et al.* 1984) and *Allium* (Dommisse *et al.* 1990) are also members of the Liliaceae for which foreign gene expression after infection by *Agrobacterium* has been described and tulip can now be added to this list.
The efficiency of transformation by *Agrobacterium* tumefaciens was rather low, which might be due to a lack of virulence inducing substances. The negative results of the virulence induction assay indicate that tulip tissue does not produce detectable amounts of these substances or produces inhibitory substances. Experiments in other laboratories, using comparable approaches, showed that some monocotyledonous species produce virulence inducing substances, e.g. wheat cell suspension cultures (Messens *et al.* 1990) or extracts from wheat and oat (Usami *et al.* 1988). However, corn seedling homogenates reduced the growth rate of *Agrobacterium* and inhibited virulence induction by acetosyringone (Sahi *et al.* 1990), and for maize, rice and barley cell suspension cultures, no or only low levels of ß-galactosidase activity could be detected (Messens *et al.* 1990). These conflicting results might be due to the way the bio-assay is performed. In this study it appeared to be necessary to grow the test strain LBA 2516 in the presence of actively metabolizing plant tissues. Filtered exudate was unable to induce the virulence genes. This observation agrees well with results previously obtained by Stachel *et al.* (1986). By performing the assay with wounded plant tissue we obtained levels of virulence induction far exceeding those reported earlier (e.g. Usami *et al.* 1988).
The wildtype *A. rhizogenes* strain LBA9402 appeared to be the most efficient strain for transformation of tulip tissues. Although this strain is highly tumorigenic and has a broad host range, the reason for this high efficiency is unknown. One possible explanation might be the deviating onc-genes of *A. rhizogenes* compared to *A. tumefaciens*, which make plant cells more susceptible to auxins (Gelvin 1990).
In general, the *Agrobacterium* gene transfer system is regarded to be the most efficient for stable transformation of plant tissues, compared to any of the direct DNA transfer methods. In this work the *Agrobacterium* method was compared with the method of particle bombardment as far as transient expression is concerned. The percentage of explants showing blue spots was the same, except for the botanical species which

showed little reaction after particle bombardment. This means that both approaches can be used.

It can be concluded that the monocotyledonous species tulip is susceptible to transformation both by *Agrobacterium* and by particle bombardment. The blue spots of GUS expression were found in a region which is highly meristematic. This gives good prospects for the introduction and stable integration in cells which are at least part of meristems. Research is now focussed on the use of this regeneration system for the production of transgenic tulips.

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chapter 6

Genetic modification of tulip via particle bombardment of floral stem segments

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Abstract

A procedure is described for the genetic modification of tulip, using particle bombardment of segments of floral stems. Shoots were formed after particle bombardment with the *bar* gene and selection on medium containing the herbicide Basta. They were cultured untill bulblets, that produced shoots again. At this stage a molecular analysis was performed to investigate the presence and expression of the transgenes.
PCR analysis demonstrated the presence of the introduced genes and RT-PCR suggested that the genes were expressed as mRNA's. The absence of free plasmids remaining from the bombardment event could be excluded by plasmid rescue experiments. Unfortunately, the size of the tulip genome (25.10^6 kb) hampered the demonstation of integration into the tulip genome. However, by using methylation sensitive and insensitive restriction enzymes, we could show that replication of introduced genes in tulip had taken place. Taking all results together strong indications have been obtained that the transformation procedure leads to transgenic tulips, although we cannot exclude to have harboured chimeric transformed tissue. The prospects for using the described regeneration and selection system for tranformation of tulip are discussed.

Introduction

For many monocotyledonous crops transformation procedures have been established over the last years. In most studies foreign genes were introduced via particle bombardment (reviewed by Vasil, 1994). Also *Gladiolus*, a monocotyledonous bulb plant like tulip, has been succesfully transformed by means of particle bombardment (Kamo *et al.*, 1995). For tulip, two methods of gene-transfer were tested, and like for other monocot species, particle bombardment appeared to be a more reproducible and efficient method than *Agrobacterium tumefaciens* mediated procedures (Wilmink *et al.*, 1992; chapter 5).
In general, for monocots, cells or protoplasts derived from embryogenic tissues are used as a target tissue for transformation. For tulip, such a regeneration system is not available yet, and a system of direct adventitious shoot formation on developing floral stem explants was used. The system has been described before by LeNard *et al.* (1987). In a seperate paper, the prospects for this regeneration system for tulip transformation were evaluated, together with the use of the Basta-resistance gene *bar* for selection (Wilmink *et al.*, 1995[a], chapter 3).
The transformation procedure described in this study is based on this regeneration system. Reporter- and selection genes were introduced in tulip floral stem explants via particle bombardments. Shoots were harvested after Basta-selection, and analyzed for the presence and expression of the introduced genes. On the basis of the results, we discuss the prospects for the use of this system.

Material and methods

Plant material: Floral stem explants of *Tulipa gesneriana* cvs. Lucky Strike and Monte Carlo were used. Bulbs were treated as described before (Wilmink *et al.*, 1995[a]; chapter 3), and used in December (dry-stored bulbs) and January until April (ice-stored bulbs). Two transgenic plants were used as positive controls in the molecular analysis: *Brassica napus* cv. Drakkar, transgenic for the *bar* gene (plasmid pthw40.seq, line SL3, P4441, Plant Genetic Systems, Ghent, Belgium) and potato, transgenic for the *amp* gene between left and right border of the T-DNA (Pereira *et al.*, 1991).
Particle bombardment: Transformation was performed by means of particle bombardment on floral stem segments using the PDS 1000 He (Bio-rad) with a helium pressure of 1800 psi, and a vacuum pressure of 27 inch Hg. Explants were bombarded three times using tungsten particles (1.1 μm), coated with 2.5 μg DNA. For these three bombardments 1.5 mg particles were used, on which the DNA was precipitated in a mixture of 1 M $CaCl_2$ and 16 mM spermidine (total volume 62.5 μl). After vortexing, centrifuging and washing in 100% ethanol, particles were resuspended in 30 μl ethanol, and 10 μl was applied per macrocarrier.
In two sets of transformation experiments performed in 1993 and 1994, petri dishes (6 cm) containing 50 freshly sliced floral stem segments were bombarded. In the 1993 series the double construct 35S*bar*Emu*gus* was used. This construct was a gift of O. Olsen, the pEmu promoter was described by D. Last *et al.*, 1991. In the 1994 series of transformations the construct Act*bar*-Act*gus* was used. The actine promoter and expression vectors were made available by R. Wu, and described by McElroy *et al.*, 1990, 1991 and Zhang *et al.*, 1991. Both constructs are visualized in Fig. 1. All constructs harbour the *ampicillin* gene (*amp*) as a bacterial selection gene, which can be co-introduced into the plant cells.

Selection and culture: After particle bombardment, tissue was incubated on selective agent free medium for one week, then transferred to medium containing 1 mg/l Basta for eight weeks (17°C, darkness). For further development, shoots were transferred to light (16 h light periods, Philips TL85 fluorescent lamps, 36 μmol/m^{2}s) after nine weeks. Further culture of shoots and bulblet formation was done in the absence of Basta, and the subsequent treatments are described in Table 1.

Histochemical ß-glucuronidase (GUS) detection: At various times after incubation on regeneration medium explants were stained overnight in 0.1 M $NaPO_4$ (pH 7.5), 0.02 % (w/v) NaN_3, 0.1 % (v/v) Triton, 0.01 M EDTA, 0.5 μM ferricyanide, 0.5 μM ferrocyanide, 0.5 mg/ml X-gluc (37°C), according to the method modified from Jefferson *et al.* (1987).

Fluorimetrical ß-glucuronidase detection: Samples (total tissue of one bombardment) were grinded in liquid nitrogen, and extracted in 3 ml. GUS buffer (0.1 M $NaPO_4$ pH 6,7; 10 mM EDTA; 0.1% v/v triton X-100; 0.1% w/v sarkosyl; 10 mM DTT). GUS activity was measured by determination of fluorescence during 24 hours. For this, 25 μl extract was mixed with 75 μl buffer, 50 μl 4-Methylumbelliferone (MU, 1 nmol) and 50 μl Methylumbelliferone Glucuronide (MUG, 4 mM). Fluorescence measurements were done with a Titertek Fluorskan II at 37°C after 4, 7, 16 and 24 hours, and expressed as increase in fluorescence per hour per mg protein.

Table 1. Procedure for selection and bulblet formation in tulip. Regeneration phases after particle bombardment of floral stem segments.

phase	temp.°C	light	medium	months
adventitious shoot formation[s]	17	-	I	2
shoot growth, 1st bulblet induction	17	+	II	3
1st bulblet growth	4	-	III	3
dormancy	17	-	III	4-5
dormancy breaking, elongation	4	-	III	3
shoot growth	17	+	II	2
2nd bulblet induction	17	+	III	2
2nd bulblet growth	4	-	III	3
dormancy	17	-	III	3
planting	9	-	-	3

media:
I: regeneration: MS, 40 g/l sucrose, 1 mg/l NAA, 1.5 mg/l 2IP
II: growth: MS, 30 g/l sucrose, 80 mg/l adeninesulfaat, 10 mg/l thiamine, 1 mg/l BA, 0.5 mg/l IBA
III: bulb induction: MS, 60 g/l sucrose, 10 mg/l thiamine, 0.5 mg/l IBA
[s] : selective medium (1 mg/l Basta in week 2-9)
MS : Murashige and Skoog medium, 1962

Construction of Actbar-Actgus: The construct Act*bar*-Act*gus* was obtained by ligating the *bar*-gene from pDE110 (Denecke *et al.*, 1989) as a *Nco*I-*Pst*I fragment into the actine-expression vector pCOR112 (Mc Elroy *et al.*, 1991) to yield Act*bar*. The *Xho*I-*Xba*I blunt-end fragment Act*gus* from pActF (McElroy *et al.* 1990) was ligated in the *Xho*I site (blunt) of Act*bar* with standard molecular techniques.

DNA isolation: For DNA isolation, a modified Dellaporta protocol was used (Dellaporta *et al.*, 1983). 50 mg of tissue was grinded in liquid nitrogen and DNA was extracted with 750 µl of extraction buffer (100 mM Tris, pH8; 50 mM EDTA, pH8; 500 mM sodium chloride, 10 mM ß-mercaptoethanol), 100 µl sodium dodecyl sulphate (the mixture was incubated at 65°C for 10 min.), and 250 µl of 5M potassium acetate (on ice, 20 min.). After centrifuging, DNA from the supernatant was precipitated with isopropanol. The pellet was dissolved in 500 µl water with 1 µg RNase, phenol:chloroform treated, precipitated with sodium acetate and ethanol, and dissolved in 50 µl water.

PCR: For PCR analysis 100 ng of tulip-DNA was denaturated at 94°C for 5 minutes. Analysis was performed on a selection of bulblets, harvested after Basta-selection. Of the experiments of 1993, approximately 100 bulblets from the first three experiments were analyzed, of the experiments of 1994, 16 bulblets from the first experiments were analyzed. Genomic DNA of *Brassica napus*, enriched with the *bar* gene, or of *Solanum tuberosum*, enriched with the *amp* gene, was taken as positive control in every PCR reaction.

Primers from Isogen were as follows (5'-3'):
Bar: CATCGTCAACCACTACATC and AGGCTGAAGTCCAGCTGCCAG (*bar*-fragment 450 bp)
Amp: TAATCAGTGAGGCACCTATCTCAGC and ATTTCCGTGTCGCCCTTATTCC (*amp*-fragment 700 bp). After 35 cycli (0.5 min. 94°C; 1.5 min. 55°C; 2 min. 72°C), samples were subjected to gel-electrophoresis. After blotting, the filters were hybridized with 15 ng of a probe for the *bar*-gene or for the *amp*-gene respectively. Probes were generated by PCR of plasmid or of genomic DNA of the control plants, Brassica for the *bar*-probe and potato for the *amp*-probe. PCR analyses were performed in a separate, plasmid-free lab, to avoid contaminations.

