Regeneration and transformation of *Alstroemeria.*

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Regeneration and transformation of *Alstroemeria.*

Proefschrift

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Bibliographic abstract: This thesis describes the first steps of genetic modification of *Alstroemeria*. A plant regeneration system through somatic embryogenesis from callus induced on immature embryos of *Alstroemeria* was achieved. This somatic embryogenesis system was also made cyclic, thus enabling a constant production of material irrespective of the availability of flowers. *Agrobacterium*-mediated and particle gun-mediated transformations were performed on callus, somatic embryos and cell suspension cultures. This resulted finally in a transformed friable cell culture in which the transformed nature was confirmed by PCR and luciferase activity.

The cover shows an *Alstroemeria* cell after particle bombardment (made by J. van Eijsden).

Stellingen

1. Vele aktief delende en embryogene cellen, welke tevens goed bereikbaar zijn voor transformatie is de belangrijkste voorwaarde om tot het ontwikkelen van een transformatie-methode te komen bij *Alstroemeria* (dit proefschrift).

2. Transformatie van meristemen of andere gedifferentieerde weefsels zullen hooguit in chimère transgene planten resulteren. Transformatie van een indirect regeneratie-proces daarentegen zal frequenter in volledig stabiele transgenen resulteren (Sato *et al.*, 1993. Plant Cell Rep.12: 408-413.)

3. Het marker gen luciferase kan goed dienen voor visuele selectie van transgeen weefsel, dat na deze assay verder gekweekt kan worden als potentiële transformant (dit proefschrift; C.J.J.M. Raemakers *et al.*, 1996. Molecular Breeding 2: 339-349).

4. Promoter onderzoek van selectie en detectie genen is een belangrijk onderdeel voor het ontwikkelen van een transformatie methode bij monocotylen (dit proefschrift).

5. *Alstroemeria* is een recalcitrant gewas in weefselkweek (dit proefschrift; dr. ir. R.L.M. Pierik, Bloemenkrant, november 1996).

6. Het werken met recalcitranten planten geeft in de regel minder problemen dan het werken met recalcitrante mensen.

7. Ik schaar mij graag achter dr. Chris Sommerville, die de uitspraak deed dat in de toekomst biotechnologie zal bijdrage aan een schone en duurzame landbouw (WUB 10-12 maart, 1998).

8. Nu de wetenschap steeds meer te weten komt, blijkt steeds vaker hoe weinig wij eigenlijk nog maar weten.

9. Democratie is de wil van het volk. Elke morgen lees ik stomverbaasd in de krant wat ik nou weer wil (Wim Kan).

10. Het leven is zo eenvoudig, de mensen hebben het zo vreemd en ingewikkeld gemaakt (Louis Couperus).

11. Geniet het leven, benut het leven,
het vliegt voorbij en duurt maar even.

Stellingen, behorende bij het proefschrift "Regeneration and transformation of *Alstroemeria*" door Carla E. van Schaik, in het openbaar te verdedigen op woensdag 24 juni 1998, te Wageningen.

Voorwoord

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In het kader van een doctoraalonderzoek hebben vele studenten hun steentje bijgedragen aan het onderzoek. In chronologische volgorde Gerthon van de Bunt, Jeroen van Eijsden, Niels Nieuwenhuizen, Jan-Willem Braakhekke, Mirjam van Delft en Peggy Kooij.

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Contents

Abbreviations

BAP	6-benzyladeninepurine
BAR	phosphinothricin-N-acetyltransferase
CR	chlorophenol red
cv	cultivar
2,4-D	2,4 di-chlorophenoxyacetic acid
4FPA	4-fluorophenoxyacetic acid
GA_3	gibberellic acid
GUS	ß-glucuronidase
IBA	indole butyric acid
LUC	luciferase
MS	Murashige and Skoog medium
NAA	α-naphthaleneacetic acid.
NPT II	neomycin phosphotransferase II
OD550	optical density at 550 nm
PCR	polymerase chain reaction
PPT	L-phosphinothricin
p35S	35S promoter
p2x35S	double 35S promoter
TIBA	triiodobenzoic acid
t35S	35S terminator
tnos	nos terminator
pUbi-1	ubiquitin promoter
X-gluc	5-bromo,4-chloro,3-indolyl-ß-D-glucuronide

Chapter 1

General introduction.

Alstroemeria culture and economical importance

Alstroemeria or Inca Lily is economically an important cut flower in the Netherlands. Figures from the Association of Dutch Flower Auctions (VBN) show that *Alstroemeria* cut flower turnover in 1997 was 91 million Dutch guilders which accounted for about 260 million flowers. The average price per stem is 0.35 Dutch guilders. *Alstroemeria* occupies the ninth place in the top ten of most important cut flowers in the Netherlands. All over the world there are about 600 hectares of greenhouse acreage in culture from which 120 hectares are situated in the Netherlands. The production is 160-300 stems per square metre per year depending on the cultivar. The increasing popularity of *Alstroemeria* with growers and consumers as a cut flower can be attributed to its extensive range of large and colourful flowers, its long post harvest life and its ability to grow at low greenhouse temperatures. The crop *Alstroemeria* is mainly cultivated for the production of cut flowers, but there are also *Alstroemeria* pot plants and garden plants on the market (Figure 1).

Figure 1A. A cut flower of *Alstroemeria* cultivar Rebecca

Figure 1B. A pot type of *Alstroemeria* cultivar Zsa Zsa

History and taxonomy of *Alstroemeria*

Inca Lily (*Alstroemeria*) was first described by reverend Father R.P. Feuillet, who travelled across South America from 1707 to 1712 (Uphof, 1952). He wrote in his journal, that this Inca Lily or Lily of Lima was cultivated in the Kings gardens of the Incas. *Alstroemeria* appeared in Europe in the 18th century. In 1754 Klas von Alströmer collected seeds of a plant from Peru and sent them to Sweden to Linnaeus. Linnaeus identified the received plant as *pelegrina* and for the memory of Alströmer he named it *Alstroemeria (Alstroemeria pelegrina)*. Previously *Alstroemeria* was included in the Amaryllidaceae and Liliaceae by Buxbaum (Buxbaum, 1951). Nowadays the genus *Alstroemeria* belongs to the family of Alstroemeriaceae. The family of Alstroemeriaceae belongs to the order Liliales, superorder Liliiflorae, division Monocotyledonae (Dahlgren *et al.*, 1985; de Hertog *et al.*, 1993). Also the genera *Bomarea, Leontochir* and *Schickendantzia* belong to the family Alstroemeriaceae (Dahlgren and Clifford, 1982).

The number of *Alstroemeria* species is not precisely determined, but there are (believed to be) more then one hundred. The species in Chile are described in a monography by Bayer (1987). The natural habitat of *Alstroemeria* is South America, mainly Chile and Brazil, but some species are found in Argentina, Paraguay, Bolivia, Peru, Ecuador and Venezuela (Aker and Healy, 1990). The ecological amplitude of the *Alstroemeria* genus is wide, ranging from the snowline of the Andes, swampy territories, mountain forests, desert sites, river valleys to coasts of the Pacific. Several of these *Alstroemeria* species, growing in very differentiated biotopic conditions of South America, constitute a considerable genetic potential, with significant importance for breeding.

Detailed morphological descriptions of *Alstroemeria* plants can be found in several reports (Healy and Wilkins, 1985 and 1986; Buitendijk, 1998). Aerial shoots arise from the underground rhizome, which grows sympodially. Each aerial shoot has at its base two axillary buds. The first axillary bud develops into a rhizome tip and grows horizontally in the ground. Later on it grows upwards and forms a new aerial shoot, again with two axillary buds at its base. The second axillary bud may stay dormant or develops a second rhizome tip (Rees, 1989). The rhizome also produces storage and fibrous roots. A detailed description of the rhizome from different species and hybrids can be found in Buxbaum (1951) and Buitendijk

(1998). Figure 2 shows a drawing of a rhizome of *A. aurea*. The shoots can be vegetative or generative, depending on the previous environmental conditions. Leaves twist 180°, reversing their surfaces. Inflorescences are simple or compound cymes, each with one to many sympodially arranged flowers.

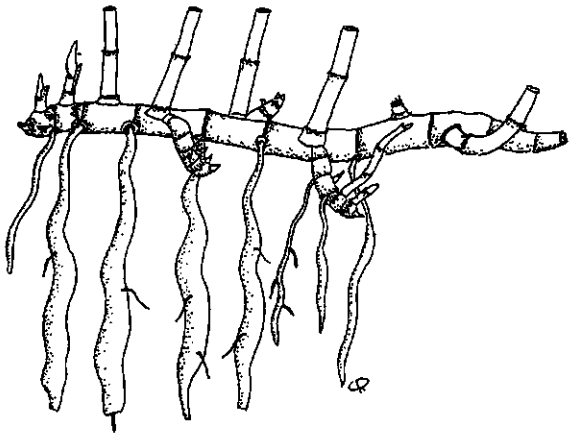

Figure 2. A drawing of a rhizome of *A. aurea* by R.J. Quené (Buitendijk, 1998).

Convential breeding of *Alstroemeria*

Breeding of *Alstroemeria* started in Europe and was carried out by Goemans in England. The hybrid "Walter Fleming" was a commercial success (Goemans, 1962). This diploid cultivar was highly sterile, but during vegetative propagation of this hybrid a tetraploid plant was found, which produced viable 2x gametes. This plant has been used as a parent to breed the "Parigo hybrids" by manual crossings (De Jeu *et al.*, 1992). The hybrid "Walter Fleming" was developed from Chilean species (*A. aurea* and probably *A. violacea),* however cytological studies showed *A. aurea* as a parent, but not *A. violacea* (Vonk Noordegraaf, 1981). The hybrid "Walter Fleming" was also known as "Orchid" or "Orchid flowering". For this reason its offspring was known as "Orchid type" cultivars. After 1960 the cultivars of Goemans were introduced in the Netherlands by the breeding company of Wülfinghoff. Breeding of *Alstroemeria* in the Netherlands was started by the breeding company of van Staaveren from 1963 and later by Wülfinghoff in co-operation with Goemans (Verboom, 1979). The first

cultivars were interspecific hybrids and consisted of diploids (2n=2x=16), triploids (2n=3x=24) and tetraploids (2n=4x=32). The polyploid forms have originated through a gradual process of sexual polyploidisation involving predominantly 2n-gametes as well as other types of functional gametes produced by the sterile diploid interspecific hybrids and triploids (Ramanna, 1992). Most of the cultivars of *Alstroemeria* seem to have originated from polyploidisation and hybridisation. Mutation breeding is used by breeders to introduce mutations in already existing cultivars. Actively growing young rhizomes can be treated with X-ray to induce mutations. Although the buds on the rhizomes most certainly have multicellular apices, most X-ray mutants show no sign of chimerism (Broertjes and Verboom, 1974). Besides inter-Chilean hybrids, also Chilean-Brazilian hybrids were produced. These cultivars were referred to as "Butterfly type", because they looked like butterflies. Nowadays more and different species are used to breed new cultivars, with the result that more intermediate cultivars enter the market. Most new cultivars are triploid, tetraploid or even aneuploid.

Interspecific hybridisation of *Alstroemeria* is hindered by post fertilisation barriers and therefore different ovule and embryo rescue cultures were developed (Buitendijk *et al.*, 1995; De Jeu and Jacobsen, 1995; Lu and Bridgen, 1996; Kristiansen, 1995; Ishikawa *et al.*, 1997; De Jeu and Calderé, 1997). Besides sexual polyploidisation, chromosome doublings were performed, for instance to overcome fertility problems of the hybrids (Lu and Bridgen, 1997). It takes approximately 7 years after the original cross has been made, before all the characteristics of a promising hybrid are evaluated. Important selection criteria for the breeders are: cut flower production (especially first cut flower quality), flower colour, flower size and number of flowers on the stem, quality of the leaves and stem, vase life, virus resistance and year round flowering. Recently a new criterion, the micropropagation ability, was added (Buitendijk *et al.*, 1992).

In vitro micropropagation of Liliales

In general regeneration *in vitro* can occur through already existing meristems (apically or axillary) or through *de novo* regeneration. *De novo* or adventitious plant regeneration can be accomplished by two different pathways: organogenesis and somatic embryogenesis (or a

combination of both pathways). In both processes the regenerated structures originate either directly from the explant or indirectly from callus induced on the explant. Also here a combination of direct and indirect regeneration is possible. Organogenesis is defined as the process in which an unipolar structure is formed, mostly of multicellular origin, which is connected by its vascular system to the parental tissue. Somatic embryogenesis is defined as a process in which bipolar structures arise. Somatic embryos are structures that resemble zygotic embryos. Somatic embryos derive from somatic cells and have a root and shoot meristem with a connecting provascular system (Flick *et al.*, 1983; Terzi and Loschiavo, 1990; Emons, 1994), and a storage organ typical for the species (Emons and De Does, 1993).

The order Liliales consists of many interesting ornamentals and other crops like *Allium, Asparagus* and Yam *(Dioscorea)*. The bulbous ornamentals like *Lilium*, tulip and *Narcissus* are mainly micropropagated through bulblet formation from axillary buds of bulb scale segments. Sometimes other explants for regeneration are described, e.g., tulip can also be micropropagated from stem slices (Hulscher and Krijgsheld, 1995). Plant regeneration through somatic embryogenesis in different genotypes of *Lilium* hybrids was developed by Haensch (1996). Bulb scales were cultured on MS medium supplemented with the auxins 2,4D or Picloram. Somatic embryos could be obtained and developed into plants, although only in a low percentage (4 of the 23 genotypes tested). In *Lilium* and tulip anther or microspore culture has been investigated. Multicellular structures were observed by van den Bulk and co-workers after microspore culture of tulip (van den Bulk *et al.*, 1994). Regeneration of haploid plants from anther cultures of the Asiatic hybrid lily "Connecticut King" and *Lilium longiflorum* was achieved by the group of DongSheng and the group of Arzate-Fernandez (DongSheng *et al.*, 1997; Arzate-Fernandez *et al.*, 1997). Successful protoplast regeneration has not yet been achieved for *Lilium*.

Gladiolus and *Freesia* have no bulbs, but corms. Micropropagation of *Gladiolus* and *Freesia* could be accomplished by shoot development of the axillary buds on the corms or shoot tips. Somatic embryos could be induced directly on cormel tissue explants or indirectly on callus. Callus could be induced on different explants like peduncle, flower and leaf (Kim and Kang, 1992).

Allium could be micropropagated through many different explants, such as the normal meristems, but also through vegetative and floral buds, (immature) embryos and roots (Kanazawa *et al.*, 1992). Also plant regeneration through somatic embryogenesis of *Allium* is possible (Buiteveld, 1998; Schavemaker and Jacobsen, 1995). This regeneration system is mostly indirect through callus culture or cell suspensions. Also regeneration of whole plants from protoplasts isolated from tissue-cultured shoot primordia or cell suspensions is accomplished (Buiteveld, 1998; Ayabe *et al.*, 1995).

Many explants of *Asparagus* could be used for the regeneration of plants *in vitro* through meristems or through somatic embryogenesis, like (lateral) bud clusters, shoot tips and young spears. Callus and cell suspension formation is achieved and from this also protoplast systems could be derived (Kohmura *et al.*, 1995; May and Sink, 1995).

Yam (*Dioscorea* spp) is a tropical food crop and *in vitro* culture could be achieved through the following explants; shoots, nodal segments, roots, leaf and petioles. Somatic embryogenesis and cell suspensions have been described by Twyford and Mantell (1996).

In conclusion several members of the Liliales could be regenerated through the use of existing meristems (axillary meristems in bulbs, corms or shoot apex), and also through *de novo* regeneration involving either organogenesis or somatic embryogenesis (directly or indirectly), which are very much dependent on the crop, genotype or the species.

Micropropagation of *Alstroemeria*

Alstroemeria plants are generally propagated through splitting of rhizomes of greenhouse grown plants. More recently micropropagation of *in vitro* grown plants was introduced by proliferation of rhizome axillary buds. This technique enables plant propagators to produce plants more rapidly, on a large scale in a relatively small laboratory, and disease free. Especially the latter aspect is of great importance and an improvement over the conventional method of multiplication. Meristem culture and virus free multiplication has been described by various research groups (Hakkaart and Versluis, 1988; Van Zaayen *et al.*, 1992). Disinfection of axillary buds from underground rhizomes is very difficult. A procedure for disinfection of *Alstroemeria* rhizome tips has been developed, based on trimming of scale leaves 3 times alternating with short immersions in disinfectants (Pederson and Brandt, 1992).

A successful application of meristem culture is dependent on a reliable detection method for viruses. The General Netherlands Inspection Service for Ornamental Crops (NAKB) is able to certify *Alstroemeria* plants. However, the development of reliable virus detection tests is still in progress (de Blank *et al.*, 1994). The multiplication rate of the micropropagation of *Alstroemeria* is still low, due to its recalcitrant nature. To improve the multiplication rate of *Alstroemeria*, many factors that influence the culture have been studied, but so far without real dramatic improvement. For maximum rhizome production *in vitro* a temperature between 13-18 °C was found to be optimal. Increasing temperature decreased the number of rhizomes. Decreases in irradiance from 20 W m^{-2} to 5 W m^{-2} and increases in day length from 8 to 20 hours, had no effect on the number of lateral rhizomes, aerial shoots or roots per explant. Good multiplication of *Alstroemeria* requires a temperature of 15 °C, an irradiance of 5 W m^{-2} with a daylength of 8 hours. Generally the cytokinin BAP is used for multiplication, the growth regulators TIBA, thidiazuron, NAA, GA_3 and paclobutrazol in the culture medium (with or without BAP) did not change the number of lateral rhizomes, shoots or roots produced. (Pierik *et al.*, 1988; Elliot *et al.*, 1993; Bond and Alderson, 1993a,b,c). Buitendijk investigated the genetic variation for rhizome multiplication rate in micropropagation of *Alstroemeria*. Heterosis for rhizome multiplication rate was revealed significantly. So, a genetic base for micropropagation of *Alstroemeria* was evident in the 23 accessions of five species and 64 interspecific hybrids (all diploid level) tested (Buitendijk, 1998).

De novo regeneration of *Alstroemeria*

When the research of this thesis started in 1991, there were only a few references on callus induction and *de novo* plant regeneration in *Alstroemeria*. Ziv *et al.* (1973) reported non embryogenic callus formation and direct plantlet regeneration from segments of sub apical inflorescence stem. Bridgen *et al.* (1989) reported somatic embryogenesis without detailed information. Gonzalez-Benito and Alderson (1992) described plant regeneration of callus derived from cultures of mature zygotic embryos of the diploid cultivar "Butterfly". Callus was induced on MS medium supplemented with 2,4D or Picloram and incubated in the dark. The maximum regeneration response from this callus, observed in a single treatment, was only 4%. In the same period, we started our research and tried to develop a more efficient regeneration

system of *Alstroemeria*. More recently Hutchinson *et al.* (1994) reported callus induction and plant regeneration, using mature zygotic embryos of a tetraploid *Alstroemeria* ("Butterfly type") as explant. The maximum frequency of regeneration was 40% on medium containing NAA and kinetin, according to these authors. Regeneration to a complete plant occurred via somatic embryogenesis in the absence of growth regulators and the plantlets grew to maturity and flowered in the greenhouse. Very recently Hutchinson and co-workers reported somatic embryogenesis in liquid cultures of a tetraploid *Alstroemeria* (Hutchinson *et al.*, 1997). Again embryogenic callus was induced from mature zygotic embryos, cultured on MS medium, supplemented with NAA and kinetin. Pre-culture of the callus on MS medium supplemented with NAA for two days was essential for cell proliferation in the liquid medium. When the embryogenic cell aggregates were transferred to a semi-solid half strength MS medium supplemented with casein hydrolysate, they successfully differentiated into plantlets.

