Development of efficient regeneration and transformation systems in *Alstroemeria*

Jong-Bo Kim

Promotoren:
Prof. dr. Richard G.F. Visser, Hoogleraar in de Plantenveredeling,

Prof.dr.ir. Evert Jacobsen, Hoogleraar in de Plantenveredeling,

Co-promotor:
Dr. ir. Krit. J.J.M. Raemakers, Onderzoeker, Laboratorium voor plantenveredeling

Promotie commissie:

Prof. Dr. Linus H. W. van der Plas, Wageningen Universiteit

Dr. Geert J.M. de Klerk, Plant Research International, Wageningen

Dr. Frans A. Krens, Plant Research International, Wageningen

Dr. Ir. Piet C.G. van der Linde, SBW International B.V, Roelofarendsveen

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Jong-Bo Kim

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To my wife, Eun-Hee,
My son Tae-Hyeon,
My daughter Su-Yeon

Contents

Abbreviations

AFLP – **A**mplified **F**ragment **L**ength **P**olymorphism
AlMV – **Al**stroemeria **M**osaic **V**irus
2,4-D – 2,4-**d**ichlorophenoxyacetic acid
BAP – 6-**b**enzyl**a**mino**p**urine
CaMV – **Ca**uliflower **M**osaic **V**irus
CEC – **C**ompact **E**mbryogenic **C**allus
CIM – **C**allus **I**nduction **M**edium
CP – **C**oat **P**rotein
CPMR – **C**oat **P**rotein **M**ediated **R**esistance
ELISA – **E**nzyme **L**inked **I**mmuno **S**olvent **A**ssay
FEC – **F**riable **E**mbryogenic **C**allus
GA –**G**ibberellic **A**cids
GUS - β-**G**l**u**curonida**s**e
HPT – **H**ygromycin **P**hospho**t**ransferase
IBA – **I**ndole **B**utyric **A**cids
LB – **L**ucia **B**roth
LS - **L**insmaier and **S**koog
LUC - **L**uciferase
MS – **M**urashige & **S**koog
NAA – **n**aphthalene **a**cetic **a**cids
NPTII – **N**eomycin **P**hospho**t**ransferase II gene
NTR – **N**on-**T**ranslated **R**egion
OD – **O**ptical **D**ensity
PAT – **P**hosphinotricin-N-**a**cetyl**t**ransferase
PCR – **P**olymerase **C**hain **R**eaction
PDR – **P**athogen **D**erived **R**esistance
PPT - **P**hos**p**hinot**t**ricine
PTGS – **P**ostt**t**ranscriptional **g**ene **s**ilencing
RM – **R**ooting **M**edium
RT-PCR – **R**everse **T**ranscriptase – **P**olymerase **C**hain **R**eaction
SH – **S**chenk and **H**ildebrandt
SIM – **S**hoot **I**nduction **M**edium
SV – **S**omaclonal **V**ariation
TDZ – **T**hi**d**ia**z**urone (N-phenyl-N'-1,2,3-thidiazol-5-yl urea)
Ubi – **Ubi**quitine
uidA - β-glucuronidase gene
X-gluc – 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

CHAPTER 1. INTRODUCTION

I. Introduction

Alstroemeria and its position in the cut flower market

During the last two decades, *Alstroemeria* has been one of the most commercially successful ornamental cut flowers in Japan, the Netherlands, the U.K., and the USA. Especially, characteristics like long vase-life, large color variety and a low energy required during cultivation have stimulated this success. The production of *Alstroemeria* flowers has been rapidly increasing in Europe and other parts of the world (Spence et al., 2000). Up to now, a huge number of cultivars have been released on the commercial market mainly as cut flower, however, *Alstroemeria* plants are also known as pot and garden plants on a small scale (Van Schaik, 1998). An overview of cultivation area, production volume, auction turnover, and the price per stem since the past decades in the Netherlands is shown in Table 1. There was an increase of almost 50% in cultivation area, production volume and auction turnover in the year 2001 compared to 1990. However, the price per stem showed a slight decrease. According to Table 1, the cultivation area had already decreased significantly from 2000 to 2003.

In the year 2003, *Alstroemeria* cut flowers ranked in the 9th position of the annual turnover (Table 2) and in the 6th position of the sales volume at the Aalsmeer flower auction, in the Netherlands (http://www.vba.nl/). During the 1990s, there has been a slight change in the top 10 of famous cut flowers at the auction in Aalsmeer, The Netherlands. The consumers purchased more cymbidium, gerbera, lily, and rose, whereas they lost more and more their interests for carnation and chrysanthemum. The other cut flowers have remained stable.

Discovery, geographical feature, and growth habit of *Alstroemeria*

In 1714, Feuillee discovered *Alstroemeria* in Chile, and he registered it under the genus *Hemerocallus*. The name *Alstroemeria* was given by Linnaeus in 1762 (Aker and Healy, 1990). Linnaeus combined the information of Feuillee and Alstroemer, and named the genus *Alstroemeria* and described three species (Buitendijk, 1998). In 1837, Herbert reported 29 species, while Kunth described 40 species in 1850 (Uphof, 1952). Later, Baker reported a total of 44 species, which he divided into two groups, the Chilean species and the Brazilian species based on geographical

distribution, with 24 and 20 species, respectively (Buidentijk, 1998). Generally, the Chilean and Brazilian species can be discriminated by evaluating the morphological differences such as leaf shape, color and shapes of flower, the fragrance, and their year-round production (De Jeu and Jacobsen, 1995a).

Alstroemeria species predominantly have their natural habit in South-America, mainly in Chile and Brazil, as mentioned above, but also in Argentina, Bolivia, Paraguay, Peru, and Venezuela, species are found (Ravenna, 1988). The center of distribution appears to be in central Chile (Bayer, 1987). Some species such as *A. pelegrina*, *A. ligtu* and *A. aurea* are widely distributed, whereas others, like *A. patagonica*, are found in more restricted areas (Aker and Healy, 1990). In general, soil temperature seems to be a crucial factor for the growth of *Alstroemeria* species. In many *Alstroemeria* species flowering is highly dependent on a period of cool soil temperature (Healy and Wilkins 1982; 1986). Cool temperatures are also important for seed germination of many species of *Alstroemeria* (Hannibal, 1942), especially the ones that grow high up in the mountains or in coastal areas. *A. campaniflora* is adapted to tropical marshy areas, whereas, *A. parvula* is found in an alpine area. Surprisingly, *A. polyphilla* and *A. graminea* are found in the desert (Aker and Healy, 1990).

Table 1. Production areas, supply volume, auction turnover and price per stem of *Alstroemeria* in the Netherlands (Centraal Bureau Voor de Statistiek)

	1990	1995	2000	2003
Cultivation area (ha)	83	118	119	98
Auction supply (no. stems million)	185	230	277	125
Auction turnover (Million Euro)	30.4	35.8	44.6	40.0
Price per stem (Euro cents)	0.37	0.35	0.33	0.30

Table 2. Top 10 cut flowers in turnover in Aalsmeer flower auction in 2001 and 2003 (Bloemenbureau Holland)

RANKING	2001		2003	
	Flower	Turnover*	Flower	Turnover*
1	Rose	653.0	Rose	681.3
2	Chrysanthemum spray	289.1	Chrysanthemum spray	299.1
3	Tulip	177.3	Tulip	185.9
4	Lily	155.9	Lily	160.0
5	Gerbera	103.8	Gerbera	105.9
6	Cymbidium	66.6	Cymbidium	65.7
7	Freesia	61.7	Freesia	60.2
8	Carnation	56.2	Anthurium	42.6
9	Alstroemeria	44.6	Alstroemeria	40.0
10	Gypsophilia	42.0	Chrysanthemum	37.7

*: Euro × 1,000,000

Botanical features of the *Alstroemeria* species

Alstroemeria plants are multiplied by splitting of fleshy rhizomes. The roots vary from thick and tuberous to thin and fibrous and produce thickened cylindrical storage roots that mainly contain starch and are edible (Bridgen et al., 1989). The *Alstroemeria* species have vegetative and generative shoots, which are initiated on the subterranean rhizomes that branch sympodically. The rhizome apex is the axillary bud of the first scale of the previous shoot. Then, each successive aerial shoot growing from the rhizome is a shoot grown from an axillary bud of the

preceding aerial shoot. The axillary bud, which is also subterranean located, is the second scale leaf of the aerial shoot, and has the potential to produce growth as another rhizome. The other leaves do not have the special meristem for growing rhizome (Garriga, 1994).

In the flower structure, the perianth consists of two whorls of three petals. The petal of the outer whorl has a different size and shape compared to the petal of the inner whorl. In some genotypes, the nectaries produce abundant drops of nectar. Moreover, spots and streaks are associated with the signaling of pollen vectors (De Jeu et al., 1992). *Alstroemeria* is predominantly an insect-pollinated crop (Dahlgren & Clifford, 1982). The ovary is pseduo-epigyn (Buxbaum, 1951), with three carpels forming a tripartite ovary in which an axial placenta is present. In each cavity of the ovary, two rows of ovules are located next to each other along the central placenta. The total number of ovules varies from 24 to 36 depending on the genotype (De Jeu et al., 1992).

The *Alstroemeria* has a protandrous flowering, which means that the anthers dehisce before the stigma is receptive. Therefore, self-pollination within a flower is difficult. In total, six anthers are situated in two whorls. Two days after anthesis, the first anther dehisces; at that moment, the style is still short and undeveloped. Four days after anther dehiscence, the anthers become dried and the filaments curl towards the lowest petal, at a distance of the developing style. Two days after all six anthers have wilted, the stigma becomes receptive, producing droplets of exudate on the papillae. This is the exact moment for pollination of the stigma. The pollen grains are only able to germinate a wet stigma. The pollen tubes grow between the papillae and within 24 hours grow through the cavity into the ovary (Chevalier, 1994). In general, after compatible fertilization, it takes about two months before the round seeds are scattered with force out of the ripe fruits.

Taxonomy and chromosome studies of *Alstroemeria*

Alstroemeria is a member of the monocotyledonous family *Alstroemeriaceae*, order Liliales, superorder Liliflorae, division Monocotyledonae (Dahlgren et al., 1985). *Alstroemeria* first belonged to the Liliaceae, and later it was included in the Amaryllidaceae (Herbert, 1837). In 1959, Hutchinson proposed to separate the genus Alstroemeria from the Amaryllidaceae into a new family of Alstroemeriaceae, comprising four genera *Alstroemeri*a, *Bomarea*, *Schickendantzia*

and *Leontochir* (Hutchinson, 1957). The classification of the genus *Alstroemeria* as described in Dahlgren et al., (1985) is generally accepted, although some *Alstroemeria* species are still considered as members of the *Amaryllidaceae*.

The chromosome number and genome composition of Alstroemeriaceae is well documented by Buitendijk (1998). The species of *Alstroemeria*, *Bomarea* and *Leontochir* are mainly diploid with a basic chromosome number of n=8 for *Alstroemeria* species and n=9 for *Bomarea* (Whyte, 1929). However, the commercial cultivars are not only diploid, but also triploid (2n=3X=24), tetraploid (2n=4X=32), and even aneuploid (Hang and Tsuchyia, 1988; Tsuchyia et al., 1987). Interestingly, the most attractive cultivars are triploid and tetraploid with big-sized flowers and a variety of colors.

Breeding history of *Alstroemeria*

Breeding programs of *Alstroemeria* have been focused on the production of cut flowers. In the early 1950s, three *Alstroemeria* species were released into Europe – *A. pelegrina*, *A. ligtu* and *A. aurea*. Since then, the interest in *Alstroemeria* as an ornamental has increased. The commercial quality of this first *Alstroemeria* was poor due to the short flowering period, bad quality of stem and leaf. Nevertheless, these first *Alstroemerias* were most probably the ancestors of the modern hybrids that were often produced after crossing with wild species.

Currently, the *Alstroemeria* cultivars can be divided into three types. One of these - the "Orchid type"- has open flowers with a long flowering period (Garriga, 1994). "Orchid type" plants are diploid (2n=2x=16) and almost sterile, whereas they are easily propagated *in vitro* (Garriga, 1994; De Jeu et al., 1992). Crossing Chilean with Brazilian species has created the "butterfly type" of plants. The "butterfly" type is allotetraploid (2n=4X) and produces viable 2X gametes (De Jeu et al., 1992). The "hybrids type" was created by several crossings between various species and cultivars.

Mutation techniques have been used for *Alstroemeria* breeding since 1970 to increase variation in flower color, stripes of the inner petal, flower size and height of plants. After irradiation of actively grown rhizomes with X-rays, a variety of mutants were obtained. Some of these mutants were selected and vegetatively propagated and then developed into a new cultivar (Broertjes & Verboom, 1974).

Up till now, more than 60 species/genotypes have been released onto commercial markets by applying conventional breeding techniques. One problem found in conventional breeding is the lack of useful genes in *Alstroemeria* germplasm for use in further breeding. The majority of the *Alstroemeria* cultivars are polyploid, which makes breeding time consuming (Chevalier, 1994). However, new cultivars have been produced by using interspecific hybridization in the last decades (De Jeu and Jacobsen, 1995b). Furthermore, cross-hybridization does not always lead to seed set, although some hybrids were produced by using embryo rescue techniques (Buitendijk, 1992). The slow process of breeding delays the introduction of new cultivars to the commercial market.

Virus diseases in *Alstroemeria*

Once the new cultivars are developed, these plants should be propagated without loss of quality. However, numerous factors have a negative influence on the quality of *Alstroemeria*. Virus diseases are the most important problem in maintaining a high quality in the plant material. It has become apparent that many serious virus diseases in the world are the direct or indirect result of human activities (Thresh, 1982). These activities are the use of monoculture in vast areas, the introduction of virus vectors into new areas, the introduction of new viruses into new areas through travel or transportation, and repeated use of the same field for the same crop (Hull, 2002).

Viruses have caused severe problems in *Alstroemeria* plants propagated by rhizome splitting. According to Van Zaayen (1995), different viruses are reported in several European countries such as England (Brunt and Phillips, 1981), Italy (Bellardi and Bertaccini, 1991) and the Netherlands (Hakkaart and Versluijs, 1985). The “butterfly-type” is generally infected with the most problematic virus in the *Alstroemeria* species, Alstroemeria Mosaic virus (AlMV). Figure 1A shows particles of AlMV in the infected *Alstroemeria* plants.

In addition, the Alstroemeria Carla virus (AlCV) and cucumber mosaic virus (CMV) have been found in the “Aurea-type” *Alstroemeria*. These two viruses have also been observed in other *Alstroemeria* groups. Recently, *Alstroemeria* plants became infected with the Tomato spotted wilt virus (TSWV), and the Impatiens necrotic spot tospovirus (INSV). However, until now, they are not very common in *Alstroemeria* cultivation. AlMV is the most common virus in *Alstroemeria* species

and belongs to the potyvirus group. Plants infected with AlMV have symptoms such as streaking on the leaves, light green and dark spots (Figure 1B) and flower-break (Chiari and Bridgen, 2002). When *Alstroemeria* plants become infected with AlMV, there is substantial variation in symptoms dependent on the cultivar, growing conditions and the time of year (Van Zaayen, 1995). This wide range of variation means that more than one potyvirus can exist and infect *Alstroemeria* (Hakkaart and Versluijs, 1985). Recently, a new potyvirus was discovered and named the Alstroemeria streak virus (AlSV) (Wong et al., 1992). However, Van der Vlugt and Bouwen. (2002) have concluded that AlMV and AlSV are strains of the same virus. Until now, unfortunately, there has been little research done on the development of AlMV-resistant lines by using either conventional breeding or genetic modification techniques in *Alstroemeria*.

Protection strategies against virus diseases

In general, strategies for the control of virus diseases in most crops have been focused on methods designed to avoid the virus infection (Fraser, 1989), breeding of resistant lines, control of vectors, or production of virus-free stocks through tissue culture (Hull, 2002). More interest is being given to a combination of these strategies. However, even this combined strategy has also proven unsuccessful in preventing virus infection or spread in crops. The first virus-free *Alstroemeria* cultivars were obtained mainly by meristem culture (Hakkaart and Versluijs, 1985). Unfortunately, the protocol described by Hakkaart and Versluijs (1985) takes four months to make virus-free stocks and contained little information on factors such as the optimal size of meristem tissues or the best method to confirm the eradication of virus. Recently, Chiari and Bridgen (2002) improved the meristem culture protocol and reported the production of virus-free *Alstroemeria* plants against AlMV. In spite of this effort, however, the meristem culture-derived plant can also be a target for AlMV and therefore become infected in the greenhouse during the culture period as well as on the commercial market due to contact with AlMV-infected sources. A long-term solution to the problems caused by AlMV could be the production of *Alstroemeria* transgenic plants that are genetically resistant or immune to the virus.

Transgenic approaches for the development of virus resistance

With the advent of gene transfer techniques and molecular identification of the virus genome structure, a number of virus-resistant crops have been produced and are in the process of being commercialized (Chowrira et al., 1998). This resistance based on virus-derived transgenes has been known to be effective against various plant viruses (Grumet, 1995). However, despite its success in many crops, there are no reports on the production of transgenic virus-resistant *Alstroemeria* plants.

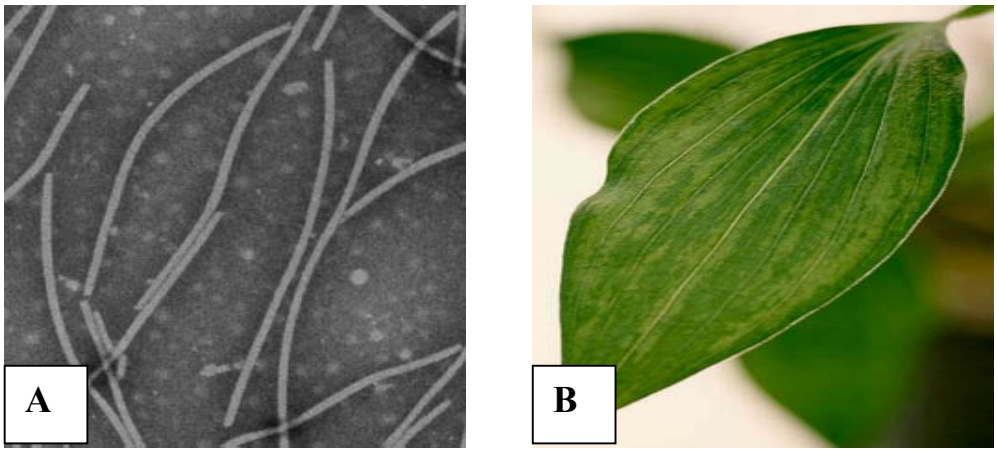

Figure 1. *Alstroemeria* infected with mosaic virus A) AlMV particles as seen in the TEM (magnification: 31,000×) (B) symptoms on leaves (Kindly provided by Ir. Inge Bouwen, Plant Research International, Wageningen UR, The Netherlands)

To obtain virus-resistant plants through genetic modification, there are three major sources of transgenes for protecting plants against viruses. The first source is "natural resistance genes", which after identification can be isolated and transferred to plant species using genetic modification. For instance, the *Rx1* gene, which confers strong resistance to PVX, has been isolated from potato and transferred to *Nicotiana benthamiana* and *N. tobacum* (Bendahmane et al., 1999). In the same way, the *N* gene, which gives resistance to TMV, found in *N. glutinosa*, was transferred to tomato (Whitham et al., 1996). In rice, the *N* gene transformed with

the particle bombardment showed hypersensitive resistance to *rice hoja blanca virus* as well (Lentini et al., 2003).

The second source is genes derived from viral sequences, also referred to as pathogen-derived resistance (PDR). PDR had developed from the phenomenon of cross-protection, which refers to the resistance of plants to virus infection if plants have a viral transgene (Sijen, 1997). It was expected that expression of the pathogen-derived gene could either prevent or inhibit the virus infection and movement process. In PDR, there are two main molecular mechanisms for its operation. One is protein-based and the other is nucleic acid-based protection (Hull, 2002). In protein-based protection, coat protein-mediated resistance is the most widely used because the nucleic sequence of many viruses has been identified and cloned. Transforming plants with viral sequences that encode the coat protein of the virus achieve it. When this protein accumulates in uninfected plants, it results in resistance by uncoating the virus particle before translation and replication (Chahal and Gosal, 2002). Coat protein-mediated resistance was first described in tobacco for TMV (Power-Abel et al., 1986). Subsequently, coat protein-mediated resistance by genetic modification has been demonstrated successfully in citrus (Febres et al, 2003), papaya (Lines et al, 2002), potato (Racman et al., 2001), pea (Chowrira et al., 1998), soybean (Wang et al., 2001), squash (Pang et al., 2000) and wheat (Sivamani et al., 2002).

Apart from the coat protein-mediated resistance, virus movement proteins can confer partial resistance (Malyshenko et al., 1993) or protection to other viruses with a similar genome organization (Beck et al., 1994). However, virus movement problems can have a detrimental effect for plant development as was reported by Hou et al. (2000). Another approach based on the protein level is the use of viral replicase proteins. Conclusions from several reports suggest that interaction between replicase proteins and other viral-encoded proteins may affect the process of the replication and cell-to-cell movement, leading to the arrest of the replication procedure (Hull, 2002).

RNA-mediated resistance, antisense-mediated, satellite RNA-mediated resistance and ribozymes-mediated resistance are examples of nucleic acid based protection. RNA-mediated, antisense-mediated and satellite RNA-mediated resistance have been widely applied and show successful resistance in several crops. In RNA-mediated resistance, the introduced viral sequences do not produce a protein, thereby the protection is due to the RNA. Unlike coat protein-mediated

resistance, the following four features have been reported in this strategy. Pang et al. (1993) found no correlation between the level of resistance and the expression level of the transgene. Secondly, RNA-mediated resistance is not dose-dependent and shows resistance at a high level of inoculum (Hull, 2002). Thirdly, the resistance is narrow based and only against viruses, which have a similar virus genome sequence as that of the inserted transgenes (Hull, 2002). Finally, transformed viral sequences may be methylated or truncated (Kohli et al., 1999). The molecular mechanism behind RNA-mediated resistance associated with the low steady states of transgene RNA and homology-dependent or post-transcriptional gene silencing might explain the narrow range of resistance. For instance, when the resistance was obtained by the transcript, and not by the protein, or if transgenic plants with a low level of viral transgene expression showed more resistance than did those plants with a high level of transgene expression, it can be assumed that the resistance generated in these cases might be due to homology-dependent gene silencing (Hull, 2002).