RNA isolation: Tissue, grinded in liquid nitrogen, was added to a 1 ml 60°C mixture of RNA extraction buffer (0.1 M lithium chloride (LiCl), 1% SDS, 100 mM Tris pH9, 10 mM EDTA) and phenol (1:1), and incubated 5 min. 500 µl Chloroform was added, and after vortexing and centrifuging, the supernatant was mixed with 1 vol. cold 4M LiCl. After overnight precipitation, the pellet was washed with 2M LiCl and 80% ethanol.

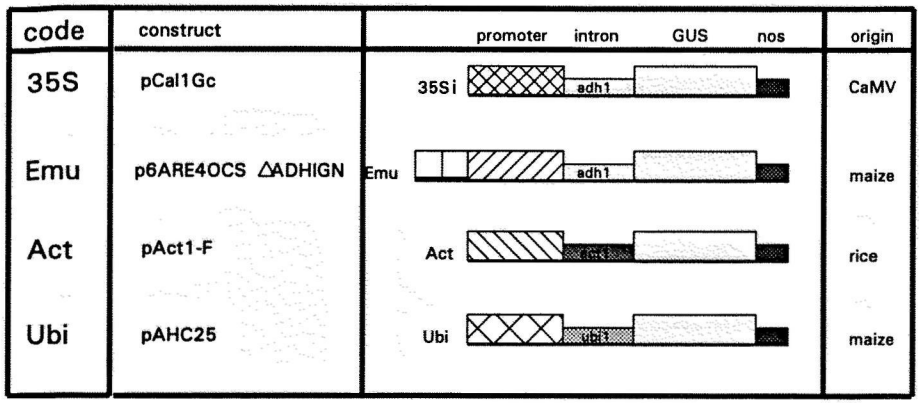
RT-PCR: 10 µg of total RNA was used for cDNA synthesis, with primer CCGGATCCTCT-AGAGCGGCCGCTTTTTTTTTTTTTTTT (5'-3'; Isogen) in a 50 µl reaction. At first, the RNA was melted at 70°C together with 1 ug primer in a 15 µl reaction for 1 min. After this, first strand buffer, RNase inhibitor (10U), DTT (0.1M), dNTP's (125µM), reverse transcriptase (400U) and water were added. CDNA synthesis took place at 37°C during one hour.
From this cDNA 5 µl was used in a PCR reaction as described above.

Plasmid Rescue: 3.5 µg of genomic DNA of putative transformants, transformed with 35S*bar*Emu*gus* was digested with *Xba*I (which cuts once inside the plasmid but fragments tulip DNA), ligated overnight in a volume of 3.5 ml, extracted with fenol-chloroform, precipitated and dissolved in 10 µl water. This DNA was transformed to electrocompetent MC1061 *E.coli* cells according to manufacturer's instructions (Bio-rad). Resistant colonies were grown on ampicilline-containing medium, and subjected to DNA isolation and restriction analysis. As a positive control, 100 ng of potato-DNA (described under *PCR*), harbouring the ampicilline-resistance gene was treated in the same way (except ligation in 100 µl).

Results

Analysis of promoter activity in tulip floral stem segments:
To select promoters that are highly active in tulip we tested the activity of various monocot promoters in tulip tissue. Floral stem segments were bombarded with various constructs, all containing a monocot intron between promoter and *gus*-gene (Fig. 1A). Two days after bombardment, tissue was grinded in liquid nitrogen, and used for fluorimetrical determination of GUS-activity. The results are shown in Table 2.
GUS-expression brought about by the various monocot promoters was in all cases higher than the reference CaMV promoter. The promoter of the *actin* gene from rice (Act) induced an activity which was two times higher than the activity of Emu, a modified promoter of the *alcohol dehydrogenase* gene from maize. Also the promoter of the maize

A



B

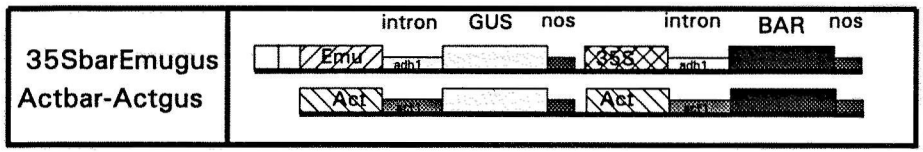


Fig. 1. Constructs used for determination of promoter-activity and particle bombardment. 35S is the CaMV 35S promoter with adh1 intron (pCal1Gc, Walbot); Emu is the maize Emu promoter (p6ARE4OCSΔADHIGN, Last *et al.*, 1991); Act is the rice *actin*1 promoter (pAct1-F, Zhang *et al.*, 1991; McElroy *et al.*, 1991), Ubi is the maize *ubiquitin* promoter (pAHC25, Christensen *et al.*, 1992). A: constructs used for determination of promoter activity in tulip tissue; B: constructs used for transformation.

Table 2. GUS-activity in tissue of floral stem segments of tulip after particle bombardment with 4 promoter-*gus* constructs. Values are given as fluorescence per ug protein (GUS-activity), with the activity brought about by the CaMV 35S promoter adjusted to 100. SEM is standard error of the mean of a series of bombardments (n = 25). The constructs used are presented in Fig. 1.

promoter	GUS activity	SEM
35S	100,0	12,9
Emu	130,1	5,5
Act	221,2	8,8
Ubi	189,6	10,8

ubiquitin gene (Ubi) was very active in tulip tissue. In the first set of transformation experiments (1993) the Emu promoter was used driving the *gus* gene, and 35S was used in front of the *bar* gene. Because of the high activity of Act, we decided to use this promoter in a new series of experiments (1994). For this, we combined Act*gus* and Act*bar* in one construct (Fig. 1B).

The development of Basta-resistant tissue:

In the first transformation season (1993) large numbers (3800) of tulip floral stem segments were bombarded with particles carrying a double construct with the *gus*-reporter gene and the *bar* gene: 35S*bar*Emu*gus* (Fig. 1B). One week after transformation, explants were transferred to regeneration medium with 1 mg/l Basta and this selection pressure was applied for two months (Wilmink *et al.*, 1995[a]; chapter 3). In course of the selection process, vital looking explants were GUS-stained. Fig. 2 shows the initiation of shoot formation after 9 weeks of culture on floral stem segments of the cultivar Lucky Strike. The blue, GUS expressing shoot primordia formed a region from which shoots would have proliferated after further incubation on regeneration medium. The GUS expression in this meristematic region shows that transgenic tissue is able to proliferate on selective medium.

Fig. 3 shows that shoots were obtained on segments that had been bombarded, but hardly on unbombarded explants. Table 3 presents the results of the transformation of two cultivars, Monte Carlo and Lucky Strike, which were selected for their good regeneration and bulbing qualities (Wilmink *et al.*, 1995[a]; chapter 3). After bombardment stem segments were able to form adventitious shoots on medium without Basta. For unknown reasons bulblet formation in Lucky Strike was surprisingly low in this control. Regeneration was almost completely inhibited by the presence of Basta in the medium. Shoots that occasionally were able to develop, died during subsequent culture. After bombardment with the *bar* gene and selection on Basta two percent of the explants of Monte Carlo and ten percent of the explants of Lucky Strike formed shoots respectively. These shoots generally were formed in clusters and not dispersed as in the regeneration control, but in similar numbers per regenerating explant (approximately 7). Developing shoots were transferred to light and further cultured as described in Table 1 in the absence of Basta.

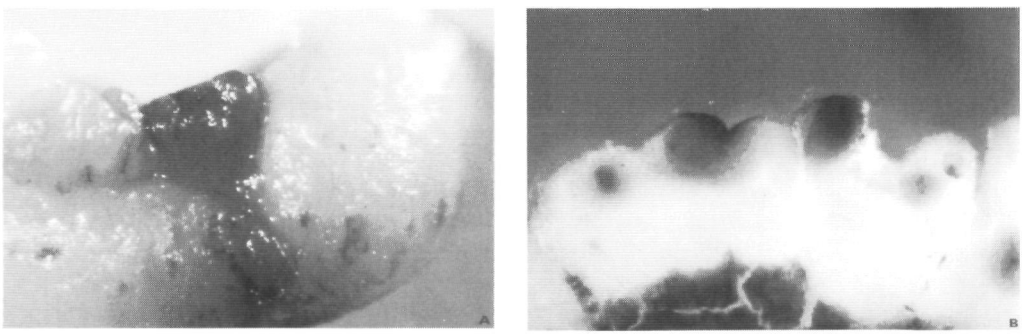


Fig. 2. Development of a bombarded tulip floral stem explant after 8 weeks of incubation on selective medium containing 1 mg/l Basta. GUS expression is shown in the regenerative area. A: explant of 1993, bombarded with 35S*bar*Emu*gus*; B: explant of 1994, bombarded with Act*bar*-Act*gus*.

Table 3. Regeneration and bulblet formation of two tulip cultivars after bombardment with the Basta-resistance gene (construct 35S*bar*Emu*gus* in 1993, Act*bar*-Act*gus* in 1994) and selection on medium with Basta. +PG-B: bombardment, no selection (regeneration control); -PG + B: no bombardment, with selection (selection control; in 1994 half of the explants were bombarded with the *gus* gene only, and selected: ±PG + B); +PG + B: bombardment and selection; % reg: the percentage of explants that regenerated shoots; %bulb: the percentage of shoots that developed one or more bulblets. The mean number of shoots per regenerating explant was approximately 7 for all treatments.

			1993	1993		1994
Treatment			Monte Carlo	Lucky Strike		Lucky Strike
Regeneration control	+PG-B	%reg	15	46	-PG-B	36
		%bulb	96	1		32
Selection control	-PG + B	%reg	1	3	±PG + B*	6
		%bulb	0	1		46
Transformation	+PG + B	%reg	2	10	+PG + B	9
		%bulb	4	10		44

*: half of the explants were bombarded with the non-selectable Act-GUS construct (pAct-F)

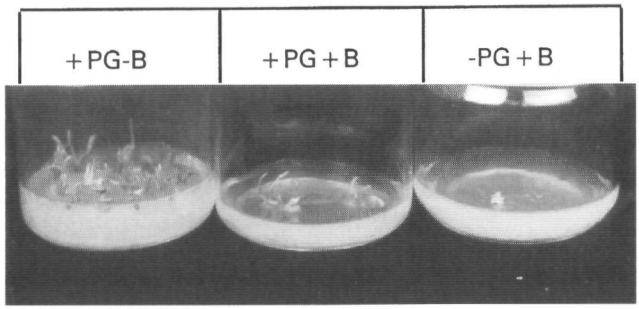


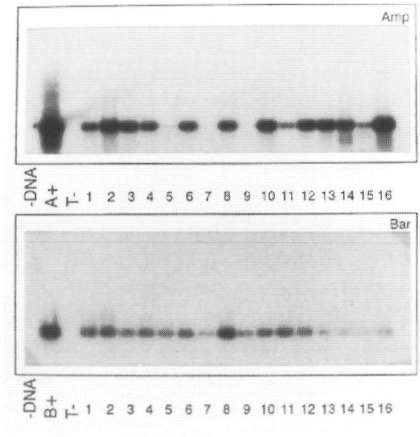
Fig. 3. Development of Basta-resistant tissue after bombardment with the *bar* gene. Left: regeneration control (+PG-B); right: selection control (-PG + B); middle: development of clusters of shoots as a result of bombardment (+PG + B). Photo was taken after 8 weeks on regeneration medium (I) with 1 mg/l Basta, and 4 weeks on growth medium (II) without Basta (Table 1).

Lucky Strike was used again in the second transformation season in 1994. Comparable numbers of explants were bombarded (3200), and similar results were obtained as in 1993. This time bulblet formation was far more efficient. On medium with Basta, unbombarded explants did not regenerate shoots. After bombardment with the non-selectable Act*gus* construct (pAct1-F) however, low regeneration and bulbing percentages were found. As in 1993, about 10% of the explants regenerated shoots after bombardment with the *bar* gene and selection. After bulblet induction, bulblet growth and dormancy induction according to the procedure described in Table 1, more than 600 bulblets were harvested. All bulblets were cold treated for dormancy breaking, and the

new shoots that developed from the bulblets at higher temperatures were used for molecular analysis. GUS expression could not be demonstrated during these periods, and neither did we find any phenotypically expressed Basta-resistance. The reasons for this will be discussed below. The remaining bulblets will be cultured further as described in Table 1 (2nd bulblet induction, 2nd bulblet growth, dormancy), and planted in soil when they are large enough. From that moment, flowering will take another 3-4 years.