The regenerative ability of explants from various organs of *Alstroemeria* plants were investigated by Gabryszewska (1995). Subapical segments of vegetative stem, segments of flower petals and parts of ovary did not regenerate into rhizomes or roots, but occasionally produced callus on medium with NAA and kinetin. Segments excised from vegetative stem sporadically developed roots on medium with NAA or IBA. Rhizome apical and axillary tips were the best among the tissue tested as initial explants for plant regeneration.

Direct shoot regeneration from excised leaf explants of *in vitro* grown seedlings of *Alstroemeria* was recently described by Lin *et al.* (1997). For this process a two-step protocol has been developed. Leaf explants with stem node tissue attached were incubated on shoot induction medium for 10 days, and then transferred to regeneration medium. The best induction medium was obtained with MS medium containing thidiazuron and IBA. The regeneration medium contained BAP. After several subcultures of the leaf explants with induced shoots, normal plantlets were formed.

So *de novo* regeneration of *Alstroemeria* is possible, but the regeneration systems are still poor in regeneration frequency and time consuming. The best regeneration frequency for *Alstroemeria* so far was around 40% with one or a few reactions per explant. The regeneration period took 3 to 4 months. So, at the start of the research of this thesis, our aim was to develop an efficient regeneration system of *Alstroemeria*, preferably a (cyclic) somatic embryogenesis

system. The idea was that such a regeneration system could be most useful for genetic transformation.

Genetic modification of Liliales

Unlike in sexual methods, the gene transfer through genetic transformation is no longer determined by the crossability of the genotypes and thus offers unlimited potential. This makes it possible to utilise for plant improvement suitable genes from either related or unrelated plants, but also from animals, fungi, bacteria or viruses. The *Agrobacterium tumefaciens* DNA delivery system is the most commonly used genetic modification technique (reviewed by Wordragen and Dons, 1992), together with the naked DNA delivery system, microprojectile bombardment (reviewed by Christou, 1995). Other alternative methods are (tissue) electroporation, micro-injection, silicon carbide whiskers and bombardment of pollen. So far there are verified methods for stable introduction of novel genes into the nuclear genomes of over 120 diverse plant species (Birch, 1997). The list of successful transfers is getting longer by the day, and examples of commercially released plants are beginning to appear. The total approvals world-wide for field experiments of genetically-engineered plants classified in 1992 was around 1250 (Law, 1995). The type of field release applications throughout the world refer to herbicide tolerance, disease resistance, virus resistance, insect resistance, quality traits, flower colour, male sterility, metal tolerance and stress resistance. Success has been achieved through the introduction of virus coat protein genes to confer viral resistance (Powell *et al.*, 1986; Beachy *et al.*, 1990; Jacquet *et al.*, 1998), the exploitation of insect toxins by transferring the Bt gene from *Bacillus thuringiensis* (Barton *et al.*, 1987; Jelenkovic *et al.*, 1998) or the trypsin inhibitor gene from cowpea (Hilder *et al.*, 1987) to induce insect resistance.

At the start of the research for this thesis, no research had ever been published about genetic modification in the family of Alstroemeriaceae. In the order Liliales, for several crops attempts have been made to develop transformation techniques. Transformation of the important bulbous ornamental, tulip, was investigated by Wilmink (1996). Genetic modification of tulip was applied by means of particle bombardment of the floral stem

segments. Putative transgenic tulips were analysed at the molecular level. Positive PCR (polymerase chain reaction) analysis was performed, but GUS expression was very poor in the transgenic tulip plants. Therefore, it was concluded that the transgenic tulip plants were chimeric. Histological analysis of the adventitious shoot formation in tulip revealed that the shoots were formed directly from the explant (young floral stems) without an intermediate callus phase. Adventitious shoot formation was initiated in the first two subepidermal cell layers of the explants (Wilmink *et al.*, 1995). Several research groups have *Lilium* successfully transformed using the vector *Agrobacterium tumefaciens* or by particle gun bombardment (also pollen were used as explant for bombardment, followed by pollination). So far, no report has described a transformation technique resulting in stable transgenic *Lilium* plants (Cohen and Meredith, 1992; Miyoshi *et al.*, 1995; Langeveld *et al.*, 1995; Tunen *et al.*, 1995; Tsuchiya *et al.*, 1996). However, at present stable transgenic *Lilium* has now been produced by particle bombardment of bulb scale segments of *L. longiflorum* (Dr. S. Langeveld, Lisse, personal communication). Particle gun bombardment was performed after preculture of the bulb scales. A total of 58 transgenic callus lines was found, which resulted after regeneration in a total of 726 bulblets. Further research is in progress regarding the level of transgene expression in these plants and their offspring in the greenhouse.

Gladiolus and *Asparagus* transformation have been investigated both by the *Agrobacterium* vector system and microprojectile bombardment (Kamo, 1997; GuangYu *et al.*, 1996; Conner and Abernethy, 1996). Transgenic *Gladiolus* was obtained by particle gun bombardment of embryogenic cell suspensions or embryogenic callus. *Asparagus* was transformed by microprojectile bombardment of embryogenic callus of *Asparagus*, resulting in stably transformed plants. Putatively transformed calli were identified from the bombarded tissue after 4 months of selection. The transgenic nature of the selected material was demonstrated by GUS expression and Southern blot hybridisation analysis (Cabrera-Ponce *et al.*, 1997). Also *Asparagus* embryogenic suspension cultures were successfully used for particle gun bombardment. Transgenic plants were obtained after bombardment with a plasmid containing NPTII (kanamycin resistance) and GUS. The combination of 1.1 μm tungsten particles and 1000 psi bombardment pressure was the best. The GUS expression was detected in the stems and cladodes of the transgenic plants. Southern analysis confirmed the integration of NPTII and GUS into the *Asparagus* genome (BaoChun and Wolyn, 1997). Also transformation by

direct DNA uptake into protoplasts and *Agrobacterium*-mediated transformation of *Asparagus* has been reported (Delbreil *et al.*, 1993).

Genetic transformation can also be an alternative tool for the improvement of *Alstroemeria*. Introduction of virus resistance by genetic modification may be very attractive for *Alstroemeria*, because virus diseases are important problems in *Alstroemeria* culture. *Alstroemeria* has been multiplied vegetatively for a long time so viruses, occurring in the crop, have also been present for years. Several viruses are described: Alstroemeria mosaic virus, Alstroemeria carla virus and cucumber mosaic virus (Phillips and Brunt, 1986). Recently more viruses were detected in *Alstroemeria*: Alstroemeria streak virus, impatiens necrotic spot virus and tomato spotted wilt virus (Van Zaayen *et al.*, 1994). The most frequently occurring virus is Alstroemeria mosaic virus, the presence of which is correlated with decrease in quality and number of flower stems (Van Zaayen *et al.*, 1992). Probably genetic modification of *Alstroemeria* provides a solution for these virus diseases, like tomato spotted wilt in transgenic tomato hybrids (Ultzen *et al.*, 1995). Resistance to the tomato spotted wilt virus was performed in these tomato plants after transformation. Inhibition of premature leaf yellowing in *Alstroemeria* cut flowers would also be an other trait for improvement by genetic modification (Hicklenton, 1991). Other characters like plant structure, flower colour, flower shape, flower timing or flower ripening are likely to be targets for genetic modification in *Alstroemeria*. The first steps in order to develop a method for genetic modification of *Alstroemeria* are carried out in the present thesis.

Aim and outline of the thesis.

The aim of the research described in this thesis was to develop a genetic modification system for *Alstroemeria*. Several steps are necessary in order to develop a transformation procedure for this crop. At first an efficient regeneration system has to be developed for *Alstroemeria*. In Chapter 2 a regeneration system based on somatic embryogenesis, derived from immature embryos of *Alstroemeria,* is described. In Chapter 3, the results of optimising the somatic embryogenesis system are presented together with methods to develop a cyclic somatic embryogenesis system, solving the restriction of the availability of large amounts of

flowers. Chapter 4 describes some of the technical prerequisites for transformation; one of them being the fact that the cells, that are potent to regenerate, need to be targeted successfully by the transformation method applied. Therefore, histology of the initiation of the somatic embryogenesis is described together with the histology of the transient GUS expression of the treated tissue after particle bombardment. Furthermore, results about utilities and concentrations of selective antibiotics and herbicides in *Alstroemeria* are presented in Chapter 4. In Chapter 5 it was investigated, which transformation method could be used for obtaining the best results in *Alstroemeria*. *Agrobacterium*-mediated and particle gun-mediated transformations were performed on callus, somatic embryos and cell suspension cultures. The evidence for transformation of the cell suspension culture was confirmed by PCR analysis and luciferase activity. In Chapter 6 the results are generally discussed.

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Chapter 2

Plant regeneration through somatic embryogenesis from callus induced on immature embryos of *Alstroemeria* L.

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Abstract

The plant regeneration ability of callus obtained from zygotic embryos of the monocot *Alstroemeria* was studied. The best explants for somatic embryogenesis were immature zygotic embryos in half ovules when the endosperm was still soft and white. Embryogenic callus was induced on callus induction medium with a success rate of 54%. The best callus induction period was 10 weeks. The morphology of embryogenic callus was nodular. Somatic embryos were formed after transfer of the callus to regeneration medium. These somatic embryos revealed later on the typical features of zygotic *Alstroemeria* embryos. The total duration of the plant regeneration protocol, from inoculation till rooted plantlets ready for transfer to the greenhouse, was 28 weeks.

Introduction

The monocotyledonous *Alstroemeria* (Inca Lily) is an important cut flower crop, having a wide range of flower colours, long vase life, and a low energy requirement in the greenhouse. *Alstroemeria* produces underground rhizomes which branch sympodially. Plants can be multiplied vegetatively either through splitting of rhizomes or through micropropagation of rhizome cuttings (Buitendijk *et al.*, 1992; Monette, 1992; Pierik *et al.*, 1988).

Recent progress in molecular and cell biology makes it possible to add genetically well defined traits to the gene pools in numerous dicot and monocot crops. Genetic transformation of monocots is possible by direct gene transfer and particle gun bombardment (Christou, 1992; Potrykus, 1991). Major prerequisites for genetic transformation are a reproducible and efficient protocol for plant regeneration, an efficient gene transfer system and a good selection system. The aim of the present study was to develop an efficient *in vitro* plant regeneration protocol for *Alstroemeria* that can be used in genetic modification studies using different gene transfer systems.

There are only a few reports on callus induction and plant regeneration in *Alstroemeria*. Ziv *et al.* (1973) reported non embryogenic callus formation and direct plantlet regeneration from segments of sub apical inflorescence stem. Bridgen *et al.* (1989) reported somatic embryogenesis without detailed information. Gonzalez-Benito and Alderson (1992) reported plant regeneration

of callus derived from cultures of mature zygotic embryos of a diploid cultivar "Butterfly". Recently Hutchinson *et al.* (1994) reported callus induction and plant regeneration using mature zygotic embryos of a tetraploid *Alstroemeria* as explant.

In this study a general protocol to regenerate plants through somatic embryogenesis from immature zygotic embryos is developed. Seed germination and germination of zygotic and somatic embryos *in vitro* were observed in order to compare these processes. Also the length of the callus induction period and the developmental stage of the zygotic embryo as explant were studied to define their influence on the somatic embryogenesis of *Alstroemeria.*

Material and methods

Material.

Flowers of the diploid (2n=2x=16) *A. inodora,* accession P007, and the tetraploid (2n=4x=32) cultivar118 were self-pollinated.

General somatic embryogenesis protocol.

Dependent on the season, 4 weeks after pollination, the developing ovaries were removed from the plants and surface sterilised (20 minutes in 1,5% Na-hypochlorite solution). The ovules were taken out of the ovaries and were cut into halves. The ovule parts containing the embryo were placed on callus induction medium. This callus induction medium consisted of MS (Murashige and Skoog, 1961) medium supplemented with 50 g l^{-1} sucrose, 0.4 g l^{-1} casein hydrolysate, 1 mg l^{-1} 2,4D, 0.5 mg l^{-1} BAP and 2 g l^{-1} gelrite. The pH was adjusted to 6.0 prior to sterilisation. Four weeks after culturing the immature embryo derived callus was removed from the ovule parts and subcultured on the same callus induction medium for 2 weeks. Then the sucrose concentration of the callus induction medium was decreased to 30 g l^{-1} sucrose and these were cultured for another 4 weeks. So, the immature embryo derived callus was grown in total for 10 weeks on callus induction medium, followed by 12 weeks on regeneration medium. Regeneration medium consisted of MS medium supplemented with 30 g l^{-1} sucrose, 0.4 g l^{-1} casein hydrolysate, 2 mg l^{-1} BAP, pH 6.0 and 2 g l^{-1} gelrite.

Callus induction took place in the dark at 21°C and regeneration took place in the light, 12/12 h photoperiod at 18°C. Callus was subcultured bi-weekly. Twenty-two weeks after culturing

regenerated plants were counted and cultured on rooting medium for 6 weeks. Rooting medium consisted of MS medium with 45 g l^{-1} sucrose, 0.75 mg l^{-1} NAA, pH 6.0 and 2 g l^{-1} gelrite.

Germination of zygotic embryos.

Mature seeds of cv118 selfings were surface sterilised and imbibed in sterilised water for 48 hours. As a result the seeds became soft and from these the zygotic embryos were excised under aseptic conditions. Besides these bipolar embryos also complete seeds were placed on MS medium supplemented with 30 g l^{-1} sucrose, pH 6.0 and 2 g l^{-1} in the light at 18°C.

Variation of callus induction period.

Four weeks after selfing the ovule parts of cv118 and P007 were cultured on callus induction medium. Total callus induction period was 7, 10, 13 or 16 weeks. The regeneration medium and culture conditions were according to the somatic embryogenesis protocol. Forty embryo derived calli per treatment were used. The total amount of regenerated plantlets of 40 calli was scored 22 weeks after culture.

Different developmental stages of the zygotic embryo.

From 1 till 6 weeks after pollination the somatic embryogenesis protocol was started. One till 4 weeks after pollination, half ovules were cultured. Five weeks after pollination the isolated zygotic embryos were cultured directly and 6 weeks after pollination the seeds were mature. The mature seeds were imbibed for 48 hours before the zygotic embryos were excised and cultured. The ovule parts and the zygotic embryos were used as explants for the general somatic embryogenesis protocol. The embryo derived calli were taken out of the ovule parts at the size of 0.1 cm. At least 70 embryo derived calli were cultured per treatment. The total callus induction period was 10 weeks and the total regeneration period 12 weeks. The ovule size, the endosperm colour and consistency of the explant were observed. The callus morphology and the number of regenerated plants per embryogenic callus were scored.

Results

Morphological observations during callus induction and somatic embryogenesis.

At the start of the somatic embryogenesis protocol the immature zygotic embryos in the ovule parts were not visible to the eye. But after 4 weeks in culture the size increased (0.1 cm) and it had a globular shape. Part of the callus became nodular four weeks after excision from the ovule parts (Figure 1A). Besides the embryogenic nodular type of callus, also non embryogenic compact and rhizogenic callus types were observed. Nodular callus formed bipolar somatic embryos four weeks after transfer to regeneration medium (Figure 1B). The somatic embryos germinated on the same regeneration medium (Figure 1C).

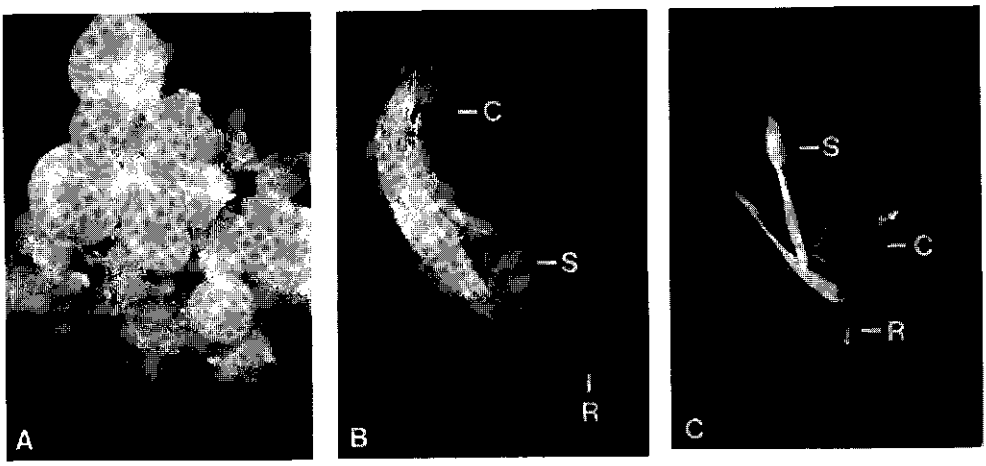

Figure 1. Callus induction and somatic embryogenesis of *Alstroemeria* cv118.

1A. Nodular immature embryo derived callus 10 weeks after culturing on callus induction medium (real size 1 cm).

1B. Somatic embryo with root(R), shoot apex(S), coming through the germination notch, and a cotyledon(C) (real size 0.2 cm).

1C. Germinated somatic embryo with root(R), shoot(S) and cotyledon(C) (real size 1.5 cm).

Morphological observations on germination of zygotic and somatic embryos.

Alstroemeria seed showed hypogeal germination. The cotyledon elongated at its base and pushed the embryo axis from the seed. Elongation of the root and epicotyl occurred. The epicotyl broke through the cotyledonary sheath, but the cotyledon remained embedded in the endosperm. The cotyledon formed a haustorium, a tissue with the function to transport the storage supply from the endosperm to the root and shoot meristem. When the endosperm was exhausted in the seed, the haustorium disappeared. The germination of bipolar mature zygotic embryos *in vitro* was almost the same as mature seed. The only difference was that the cotyledon *in vitro* was green and did not develop a haustorium as seen in seed (Figure 2B). The germination of a somatic embryo was morphologically similar to mature zygotic embryos *in vitro*; root, shoot apex through the cotyledonary sheath and the cotyledon (Figure1). The root development of the somatic embryo was generally poor, probably because of the presence of BAP in the regeneration medium.

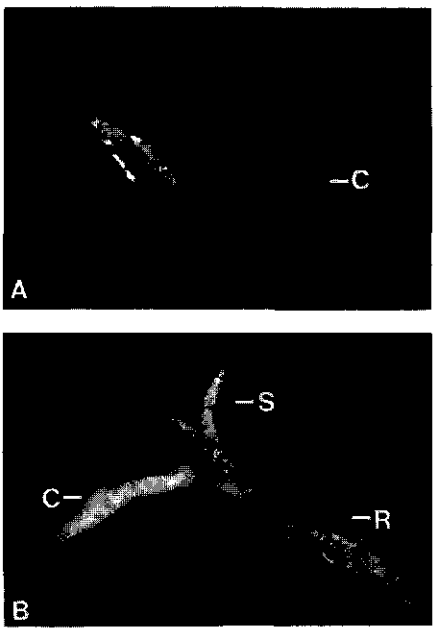

Figure 2. Germination of a mature zygotic embryo of *Alstroemeria* cv118 *in vitro*.