The antisense-mediated resistance is based on a strategy first developed to control fruit ripening (Smith et al., 1990) and virus resistance (Elmer and Rogers, 1990) in tomato. For this, the cDNAs representing viral RNA genomes were cloned in an antisense orientation behind an appropriate plant promoter and transferred to plants. Antisense RNA can control gene expression. RNA production of the coat protein will therefore be inhibited by this antisense sequence, and will arrest the production of new virus particles in plant cells.

Finally, several RNA viruses have small RNA molecules called satellite RNAs, which affect the severity of infection by a virus. These satellite RNAs are entirely dependent on their helper virus for the replication and encapsidation (Kuwata et al., 1991; Simon, 1988). Generally, the presence of a satellite RNA can control the severity of infection caused by its helper virus (Tien and Wu, 1991), thereby reducing damage, although severe and different levels of damage can be induced in some cases. Using this strategy, Kim et al. (1997) observed that severity of infection was attenuated in the offspring of hot pepper.

The final source of transgenes for protecting plants against viruses is genes from various sources that inhibit or interfere with the target virus. These include pathogen-related proteins, virus-specific antibodies, ribosome-inactivating proteins, antisense to β-1,3-glucanase. However, some of these sources showed no resistance or only limited application in a small number of crops. Table 3 outlines

target genes associated with different strategies. Of all these strategies, coat protein-mediated resistance has been widely applied and is one of the most successful strategies for producing virus-resistant plants (Wilmink, 1996).

In this thesis, RNA-mediated resistance was chosen to obtain *Alstroemeria* transgenic plants, which are resistant to ALMV and not a strategy based on coat protein-mediated resistance, because it is then difficult to distinguish between resistance and expression of potyvirus.

Somatic embryogenesis in *Alstroemeria* and other monocotyledonous species

The availability of efficient regeneration systems is essential for the application of genetic modification. As compared to dicotyledonous ornamentals, monocotyledonous ornamentals seem to be rather recalcitrant. *Alstroemeria* is not an exception. In the past decades, multiplication of *Alstroemeria* via tissue culture was carried out mainly by rhizome splitting (Lin, 1998). Once rhizomes have been produced from shoot clusters (Figure 2A), plants will form healthy roots and will be established in the greenhouse within 3-4 months (Figure 2B). Breeding companies and farmers use rhizome splitting in commercial propagation. Some *Alstroemeria* species, especially the "Butterfly type" have shown significantly low propagation efficiency (Buitendijk, 1992). Therefore, a "Butterfly type" was used in this study. Another important *in vitro* technique is the embryo rescue system. It was developed to solve crossing-barriers and produced hybrids in *Alstroemeria* (De Jeu, 1992). A large number of new cultivars have been developed through embryo rescue. However, neither embryo rescue techniques nor rhizome division is suitable for genetic modification due to the low efficiency and the non-adventitious character of the regeneration system. Adventitious regeneration is a precondition for genetic engineering.

Regeneration procedures have to be developed to be able to produce genetically modified plants. The regeneration system might also be used for plant propagation. Adventitious regeneration was first reported by Ziv et al. (1973). They obtained plants from apical inflorescences via direct plant regeneration. Since then, a large number of publications have appeared.

In the late 1980s, Bridgen et al. (1989) reported on somatic embryogenesis and Gonzales-Benito and Alderson (1990, 1992) presented plant regeneration via callus tissues, which were induced from mature zygotic embryos. However, the

regeneration efficiency of the system described was too low to be used for genetic transformation. A few years later, Hutchinson et al. (1994) described callus induction and plant regeneration from mature zygotic embryos with a 40% rate of regeneration frequency. In addition, they reported another regeneration system from callus using liquid culture (Hutchinson et al., 1997). Furthermore, Van Schaik et al, (1996) obtained plants from callus that was induced on immature zygotic embryos with 41-54% regeneration rates, depending on the used cultivars. More recently, plants were regenerated from seedling-derived (Lin et al., 2000a) and ovary-derived FEC (Akutsu et al., 2002) via somatic embryogenesis. In both cases FEC was derived from generative tissue and does lead to loss of the original genotype, since *Alstroemeria* is a vegetative propagated crop. Sage et al. (2000) also supported the view that it is necessary to develop an efficient embryogenic culture system from vegetative tissues in order to propagate elite new *Narcissus* genotypes. Thus, the two systems described by Lin et al. (2000a) and Akutsu et al. (2002) would be difficult to immediately combine with a genetic transformation protocol system in *Alstroemeria*.

Table 3. Summary of the various strategies used to obtain virus resistance in plants

Resistance type	Target gene	Reference
Natural	*N* gene	Whitham et al., 1996; Lentini et al., 2003 Fedorowicz et al., 2005
	*Rx*1gene	Bendahmane et al., 1999
PDR (Pathogen-derived resistance)	Coat protein	Powell-Abel et al., 1986; Sivamani et al., 2002; Tripathi et al., 2004; Kamo et al., 2005;
	Viral movement protein	Cooper et al., 1996
	Viral replicase	Golemboski et al., 1990; Praveen et al., 2005
	RNA-mediated	Reviewed by Prins and Goldbach, 1996 Chen et al., 2004; Higgins et al., 2004
	Antisense RNA	Reviewed by Tabler et al., 1998
	Ribozymes	Reviewed by Tabler et al., 1998
	Satellite-mediated	Harrison et al., 1987
	DI nucleic acid-mediated	Kollar et al., 1993
Other sources	PR protein	Hooft van Huijsduijnen et al., 1986
	β-1,3'glucanase	Beffa et al., 1996
	Virus specific antibody	Hiatt et al., 1989
	Ribosome-inactivating proteins	Reviewed by Wang and Tumer, 2000
	Ribonuclease gene pac-1	Watanabe et al., 1995
	2',5'-oligoadenylate synthase	Truve et al., 1993

On the other hand, in the monocot species, an efficient regeneration system through somatic embryogenesis has been reported by using compact embryogenic callus (CEC – type I callus in maize) in *Asparagus* (Limanton-Grevet and Julllien, 2000), *Lily* (Godo et al., 1998), and *Oil palm* (Schwendiman et al., 1988). CEC callus is comparable with type I callus in maize. It was found in *Anthurium* (Kuehnle et al., 1992), *Gladiolus* (Stefaniak, 1994), and *Agapanthus* (Suzuki et al., 2002) by using friable embryogenic callus (FEC – type II callus in maize). However, of these two types of callus structures, FEC has proven to be an ideal target for genetic transformation due to its high rate of efficiency in transformation (Lin et al., 2000b; Raemakers, 2001). Therefore, the development of an efficient regeneration system from the vegetative tissue (leaves with axil tissue) was studied in this thesis as well.

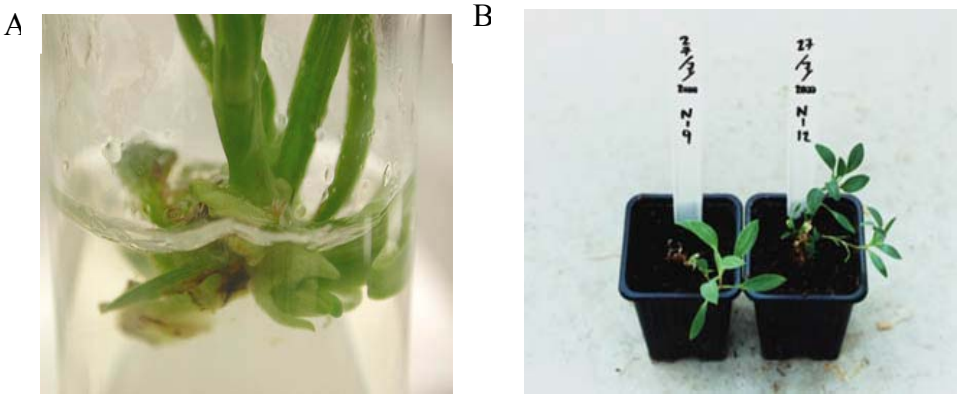

Figure 2. Rhizome formation and regeneration in *Alstroemeria*

A) rhizome formation B) 3 months after rhizome culture in the greenhouse

Overview of genetic modification in *Alstroemeria* and monocot ornamentals

Alstroemeria, like other monocot ornamentals have been generally recalcitrant to genetic transformation techniques that are routinely applied in dicotyledonous plants. FEC induced from stem tissue of seedling plants was transformed with particle bombardment by pAHC18 that contained the luciferase gene as a reporter

gene (Lin et al., 2000b). Plants were obtained from 10 independent lines. After 1 year of maintenance, however, only a few plants were still luciferase-positive. Furthermore, FEC was also used to transform using *Agrobacterium tumefaciens* (Van Schaik, 1998), however, no transgenic plants were produced. She concluded that FEC might be an alternative source for genetic modification and an ideal explant without severe somaclonal variations provided young FEC was used for transformation. To obtain transgenic *Alstroemeria* plants via either *A. tumefaciens* or particle bombardment, an efficient regeneration system and the optimization of parameters influencing the transformation process should be prepared. Therefore, much attention has been directed to the optimization of regeneration protocols and the production of transgenic plants in this Thesis.

Furthermore, in many monocotyledonous ornamentals, particle bombardment and *Agrobacterium*-mediated transformation have been used for the production of transgenic plants with improved agricultural traits (Table 4). Particle bombardment has been applied in *Alstroemeria* (Lin et al., 2000b), *Gladiolus* (Kamo et al., 1995; 2000; 2005), *Dendrobium* (Kuehnle and Sugii, 1992), *Lily* (Watad et al., 1998), and *Tulip* (Wilmink et al., 1995). *Agrobacterium*-mediated transformation has been applied in *Alstroemeria* (Akutsu et al., 2004a; 2004b), *Anthurium* (Chen and Kuehele, 1996), *Cymbidium* (Yang et al., 1998), *Iris* (Jeknic et al., 1999), and *Phalaenopsis* (Chai et al., 2002; Belarmino and Mii, 2000). However, in spite of several successful reports, *Agrobacterium*-mediated transformation is still cumbersome for quite a number of monocot ornamentals. In the past decades, most of the reports on the transformation of monocot ornamentals used the GUS gene as a reporter gene because of its accuracy, fast and convenient characteristics.

Scope of this research

Due to a lack of useful genes in existing cultivars, the development of new breeding systems with biotechnological techniques is needed for *Alstroemeria*. Genetic modification of elite genotypes can be used to transfer useful genes within a short period of time. To establish these techniques in *Alstroemeria*, an efficient regeneration protocol and transformation system must be developed. With these systems in place, transgenic *Alstroemeria* plants could be obtained. Therefore, four objectives were studied and will be discussed here. The first objective was to develop an efficient regeneration system in *Alstroemeria* species that could be

combined with transformation. Secondly, a reliable transformation system using either *Agrobacterium tumefaciens* or particle bombardment in *Alstroemeria* should be developed and optimized for further application. Thirdly, based on an established regeneration and transformation system, virus-resistant *Alstroemeria* plants should be obtained and analyzed. Finally, since somaclonal variation is known to occur in the tissue culture process, transgenic *Alstroemeria* plants as well as plants from different regeneration systems should be assessed for this occurrence and level of somaclonal variation.

Chapter 2 describes the induction of compact embryogenic callus (CEC) and friable embryogenic callus (FEC) from vegetative tissue, and the subsequent regeneration of plants.

Chapter 3 describes for the first time in *Alstroemeria* a protoplast culture system and regeneration of plants. For this, leaves with axils, compact callus, and friable embryogenic callus tissues were compared. Moreover, somaclonal variation occurring in the protoplast culture system is discussed.

Chapter 4 describes the co-transformation system and discusses the optimization of the particle bombardment system. Further, the production of virus-resistant *Alstroemeria* through the optimized particle bombardment protocol is described.

In Chapter 5 *Agrobacterium*-mediated transformation using FEC is described and optimized by an investigation into several parameters such as the bacterial culture period, concentration of bacterial suspension, co-cultivation period, infection time and temperature.

Finally, a general discussion on somatic embryogenesis, protoplast culture and transformation in *Alstroemeria* is given in Chapter 6.

Table 4. Examples of the production of transgenic plants in monocotyledonous ornamentals

Species	Methods* (used explant**)	Reporter gene***	Selectable marker***	Reference
Tulip	P.B	GUS	N.A	Wilmink et al., 1995
Tulip	A.T and P.B. (FSS)	GUS	N.A	Wilmink et al., 1996
Gladiolus	P.B. (Callus)	GUS	PPT	Kamo et al., 1995, 2000
Gladiolus	P.B. (Callus)	N.A	PPT	Kamo et al, 2005
Lily	A.T (Scale slices)	N.A	N.A	Cohen and Meredith, 1992
Lily	P.B. (Callus)	GUS	PPT	Watad et al., 1998
Lily	P.B. (Pollen)	GUS	N.A	Tanaka et al., 1995
Lily	P.B.	GUS	N.A	Wilmink et al., 1995
Iris	A.T (SC)	GUS	Hyg and Gen	Jeknic et al., 1999
Freesia	P.B (Pollen)	GUS	N.A.	Tanaka et al., 1995
Anthurium	A.T. (Root)	N.A	Kan	Chen et al., 1997
Anthurium	A.T. (Internodes)	GUS	Kan	Chen and Kuehnle, 1996
Dendrobium	A.T (PLB)	N.A	Kan	Kuehnle and Sugii, 1992
Cymbidium	P.B. (PLB)	GUS	Kan	Yang et al., 1999
Phalaenopsis	A.T. (PLB)	GUS	Hyg and Kan	Chai et al., 2002
Phalaenopsis	A.T. (SC)	GUS	Hyg and Kan	Belarmino et al., 2000
Alstroemeria	P.B. (FEC)	Luc, GUS	PPT	Lin et al., 2000b
Alstroemeria	A.T. (FEC)	GUS	Hyg and Kan	Akutsu et al., 2004a
Alstroemeria	A.R. (FEC)	GUS	Hyg and Kan	Akutsu et al., 2004b

*: P.B; Particle bombardment, A.T: *Agrobacterium tumefaciens*-mediated transformation, A.R: *Agrobacterium rhizonenous*-mediated transformation, E: Electroporation

**: CEC (Compact embryogenic callus), FEC (Friable embryogenic callus), FSS (Floral stem segment), PLB (Protocorm like body), SE (Somatic embryo), ZE (Zygotic embryo), SC (suspension culture)

***: Hyg (Hygromycin), Gen (Geneticin), Kan (Kanamycin), Pat (Luc (Luciferase), Gus (β-gluculonise), N.A.: non-applicable

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CHAPTER 2. Efficient somatic embryogenesis from leaves with axil tissue in *Alstroemeria*

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Efficient somatic embryogenesis from leaves with axil tissue in *Alstroemeria*

Kim. J. B[1,2]., · C. J. J. M. Raemakers[1*] · E. Jacobsen[1] · R.G. F. Visser[1]
[1] Laboratory of Plant Breeding, The Graduate School of Experimental Plant Sciences, Wageningen University, 6700 AJ, P.O.BOX 386, Wageningen, The Netherlands
[2]Turfgrass & Environment Research Institute, Samsung Everland Inc. #1 Bu-kok, Gunpo, Kyong-ki, 435-737, Korea

* Author for correspondence: e-mail address – krit.raemakers@wur.nl
Telephone: +31-317-484048
Fax: +31-317-483457

Abstract

In *Alstroemeria* high frequencies of compact embryogenic callus (CEC) induction (~40%) and friable embryogenic callus (FEC) induction (~15%) were obtained from leaves with axil tissue on Schenk and Hildebrandt medium, supplemented with 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA. Both types of callus were maintained on modified Murashige and Skoog medium supplemented with 5 mg l^{-1} Picloram. On this medium, CEC also formed FEC (indirect induction).

In general, 35% of FEC and 74% of CEC-derived somatic embryos regenerated into plants on MS medium containing 0.5 mg l^{-1} BA, 25 g l^{-1} sucrose, solidified with 2.75 g l^{-1} Gelrite within 6 weeks. Approximately 500 *in vitro* plants were produced after 12 weeks (CEC) and after 16 weeks (FEC) of culture on regeneration medium using 1g of callus via a three-week subculture regime. Regenerated plants were well established in the greenhouse and showed normal flowering. This is the first time, that in *Alstroemeria* somatic embryogenesis from adult plants is achieved.

Abbreviations: CEC – compact embryogenic callus; CIM – callus induction medium; FEC – friable embryogenic callus; LS – Linsmaier and Skoog; MS – Murashige and Skoog; RM – regeneration medium; SE – somatic embryo; SH – Schenk and Hildebrandt; SIM – shoot induction medium

Introduction

Alstroemeria is an important ornamental in the world (Van Zaayen, 1995). Nowadays, it is used as a cut flower and, recently, also as a pot plant. *Alstroemeria* has a long vase-life and a variety of flower colors. In addition, it requires a low input of energy during the growth (Blom and Piott, 1990; Chekpkairor and Waithaka, 1988; Van Schaik et al., 1996).

Conventional breeding techniques have been used in *Alstroemeria* to create new and attractive cultivars with new colors, longer vase life, high yield of flowers and resistance to diseases. However, the genes for many agriculturally useful traits are not present in the *Alstroemeria* gene pool. In those cases, genetic modification can be used. However, genetic modification requires regeneration protocols with high efficiency and reproducibility. Plant regeneration in *Alstroemeria* has been mainly accomplished via somatic embryogenesis (Akutsu and Sato, 2002; Gonzalez-Benito and Alderson, 1992; Hutchinson et al., 1994, 1997; Lin et al., 2000a; Van Schaik et al., 1996) rather than by organogenesis (Lin et al., 1997). In general, two types of embryogenic callus have been observed in *Alstroemeria*: compact embryogenic callus (CEC) and friable embryogenic callus (FEC). CEC was induced on zygotic embryos cultured on auxin supplemented medium (Van Schaik et al., 1996), and is a hard type of callus that consists of embryogenic aggregates that are bigger than 0.5 mm.

FEC was initiated from seedling explants (Lin et al., 2000a) or from ovules (Akutsu and Sato, 2002) cultured on auxin supplemented medium and is a soft type of callus that consists of embryogenic units, which are smaller than 0.1 mm in diameter. CEC used for genetic modification did not result in the production of transgenic plants (Van Schaik et al., 2000). On the other hand, FEC has been used successfully for the production of genetic modified alstroemeria plants (Lin et al., 2000b). Also in crops as cassava (Munyikwa et al., 1998; Raemakers et al., 2001), *Gladiolu*s (Kamo et al., 1995), oat (Somers et al., 1992; Tobert et al., 1995), rose (Robinson and Firoozababy, 1993), and wheat (Brisibe et al., 2000), FEC has been used successfully to obtain genetically modified plants. However, because *Alstroemeria* is a highly heterozygous and vegetatively propagated crop, FEC derived from seedling tissue or ovules cannot be used for improvement of an existing variety. Due to this reason, the development of FEC from clonal tisse should be established. Unfortunately, no efficient regeneration system via somatic

embryogenesis from clonal tissue has been described in *Alstroemeria*. Therefore, the aim of this investigation was to determine whether it is possible to induce FEC from adult plants.

Materials and methods

Plant material and selection of explants

VV024-6 was selected from a previous study (Lin et al., 1998) due to its high multiplication rate and used all further experiments in this chapter. Plants were maintained by a 4-week subculture regime on a regeneration medium (RM) consisting of MS (Murashige and Skoog, 1962) basal salts plus vitamins, 0.5 mg l^{-1} BA, 40 g l^{-1} sucrose and 2.2 g l^{-1} Gelrite. Plants were cultured at 18 ˚C under a 16 h (light)/8 h (dark) photoperiod provided by Philips white fluorescent lights at 40 $\mu mol.m^{-2}s^{-1}$. The callus induction and maintenance experiments were done at 18 ˚C in darkness. Somatic embryos and plants were then cultured in light. All media used in this study were adjusted to pH 6.0 using 1N KOH.

In the first experiment, leaves with axil tissue (Lin et al., 1997) and node tissues, both derived from *in vitro* plants, were compared with respect to embryogenic callus induction. The leaf explants were prepared by using the C2 method as described by Lin et al. (1997). Leaves with axil tissue and node tissues were first cultured on shoot induction medium (SIM) consisting of MS basal salts and vitamins, 2.2 mg l^{-1} TDZ, 0.1 mg l^{-1} IBA, 30 g l^{-1} sucrose and 7.5 g l^{-1} Microagar (Duchefa Biochemie B.V., Haarlem, The Netherlands) for 10 days (Lin et al., 1997). Explants were then transferred to callus induction medium (CIM). CIM medium contains MS basal salts and vitamins, 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA, 30 g l^{-1} sucrose, and 8 g l^{-1} Microagar (Duchefa Biochemie B.V., Haarlem, The Netherlands) (subcultured after 4 weeks). Formation of FEC and CEC was evaluated after 8 weeks of growth. The explants were then transferred to RM and evaluated for the production of somatic embryos 6 weeks later.

Induction of somatic embryogenesis from leaves with axil tissue

Several factors were studied to improve induction of CEC and FEC using leaves with axil tissue as explants. In the second experiment, leaves with axils were

pre-cultured on SIM medium for 10 days before being transferred to CIM which consisted of different basal media: CIM-MS (Murashige and Skoog, 1962), CIM-N6 (Chu et al., 1975), CIM-LS (Linsmaier and Skoog, 1965), and CIM-SH (Schenk and Hildebrandt, 1972).

In the third experiment, the effect of leaf age on induction of CEC and FEC was investigated. The leaves with axil tissues were numbered on a grid from top to bottom (1 to 5), cultured for 10 days on SIM, and transferred to CIM-SH supplemented with 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA.

The effect of different gelling agents added to CIM-SH medium on induction of CEC and FEC, somatic embryos and shoot development was tested in the fourth experiment. Leaves with axil tissues were cultured for 10 days on SIM (solidified with 7.5 g/l Microagar) then transferred to CIM-SH supplemented with 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and solidified with 5 g l^{-1} Plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands), or 2.75 g l^{-1} Gelrite or 7.5 g l^{-1} Micro agar. After 8 weeks, the explants were transferred to RM medium, solidified with 2.2 g l^{-1} Gelrite.