Molecular analysis of herbicide-resistant shoots:
PCR
PCR analysis was performed on leaf tissue that originated from bulblets which had developed after 1st bulblet growth, dormancy, and cold treatment for dormancy breaking (Table 1). This means that the first molecular analysis was done approximately 15 months after transformation. For analysis, a selection of bulblets was made; for the experiments of 1993 approximately 100 bulblets were analyzed. The presence of the *bar* and the *amp* gene was investigated with primers specific for both genes. To exclude the presence of PCR artefacts in the reactions, the PCR products were subjected to Southern blotting and hybridization with plasmid derived *bar*- resp. *amp*-fragments. Fig. 4A shows part of the results for material obtained from transformations in 1993 (*bar* and *amp*). The numbers 1 to 16 represent PCR products of DNA isolated from independently produced tulip bulblets, from which a developing leaf was analyzed. All were positive for the presence of the *bar* gene, while most of them also contained *amp* sequences. In these PCR-based hybridization experiments, adventitious shoots obtained from control experiments were analyzed simultaneously. The regeneration control (+PG-B) was bombarded with the *bar* gene but not selected. Surprisingly, also these plants showed the presence of both *bar* and *amp* in the PCR reaction (data not shown). DNA isolated from unbombarded floral stem segments always was negative in these PCR reactions (lanes marked T- in Fig. 4A). As a positive control, Basta-resistant Brassica or potato plants, harbouring the *bar* or *amp* gene respectively, were analyzed in all PCR experiments. These controls always were positive (B^+ and A^+ in Fig. 4A).
In 1994, analysis of the selected fourteen bulblets showed similar results, as shown for the presence of the *amp* gene in Fig. 4B. The numbers 1 to 14 again represent independent tulip bulblets, which all show the presence of the *amp* gene. In contrast to 1993, the regeneration control (-PG-B, R) in 1994 transformations was unbombarded. As expected, no amplified *amp* fragments were found. In this season, some bulblets could be harvested from one of the selection controls, which had been bombarded with the *gus* gene only, and selected with Basta (+PG+B^*, A). Leaf material from these bulblets was positive for *amp* (present on the plasmid pActF, which was used for these experiments, Fig. 4B), and negative for *bar* (not shown). The positive control (potato, A^+) and the negative control (no DNA, -) both show the proper response.

RT-PCR
RT-PCR was used to investigate the expression of the transgenes in tulip tissue. Plants that had shown a positive PCR reaction on isolated DNA were subjected to RT-PCR.



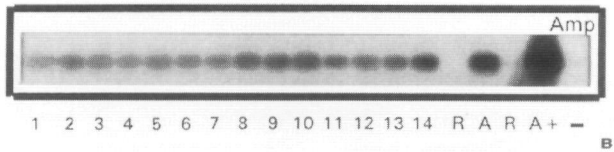


Fig. 4.
PCR analysis of leaf tissue, developed from Basta resistant bulblets.
A: experiments of 1993; -DNA: negative control, no DNA added; A^+, B^+: positive controls, resp. potato, transgenic for *amp*, and *Brassica napus*, transgenic for *bar* (Material and Methods); T-: DNA from unbombarded tulip; numbers 1 to 16: independent tulip transformants.
B: experiments of 1994; numbers 1 to 14: independent tulip transformants; R: regeneration control, DNA from unbombarded tulip; A: DNA from tulip tissue, bombarded with pAct1-F (Fig. 1A); A^+: positive control, potato, transgenic for *amp*; -: negative control, no DNA added.

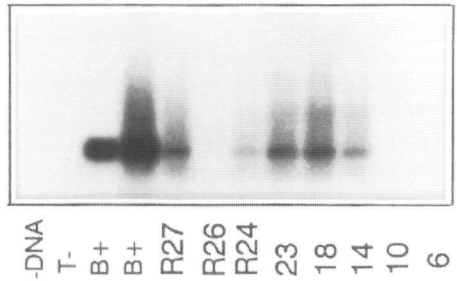


Fig. 5.
RT-PCR on RNA of leaf tissue, developed from Basta resistant bulblets.
-DNA: negative control, no DNA added; T-: RNA from unbombarded tulip; B^+: positive control, RNA from *Brassica napus*, transgenic for *bar*; R27, R26, R24, 23,18,14,10,6: RNA of independent tulip transformants; R-numbers were bombarded shoots which had not been subjected to selection.

Total RNA was isolated and was free of contaminating DNA as judged from a negative direct PCR reaction (data not shown). RNA preparations were first subjected to a reverse transcriptase reaction using a poly-dT primer, followed by a PCR reaction with *bar* primers. PCR products were Southern blotted and hybridized with a *bar* probe (Fig. 5). Five out of eight samples of putative transgenic plants showed a clear *bar*-PCR product indicating that the *bar* gene was expressed in these tissues. The fragment co-migrates with the PCR product of RNA isolated from *Brassica napus* plants, transgenic for the *bar* gene. The specificity of the reaction was convincingly demonstrated by the negative PCR reaction of samples from unbombarded tissue and samples without DNA (Fig. 5). We also investigated RNA expression by Northern analysis, byt this was not succesful (data not shown). This might indicate very low levels of expression.

Plasmid rescue

PCR reaction on both DNA and RNA isolated from transformed tulip tissue indicated the presence and expression of transferred genes. To demonstrate real integration of the genes in the tulip genome, we attempted to isolate flanking genomic DNA by means of plasmid rescue. To this end, genomic DNA of putative transformants was cleaved with XbaI, which cleaves the introduced plasmid only once. Fragments were ligated and transformed into electro-competent *E.coli* cells (strain MC1061). As a positive control, we used 100 ng (140.000 genomic copies) of DNA isolated from potato plants harbouring a T-DNA insert with the *amp*-gene and bacterial origin between left and right border. After restriction, ligation, transformation to *E.coli* and selection on ampicilline, we obtained only colonies, which contain part of the T-DNA insert flanked by genomic potato sequences (results not shown).

To investigate whether tulip DNA might inhibit plasmid rescue, we mixed 100 ng of potato DNA with 3.5 μg of tulip DNA (140.000 genomic copies), and used the same procedure as described above. This resulted in similar numbers of ampicilline resistant colonies, indicating that the tulip genomic background is not restrictive for the plasmid rescue procedure. However, after testing several putative tulip transformants, we did not succeed in isolating flanking DNA sequences. Only small 2kb plasmids containing the *amp*-gene and bacterial origin were harvested.

It is important to note that no plasmids were rescued from untreated (unrestricted, unligated) chromosomal DNA of tulip, indicating the absence of free plasmid-DNA in the transformants.

Replication of the introduced genes in tulip cells

In order to prove that the PCR-positive signals in the putative transformants indeed were caused by integrated plasmid DNA and not by free plasmid DNA, remaining from the bombardment event, we analyzed the methylation state of the DNA by using the restriction enzymes *Dpn*I and *Dpn*II. Both enzymes recognize GATC-restriction sites, but while *Dpn*I is A-methylation-dependent, *Dpn*II is inhibited by methylation of the A-residue in GATC-sites. The plasmids which were introduced into tulip cells were grown in *E.coli* strain JM101, which is wild-type for the *dam* locus, and methylates A residues in GATC

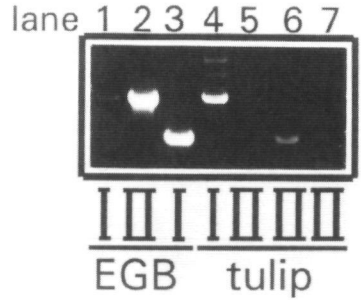


Fig. 6.
Replication of the *amp* gene in tulip tissue, determined with the restriction enzymes DpnI and *Dpn*II; PCR analysis with *amp* primers was performed on restricted plasmid and tulip DNA. I: *Dpn*I; II: *Dpn*II; EGB: 35S*bar*Emu*gus* plasmid DNA, used for transformation.

sites. In contrast, in eukaryotes these sites are never methylated. By restriction analysis we confirmed that plasmids were indeed cleaved by *Dpn*I and not by *Dpn*II, while tulip DNA was cleaved by *Dpn*II and not by *Dpn*I (results not shown). Because the *amp* gene contains a GATC site between the primers used for PCR reactions, we were able to show the methylation status of the introduced gene from the presence or absence of PCR products. From Fig. 6 it is clear, that indeed the *amp*-gene is hardly detectable in *Dpn*I cleaved plasmid DNA, while it is present in *Dpn*II treated DNA (lane 1, 2). The faint *amp* signal that is present in lane 1 (*Dpn*I cleaved plasmid) might be due to some uncleaved molecules still present in the restriction reaction. For tulip, however, the *amp*-gene was only detectable in *Dpn*I treated DNA (lane 4). The faint signal in *Dpn*II treated tulip DNA (lane 5) is due to uncleaved molecules, because after isolating the cleaved DNA from gel prior to PCR reaction, the signal was not present anymore (lane 7). This means that the DNA that we had isolated from the putative tulip transformants, was not methylated anymore, because it was cleaved by *Dpn*II and not by *Dpn*I, and hence must have been replicated in the tulip cells. As a positive control, in lanes 3 and 6, PCR products of the *bar* gene are shown from restricted DNA (EGB/*Dpn*I and tulip/*Dpn*II). The *bar* gene does not contain a GATC site, and should be intact in the restricted DNA. The presence of the *bar* product indicates, that the absence of *amp* products in restricted DNA is not due to DNA breakdown.

Discussion

In this paper we describe the development and evaluation of a system for genetic modification of tulip by means of particle bombardment. By using a PCR approach it is shown that introduced genes were still present in tulip tissue after prolonged culture. By means of plasmid rescue it was also shown that the positive PCR signals were not due to the presence of free plasmid DNA remaining from the transformation event. Moreover, a low level of expression at the RNA level was found, as suggested by RT-PCR analysis. Analysis with methylation-sensitive restriction enzymes suggests that the genes have replicated in tulip cells. At this moment we have not yet been able to demonstrate that

the transferred genes have indeed integrated into the tulip genome. This is due to the fact that the genome of tulip is too large to allow direct investigation of integration by Southern blot analysis. On the other hand stable transmission of the transgenes to the next generation awaits flowering of the putative transgenic plant. This will take about four years. Nevertheless, the results that were obtained strongly indicate, that we have been able to introduce genes into tulip cells, and to regenerate these cells to transgenic shoots and bulblets.

This is the first description of genetic modification of tulip. Because of the peculiar life cycle of tulip, its large genome size and the regeneration protocol that has been used, it is necessary to evaluate critically some specific aspects of the regeneration/transformation system developed. Especially the development of so many PCR-positive plants and bulblets irrespective of the use of selection pressure and the silencing of the GUS expression need further evaluation.

Regeneration and selection.

The regeneration system that has been applied is rather uncommon for monocots, because we used a system of direct adventitious shoot formation instead of embryogenic callus as used for most monocots e.g. maize. The shoots that developed on floral stem segments, originated from multicellular regions, as shown in a seperate paper (Wilmink *et al.*, 1995[a]; chapter 3). These adventitious shoots gave rise to the formation of bulblets, originating from a number of cells of the shoot base (Chanteloube *et al.*, 1993). The bulblets in turn develop a new shoot, which provided the material for PCR-analysis. This regeneration system includes three phases in which a number of cells are involved in the formation of new tissue. Such a regeneration system might easily lead to the production of chimeric transformed shoots.

The selection system was designed to prevent growth of untransformed cells, without killing too much of the surrounding tissue (Wilmink *et al.*, 1995[a]; chapter 3). Shoots that had developed on selective medium were subcultured for outgrowth without further application of selective pressure. The development of shoots on selective medium after particle bombardment was completely normal. No shoots developed on unbombarded explants on selective medium. However, because low numbers of shoots were able to regenerate on selective medium after bombardment with the non- selectable *gus* gene, a slightly positive effect of bombardment on regeneration cannot be excluded.