2A. Bipolar mature zygotic embryo (real size 0.2 cm)

2B. Germinated zygotic embryo with root (R), shoot(S) and cotyledon(C) (real size 1.5 cm).

Effect of the callus induction period on plant regeneration.

A callus induction period of 7, 10, 13 and 16 weeks was studied using the somatic embryogenesis protocol with P007 and cv118. The total amount of regenerated plantlets of 40 embryo derived calli was scored. The best regeneration capacity was observed after 10 weeks on callus induction medium. After 16 weeks the regeneration ability was almost decreased until zero (Table 1). There was a clear difference found in frequency of plant regeneration between P007 and cv118.

Table 1. The effect of different callus induction periods on the total number of regenerated plantlets through somatic embryogenesis of 40 embryo derived calli of *A. inodora* P007 and cv118 after selfing.

	P007	cv118
period	total plants	total plants
7 weeks	9	16
10 weeks	19	32
13 weeks	12	6
16 weeks	1	0

Zygotic embryos of different developmental stages.

Immature and mature zygotic embryos of different developmental stages were used as explant for somatic embryogenesis. The immature embryos are too small for visual detection. But the endosperm consistently developed from liquid through soft to hard and tells something about the development stages of the embryo. The best results were obtained from immature zygotic embryos in ovule parts with white soft endosperm. Immature zygotic embryos in ovule parts with liquid clear endosperm gave less embryogenic callus. But the mature zygotic embryos from ovules with hard endosperm gave no embryogenic callus at all. All those mature zygotic embryos germinated directly on callus induction medium (Table 2). Immature zygotic embryos cultured 3-4 weeks after pollination gave the highest percentage of embryogenic callus.

Table 2. *In vitro* responses of cultured zygotic embryos of different developmental stages of *A. inodora* P007 and cv118.

	P007					
weeks after pollination	1	2	3	4	5	6
ovule size(mm)	2	3	4	4	4	4
end. consistency	l	l	s	s	h	h
end. colour	c	c	w	w	w	w
dead(%)	72	80	66	34	0	0
emb. callus(%)	19	14	21	54	0	0
non emb. callus(%)	7	1	2	10	0	0
direct germ.(%)	2	5	11	2	100	100
pl./emb. callus	0.7	0.9	0.9	1.3	0	0
	cv118					
ovule size(mm)	3	4	5	5	5	5
end. consistency	l	l	s	h	h	h
end. colour	c	c	w	w	w	w
dead(%)	39	55	36	9	0	0
emb. callus(%)	34	34	41	4	0	0
non emb. callus(%)	27	11	23	30	0	0
direct germ.(%)	0	0	0	57	100	100
pl./emb. callus	0,6	0,6	1,1	2,0	0	0

endosperm consistency l=liquid, s=soft, h=hard

endosperm colour c=clear, w=white

emb=embryogenic germ=germination pl=plants

Discussion.

In general, explants of monocots that contain immature, meristematic cells develop totipotent callus. Plant regeneration can follow two different pathways: organogenesis or somatic embryogenesis. Extensive evidence is now available on the regeneration of plants via somatic embryogenesis in a wide variety of monocots, including cereals and other monocots (Bhaskaran and Smith, 1990; Vasil, 1987).

In this report a plant regeneration protocol through somatic embryogenesis of *Alstroemeria* is described. The effect of the callus induction period and the developmental stage of zygotic embryos on the regeneration frequency was investigated. The plant regeneration frequency was assayed by counting the number of regenerated plants. A callus induction period of 10 weeks was optimal for the regeneration frequency of this somatic embryogenesis protocol. After a callus induction period of 16 weeks the callus was no longer embryogenic. It is a known fact that the embryogenic capacity can be lost during prolonged subculture, especially in cell suspension cultures (Lörz, 1988). The effect of the developmental stage of the zygotic embryo on the regeneration frequency of *Alstroemeria* was clearly observed. Embryogenic callus was only obtained from immature zygotic embryos. The mature zygotic embryos, excised of hard endosperm, germinated directly on callus induction medium. Immature zygotic embryos from different ages formed more or less frequently embryogenic callus. The immature zygotic embryos in the half ovules with soft white endosperm 3 or 4 weeks after pollination were the best explants for initiation of somatic embryogenesis. So a strictly defined stage of embryo development has to be used as explant for the best response in *Alstroemeria*. Also in some other monocots only immature zygotic embryos of a strictly defined stage could be used as explant, like wild barley (Rotem-Abarbanell and Breiman, 1989) and oil palm (Teixeira *et al.*, 1993).

Gonzalez-Benito (1990) and Hutchinson *et al.* (1994) reported callus induction and plant regeneration from **mature** zygotic *Alstroemeria* embryos. Gonzalez-Benito reported 4% plant regeneration of a diploid cultivar "Butterfly" after callus induction on a 2,4D medium. Hutchinson described 40% plant regeneration of a tetraploid complex hybrid between *A. pelegrina* and *A. psittacina* after callus induction on a medium containing NAA and kinetin. The auxin 2,4D was also tried, but caused excessive browning and death of the explants. In our experiment this problem was overcome by using ovule parts with immature embryos to start the

callus induction, a method inspired by the half-ovule culture technique for the production of interspecific hybrids in *Alstroemeria* (Buitendijk *et al.*, 1995). The best result in our somatic embryogenesis protocol was given by **immature** zygotic embryos. Maximum 54 % plant regeneration from a diploid species *A. inodora* P007 and 41% plant regeneration from a tetraploid cv118 were obtained. Genotypic differences for regeneration ability via somatic embryogenesis is a normal phenomenon (Raemakers *et al.*, 1993). Mature zygotic embryos germinated directly on our callus induction medium, probably because of a hormone concentration, which was too low for callus induction.

Alstroemeria seed showed hypogeal germination like *Asparagus* (Esau, 1977). The germination of zygotic and somatic *Alstroemeria* embryos *in vitro* is morphologically similar (Figure 1 and 2). During the germination of both embryos *in vitro* the cotyledon does not develop a haustorium as it normally develops in the seed. The function of a haustorium *in vitro* is probably superfluous, because the embryo is surrounded by medium.

For *Alstroemeria* this is the first time that immature zygotic embryos in ovule parts are reported as explant for plant regeneration. Immature zygotic embryos gave a good response, but the regeneration frequency is still low. However, the regeneration frequency of some other monocot species, like rice (Rueb *et al.*, 1994), *durum* wheat (Bennini *et al.*, 1988) and barley (Goldstein and Kronstad, 1986) is in the same range. Optimisation of this plantlet regeneration protocol in *Alstroemeria* is now in progress.

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Chapter 3

Primary and cyclic somatic embryogenesis in *Alstroemeria* L.

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Abstract

Primary and cyclic somatic embryogenesis was developed for regeneration of a tetraploid cultivar of *Alstroemeria*. Factors influencing the efficiency of the primary and cyclic somatic embryogenesis were investigated. Of the many tested parameters only the presence of a low sucrose concentration in the regeneration medium (10 g l^{-1}) was a positive improvement. The addition of a low sucrose concentration had a positive effect on the amount of somatic embryos formed and the germination of the somatic embryos. Cyclic somatic embryogenesis was performed and followed for six cycles. However, despite the fact that the callus induction period could be shortened considerably (from 10 weeks to 6 weeks) the average amount of somatic embryos per cycle was not increased. Regenerants from the somatic embryos of the different cycles were obtained and were cultured in the greenhouse. Like the control, more then seventy percent of the regenerants gave seed set after self-pollination. Somaclonal regenerants did not show undesired variation, like chlorophyll deficiency, dwarf growth, aberrant flowers or plant morphology. Despite the rather low, but constant, level of somatic embryos produced it is clear that cyclic somatic embryogenesis can be used for mass production of *Alstroemeria* L.

Introduction

An efficient plant regeneration system is a prerequisite for genetic transformation. *Alstroemeria* can be multiplied vegetatively either by rhizome division or by micropropagation of rhizome cuttings (Pierik *et al.*, 1988; Pedersen and Brandt, 1992; Bond and Alderson, 1993; Gabryszewska, 1995). Progress towards micropropagation of this species has been slow due to its recalcitrant nature. To date, there are only few reports of callus induction and plant regeneration from tissue cultures of *Alstroemeria*. In recent studies, callus induction and plant regeneration from mature zygotic embryos has been reported (Gonzalez-Benito and Alderson, 1992; Hutchinson *et al.*, 1994a and b). We have developed a regeneration system through somatic embryogenesis from immature zygotic embryos (Van Schaik *et al.*, 1996). Given its long breeding time and virus problems it would be beneficial to improve *Alstroemeria* by genetically modification of elite cultivars. This requires the

establishment of a good, reliable and efficient transformation protocol. Before a transformation protocol for the monocotyledonous ornamental *Alstroemeria* can be effective, it is essential that an efficient regeneration protocol is established. For transformation experiments a large amount of material is needed, because normally the transformation frequency of monocots is low. That is why in the present study, factors influencing the efficiency of primary somatic embryogenesis of *Alstroemeria* were investigated and a cyclic system was developed, in order to become independent of immature embryos for which flowers are needed. Also to produce a large amount of material for transformation experiments. An other advantage of a cyclic system is the possibility to gain transgenic somatic embryos from chimeric transgenic origin. Also this cyclic system was evaluated for its performance and factors influencing its efficiency. Finally regenerants obtained from the somatic embryogenesis process were analysed for the appearance of undesired somaclonal variation and seed set.

Materials and methods

Plant material and regeneration protocol

Sucrose concentration in the regeneration medium

Selfings were made on the tetraploid (2n=4x=32) cultivar 118 and primary somatic embryogenesis was performed according to the standard regeneration protocol (Van Schaik *et al.*, 1996). Only in the maturation and germination period (together the regeneration period) the regeneration medium with the standard sucrose concentration of 30 g l^{-1} was compared with the concentrations 1, 7, 10, 15, 20, 30, 40, 45, 50 and 60 g l^{-1}. Fifty calli were investigated on each sucrose concentration (five replicates of 10 calli). The average number of somatic embryos and the germination of these somatic embryos were scored.

Cyclic somatic embryogenesis

In the primary cycle the callus induction period was 10 weeks, and the maturation period was 4 weeks. Callus induction took place in the dark at 21 °C and the 4 weeks maturation took place in the dark at 18 °C. Then somatic embryos were isolated from the callus. At this stage

a second cycle could be started on callus induction medium or the somatic embryos were germinated on regeneration medium (germination period). Germination took place in the light at 18 °C. In the second and following cycles, the callus induction period was 6 weeks (see Figure 1).

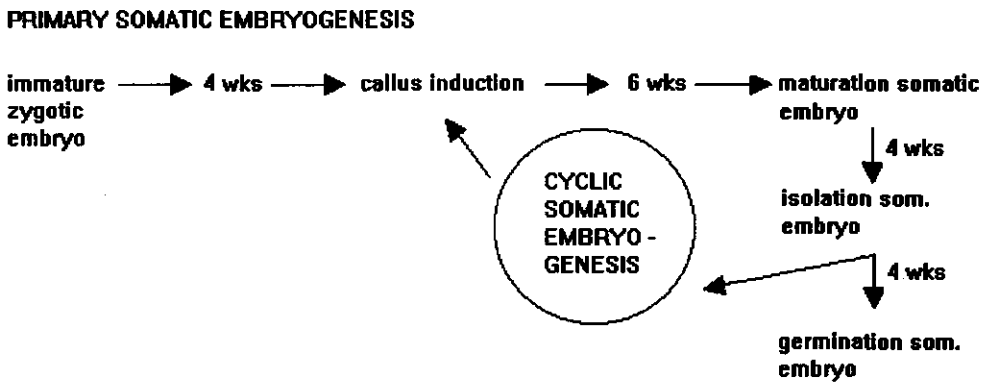

Figure 1: Schematic representation of primary and cyclic somatic embryogenesis of *Alstroemeria.*

In the first cyclic experiment the first and second cycle were investigated. From 40 calli somatic embryos were isolated and placed on regeneration medium for germination (first cycle with isolation of somatic embryos). Also 40 calli with somatic embryos were placed directly on regeneration medium for germination (first cycle without isolation of the somatic embryos). For the second cycle the somatic embryos from another 40 calli were placed for 6 weeks on callus induction medium, followed by 4 weeks maturation in the dark. The calli with somatic embryos and the isolated somatic embryos were placed for germination on regeneration medium in the light for 4 weeks. During embryogenesis the embryogenic, non-embryogenic and dead calli were scored, together with the amount of somatic embryos isolated and the amount of plants which were obtained after germination.

Cyclic somatic embryogenesis was performed by growing the embryos for 6 weeks on callus induction medium followed by 4 weeks on regeneration medium, after which the embryos were transferred again to callus induction medium. The developmental stage of the somatic embryos, used as explants in the cyclic somatic embryogenesis should be preferably old enough to have a clear germination notch, but not yet an open germination notch. In the second cyclic experiment the callus induction took place on the standard MS medium (Murashige and Skoog, 1962) with 1 mg l^{-1} 2,4 D, 0.5 mg l^{-1} BAP, pH 6.0, 30 g l^{-1} sucrose and 2 g l^{-1} gelrite in the dark at 21 °C. This standard callus induction medium was compared with the following callus induction medium containing 4 mg l^{-1} 4FPA and 1 mg l^{-1} BAP. More then 200 immature zygotic embryos were placed on each callus induction medium. Regeneration took place at 18 °C on standard MS medium with 10 g l^{-1} sucrose, 2 mg l^{-1} BAP pH 6.0 and 2 g l^{-1} gelrite. Each cycle was started with around 100 somatic embryos and another 100 somatic embryos were placed on germination medium for regeneration. In six following cycles the percentage embryogenic callus, the number of somatic embryos per embryogenic calli and their germination percentages were scored. Plantlets from each cycle and control plants (selfings of cv118 through seed) were after rooting on rooting medium (MS medium with 45 g l^{-1} sucrose, 0.75 mg l^{-1} NAA, pH 6.0 and 2 g l^{-1} gelrite) cultured in the greenhouse and somaclonal variation was monitored. Characteristics were scored concerning stem, leaves, flower colour and flower morphology (25 UPOV characteristics, UPOV = international union for the protection of new varieties of plants). Plants from the first 4 cycles were self-pollinated and scored for seed set, together with selfings of cv118 as control.

Results

Influence of sucrose concentration on somatic embryogenesis

A number of factors (see discussion) were investigated in order to influence the efficiency of somatic embryogenesis in the primary and cyclic system. No clear factors could be detected except the sucrose concentration in the regeneration medium. Variation of this factor showed clear effects. The optimum sucrose concentration in the regeneration medium was 10 g l^{-1}. This sucrose concentration gave a high average number of somatic embryos, which germinated at a percentage of 54% (see Table 1).

Table 1. Influence of sucrose concentration in the regeneration medium on the average numbers of *Alstroemeria* somatic embryos/embryogenic calli formed and their germination percentage. Per concentration 40 calli were tested.

sucrose concentration ($g\,l^{-1}$)	average number of somatic embryos per embryogenic calli	germination of somatic embryos (%)
1	0.0	0
7	2.4	54
10	3.1	54
15	3.2	42
20	2.6	47
30	1.6	38
40	1.2	26
45	1.7	25
50	1.2	25
60	0.9	25

Cyclic somatic embryogenesis

In the first cyclic somatic embryogenesis experiment, the first two cycles were investigated. In the first cycle for germination, the effects of leaving the somatic embryos on the callus or isolation of them were compared. Leaving the somatic embryos on the callus, a group of 40 calli gave at the end a total of 46 plants. Experiments with the isolated somatic embryos from an other group of 40 calli, almost the same number of regenerants after germination was obtained. From these 40 calli 117 somatic embryos were isolated, however only 38% of these somatic embryos resulted in a complete plant, 13% died and 49% did not result in the development of a normal plant (no entire shoot, only a root and cotyledon). So in total 44 plants could be regenerated.

From a third group of 40 calli 95 somatic embryos were isolated and placed on callus induction medium for the second cycle. At the end a total of 131 plants were formed and the

production of cyclic embryogenic callus in the second cycle also remained between 50-60%. Also the average number of somatic embryos produced in the first and second cycle was comparable (average around 4 somatic embryos).

Table 2. Production of embryogenic callus (%), average number of somatic embryos/embryogenic calli and the germination of the somatic embryos of cyclic somatic embryogenesis of *Alstroemeria* cv 118. In each cycle 100 somatic embryos were tested.

	callus induction medium with 2,4D			callus induction medium with 4FPA		
cycle	% of embryogenic calli	average number of somatic embryos	% of germination of somatic embryos	% of embryogenic calli	average number of somatic embryos	% of germination of somatic embryos
1	24	4,3	40	20	5,5	42
2	27	6,0	36	22	6,1	53
3	50	5,6	41	32	5,6	54
4	40	4,1	74	39	3,0	51
5	27	4,7	78	26	5,0	55
6	16	1,8	40	15	2,5	58

Table 2 shows that the average number of somatic embryos per embryogenic callus in the first 5 cycles of the somatic embryogenesis is around 5. The standard deviation is high, therefore, there is only a significant difference between the first 5 and the sixth cycle with callus induction medium containing 2,4D. No clear differences could be determined between the two different callus induction media, only the germination rate after callus induction medium with 2,4D appeared to be more variable then on medium with 4FPA. After 5 cycles there was a clear decrease in the average number of somatic embryos on both media. The reason to explain this difference is that after 4 weeks somatic embryos in a suitable developmental stage was too low.

Seed set of the regenerants

Regenerants were after culture on rooting medium transferred and grown in the greenhouse. Fasciation occurred not only in the regenerants, but also in the control plants. The phenotypical variation was clear between regenerants of different calli, different pink flower colours were seen. However, the phenotypical variation in the regenerants was the same as in the selfings of cv118 for the 25 UPOV characteristics scored. Somaclonal variation between the regenerants from one and the same callus was restricted (see Figure 2). No gross somaclonal variants with clear chlorophyll deficiency, dwarf growth, modification of flowers or clear morphological difference of plants were formed. The regenerated plants from the first 4 cycles and control plants were self-pollinated to investigate seed set. Table 3 shows the percentage of plants which set seed. The regenerants and the control plants were scored and in both groups there was about 70-80% seed set. The plants, which did not set seed showed sometimes abnormal anther development.

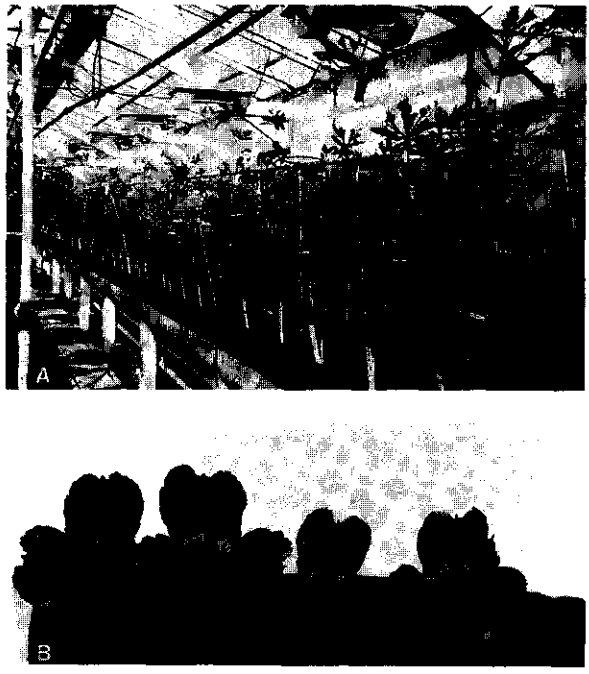

Figure 2. Regenerants of cyclic somatic embryogenesis of *Alstroemeria* cv118.