In the last experiment, leaves with axil tissues, precultured for 10 days on SIM, were cultured on CIM-SH with different auxin/cytokinin combinations (2 mg l^{-1} 2,4-D or 2 mg l^{-1} Picloram; with and without 0.5 mg l^{-1} BA). After 6 weeks of culture the initiated CEC and FEC were placed on RM for development of somatic embryos. In all experiments, the callus response was evaluated 6 weeks after culture on CIM. Somatic embryo production and plant formation were evaluated after 6 and 12 weeks weeks of growth on RM, respectively.

Plant regeneration

CEC and FEC maintained in a 3-weeks subculture regime on PCA medium (Sofiari et al., 1998) were transferred to RM for plant regeneration. After 4 weeks the medium was refreshed and after 8 weeks somatic embryos were isolated and transferred to fresh RM and cultured for an additional 4 weeks. Primary and secondary embryos with two or more normal shoots considered to be regenerated into plants. These plants were transferred to a rooting medium composed of MS basal salts and supplemented with 0.5 mg l^{-1} NAA, 45 g l^{-1} sucrose, and 2.2 g l^{-1} Gelrite (pH 6.0). Rooted plants were transferred to the greenhouse.

Statistical analysis

The experiments were repeated at least three times. The data are presented as the mean ± standard error (SE). The data were analyzed using least significant difference (LSD) test (P=0.05) for multiple comparisons (SPSS for Windows version 10.0' statistical software).

Results

Selection of proper explants for somatic embryogenesis

In the first experiment, the response of leaves with axil tissue was compared with that of nodal tissues. After 2 weeks of culture on CIM, callus appeared at the wound sites in leaves with axil tissue and nodal tissue. In some cases, callus was formed in the axil at the point of attachment to the stem (Figure 1A). Three different types of callus were observed simultaneously: soft and watery callus (non embryogenic callus: NEC); compact embryogenic callus (CEC), and friable embryogenic callus (FEC). The FEC was yellowish, tiny, round-shaped, fast growing and could easily be divided in single units. The CEC (Figure 1B) was initially white and later turned yellow. Further, the CEC showed rather slow growing and difficulty in dividing in singlw units. The soft, watery callus turned brown and died soon after initiation. Approximately 45% of the leaves with axil tissue explants initiated CEC compared to 15% of the node tissue explants. FEC was observed on 8% of the leaves with axil tissue compared to 2% of the nodes. After 8 weeks of culture on CIM, the CEC and FEC types were transferred to RM. CEC produced either new CEC or agglomerates of somatic embryos (Figure 1B), on its surface. FEC initially produced CEC, which than behaved similar to CEC. Somatic embryos induced from both CEC and FEC cultures were developed into plants (Figure 1C) and flowered in the greenhouse (Figure 1D).

Optimization of the induction of CEC and FEC from leaves with axil tissue

In the next experiments, several parameters were varied using leaves with axil tissue as explant source. The aim of these studies was to optimize the formation of both types of embryogenic callus. The second experiment examined the effect of 4

different basal media. The CIM-SH medium (at 41%) and the CIM-LS medium (at 14%) resulted in the highest and the lowest frequencies of CEC induction, respectively (Table 1). Also, the CIM-LS medium had the lowest frequency for FEC initiation. For the other 3 media (CIM-MS, CIM-N6 and CIM-SH), between 15 and 19% of the explants produced FEC. Based on these results, CIM-SH was selected for further experimentation.

Figure 2 shows the effect of the leaf age on CEC and FEC induction. Both CEC and FEC induction decreased with increased leaf age. Nearly 70 % of the first leaves initiated CEC compared to less than 10% of the fifth leaves. For the initiation of FEC, these figures were more than 10 % for the first leaf compared to less than 2% for the fifth leaf.

In the next experiment, the CIM-SH medium was solidified with different types of agar. Microagar and gelrite showed significantly better results than plant agar with respect to the initiation of CEC. For FEC initiation, microagar had the best results. However, for the frequency of somatic embryo initiation from FEC cultures, gelrite was significantly better than the other 2 types of agar. Gelrite also gave the best results for plant regeneration from somatic embryos (Table 2). In case of CEC, the frequency of somatic embryo induction was less than 40% and the frequency of shoot formation ranged from 20 to 25%.

In the next experiment leaves with axil tissue, pre-cultured on SIM medium, were cultured on CIM-SH with different growth regulators. All 4 media induced the formation of both CEC and FEC. The addition of BA to both picloram and 2, 4-D slightly increased the formation of CEC. A medium supplemented with 2, 4-D was more efficient in producing CEC than a medium supplemented with picloram. The addition of BA to 2, 4-D had a slight positive effect on the induction of FEC whereas the addition of BA added to picloram doubled the formation of FEC. Also for FEC induction 2, 4-D is more efficient than picloram.

In summary, about 40% of the leaves with axil tissue produced CEC while 10-20% of the explants showed FEC formation. Almost 50% of leaves with axil tissue produced directly somatic embryos without callus phase. However, more than 60% of the somatic embryos induced directly from explants deteriorated or developed severe browning. The rest of 40% of the somatic embryos were developed into plants and showed a considerable variation in leaf morphology. However, nearly 60% of the cultured FEC clumps produced somatic embryos, and 35% of these germinated into plants, showing less variation, as compared with plants from direct

somatic embryogenesis.

Maintenance and proliferation of embryogenic callus lines

After 3 months of culture, a total of 150 CEC and 100 FEC lines were selected from 400 leaves with axil tissue explants. The lines were maintained on either PCA or MS medium supplemented with 5 mg/l picloram. CEC and FEC did not produce somatic embryos on either media. About 15% of the CEC also produced FEC. In all cases, FEC initiated new FEC without further organization into CEC. The fresh weight of CEC was increased by 115% on MS and by 190% on PCA after 3 weeks of culture. For FEC, these figures were 75% and 136%, respectively. Based on these results, PCA medium was chosen for FEC and CEC maintenance. CEC and FEC were maintained without any problems for more than a year.

Somatic embryo germination and plant establishment in the greenhouse

CEC and FEC lines either induced directly from leaf with axil tissues or indirectly from CEC were maintained for 4 months on PCA medium before culturing for plant regeneration. In general, FEC did not initiate somatic embryos directly on RM. About 80% of the FEC units developed into CEC units after 3 weeks of culture. These CEC units were both relatively large and white in color or were small and yellow. Only the yellow CEC units had the capacity to regenerate somatic embryos. Again, 3 weeks later, (pre-) globular embryos were developed from CEC units. Up to 6 globular somatic embryos were initiated from one CEC unit. These embryos were isolated from the CEC and cultured on fresh regeneration medium for further development. After two weeks, the shoot apex broke through the cotyledon, developing into a shoot. Vigorously growing shoots were dissected once, they had reached 2-4 cm in length, then were transferred to rooting medium. When roots developed after 3–5 weeks of culture, plants with a size of 4-6 cm and with 2-4 roots were transferred to the greenhouse. Rhizomes were also formed after 4-6 weeks of culture. Approximately 90-95% of the plants survived and flowered normally 3-4 months later.

For the FEC lines, two to three weeks more were required for the germination of somatic embryos into shoots. This is due to the development of FEC into CEC before following the procedures outlined above.

Discussion

In monocot ornamental crops, embryogenic cultures have been used as target tissues for genetic modifications (Kamo et al., 1995; Lin et al., 2000b; Van Schaik et al., 2000). Zygotic embryos (Hutchinson et al., 1994; Gonzalez-Benito and Alderson, 1992; Van Schaik et al., 1996) have previously been used for the initiation of compact embryogenic cultures in *Alstroemeria*. Only Lin et al. (2000a) and Akutsu et al. (2002) obtained FEC from stem and ovule tissues of seedlings, respectively. In *Alstroemeria*, both CEC and FEC cultures have been used for genetic modification. In the case of CEC, however, only chimeric transgenic plants were produced at a very low frequency (Van Schaik et al., 2000). However, FEC, combined with genetic modification, has resulted in completely genetically modified plants (Lin et al., 2000b).

The approach described by Lin et al. (2000a) had several disadvantages including the low frequency of FEC initiation, lack of repeatability, and the long period of time required to initiate FEC. Furthermore, ovule-derived calli were developed into plants via somatic embryogenesis with a high efficiency (Akutsu et al., 2002). However, FEC derived from seedling tissue cannot be used for the improvement of an existing cultivar via genetic modification because *Alstroemeria* is a heterozygous and vegetatively propagated crop. Other disadvantages are the excessive greenhouse space required for the production of explant material and the possible effect of seasonal variations on the embryogenic response. Clonal tissue was used for the initiation of embryogenic callus in *Gladiolus* (Kamo et al., 1990; Stefaniak, 1994), *Freesia* (Wang et al., 1990), and *Asparagus* (Limanton-Grevet et al., 2000). To our knowledge, this is the first report, showing that *Alstroemeria* clonal tissue has the capacity to form CEC and FEC. Several factors influencing callus induction and somatic embryo production, such as basal medium composition, hormone combinations, gelling agents, leaf age and medium were investigated for optimization. In *Alstroemeria*, SH (Schenk and Hildebrandt, 1972) salts and vitamins resulted in better induction rates of callus and somatic embryo production than the other tested salts and vitamins mixtures. Also germination of somatic embryos was improved when BA was added to the callus induction medium. Similar trend was observed by Lin et al. (2000). In the cases of the legume *Astragalus* (Luo et al., 1999) and the monocot bermudagrass (Mukesh et al., 2005), the addition of a low concentration of BA to a high concentration of 2,4-D

positively influenced the formation of embryogenic callus and somatic embryos.

The leaves in the first and second positions with axil in the shoots produced the highest frequency of embryogenic callus. Lin et al. (1998) observed a similar effect of the age of the leaves of *Alstroemeria* when used for the induction of organogenesis. Also in *Dianthus* (Van Altvorst et al., 1995), *Hordeum* (Becher et al., 1992), oat (Chen et al., 1995), and *Miscanthus* (Holme et al., 1996) young leaves give better results with respect to embryogenic callus induction than older leaves.

Generally, there are two methods to obtain FEC lines using leaves with axil tissue for *Alstroemeria*. The first one is directly from leaves with axil tissue without formation of CEC formation. The second method is indirect: FEC is induced from CEC cultured on PCA medium. Indirectly-induced FEC generally shows better callus growth, somatic embryo yield and germination than directly-induced FEC (results not shown).

In conclusion, the results described here demonstrate that embryogenic callus formation was induced with high efficiency on SH medium supplemented with 2 mg/l 2,4-D, 0.5 mg/l BA, 30g/l sucrose, and solidified with 2.75g/l Gelrite, at pH 6.0 following 10 days of culture on SIM. Two embryogenic callus types, CEC and FEC, can be obtained from vegetatively propagated plants within 3 months of culture. Both callus types were able to regenerate somatic embryos and subsequently plants at a high frequency. In total, more than 500 plants with healthy roots were produced from the selected CEC and FEC lines. Particularly, the FEC lines may be superior material for use in genetic modification as was shown by Lin et al. (2000b).

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Table 1. The influence of different basal salts with vitamin mixtures on callus induction of VV024 in *Alstroemeria*

Medium	% of explants with CEC	% of explants with FEC
SH	40.6±8.5a[1]	14.6±2.4a
N6	23.1±4.3b	16.8±2.1a
MS	15.7±6.9b	18.6±3.3a
LS	14.4±3.3b	5.4±1.1b

3 replicates (40 explants) per treatment, data collected after 8 weeks of culture, means in a column followed by the same letter are not significantly different at the 5% level as determined by LSD.

Table 2. Effect of different gelling agents on callus induction, somatic embryo production and regeneration of plants from FEC in *Alstroemeria* genotype VV024.

Gelling agents (g/l)	% CEC[1]	% FEC[1]	% of somatic embryos/48 FEC clumps	% of shoot for-mation/100 FEC-derived SE
Plant Agar (5)	28.0±4.3b	7.5±1.1b	45.3±8.8b	21.6±2.5b
Gelrite (2.75)	37.0±3.8a	15.5±4.4ab	60.6±7.1a	35.0±6.9a
Microagar (7.5)	36.0±2.9a	22.5±3.7a	48.5±11.3b	19.3±5.4b

[1] SH medium supplemented supplemented with 2mg/L 2,4-D and 0.5mg/L BA used, 3 replicates (40 explants) per treatment and data collected after 8 weeks of culture, means in a column followed by the same letter are not significantly different at the 5% level as determined by LSD.

Table 3. Effect of different growth regulators combinations on callus induction and somatic embryo (SE) development in *Alstroemeria* genotype VV024.

Hormone combination[1]	% CEC[2]	% FEC[2]	# of SE / 100 units of CEC[3]	# of SEs / 100 clumps of FEC
2,4-D 2 mg/l + BA 0.5 mg/l	37.4±3.4a	19.5±4.2a	132.8±18.4a	85.5±8.9a
Picloram 2 mg/l + BA 0.5 mg/l	19.8±4.4b	12.0±5.4ab	46.8±6.1c	55.7±3.5b
2,4-D 2mg/l	31.4±2.5ab	15.4±3.3a	98.8±9.3b	47.3±6.3b
Picloram 2 mg/l	18.5±4.7b	5.3±3.8b	20.5±5.0d	23.4±4.1c

[1] SH medium used, [2] 3 replicates (40 explants) per treatment and data collected after 8 weeks of culture, means in a column followed by the same letter are not significantly different at the 5% level as determined by LSD.

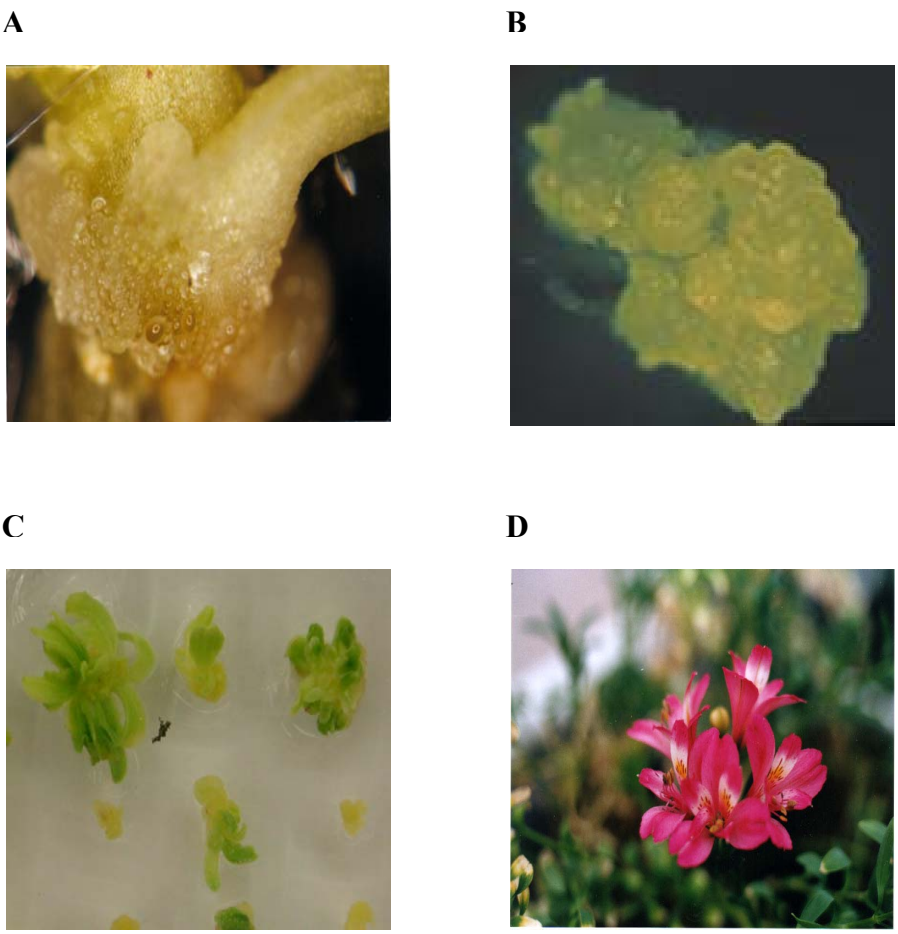

Figure 1. Somatic embryogenesis from leaves with axil tissues in *Alstroemeria* A. friable embryogenic callus (FEC) induced from leaves with axil tissue, B. Yellow compact embryogenic callus (CEC), C. regeneration of somatic embryos from FEC, D. flower formation in plant regenerated from FEC.

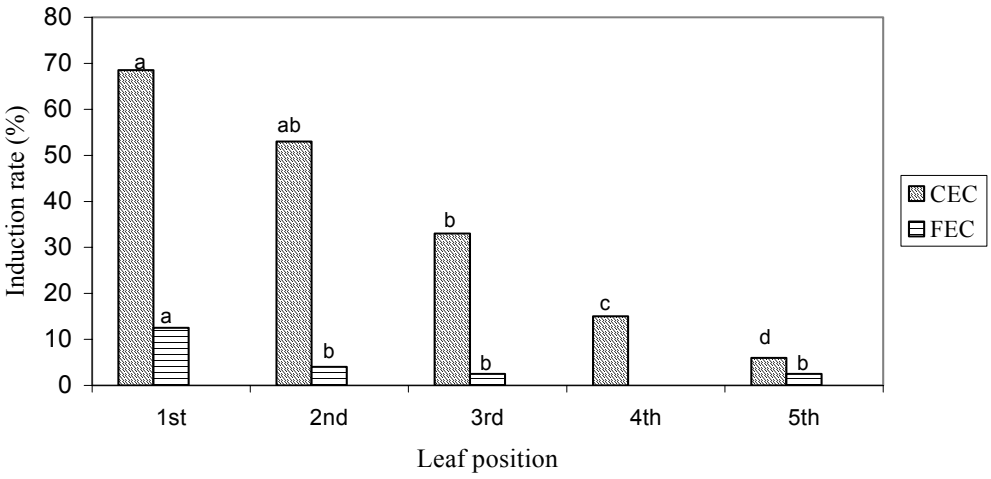

Figure 2. The effect of age of leaves with axil explants on the induction of compact embryogenic callus (CEC) and friable embryogenic callus (FEC) of VV024 in *Alstroemeria* (3 replicates with 40 explants per treatment and data collected after 8 weeks of culture; columns with different letters are significantly different from each other at the 5% level as determined by LSD).

CHAPTER 3

Isolation of protoplasts, culture and regeneration into plants in *Alstroemeria*

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Isolation of protoplasts, culture and regeneration into plants in *Alstroemeria*.

Kim J.B[1), 2)], Bergervoet J.E.M[1)], Raemakers C.J.J.M.[1)*], Jacobsen E[1)], Visser R.G. F[1)].

[1]Laboratory of Plant Breeding, The Graduate School of Experimental Plant Sciences
Wageningen University, P.O.BOX 386, 6700 AJ, Wageningen, The Netherlands

[2]Current address; Turfgrass & Environment Research Institute, Samsung Everland Inc.,
#1 Bu-kok, Gunpo, Kyong-ki, 435-737, Korea,

[*]Author for correspondence: e-mail - krit.raemakers@wur.nl
Telephone: +31-317-484038, Fax: +31-317-483457

Abstract

An efficient system for the regeneration of plants from protoplasts was developed in *Alstroemeria*. Friable embryogenic callus (FEC) proved to be the best source for protoplast isolation and culture when compared with leaf tissue and compact embryogenic callus. Protoplast isolation was most efficient when FEC was incubated for 5 min under vacuum in an enzyme solution consisting of 4% Cellulase, 0.5% Driselase and 0.2% Macerozyme, followed by culture for 12-16 h in the dark at 24°C. Cell wall formation and colony formation were better in a liquid medium than on a semi-solid agarose medium. Micro-calluses were formed after 4 wk of culture. Ninety percent of the micro-calluses developed into FEC after 12 wk of culture on proliferation medium. FEC cultures produced somatic embryos on regeneration medium and half of these somatic embryos developed shoots. Protoplast-derived plants showed more somaclonal variation than vegetatively propagated control plants.

Key words: *Alstroemeria*, Callus, Ornamental, Protoplasts, Regeneration, Somatic embryos.

Introduction

Alstroemeria is a popular ornamental plant in the Netherlands, USA, UK, Canada, and Japan. Due to increasing interest in *Alstroemeria*, there is a continuous demand for new cultivars. Conventional breeding techniques such as cross-hybridization, mutation breeding and selection of elite lines from seedlings have been employed and have contributed considerably to the production of new and improved *Alstroemeria* cultivars. Consequently, more than a hundred elite cultivars have been released for the commercial market since the 1950s.

However, for traits such as resistance against bacteria, fungal and virus diseases, flower scent, and abiotic tolerances (cold, drought and heat), little variation is available in the existing *Alstroemeria* cultivars. Genetic variation can be increased by using either hybridization techniques or genetic modification. Unfortunately, hybridization of *Alstroemeria* cultivars with other *Alstroemeria* species is difficult due to incompatibility. Although in some cases cross-incompatibility was overcome by ovule culture, breeders are still looking for new techniques to transfer genes of interest to current *Alstroemeria* cultivars. One such technique, used successfully in other crops to overcome sexual incompatibility, is protoplast fusion (Assani et al., 2005). Protoplast fusion requires the development of a regeneration system from protoplasts. Furthermore, an efficient and reliable regeneration system from protoplasts opens up new opportunities for genetic transformation via direct DNA uptake or electroporation, as was shown in many other crops (Potrykus, 1990). For instance, transgenic rice and citrus plants have been produced from protoplasts by PEG-mediated transformation (Lin et al., 1995; Fleming et al., 2000). Somatic hybridization and genetic modification may enable us to enhance the genetic variation of ornamental plants and improve them by employing these techniques in combination with conventional breeding (Nakano et al., 1995).

In monocotyledonous ornamentals, protoplast culture systems have been described in *Agapanthus* (Nakano et al., 2003), Iris (Shimizu et al., 1996), Lily (Mii et al., 1994), and *Phalaenopsis* (Chen et al., 1991). Until now, there is no report on plant regeneration from protoplasts in *Alstroemeria*. Therefore, the objective of this study was to develop a reliable system for regenerating *Alstroemeria* plants from protoplasts. Successful plant regeneration from protoplasts was achieved from a friable embryogenic callus culture. Viable plants

were obtained, which flowered, but showed more somaclonal variation than control plants.