A selective system was used in which resistance was conferred by means of detoxification of the selective agent, which systems generally give rise to some cross-protection (Wilmink and Dons, 1993; chapter 2). This might enable outgrowth of non-transformed cells in the presence of selective pressure, and promote chimeric shoots to develop.

Considering all this, it cannot be excluded that the transgenic plants obtained were formed from chimeric tissue, due to an inappropriate selection system. This suggests the use of a higher concentration of Basta during a longer period of growth, or a non-detoxifying selective agent, like glyphosate or chlorsulfuron.

Expression of monocot promoter constructs.

Monocot promoter constructs with the rice *actin* promoter or the maize *ubiquitin* promoter directed a twofold increase in gene expression in tulip floral stem explants compared to the CaMV *35S* promoter, and a 1.5 fold increase in expression compared to the *Emu* promoter. This is comparable to, but not completely equal to results described earlier (Wilmink *et al.*, 1995[b]; chapter 4), in which leaf tissues were used. Promoters might respond slightly different in various plant tissues.

In the transformed tissues, GUS expression was observed in young stages of shoot development, but not in stages older than nine weeks. Phenotypically expressed Basta-resistance in the bulblet stage was tested by screening for Basta-resistance of explants on medium with the pH indicator chlorphenol-red (Kramer *et al.*, 1993), and could not be demonstrated, irrespective of the promoter that was used in front of the *gus* or the *bar* gene. The reasons for this are unclear, it suggests that expression of the transgene is too low to direct any visible expression. The low amounts of expression were confirmed for the *bar* gene by RT-PCR analysis (Fig. 5), and might either originate from only a few cells in chimeric tissue, or from tissues in which expression has been silenced in most of the cells. Gene silencing has been reported in a number of studies (reviewed by Flavell, 1994; Finnegan and McElroy, 1994; Matzke and Matzke, 1995), and might either act transcriptionally (Matzke *et al.*, 1994) or post-transcriptionally (de Carvalho Niebel *et al.*, 1995). Promoter methylation and silencing might be induced when multiple gene insertions have occurred, as reported before for transformation by means of direct gene transfer.

Integration of the introduced genes.

Putative transformed tissues were analyzed by means of PCR. This technique detects the presence of a gene, but does not give information about the integration of the transgenes into the host genome. It is well known, that positive PCR signals can result as some kind of artefact, and a lot of precautions should be taken to exclude that. In this study we took precautions against plasmid or product contaminations by spatial separation of PCR analysis and other lab handling. By including negative control samples in the PCR reactions, we showed that this approach was successful. On the other hand, particle bombardment results in large amounts of unintegrated plasmid in the target cells. We tried to overcome this problem by delaying analysis of putative transformants until secondary and even tertiary tissue (i.e. bulblets and shoots from these bulblets) had developed from the primary shoots. Twelve to 15 months after bombardment, the chance that non-integrated plasmids would still be present in tulip cells was considered to be neglectable. However, the large number of PCR-positive plants that was obtained with or without selection made it necessary to assess very carefully the presence and activity of introduced genes.

For this, three approaches were used. First, RT-PCR analysis showed that the *bar*-gene was expressed. The level of transcription seems to be very low, since we were not able to detect transcripts by using Northern analysis. Besides, it cannot be excluded, that this RNA expression originates from unintegrated plasmids. Secondly, to demonstrate integration, an attempt was made to isolate flanking tulip genomic sequences by means of plasmid rescue. Although it was found that the presence of tulip DNA was not

inhibitory for the rescue of *amp*-containing sequences, we were not successful in isolating flanking tulip DNA. However, by means of this approach, the presence of intact plasmids in the tulip genomic DNA could be excluded. Thirdly, strong indications have been obtained that the introduced plasmids replicate in tulip cells, by determining the methylation state of the DNA. This approach was described before to indicate integration of foreign DNA in barley cells by Ritala *et al.* (1994).

All these approaches however, are no firm proof of integration of foreign DNA in the tulip genome. For this, Southern blotting or progeny analysis remain the methods of choice. Due to the large size of the tulip genome (25.10^6 kb, 35 times the potato genome), a lot of difficulties were met in demonstrating the presence of endogenous genes by means of Southern blotting. This means that for the final proof of transformation, we can only wait for progeny analysis. The bulblets that were obtained will flower after approximately 4 years of further propagation.

Prospects for improvement of the system

In order to make the procedure of adventitious shoot formation more efficient for use in transformation, we suggest that some adaptations of the regeneration and selection system are necessary. In order to prevent the development of chimeric transformed tissue, a more stringent selection system should be applied, using higher concentrations of Basta during longer periods of growth, or using other selective agents like glyphosate or chlorsulfuron. The regeneration system itself can be adapted as well. It is plausible that a clonal regeneration system, in which regeneration is induced from one single transformed cell, should be established for tulip. In such a regeneration system non-transformed cells would not be involved in the formation of new tissue, and fully transformed shoots can be obtained. Untill now, reproducible clonal regeneration systems are not available for tulip, but good prospects are offered for somatic embryogenesis or embryogenesis from microspores.

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chapter 7

Summary and General conclusions

This thesis describes the development and evaluation of a procedure for genetic modification of the monocotyledonous, bulbous ornamental crop tulip (*Tulipa gesneriana* L.). In this chapter, we will summarize the results obtained and make general conclusions on the prospects for use of this system in the future.

Regeneration and transformation

The regeneration system used was a system of direct adventitious shoot formation on floral stem segments. With this regeneration procedure, we tested the effects of various selective agents, antibiotics and herbicides, on the regenerative capacity of floral stem segments. The herbicide Basta offered best perspectives for use in a transformation procedure when compared to antibiotics, because there was a clear effect on the regeneration of unbombarded explants, while tissue death was limited.

The use of two transformation methods, *Agrobacterium*-mediated gene transfer and direct gene transfer by particle bombardment was investigated. From these experiments, we concluded that efficiencies obtained with *Agrobacterium* were low and cultivar-dependent, while particle bombardment was the most reproducible method for transformation of tulip.

For transformation, floral stem segments were bombarded with the Basta-resistance gene *bar* and subjected to various periods of tissue culture. Because the floral stem segments are regenerative only during a limited period of the year, two extensive transformation experiments were performed, in 1993 and in 1994. Meanwhile, histological analysis of the origin of adventitious shoots was performed, and showed that they originate from multicellular regions. Because this might cause the formation of chimeric transformed tissue, we hypothezised that the use of a strong promoter in front of the selection gene might be beneficial for the selective advantage of transformed cells. The activity of the CaMV 35S promoter and three monocot promoters was tested by bombarding floral stem explants with the various promoters fused to the *gus*-reporter gene and subsequent determination of GUS-expression. On the basis of these experiments, the rice *actin* promoter was chosen for further experiments.

After bombardment, explants were incubated and adventitious shoot formation took place in the presence of the selective agent Basta. In both transformation experiments bombardment of the explants resulted in the formation of Basta-resistant shoots. No shoots were able to develop from unbombarded explants in the presence of the selective agent. Basta-resistant shoots were further grown in the absence of the selective agent and the first bulblets were produced. After dormancy and cold treatment of the bulblets, new shoots were formed from these bulblets, and these new shoots provided the material for molecular analysis. This analysis was performed 12-15 months after transformation. Shoots were analyzed for the presence and expression of the introduced *bar* gene, as well as for the presence of the co-introduced *gus* gene and the ampicilline resistance gene *amp*. All genes were present on the introduced plasmid, *amp* as a bacterial selection gene.

Molecular analysis of putative transgenic plants

First analysis was done by applying the polymerase chain reaction (PCR) to DNA isolated from the shoots that developed from the bulblets. In these experiments, precautions were taken to exclude PCR artefacts, all PCR handlings were separated from other lab handlings and the proper negative controls were included. For DNA, isolated from material obtained in both transformation seasons, the PCR analysis was positive both for the *bar* and for the *amp* gene, for all putative transformants. This indicated that the introduced genes were present in the tulip shoots. To demonstrate transcription, RNA was isolated, and cDNA was made. PCR analysis of this cDNA again was positive for most of the putative transformants and indicated that the *bar* gene was expressed, at least at low levels. Remarkably however we observed that also for those shoots which had been bombarded but not been subjected to Basta-selection, a positive signal was found.

Expression at the protein level was analyzed for GUS and for BAR. GUS expression was nicely demonstrated in primordia and young shoots, but could never be shown in shoots older than two to three months. The same was observed for Basta resistance, which could only be demonstrated in young stages of shoot formation, indicating that no or at most low enzyme activity was present.

To examine further what happened to the introduced genes in the cells, we investigated, whether the genes had replicated in the tulip cells. The restriction enzymes *Dpn*I and *Dpn*II discriminate between methylated and non- methylated DNA sequences. Using these enzymes for digestion of tulip DNA, we were able to demonstrate a shift in the methylation pattern of the *amp* gene, indicating that the gene had replicated after introduction in tulip cells.

Taking all these results together we can conclude that strong indications have been obtained for the presence of the introduced genes in tulip cells. However, these results do not establish conclusive evidence for integration into the nuclear genome of tulip. Because the tulip genome is too large (25.10^6 kB) for Southern blotting, we attempted to demonstrate integration of the genes by means of rescueing the introduced DNA together with flanking genomic sequences from the tulip DNA (plasmid rescue). In reconstruction experiments we could demonstrate that in principle, this method should work for tulip DNA if at least one copy of the introduced gene per genome had integrated. Several attempts have been made, but we did not succeed in rescueing plasmids with additional tulip sequences. A reasonable explantation for this might be, that we were dealing with copy numbers less than one. In these experiments we could also show that it was impossible to harvest any plasmid from unrestricted and unligated tulip genomic DNA. This strongly suggests that the positive PCR results on genomic DNA were not due to the presence of free plasmid remaining from the bombardment event.

Evaluation

The results of the molecular analysis of putative transgenic tulips are not conclusive about the integration of the introduced DNA. An obvious explanation for the positive results when using the PCR test might be the presence of plasmid DNA in a non-

integrated form as a result of the particle bombardment. However, a number of arguments do not support this assumption. First, the molecular analysis was performed one and a half year after transformation and free plasmids are not expected to survive such a long period. This holds particularly for a plant like tulip with its complicated vegetative growth, from which DNA was isolated from secondary shoots developed from the first bulblets. Secondly, evidence was presented for RNA expression and replication of the introduced genes in tulip cells. Thirdly, it was not possible to rescue any plasmids from tulip genomic DNA, which would have been rather easy if free plasmids would have been present.

This leads to the conclusion that the introduced DNA is indeed integrated. The problems that were encountered concerning the demonstration of integration of the DNA and the low level (or even absence) of expression at the protein level, need further discussion. To explain this, we hypothezise that the Basta-resistant shoots have the *bar* and *gus* gene only in part of their tissue. To support this hypothesis we refer here to a reconstruction PCR analysis that was performed to get an impression about the sensitivity of the method (Fig. 1). In these experiments we added various amounts of plasmid DNA to fixed amounts of tulip DNA prior to PCR reaction. The strength of the signal reflects the amount of templates added. When comparing the strength of the signal obtained from equal amounts of DNA of putative transformants obtained after selection, we can conclude that the gene is present, but in numbers lower than 1 copy per genome. Two of the transformants possess approximately 0.01 copy of the gene per genome, while the others have 0.1 to 1 copy per genome present. Although the use of PCR reactions for quantitative estimations should be considered cautiously, these results do indicate

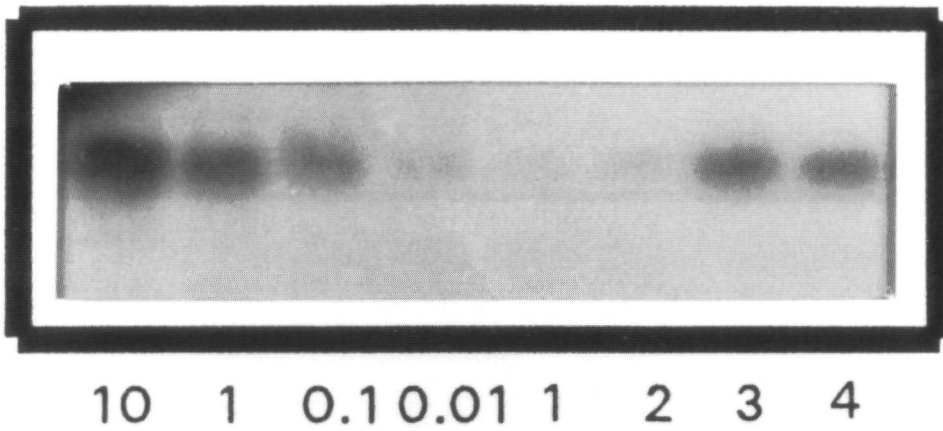

Fig. 1.
"Reconstruction" PCR analysis of the presence of the *amp* gene in tulip transformants. In lane 1 to 4 resp. 10, 1, 0.1 and 0.01 copy of the *amp* gene were added per copy of the tulip genome of DNA of unbombarded tulip shoots. Numbers 1 to 4 (lanes 5 to 8) represent independent tulip transformants, in which equal amounts of genomic DNA were added, comparable to lanes 1 to 4.

that we obtained shoots, which have the introduced genes present only in part of their cells. The suggestion that chimeric transformed shoots have developed is in line with our observation that adventitious shoots originated from multiple cells. If one or few of these cells were transformed by means of particle bombardment, it is very likely that chimeric shoots have developed.