2A. Regenerants of cyclic somatic embryogenesis in the greenhouse.

2B. Flowers of somaclones of 1 callus, which regenerated after the primary cycle.

Table 3. Percentage of regenerated plants which can set seed per cycle of cyclic somatic embryogenesis of *Alstroemeria* cv118. Controls were selfings of cv118.

cycle	number of regenerants	number of plants which set seed	percentage (%)
control	38	30	79%
1	59	48	81%
3	68	39	57%
4	54	46	85%

Discussion

An efficient regeneration protocol is a prerequisite for transformation, therefore, the influence of different factors on the somatic embryogenesis of *Alstroemeria* was investigated to improve the primary and cyclic somatic embryogenesis. Factors such as adding amino acids (L-proline, L-glutamine, L-tryptophan) in the callus induction and/or the regeneration medium, made no difference or were even negative for the regeneration (data not shown). However, Chowdhry *et al.* (1993) described a positive effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice. Also addition of different auxins in the callus induction medium, or addition of ABA in the regeneration medium did not improve the standard regeneration protocol of *Alstroemeria.* Solid or liquid medium made no difference. Dehydration treatments did not promote somatic embryogenesis of *Alstroemeria.* In rice a simple dehydration treatment promoted plant regeneration of rice callus (Tsukahara and Hirosawa, 1992). Of the many factors that were tested in *Alstroemeria*, only the low sucrose concentration (10 g l^{-1}) in the regeneration medium gave a clear positive effect on the number of somatic embryos formed and their germination percentage. In maize the sucrose concentration of the growth medium appeared also very important, but the maturation of maize somatic embryos was optimal at a high concentration of 60 g l^{-1} sucrose instead of a low one (Emons *et al.*, 1993). As already described by Van Schaik *et al.* (1996) *Alstroemeria* seed showed hypogeal germination. The first cotyledon of *Alstroemeria* is not a real storage organ as it is in maize. Probably this explains the different optimum sucrose concentrations

for the maturation of the somatic embryos of *Alstroemeria* and maize. A low sucrose concentration is more often a positive condition for regeneration, as shown in the case of germination of soybean somatic embryos (Lazzeri *et al.*, 1988).

Isolated somatic embryos or embryos still present on the callus gave almost the same number of regenerants after germination induction in the primary cycle. So leaving the somatic embryos in contact with the callus or isolation of the somatic embryos from the callus had no effect on the final amount of regenerants formed. In the first cyclic experiment only 2 cycles were investigated. The average amount of somatic embryos per embryogenic callus and the percentage of embryogenic callus formed was not much different in the first and second cycle. But a clear difference was that in a relatively short time, 3x times more regenerants could be produced (start 40 calli: primary cycle after 22 weeks 46 plants, 2 cycles after 32 weeks 131 plants).

In the second cyclic experiment 6 cycles were performed with 2 different callus induction media. In separate pilot studies, the optimum concentration for callus induction medium with 2,4D and 4FPA was determined. The optimal concentration of 4FPA was relatively high, compared with that for 2,4D. The cyclic experiments were performed at the optimal concentrations. The average number of somatic embryos in the first or following cycles was not significantly different from each other, except from the sixth cycle with 2,4D. The auxin 4FPA was compared with 2,4D, because Yasuda and co-workers (Yasuda *et al.*, 1990) described 4FPA as a potent auxin in subcultures. In rice 4FPA-induced callus retained their regenerative ability for at least 15 month with 9 subcultures, while callus induced by 2,4D lost their regenerative ability after 3 subcultures. Also 4FPA-induced callus showed a higher regeneration ability than 2,4D-induced callus did. In *Alstroemeria* there was no clear difference between the performance of 2,4D and 4FPA and with both auxins the average amount of somatic embryos decreased in the 6^{th} cycle. Thus it is advised to terminate the experiments after the 5^{th} cycle or to determine more carefully which somatic embryos are most optimal developed to be used in a cyclic system.

The second or following cycles did not produce more somatic embryos per callus then the primary cycle in *Alstroemeria*, so we could not make the somatic embryogenesis system more efficient by a cyclic step. In some cases higher efficiency occurs in cyclic embryogenesis, for instance in cassava where in all successive cycles the embryo explants produced significant

more embryos than leaf lobe explants (Raemakers *et al.*, 1993). However in the monocotyledonous crop leek the average number of somatic embryos per initial was 24 in the primary cycle and in the next cycles nearly 50% of this amount (Schavemaker and Jacobsen, 1995). Moreover, the process of cyclic somatic embryogenesis in monocotyledonous crops is mostly performed through (pre)globular embryos in liquid medium (often called cell suspensions) (Vasil and Vasil, 1981).

Most of the plants regenerated from cyclic somatic embryogenesis were able to produce seeds. The cv118 is a complex interspecific hybrid, so that is why we did not expect a 100% seed set of the selfings of cv118. The *in vitro* pollen vitality of cv118 self is only 15% (personal communication M.J. de Jeu). An average of seventy-four percent of the regenerated plants produced seed, which is a rather good result and comparable with the control. It is important to detect if the regenerants set seed, because after a transformation technique is applied in the somatic embryogenesis regeneration system, the transgenic regenerants will be used as breeding parents in order to bring the novel gene into the gene pool for normal breeding. Gross somaclonal variation between the regenerated plants from one callus was not registrated. Characteristics especially concerning stem, leaf and flower colour and morphology were scored. Of course the number of regenerants from one callus was very small (1-6 plants), so it is not possible to make conclusions about the somaclonal variation of the regenerants, except that no clear mutations or large variations were detected.

The reason to optimise the primary and cyclic somatic embryogenesis system of *Alstroemeria* is that this needs to be very effective if we want to use it in genetic transformation studies. A low sucrose concentration in the regeneration medium was a positive factor for improvement. A cyclic system of *Alstroemeria* is possible, but the average amount of somatic embryos per embryogenic callus could not be improved. The regeneration experiments described here indicate that mass production of certain genotypes is possible through cyclic somatic embryogenesis in a short time. The cyclic method, which is independent of the flowering season is faster then the same amount of separate primary cycles, to produce high numbers of *Alstroemeria* plants.

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Chapter 4

Prerequisites for genetic transformation in *Alstroemeria* L.

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Abstract

The successful application of plant biotechnology to *Alstroemeria* improvement will largely depend on the availability of an efficient regeneration system and its accessibility to transformation. In the present study a number of factors required for successful genetic transformation have been studied. What is the origin of regeneration? How far do particles penetrate the plant tissue after particle bombardment? What are the optimal selection conditions for somatic embryogenesis of *Alstroemeria* after transformation?

Histological studies of the callus tissue revealed that the outermost layers of the protoderm divided repeatedly. Most of these cells became bulky with large vacuoles, but some cells remained small after division and formed a nodule or a proembryo. Later on globular stage somatic embryos originated, thus showing that the origin of somatic embryos was on the callus surface. Histology of *Alstroemeria* callus showing transient GUS expression, following particle bombardment, showed GUS positive cells up to 8 cell layers beneath the callus surface. These results show that the origin of regeneration and the cells which become transformed as judged by GUS expression, are found in the same cell layers just below the callus surface.

Like other monocotyledonous plants *Alstroemeria* is relatively insensitive to kanamycin, which is generally used as a selective agent. The selective agent PPT proved to be a more powerful selective agent in *Alstroemeria* callus.

Introduction

Alstroemeria (Inca lily) is an important cut flower in the Netherlands. This fact has stimulated an interest in developing a regeneration and transformation protocol for this monocotyledonous ornamental. Before gene transfer can be effective, it is essential that an efficient regeneration protocol is established. Furthermore, it is important to establish which cells are competent to regenerate and whether their source is unicellular or multicellular, because individual cells of these cell layers have to be accessible to the transformation protocol (Christou, 1995). On the other hand, it is very important to establish which cells are

accessible by particle bombardment. Another utmost important prerequisite for transformation is a good selection system at cellular and tissue culture level.

Alstroemeria can be multiplied vegetatively either by rhizome division or by micropropagation of rhizome cuttings *in vitro* (Pierik *et al.*, 1988; Bond and Alderson, 1993). In previous studies callus induction and plant regeneration from mature zygotic embryos has been reported (Gonzalez-Benito and Alderson, 1992; Hutchinson *et al.*, 1994 and 1997). We have developed a regeneration system through somatic embryogenesis from immature zygotic embryos (Van Schaik *et al.*, 1996a). In the present study prerequisites for genetic transformation in *Alstroemeria* were investigated. In order to determine the origin of the somatic embryos in our regeneration system and the hit-place after particle bombardment, histogenesis of the somatic embryos was investigated. The optimal selection system after transformation of *Alstroemeria* was determined. In addition, mature zygotic and somatic embryos were compared by scanning electron microscopy.

Material and methods

Plant material and standard regeneration protocol

Selfings were made on the tetraploid (2n=4x=32) cultivar 118 (parentage unknown) in the greenhouse. Immature embryo derived callus and somatic embryogenesis were performed according to the protocol of Van Schaik *et al.* (1996a).

Particle bombardment

For the transformation experiments the PDS-1000He biolistic device (Biorad) was used. Callus explants (30 days after initiation) were bombarded twice using tungsten particles (1.1 μm) at a helium pressure of 1300 psi combined with a vacuum level of 25 inches Hg. Plasmid pBI221 containing the GUS gene, regulated by the 35S promoter and the nos terminator, was coated on the particles using a procedure modified by Klein *et al.* (1987).

Histochemical GUS assay

Four days after bombardment the explants were analysed for *in situ* localisation of GUS activity using X-gluc as a substrate (Jefferson *et al.*, 1987).

Light microscopy

Callus and somatic embryos were collected between 0 and 75 days at 3 and 4 day intervals. Also callus after particle bombardment and GUS assay was collected. The explants were fixed in 5% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) for 1 h, rinsed in buffer, dehydrated in an ethanol series and embedded in HistoResin (Reichert-Jung commercial embedding kit). Serial sections of 5-7 μm were made using a Reichert-Jung Autocut 2055 microtome. The sections of the somatic embryogenesis were stained with 0.25% aqueous toluidine blue and the sections after particle bombardment were stained with 0.2% safranine red. Some sections were also treated with an IKI-solution (2 gram KI and 0.2 gram I_2 in 100 ml distilled water) for staining starch. The sections were studied and photographed under bright-field conditions using a Zeiss Axiophot light microscope.

Scanning electron microscopy

Mature zygotic and somatic embryos were fixed and dehydrated similarly to those prepared for light microscopy. Thereafter, the specimens were prepared for scanning electron microscopy by critical point drying and sputtered with gold and palladium. The specimens were examined at 10 kV and photographed with a JEOL 5200 SEM.

Kanamycin and L-phosphinothricin (PPT) dose response

After six weeks (T=42 days, culture initiation as half ovule) on callus induction medium the calli were transferred to callus induction medium containing the antibiotic kanamycin (0, 10, 20, 30, 40 mg l^{-1}) or the herbicide PPT (0, 0.5, 1, 5, 10, 20 mg l^{-1}). Three replicate callus samples and 10 calli per treatment were used. In the experiments with selective agent PPT no casein hydrolysate was added. After 4 weeks on callus induction medium with a selective agent the fresh weight of the calli was measured. The calli were cultured during 12 weeks on regeneration medium with the same concentration selective agent. Observation of regeneration took place.

Selection on L-phosphinothricin (PPT) and chlorophenol red (CR)

At different time intervals in the somatic embryogenesis calli were placed on culture medium with different PPT concentrations (0-0.5-1-2-5-10 mg l^{-1}) combined with 25 mg l^{-1} CR. Small

petridishes were used with 4 calli in each petridish. Five petridishes per PPT concentration were scored. The degree of colour change in each petridish was assayed after 1 week. Phosphinothricin (PPT) inhibits glutamine synthetase in plant cells, resulting in an accumulation of ammonia that can be toxic (Wilmink and Dons, 1993). The pH indicator CR changes from red through orange to yellow as pH decreases from 6.0 to 5.0 (Kramer *et al.*, 1993). Normally growing *Alstroemeria* callus acidified the surrounding medium, but with PPT in the culture medium the pH of the medium remains high (accumulation of ammonia). This means that normally medium with CR will change into yellow, but with the proper concentration of PPT it remains red.

Results

Histology of somatic embryogenesis

According to the standard protocol, 4 week-old immature zygotic embryos were initiated to form callus. Figure 1 represents a section through an immature zygotic embryo inside an ovule four weeks after pollination at initiation time (T=0). At this stage the immature globular zygotic embryo consisted of about 100 cells and was about 200 µm in length. Differentiation of the embryo and suspensor was evident. In the endosperm, the process of cellularization had started. After four weeks on induction medium in general a callus of 1-2 mm was formed and that callus was separated from the half ovule (T=28). At that stage different callus types were detectable: rhizogenic, compact and nodular callus. The rhizogenic callus consisted of callus with root hairs and the compact callus was a massive type (not shown). Attention was focused on the nodular callus, which was an embryogenic callus as it formed new nodules (Figure 2 and 3) which regenerated through somatic embryogenesis. The cell divisions started in the outermost layers of the protoderm. In these cell layers cell divisions occurred in all directions (Figure 3A). Most of these dividing cells became large, vacuolated and contained a lot of starch. These bulky cells gave the nodular callus a rough appearance. Between these large vacuolated cells a few meristematic cells remained, which were able to form a nodule or a proembryo (see Figure 3B and 3C). The proembryos formed globular somatic embryos (Figure 4). These globular somatic embryos could transform into a mature somatic embryo during the regeneration period.

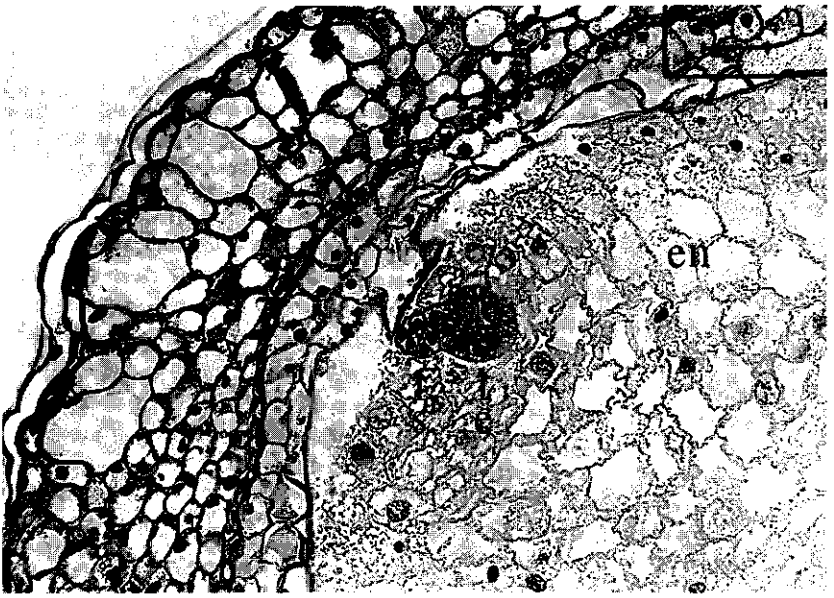


Figure 1. Section through an immature zygotic embryo inside an ovule before culture T=0 (bar represents 100 μm). e=embryo, s=suspensor, en=endosperm

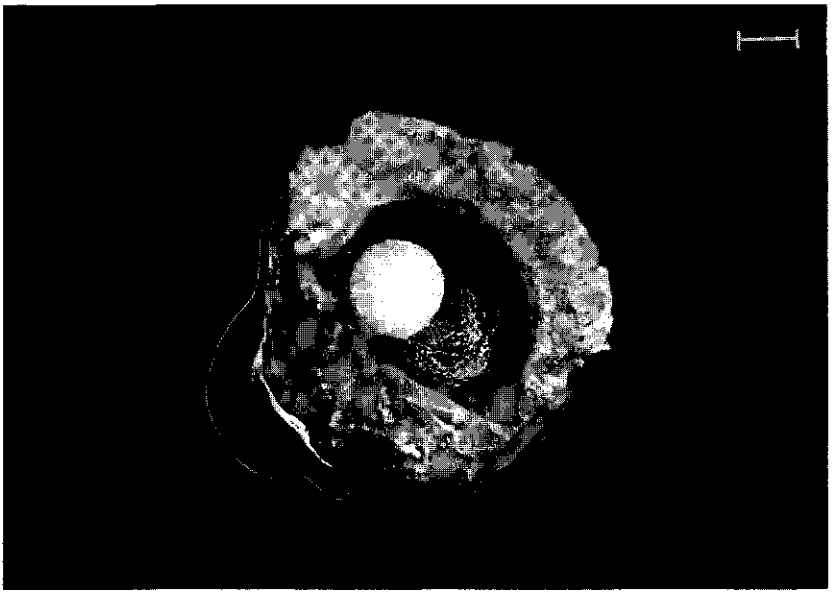


Figure 2. Nodular callus inside the half-ovule after 4 weeks on callus induction medium (bar represents 400 μm).

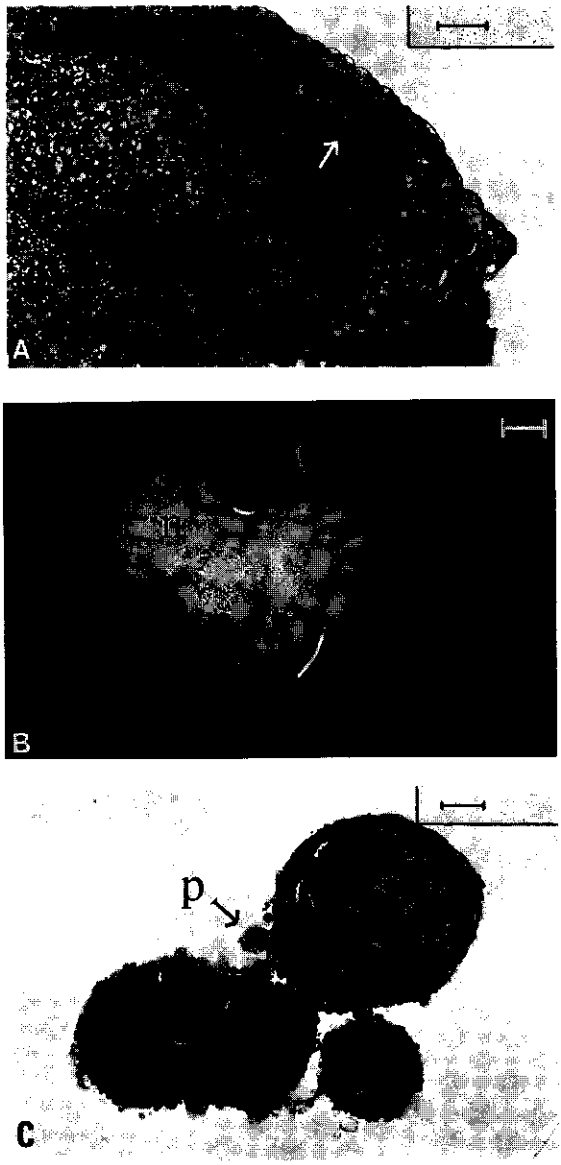
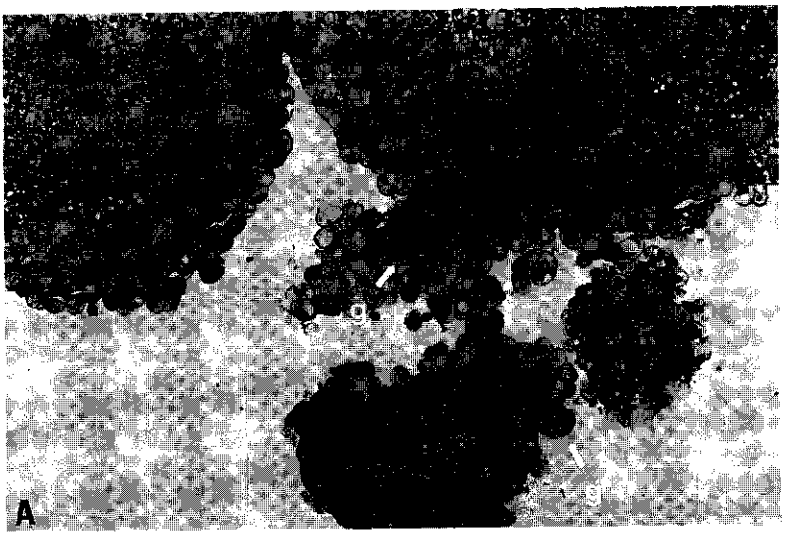


Figure 3. Callus of somatic embryogenesis of *Alstroemeria*. cv118

3A. Detail of *Alstroemeria* callus T=48 days after initiation of somatic embryogenesis. (bar represents 100 μm). The first divisions in the cell layers are visible (arrow) .