Materials and methods

Plant material, callus induction and its maintenance

In-vitro grown tetraploid *Alstroemeria* plants of VV024 were used in this study. Plants were kept on regeneration medium (RM), which consisted of MS (Murashige and Skoog, 1962) basal salts plus MS vitamins, 2.2 μM 6-benzylaminopurine (BA), 40 g/l sucrose, and 2.5 g/l Gelrite (pH 6.0). Cultures were maintained by a 4-wk subculture regime at 18°C under a photoperiod of 16 h. Friable embryogenic callus (FEC) induction was initiated by subculture of the top three leaves including axil tissue on shoot induction medium (SIM), which consisted of MS basal salts plus MS vitamins, 10 μM thidiazuron (TDZ), 2.5 μM indole-3-butyric acid (IBA), 30 g/l sucrose, and 7.5 g/l Microagar (Duchefa Biochemie B.V., Haarlem, The Netherlands) (pH 6.0) as described by Lin et al. (1997). After 10 d of culture on SIM, leaves with axil tissue were transferred to callus induction medium (CIM), that consisted of SH (Schenk and Hildebrandt, 1972) basal salts and SH vitamins, 9.1 μM 2, 4-dicholorophenoxyacetic acid (2,4-D), 2.2 μM 6-benzylaminopurine (BA), 30 g/l sucrose, and 7.5 g/l Microagar (pH 6.0). On CIM, the explants were cultured for 9 wk with a 3-wk subculture regime to stimulate compact embryogenic callus (CEC) formation. CEC was transferred to solid (7.5 g/l Microagar) PCA medium (Sofiari et al., 1998) containing 41.5 μM Picloram with a pH 6.0. Every 4 wk the tissue was subcultured in order to obtain FEC. FEC clumps were isolated and maintained on solid PCA medium with a 4-wk subculture. All cultures for callus induction and FEC maintenance were placed at 18°C in darkness unless otherwise stated.

Factors affecting protoplast isolation

In initial experiments, protoplasts were isolated from three different types of explants (CEC, FEC, and leaf with axil tissue precultured for 1 wk on SIM). For this 0.5 g of fresh material was placed in 10 ml pre-plasmolysis solution (0.45 M mannitol) for 1 h at room temperature in darkness. Then, the plant tissues were

transferred to 5 ml cell wall degrading enzyme solution with or without vacuum treatment (5 min at 28 inch Hg partial vacuum) and placed on a rotary shaker (40 rpm) for 12 h at 24°C in darkness. Three different enzyme solutions (pH 5.5) were used: A: Cellulase Onozuka R-10 1.0% (w/v), Macerozyme R-10 0.1% (w/v), B: Cellulase Onozuka R-10 2.0% (w/v), Macerozyme R-10 0.2% (w/v), C: Cellulase Onozuka R-10 4.0% (w/v), Driselase 0.5% (w/v), Macerozyme R-10 0.2% (w/v). After the enzyme treatment, the protoplasts were filtered through a mesh (45 μm and 83 μm) and centrifuged at 600 rpm for 3 min. The pelleted protoplasts were resuspended in 10 ml washing solution which consisted of 7.25 g/l KCl, 13.75 g/l $MgCl_2.6H_2O$ and 10.25 g/l $CaCl_2.2H_2O$ (pH 6.0) and centrifuged again. Then, the supernatant was discarded, and the protoplast pellet was purified by resuspension in a 14% sucrose solution that was overlayed with washing solution. Following centrifugation for 3 min at 600 rpm, protoplasts were collected with Pasteur pipettes at the boundary phase between the sucrose solution and the washing solution, transferred to 10 ml sterile centrifuge tubes and washed twice with washing solution. The washed protoplasts were resuspended in a liquid culture medium containing MS basal salts plus MS vitamins, 4.5 μM 2,4-D, 0.5 μM 2-isopentenyladenine (2-iP), 20 g/l sucrose, at a density of 10^5 cells/ml. The viability of the isolated protoplasts was measured by staining with fluorescein diacetate (0.5 mg/ml fluorescein diacetate in acetone, mixed with washing solution at a ratio of 1:50). The protoplasts were counted under a fluorescence Axiophot photo-microscope using an aniline blue filter.

In subsequent experiments, protoplasts were isolated from 0.5 g of a 10-d-old yellow FEC culture incubated in enzyme solution C under a vacuum for 5 min at 28 inch Hg partial vacuum as described above. To determine the optimal length of the enzyme treatment, FEC was incubated for 4, 8, 12, 16 or 20 h in enzyme solution. In another experiment, to determine the optimal temperature, FEC cultures were incubated for 16 h in enzyme solution C at 18, 24 and 30°C.

Regeneration of plants from protoplasts

Isolated protoplasts were cultured in Petri dishes (5 cm diameter), with either liquid or agarose-solidified (Sea Plaque agarose 0.2 %) PCA medium. After 1 week of culture in the dark, protoplasts were moved to light conditions (16 h). Plating efficiency was estimated as the percentage of cell colonies larger than 0.2 mm in

diameter that developed in a Petri dish 2 wk after protoplast isolation. Visible cell colonies were then transferred to fresh liquid PCA medium. Six to 8 wk after plating, visible micro-calluses were transferred onto solid PCA medium. The micro-calluses were subcultured every 2 wk.

After 3-4 mo of culture, FEC clumps with a diameter of 3-4 mm were placed on RM, and cultured at 18°C with a 16 h photoperiod to promote the formation of somatic embryos and shoot development. Cultures were refreshed every 3 wk. Shoots of 2-3 cm high with embryonic tissue were excised from the somatic embryo clumps and transferred to rooting medium, which consisted of MS basal salts plus MS vitamins, 2.7 μM α-naphthaleneacetic acid (NAA), and 45 g/l sucrose, and solidified with 2.5 g/l Gelrite (pH 6.0). Plants with healthy roots were transferred to the greenhouse. The protoplast-derived plants were compared with control plants with respect to plant height, leaf length, leaf width, stem thickness, flower stalk number, flower stalk length, flower number and flower color intensity. Two cm^2 of the blade of young leaves of plants grown in the greenhouse were collected and analyzed using a flow cytometer (Plant Cytometry Services, Schijndel, The Netherlands) to determine the ploidy level of the plants.

Data analysis

The data shown represent the mean ± standard error (SE). In some experiments statistically significant differences were determined with the least significant difference test (LSD) at 0.05 level of probability.

Results

Factors affecting protoplast isolation

CEC, FEC and leaf tissues were incubated in 3 different enzyme mixtures with or without vacuum treatment. Table 1 shows that in all tested treatments, FEC gave a higher protoplast yield than did CEC or leaf tissue. Enzyme mixture C gave the highest yields, whereas enzyme mixture A gave the lowest yield of protoplasts. The combination of FEC culture with enzyme solution C yielded ca. 12.9×10^5 protoplasts/g FW, which was 2.7 times higher than that of the CEC and 160 times higher than that of leaf tissue.

Although there was a clear difference in yield, there was no substantial difference between CEC and FEC with regard to the viability of isolated protoplasts (Table 1). Protoplasts released from leaf tissue showed a low viability. Vacuum treatment produced approximately twice as many protoplasts as did non-vacuum treatment in both CEC and FEC. The vacuum treatment had no significant effect on the viability of the protoplasts.

Based on the above-described results, FEC was used in a series of experiments to assess the effect of temperature and duration of enzyme treatment on protoplast yield and viability. It was observed that protoplast yield increased with longer enzymatic digestion periods, while protoplast viability declined (Table 2). These results suggested that the optimum incubation time for obtaining a high protoplast yield is around 16 h. Table 3 shows that 24°C was the optimal temperature to produce a high yield of viable protoplasts.

Regeneration of plants from protoplasts

Protoplasts isolated from FEC were transferred to either agarose-solidified or a liquid PCA medium (Figure 1A). As Table 4 indicates, there were no significant differences in the viability of protoplasts, the time needed for the first division or micro-calluses formation. However, a higher frequency of cell division was obtained by employing liquid medium as compared to solid medium.

A higher number of FEC clumps were produced by the liquid medium than by the agarose-solidified medium 3 mo. after protoplast isolation (Table 4). The first protoplast division occurred within 3-4 d, and small colonies were observed microscopically after 2 wk of protoplast isolation (Figure 1B, C). Protoplasts underwent further division and formed cell clumps that consisted of 2-10 cells 10-15 d after culture on liquid medium and 13-20 d after culture on agarose-solidified medium (Figure 1D). After 1 wk of culture, significant differences were observed between the agarose-solidified and liquid medium. In agarose-solidified medium, browning occurred, which was not observed in liquid medium. Additionally, a higher frequency of cell division and a higher number of FEC clumps per 10^5 protoplasts were observed in liquid medium than in agarose-solidified medium.

When colonies reached a size of 1 mm in diameter, they were transferred to fresh medium, and 3-4 wk later micro-calluses of 2-4 mm were observed. The transition of micro-calluses to FEC required additional 4-8 wk. In some cases, FEC

developed directly from the surface of the micro-calluses. In other cases, CEC was first formed on the surface of the micro-calluses, and FEC clumps were then produced on the surface of CEC. A total of 375 FEC colonies were obtained from 10^5 protoplasts/ml in liquid medium and 225 colonies from 10^5 protoplasts/ml on solid medium.

FEC colonies were transferred to RM for somatic embryo production and germination (Figure 1E). Within 4-8 wk, 30-35% of FEC colonies produced somatic embryos. About 35-40% of germinated somatic embryos produced secondary embryos (Figure 1F), while the rest of them showed a swollen yellow globular structure. After 4-6 wk of culture on RM, 22-25% of the cultured somatic embryos germinated and formed either single or multiple shoots (Figure 1G). Shoots of 2.5-3 cm in length were transferred to rooting medium. Healthy roots were obtained within 4-5 wk. Plants were transferred to the greenhouse. In total, 45 plants from 15 protoplast clones were compared with *in vitro*-maintained control plants. The plants started to flower 11-13 wk after being transferred to the greenhouse (Figure 1H). There were no distinct differences in traits such as the number of flowers per stem, number of flower stalks, flower stalk length, or stem thickness between the protoplast-derived plants and the control plants. However, there was a substantial variation in morphological traits such as leaf shape, flower colour and shape. Figure 2A shows the typical leaf shape of *Alstroemeria* VV024 control plants. About 25% of the protoplast-derived plants had either or a sharp long leaf (Figure 2B) or a round type of leaf (Figure 2C).

Figure 2D shows a typical type of flower of a control *Alstroemeria* plant. Twenty percent of the protoplast-derived plants had flowers with a more intense colour than the control plants (Figure 2E). And in 35% of the protoplast-derived plants the colour of the flowers was less intensive (Figure 2F). Additionally, 2-3% of the plants regenerated from protoplasts were produced nearly white flowers (Figure 2G).

A typical flower of the control plants has 6 petals, whereas 20-30% of the protoplast-derived plants had 7 petals (Figure 2H) and 4% had 8 petals (Figure 2I). Moreover, strange shapes of flowers were found in protoplast-derived plants (Figure 2J, K). Black spots on the inner petal were observed more frequently in the protoplast-derived plants than in the control plants (Figure 2L). All 15 protoclones were evaluated for their ploidy level by flow cytometry. As compared with the control plants (Figure 3A), two of the 15 protoclones had similar DNA content

(Figure 3B). However, the rest thirteen of the 15 protoclones had lower DNA content as compared to the control plants (Figure 3C).

Discussion

In this study, plants were regenerated from *Alstroemeria* protoplasts. This culture system could be used for a variety of breeding purposes. Factors, including source of protoplasts, composition of the enzyme mixture, incubation period and temperature during enzyme treatment, and culture medium used after protoplast isolation, influenced the efficiency of protoplast isolation and culture. These factors were optimized for the development of a protoplast culture system.

Leaves with axil tissue were previously selected as the best explant for callus induction in *Alstroemeria* (Lin et al., 1997), and this tissue was also used as a source for protoplast isolation and culture. However, the efficiency of protoplast isolation from leaf tissue was low, whereas that from FEC was high. FEC also performed better in protoplast production than did CEC. This is probably due to the fact that CEC has a harder structure and because fewer cells are probably exposed to the enzyme solution. Because FEC proved to be the best source for protoplast isolation in *Alstroemeria*, it was used in further experiments. FEC has also been used successfully for protoplast culture in other crops such as Banana (Assani et al., 2001) and Cassava (Sofiari et al., 1998), and in ornamentals such as Iris (Shimizu et al., 1996) and Primula (Mizuhiro et al., 2001). Before treatment with an enzyme solution, FEC cultures were transferred to a pre-plasmolysis solution to avoid protoplast bursting from internal turgor.

An incubation period consisting of 16 h enzyme treatment increased the number of protoplasts and their viability in *Alstroemeria*. Luo et al. (1998) also observed that 12-16 h of enzyme treatment increased protoplast yield in *Astragalus adsurgens*. In mango, a 24-h enzyme treatment was used (Ara et al., 2000). In Iris (Shimizu et al., 1996), however, embryogenic calluses were treated with an enzyme solution for only 2-3 hr.

In addition to the explant source and incubation time, the enzyme combination used was also an important factor influencing protoplast yield and viability. A high concentration of enzymes negatively influenced the viability of the protoplasts. However, this was partly compensated by the higher yield of protoplasts.

Temperature is another main factor in the isolation of protoplasts. In general, protoplasts of many crops are obtained by incubation at 24-27°C. In our study, 24°C was the best temperature for protoplast yield and viability.

A liquid medium was better than an agarose-solidified PCA medium for further growth of isolated protoplasts, although in many crops agarose-solidified media are used. In this study, browning occurred more frequent in an agarose-solidified medium than in a liquid medium. This browning is probably caused by the oxidation of phenolic compounds, which are released from cultured plant cells into the medium (Saxena and Gill, 1986). In a liquid medium, these toxic compounds might be diluted, thus resulting in less browning than an agarose-solidified medium does.

Changes were observed in the morphological traits of plants regenerated from protoplasts. Larkin and Scowcroft (1981) first described this phenomenon and named it somaclonal variation (SV). They concluded that SV could be used in a breeding program as a new source of genetic variation. This term was also used to describe the phenotypic variation observed in plants derived from plant tissue or cell culture (Lee et al., 1999). During the tissue culture of many plants, SV is a common phenomenon (Kaeppler et al., 2000). Several morphological traits of the plants regenerated from protoplasts of *Alstroemeria* were examined to determine whether protoplast-derived plants varied from control plants. Variation was observed in the morphology of the flower and the leaves. This variation was both positive and negative. In conclusion, our results show that FEC of *Alstroemeria* is the best source for protoplast isolation and culture, as shown for other crops. Although SV has been detected in the morphology of protoplast-derived plants, the system presented here should accelerate the use of somatic hybridization by protoplast fusion to develop genotypes that can be used as parents for an *Alstroemeria* breeding program.

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Table 1. Comparison of protoplast yield and viability between different explant sources of *Alstroemeria* VV024 (FEC: friable embryogenic callus; CEC: compact embryogenic callus).

		Source					
Enzyme[1]	Vacuum	FEC		CEC		Leaf	
		Yield[2]	Viability (%)	Yield[2]	Viability (%)	Yield[2]	Viability (%)
A	YES	3.5±0.8*	65.1±6.5	1.8±0.2	75.0±6.7	0.01±0.0	5.0
A	NO	1.9±0.5	46.2±3.3	0.5±0.1	72.5±5.5	0.0±0.0	N.A
B	YES	5.8±1.1	48.5±5.1	2.5±0.3	64.0±9.4	0.2±0.0	2.5
B	NO	3.4±1.3	45.1±3.3	2.0±0.4	52.5±3.3	0.3±0.0	7.5
C	YES	12.9±2.4	55.5±8.4	4.8±0.9	57.5±4.8	0.8±0.2	3.3
C	NO	7.4±1.5	61.5±9.9	2.2±0.3	55.0±12.5	0.5±0.1	N.A

[1] Enzyme solutions: A=Cellulase Onozuka R-10 1%, Macerozyme 0.1%, B=(Cellulase Onuzuka R-10 2%,
Macerozyme 0.2%), C=Cellulase Onozuka R-10 4%, Driselase 0.5%, Macerozyme 0.2%
[2] $\times 10^5$ protoplasts per 1 g FW.
*: Means±SE of 4 replicates.

Table 2. The effect of different periods of enzyme treatment on protoplast yield and viability of *Alstroemeria* VV024.

	Incubation time (h)					
	4	8	12	16	20	24
Protoplast yield[1]	0.5±0.1d	4.1±0.6c	9.5 ±2.5b	14.4±2.2ab	15.1±4.2a	19.2±5.8a
Viability (%)	85.1±3.7a	76.5±5.9a	72.5±6.0a	62.3±9.9b	55.1±7.5b	42.3±3.1c

[1]: $\times 10^5$ /g F.Wt. callus,
Means±SE of 4 replicates; same letter within rows indicates no significant difference (0.05).

Table 3. The effect of temperature during enzyme treatment on protoplast yield and viability of *Alstroemeria* (enzyme solution C=Cellulase R-10 4%, Driselase 0.5%, and Macerozyme 0.2%).

Source	Temperature					
	18		24		30	
FEC	Yield[1]	Viability (%)	Yield[1]	Viability (%)	Yield[1]	Viability (%)
	1.4±0.4b[2]	22.5±7.7b	18.4±5.9a	77.5±14.5a	10.5±3.3ab	35.0±9.4b

[1]$\times 10^5$ /g F.Wt. callus,
Means±SE of 3 replicates; same letter in one column indicates no significant difference (0.05).

Table 4. Differences in viability, cell division and micro-calluses formation of protoplasts cultured on agarose-solidified or liquid medium.

Medium	Viability (%)	First division (Days)	Division frequency (%)[1]	Micro-calluses formation (%)[2]	# of FEC clumps / 10^5 protoplasts
Agarose	65.0±4.5a[3]	3	24.7±2.4b	34.0±5.7a	225b
Liquid	71.5±8.4a	4	39.5±7.1a	42.5±8.2a	375a

[1] Division frequency: # protoplasts dividing/total # protoplasts) × 100
[2] Micro-calluses formation: # visible micro calluses (2-4 mm)/200 micro colony) after 4 wk of culture
Means±SE of 3 replicates; same letter within columns indicates no significant difference (0.05).

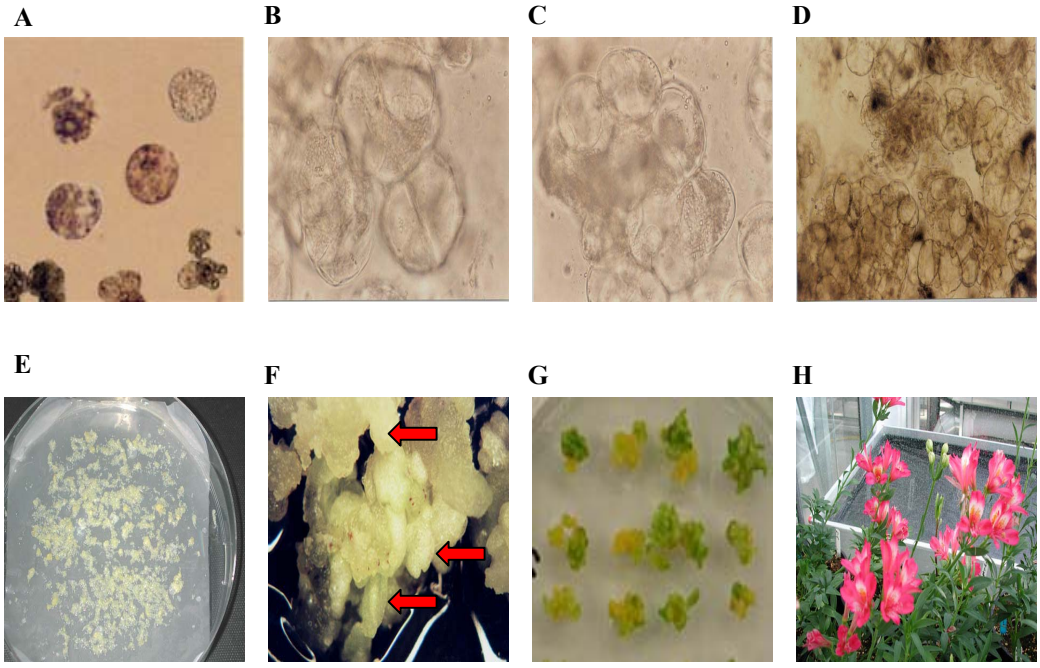
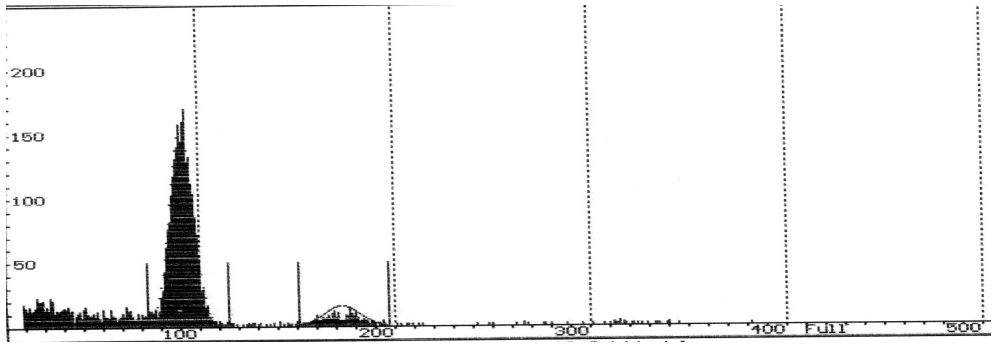


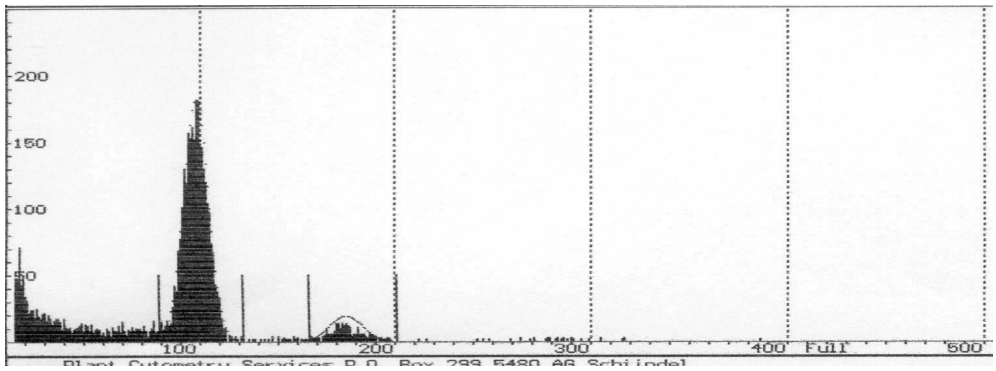
Figure 1. General overview of protoplast culture and regeneration procedure developed for *Alstroemeria*. A) Isolated protoplasts from friable embryogenic callus, B) first cell division of protoplasts, C) cell clump formation, D) micro-calluses formation 2 wk after culture, E) formation of FEC, F) somatic embryo initiation (red arrows) and germination, G) regenerated plants from protoplasts 16-18 wk after culture, H) flowering plants in greenhouse

Figure 2. Variation in morphology of leaf and flower in protoplast-derived *Alstroemeria.* A) Leaf of control plants, B) long type of leaf, C) round type of leaf, D) flower of control plant, E) flower with intense color, F) flower with weak color, G) albino-like flower, H) flower with 7 petals, I) flower with 8 petals, J) aberrant shape of flower, K) another aberrant shape of flower, L) flower with higher number of black spots on inner petal than in control plant.