The development of chimeric shoots implies the division of non-transformed cells in the presence of the selective agent, while the selective system we applied was designed to prevent this. The chance that chimeric shoots were able to develop later on was enhanced by the further culture in the absence of selective agents. This leads to the conclusion that the selection system was not tight enough, which might also be due to the nature of the Basta-resistance mechanism, that allows cross protection of untransformed cells. Resistance to the active component of Basta, PPT, is conferred by means of acetylation, brought about by the *bar*-gene product. Such detoxifying enzymes can cause a medium detoxification, which then serves as a substrate for untransformed cells. Apart from this, it cannot be excluded that the BAR-enzyme itself is able to pass the membranes into adjacent cells, making them resistant without having the resistance gene. This assumption of transport of proteins between tulip cells can be supported by histochemical data on GUS-expression. These indicate that epidermal and subepidermal cells of floral stem explants of tulip exert high levels of diffusion of the GUS-activity. Although it is well-known that the GUS-reaction produces diffusible intermediates, the diffusion we observed for tulip (sub)epidermal cells is extraordinary in comparison with cells of other plant species, or cells of different tulip tissues. This is illustrated in Fig. 2.

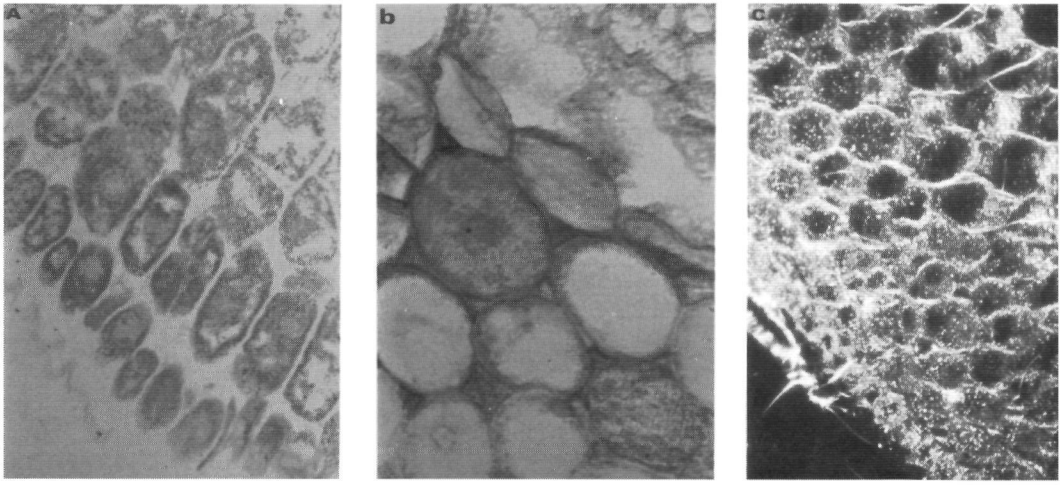

Fig. 2.
Histological pictures of diffusion of GUS-activity in tulip epidermal and subepidermal cells after bombardment with the *gus* gene. A: 20 days after bombardment. B: cells of central regions of the floral stem explant show less diffusion. C: dark field exposure of GUS-activity and diffusion after 30 days incubation on selection medium.

The occurrence of cross-protection or diffusion fits with an observation we made on regeneration after particle bombardment and subsequent selection. When no selective pressure is applied shoots developed dispersed over the explant. After transformation and subsequent selection however, Basta-resistant shoots developed in clusters, only on specific locations of the explants. This different mode of regeneration might indicate that cells are able to proliferate as a result of protection by neighbouring resistant cells.
The formation of chimeric transformed shoots would also give answers to some other intriguing questions. First, the high efficiency of tranformation, as demonstrated by PCR even in shoots which had not been selected for the presence of the resistance gene. When shoots develop from multicellular regions, and a high-efficiency transformation protocol is used, all shoots are likely to include some transformed cells. Since PCR-analysis is very sensitive, the low number of transformed cells will cause a positive PCR-signal. Secondly, the negative results on BAR and GUS expression indicate that at most low levels of protein are expressed. When only a few cells in the tissue are expressing the proteins, this might be too low to confer Basta-resistance. However, also the very slow growth of tulip shoots and bulblets provides an explantation for low protein expression levels, which might be even too low to detect visible GUS expression. Finally, chimerism might also explain the unsuccessful plasmid rescue. When the gene is present in copy numbers lower than one per genome, higher amounts of DNA have to be used in the reactions in order to rescue genomic sequences. We did not check whether the procedure still works with extremely high amounts of DNA, but it seems quite likely that the lowest detection limits were not yet met.

Future investigations
The system of direct adventitious shoot formation that we used for transformation of tulip has proven to be valuable, since gene transfer efficiency by particle bombardment was very high, and shoots were able to develop from bombarded explants. However, the problem of the development of chimeric shoots demands further adaptation of the system, especially with respect to the selection system. To prevent the development of non-transformed cells, a more stringent selective system is necessary, providing that the regeneration process is not hampered. One option is, to use higher concentrations of Basta during longer periods of growth. Another possibility would be, to use a different kind of selective system, in which the active component is not detoxified, but resistance is conferred by other means, like glyphosate or chlorsulfuron.
The regeneration system itself can be adapted as well. In course of this research we have also observed that it is possible to induce secondary shoot formation on explants from primary adventitious shoots, which offers opportunities to reduce the degree of chimerism. When secondary shoots are induced on medium with the selective agent, only regions that contain transformed cells will develop secondary shoots. After several cycles of subculture, fully transformed shoots are likely to be obtained, after which bulblet induction can be induced. In this way, fully transformed bulblets might be obtained. If this system does not result in homogeneously transformed tissue, the only alternative will be to establish a clonal regeneration system, in which regeneration is

induced from one single transformed cell. Such a system would be more in line with results found for other monocot crops like for instance maize, rice and wheat, for which only single cell regeneration systems are used for transformation. Although for monocots direct regeneration has never been applied for transformation, for dicots such systems of adventitious shoot formation are suitable for use in transformation experiments without harvesting too many chimeric shoots. Because our results differ from results obtained with dicots, it seems plausible that some differences may exist between monocots and dicots with respect to the use of a system of direct regeneration for transformation.
Untill now, reproducible clonal regeneration systems are not available for tulip, but good prospects are offered for somatic embryogenesis or embryogenesis from microspores.

Concluding remarks
This thesis describes the first results on the development of a transformation procedure for tulip. Especially for a crop like tulip, with its long generation cycle and its susceptibility for diseases, genetic modification is of great importance, and will have a great impact for breeding. The results described here indicate, that gene transfer into tulip cells can succesfully be performed using the particle bombardment technique. This can be concluded from the fact that the introduced genes are still present after prolonged periods of tissue culture and genes replicate in tulip cells. However, integration of introduced DNA in the tulip genome was not yet proven, and some problems remain regarding the regeneration and selection process. We are quite confident that these problems can be solved in the near future, for instance by using a more stringent selection system and by adapting the regeneration procedure or by establishing a new, clonal system for regeneration. We conclude that the first steps have been made towards genetic modification of tulip and that after further improvement of the developed transformation system, tulip will become a crop in which new traits can be introduced.

chapter 8

Prospects for improved integration of transferred DNA into the genome of monocots

Annemiek Wilmink, Ingrid van der Meer and Hans Dons

Introduction

Monocot crops have long been recalcitrant for transformation, mainly because most are not susceptible to *Agrobacterium*, but also because methods for direct gene transfer are hampered by difficulties in setting up protoplast regeneration systems. The introduction of the particle bombardment technique circumvented both these problems, and transgenic plants have subsequently been obtained for various monocot crops. However, the process of gene integration after direct gene transfer is poorly controlled. It has often been postulated that integration frequencies are low, that rearrangements occur and that single copy insertions are rare (Gharti-Chhetri *et al.*, 1992; Cherdshewasart et al, 1993). For tulip we are likely to be confronted with similar problems. Although the presence of the *bar* gene has been demonstrated by means of PCR, and a low level of expression could be demonstrated by means of RT-PCR (Chapter 6), the integration of the gene in the host genome has not been proven. Because of the problem of the size of the tulip genome we still know nothing about integration efficiency, multicopy insertions, deletions and rearrangments.
For many monocots, data concerning transformation frequencies and integration patterns have been published. However, only for maize, and to a lesser extent also for rice, data are available from a number of different research groups. Therefore, a case study was made on maize transformation to evaluate the results of particle bombardment in this monocot. We will not discuss the various target tissues and conditions that have been applied in these studies, but rather evaluate the molecular proof of integration and expression of the genes introduced by particle bombardment. On the basis of these results we will try to draw conclusions concerning the quality of gene integration after particle bombardment and discuss how it could be improved. Finally, we will summarize some new approaches that might be useful in establishing a system for guided integration of genes.

Maize: a number of transformation methods have been applied

Maize has been shown to be a host for *Agrobacterium*, but only in the case of agroinfection experiments using the Maize Streak Virus (Grimsley *et al.*, 1987). Although some preliminary results have been described so far, this has not resulted in the establisment of a transformation system for maize using *Agrobacterium*. Gould *et al.* (1991) obtained the first maize transgenics by infection of shoot apices with *Agrobacterium*. Since then, attempts to use *Agrobacterium* have not been successful except for the detection of GUS-positive spots on shoots of young maize seedlings after infection (Shen *et al.*, 1993). Prospects were greater for direct gene transfer techniques, especially since protoplasts can now be regenerated from certain maize genotypes. Electroporation of maize protoplasts resulted in stably transformed cells (Fromm *et al.*, 1986) and transgenic plants were obtained by Rhodes *et al.* (1988). Also PEG-mediated transformation of protoplasts resulted in transformed maize plants (Omirulleh *et al.*, 1993; Golovkin *et al.*, 1993). Some other alternatives were also assessed, for instance incubation of pollen

with DNA during fertilization (Ohta, 1986), electroporation of intact tissues (D'Halluin *et al.*, 1992; Songstad *et al.*, 1993) and the use of silicon carbide whiskers (Frame *et al.*, 1994). In some cases, transgenic plants have been obtained, but none of these methods however, has yet had such an impact on the development of a transformation procedure for maize as the use of the particle gun.