3B. Morphological observation of nodular callus T=65 days (bar represents 250 μm).

3C. Micrograph showing a section through the same nodular callus. Arrow shows proembryo. (bar represents 300 μm).i=original immature embryo, n=nodule, p=proembryo



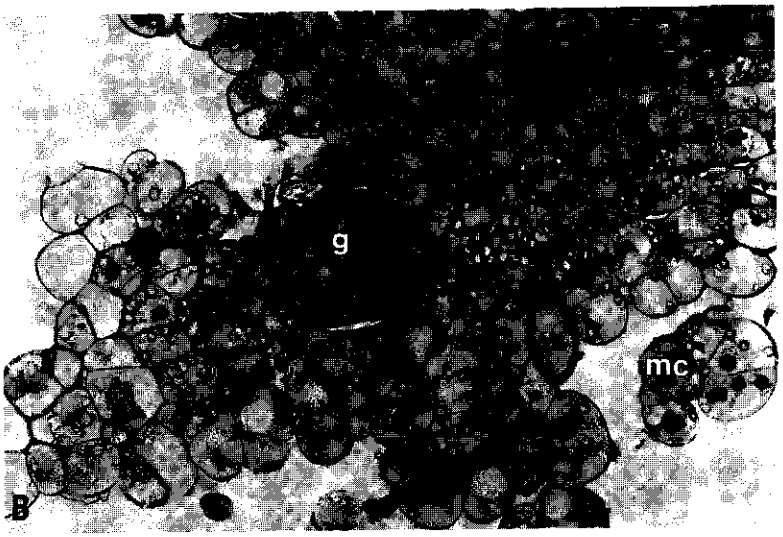


Figure 4. Section through active callus protoderm in a later stage of the somatic embryogenesis of *Alstroemeria* (T=77 days).

4A. Micrograph showing active callus protoderm with two globular somatic embryos (arrows) (bar represents 200 μm).

4B. Detail of a globular somatic embryo, also meristemeric cells visible. (bar represents 60 μm). g=globular somatic embryo mc= meristemic cells

Comparison of mature zygotic and somatic embryos

The morphology of a zygotic and somatic embryo was investigated in more detail, especially the germination notch, which is the most important part of the embryo (see Figure 5). Both embryos consisted of a cotyledon, a root meristem and a shoot meristem in the germination notch. The shoot meristems are inside the germination notch. The cotyledon had a different cell type compared with the hypocotyl region in both the zygotic and somatic embryo. In the cotyledon of the somatic embryo stomata, a clear evidence for a leaf origin, were visible. Stomata were only visible in older zygotic embryos as well. The somatic embryo had the same structural and functional parts as the zygotic embryo. However, the size of the somatic embryo was highly variable, ranging from the size of a normal mature zygotic embryo (2 mm) to much larger (12 mm). Also the germination notch of the somatic embryo in general was more open and the shoot apex more developed then the germination notch and shoot apex of the mature zygotic embryo.

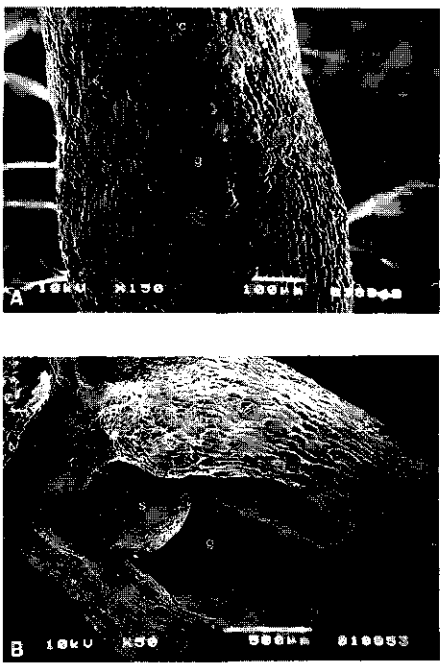


Figure 5. Scanning electron micrograph showing the germination notch of a mature zygotic embryo (A) and a mature somatic embryo (B).

g = germination notch, s = shoot apex, c = cotyledon

Histology after particle bombardment

Fifteen calli from different bombardments were randomly picked after the GUS assay for histological study. Transient GUS expression was detected as blue crystals in sections of glutaraldehyde-fixed material, which were not seen in the controls. GUS crystals could be found up to 8 cell layers deep into the tissue (see Figure 6). Most GUS spots displayed a gradient in which the centrally located cells on the surface (direct hit place) had the darkest colour and the most assay product. The Figure on the cover page shows beautifully a cluster of particles in an active dividing cell, even the hole in the cell wall is visible.

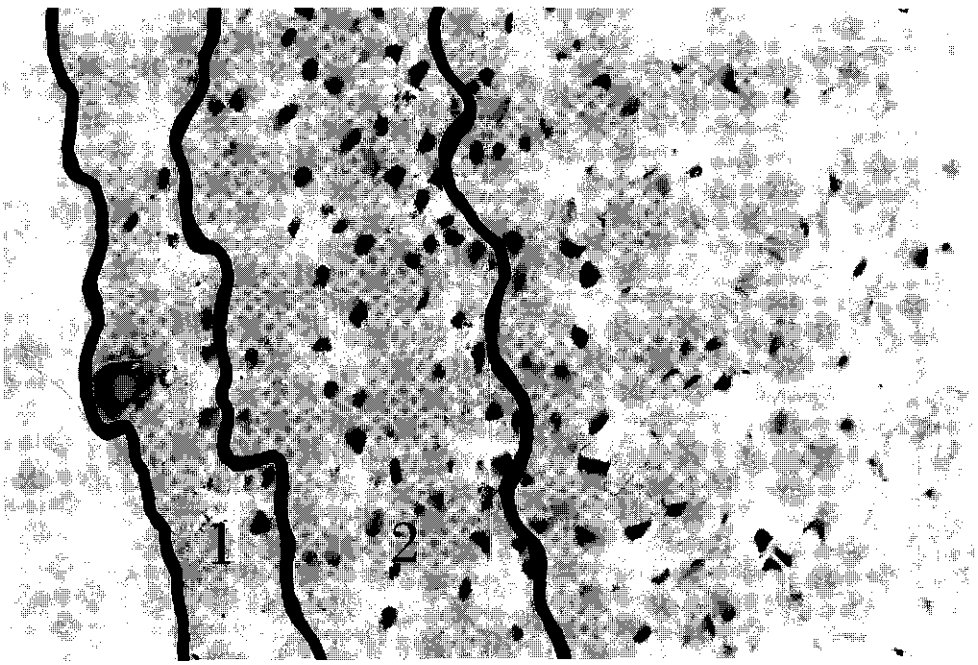


Figure 6. Micrograph showing a section through *Alstroemeria* callus, which was bombarded with the particle gun, followed by a GUS assay. The blue GUS crystals are visible in the direct hit spot (very blue=1) and the deeper layers (blue=2). Bar represents 100 μm.

Optimal selection system

As has been already described by others, kanamycin is not the most favoured selective agent for monocots. For *Alstroemeria* the same results were found (see Table 1). A concentration of 30 mg l^{-1} kanamycin could block the regeneration of the somatic embryogenesis, but did not block the callus growth. Therefore we studied the effect of phosphinothricin (PPT) as selective agent. Table 1 and 2 shows that 1 mg l^{-1} PPT is sufficient as selective agent in young *Alstroemeria* material (till 49 days after initiation) for blocking callus growth as well as regeneration. Older material (50 days or more after initiation) needs higher concentrations of PPT (5-10 mg l^{-1}) for the same effect. The colour is then changed into red with CR in the medium. These results show that PPT can be used at relatively low concentrations in transformation experiments.

Table 1. Inhibitory effect of kanamycin and PPT on *Alstroemeria* callus growth, measured by decrease in fresh weight per 0.1 gram starting callus, after 4 weeks on callus induction medium with kanamycin or PPT. Plantlet regeneration was observed after 12 weeks on regeneration medium containing the same concentration selective agent.

	kanamycin		**PPT**	
concentra-tion (mg l^{-1})	weight decrease	regeneration	weight decrease	regeneration
0	0 a	+	0 a	+
0.5	0 a	+	0.9±0.2 b	-
1	0 a	+	1.0±0.1 b	-
5	0 a	+	1.1±0.1 b	-
10	0 a	+	1.1±0.2 b	-
20	0.3±0.2 a	+	1.2±0.1 b	-
30	0.2±0.1 a	-		
40	0.2±0.1 a	-		

-letters indicate significant differences p=0.05

Table 2. Chorophenol red assay with *Alstroemeria* callus and somatic embryogenic material of different time after initiation (T in days).

PPT (mg l^{-1})	0	0.5	1	2	5	10
time after initiation						
T=28	yellow	orange	red	red	red	red
T=49	yellow	orange	red	red	red	red
T=70	yellow	yellow	yellow	orange	red	red
T=91	yellow	yellow	orange	orange	orange	red

Discussion

One prerequisite for a transformation protocol is to know if the cells that are able to regenerate are at a place which can be treated for transformation. For this reason histology of the somatic embryogenesis process in *Alstroemeria* and the histology after particle bombardment of these embryogenic calli were investigated. Our callus induction and regeneration protocol was started with an immature zygotic embryo as explant. The immature zygotic embryo was globular and consisted of about 100 cells at the beginning of the *in vitro* culture. The endosperm was still nuclear, but cellularization had started. This is in agreement with earlier observations of Buitendijk *et al.,* (1995) and De Jeu and Jacobsen (1995). These authors described an embryo rescue protocol for the production of interspecific hybrids in *Alstroemeria*. In their study a high sucrose concentration at the start of the embryo rescue culture was essential, as we also found to be essential at the beginning of our somatic embryogenesis. So, it can be expected that in this phase the explants contain a high amount of starch. Later on, when the MS medium was supplemented with only 30 g l^{-1} sucrose, starch was still present, and accumulated inside the bulky cells with large vacuoles. New globular stage somatic embryos originated between these bulky cells. According to the literature, the surrounding cells synthesise and store considerable amounts of starch both in case of organogenesis or embryogenesis (Mangat *et al.,* 1990; Barciela and Vieitez, 1993). It appears

that starch metabolism might satisfy the high energy requirements of dividing meristemic cells (Redway, 1991).

Characteristics of meristemic cells are thin walls, dense cytoplasm, small vacuoles and often rich in starch (Emons, 1994). Most of the dividing cells in the outermost layers of the protoderm of *Alstroemeria* become bulky with large vacuoles and a few meristemic cells were found between these bulky cells. So it is reasonable to believe that only those few meristemic cells become globular somatic embryos, because the regeneration frequency per explant is very low (Van Schaik *et al.*, 1996a and b). However, according to our data, it cannot be excluded that the bulky cells also are involved in the formation of globular somatic embryos. Bulky cells are, as mentioned in the histology of somatic embryogenesis, induced in *in vitro* stem fragments of cork oak (*Quercus suber*) cultured *in vitro* (El Maâtaoui *et al.*, 1990). In cork oak the somatic embryos were formed in groups around the edges of some calli. In this paper histological examinations showed that somatic embryos, originated potentially from two different types of cells: either a characteristic embryogenic cell or a highly bulky cell with large vacuoles.

For transformation techniques it is important to establish in which cell layers the regeneration process is initiated. The precise location of the earliest cell divisions was studied histologically. In *Alstroemeria* it seemed that a small number of cells of the outermost layers of the tissue were involved in the regeneration process. The formation of young globular somatic embryos took place on the surface of the callus. The origin of the somatic embryos of *Alstroemeria* was probably from a limited number of cells, because the meristemic cells and globular stage somatic embryos were more or less isolated from the surrounding cells and the meristemic cells consisted of only few cells and the globular somatic embryos consisted of about 40 cells. This is useful information for the development of a transformation technique, but their surfacial origin is even more important. Single cell origin of somatic embryogenesis has been reported in rice and maize, but also multiple cells origin has been reported in sunflower and *Stylosanthus scabra* (Jones and Rost, 1989; Van Lammeren, 1988; Bronner *et al.*, 1994; Dornelas *et al.*, 1992).

This means that the cell layers where regeneration occurs could be reached by our applied transformation technique. For certainty, the histology after particle bombardment was also investigated. Transient GUS expression was found as deep as 8 cell layers of the callus. This

is in accordance with the histology of transient GUS expression in pearl millet (*Pennisetum glaucum* (L.) R. Br.) (Tayler and Vasil, 1991). In pearl millet GUS positive cells could even been seen up to 12 cell layers beneath the epidermis. This can be due to the bombardment conditions. So, in theory particle gun mediated transformation is possible, because both regeneration and transformation take place in the same cell layers of our *Alstroemeria* callus system. However, the frequency of cells involved in both processes is low.

Alstroemeria callus, as in many other monocots (especially Gramineae), exhibits a high level of natural tolerance to the antibiotic kanamycin (Hauptmann *et al.*, 1988). High levels of kanamycin (up to 300 mg l^{-1}) did not completely inhibit callus growth of *Alstroemeria* (data not shown). However, the regeneration of somatic embryos was completely inhibited by 30 mg l^{-1} kanamycin. In the forage grass Caucasian bluestem (*Bothriochloa caucasia*) similar results were found. High levels of kanamycin did not completely inhibit callus growth. However, the regeneration of healthy plantlets was completely inhibited by kanamycin even at low levels (Franklin *et al.*, 1990). Low concentrations of PPT in the culture medium did inhibit both callus growth and regeneration of the somatic embryos of *Alstroemeria*. The pH indicator CR showed clearly the effect of PPT in the culture medium. PPT is a good selective agent in low concentrations (1 mg l^{-1}) for young *Alstroemeria* callus and in higher concentrations (2-10 mg l^{-1}) for older and bigger explants.

In conclusion histological monitoring of immature zygotic embryos as explants in a somatic embryogenesis protocol showed that the protoderm cells divided in all directions on callus induction medium. Most of these cells became bulky with large vacuoles, containing plastids with starch granules. But some cells remained small after division with condensed cytoplasm and formed a nodule or a proembryo, which resulted in a globular stage somatic embryo. Transient GUS expression was found after particle bombardment in the same cell layers of the callus surface. A good selection system was established for transformation of somatic embryos of *Alstroemeria*. Optimisation of the regeneration protocol and development of a transformation protocol is still in progress.

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Chapter 5

Transformation of monocotyledonous *Alstroemeria* L.

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Abstract

The monocotyledonous ornamental *Alstroemeria* was subjected to genetic transformation using *Agrobacterium*-mediated and particle gun-mediated techniques. We report that *Alstroemeria* is a host for *Agrobacterium,* based on the expression of the intron containing reporter gene ß-glucuronidase (GUS) after inoculation with a disarmed strain of *A. tumefaciens*. Factors influencing transient gene expression in *Alstroemeria* callus after transformation with *Agrobacterium* or particle gun (PDS 1000/He device) were optimised. Wounding of the callus with a needle, prior to inoculation with a high *Agrobacterium* concentration during a three day cocultivation regime were considered to be the best *Agrobacterium*-mediated transformation conditions. Optimal GUS expression was found after particle bombardment, when the target tissue was close to the microcarrier launch assembly, when the rupture disc-breakage point was between 650-900 psi and when the GUS gene was under control of the ubiquitin promoter.

Somatic embryogenesis of *Alstroemeria* combined with particle gun-mediated transformation resulted in transgenic chimeric plants. A friable cell suspension was made from callus isolated from cyclic somatic embryo culture. It was possible to isolate small embryogenic units at low frequency, which resulted in a friable cell suspension. This cell suspension was subjected to transformation with the particle gun. A complete stably transformed cell suspension was detected after bombardment and visible LUC selection. PCR analysis as well as LUC activity demonstrated the stable presence of the introduced gene in this friable cell suspension line.

Introduction

Alstroemeria (Inca Lily) has gained world-wide importance as a cut flower crop due to a low energy requirement in the greenhouse, long vase life and a wide range of attractive flower colours (Monette 1992). *Alstroemeria* can be multiplied vegetatively either through splitting of rhizomes or through micropropagation of rhizome cuttings (Bond and Alderson, 1993; Buitendijk *et al.*, 1992). Callus induction and plant regeneration can take place from mature and immature zygotic embryos (Gonzalez-Benito *et al.*, 1992; Hutchinson *et al.*, 1994; Van

Schaik *et al.*, 1996). Direct shoot regeneration from excised leaf explants of *in vitro* grown seedlings of *Alstroemeria* is possible (Lin *et al.*, 1997). All these plant regeneration protocols are still not very efficient and often labour intensive. *Alstroemeria* is a monocotyledonous species belonging to the family Alstroemeriaceae. The family Alstroemeriaceae belongs to the order Liliales, superorder Liliiflorae (Dahlgren *et al.*, 1985). The two most widely used techniques for the introduction of new genetic information into plant cells are based on the natural gene transfer capacity of *Agrobacterium tumefaciens* or on direct gene transfer by particle gun bombardment (van Wordragen and Dons, 1992; Christou, 1992). Transformation of dicotyledonous plants with *Agrobacterium tumefaciens* is well established, and has produced stable transgenic plants expressing a variety of foreign genes. This has not been the case for monocots in general, although more and more evidence is emerging that also monocots like rice and maize can be transformed using *Agrobacterium tumefaciens* (Hiei *et al.*, 1994). A few representatives of the families Liliaceae, Amaryllidaceae, Iridaceae, Dioscoreae have been reported to be transformable by *Agrobacterium* (Delbreil *et al.*, 1993; Mooney *et al.*, 1992; Raineri *et al.*, 1990; Schäfer *et al.*, 1987; Wilmink *et al.*, 1992). Six families of the order Liliales were susceptible to *Agrobacterium tumefaciens* (De Cleene, 1985).