A) Control plant (VV024)



B) Protoclone –1



C) Protoclone-2

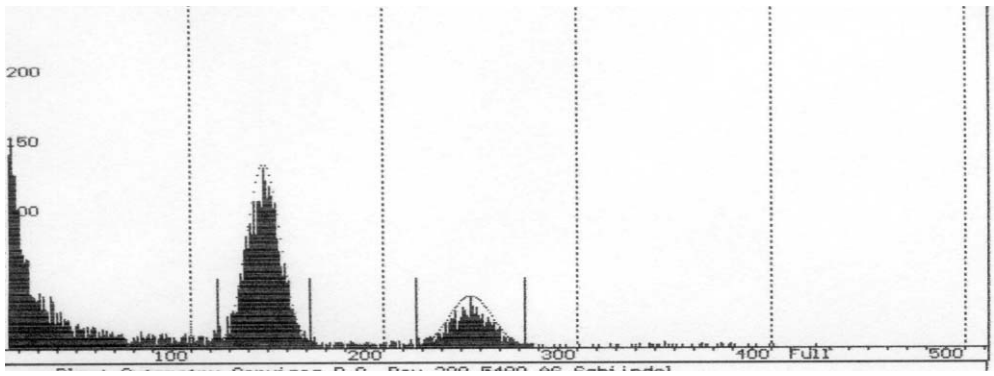


Figure 3. Histograms of flowcytometric analysis of *Alstroemeria* plants from control (A) and protoplasts-derived plants (B and C)

CHAPTER 4

Production of transgenic *Alstroemeria* plants containing virus resistance genes

In preparation for submission

Production of transgenic *Alstroemeria* plants containing virus resistance genes

Kim. J. B[1,2] · C. J. J. M. Raemakers[1*] · R. A. A. Van der Vlugt[3] · I. Bouwen[3] · E. Jacobsen[1] · R. G. F. Visser[1]

[1]Laboratory of Plant Breeding, The Graduate School of Experimental Plant Sciences Wageningen University, P.O.BOX 386, 6700 AJ, Wageningen, The Netherlands

[2]Current address; Turfgrass & Environment Research Institute, Samsung Everland Inc., #1 Bu-kok, Gunpo, Kyong-ki, 435-737, Korea,
E-mail address: jb1011.kim@samsung.com

[3]Plant Research International B.V., P.O.BOX 16, 6700AA, Wageningen, The Netherlands

[*]Author for correspondence: e-mail - krit.raemakers@wur.nl
Telephone: +31-317-484038, Fax: +31-317-483457

Abstract

Alstroemeria plants were transformed with viral sequences [coat protein (CP) gene and 3'-nontranslated region (3'-NTR)] of *Alstroemeria mosaic virus* (AlMV) using an improved particle gun-mediated transformation system. Friable embryogenic callus (FEC) induced from the leaves with axil tissues of *Alstroemeria* plant was used as target tissue. As a result, 2 bombardments with a pre- and post bombardment culture (8 hr before and 16 hr after transformation) resulted in the greatest efficiency of transformation and recovery of transgenic lines.

FEC was transformed with the viral sequence to induce RNA-mediated resistance and the *bar* gene as a selectable marker. In the initial stage, more than 300 independent transgenic callus lines were obtained. Then, the first 155 PPT-resistant FEC lines were selected within 2-3 weeks. After stringent PPT selection, 44 transgenic lines remained from 155 PPT-resistant FEC lines. Around 700 somatic embryos from 44 independent transgenic lines developed into shoots, which were transferred to the greenhouse were they were challenged with the virus.

After challenging with AlMV, 25 lines from a total of 44 transgenic lines showed some degree of virus resistance. PCR analysis confirmed the presence of the viral sequence and the *bar* gene. The phenomenon of virus resistance was observed at different levels when transgenic *Alstroemeria* plants containing the viral sequences were evaluated for infection after challenging with AlMV. Establishment of an efficient transformation system in *Alstroemeria* will allow the insertion of transgenes to acquire resistance to viral and fungal pathogens. This is the first report on the production of transgenic virus-resistant *Alstroemeria* plants, opening a new alternative for the control of virus diseases in *Alstroemeria*.

Keywords *Alstroemeria* · *Alstroemeria Mosaic Virus* · Friable embryogenic callus · GUS · Luciferase · Particle bombardment · Regeneration · Somatic embryo · Virus resistance

Introduction

Alstroemeria is a popular ornamental, grown for the cut flower industry or as indoor potted plants. *Alstroemeria* plants are primarily vegetatively propagated, therefore, viral infections are a serious concern. Viruses have caused considerable losses in both yield and quality in many crops (Gielen, 1995). As a rule, strategies for the control of viral diseases are control of vector groups by the use of insecticides, meristem culture, phytosanitary cultural practices and introduction of resistant cultivars. Unfortunately, each of the above has its problems (Dasgupta et al., 2003).

Although several reports have appeared describing the attempts to produce virus-free *Alstroemeria* plants using a meristem culture (Chiari and Bridgen, 2002), the presence of viruses continues to be an important problem in *Alstroemeria* species, as meristem-derived *Alstroemeria* plants can be re-infected during harvest or transport. A number of viruses are currently considered critical in *Alstroemeria*. Examples include *Alstroemeria carla virus* (AlCV), *Alstroemeria mosaic virus* (AlMV), *Cucumber mosaic virus* (CMV*), Tomato spotted wilt virus* (TSWV), *Alstroemeria streak virus* (AlSV) and *Impatiens necrotic spot virus* (INSV), as described by Van Zaayen (1995).

Of these viruses, AlMV is considered to be the most endemic and is difficult to detect and prevent in *Alstroemeria* culture. AlMV is a species of the potyvirus genus in the family *Potyviridae*, one of the largest and most widely distributed families of plant viruses. The potyvirus genus is known for its ability to cause severe damage in a large variety of crops (Hollings and Brunt, 1981). Virus symptoms vary depending on virus strain, variety of *Alstroemeria* infected and environmental factors present. A typical symptom of AlMV is the streaking of leaves, light green or darkened elongated spots, which develop along the veins and flower-breaks (Chiari and Bridgen, 2002).

Similar to other crops, no effective physical or chemical treatments to cure plants from virus diseases have been reported for *Alstroemeria*. This is mostly due to three critical drawbacks in the development of virus-resistant *Alstroemeria* plants. The first drawback is the difficulty in detecting viral symptoms, which results in many breeders and farmers to have infected mother stock. The second drawback is the re-infection of previously virus-free *Alstroemeria*, produced by meristem culture. Re-infection may occur, during propagation activities through

rhizome splitting, harvest, transport and vector activity. The third drawback is the limited number of resistant genotypes in the germplasm collection, which restricts the number of potential crosses for the generation of elite varieties. Due to these complications, a virus-resistant *Alstroemeria* cultivar has not yet been developed.

Potential resistance to plant viruses can be provided by either expressing part of the viral genome or virally associated sequences, now known as pathogen-derived resistance (PDR) (Satyavathi et al., 2005). PDR can be divided into two groups: Coat protein-mediated resistance (CPMR) in which the production of coat protein results in resistance, and RNA-mediated resistance in which the accumulation of the viral sequences leads to resistance (Satyavathi et al., 2005). CPMR might confer incomplete resistance to a wide range of virus, whereas, RNA-mediated resistance might confer substantially higher levels of virus resistance to a narrower range of viruses (Beachy, 1997).

Despite the numerous strategies for developing transgenic virus resistant plants, there has been only one clear commercial success: the enhanced virus-resistance in papaya by stable expression of a viral gene (papaya ring spot virus [PRSV]) (Campbell et al., 2004).

In order to introduce the PDR concept into an *Alstroemeria* breeding program, an efficient and reliable genetic transformation system is required. In addition to the virus-resistance trait, genetic transformation systems may provide opportunities to improve other agriculturally important traits such as bacteria or fungal disease resistance and abiotic stresses in breeding stocks or directly in elite genotypes. Due to these advantages, genetic transformation is regarded as a promising tool in agricultural breeding programs. It may also lead to the simultaneous introduction of multiple genes (Campbell et al., 2004), thereby becoming important in monocot breeding programs for vegetatively propagated ornamentals such as *Alstroemeria*, crocus, gladiolus, lily, narcissus and tulip.

The primary objective for this study was to further optimize the particle gun-mediated transformation system by application of osmotic treatments and re-transformation with two different two constructs. The second objective is the production of successful transgenic virus resistant *Alstroemeria* plants.

Materials and methods

Plant material

Friable embryogenic callus (FEC) induced from *Alstroemeria* leaves with axil tissues (VV024, tetraploid cultivar from Van Staaveren B.V, The Netherlands) was used in the experiments as described in Chapter 2. FEC cultures were refreshed every four weeks on PCA medium (Sofiari, et al., 1998) in the dark at 18˚C. The pH was adjusted to 6.0 prior to autoclaving at 121˚C and 120 kPa for 15 min, unless stated otherwise.

FEC was selected as described in chapter 2, in order to perform particle bombardment. For the particle bombardment, 6-month-old FEC was used in all experiments unless otherwise stated.

Plasmids for optimizing particle bombardment, co-transformation and virus resistance experiments

To optimize the results of particle bombardment and co-transformation experiments, pAHC25 and PBL9780 were used. The plasmid pAHC25 (Christensen and Quail, 1996) contains the *uid*A gene coding for β-glucuronidase (GUS) and the *bar* gene coding for resistance to phosphinotricin (PPT), both under the control of the maize ubiquitin promoter (Figure 1A). The plasmid PBL9780 contains the *luc* gene coding for luciferase gene and the *bar* gene coding for resistance to PPT was constructed by ligating plasmid pAHC18 and pAHC25 (Christensen and Quail, 1996), together after restricting them with *AatIII* and *SstI*.

Total DNA was isolated from an *Alstroemeria* plant infected with AlMV. Using this total RNA, first strand cDNA-synthesis was initiated using general oligo-dT primer P5902 (Van der Vlugt et al., 1999). Then, cDNA was amplified with primer combination AlMV-UP02 (upsteam) and P9502 (downstream) (Table 1). This generated a DNA fragment of approximately 1,300 bp which was isolated from agarose gel and cloned in a pGEM-T vector. This resulted in plasmid pAlMV-PV, which was used to amplify the viral coat protein (CP) and 3’ non-translated region (3’-NTR) fragment from pAlMV-PV. At the 5’-end of each of these two primers a short non-virus specific sequence was included to facilitate cloning of the PCR fragment in plant transformation vector pAHC25. For AlMV CP-UP2, this

sequence consists of a *SacI* recognition site (GAGCTC) preceded by an extra three nts (TTA). For AlMV Tot-DW, the non-virus specific part consisted of a GGG triplet to reconstruct a *Sma1* cleavage site upon directional ligation of the PCR-fragments in the *SacI* / *Sma1* digested pAHC25 vector. Using these two primers in combination, both the CP and 3'-NTR of AlMV was amplified from pAlMV-PV and checked for the expected size by agarose gel elctrophoresis. PCR-DNA was subsequently purified using the Boehringer High Pure PCR product purification Kit. Purified PCR-fragment was digested with *SacI* and again purified with the Pure PCR columns. RFLP analysis using restriction enzymes *DdeI*, *NsiI* and *BstOI* confirmed the integrity of the PCR fragment.

Vector pAhC25 was digested with *SmaI*/*SacI* to remove the GUS gene (pos. 2022-3910) and ligated with the AlMV CP-3'NTR PCR-fragment with an expected size of 1258 nts yielding plasmid pXYZ000 (Figure 1B). The resulting plasmid was transformed to *E. coli* JM109. Recombinants were checked for the presence of the desired insert by PCR directly on bacterial cells and subsequent gel electrophoresis. DNA from p XYZ000 was used for plant transformation.

DNA coating and particle bombardment process

The Promega Wizard Midiprep DNA purification kit was used to isolate plasmid DNA. The final DNA concentration was 1.1 μg/ml in sterilized distilled water. Twenty-five μg of plasmid DNA was mixed with 15 mg gold particles (size 1.6 μm). The centrifuge tube was vortexed for 1 min and centrifuged at 11,000 rpm for 10 seconds in an Eppendorf centrifuge. The following ingredients were then added individually: 30 μl 5 M NaCl, 5 μl 2 M Tris-HCl (pH 8.0), 100 μl 0.1 M spermidine, 100 μl 25% PEG1550, 100 μl 2.5 M $CaCl_2$, and 965 μl sterile double-distilled water. After centrifugation, the coated gold particles were washed with 1ml sterilized water, centrifuged again, followed by resuspension of the pellet om 10 ml 100% ethanol. Finally, 163 μl of suspended DNA-coated gold particles were pipetted on a macrocarrier (BioRad, California, U.S.A) and used for bombardment.

For each bombardment, approximately 200 mg of FEC clumps grown on PCA medium were spread evenly in a circle with a 2.5 cm diameter in the center of a Petri dish (60×15 mm) containing PCA medium. The dishes were placed in the vacuum chamber of a Biolistic Delivery System (PDS-1000/He, BioRad, California, U. S. A), 5 cm from the stopping plate. The helium pressure was set at

1350 PSI with a partial vacuum of 28 inch Hg. After the FEC was bombarded, the clumps were transferred to a selection medium (SM) consisting of Murashige and Skoog (1962) (MS) basal salts with vitamins, 0.5 mg/l BA, 30 g/l sucrose, 2.75 g/l Gelrite (pH 6.0) and 20 mg/l PPT.

Optimizing the process of particle bombardment

A number of experiments were designed without the virus construct but with plasmids pAHC25 and PBL9780 to optimize the particle bombardment procedure. First, the effect of different shooting positions on transient *luc*/*gus* gene expression was studied. Tissue was bombarded either once or twice, with the Petridish rotated 90° after the first bombardment. Next, to determine the transient gene expression and transformation efficiency, luciferase and GUS assays were performed 4 weeks after bombardment. Data on the number of transgenic lines per 100 mg of FEC was recorded 8 weeks after transformation.

In the second set of experiments, the effect of osmotic treatment was tested. Bombarded FEC cultures were transferred to SM supplemented with 0.2 M mannitol under the following culture regimes: A) osmoticum 4 hr before, and 8 hr after transformation; B) osmoticum only 4 h before; C) osmoticum only 8h after; D) osmoticum only 8 h before and 16 h after; E) osmoticum 8 h before; F) osmoticum 16 h after; Control) no osmoticum in the SM)). After the second and eighth week of bombardment, gene expression was measured.

In another set of experiments, the different osmotic treatments were compared as follows: medium I containing 0.2 M mannitol; medium II containing 0.2 M sorbitol; medium III containing 0.2 M mannitol and 0.2 M sorbitol. FEC cultures were transferred to the above-indicated media and cultures for 8 h prior to bombardment or 8 h prior and 12 h after bombardment. For this experiment, 96 randomly selected FEC clumps (ca 1-1.5 mm in diameter) were placed on SM medium prior to the described treatment. For each treatment, 5 petridishes (90 × 15 mm, Greiner) were bombarded.

GUS assay

GUS gene expression was assayed by incubating the samples in 5-bromo-4-chloro-3-indoyl-D-glucuronic acid (X-Gluc) solution for 16h at 37˚C,

supplemented with 10 mM EDTA and 0.1% Triton X-100. GUS assay was performed at 7, 14, 30 and 50 days after bombardment.

Luciferase assay

Bombarded FEC and somatic embryos were assayed 7, 14, 30, and 50 days after bombardment. To verify the expression of the luciferase gene, FEC and somatic embryos were sprayed with 0.15 mg/l of luciferin aqueous solution, placed in a dark room and measured by a luminometer equipped with CCD camera (Hamamatsu, Japan), linked to a personal computer. The amount of photons generated by transformed material was recorded automatically. After each measurement, FEC and somatic embryos were transferred to fresh medium for further growth and development.

Development of transgenic AlMV resistant *Alstroemeria* plants

In this experiment, six-month-old FEC cultures were used for particle bombardment. Ten days before particle bombardment, approximately 200 mg of fast-growing yellow FEC on PCA medium (9 cm Petri dish) were broken into small pieces (*ca*. 2-5 mm), and placed in a 2.5 cm diameter circle on a Petri dish. Two days after bombardment, FEC cultures were transferred to regeneration medium (RM). Ten days later, cultures were transferred to RM supplemented with 20 mg/l PPT. Yellow, fast-growing FEC calli were dissected into small pieces for stringent selection with PPT. The selected FEC cultures were grown at 18˚C under a 16 h /8 h (light/dark) photoperiod for 2-4 months and transferred to fresh RM at regular monthly intervals. Transgenic shoots were produced from the FEC culture and transferred to the same selective RM medium. Shoot clusters developed into plantlets 6-8 months after transformation. Putative transgenic plants were planted in soil in the greenhouse and maintained at 15-23˚C (day/night).

Selection and regeneration of bombarded FEC and somatic embryos by using the *gus* gene, luciferase gene, and PPT as a selection marker

For pAHC25 transformation, immediately after bombardment, FEC and somatic embryos were placed on SM as described before. Four or five days after

bombardment, they were assayed for GUS activity. It was repeated 15, 21, 50 and 100 days after bombardment.

For PBL9780 transformation, immediately after bombardment, bombarded cells were transferred to SM (selection medium), supplemented with Murashige and Skoog (1962) (MS) basal salts with vitamins, 0.5 mg/l BA, 30 g/l sucrose, 2.75 g/l Gelrite (pH 6.0) and 20 mg/l PPT. A week after bombardment, they were assayed for luciferase activity. Then, only the luciferase positive clumps were selected and transferred to fresh SM for further growth. This procedure was repeated 15, 21, 50 and 100 days after bombardment. Transformation efficiency (%) was measured by dividing the number of luciferase positive clumps with the total number of bombarded tissues. A luciferase-positive clump obtained 4 weeks after transformation was regarded as an individual line and was transferred to SM for regeneration. All transgenic lines were refreshed by a 4-week interval unless stated otherwise.

During the selection process, concentrations of PPT in SM varied. In the first subculture one week after bombardment, *gus* or *luc* positive transgenic lines were placed on SM containing 20 mg/l PPT. In the first and second round of subculture, vigorously growing light yellow FEC was selected and transferred to fresh SM. For the third and fourth round of subculture, the concentration of PPT was decreased to 10 mg/l. At this time, most of the non-resistant clumps had died. In the final round of subculture approximately 5-6 months after bombardment, surviving clumps, which were still *gus*/*luciferase* positive were transferred to SM without PPT and they developed into plantlets within 4-6 weeks.

Molecular characterization of regenerated transgenic lines

Polymerase chain reaction (PCR) analysis was conducted to demonstrate the presence of specific *bar* gene sequences and viral sequences in putatively transformed plant tissue. Genomic plant DNA was extracted from 250 mg of the regenerated putative transgenic lines as described by Lin et al. (2000). DNA (2 μg) from transgenic *Alstroemeria* plants was analyzed by PCR reaction. The forward primer sequence used in the PCR analysis for detecting the *bar* gene (244 bp) was 5'-CGCAGGAACCGCAGGAGTGGAC-3', and the reverse primer was 5'-CTCTTGAAGCCCTGTGCCTCCA-3'. To detect the viral sequence (CP+3'-NTR; 1258 bp), the forward primer was 5'-

*TTAGAGCTC*GAGTTGGACGATGATTTTGA-3', and the reverse primer was 5'-*GGG*TGAGTCACCGTAACGATAGAAT-3' (see Table 1). Reactions were carried out using 30 pmol of each primers, 300 μM dNTP, 0.25 units *Taq* polymerase in 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.3. The PCR program is 94°C initial melting for 5 min, followed by 35 cycles of 94°C for 30 sec / 51°C for 30 sec, 72°C for 30 second. The final step was was an extension cycle at 72°C for 7 min.

Preliminary test to optimize the AlMV inoculation conditions

The effects of the concentration of AlMV inoculum and an appropriate frequency of inoculation were investigated to obtain a sufficient high infection rate with minimal damage to *Alstroemeria* plants. Before inoculation, plants were dusted with 400-mesh carborundum powder. When the inoculum was prepared by grinding 5 systemically virus-infected leaves of *Nicotiana benthamiana* with a solution of 10 ml 0.3 M phosphate buffer (pH 7.0), it was marked as 1×. When 5 ml of phosphate buffer solution was used, it was marked as 2×. For the preparation of inoculum, one-week old AlMV-infected leaves of *N. benthamiana* were used.

Further, an additional (2^{nd}) inoculation was performed 4-5 days after the first inoculation on the same leaf inoculated in the first round. The third and the fourth inoculation were repeated at the same interval after each inoculation on the same leaf.

Virus inoculation of transgenic *Alstroemeria* plants in the greenhouse

After the preliminary experiment, *N. benthamiana* (as a positive control; 5 plants), non-transformed *Alstroemeria* (5 plants; as a negative control) and transgenic *Alstroemeria* plants were mechanically inoculated using carborundum powders (400 mesh) and the sap of AlMV-infected *Nicotiana benthamiana* leaves (2× concentration: 5 ml of phosphate buffer solution). Five to ten minutes after inoculation, the infected *Alstroemeria* plants were washed thoroughly with tap water to prevent the carborundum powder from drying.