Particle bombardment: the most reproducible method to obtain transgenic maize

The first positive results on the use of particle bombardment for maize cells were obtained by Klein *et al.* (1988), who demonstrated transient expression of the *chloramphenicol acetyltransferase* gene in maize cell suspension cells. Since then, much effort has been put into optimizing this gene transfer system, and transgenic plants have since been obtained by numerous groups. In Table 1 the results on gene integration in maize after particle bombardment are summarized. These will be discussed briefly:
Gene integration. In all transgenic maize plants mentioned in the table, the presence of the introduced gene has been demonstrated by means of Southern blotting. However, for direct gene transfer, it is important to rule out the possibility of detecting plasmid DNA that is still present as a result of the bombardment event. This can be done by using unique restriction sites on the plasmid to obtain definite proof of integration, and at the same time gaining an indication of the number of copies present in the genome. Integration has been demonstrated in this manner only in the more recent papers on transformation of maize (Walters *et al.*, 1992; Murry *et al.*, 1993; Koziel *et al.*, 1993; Vain *et al.* 1993).
Expression. mRNA and protein expression directed by the introduced DNA sequences detected in both the transgenic plants and their progeny demonstrated stable integration of the selection genes in the transgenic maize plants obtained in these studies.
Copy numbers. Copy numbers of the transferred genes were estimated in most cases by means of reconstruction experiments, occasionally using Southern blots. These appeared to be variable, in most transgenics more than one gene copy was found. This might be one of the reasons for the reduced fertility of transformed plants observed by Fromm (1990), Spencer (1992), Walters (1992) and Murry (1993). Also, Mendelian segregation was found to be disturbed in these reports, probably as a result of the presence of multiple integration sites.
Rearrangements. When evaluating the Southern blots, many hybridizing fragments were frequently found, even when internal fragments were used for hybridization. These hybridizing fragments were not a result of aspecific hybridizing genomic fragments, because they were absent from untransformed controls. Of course some of these bands may be due to incompletely digested DNA, but considering the nature and abundance of these extra bands in most studies, it seems more likely that tandem integrations and rearrangements have occured.

Table 1. Literature reports on transgenic maize, obtained after particle bombardment. Evaluation of foreign gene integration in the various studies.

reference	S.blot internal fragment	S.blot integration	PCR	expr. sel. gene	progeny	copynr.	rearrange-ments	stable: transient ratio %	transf. frequency transform./ bombardment
Klein, '89	+	-	nd	+		1-8^{r}	+		1
Spencer, '90	+	-	nd	+		1-20^{r}	+	5-10	10-20
Fromm, '90	+	+*	+	+	+^{f}		1 site		0.2
Gordon Kamm, '90	+	-	nd	+	+	1-20^{r}	+	0.1	1.35
Spencer, '92	+^{o}	-, +$^{+}$		+	+$^{fm+}$	1^{+}-6	+		
Walters, '92	+^{c}	+^{c}	+	+	±^{fm}	<5^{r}	+?		0.12
Murry, '93	+	+	+	+	+^{fm}	2-5^{r}	tandem?		0.1
Koziel, '93	+	+	+	+	+	few	+? 1 site		0.4
Vain, '93	nd	+	nd	+	+	5-10	+	0.04	
Register, '94	+^{c}	-	+	+, -	+	1->10^{r}	+; tandem		0 5-1
Wan, '95	+	+	nd	+	+	1-50^{r}	+; tandem		2.4

*: determined with uncut DNA
^{r}: determined from reconstruction
^{o}: deviating fragment size
^{m}: deviating mendelian segregation
^{f}: reduced fertility
^{c}: callus
$^{+}$: in some of the cases

Transformation frequency. In most studies, transformation frequencies have been measured, mostly as the number of transformants per particle gun bombardment with a fixed amound of DNA, or per number of bombarded cells, but also per number of transiently expressing cells. It is difficult to compare the efficiencies, because so many different parameters have been used. In general, 0.1-1 transformants are obtained per bombardment, although Spencer *et al.* (1990) reported efficiencies of 10-20 transformants per bombardment. Stable to transient ratios vary between 0.04% and 0.1%, while Spencer again reported higher ratios. Therefore we can conclude that although integration frequencies are low, as judged from the stable to transient ratios, the number of transformants obtained per bombardment is rather high. This means that particle bombardment can be considered to be a rather efficient method of gene transfer, not due to high integration frequencies, but rather to the high numbers of cells that receive DNA-coated particles.

Evaluation: are rearrangements and multiple integrations specific for particle bombardment?

From the previous section it can be concluded that complex integration patterns, rearrangements and non-Mendelian segregation ratios are common in the various transgenic plants of maize obtained by particle bombardment. Such problems have also been encountered in tobacco after transformation using particle bombardment. Tomes *et al.* (1990) analyzed the progeny of two tobacco transformants derived by microprojectile bombardment, and concluded that the plasmid DNA had integrated in a complex manner, suggesting that rearrangements had occurred. Only one of the transformants showed Mendelian segregation ratios.
Integration of genes after *Agrobacterium*-mediated transformation was investigated by Deroles and Gardner (1988). Extreme rearrangements had occurred in only three of the 96 petunia plants, while 52 plants contained some unpredictable fragments. From 93 analyzed transgenic plants, 35 showed a single copy integration, while 30 plants had 3-6 copies of the kanamycin resistance gene. Of the 35 single copy plants, 21 had an intact copy of the T-DNA insert, while the others were rather simple deletion variants.
This study shows that, although high percentages of transgenic plants have one intact T-DNA, *Agrobacterium* mediated gene transfer can also lead to several aberrant integrations, both in terms of copy number and rearrangements. Problems with direct gene transfer however, seem to be more severe. This is probably caused by the naked structure of the plasmid-DNA and the lack of recombination sites analogous to the left and right T-DNA borders.
To elevate integration efficiencies, it seems useful to evaluate systems that control the transgene integration event.

Evaluation of systems to improve integration

To circumvent integration problems, more effort might be put into the improvement of the integration process. One approach is to establish a system of integration via homologous recombination rather than via illegitimate recombination, in order to direct genes to specific locations of the genome. This approach is commonly used in animal cells, but in plant cells, efficiencies are still extremely low. Additionally, the use of prokaryotic recombinase systems in plant cells has been investigated. Here we also evaluate another system to improve integration efficiencies, using transposable elements.

Homologous recombination

Animal and yeast cells. Much data on homologous recombination have been obtained from studies using animal and yeast cells. This type of integration can be achieved by using the same sequences as present in the target site in the plant genome for the construction of the recombination vector. After gene transfer, the introduced DNA is expected to locate the homologous sequences and to integrate via homologous recombination. In this way, a gene can be inserted in one copy into a previously determined site on the chromosome. In particular, mouse embryonic stem cells have frequently been used to study integration processes (reviewed by Wilder and Rizzino, 1993). In this system, factors that affect the frequency of integration by means of homologous recombination have been investigated. Depending on the gene to be targeted, frequencies may range from 10^{-4} to 10^{-2} (ratio homologous to illegitimate). Many attempts have been made to increase or enrich for the efficiency of homologous recombination, e.g. the use of positive-negative selection systems (PNS, Mansour *et al.*, 1988). In these systems, stable integration of the vector is selected by means of a positive selection marker gene (for instance a *neo* gene, conferring resistance to G418). In combination with this, homologous integration events are monitored by switching off a negative selection marker gene, for instance, the HSV-thymidine kinase gene (*tk*). When this gene is present, cells are sensitive to gancyclovir and will die when exposed to this selective agent. The negative selection gene is inserted in the targeting vector outside the region carrying the homologous sequences. Integration of the gene of interest via a double homologous cross-over event will cause depletion of the negative selection marker. In contrast, cells in which illegitimate recombination has taken place will still contain the negative selection marker gene and subsequently die (Fig. 1). PNS systems enrich for homologous recombination events. However the degree of enrichment varies, from 10-fold (Wilder and Rizzino, 1993) to 100- or 2000-fold (Pascoe *et al.*, 1992; Mansour *et al.*, 1988).

A major improvement in the efficiency of targeting systems can be achieved by enlarging the size of the homologous sequences in the targeting vector. Thomas and Capecchi (1987) showed that increasing the size of the homologous sequences from 4 to 9 kb yielded 10-fold higher targeting frequencies in mouse embryo-derived stem cells. A 100-fold increase was obtained by increasing the length of homologous DNA from 2 to 10

kb (Deng and Capecchi, 1992). Similar observations have been made by Hasty *et al.* (1991). They reported an increase in targeting efficiency of 190-250 times when the region of homology was enlarged from 1.3 to 6.8 kb.
Additionally, the source of homologous DNA that is used for constructing the targeting vector is of great importance. Homologous sequences should preferentially be isolated from target cell lines (te Riele *et al.*, 1992). This isogenic DNA provides long regions of complete homology, which enhances the efficiency of homologous recombination. Partially homologous DNA, with sequence heterogeneities, gives rise to mismatches in the homologous regions causing mutations after recombination.
Plant cells. Homologous recombination in plant cells was reviewed by Puchta *et al.* (1994). Experiments were performed for instance by Baur *et al.* (1990), who cotransformed *Nicotiana tabacum* protoplasts with two plasmids, containing nonoverlapping deletions in the *aph*(3')II gene. Restoration of the gene, leading to kanamycin resistance, was positively correlated with the length of overlapping homology ranging from 6 to 405 bp of the truncated *aph*(3')II gene. The frequency of recombination compared to the frequency of random integration increased from negligible levels of 0.18% for 6 bp homology up to 32.4% for 352 bp of homology. A region with 53 bp of sequence homology was the minimum necessary for efficient recombination (1-6%). A similar approach, using the *ß-glucuronidase* gene, was used by Puchta and Hohn (1991) to demonstrate a positive correlation between the length of overlapping homology and, in this case, restored GUS-activity. Using a transient expression assay, the need for recombined plasmids to integrate into the genome was circumvented. The requirement of a minimal length of 456 bp homologous DNA was demonstrated, while recombination increased when homologous regions up to 1200 bp were used. Extrachromosomal homologous recombination occurs early after transfection, as was demonstrated by Puchta *et al.* (1992) using the same GUS-restoration assay. Thirty minutes after transformation enzyme activity which required recombination was detected in the protoplasts. In addition, Puchta *et al.* (1993) showed that double strand breaks enhanced extrachromosomal homologous recombination in plant cells. When introduced plasmids were cleaved *in vivo*, adjacent to their homologous sequences, GUS-activity, indicative for extrachromosomal recombination, was strongly increased. They hypothesized that *in vivo* induction of transient breaks at specific sites in the plant genome could target foreign DNA to these sites via homologous recombination.

Gene targeting in plant cells

Plastid DNA
While the use of homologous regions for targeting introduced DNA into the genome is common in research on animal cells, this process is not commonly used in plant cell transformation.
One of the first studies in which large target sequences have been used, was a study of stable transformation of plastids in higher plants. Svab *et al.* (1990) incorporated a stretch of 3.7 kb of plastid DNA encoding the 16S rRNA into the DNA to be inserted