The most commonly used direct DNA delivery method is particle gun bombardment. Microprojectile bombardment employs high velocity metal particles to deliver biologically active DNA into plant cells (Sanford, 1988). Christou *et al.* (1988) demonstrated that the process could be used to deliver biologically active DNA into living cells and result in the recovery of stable transformants. Nowadays many crops, especially monocots, can be transformed utilising particle bombardment (Christou, 1995; Vain *et al.*, 1995; Brettschneider *et al.*, 1997). The first part of the present study investigates optimal transient expression of the ß-glucuronidase (GUS) gene after transformation with *Agrobacterium* or particle bombardment in *Alstroemeria* callus. In the second part of the present study particle gun experiments were performed on somatic embryos and cell suspensions of *Alstroemeria* with the aim to produce stable transgenic plants.

MATERIALS AND METHODS

Materials and methods for *Agrobacterium*-mediated transformation

For the disarmed *Agrobacterium tumefaciens* inoculations mature seeds of selfings of *A. aurea* accession A002 and A003 (2n = 2x = 16, Department of Plant Breeding), a free pollinated population of the tetraploid hybrid K030 (2n = 4x = 32) and an open pollinated population of *A. aurea* accession A904 were used (Wülfinghoff b.v.). Callus induction was performed using a protocol modified from Gonzalez-Benito and Alderson (1992). The mature seeds were surface sterilised and imbibed in sterilised water for 48 hours. As a result the seed became soft and from these the zygotic embryos were excised under aseptic conditions. The zygotic embryos were placed on callus induction medium and were subcultured biweekly. The callus induction medium (2D:1) consisted of MS medium supplemented with 30 g l^{-1} sucrose, 0.2 g l^{-1} casein hydrolysate, 2 mg l^{-1} 2,4D, 1 mg l^{-1} BAP and 2 g l^{-1} gelrite. The pH was adjusted to 6.0 prior to sterilisation. The callus was cultured in the dark at 21°C and used within 2 to 6 weeks after subculture. After 6 weeks on callus induction medium the callus was placed on regeneration medium and was cultured in the light with a 12 h photoperiod at 18 °C. Regeneration medium consisted of MS (Murashige and Skoog, 1962) medium supplemented with 10 g l^{-1} sucrose, 0.2 g l^{-1} casein hydrolysate, 2 mg l^{-1} BAP, pH 6.0 and 2 g l^{-1} gelrite.

The disarmed strain C58C1 (Koncz and Schell, 1986) was kindly supplied by Dr. H. Dons, CPRO-DLO, Wageningen. This strain contained the binary plasmid p35SGUSintron, harbouring the NPTII gene regulated by the nos promoter, and the intron containing β-glucuronidase(GUS) gene, controlled by the CaMV 35S promoter (Vancanneyt *et al.*, 1990). The strain was grown in Luria broth (LB) medium (10 g l^{-1} trypton, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, 1 g l^{-1} glucose) for 18 hours at 30 °C. The LB medium was supplemented with 200 μM acetosyringon and the appropriate antibiotics. Prior to inoculation, bacteria were pelleted and resuspended to an OD550 of 0.1 in MS medium containing 200 μM acetosyringon, 30 g l^{-1} sucrose and pH 5.5. For 10 minutes the calli were soaked in this MS (Murashige and Skoog, 1962) medium with *Agrobacterium* after wounding. Wounding was achieved with a sterile needle, rolling in sterile carborundum powder or bombardment using a biolistic PDS 700/He device, with a vacuum pressure of 28 inch Hg and tungsten particles (average

diameter 1.6 µm). After two days of cocultivation the bacteria were killed off in liquid callus induction medium (2D:1) supplemented with antibiotics, 300 mg l^{-1} cefotaxim and 200 mg l^{-1} vancomycin. After 5 days the calli were transferred to solid medium with the same composition.

Two weeks after the *Agrobacterium* inoculation the GUS activity was determined using the histochemical assay according to Jefferson *et al.* (1987). GUS positive calli and the total amount of blue spots were counted .

Materials and methods for particle gun-mediated transformation

Plant material

Alstroemeria cultivar 118 (2n=4x=32) was self-pollinated to start somatic embryogenesis (Van Schaik *et al.*, 1996). After 4 weeks globular calli were isolated from the half ovules and after 1 to 4 days the particle bombardments took place.

Cell suspensions were made from friable callus of cv118. This friable callus was selected during the fifth cycle of the cyclic somatic embryogenesis (Chapter 3). Small embryogenic units were isolated and were brought in liquid medium for suspension. Selection of fast growing suspension cells was made. The cell suspensions with a Settled Cell Volume of 2 to 4 (in 11 days) were selected. This cell suspension was maintained in a medium consisting of MS salts and vitamins, 0.1 g l^{-1} myo-inositol, 20 g l^{-1} sucrose, 18.2 g l^{-1} d-mannitol, 0.48 g l^{-1} MES, 0.1 g l^{-1} casein hydrolysate, 0.08 g l^{-1} adenine sulphate, 0.5 mg l^{-1} d-calcium-panthotenate, 0.1 mg l^{-1} choline chloride, 0.5 mg l^{-1} ascorbic acid, 2.5 mg l^{-1} nicotinic acid, 1.0 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} folic acid, 0.05 mg l^{-1} biotin, 0.5 mg l^{-1} glycine, 1 mg l^{-1} L-cysteine, 0.25 mg l^{-1} riboflavine, 10 mg l^{-1} Picloram and pH 6.0. The medium was refreshed every 3 to 4 days and after 12 days the content of each flask was divided (50 ml liquid medium in 200 ml flask, 18 °C), and fresh medium was added.

Plasmids

pAHC15, pAHC18 and pAHC25 (Table 1) were kindly provided by Prof. P.H. Quail (Christensen and Quail, 1996; Christensen *et al.*, 1992). pJIT65 was kindly provided by Dr. J.F. Guerineau, John Innes Institute, UK)(Guerineau and Mullineaux, 1993). Plasmid +3 was

constructed and kindly supplied by T. Schavemaker, Department of Plant Breeding, Netherlands. pDC2 was obtained from Plant Genetic Systems, Belgium. Details of the plasmids are shown in Table 1. All plasmids were isolated using the Wizard ™ Maxiprep DNA purification system of Promega.

Table 1. Composition of the plasmids containing the chimeric gene constructs, used in particle gun experiments.

expression vector	gene construct	references/source
pAHC15	pUbi:GUS:tnos	Christensen & Quail, 1996
pAHC18	pUbi:LUC:tnos	Christensen & Quail, 1996
pAHC25	pUbi:GUS:tnos/pUbi:BAR:tnos	Christensen & Quail, 1996
+3	p35S:GUS:t35S/p35S:BAR:tnos	Schavemaker
pJIT65	p2x35S:GUS:t35S	Guerineau & Mullineaux, 1993
pDC2	pTR2':GUS:t35S/pTR1':NPTII:tnos/ p35S:BAR:tnos	Plant Genetic Systems

BAR	=	phosphinothricin-N-acetyltransferase	pUbi	=	ubiquitin promoter
GUS	=	ß-glucuronidase	p2x35S	=	double 35S promoter
LUC	=	luciferase	p35S	=	35S promoter
NPTII	=	neomycin phosphotransferase II	tnos	=	nos terminator
pTR1'or 2'	=	TR1'or 2' promoter	t35S	=	35S terminator

DNA coating

A method adjusted from McCabe *et al.* (1988) was used to coat DNA on gold particles. Twenty µg of DNA was continually mixed by vortexing with 10 mg of gold particles (1.0 µm in diameter, Biorad), 3 µl 5 M NaCl, 0.5 µl 2 M tris HCl pH 8.0, 96.5 µl aqua dest, 100 µl 25% PEG 1550, 100 µl 0.1 M spermidine and 100 µl 2.5 M $CaCl_2$. This mixture was vortexed for 10 minutes. After centrifugation the pellet was resuspended in 10 ml of absolute

alcohol and was given sonication for a very short time. One hundred sixty µl of the gold suspension was pipetted on a macrocarrier by using the macrocarrier holder upside down (Biorad). After 5 minutes the macrocarrier holder was removed. The macrocarrier covered with a thin layer of gold beads was dried in an oven (10 minutes, 40 °C) and used for bombardment.

Particle bombardment procedure

Experiments for optimisation of transient expression

Particle bombardment was carried out using the Biolistic PDS-1000/He device (Kikkert, 1993). Petridishes with 40 calli were bombarded twice with 1 µm gold particles at a helium pressure of 1100 psi combined with a vacuum level of 25 inches Hg. The particles were coated with plasmid pAHC25. First the coating of the particles with different plasmids, containing the GUS gene, was the variable factor (see Table 1). Furthermore the distance between the microcarrier launch assembly and the target tissue was a variable factor. The particles were coated with plasmid pAHC25, pressure 900 psi. The target tissue can be placed on 4 different levels in the PDS-1000/He device. Position 1 is very close to the launch assembly and position 4 is the furthest away from the launch assembly (position 1=5 cm, position 2=8 cm, position 3=11 cm and position 4=14 cm). The helium pressure was also as variable factor studied (plasmid pAHC25 used, position 1).

Experiments for stable gene expression

In the first experiment 860 calli and in the second experiment 500 calli of cv118 were bombarded with plasmid +3, pDC2 or pAHC25. The callus induction medium was without casein hydrolysate and with L-phosphinothricin (PPT) (first 1, later 5-10 mg l^{-1}). In the first experiment after 6 months the selection was stopped. In the second experiment selection with PPT in the medium was continuous.

In a third experiment around 2000 calli were bombarded with plasmid pAHC18. In the fourth experiment suspension cultures (58 petridishes, 1 g per petridish) were used for bombardment with pAHC18 which could be selected visibly using a luminometer.

ß-glucuronidase(GUS) assay

Two days after bombardment the tissues were analysed for *in situ* localisation of GUS activity (Jefferson *et al.*, 1987). Transformation efficiency was expressed as the number of blue spots per bombarded petridish (which contained 40 calli). Each blue spot (a single cell or an aggregate of cells) was scored as one "expression unit" (Klein *et al.*, 1988).

Chorofenol red (CR) assay

Small pieces of the explant were cultured in liquid MS medium with 25 mg l^{-1} CR and 10 mg l^{-1} PPT. An explant expressing the BAR gene coloured yellow and was considered transgenic. A non transgenic explant coloured purple/red.

Luciferase (LUC) assay

Tissue cultured in liquid medium was collected on solid medium. The tissue was sprayed with 0.25 mg ml^{-1} luciferin (Promega, E160) and assayed for LUC activity with a VIM intensified CD camera and Argus-50 photon counting image processor (Hamamatsu Phototonic Systems, Japan), situated at the Department of Plant Physiology, Wageningen.

Data analysis

Data were taken as an average from 2 to 4 replications. Significance of differences among treatment means was analysed by LSD at 5% probability.

PCR analysis

Genomic DNA was isolated for PCR analysis from the LUC-positive suspension culture. Primers LUC1 (ACG GTT TTG GAA TGT TTA CTA C) and LUC2 (CGG TTG TTA CTT GAC TGG CGA C) were synthesized (Eurogentec) and yielded a 792 bp fragment corresponding to an internal portion of the luciferase gene (Ow *et al.*, 1986). A 50 µl PCR reaction mix contained the primers (50 pM), Taq polymerase (0.2 U), dNTP's (80 µM of each), 1xPCR reaction buffer and 50 ng of the isolated DNA. The reaction mix was overlaid with 50 µl mineral oil. PCR conditions were 92 °C initial melting, for 10 min., 30 cycles of 92 °C/2 min. and 47 °C/2min and a 72 °C/2 min. final extension.

RESULTS

Agrobacterium-mediated transformation

Effect of wounding prior to Agrobacterium inoculation.

Based upon pilot studies disarmed strain C58C1 was chosen to perform further research to optimise *Agrobacterium* transformation, because this strain had given the best results so far (see discussion). This strain contained the binary plasmid p35S-GUSintron, harbouring the NPTII gene regulated by the nos promoter.

Expression of the GUS gene in *Alstroemeria* callus after different types of wounding was studied. Wounding was accomplished by particle gun bombardment, needle pinching or carborundum powder. Table 2 shows the number of calli with blue (GUS) spots after wounding with a needle or with the particle gun (pressure 2000 psi). Also lower pressures (450, 900 and 1300 psi) were used with the particle bombardments without positive results (data not shown). Another way of wounding the calli was to roll them in carborundum powder. This type of wounding was also without positive results, no GUS expression was found. Likewise controls with and without wounding gave as expected no positive results.

Table 2. Effect of wounding with the particle gun (pressure 2000 psi) or with a needle on the number of GUS positive *A. aurea* calli.

species	total number of calli examined	calli with GUS expression
		wounded with the particle gun
A002	37	0
A003	84	0
		wounded with a needle
A002	39	3
A003	87	18

Effect of cocultivation period on GUS expression.

Another important factor for *Agrobacterium* transformation is cocultivation time. Table 3 shows the effect of longer cocultivation times of *Alstroemeria* callus on GUS expression (wounded with a needle). After inoculation with *A. tumefaciens* a cocultivation time of 3 or 4 days was significantly better then a period of 2 days. In practice, a 3 days cocultivation period seems to be the best, because this period gives the bacterium sufficient time to transfer T-DNA and it is still possible to kill off the bacteria in liquid medium, supplemented with antibiotics.

Table 3. The effect of cocultivation time on GUS expression of *Alstroemeria* hybrid calli.

cocultivation time	total number of calli analysed	number of calli with GUS expression	total number of GUS spots
2 days	83	8 (10%)a	11 a
3 days	81	25(31%)b	80 b
4 days	82	33(40%)b	103 b

-letters indicate a significant difference p=0.05

Effect of Agrobacterium concentration on the GUS expression

Prior to inoculation, bacteria were pelleted and resuspended to an OD550 of 0.1 or 0.5. These bacteria concentrations were used for *Agrobacterium* transformation with a cocultivation time of 3 days. In Table 4 the effect of these bacteria concentrations on the number of GUS positive *A. aurea* calli is shown. Increasing the *Agrobacterium* inoculation concentration five fold gave a significantly higher number of GUS positive calli (see Figure 5, page 82).

Table 4. The effect of *Agrobacterium* concentration (in OD550) on the GUS expression of *A. aurea* calli.

OD550	total number of of calli analysed	number of calli with GUS expression	total number of GUS spots
0.1	81	26 (32%)a	79 a
0.5	70	42 (60%)b	144 b

-letters indicate a significant difference p=0.05.

In these experiments not all the calli were used for GUS-expression studies The further cultured calli were finally tested for GUS-expression 4 or 8 weeks after inoculation, but no GUS expression could be found.

Particle gun-mediated transformation

Several factors were investigated in order to optimise the transient expression after particle gun bombardment in *Alstroemeria* callus (see discussion). No clear factors could be detected except the three shown factors; promoter effect, distance to the target tissue and bombardment pressure.

The promoter effect was studied with a first set of experiments with a pressure of 650 psi and the target tissue in position 1. Figure 1 shows that the plasmids pAHC15 and pAHC25, both with the GUS gene driven by the ubiquitin (Ubi-1) promoter, were significantly better than the other tested GUS genes, driven by the 35S, 2x35S or TR2' promoter, respectively. The Ubi-1 promoter was 8x better in expressing the GUS gene than the 35S promoter in *Alstroemeria* callus. Figure 6 shows transient GUS expression driven by the Ubi-1 promoter in *Alstroemeria* callus.

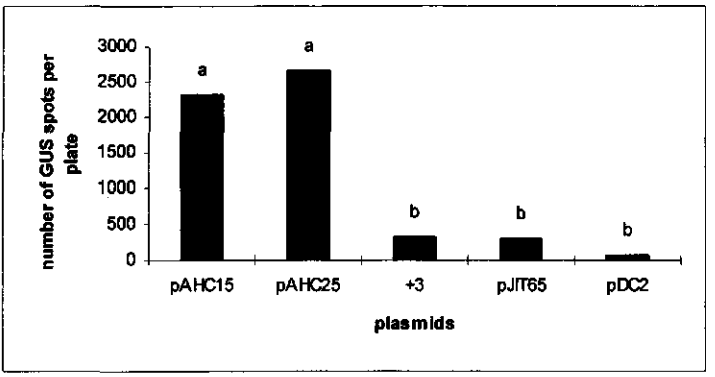

Figure 1. Effect of the promoter on the level of transient GUS expression in *Alstroemeria* callus. (See Table 1 for a full account of the compositions of the different plasmids).

In the second set of experiments the distance between the microcarrier launch assembly and the target tissue was studied. The target tissue was placed on the four possible positions in the biolistic vacuum chamber. Figure 2 clearly shows that position 1, the place closest to the microcarrier launch assembly, gave the most spots with transient GUS expression.

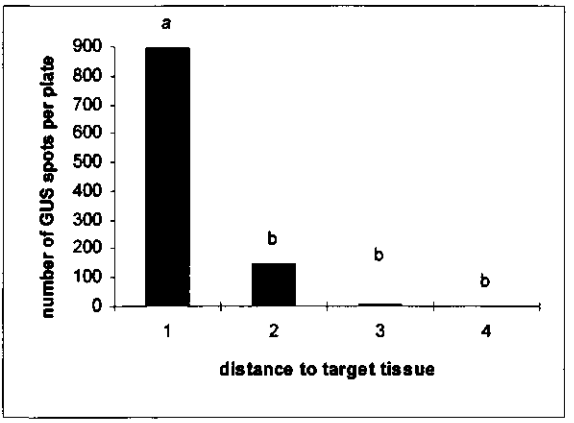

Figure 2. Effect of the distance between the microcarrier launch assembly and the target tissue on the GUS expression. Position 1(=5 cm) is close to the microcarrier launch assembly and positions 2(=8 cm), 3(=11 cm) and 4(=14 cm) are each 1 step further away. Letters indicate significant differences p=0.05

The third set of experiments showed the influence of the pressure during the particle bombardment on the number of GUS positive spots. The gold particles should possess adequate momentum to penetrate the target tissue. Different types of rupture discs have been used to deliver the gold particles different accelerations. These different types of rupture discs have different strengths and will break at different pressures. Figure 3 shows the effect of varying pressures on the GUS expression. GUS expression, determined by in number of spots per plate, was found to be the highest when particle bombardments with a pressure of 650 or 900 psi were made with the target tissue placed on position 1.

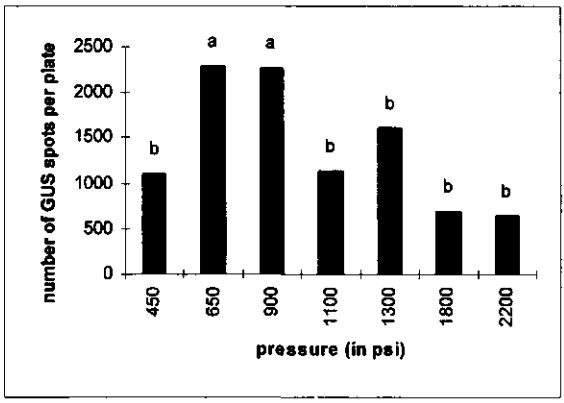

Figure 3. Effect of the pressure (in psi) during particle bombardment on the GUS expression.