For the first inoculation of transgenic plants, the inoculum was used at 5 ml of phosphate buffer solution per 10 young *Alstroemeria* plants (at the 8-10 full leaf stage). The second inoculation was carried out 5 days after the first inoculation

(day zero: 0 dpi). All 44 putative transgenic lines were inoculated twice. Whereas, the third inoculation was performed for 17 transformed lines out of 44 transgenic lines 5 days after the second inoculation, to elucidate the relationship between an additional inoculation and the level of resistance in the putative transgenic *Alstroemeria* plants.

To detect the presence of AlMV and the transcription expression of the AlMV transgene, in the inoculated *Alstroemeria* plants polyclonal antibodies against the AlMV were used. DAS-ELISA (Double Antibody Sandwich –Enzyme-Linked Immunosorbent Assay) was carried out, according to a modified protocol (Clark and Adams, 1977). Absorbance at 405 nm was taken 30 min using a microplate autoreader ELISA EL-808 (Bio-TEK Instruments, Winooski, USA). All inoculated plants were checked for the presence of virus by DAS-ELISA after 2, 4 and 6 weeks after inoculation. Further, samples were recorded as ELISA-positive when their absorbance value was at least two times greater than the mean absorbance value of healthy control plants, as described by Lines et al. (2002).

Statistical analysis

The data are presented as the mean ± standard error (SE) and analyzed using least significant difference (LSD) test (P=0.05) for multiple comparisons (SPSS for Windows version 10.0' statistical software).

Results

Optimization of particle bombardment

In the first experiment, FEC samples were bombarded once or twice with either pAHC25 or PBL9780. The results of a single bombardment were compared to the results of a double bombardment with a 90° rotation. The distance to objects (5.5 cm), the helium pressure (25 Hg), and the gas pressure for the micro-carrier (1350 psi) were set according to the procedures described by Lin et al. (2000). Transient luciferase and GUS expression were detected at high levels, respectively (Figure 2A and B). In the case of plasmid pAHC25, 7.5% of the twice-bombarded FEC clumps showed blue foci, whereas the clumps with single bombardment showed only 2.3% (Table 2). Additionally, a 90° rotation with double bombardment led to

a higher frequency (6 times) of luciferase gene expression in PBL9780 than the control treatment. After 8 weeks of bombardment, more than 60 independent transgenic lines were obtained for pAHC25 and nearly 150 independent transgenic lines were obtained for PBL9780, all of which were resistant to PPT (Figure 2C) and demonstrated either GUS or luciferase activity.

In the second experiment, FEC cultures were exposed to 7 different culture periods with osmotic treatment (0.2 M mannitol). The highest transient gene expression was obtained in regime D (8 h before and 16 h after transformation) in both pAHC25 and PBL9780 (Figure 3). Compared with the control, at least three times more GUS foci and photons were observed in regime D.

In the next experiment, bombarded FEC cultures were transferred to SM medium with three different osmotic treatments (A: 0.2 M mannitol; B: 0.2 M sorbitol; C: 0.2 M mannitol and 0.2 M sorbitol) for 8 h prior to and for 16 h following bombardment. Osmotic treatment combinations were then compared with the control. High numbers of transient and stable transgene expressions were observed for treatment C, in which 0.2 M mannitol and 0.2 M sorbitol were used in the osmotic pre-culture (Table 3).

According to Table 3, the combination of mannitol and sorbitol in osmotic culture resulted in the highest transformation efficiency in both pAHC25 (8.5%) and PBL9780 (14.5%). This osmotic treatment (treatment C) led to less browning than present in the other treatments and had a positive effect on the recovery of transgenic lines. In the control treatment, only 10% of the FEC clumps produced somatic embryos. However, by using 0.2 M mannitol and 0.2 M sorbitol, the frequency of somatic embryos increased to 36.5% (pAHC25) and 22.9% (PBL9780). Of the somatic embryos produced, at least 60% germinated (Figure 2D).

Production of virus-resistant plants through particle bombardment

The optimized bombardment protocol previously described in this chapter was used to produce plants containing virus-resistance genes. From sixteen independent transformation experiments with pXYZ000, 357 independent transgenic callus lines were obtained. These lines proliferated well on selection medium (SM) containing various PPT concentrations for 6-8 months following bombardment. After this series of subcultures, resistant pieces of FEC were separated from

necrotic tissue. The first PPT-resistant FEC cultures (155 lines) were observed 2-3 weeks after bombardment.

Putative transgenic somatic embryos were produced on SM from bombarded FEC cultures 5-6 weeks after transformation. After further growth, they were allowed to germinate for an additional 4 weeks on regeneration medium (Materials and methods). When PCR analysis was conducted to calculate the proportion of transgenic lines in all subcultured PPT positive transgenic callis lines on SM, at least 70% of the lines on SM showed positive result by PCR (data not shown). Stringent PPT-based selection of transgenic lines was completed 8 months after bombardment.

Following this stringent selection procedure, 44 putative transgenic lines were produced and transferred to PPT-free regeneration medium (RM). From these 44 lines, a total of 849 germinated embryos (GE) with a couple of shoots were obtained after 4-5 months of culture on PPT-free RM. For further growth and development, 710 healthy GE with two or more additional shoots were transferred to rooting medium to initiate root formation. After 4-5 weeks of culture on rooting medium, plants had healthy roots and they were transferred to the greenhouse 9-10 months after transformation. Here, they were acclimatized and allowed to grow until the 8-10 leaf stage and then challenged with AlMV.

Only 35% (248 out of 710 from 44 independent transgenic lines) of the transgenic *Alstroemeria* plants survived transfer to the greenhouse. The remaining 65% (462 out of 710) transgenic plants died due to contamination by fungal disease (40%: 189 out of 462) or lack of root formation (60%: 273 out of 462). Three to four months after transplantation to the greenhouse, approximately 7 % of the transgenic plants in the greenhouse flowered.

Preliminary test for the virus inoculation procedure

Alstroemeria plants were infected after the top three to four young leaves had been mechanically inoculated three or four times with the AlMV inoculum. In preliminary experiments, there was no significant difference between different time points in the level of virus infection done in September and that done in December 2001 (data not shown). However, twice the concentration of virus inoculum with two rounds of inoculation resulted in sufficient infection and less physiological damage to the plant itself (Table 4). This protocol was used in all experiments with

the transformed *Alstroemeria* plants. Nearly 90% of the control plants (9/10) were ELISA positive at 14 dpi and 100% at 28 dpi. The ELISA positive plants exhibited typical symptoms of AlMV infection such as yellowing in the tip of leaves in the beginning and finally necrosis with light greenish-yellow (or white) streaks or sections.

Characterization of virus resistance in transgenic *Alstroemeria* plants

Virus inoculation was performed with 150 transgenic *Alstroemeria* plants derived from 44 independent transgenic lines in the greenhouse, as described in the materials and methods section. Non-transformed *Alstroemeria* plants infected with the virus were used as a positive control (PC) and non-transformed plants without contact with the virus were used as a negative control (NC). Moreover, *N. benthamiana* was also used as a positive control. The AlMV-infected control *Alstroemeria* plants and *N. benthamiana* produced a highly ELISA positive value (>2.0), while the negative control plants showed a very low value. When ELISA OD^{405nm} readings of either transgenic or non-transgenic plants were two times larger than that of the non-infected control plant, they were regarded susceptible. ELISA OD^{405nm} values in the ELISA readings for inoculated transgenic *Alstroemeria* plants ranged from 0.08 to 1.28, whereas it was 2.0 for the positive *Alstroemeria* control plants and 3.23 for *N. benthamiana*. Transgenic lines were recorded as described in materials and methods section, depending on OD^{450nm} readings [susceptible (ELISA positive): measured value > 0.230; resistant (ELISA negative): measured value <0.230]].

Most of the infected plants showed no visible symptoms until 14 dpi (dpi: day post infection), and 30 out of 44 lines were regarded as resistant based on the ELISA OD^{405nm} readings. The 10 most resistant and 10 susceptible lines were subsequently selected and maintained for another 4 weeks without additional inoculation. At 56 dpi, these lines were again evaluated for the presence of AlMV by ELISA assay. As a result, only 5 lines (line 8, 10, 11, 19 and 20) were still resistant, whereas the rest of the 10 most resistant lines proved infected with AlMV 8 weeks after inoculation (Figure 4).

With regard to the symptoms, there was also no positive correlation between the level of virus-resistance and symptom expression. Seven susceptible lines showed the symptoms as demonstrated in Figure 5A. The rerst three susceptible lines

showed an intermediate growth between figure 5B and 5C (data not shown). However, negative control plants showed vigorous growth (Figure 5B) and positive control plants showed weak growth (Figure 5C). Infected *N. benthamiana* showed yellowing and partly necrosis of leaves (not shown). Ten to twelve weeks after inoculation, approximately 60% of the inoculated transgenic *Alstroemeria* plants were dead for unknown reasons, although they grew well in the first 4-6 weeks after inoculation. Moderate morphology and growth of transgenic line as seen in Figure 5C, was also observed during the virus challenging. The rest 40% of trangenis *Alstroemeria* plants were survivided more than 20 weeks after inoculation.

PCR analysis was performed to determine the presence of the *bar* gene as a selectable marker and the viral sequence (Figure 6A: the viral sequence, 6B: the *bar* gene). Transgenic plants that yielded DNA products of corresponding size were considered PCR positive.

Discussion

Although conventional breeding has contributed a lot to ornamental breeding, the demand for genetic transformation techniques is present to alter single traits in many ornamental crops. The success of genetic modification is influenced by several factors, such as available promoter and gene cassettes for proper gene expression, explant source, gene transfer method, selection procedure and regeneration capacity (Hiei et al, 1997; Smith and Hood, 1995). Since Sanford et al. (1987) developed particle bombardment, successful transformations via a particle gun have been carried out in cymbidium (Yang et al., 1999), gladiolus (Kamo et al., 2000), lily (Watad et al., 1998), sugarcane (Butterfield et al., 2002), and tulip (Wilmink et al., 1992).

The effect of re-transformation after rotating the Petri dish by 90° and the effect of osmotic treatment were critical in optimizing *Alstroemeria* genetic transformation. Firstly, the effect of the second bombardment, during which the position of the Petri dish had been changed, increased transformation efficiency. Ultimately, this re-transformation requires fewer explants for bombardment. When 100 mg FEC was bombarded with pAHC25 plasmid by using this “rotating” method, more than 100 transgenic lines with stable gene expression could be

produced 6 weeks after transformation. This efficiency is higher than that of presently available transformation system in *Alstroemeria*.

Secondly, in spite of the negative influence of osmotic treatment on transient gene expression in broccoli (Puddephat et al., 1999), osmotic treatments were effective in transient gene expression in *Alstroemeria*. In sugarcane, pre-incubation of callus cultures on a medium supplemented with 0.2 M mannitol and 0.2 M sorbitol for 4 h enhanced transformation (Bower et al., 1996). Similar results were observed in peach (Ye at al., 1994), tobacco (Russel et al., 1992), and maize (Frame et al., 2000). This might be due to the fact that osmotic treatment is attributed to play a role in the plasmolyzing cell before bombardment (Vain et al., 1993) and that turgor pressure is decreased, which might prevent plant cells from bursting (Frame et al., 2000). Osmotic treatments also stabilize cell membranes to enhance the recovery of lesions produced by the particle bombardment process (Russel et al., 1992). Additionally, pre-treating FEC cultures with osmoticum (8 h, in this experiment) are believed to initiate plasmolysis of the plant cells so that fewer cells are damaged during the particle bombardment process (Vain et al., 1993). Less browning of FEC cultures in our experiments showed there was less damage to bombarded tissues; something which was also observed in triticale (Zimny et al., 1995). Moreover, a more stable transformation frequency was observed as described in Brettschneider et al. (1997). Therefore, osmotic treatment is needed for *Alstroemeria* genetic modification via particle bombardment to enhance the transformation efficiency and its stable gene expression. We conclude that the use of both sorbitol and mannitol in the particle bombardment-mediated transformation is likely to improve expression in FEC culture or multiple cells of *Alstroemeria*.

FEC cultures induced from the leaves of axil tissues of *Alstroemeria* plants were transformed with the viral sequence derived from an AlMV strain following particle bombardment. Virus resistant transgenic plants have been obtained by transferring an untranslatable RNA from several potyviruses, such as in *Tobacco etch virus* (Dougherty et al., 1994) and PVY (Van der Vlugt et al., 1992). In addition, the use of this kind of untranslatable coat protein construct stimulates an efficient use of ELISA for virus detection, because no background transgene expression will be present (Lines et al., 2002). The presence and absence of expression of the viral sequence have been analyzed through PCR analysis and ELISA.

Of 44 independent transgenic lines obtained in this study, a large number of lines were PCR^+ / PPT^+, but some of them had ELISA positive values. In transgenic *Alstroemeria* plants, considerable differences in reaction to virus infection between different lines could be observed. However, it is hard to determine if the variations in the transformed lines including resistance level and morphology patterns are associated with AlMV infection. It might be due to either sample variation existing between independent transgenic lines or other physiological parameters. The overall trend in the resistant transgenic lines was that the resistance observed in these lines by the ELISA assay was not correlated with plant development and morphology.

The results represented in Figure 4 indicate that a considerable number of RNA-mediated transgenic *Alstroemeria* plants had a short-term tolerance but not long-term tolerance to AlMV.

There have been a number of strategies to obtain virus resistance in agricultural crops. Generally, two mechanisms are widely known to exhibit resistance to virus infection (Lomonossoff, 1995). Coat-protein-mediated and RNA-mediated resistances have been introduced to the development of virus-resistant transgenic plants. In this study, however, only RNA-mediated resistance was introduced because coat-protein-mediated resistance often shows a difficulty in discriminating resistance and the expression level of potyvirus. RNA-mediated resistance that normally does not need protein expression approached to the resistance via the transcribed RNA often shows resistance to very high level of virus or even leads to near immunity (Wang et al., 2001).

Thus, using RNA-mediated strategy, transgenic *Alstroemeria* plants, which were resistant to AlMV, were generated by particle bombardment. This is the first report to show that RNA-mediated resistance is potentially applicable to obtain resistant *Alstroemeria*. Resistance seems to originate from the presence of CP RNA sequence rather than on the accumulation of viral CP (Masmoudi et al., 2002).

Tolerance to AlMV infection in some of our transgenic *Alstroemeria* plants has been obtained in this study, but more efficient transformation system to obtain a higher degree of virus resistance needs to be further assessed. There was also severe inhibition for rooting and overall growth, which might be caused by the presence of PPT in the selection medium. Therefore, other selection methods based on non-chemical should be tested to minimize the risk of somaclonal variation,

inhibition of growth and plant development in the production of transgenic virus-resistant *Alstroemeria* plants.

In summary, a particle bombardment protocol in *Alstroemeria* was optimized by re-transformation and the use of osmoticum treatments. Finally, results described in this paper suggest that transgenic *Alstroemeria* plants with a virus resistance against AlMV strain through RNA-mediated resistance can be produced.

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Table 1. PCR primer sequences used in cloning of the AlMV CP and 3'-NTR. The parts in italics in the AlMV specific primers are linker sequences and are not virus specific.

Primer	Sequence
AlMVCP-UP02	5'-*TTAGAGCTC*GAGTTGGACGATGATTTTGA-3'
AlMV-TotDW	5'-*GGG*TGAGTCACCGTAACGATAGAAT-3'
P9502	5'-GGATCCTTTTTTTTTTTTTTTT-3'

Table 2. Effect of shooting position and time on transient gene expression

Plasmids	% of luc or gus/ PPT positive FEC clumps[1]		# of transgenic lines / 100 mg FEC[2]	
	Control	90 degree rotation	Control	90 degree rotation
PAHC25	2.3±0.2a[3]	7.5±1.1b	37.5±7.5a	64.5±8.6b
PBL9780	1.5±0.1a	9.4±2.4b	48.5±2.2a	147.5±15.9b

1. Data was measured 4 week after transformation
2. Data was measured 8 weeks after transformation
3. Means followed by different letters are significantly different at the P=0.05 level.

Table 3.Effect of osmotic treatments on transient gene expression and recovery of transgenic lines

Plasmids	Treatment[1]	% of PPT positive FEC[2]	% of luc^{+}/gus^{+} FEC[2]	% of browning[3]	# of somatic embryos / 100 FEC clumps[3]
PAHC25	A	23.5±1.7c[4]	3.1±0.2b	2.5±0.6b	13.5±0.5b
	B	31.4±1.3b	2.6±0.4b	3.3±0.7b	14.4±4.1b
	C	43.4±4.7a	8.5±1.7a	1.2±0.1b	36.5±3.6a
	Control	26.5±1.1c	3.5±0.3b	10.1±0.6a	11.4±2.1b
PBL9780	A	18.5±3.3a	5.5±1.6c	2.2±0.1b	12.2±3.5c
	B	22.6±4.9a	6.4±0.1b	5.3±0.9a	15.6±2.8b
	C	26.9±3.2a	14.5±1.5a	1.5±0.1b	22.9±6.2a
	Control	19.4±2.2a	4.2±0.4c	8.5±2.8a	9.5±2.4c

1: Treatment A (0.2 M mannitol), B (0.2M sorbitol), C (0.2M mannitol + 0.2M sorbitol), control (no osmoticum)
2: Data was measured 6 (PPT) and 8 weeks (LUC/GUS assay) after transformation by counting the number of positive clumps
3: Data was measured 12 weeks after transformation by counting the number of tissues with browning and somatic embryos, respectively.
4.: Means followed by different letters are significantly different at the P=0.05 level.

Table 4. Optimization of inoculation procedure with AlMV for *Alstroemeria* VV024

Concentration of inoculum[1]	Frequency of inoculation[2]	Damage to plants[3]	ELISAreadings (O.D ^{405nm})[4]
Non-inoculation	0	-	0.04±0.01
1X	2	-	0.24±0.07
1X	3	+++	0.51±0.22
1X	4	-	0.97±0.39
2X	2	-	0.88±0.17
2X	3	++	1.28±0.15
2X	4	++	1.31±0.28

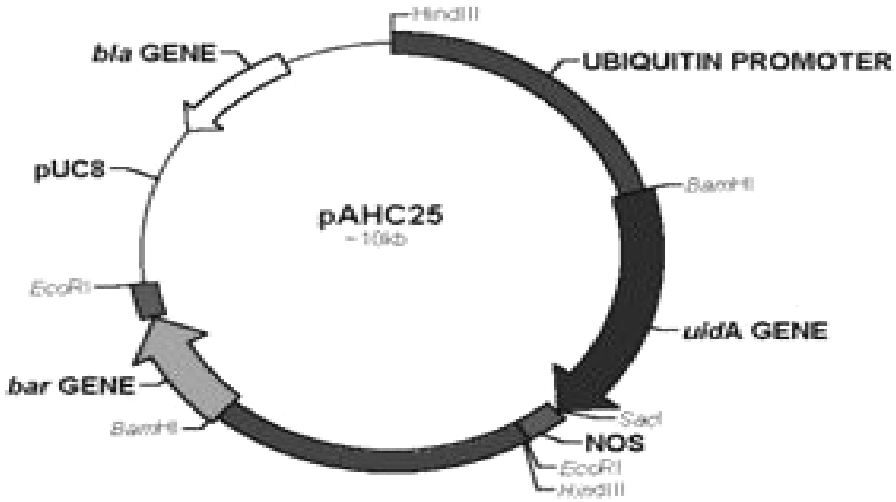
[1]: When the virus inoculum is prepared with an infected *N. benthamiana* leaf with a size of 5 cm and 10 ml buffer, it is called 1×. If the inoculum prepared 5 ml buffer with the same infected leaf, it would be called 2×.

[2]: The second inoculation was performed 4-5 days after the first inoculation. The third inoculation was conducted 5 days after the second inoculation. The fourth inoculation was done as the interval of third inoculation.

[3]: +++; nearly dead with necrosis, ++; severe yellowing in leaves, +; small or partly yellowing in the leaf, -; no damage

[4]: O.D data presented as the mean of six replicates, and measured 2 weeks after the final inoculation.