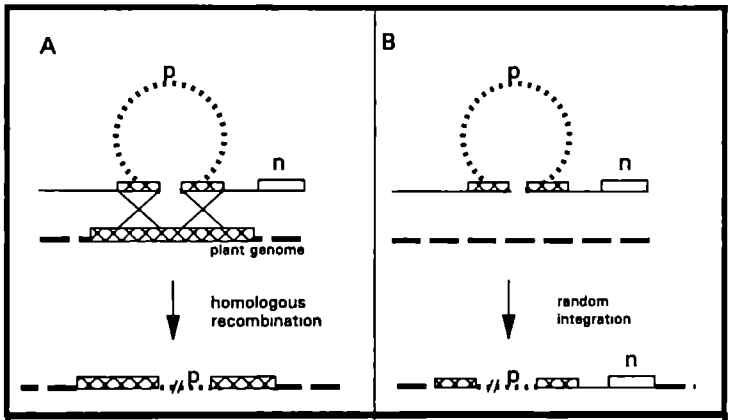

Fig. 1

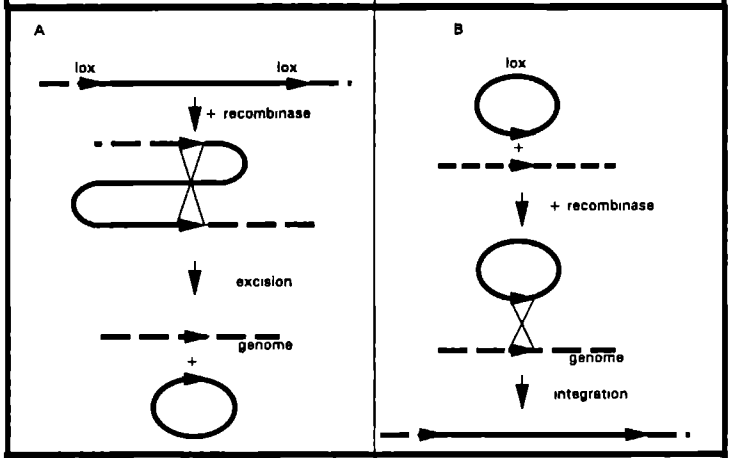

Fig. 2

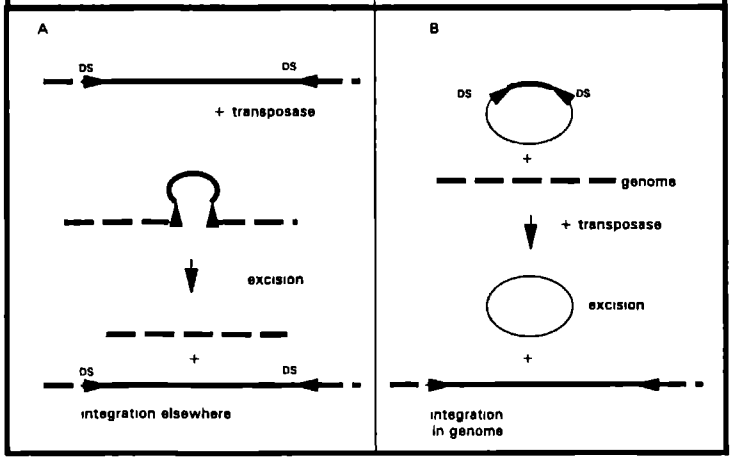

Fig. 3

Fig. 1. Positive-negative selection system (PNS).
The use of PNS to distinguish insertion via homologous recombination (A) from random integration (B). P: positive selection marker; N: negative selection marker; homologous regions are indicated by hatched boxes and stippled circles. Genomic DNA is indicated by broken lines. After homologous recombination the negative selection marker is not integrated in the genome because it is placed outside the homology region, in contrast to illegitimate recombination, where both the positive and negative selection marker genes are integrated.

Fig. 2. Recombinase system.
The Cre-*lox* recombinase system. A: excision of the DNA between the lox sites by recombination induced by the Cre-recombinase protein. B: the reversion of the reaction: *lox* site specific integration of DNA by the action of the recombinase protein. The *lox* site is indicated by an arrow, genomic DNA by broken lines.

Fig. 3. Transposase Mediated Integration (TMI).
A: transposase-induced excision of DNA between DS borders followed by integration elsewhere in the genome. B: transposase-induced excision of DNA from an introduced plasmid followed by integration in the genome. The DS borders are indicated by arrows, genomic DNA by broken lines.

into *Nicotiana tabacum* plastids. Although the efficiency of transformation was low (one "transplastomic" clone per 50 bombardments, as opposed to 2-6 nuclear transformants per bombardment), the introduced rDNA had integrated into the plastid rDNA by homologous recombination and had replaced the wildtype 16S rDNA. Staub and Maliga (1992) showed that integration of a long, uninterrupted region of homologous DNA is a more likely event than integration of small fragments. Their plasmid contained a 6.2 kb region from the tobacco plastid genome. Wild-type sequences were replaced and almost all of the 6.2 kb plastid DNA was incorporated into the tobacco chloroplast genome. A 100-fold increase in plastid transformation frequency was reported by Svab and Maliga (1993) when the dominant *aadA* gene, conferring resistance against spectinomycin and streptomycin, was used for selection instead of the mutant 16S rRNA gene, which is recessive with respect to spectinomycin resistance. Using PEG treatment of protoplasts, O'Neill *et al.* (1993) reported a chloroplast transformation efficiency of 2×10^{5}, and they observed homologous integration of the introduced 16S rRNA gene in the only recombinant that was studied. Maliga (1993) concluded that plastid transformation can become a powerful technique for plant transformation, because of features like maternal inheritance, integration at specific loci and amplification of introduced genes in cells, which permits overexpression of certain proteins.

Nuclear DNA

Offringa *et al.* (1992) reviewed the most recent developments concerning gene targeting in plant nuclear DNA, and concluded that defective marker genes were restored by means of homologous recombination at frequencies of 10^{-7} to 10^{-4}. These frequencies were much higher (10^{-5} to 10^{-2}) for human and animal cells. One possible explanation might be that the targeted stretches of homology used in animal systems were generally much longer than those used in plant systems (0.4 - 3.6 kb). This is below the critical length of 4.2 kb reported to be necessary for animal cells by Hasty *et al.* (1991).

Another explanation might be that there is a different recombinational system active in plant cells as compared to animal cells.

The use of prokaryotic and lower eukaryotic recombinase systems

Although the use of homologous sequences to target genes into a specific site in the genome is an obvious way to improve integration, the efficiency of targeting in plants remains low. Target events have to be selected against a high background of random integration events. Due to this low frequency, external site-specific recombinase systems have been investigated for their potential to target incoming DNA to a previously inserted target site (Kilby *et al.*, 1993). In prokaryotes and lower eukaryotes, several recombinase systems have been identified that promote recombination between two specific target sequences. These systems also function in higher organisms such as mammalian cells (O'Gorman *et al.*, 1991) and plant cells (Dale and Ow, 1990; Ow *et al.*, 1994). This is true for the Cre-*lox* system from bacteriophage P1, the Gin-*gix* system from phage Mu, the R/RS system of pSR1 of *Zygosaccharomyces rouxii* and the yeast FLP/FRT system (Odell *et al.*, 1990; Maeser and Kahmann, 1991; Onouchi *et al.*, 1991; Lyznik *et al.*, 1993).

Recombinase systems consist of a recombinase protein and a short target sequence (34 bp in the case of the Cre-*lox* system). When these target sequences flank a chromosomal segment, this segment will be excised. This action is reversed when the target sequences are located on different molecules, leading to insertion instead of excision. If one of the target sequences is located on a chromosome and the other one is located on a transfered plasmid, this plasmid will integrate into the genome (Fig. 2). In this way, foreign DNA can be introduced at specific locations in the chromosome, predetermined by the location of the first target sequence. However, the insertion of this first sequence still remains a random process.

When excision frequencies were studied, the frequency of recombinase-mediated recombination appeared to be much higher than that of homologous recombination, as shown by Onouchi *et al.* (1991) for the R/RS system. In their study, 40% of tobacco cells that received the R gene were altered to a GUS positive phenotype because of a very precise excision of sequences interrupting the *gusA* gene located on the chromosomal DNA. The FLP/FRT system was able to induce extrachromosomal recombination in maize and rice at frequencies up to 90% when a strong promoter in front of the recombinase gene was used (Lyznik *et al.*, 1993). In tobacco, efficient FLP-induced excision of FRT-flanked sequences from the genome was reported by Lloyd and Davis (1994).

The ability of the Cre-*lox* recombinase system to induce site-specific recombination in plants has been investigated in a number of studies. Extra-chromosomal recombination in tobacco cells was first demonstrated by Dale and Ow (1990). Odell *et al.* (1990) showed that the Cre recombinase protein was able to excise a *lox*-flanked DNA segment from the tobacco genome. In this case, the *cre* gene was also stably integrated in the genome as a result of crossing with, or retransformation of, *lox*-plants. The excision

event was detected in 50-100% of the plants containing both elements. This recombinase system appears to work very efficiently in plant cells, opening the way to the inversion of the reaction which will lead to an efficient integration of a plasmid into a previously inserted *lox*-site in the genome. In this way, the integration process will be improved because it is controlled by the recombinase protein. Only one copy will be inserted, there will be no deletions or rearrangements, and the variability in expression levels due to "position effects" will be reduced because the introduced DNA is always targeted to the same predetermined locus. However, Cre-*lox* recombination events are reversible as long as the Cre protein is present. This can lead to instability of the insertion amongst the transformants. There are several ways to circumvent this problem. One is to separate the *cre* gene from the *lox* sites by means of sexual segregation after individual integration at different loci. Other possibilities are to achieve only transient expression of the *cre* gene, to use inducible plant promoters in front of the *cre* gene or to introduce the purified Cre protein into cultured cells together with the targeting vector by means of particle bombardment or lipofection (Baubonis and Saur, 1993). Qin *et al.* (1994) prevented *cre* expression by seperating the *cre* gene from its promoter during the recombination process.
It can be concluded that the use of the Cre-*lox* recombinase system opens up many possibilities to improve the integration of transgenes, and to direct them to specific genomic locations obtained through selection from a range of random integration events of the *lox* sites. For most applications, the use of this procedure implies a requirement for two different transformation events, but will however, result in integration patterns that will be very reproducible.

The use of transposable elements to promote integration

Transposable element systems resemble the recombinase systems in a number of ways. They generally consist of two elements: a gene encoding a protein, the transposase (like the recombinase), and two short border repeats that are target sequences for the action of the protein. The protein causes the DNA segment between the border repeats to be excised from one location in the genome and to be integrated at another location (transposition). In a similar way, transiently-expressed transposase can excise genes that are cloned between the border repeats, even when these genes are not stably integrated into the genome (Houba-Hérin *et al.*, 1990; Shimamoto *et al.*, 1993).
It is tempting to speculate on the potential of the Ac-Ds system in improving the integration process of genes after direct gene transfer and in elevating integration frequencies. The recombinase systems described above depend on two transformation events, the first of which could be a bottle-neck in plants that are not easy to transform, rendering the whole system less efficient than expected. Ac-Ds-mediated transformation might be expected to improve the efficiency of direct gene transfer. When cells are transformed with two plasmids, one containing the gene encoding the transposase protein and the other containing the gene of interest between transposon borders, the transposase protein can excise the gene of interest from the plasmid and promote

integration somewhere in the plant genome (Fig. 3). As long as the transposase remains active in the plant cell, the integration of the gene is unstable. However, the transposase gene, immobilized by deleting the borders, will only rarely become integrated in the genome by random (illegitimate) integration. After cell division, the majority of the daughter cells will only contain the gene of interest and will have lost the transposase gene. This results in a stably integrated gene in cell lines originating from the primary transformed cells.
Shimamoto *et al.* (1993) used the Ac-Ds system to show that Ds elements can be activated by cotransfected Ac transposase and integrated stably into the rice genome, while the Ac gene is only transiently expressed. In their system, the excision probably took place after integration of the transfected Ds containing plasmid. It is well-established, that transposons are mainly activated in replicating DNA. Therefore, Sugimoto *et al.* (1994) inserted a *DS::HPT* element into a viral MiSV vector, and studied excision and integration into the rice genome. Excision was observed, only when the MiSV vector was able to replicate, and when the transposase gene was co-transfected. However, Houba-Hérin *et al.* (1994) showed convincing evidence that the Ac-Ds system is indeed active, also from non-replicating DNA: Ds can efficiently transpose from extrachromosomal DNA to plant chromosomes when the Ac protein is transiently expressed. In the majority of the transgenic lines, the Ds-flanking sequences of the transferred plasmid are missing, and a limited number of copies is integrated in the genome. They showed that transposition occurs from extrachromosomal DNA to the plant genome, because there are no empty donor sites present in the transgenic plants genomic DNA, which would indicate transposition from one site in the chromosome into another, and by recovering intact plasmids that lacked the Ds insertion from the transformed protoplasts.