Isolation cell suspensions

Friable embryogenic callus lines were selected in the fifth cycle of the cyclic somatic embryogenesis procedure. A total of 28 lines were selected. Only two of these lines had a Settled Cell Volume (=SCV) of 2 to 4 in 11 days. The other callus lines had a SCV of 1.5 or less in 11 days. These 2 callus lines of cv118 were used for bombardment experiments, because these two callus lines gave the most transient LUC expression after bombardment (factor 10-50 more then slow growing callus lines). The callus of both lines was bright yellow, friable and very fast growing in liquid medium (see Figure 4).

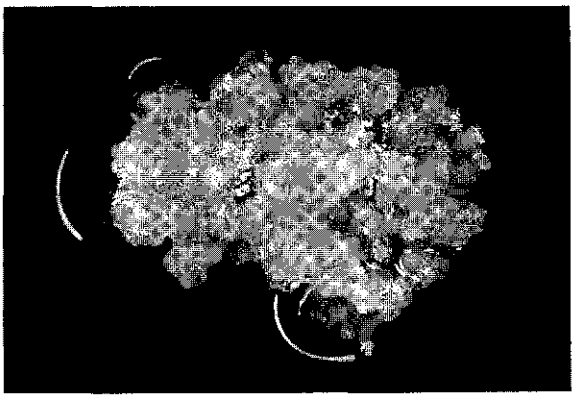

Figure 4. Friable callus of cv118 selected for producing a cell suspension culture.

Stable gene expression using GUS and LUC reporter genes

Somatic embryogenesis was performed according to the standard protocol. In the first particle gun experiment regeneration of somatic embryos was not obtained under selection pressure of PPT. Regenerants could be obtained without PPT in the medium. From 860 bombarded calli finally three regenerated plants gave positive results after the GUS-assay and the CR-assay. GUS-activity occurred mostly in the stem, especially in meristemic areas like nodes and top meristem, but never in the leaves. The GUS expression was blue, but not very intense (see Figure 7). After the second and third experiment with somatic embryos no transgenic plants could be found. However in the third experiment 2% LUC activity was still found after 3 months, but this callus did not regenerate into plants.

After selection of friable small embryogenic units, cell suspensions were made by transferring this callus to liquid medium. Selection of cell suspension with a high Packed Cell Volume had taken place. The fourth experiment with these suspension cultures gave a clear positive result with the LUC-assay and PCR analysis (see Figure 8 and 9). Two of the 58 bombarded petridishes gave finally LUC-positive callus. The LUC-positive spots were visible selected, multiplied and placed on different regeneration media there after. Only one LUC-positive callus spot became a complete transgenic cell suspension (line 5.3.3). Despite numerous attempts no plants could be regenerated from this transgenic friable cell suspension. The culture themself was completely transformed and remain LUC positive until this day.

Figure 5. Transient GUS positive *A. aurea* callus after *Agrobacterium tumefaciens* (C58C1) mediated transformation.

Figure 6. Transient GUS expression in *Alstroemeria* callus with pAHC25 (contains ubi-GUS).

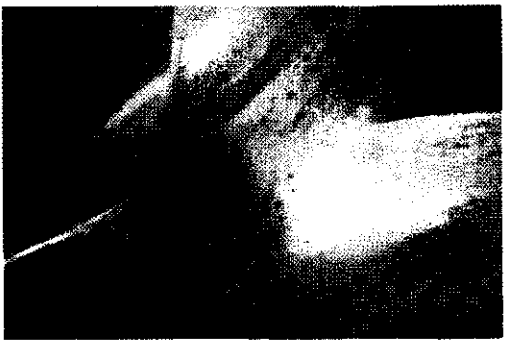

Figure 7. GUS-expression in a chimeric transgenic *Alstroemeria* cv118 regenerant.

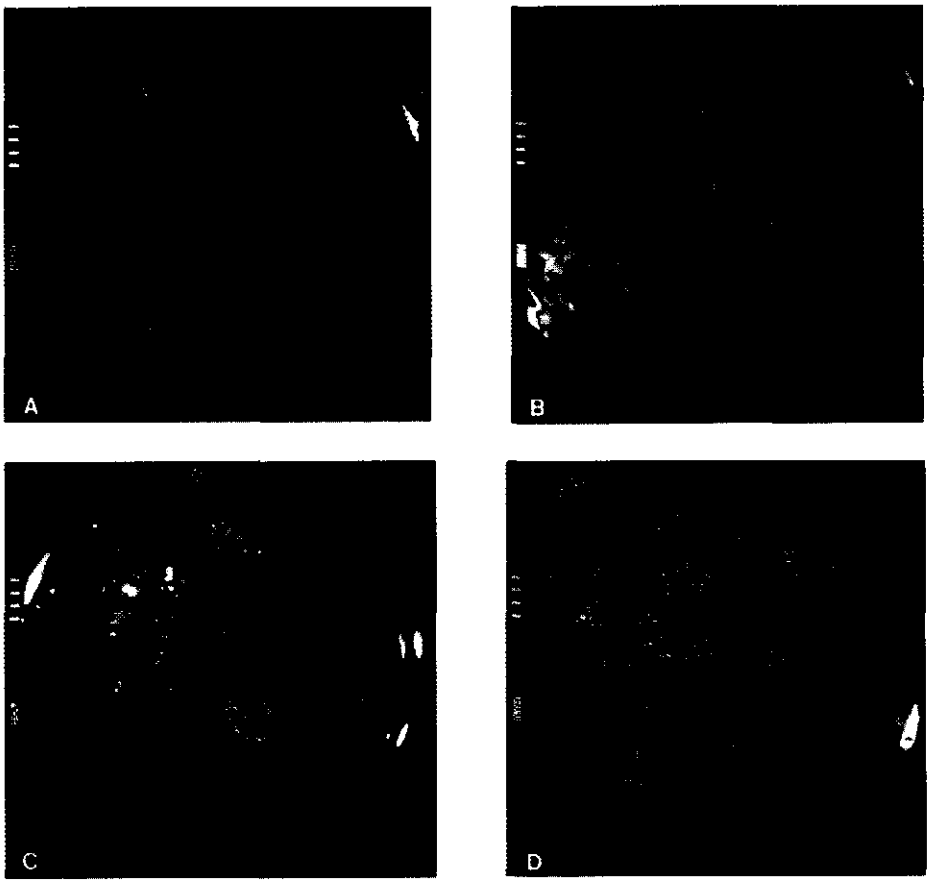

Figure 8. Luciferase activity of an *Alstroemeria* suspension culture (line 5.3.3), bombarded with plasmid pAHC18 (ubiLUC) and constantly visible selected.

A. 6 weeks after bombardment, B. 10 weeks after bombardment

C. 12 weeks after bombardment, D. 16 weeks after bombardment.

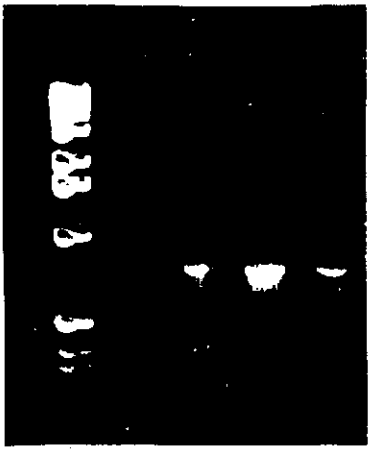

Figure 9. PCR analysis of transgenic *Alstroemeria* suspension culture

Lane 1 depicts 1 kb DNA ladder; lane 2 contains non-transgenic *Alstroemeria* suspension culture; lane 3 contains the products of the PCR made on the plasmid pAHC18; lane 4 contains the products of the PCR of DNA isolated from a transgenic cassava plant (positive control) (Raemakers *et al.*, 1997); lane 5 contains the PCR products from the transgenic *Alstroemeria* suspension culture (line 5.3.3).

Discussion

The plant pathogenic soil bacterium *Agrobacterium* has developed a sophisticated mechanism for stably integrating part of its extrachromosomal DNA (T-DNA) into the nuclear genome of recipient plants (Gelvin, 1990). To cover a wide host range the disarmed octopine strain LBA4404, the disarmed nopaline strain C58C1 and the disarmed agropine/ succinamopine strain EHA101 were used in preliminary experiments. Strain C58C1 was chosen to perform further research to optimise *Agrobacterium* transformation, because so far strain C58C1 had given the highest number of blue spots (data not shown).

Here experiments have been performed with the disarmed strain C58C1 and according to the GUS expression, transformation of young *Alstroemeria* callus is the best. We used 9

different *Alstroemeria* genotypes in our *Agrobacterium* transformation experiments. The number of transformation events varied considerably between the genotypes. So, clearly the transformation events is genotype dependent, however, almost all genotypes (8 of the 9) gave a low number of GUS spots (data not shown). As expected, the transformation events varied also considerably between different experiments. *Agrobacterium* transformation requires optimal conditions for both plant and pathogen.

Different types of wounding of *Alstroemeria* callus prior to *Agrobacterium* inoculation were studied. The best way of wounding seems to be pinching with a needle. Wounding after rolling in carborundum powder or particle gun bombardment was probably too severe for the calli. Also pinching of the calli should be done with caution, ten times per callus is sufficient. Bidney *et al.* (1992) showed however, that microprojectile bombardment of tobacco plant tissue increased transformation frequency by *Agrobacterium tumefaciens.* So, probably for each plant, the optimum wounding procedure, before inoculation, has to be developed. A three days cocultivation time together with an *Agrobacterium* inoculation concentration of 0.5 (OD550) were the best transformation conditions according to the factors studied. A longer cocultivation time or a higher *Agrobacterium* concentration (OD550 1.0 data not shown) gave severe problems with killing off the bacteria. Even a longer period in liquid medium with sufficient antibiotics did not kill off the bacteria of C58C1. Also the growth condition of the callus is affected by a longer period in liquid medium. The callus suffers enormously from the extreme conditions. The results in this report demonstrate for the first time that the monocotyledonous *Alstroemeria* is susceptible to *A. tumefaciens* transformation. The blue spots of β-glucuronidase activity show that the GUS gene is introduced, correctly spliced and transiently expressed. Stably transformed callus was not found after *Agrobacterium*-mediated transformation. The reason for the loss of GUS expression requires further investigation.

Recently an *A. tumefaciens* strain that carried a so-called 'super-binary' vector gave high transformation frequencies of various cultivars of japonica rice (Hiei *et al.* 1994). In the near future, this type of strain seems to be promising to perform successful transformation experiments with other monocots.

This paper describes also for the first time application of the biolistics system on *Alstroemeria* callus. *Alstroemeria* calli were used for the improvement of experimental

conditions of the biolistic device. The experiments described in this paper revealed that the best GUS expression of *Alstroemeria* callus was obtained under the following conditions. The target tissue position closest to the microcarrier launch assembly was the best position during particle bombardment. The best acceleration was given to the particles with a rupture disc-breakage point of 650 or 900 psi. The best promoter studied was the Ubi-1 promoter, this promoter was 8 times better than other tested promoters.

Several other experimental conditions for the biolistic delivery of DNA were studied such as the DNA concentration, culture time and culture age of the calli and osmotic strength of the medium. None of these experimental conditions enhanced the particle bombardment-mediated transient GUS expression of *Alstroemeria* callus (data not shown). However in the literature there was an optimal effect found for these factors in other crops (Christou, 1992; Seki *et al.*, 1991; Vain *et al.*, 1993). Also we tried to synchronise the growth of the cells of the *Alstroemeria* callus, with the idea that in a certain phase of the cell cycle the delivery of exogenous DNA might favour the integration of DNA into the chromosomes. For this study, the callus was placed on ice and after different time intervals of recovery at room-temperature the callus was used for particle bombardments. No positive influence of synchronised cells of *Alstroemeria* on transient expression was detected. However, the efficiency of transformation of synchronised tobacco protoplasts was twice the efficiency of unsynchronized protoplasts (Meyer *et al.*, 1985). The absence of the nuclear membrane in mitotic cells favours delivery of exogenous DNA, introduced into the cytoplasm, to chromosomal DNA. (Okada *et al.*, 1986).

In *Alstroemeria* callus the positive effect of the Ubi-1 promoter on transient GUS expression was also found in other experiments with the luciferase (LUC) gene as a genetic marker (data not shown). The transient expression of GUS and LUC was followed in time after bombardment with a plasmid, containing both marker genes. Both genes reacted similar in the bombarded calli, however the GUS enzyme was much more stable in time compared to the luciferase enzyme. GUS expression could still be found up to 48 days after bombardment, while the luciferase activity disappeared after 28 days (data not shown).

Poor stable expression was found in potentially chimeric transgenic plants after the first set of particle gun experiments followed by somatic embryogenesis. GUS expression was only

found in meristemic areas in these plants. These three plants could be regenerated after terminating the PPT selection in the regeneration medium. During micropropagation of these plants the GUS expression was lost, together with the performance of the BAR gene (followed by CR assay). This is an indication that these plants were chimeric. Therefore, these plants were cut and cultured on leaf regeneration medium according to Lin *et al.* (1997) with 14 mg l^{-1} PPT to try to develop stable transgenics. However no regenerants were obtained on this selective direct shoot regeneration medium.

In other particle gun-mediated experiments PPT-selection was continuously applied during somatic embryogenesis. After these experiments no transgenic plants were found. At that time of the research we were able to use the LUC gene in combination with the luminometer. The LUC assay is non-destructive and the LUC assay can be performed in time to monitor the development of the LUC activity. So, by experiments performed with plasmid pAHC18 (ubi-LUC), visible selection could take place. Only some LUC-activity was still found after 3 months in non-regenerable callus. On the other hand suspension cultures could be stably transformed with pAHC18. A clear positive LUC-activity and a PCR analysis indicated this. These results show that the combination of somatic embryogenesis of *Alstroemeria* and particle gun transformation is not efficient enough to produce stable transgenic plants. A total of 2500 calli were bombarded, from which 50% (1250) gave rise to embryogenic calli with an average of 3 somatic embryos. Only 40% of these somatic embryos became a good functional plant. So 1250 calli with 1-2 embryogenic places on this callus were bombarded. Stable transformation occurred on average in 2% of the calli. So in 25 calli the regeneration and transformation process had to take place simultaneously on the same spot, to produce a stable transgenic plant. The chance that this will happen is small, because these high numbers of explants are not easily obtained in the present culture systems of *Alstroemeria*. Therefore, a suspension culture with a rapid multiplication rate should be a better choice for bombardment, because many more dividing cells can be targeted by the particles. Stable gene expression can be selected and selectively multiplied and there after regeneration can take place. In cassava the same phenomenon was described by Raemakers and co-workers (Raemakers *et al.*, 1997). Transformation of cassava embryogenic cultures with particle bombardment resulted in transgenic chimeric embryos. These transformed sectors were lost after repeated cycles of secondary somatic embryogenesis. A less organised system of somatic embryogenesis is the

so-called friable embryogenic callus (FEC). In cassave this FEC regeneration system was combined successfully with particle bombardment. Unfortunately our *Alstroemeria* suspension culture was no longer embryogenic. Probably the selection of friable callus in the cyclic somatic embryogenesis and the selection of a fast growing cell suspensions had taken too long (more then 1 year).

Optimisation of *Agrobacterium*-mediated and particle gun-mediated transformation has occurred in the callus of *Alstroemeria*. Many particle gun-mediated transformation experiments were performed under optimal conditions. Finally, only chimeric transgenic plants were found. This paper shows that the combination of somatic embryogenesis and particle gun transformation is not a perfect combination. The efficiency of both processes is too low to provide a better chance of producing stable transgenics. A fast growing cell suspension culture is a much better regeneration system for particle gun-mediated transformation. In view of this, future research should focus on producing friable embryogenic cell suspensions or a *de novo* regeneration system originating from epidermal or subepidermal cells in order to combine these regeneration systems with particle gun-mediated transformation.

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Chapter 6

General discussion.

Regeneration

Genetic modification has the potential to overcome barriers in conventional breeding. That is the reason why many research groups are interested in developing genetic modification techniques. Transformation research in the Liliales has been performed only by a few research groups around the world. In contrast to transformation research for cereals, especially important food crops like maize and rice, where many research groups in the world are involved already for a long period. So much more manpower is invested in transformation of cereals, compared to the ornamentals in the order Liliales. This large input resulted in several transformation protocols for maize and rice, both with *Agrobacterium* and with particle bombardment (Christou, 1995; Hiei *et al.*, 1994). In spite of less manpower, recently transformation protocols have been developed for several crops, belonging to the order Liliales like *Lilium*, tulip, *Gladiolus* and *Asparagus*. These transformed crops involve many regeneration systems.

When our research was started only a few reports on regeneration of *Alstroemeria* were available. Therefore, a regeneration system was firstly developed (Van Schaik *et al.*, 1996). This somatic embryogenesis system started with immature zygotic embryos in the half ovule as explant. A maximum of 54% plant regeneration from the diploid species *A. inodora* P007 and of 41% plant regeneration from the tetraploid cultivar 118 was obtained, with an average of 3 somatic embryos per embryogenic callus. The developmental stage of the immature embryo and the callus induction period was of great importance in our somatic embryogenesis system (see Chapter 2). A disadvantage of this regeneration system was that, owing to its sexual origin the progenies segregate and therefore, it is not possible to introduce genes directly into the existing cultivars. Despite the fact that this regeneration system was more successful than the systems described so far, the regeneration system was dependent on the availability of flowers (giving a restriction to the number of calli that can be used per experiment), it was labour intensive (bi-weekly culturing, isolating the calli out of the ovules) and it needed a long period of culture (from inoculation till rooted plantlets ready for transfer to the greenhouse, was 28 weeks).

The system for embryogenesis was optimised. Optimisation of this system was not very easy, although a lot of efforts were made using different kinds of stimulants. Only a low

sucrose concentration (10 mg l^{-1}) in the regeneration medium gave an improvement to the development and germination of somatic embryos. The somatic embryogenesis system could be made cyclic, but this was only efficient up to the 5th cycle (Chapter 3). Initially, it was believed that somatic embryogenesis was a most suitable regeneration system for transformation, especially via indirect somatic embryogenesis. In the callus phase, it was hoped, that a proper selection of transgenic cells might be possible.

Regeneration systems of *Alstroemeria* have recently been described by others (Hutchinson *et al.*, 1994 and 1997; Lin *et al.*, 1997). The difference between our system and the one developed by Hutchinson and co-workers was, that they started with a mature zygotic embryo as explant and that they used different hormones (NAA and kinetin). The results of both systems are very much alike. Lin *et al.* (1997 and 1998) developed a direct organogenesis system for *Alstroemeria* in which shoot regeneration from excised leaf explants of in vitro grown seedlings was described. At present, however, there are only a few explants of *Alstroemeria*, which can be used for regeneration. Compared to other Liliales, *Alstroemeria* is an extremely recalcitrant crop, regarding the regeneration possibilities.

Transformation

We focused on our somatic embryogenesis system for transformation experiments. Therefore, the somatic embryogenesis system was investigated histologically (Chapter 4). The purpose was to determine, whether the cells capable of regeneration were accessible for transformation, especially by particle gun bombardment. The cells capable of regenerating showed transient GUS expression after particle bombardment, thus proving that in the same cell layers close to the callus surface, transformation would be possible. So, theoretically, the combination of somatic embryogenesis and particle gun bombardment seemed a good one for the development of a transformation protocol. Another prerequisite for transformation is a good selection system for transformed cells. The herbicide PPT proved to be a successful selection agent in *Alstroemeria* callus after several tests in which kanamycin was included. Also LUC detection gave the possibility to select visible.