A) Plasmid pAHC25



B) pXYZo0

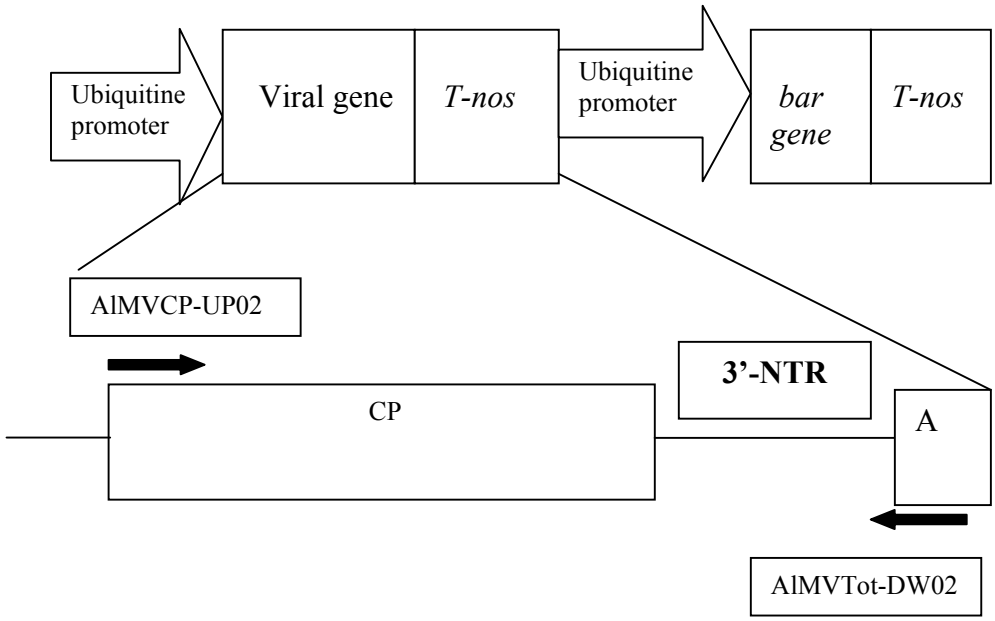
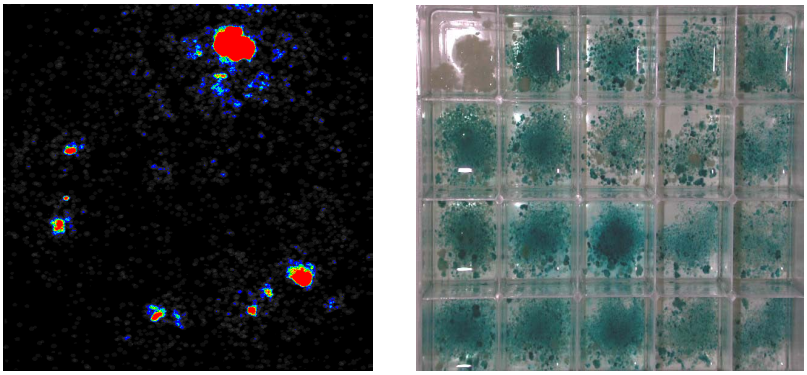


Figure 1. Transgene expression cassette (A: pAHC25 B: Viral construct)
(Arrows indicate the positions of the two primers on plasmid pAlMV-PV)

A) B)



C) D)

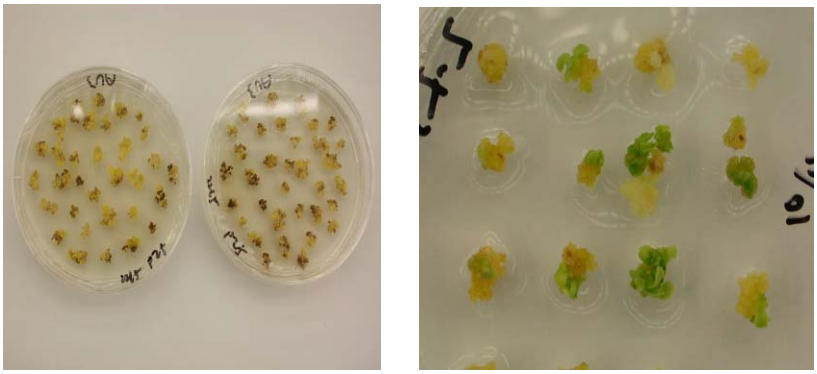


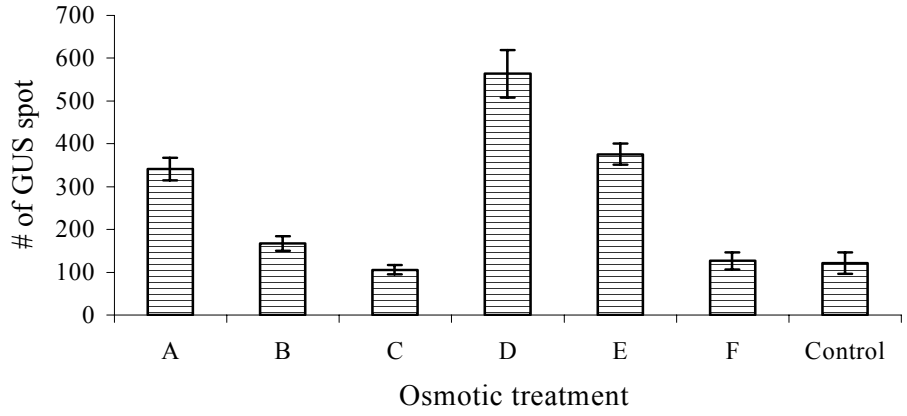
Figure 2. Transient gene expression of luciferase and GUS gene in transgenic *Alstroemeria*

A) luciferase expression of FEC clumps 1 week after transformation (red: high expression, blue: low expression)

B) GUS gene expression of FEC clumps 2 weeks after transformation

C) PPT-resistant FEC clumps 6 weeks after transformation

D) germination of transgenic somatic embryos 14 weeks after transformation using the stringent PPT selection



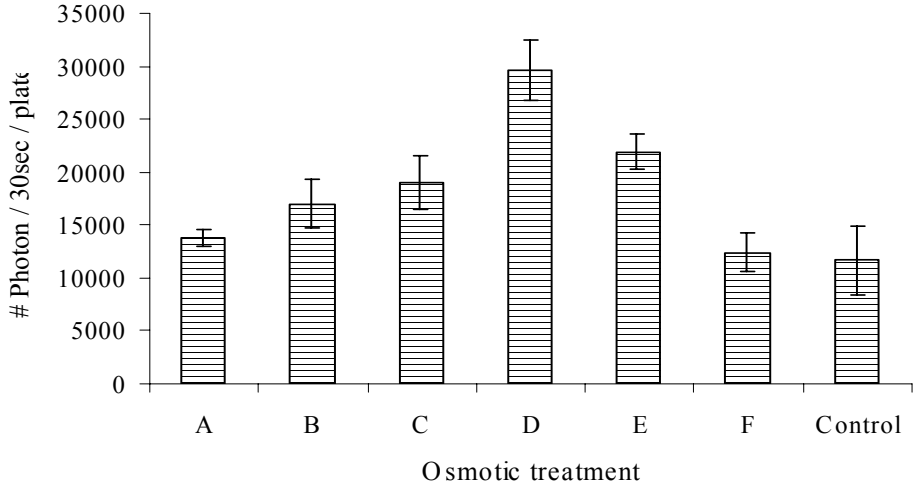


Figure 3. Effect of different culturing regimes with osmoticum.

(A: 4 h before and 8 h after; B: 4 h before; C: 8 h after; D: 8 h before and 16 h after; E: 8 h before; F: 16 h after; Control: no osmotic treatment)

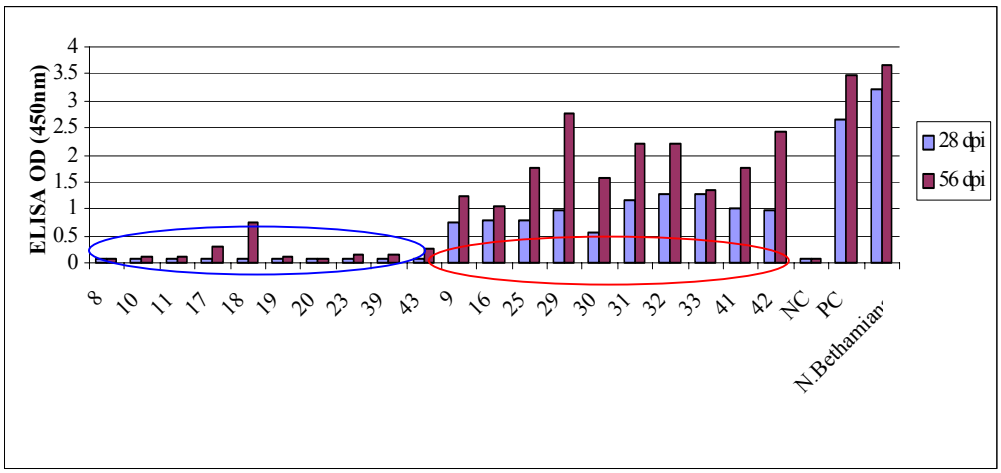


Figure 4. Evaluation of AlMV accumulation in transgenic *Alstroemeria* lines at 28 and 56 days post inoculation. The data indicate the average of ELISA (OD^{405nm}) values determined for the mean of 3 plants for transgenic lines (NC: Negative control, PC: Positive control, blue circle – resistant lines, red circle – susceptible lines).

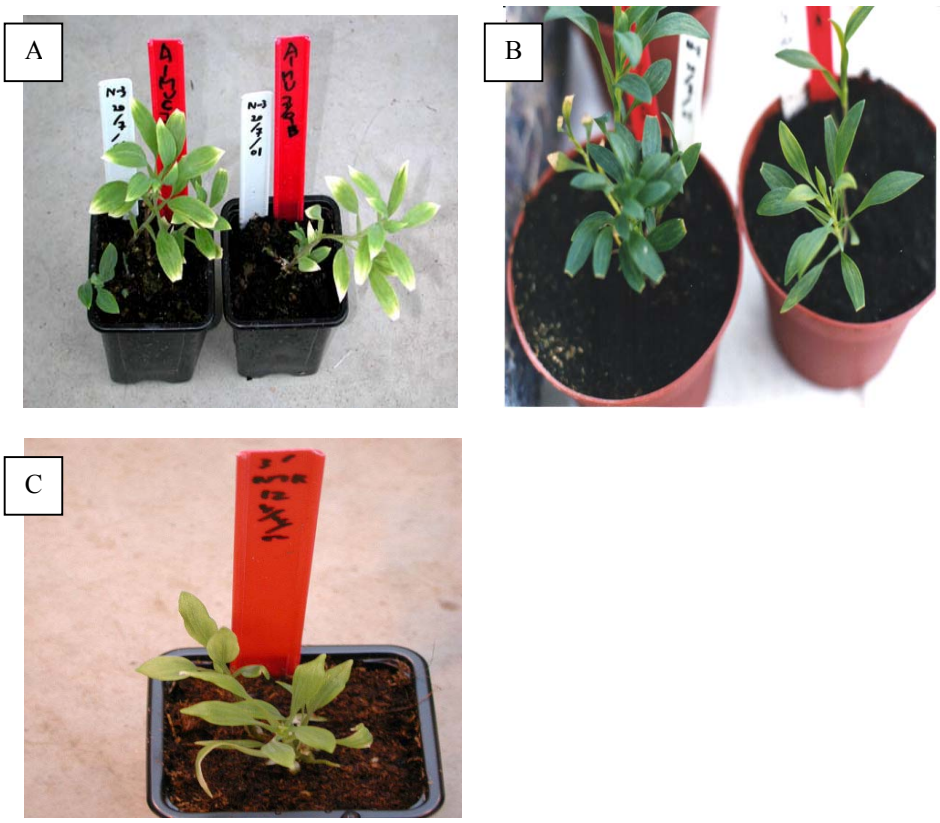
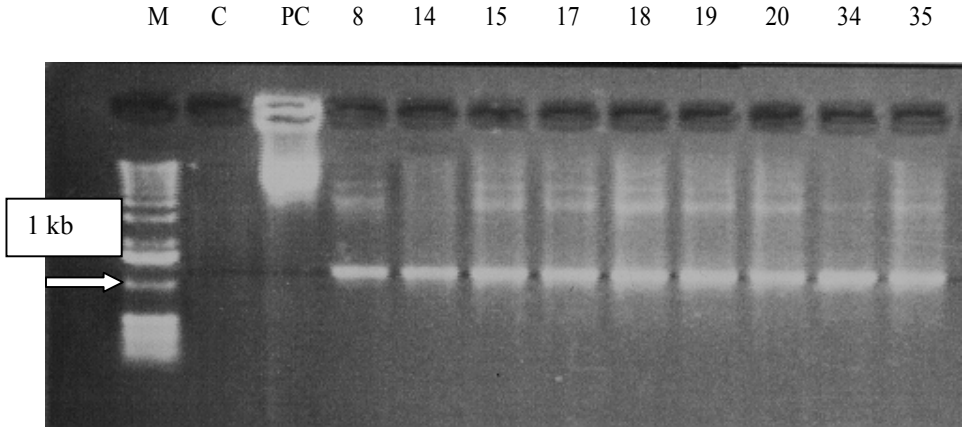


Figure 5. Corresponding photographs to the level of morphology evaluation 6 weeks after inoculation (A: inoculated control, B: non-inoculated control, C: moderate growing transgenic lines)

A) Viral (CP+3'NTR sequence: 1258 bp)



B) *bar* gene (244 bp)

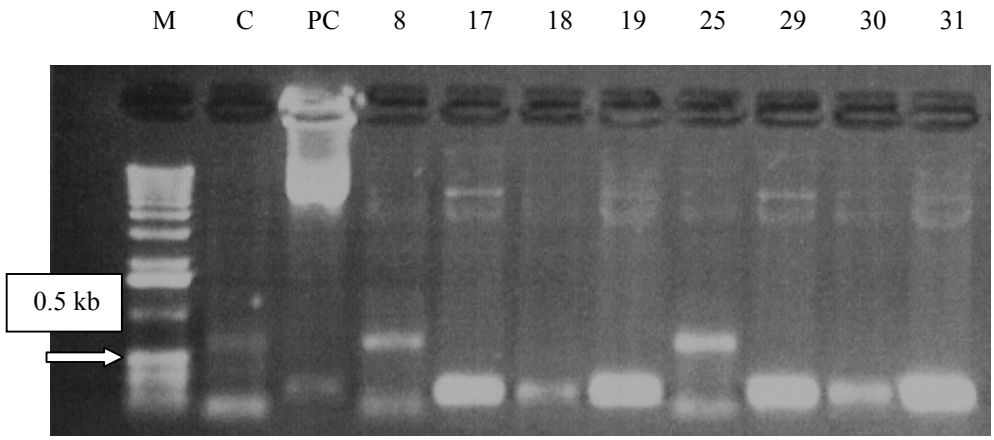


Figure 6. PCR analysis of the transgenes (viral sequence and *bar* gene) in the transformed *Alstroemeria* plants (black arrows indicate the corresponding band, and white arrow indicate the size of marker). M: Marker, C: Control (non-transformed *Alstroemeria* plant), PC: Positive control (pAHC25)

Lines for A) – AV 8, 14, 15, 17, 18, 19, 20, 34, 35 (all transgenic lines from resistance group)

Lines for B) – AV 8, 17, 18, 19, 25, 29, 30, 31 (lane 1-4 from resistance group, 5-8 from susceptible group)

CHAPTER 5. Efficient production of transgenic *Alstroemeria* plants by using *Agrobacterium tumefaciens*

In preparation for submission

Efficient production of transgenic *Alstroemeria* plants by using *Agrobacterium tumefaciens*

Jong-Bo, Kim[1,2], Krit Raemakers*[1], Evert Jacobsen[1], Richard G.F. Visser[1]

[1]Laboratory of Plant Breeding, The Graduate School of Experimental Plant Sciences

Wageningen University, 6700 AJ, Wageningen, The Netherlands

*: author for correspondence, e-mail address: krit.raemakers@wur.nl

[2]Turfgrass & Environment Research Institute, Samsung Everland Inc.

#1 Bu-kok, Gunpo, Kyong-ki, 435-737, Korea

Abstract

A highly efficient and reproducible protocol was developed to obtain transgenic *Alstroemeria* plants by combining *A. tumefaciens* with friable embryogenic callus (FEC). To develop this transformation method, factors such as infection time, cocultivation period, effect of acetosyringone, different dilution concentrations of the bacterium, and temperature during cocultivation were evaluated. A protocol was developed in which transient GUS expression activity was observed ranging from 25% to 55% out of the co-cultivated FEC cultures, when FEC cultures were infected for 30 min with 50 μM acetosyringone, 1:10 dilution, and then co-cultivated at 24˚C in the dark for 7 days with *Agrobacterium* strain LBA4404 (pTOK233) that carried *gus*, *nptII*, and *hpt* genes. Seven independent experiments produced a total of 1300 transformed somatic embryos with shoots from 3.5 g of FEC. Of these germinated embryos, half of them developed into *in vitro* plants. Thus, on average, 500 mg of FEC infected with *A. tumefaciens* produced *ca*. 80–100 transgenic *in vitro* plants within 6-8 months via a selection process with 2.5-20 mg/l hygromycin. Additionally, luciferase-based selection showed to be less detrimental to the transgenic lines than was herbicide-based selection. Transformation efficiency was 18.6% for the luciferase-based selection and 7.6% for the PPT-based selection, although with luciferase-based selection about a quarter of the lines were escapes. The *nptII* and *uid*A genes were detected by PCR analysis in 9 of the 19 tested lines. The results indicate that the system developed here can be used as an alternative to particle bombardment of *Alstroemeria*.

Keywords: *Agrobacterium*, *Alstroemeria*, Friable embryogenic callus, Luciferase, Regeneration, Transformation

Introduction

Alstroemeria is a monocotyledonous plant species and has its centers of origin in Chile and Brazil (Aker and Healy, 1990). Breeding activities have resulted in modern hybrids of *Alstroemeria* with large and beautiful flowers (Anastassopoulos and Keil, 1996). Biotechnological strategies such as recombinant DNA technologies and genetic transformation could provide efficient approaches in *Alstroemeria* breeding, but the lack of a reliable regeneration system to generate transgenic plants has hindered the use of genetic transformation in *Alstroemeria* improvement.

In broad terms, the two most commonly used gene transfer techniques are *Agrobacterium tumefaciens* and particle bombardment (Songstad et al., 1995). However, a drawback of particle bombardment is the complex and unpredictable pattern of DNA integration.

On the other hand, *Agrobacterium*-mediated transformation shows low or single copy insertions more often than does particle bombardment. Therefore, it seems more beneficial to develop an efficient *Agrobacterium*-mediated transformation system for *Alstroemeria*. Accordingly, in many other crops, *Agrobacterium*-mediated transformation is the preferred system for gene transfer due to its low copy integration, relatively precise DNA transfer, highly efficient transformation rate and its inexpensive nature (Hiei et al., 1994).

In a large number of dicotyledonous plants, routine procedures are now available for genetic modification via *Agrobacterium tumefaciens*. However, many monocotyledonous plants are still recalcitrant to *Agrobacterium*-mediated transformation since they are a non-host for *Agrobacterium tumefaciens* (Zheng, 2000). Transgenic monocotyledonous ornamental plants have been obtained by transforming friable embryogenic callus (FEC) lines in *Anthurrium* (Chen et al., 1996), *Iris* (Jeknic et al., 1999), *lily* (Langeveld et al., 1995), *Phalaenopsis* (Belarmino et al., 2000), *Tulip* (Wilmink et al., 1992), *Gladiolus* (Kamo et al., 1995), and *Hyacinth* (Santie et al., 2000).

In *Alstroemeria*, transgenic plants have been produced by particle bombardment (Lin et al., 2000), using either visual-based (luciferase gene) or chemical-based selection (*bar* gene). Neither selection method produced transgenic plants efficiently, either because of the gene transfer technique or the selection regime used. In addition to the particle bombardment, *Agrobacterium tumefaciens* (Akutsu

et al., 2004a) and *Agrobacterium rhizogenes* (Akutsu et al., 2004b) were used for the development of a transformation system in *Alstroemeria* plants. In these protocols, however, ovule-derived embryogenic calli were used as the explant source for transformation, and thereby transgenic plants were regenerated via organogenesis. Additionally, GUS activity was not observed in the leaves of transformed *Alstroemeria* plants, indicating the chimeric or unstable nature of these transformed plants.

Agrobacterium-mediated transformation is influenced by several factors such as explant type, bacterial strain, temperature, wounding methods, virulence genes, acetosyringone (AS), infection time, and cocultivation period. These factors were optimized in this study to develop a routine transformation method in *Alstroemeria*.

Materials and methods

Plant materials and callus culture

In vitro-grown *Alstroemeria* plants of VV024 were subcultured every 4 weeks on a regeneration medium (RM) containing Murashige and Skoog (1962) salts plus vitamins supplemented with 0.5 mg/l BA, 40 g/l sucrose and 2.5 g/l Gelrite (pH 6.0). Leaves with axil tissues were cut from young shoots of *in vitro*-grown plants using the protocol of Lin et al (1997). Excised explants were then transferred to callus induction medium (CIM) containing Schenk and Hildebrandt (1972) basal salts plus vitamins, supplemented with 2 mg/l 2,4-D, 0.5 mg/l BA, 30 g/l sucrose and 7.5 g/l micro agar and cultured in the dark. After 6-8 weeks of culture on CIM, yellow compact embryogenic calli (CEC) were formed on the surface of the leaf. The CEC were isolated and transferred to PCA medium (Sofiari et al., 1998) to initiate friable embryogenic callus (FEC). Induced FEC was isolated from the surface of CEC a month later, and maintained on PCA medium with a 4-week subculture scheme. All media were adjusted to pH 6.0 and cultures were maintained at 18 ˚C with a photoperiod of 16 h and an irradiance of 40 μmol m^{-2} s^{-1}, unless stated otherwise.

Experiment 1: selection of appropriate explants for transformation

In order to identify the appropriate type of explants, the virulent *Agrobacterium tumefaciens* strain LBA4404 (pTOK233) was used. LBA440 (pTOK233) was chosen because it resulted in the highest transient GUS expression (unpublished results) as compared to EHA101 (Hood et al., 1986), C58C1 C58 (Koncz et al., 1986), and MOG101 (Hood et al., 1993). CEC, FEC, and leaves with axil tissues were compared in transient GUS expression. Transient GUS expression was calculated by counting blue foci on explants 3 days after transformation. Stable GUS expression was recorded by counting blue spots on explants and counting explants with *gus* positive spots 6 weeks after transformation. After that, explants were transferred to RM and cultured for 3 to 4 months with a 4-week subculture regime to produce transgenic somatic embryos and, subsequently, plants. Once CEC, FEC or leaves with axil tissue produced shoots, they were transferred to RM for further growth. Nine months after transformation, the regeneration rate of transgenic lines from the different explant types was recorded. In the above-mentioned experiments, *Agrobacterium* cultures were grown in LB medium with the appropriate antibiotics for 2 days on a gyratory shaker (120 rpm) at 30˚C.

Prior to inoculation, the bacterial cells were pelleted by centrifugation at 3,000 rpm for 10 mins, and re-suspended in liquid MS medium, supplemented with 100 μM acetosyringone (AS) and 0.5 mg/l BA (OD_{550} = 1.0-1.2).

General procedure of *Agrobacterium-mediated* transformation

LBA4404 (pTOK233) and FEC were chosen for further experiments. 500 mg of FEC clumps was mixed with 1 ml of *A. tumefaciens* suspension. Ten minutes later, FEC clumps were washed with liquid MS medium and blotted dry on sterilized filter paper. The FEC clumps were cultured in the dark on RM supplemented with 100 μM AS for 7 days. Then, FEC clumps were washed with liquid RM supplemented with 300 mg/l cefotaxime (claforan; Duchefa, Haarlem, The Netherlands) and 250 mg/l vancomycin (Duchefa, Haarlem, The Netherlands) dried and transferred to RM containing 20 mg/l hygromycin as a first selection medium. FEC cultures were maintained in fresh medium for 3 weeks.

After 6 weeks, putative transgenic calli were transferred to a second selection medium containing 7.5 mg/l hygromycin. Somatic embryos were transferred to

fresh RM medium. After another 4 weeks of culture on selection medium, transgenic shoots with somatic embryos were isolated and transferred to a third selection medium containing 2.5 mg/l hygromycin. After another 4-6 weeks on this selection medium, shoot clumps were formed, and transferred to rooting medium, supplemented with MS basal salts plus vitamins, 0.5 mg/l NAA, and 45 g/l sucrose. Plants from a single resistant callus clump were regarded as clones. All media were supplemented with 200 mg/l cefotaxime and 100 mg/l vancomycin to eliminate *Agrobacterium tumefaciens*. Plantlets with well-developed roots were transferred to the greenhouse.