Conclusions

During the last decade, many methods have been developed for the introduction of foreign DNA into plant cells. Methods for direct DNA transfer are considered to result in low integration frequencies because the process of integration is poorly controlled. This process has been extensively studied in mammalian cells, and the level of targeted integration could be enhanced considerably by means of homologous recombination. The size of the homologous sequence showed to be an important factor involved in improving targeting frequencies, while the use of PNS systems facilitated the selection of homologous recombination events.
The importance of the length of homology varies for different organisms. In *E. coli*, the length of overlap required for intrachromosomal recombination was only 20 bp, while in mammalian cells, at least extrachromosomal recombination is promoted by a length of overlap of several hundred base pairs. This phenomenon was discussed by Puchta and Hohn (1991). They hypothesized that in plants as well as in mammals, the genome is stabilized by a tight homology requirement for efficient homologous recombination. This is necessary because the genomes of eukaryotes, and particularly of plants, are generally

much larger than the genomes of prokaryotes. Moreover, in plants long stretches of repetitive DNA are present. If longer regions of homology were not required for recombination, these repetitive stretches could be substrates for homologous recombination leading to a continuous rearrangement of the plant genome, which would be highly undesirable.
In plant cells homologous recombination studies have always been hampered by lower efficiencies than are obtained in mammalian cell systems. To enhance these efficiencies the use of longer stretches of homology was shown to have a positive effect, but the use of very long sequence homologies (several kb's) has not been investigated yet for nuclear DNA. Especially because this turned out to be beneficial for integration of foreign DNA in plastids, the use of such long sequences should be considered for gene targeting. Additionally, the use of PNS systems for plant cells have not yet been studied extensively, partly because a tight negative selection system for plant cells still has to be established.
Specific recombinase systems might be of great use to elevate the frequency of precise integration. Nevertheless, an initial, non-guided integration event (of the *lox* site) has to be achieved, which causes problems regarding recalcitrant crops. In this case, when the frequency of precise integration itself has to be elevated without the need for targeting, the use of transposase systems for a controlled integration might be helpful. Since results on Transposase Mediated Integration show that precise integration is promoted and that transformation frequencies are expected to be elevated (Houba-Hérin *et al.*, 1994), this might prove to be a valuable technique for integration of transgenes in recalcitrant crops, particularly when methods for direct gene transfer are used.

Perspectives for tulip transformation

For tulip, the use of homologous recombination and bacterial recombination systems have been considered. However, these systems both depend on two transformation events, and therefore do not seem to be the best choice for a crop for which the primary integration event might be restrictive, and selection for transformants takes a number of years.
Of the systems described above, the use of Transposase Mediated Integration is very promising for use in tulip. When the transposase gene is expressed transiently, it may cause a higher integration frequency and a stable integration of DS-flanked genes into the tulip genome. Especially for the method of direct adventitious shoot formation we applied, as described in the previous chapters, this could result in higher numbers of simultaneously transformed cells, which enlarges the chance to harbour fully transformed shoots.

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Samenvatting

Dit proefschrift beschrijft de resultaten van 5 jaar onderzoek op het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO). Doel van het onderzoek was het opzetten van een methode voor genetische manipulatie van het bolgewas tulp. Voor de veredeling is tulp een lastig gewas, omdat het een zeer lange reproduktiefase heeft, van zaad tot bloem duurt circa 5 jaar. Het inkruisen van nieuwe eigenschappen, het selecteren van de juiste eigenschappen, en het op de markt brengen van een nieuwe partij kan dan ook 25 jaar in beslag nemen. De introductie van nieuwe eigenschappen, met name ziekte-resistenties, is echter juist voor tulp van groot belang, omdat het gewas vatbaar is voor vele ziekten. Hierdoor worden bij de teelt flink wat gewasbespuitingsmiddelen en grondontsmettingsmiddelen gebruikt. In het Meerjarenplan Gewasbescherming (1989) werd terugdringing van het gebruik van deze middelen geëist, hetgeen voor de bollenindustrie betekende, dat er een snelle oplossing moest worden gevonden voor de ziekte-problematiek.
Genetische manipulatie, oftewel "transformatie", is een techniek waarbij geïsoleerde genen, die een bepaalde eigenschap tot gevolg hebben, worden geïntroduceerd in het erfelijk materiaal van een plantecel. De getransformeerde plantecel heeft dan een nieuwe eigenschap. Vervolgens wordt deze cel geselecteerd op aanwezigheid van het nieuwe gen, en opgekweekt tot een nieuwe plant. Voor het opzetten van een methode van genetische manipulatie maakt men gebruik van specifieke genen, met name herbicide-resistentie genen. Door het herbicide in het groei-medium toe te voegen, kunnen alleen de resistente, dus getransformeerde cellen uitgroeien.

Allereerst werden twee methoden van gen-overdracht getest, en werd gekozen voor het introduceren van genen met behulp van een "particle gun". Met dit apparaat worden stukjes weefsel beschoten met wolfraamdeeltjes die zijn gecoat met DNA dat het gewenste gen bevat.
Getransformeerde cellen moeten vervolgens door regeneratie uitgroeien tot een volledige plant. Daartoe werd de methode van adventieve scheutvorming op stengelplakjes toegepast. Beschoten stengelsegmentjes van tulp werden op regeneratiemedium geïncubeerd en de nieuwe scheutjes werden na 8 à 10 weken gevormd. Histologische analyse toonde aan, dat de scheuten niet uit één cel ontstaan, er zijn meerdere cellagen betrokken bij het proces van scheutvorming. Om scheuten te verkrijgen die volledig getransformeerd zijn, moeten transgene cellen worden bevoordeeld met behulp van een selectiemiddel, b.v. een herbicide, dat de groei van normale cellen remt. Transgene cellen, die getransformeerd zijn met een resistentie-gen tegen het selectiemiddel, kunnen zich ongeremd vermeerderen.
De aard van het selectiemiddel is heel belangrijk in het transformatie onderzoek. Voor tulpweefsel werden goede resultaten verkregen met het herbicide Basta: de groei van niet-transgene cellen werd geremd, terwijl getransformeerde cellen uitgroeiden tot een nieuwe scheut.

Stengelexplantaten van de cultivar Lucky Strike werden beschoten met het Basta-resistentie gen en op Basta bevattend regeneratie-medium geïncubeerd. Na 2 maanden regeneratie en selectie ontstonden er Basta-resistente scheuten op de beschoten explantaten, terwijl de onbeschoten explantaten vrijwel geen scheuten hadden gevormd. Deze Basta-resistente scheuten werden verkregen in twee opeenvolgende jaren in reproduceerbare aantallen in verschillende experimenten. Ze werden doorgekweekt en gedurende een koude-periode aangezet tot bolvorming. Dit vermogen een bol te vormen bleek cultivar afhankelijk, maar van de cultivar Lucky Strike was een groot deel van de scheuten in staat een bol te vormen.
Dat er zich inderdaad transgeen weefsel kan ontwikkelen op regenererende stengelexplantaten bleek ook uit histochemische analyse van getransformeerde explantaten. Cellen die zijn getransformeerd met het *gus*-gen, dat de informatie bevat voor de productie van het enzym ß-glucuronidase, kleuren blauw na een bepaalde chemische reaktie. Explantaten, die waren getransformeerd met het Basta-resistentie gen èn het *gus* gen, werden na 2 maanden regeneratie en selectie op deze wijze behandeld. Blauwe scheutprimordia en jonge scheutjes waren zichtbaar, hetgeen betekent dat transgene cellen inderdaad kunnen uitgroeien tot transgene scheuten.
Bolletjes van Lucky Strike, ontstaan uit stengelexplantaten na beschieting, werden geoogst, en in weefselkweek geïnduceerd tot nieuwe scheutvorming. Deze "uitlopende" scheuten werden gebruikt voor DNA-analyse. De aanwezigheid van het geïntroduceerde Basta-resistentie gen werd aangetoond met moleculaire technieken: uit PCR-analyse bleek het gen in alle gevormde bolletjes aanwezig. Hoewel het aantonen van integratie van het gen in het genoom problematisch was, vanwege de grote hoeveelheid erfelijk materiaal die van nature in tulpecellen aanwezig is, kon toch een aantal mogelijke verstoringen in de reaktie worden uitgesloten, zodat geconcludeerd kon worden dat het nieuwe gen aanwezig is in het DNA van tulp. Uit de sterkte van de signalen bleek echter, dat het gen slechts in een deel van het weefsel aanwezig is. Dit betekent dat in principe de gebruikte methode van genoverdracht geschikt is voor tulp, maar dat er in de kweek van de bolletjes nog enkele stappen moeten worden ingebouwd die de ontwikkeling van partieel transgeen weefsel voorkomen.

Als iemand mij vroeger had voorspeld dat ik 5 jaar van mijn leven zou besteden aan één onderwerp, had ik die persoon uitgelachen. Als hij vervolgens zou hebben verteld dat dat ene onderwerp een *tulp* was, had ik nog harder gelachen, en als hij mij tenslotte had verteld dat ik daarover een proefschrift zou schrijven, dan had ik hem niet langer serieus genomen. Toch ligt dit boekje hier nu, en ik weet niet meer waar het nou mis ging.
Achteraf bezien kan ik eigenlijk maar één drijfveer ontdekken: het was simpelweg **leuk**. Hoewel ik die tulpen soms wel kon schieten, was het de werkomgeving, dus met name de collega's van Ontwikkelingsbiologie, die het werk zo aangenaam maakten. Voor de stimulans, en voor de lol, denk ik met plezier aan hen terug.

Net als boven een artikel, zouden er eigenlijk op een proefschrift meerdere namen moeten staan. Want je doet het natuurlijk niet alleen. In mijn geval was de medewerking van Hans Dons, die als co-promotor al mijn schrijfsels met veel geduld ombouwde tot publiceerbare artikelen, van onschatbare waarde. Bovendien was hij degene die altijd de lijn in het onderzoek wist te houden, en wees op dingen die niet onopgemerkt mochten blijven. Op de achtergrond speelde Lous van Vloten-Doting, als promotor, een constante rol, ondanks drukke werkzaamheden wist ze toch regelmatig tijd vrij te maken voor welkome input "van buiten".

Voor het lab-werk is de hulp van Bernadette van de Ven, en in het laatste jaar van Reinoud Bouwer, onontbeerlijk geweest. Het dagenlang snijden, inzetten en overzetten van duizenden stengelplakjes was dankzij het gezellig "gebep" met Bernadette helemaal niet vervelend. Zij en Reinoud hebben beide ten volle de vele beperkingen van een moeizaam handelbaar gewas ervaren, en toch nooit de motivatie en de moed verloren. Daarnaast waren er ook nog Jan Custers en Yvonne Nöllen, vooral verantwoordelijk voor tijdrovend regeneratie-werk, en Wim Eikelboom, die ons voorzag van al het plantmateriaal dat we wilden, ook al moest het uit Nieuw-Zeeland komen.

Ook thuis is uiteraard de "tulpetijd" niet ongemerkt voorbijgegaan. Voor Sanne en Renze betekenden mijn drukke werkzaamheden dat ze het vaak zonder moeder moesten stellen, en soms wel te vroeg tot zelfstandigheid werden gedwongen. Een lange reeks oppassen hebben ze zien komen en gaan totdat eindelijk Ellen weer stabiliteit bracht in huis, en opving waar ik tekort schoot.
Ook oma, vaak paraat tijdens vakanties en cursussen, is onmisbaar geweest.
Tenslotte Corné, last but not least: het kostte vele kilometers om de zaken thuis mede draaiende te houden, maar dat maakte voor mij dit werk mogelijk, zelfs op onmogelijke momenten.

Voor allen: Bedankt!!

Annemiek Wilmink werd geboren op 25 april 1954 in Haaksbergen, en behaalde haar Atheneum-A diploma in 1972. Dat deze A echter eigenlijk een B had moeten zijn werd pas gaandeweg duidelijk, en in 1982 werd het roer omgegooid Via het "voorbereidend jaar Biologie" werden de hiaten in de wis- natuur- en scheikunde aangevuld, zodat in 1983 kon worden begonnen met de studie biologie aan de RU Utrecht (deeltijd-opleiding). Tijdens deze studie werden Sanne en Renze geboren (1983 en 1986). Het doctoraal diploma werd behaald in 1990. Praktijk ervaring gedurende de studie werd opgedaan bij de vakgroep Botanische Ecologie en vooral bij Moleculaire Celbiologie in Utrecht.
Tot die tijd had zij nooit iets met tulpen te maken gehad, afgezien van een paar exemplaren uit eigen tuin. In 1990 veranderde dit met de aanvang van het project "Genetische manipulatie van tulp: het opzetten van een transformatie-procedure" op het Centrum voor Plantenveredelings- en Reproductieonderzoek in Wageningen, welk onderzoek staat beschreven in dit proefschrift, en in onderstaande artikelen.
Het project werd afgerond in 1995, en sindsdien is Annemiek werkzaam bij de afdeling Moleculaire Biologie, CPRO-DLO.

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