We investigated the possibilities of *Agrobacterium*-mediated transformation of *Alstroemeria* with transient GUS expression to optimise the protocols. Transient expression of the GUS

(intron) gene was demonstrated after inoculation with disarmed strains of *A. tumefaciens*. This is already quite unique for a monocotyledonous plant. However, stably transformed *Alstroemeria* callus was not obtained. With the development of new *Agrobacterium* strains, especially for monocots, it might be worthwhile to keep trying for *Agrobacterium*-mediated transformation of *Alstroemeria*. *Agrobacterium*-mediated transformation seems to have certain advantages over particle gun-mediated transformation. In *Agrobacterium* transformed plants copy number is usually low and gene silencing rare. In particle bombardment 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).

However, microprojectile bombardment seems at the moment to be the only available transformation method in most monocotyledonous plants (Christou, 1995). Therefore, we focused on particle gun. The particle gun-mediated transformation for *Alstroemeria* was optimised. Analysis of transient GUS and luciferase expression showed that the explants needed to be close to the microcarrier launch assembly, optimum rupture disc-breakage point between 650-900 psi and that the best promoter tested was the ubiquitin promoter. The advantage of the LUC marker gene over the GUS gene was the non-destructive nature of the LUC assay and the fact that LUC activity can be tested repeatedly in time. Furthermore, in contrast to the LUC protein, the GUS protein is very stable making it difficult to assess the actual transformation efficiency. So the LUC protein gives a more reasonable estimate. We performed many transformation experiments and started in pilot experiments with bombarding meristems of *Alstroemeria* rhizomes. Only a low number of GUS spots or stripes were detected after one month on the meristems. The combined somatic embryogenesis system and the particle gun-mediated gene transfer did not result in stably transformed *Alstroemeria* plants, but in chimeric ones (Chapter 5). These plants lost their chimeric character by the sympodial growth of the rhizomes. But in the combination cell suspension and particle gun a solidly transformed cell suspension was obtained. The cell suspension was proven to be transgenic by PCR analysis and a strong LUC activity. Therefore, we have to conclude that the somatic embryogenesis was not efficient enough to combine with particle gun bombardment.

The combination of regeneration and transformation

In general, monocots are poorly susceptible to *Agrobacterium*. For monocots direct gene transfer by means of particle bombardment offers more perspectives. It is postulated that the advantages of direct gene transfer with the particle gun are more genotype-independency and the ability to engineer organised and potentially regenerable tissue (Christou, 1995). Particle gun-mediated transformation of meristems results mostly in chimeric plants, because of their multicellular origin (McCabe *et al.*, 1988; McCabe and Martinell, 1993). However, there are research groups trying to transform certain layers of a plant. For instance the group of Potrykus developed a special micro-targeting device to transform meristems and embryogenic structures (Sautter *et al.*, 1991). The phenomenon that transformation of organised somatic embryogenic cultures with particle bombardment resulted in chimeric transformed embryos or plants was described earlier by Raemakers and co-workers (Raemakers *et al.*, 1997). The transformed sectors of these chimeric cassava somatic embryos could not be enlarged by a new cycle of embryogenesis. In *Alstroemeria* we also found chimeric plants after transformation of our somatic embryogenesis system. An *Alstroemeria* cell suspension culture on the other hand could be stably transformed using the particle gun. The group of Raemakers was also able to transform a less organised system of somatic embryogenesis, the so-called friable embryogenic callus. This callus seemed to be more suitable tissue for transformation experiments, resulting in completely transformed cassava plants, which were stable with respect to expression of the introduced genes. So, the stated advantage of particle gun that it enables the transformation of organised regenerable tissue is not always true. It very much depends on where regeneration exactly takes place.

Sato and co-workers described in their article stable transformation via particle gun in two different soybean regeneration systems (Sato *et al.*, 1993). The first system was multiple shoot proliferation from shoot tips obtained from immature zygotic embryos, the second was somatic embryogenesis from a long term proliferative suspension culture. Bombardment of shoot tips by the biolistic method produced GUS-positive sectors in 30% of the regenerated shoots. However, none of the regenerants which developed into plants continued to produce GUS positive tissue. Bombardments of embryogenic suspensions readily produced non-chimeric, GUS-positive regenerated plants. Histological analysis indicated the entry of

particles into the first two cell layers of either shoot tips or somatic embryos of soybean. We investigated the GUS expression of bombarded *Alstroemeria* tissue and found GUS crystals as deep as 8 cell layers from the callus surface. On the other hand our histological observations also suggested that transient GUS expression was concentrated in cells at the callus surface of the target tissue after bombardment. GUS is a very stable protein and around 1-2% of the transient expression becomes stable expression in *Alstroemeria*. So it is possible that only these cells on the surface can become stably transformed. Soybean histological studies indicated that single epidermal cells were responsible for initiating secondary somatic embryos. Transformed single epidermal cells could initiate secondary somatic embryogenesis which resulted in either embryo proliferation or embryo conversion into non-chimeric transformed plants. Work with these two different regeneration systems and the biolistic gun has shown that the successful production of transformed plants is dependent on which cells regenerate and the appropriate targeting of DNA-coated particles in these cells. In the soybean investigation it was very clearly demonstrated that cells deeper than the first two cell layers appeared to be necessary for the development of regenerated plants by multiple shoot proliferation. Combined multiple shoot proliferation and biolistic bombardments resulted, therefore, in chimeric plants (Sato *et al.*, 1993). Tulip transformation resulted also in chimeric plants, because the regeneration system was organogenesis with more cell layers involved in regeneration of transgenic plants (Wilmink, 1996).

The knowledge on which regeneration system is to be used for particle bombardment is highly important. It is logical to see that in many monocot transformations the most used regeneration system for particle bombardments is embryogenic cell suspension culture or friable embryogenic callus (Klein *et al.*, 1989; Fromm *et al.*, 1990; Gordon-Kam *et al.*, 1990; Finer *et al.*, 1990; Hagio *et al.*, 1991; Somers *et al.*, 1992; Cao *et al.*, 1992). Also within the Liliales, transformation of *Asparagus* and *Gladiolus* was successful using cell suspensions as explants. *Lilium* transformation used bulb scales, but after bombardment a stably transgenic callus was formed first. This callus was used to regenerate stably transgenic plants. If somatic embryogenesis is used successfully, in combination with transformation, it is mostly a very unorganised system of embryogenesis or it originates directly from single epidermal cells. In several species single cell origin of somatic embryos from epidermal cells is common (Maataoui *et al.*, 1990; Maheswaran and Williams, 1985). In our *Alstroemeria* somatic

embryogenesis system, the origin of the regenerants was found to be from the surface of the callus, but probably not from single cell origin of the epidermis. The regeneration frequency was very low, also the transformation frequency after particle gun bombardment was very low. Probably these are the reasons why only chimeric plants were found after combined *Alstroemeria* somatic embryogenesis and particle gun bombardment transformations. The prospects for the use of embryogenic cell suspension cultures for *Alstroemeria* transformation are promising. In a cell suspension there are many active embryogenic cells, which are a good explant for transformation. The efficiency of regeneration of the cell suspension is of less importance, because a complete stable transformed cell line is the first step. Later the cell suspension only needs to regenerate one or two plants. Only limited research has been done by us on the development of *Alstroemeria* embryogenic cell suspension cultures. We mainly focused on the somatic embryogenesis system. The original hypothesis that regeneration of the surface of the callus was present, and that cyclic somatic embryogenesis could be performed to overcome chimeric problems proved not to be true. Furthermore, we wanted to have the callus phase as short as possible. However, it appears that the origin of regeneration best can be epidermal or subepidermal, because the delivery of particles after bombardment is not very deep in the tissue (of course depending on the particle gun parameters). In the future embryogenic cell suspension cultures or a very efficient *de novo* regeneration system from single epidermal cells need to be developed in *Alstroemeria*. One of these regeneration systems probably can be combined successfully with particle gun transformation. A disadvantage of developing cell suspensions is somaclonal variation. Already, after normal micropropagation of certain *Alstroemeria* cultivars, somaclonal variation appeared (Anastassopoulos and Keil, 1996). An other disadvantage is that particle gun-mediated transformation is no longer genotype-independent, because the development of cell suspensions is in general highly genotype-dependent.

In conclusion, the prospects to develop a transformation system of *Alstroemeria* are encouraging. My successor at the Department of Plant Breeding on transformation of *Alstroemeria*, H.S. Lin, succeeded already in developing LUC positive plants. He used younger cell suspension cultures for bombardment, which were still embryogenic. This opens up very good prospects that in the near future genetic modification of *Alstroemeria* can solve problems like virus diseases or premature leaf yellowing in *Alstroemeria* cut flowers.

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Summary

Alstroemeria or Inca Lily is an economically important cut flower in the Netherlands. The monocotyledonous ornamental *Alstroemeria* is mainly cultivated for the production of cut flowers, but there are also *Alstroemeria* pot plants and garden plants on the market. The increasing popularity for *Alstroemeria* can be attributed to its wide range of flower colours, long vase life and low energy requirement in the greenhouse. Since 1960 many efforts have been made to create elite cultivars by conventional breeding in the Netherlands. Nowadays a novel technique, called genetic modification opens the possibility to add or alter traits which cannot easily be achieved by conventional breeding. The aim of this thesis was to develop a regeneration and transformation procedure for genetic modification of *Alstroemeria*, which could be used routinely. The most important prerequisites for genetic modification were investigated during this thesis. The first prerequisite for complete stable transformation is an efficient regeneration system. When we started this research only a very inefficient plant regeneration system was described by Gonzalez-Benito. This plant regeneration system had a regeneration frequency of 4% and used mature zygotic embryos as explants. We developed a more efficient and embryogenic regeneration system with immature zygotic embryos as explants with a regeneration frequency of 40-50% (see Chapter 2). In Chapter 3 we tried to optimise our somatic embryogenesis system and turned it into a cyclic system, thus becoming less dependent on flowering plants.

For genetic modification a regeneration system not only needs to be embryogenic and efficient, but the cells capable of regeneration need to be accessible to transformation. Histological observations revealed that the cells capable of regeneration and the transient gene expression after particle bombardment were located in cells at the surface of the callus. So regeneration and transformation took place in the same cell layers of the *Alstroemeria* callus. Another prerequisite, namely selection for transformed cells was investigated in Chapter 4. Transformed cells need to be selected between non-transformed cells, to prevent overgrow by the non-transformed cells. The herbicide PPT was found to be a good selective agent in the somatic embryogenesis system.

The two main transformation techniques nowadays are transformation by the vector *Agrobacterium tumefaciens* or direct gene transfer by the particle gun. The plant pathogenic

soil bacterium *Agrobacterium* has developed a sophisticated mechanism for stably integrating part of its extrachromosomal DNA (T-DNA) into the nuclear genome of receptive plants. The particle gun is a direct gene transfer technique, small particles coated with DNA accelerate by helium pressure to penetrate the target tissue. Both transformation techniques were investigated in *Alstroemeria* (see Chapter 5). The marker genes ß-glucuronidase (GUS) and luciferase (LUC) were used. The GUS assay, which is a destructive test, shows expression in the target tissue by a blue product. Later during our research we could use the non destructive LUC assay. This LUC gene from the firefly is capable of emitting light which can be detected by a very sensitive camera. By using both marker genes we optimised the two different transformation protocols. We were unable to find stably transformed callus by the *Agrobacterium*-mediated transformations. The combined transformation of the somatic embryogenesis system with particle gun bombardment revealed only chimeric transformed plants. On the other hand, the particle gun-mediated transformation of *Alstroemeria* cell suspension cultures resulted in a complete stably transformed cell suspension culture. This cell suspension had a clear positive LUC activity and PCR positive result, indicating that the culture was indeed transformed. Unfortunately, despite many attempts, we were not able to regenerate plants from this transgenic culture. Most likely, one of the reasons for failing to do so was the long time period, which this culture had to be maintained *in vitro*.

We concluded from our transformation experiment, that it is very important to know where the origin of regeneration exactly takes place. In our *Alstroemeria* somatic embryogenesis system the origin of the regenerants was from the surface of the callus, but not from single epidermal cells. Probably this is the reason why only chimeric plants were found after combined *Alstroemeria* somatic embryogenesis and particle gun bombardment transformations. The prospects for the use of embryogenic cell suspension cultures for *Alstroemeria* transformation are good. So in the near future, *Alstroemeria* transformation research has to focus on the development of embryogenic cell suspensions or a *de novo* regeneration system from single epidermal or subepidermal cells. The advantage of a cell suspension is that in a cell suspension there are many active cells (good explants for transformation) and that first a stable transformed cell suspension can be formed. The regeneration efficiency of the cell suspension is then of less importance.

Samenvatting

Alstroemeria (ook wel Inca lelie genoemd) is economisch een belangrijk snijbloemgewas in Nederland. *Alstroemeria* is een monocotyl siergewas en wordt hoofdzakelijk geteeld voor de produktie van snijbloemen, maar er zijn ook *Alstroemeria* potplanten en tuinplanten op de markt. De toenemende populariteit van *Alstroemeria* kan toegeschreven worden aan de volgende feiten; het brede assortiment aan bloemkleuren, zijn lange vaasleven en zijn lage energiebehoefte in de kas. Sinds 1960 zijn vele inspanningen verricht om elite cultivars te veredelen in Nederland. Tegenwoordig is er voor de veredeling van gewassen, dus ook voor *Alstroemeria* een nieuwe techniek beschikbaar, de zogeheten genetische modificatie. Deze techniek maak het mogelijk om nieuwe eigenschappen toe te voegen of de bestaande te veranderen, daar waar de traditionele veredeling dat moeilijk kan bewerkstelligen. Het doel van dit proefschrift was om een routinematige procedure te ontwikkelen voor genetische modificatie van *Alstroemeria*. De meest belangrijke voorwaarden voor transformatie werden onderzocht gedurende dit onderzoek. Een eerste voorwaarde voor transformatie is een efficiënt en embryogeen regeneratie systeem van *Alstroemeria*. Toen aan dit onderzoek werd begonnen was er alleen door Gonzalez-Benito een zeer inefficiënt regeneratie systeem beschreven bij *Alstroemeria*. Dit regeneratie systeem had een regeneratie frequentie van 4% en maakte gebruikt van volwassen zygotische embryo's als uitgangsmateriaal. Wij ontwikkelden een veel efficiënter regeneratie systeem uitgaande van onvolwassen zygotische embryo's. In hoofdstuk 3 werd dit somatische embryogenese systeem geoptimaliseerd en werd dit systeem cyclisch gemaakt, waardoor de afhankelijkheid van bloemen minder werd en meer materiaal geproduceerd kon worden.

Het regeneratie systeem moet echter niet alleen efficiënt zijn, maar de cellen, welke kunnen regenereren, moeten ook toegankelijk zijn voor transformatie. Histologische waarnemingen lieten zien dat de oorsprong van de regeneratie en de transiënte genexpressie, na beschieting met goud kogeltjes, was gelokaliseerd in het callus oppervlak. Dus de regeneratie en transformatie vonden plaats in dezelfde cellagen van het *Alstroemeria* callus. Een andere voorwaarde voor transformatie is een goed selectie systeem. Selectie werd onderzocht en beschreven in hoofdstuk 4. Getransformeerde cellen moeten worden geselecteerd tussen niet-getransformeerde cellen, om overgroei van getransformeerde cellen te voorkomen.

Phosphinothricine, een herbicide, werkte goed als selectief agens in ons somatische embryogenese systeem.

Tegenwoordig zijn de volgende twee transformatie-technieken de meest gebruikten. Transformatie door de vector *Agrobacterium tumefaciens* of directe genoverdracht door het "deeltjeskanon" (particle gun). De plant pathogene bodembacterie *Agrobacterium* heeft een zeer verfijnd mechanisme ontwikkeld voor stabiele integratie van een deel van zijn extrachromosomale DNA (het zogeheten T-DNA) in de kern van cellen van vatbare planten. Het deeltjeskanon representeert een directe vorm van genoverdracht. Kleine (wolfraam of goud) kogeltjes met een laagje DNA worden versneld door heliumdruk om het beschoten weefsel binnen te kunnen dringen. Beide transformatie-technieken werden toegepast en onderzocht bij *Alstroemeria* (hoofdstuk 5). De marker genen ß-glucuronidase (GUS) en luciferase (LUC) werden hierbij gebruikt. De GUS-toets is een destructieve test en toont de GUS-expressie na een biochemische reactie in het beschoten weefsel aan door een blauwe kleur. Later in het onderzoek kon gebruik worden gemaakt van de niet-destructieve LUC-test. Het LUC gen, afkomstig, van het vuurvliegje, is in staat om licht uit te stralen wat gedetecteerd kan worden door een zeer gevoelige camera. Door gebruik te maken van beide marker genen werden de twee transformatie protocollen bij *Alstroemeria* geoptimaliseerd. Geen stabiele transgene calli werden gevonden na transformatie met *Agrobacterium*. De combinatie somatische embryogenese met het deeltjeskanon resulteerde alleen in chimère transgene planten. Echter de combinatie celsuspensie en het deeltjeskanon resulteerde in een compleet stabiele transgene celsuspensie. Deze celsuspensie bezat een duidelijke LUC-aktiviteit en gaf een positieve PCR analyse te zien.

Geconcludeerd werd dat het van zeer groot belang is om te weten waar de oorsprong van de regeneratie precies in het weefsel is gelegen. Bij somatische embryogenese van *Alstroemeria* was de oorsprong van de regeneratie gelegen in de buitenste cellagen van het callus, maar was niet afkomstig van één enkele epidermiscel. Waarschijnlijk is dat de reden waarom alleen chimère planten werden gevonden na het beschieten van het somatische embryogenese systeem. De vooruitzichten in de nabije toekomst om tot volledige genetische modificatie te komen zijn zeer goed. Embryogene celsuspensies zijn waarschijnlijk een geschikt regeneratie systeem voor de produktie van stabiele niet-chimere *Alstroemeria* planten. Het voordeel van een celsuspensie is dat er eerst een getransformeerde celsuspensie geselecteerd kan worden,

waaruit een stabiele transgeen geregenereerd kan worden. De efficiëntie van regeneratie kan dan zelfs zeer laag zijn als er uiteindelijk maar een plant wordt gevormd.

Zo is het te verwachten dat in de nabije toekomst genetische modificatie van *Alstroemeria* kan worden uitgevoerd om problemen zoals virusvatbaarheid en bladvergeling aan te pakken en mogelijk op te lossen.

Curriculum vitae

Carla Ellen van Schaik werd geboren op 23 november 1961 te Amsterdam. In 1980 behaalde zij haar diploma Gymnasium-ß aan het Hermann Wesselink College te Amstelveen, waarna zij in hetzelfde jaar begon met de studie biologie aan de Vrije Universiteit te Amsterdam. Haar doctoraalvakken waren fytopathologie, genetica, plantenfysiologie en microbiologie. In 1988 verkreeg zij de titel doctorandus cum laude en tegelijkertijd haar onderwijsbevoegdheid voor biologie.

Vanaf juli 1988 was zij in dienst bij het veredelingsbedrijf Van Staaveren. Zij is aldaar begonnen als research medewerkster. Van 1991 tot 1996 was zij full-time, dan wel part-time betrokken bij het research project "Regeneratie en transformatie van *Alstroemeria*". De resultaten van dit onderzoek staan beschreven in dit proefschrift. Vanaf januari 1998 is zij werkzaam bij Van Zanten Research b.v.