Experiment 2: optimization of several factors important for transgene expression

The various factors which were evaluated were: dilutions of *Agrobacterium* suspension in LB liquid medium (1:100, 1:50, 1:25, 1:10, 1:5 vol:vol), infection period (5, 15, 30, 60, 120 min), length of the co-cultivation period (2, 3, 5, 7, 10 days), acetosyringone concentration (0, 25, 50, 100, 200 μM), and temperatures (18, 24, 30˚C).

Experiment 3: effect of different antibiotics on selection efficiency and regeneration

After having been washed, the infected FEC cultures were transferred to two different selection regimes, one containing 20 mg/l hygromycin and the other 200 mg/l kanamycin. Cultures were maintained by a 3-week subculture scheme.

Experiment 4: comparison of luciferase-based selection with PPT-based selection

To compare the luciferase-based selection and PPT-based selection in transformation efficiency, *Agrobacterium* strain AGL1 (Lazo et al., 1991), that harboured the binary plasmid pSB101, which contains the bar gene under the control of the nopaline synthase (NOS) promoter, and the luciferase gene under control of the cauliflower mosaic virus (CaMV) 35S promoter was used. After the transformation of 250 mg FEC, cultures were transferred to two different selection

media (selection medium I (SM I): without PPT; selection medium II (SM II): PPT 15 mg/l). Eight weeks later, the concentration of PPT in the SM II was decreased to 2.5 mg/l. All these cultures were refreshed every 4 weeks for in total 16 weeks. Luciferase activity was determined at 1, 3, and 16 weeks after transformation. To measure luciferase expression, a luminometer was used as described by Schavemaker (2000). FEC cultures were first sprayed with 0.15 mg/l of ice-stored luciferin aqueous solution and then immediately measured with the luminometer in a dark room. This luminometer consists of an intensified CCD camera (Hamamatsu, Japan) with a Nikon 35mm lens, connected to a personal computer.

After 16 weeks of transformation, all FEC cultures that were cultured on SM I and SM II medium were transferred to SM II medium and escapes were recorded 4 weeks after culture on SM II medium. FEC cultures that had been cultured on SM II medium were also measured for luciferase activity to determine whether they also contain and express the luciferase gene.

GUS assay

To detect GUS gene expression of putative transgenic tissues, FEC, CEC, somatic embryos, leaves and roots were isolated and incubated for 16 hr at 37˚C in a solution of X-Gluc, consisting of 0.1% (w/v) 5-bromo-4-chloro-3-indoyl-β-glucuronic acid, 100 μM sodium phosphate (pH 7.0), 0.5 μM potassium ferrocyanide, and 10 μM ethylene diamine tetra-acetic acid (EDTA), as described earlier (Jefferson et al., 1987). Tissues were then soaked in 70% ethanol for a day to remove chlorophyll. In each assay non-transformed plants were used as a control.

Molecular characterization of initial transformants

In order to identify putative transformants, a PCR assay was performed. DNA was isolated from fresh young leaf tissue (250-300 mg) which was collected in a 2 ml tube containing 250 μl nuclear lysis buffer (0.2 M Tris-HCl, 0.05 M EDTA, 2 M NaCl, 2% CTAB, pH 7.5), 200 μl extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 0.35M Sorbitol, 20 mM NaBisulfate, pH 7.5) and 25 μl 5% Sarkosyl in 96 wells Costar plates (Corning Inc., Corning, NY, U.S.A). The tissue was grinded for 1 min with metal balls by using a Retsch machine (Retsch Inc., Haan, Germany). The grinded mixtures in the same tubes were transferred to a 65°C water bath for

60 min. After that, the tubes were placed on ice for 15 min to cool, and then 250 µl of ice-cold chloroform isoamyl alcohol (24:1) was added, and mixed by inversion for 2-3 min. The tubes were centrifuged at 3,500 rpm for 10 min. Supernatant (200 µl) was transferred to new tubes, which already contained 200 µl isopropanol. After inversion of the tubes for 1 min, the DNA was pelleted by centrifugation at 3,500 rpm for 60 min. Following drying, the pellet was dissolved in 100 µl TE buffer with 0.5 µg RNAase. Isolated DNA samples were stored at – 20°C before use.

The *nptII* gene was amplified by polymerase chain reaction (PCR) using the 5'-TCGGCTATGGGGCACAACAGA-3' and 5'-AAGAAGGCGATAGAAGGCGATGCG-3' primers that amplify a 722-bp fragment corresponding to the coding region of the *nptII* gene. For the detection of the *uid*A gene, 5'-CTACACCACGCCGAACACCT-3'and 5'-CGCTTGGGTTTTTGTCA-3' primers that amplify a 710-bp fragment corresponding to the coding region of *uid*A gene were used. The PCR reaction contained 2 µl of genomic DNA, 50 pmols of each primer, 0.25 mM of each dNTPs, 2.5 units of *Taq* DNA polymerase. The PCR process was as follows; one cycle of 2.5 min at 94°C for an initial melting process; 30 cycles of 2 min at 94°C, 1.5 min at 62°C and 1.5 min at 72°C. The PCR products were separated by electrophoresis on 1.0% (w/v) agarose gel and visualized by 3 µl of ethidium bromide.

Results

Description of starting material

Leaves with axil tissue of the tetraploid *Alstroemeria* cultivar VV024, were cultured to induce yellow CEC as described in Chapter 2. This CEC was either maintained on callus induction medium (CIM) or cultured on PCA medium (Sofiari et al., 1998) for the induction of FEC (Figure 1A). Leaves with axil tissue were transferred to shoot induction medium, as described by Lin et al (1997) and cultured in darkness for 10 days. In the initial experiments, 1, 2 and 3 day old bacterial cultures were compared to determine the proper bacterial culture period before transformation. Two days of bacterial culture showed the highest transient GUS expression of the three periods (Figure 1B), although there were no

significant differences in transient GUS gene expression between 2 and 3 days of bacterial culture. Two and three days of bacterial culture resulted in at least 8 times the number of blue spots in 250 mg of FEC (data not shown), as observed for 1 day of bacteria culture. A period of 2 days bacterial culture was therefore selected for the rest of the experiments.

Selection of proper initial explant and *Agrobacterium* strain

CEC, FEC and leaves with axil tissues were infected with *Agrobacterium* strain LBA4404 (pTOK233). There was no statistical difference in transient GUS expression 3 days after transformation, between CEC and FEC, whereas leaves with axil tissue showed a low level of blue foci (Table 1). In general, 24% of FEC and 18% of CEC, while, only 4% of leaves with axil tissues contained blue foci. Six weeks after transformation, FEC continued to maintain GUS expression in 23 of the 96 FEC cultures. However, CEC lost half of the blue foci that they had in the transient GUS expression and leaves with axil tissue showed only two to five blue foci per 96 explants. In addition, transgenic plants were obtained from 35% of FEC, 28% of CEC and 1% of leaves with axil tissue. Thus, FEC was selected for further transformation experiments.

Parameters affecting transient and stable gene expression

In the first series of experiments, FEC were inoculated with LBA4404 (pTOK233) and co-cultivated for varying periods (2, 3, 5, 7, and 10 days).

The number of blue foci varied from less than 40 (2 days) to almost 200 (7 days) (Figure 2A). A seven-day co-cultivation period was selected as optimal, due to its high number of GUS spots and the least overgrowth by *Agrobacterium*. One-way ANOVA analysis of all treatments indicated that the 5 and 7 day co-cultivation periods were significantly superior to 2- or 3-day co-cultivation period.

A bacterial solution, which had been diluted 10 times, used for transformation gave a significantly higher number of blue foci than solutions, which had been diluted 100 times (Figure 2B). A bacterial solution, which had been diluted 5 times, showed extensive overgrowth of bacteria in the later selection procedure.

Different concentrations of AS also caused significant differences in transient GUS expression. The best result was obtained when 50 μM. AS was added to the

co-cultivation medium (Figure 2C), resulting in a three times higher number of blue foci in 250 mg FEC than the control treatment.

In order to explore the effect of infection time on transient GUS expression, 5 different infection times (5, 15, 30, 60, and 120 min) were tested during the transformation procedure. Approximately 150 GUS spots were observed after 30 min of infection, whereas 5 min of infection showed a low level of transient GUS expression (Figure 2D).

FEC were infected with LBA4404 (pTOK233) and co-cultivated at three different temperatures (18, 24, and 30˚C) for 7 days. Transient GUS expression revealed that 24˚C was the best temperature for co-cultivation with *Alstroemeria*. At this temperature nearly 300 blue foci were visible 1 week after transformation. At 18˚C, just over 160 blue foci were visible and only 80 blue foci at 30˚C.

In total, 25% of FEC clumps were transformed at 24˚C, whereas only 6% of FEC clump were transformed at 30˚C. The co-cultivation at 24˚C produced 408 GUS^+ /$hygromycin^+$ independent transgenic lines 8 weeks after transformation. The number of independent transgenic lines produced at 18˚C was around 85% of the number produced at 24˚C, and only 22 independent transgenic lines were produced at 30˚C (Table 2).

Selection and regeneration of transgenic plants

Fifteen mg/l of hygromycin and 200 mg/l of kanamycin were enough to kill non-transformed callus. After culture for a month on this high concentration of hygromycin all calli turned brown or even black and died (Figure 1C). However, a few FEC explants survived on 200 mg/l of kanamycin in the selection medium (data not shown). Kanamycin-based selection resulted in 239 independent resistant lines compared to 167 independent transgenic lines for hygromycin-based selection. Survived resistant FEC clumps developed into shoots (Figure 1D). With hygromycin selection, 86.8% of resistant FEC clumps were GUS positive compared to 46.8% for kanamycin selection. The regeneration processes in the hygromycin and kanamycin selection regimes did not differ significantly (Table 3). Both hygromycin-based and kanamycin-based selection resulted in shoots of which only a low frequency formed roots, as compared to non-transformed plants with a rooting frequency of 85-90%. Approximately 19 (hygromycin-based)-23% (kanamycin-based) of transgenic germinated somatic embryos formed roots in the

in vitro stage and germinated (Figure 1E), when they were selected on a SM medium containing either hygromycin or kanamycin. Stable GUS expression was observed 32 weeks after transformation various tissues such as somatic embryos (Figure 1F), leaves, meristematic tissues, and root segments (Figure 1G). Finally, plants produced roots 8 month after transformation (Figure 1H), and flowered in the greenhouse (Figure 1I).

Comparison of luciferase-based selection with PPT-based selection

AGL1 was used to determine the possibility of a selection system without a selectable marker. One day after transformation, no transient expression of the luciferase gene was detected by the luminometer. However, 2 days later, the transient luciferase activity of FEC was detected. Figure 3A shows luciferase positive FEC 1 week after transformation. The highest number of photons detected at this stage ranged from 20,000-52,500 (per 30 seconds). Three weeks after transformation, the areas of luciferase positive tissue decreased (Figure 3B) and at this stage, the number of photons detected was around 13,000–16,000 (per 30 seconds). Two months after transformation, the average number of photons was reduced to between 6,000 and 8,000. In addition, on average, 4.5 out of 25 FEC clumps showed a luciferase-positive response 16 weeks after transformation (Figure 3C). In total, 24 of the in total 96 FEC clumps (25%) were luciferase positive, whereas only 8 PPT positive clumps (8%) were produced from PPT-based selection, 4 weeks after transformation (Table 4). However, 26% of luciferase positive clumps were escapes, whereas only 8 % of PPT positive clumps were escapes. Furthermore, albino-like somatic embryos were produced from the ppt selection at a frequency of 7%, and transgenic FEC lines with browning were also observed at a frequency of 13% in PPT-based selection. Table 4 also shows that luciferase-based selection showed no albino-like somatic embryos and a low frequency of browning in FEC culture. Generally, the transformation efficiency by luciferase-based selection was 19% and 8% for the PPT-based selection.

Molecular analysis of transgenic *Alstroemeria* plants

PCR analysis was performed using primers to amplify the *nptII* gene and *uid*A gene. First, the 722-bp *nptII* gene fragment was amplified in 19 randomly selected

hygromycin resistant *Alstroemeria* lines. These 19 lines were selected from 145 transgenic lines (gus+/kan+/hyg+). Fragments of the expected size (722 bp) were amplified in 12 lines out of 19 lines tested (Figure 4A). Among these 12 PCR-positive lines, ten lines were selected and subjected to PCR analysis to determine the presence of *uid*A gene. As a result, nine out of ten assayed lines showed the amplification of a fragment corresponding to the coding region of *uid*A gene (Figure 4B). Thus, it was demonstrated that at least 9 lines carried the *hpt*, *nptII* and *uid*A genes out of the randomly selected 19 transgenic lines.

Discussion

An efficient protocol for stable transformation via *Agrobacterium tumefaciens* has been developed in *Alstroemeria*. In general, monocotyledonous plants are difficult to transform by *Agrobacterium tumefaciens* (Smith and Hood, 1995). Previous studies by Van Schaik et al. (2000) and Lin et al. (2000) showed that *Alstroemeria* could be transformed using particle bombardment.

Several factors influenced the transformation efficiency and final production of transgenic plants. The most important ones were the types of explant source and the *Agrobacterium* strain used. Poulsen (1996) reported similar results for genetic modification of *Brassica* species. In general, FEC cultures have been shown to have a high proliferation rate and regeneration capacity (Suzuki et al., 2001). In addition, FEC has been used for successful transgenic plant production via *A. tumefaciens* in several monocotyledonous plants (Cheng et al., 1997; Delbreli et al., 1993; Han et al., 2005; Suzuki et al., 2001).

Up to now, CEC and FEC, unlike leaves with axil tissues, were the explants that could be easily transformed in large quantities and selected visually on selection medium. For leaves with axil tissues, although two putative transgenic plants with transient transgene expressions were produced, there was no transgenic plant with stable transgene expression after 12 weeks of transformation. Only, it was possible to detect transient GUS gene expression on the transformed tissues from leaves with axil tissue 4 and 8 weeks after transformation. Lin (1998) had also conducted particle bombardment to obtain transgenic *Alstroemeria* plants from leaves with axil tissue, but only transient transgene expression was observed, and the expression was absent two months after transformation.

A comparison of CEC and FEC in the transformation efficiency showed that FEC cultures performed better in many crops. Therefore, FEC, infected with LBA4404 (pTOK233), was used as target material, because tested with several strains had shown that LBA4404 (pTOK233) performed most efficiently. Although only a very small number of transgenic *Alstroemeria* plants were obtained from C58C1, EHA101 and MOG101, these strains are important and should be optimized in the future because these three strains can be combined with genes of interests which is much more difficult for LBA4404 (pTOK233).

In general, 2 or 3 days of co-cultivation have been used for *Agrobacterium*-mediated transformation in the majority of plant species. In *Alstroemeria*, an extended co-cultivation of 7 days with FEC cultures gave better results. Similar results were also found in barley (Trifonova et al., 2001), begonia (Kishimoto et al., 2002), cyclamen (Aida et al., 1999), flax (Dong et al., 1991), and leighton (Suzuki et al., 2001). Better performance by extending the co-cultivation period may be explained by the increased chance of contact between plant cells and *A. tumefaciens* as well as by the increased number of plant cells which can be infected during a prolonged period (Dong et al., 1991; Potrykus et al., 1990).

A 10 times dilution of the bacterial suspension has shown the most positive GUS expression compared to other dilutions. It could be explained by the fact that excess numbers of bacteria can stress plant cells and thus decrease transient gene expression (Curtis et al., 1999).

AS has been thought to stimulate the transformation efficiency in numerous crops when added to an *Agrobacterium* culture medium (Gelvin and Liu 1994; James et al., 1993; Sheikholeslam and Weeks, 1987) or a co-cultivation medium (Godwin et al., 1991; Holford et al., 1993). Also in *Alstroemeria*, it can be concluded that the AS treatment is beneficial for the T-DNA transfer process.

Infection time was also a critical factor for improving transformation efficiency. In lettuce, a 10-min infection period enhanced transformation more efficiently than did a period of 2-3 seconds (Curtis et al., 1994). The number of transformed shoots was similar for infection periods of 5, 10 and 20 min in *Datura* (Curtis et al., 1999). In *Alstroemeria*, there was a significant difference between five different infection periods. Less than 10 min of infection time was not enough for the T-DNA delivery process. In contrast, longer than 30 min of infection time showed vigorous bacterial growth during the selection process.

One of the most important factors influencing transformation efficiency is the temperature during co-cultivation (Dillen et al., 1997; Salas et al., 2001). In the present study, it was concluded that 24˚C is the best temperature for *Agrobacterium*-mediated transformation of *Alstroemeria*.

Since efficient selection is a prerequisite to produce transgenic plants successfully via *Agrobacterium tumefaciens*, hygromycin-based selection and kanamycin-based were compared. In most cases of transformation, selectable marker genes are essential for recovering transformed cells and regeneration of transgenic plants (Huang et al., 2004). In rice transformation, hygromycin was first used as a selectable marker (Hiei et al., 1994). Apart from rice, hygromycin-based transformation systems in several grasses have shown a good performance (Bettany et al., 2003; Chai et al., 2004; Zhang et al., 2003). In this tudy, hygromycin supplemented selection medium (SM) resulted in fewer escapes than did kanamycin supplemented SM (data not shown). Unfortunately, both hygromycin and kanamycin resulted in *Alstroemeria* plants, which rooted poorly. However, luciferase-based selection showed twice as much root formation and a higher survival rate compared to the chemical-based selection (data not shown).

A total of 10 individual transgenic lines from 19 transgenic lines (hyg+/kan+/gus+) have been subjected to PCR analysis to confirm the presence of the transgenes (both *nptII* and *uid*A) in the genome of *Alstroemeria*. Of these 10 transgenic lines, 9 lines were transformants, as determined by chemical selection, high and stable GUS expression and PCR analysis.

FEC was also successfully transformed with a high efficiency by using luciferase and PPT-based selection system. Although luciferase-based selection has many advantages, this system is labor-intensive and requires expensive equipment (Lin et al., 2000). However, luciferase-based selection has shown its applicability in this study, although it showed more escapes than did PPT-based selection. As undesirable variation in leaf shape and less production of roots were observed in the chemical-based transformation system, a luciferase-based selection system would be an alternative for transformation in an ornamental breeding program with regard to less damage and phenotype variation in the regeneration stage compared with the chemical-based selection.

In conclusion, the results presented in this study demonstrate that LBA4404 (pTOK233) and AGL1 can be applied efficiently for the production of transgenic *Alstroemeria* plants. Together with Akutsu et al. (2004a; b), we have shown that an

Agrobacterium-mediated transformation has now become a routine process in *Alstroemeri*a.

Further, we show that the optimization of primary factors influencing T-DNA transfer and transgene expression is crucial for the genetic modification of *Alstroemeria* by *A. tumefaciens*. Taken together, our protocol successfully produced approximately 80-100 transgenic *Alstroemeria* plants from 500 mg of the infected FEC within 8 months with an average transformation efficiency of 14%.

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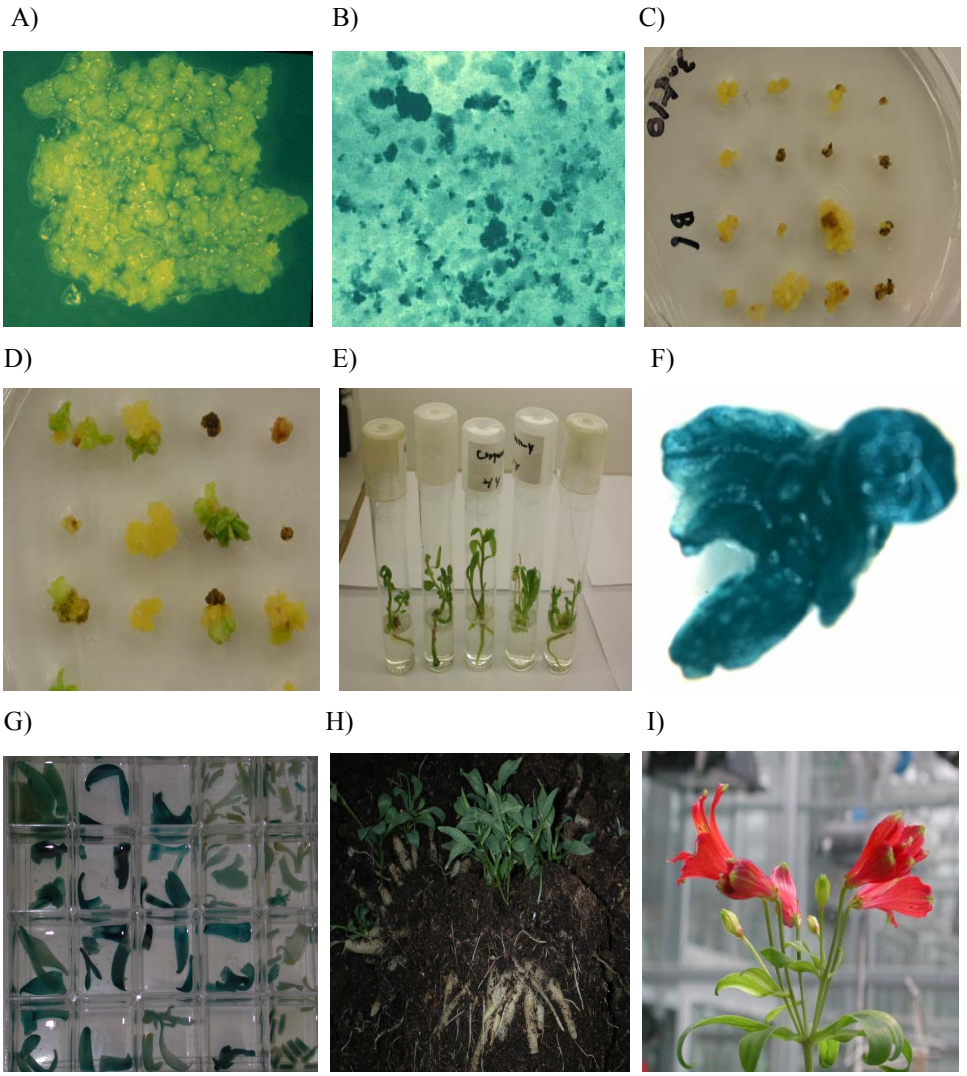


Figure 1. Production of transgenic *Alstroemeria* plants via *Agrobacterium tumefaciens* with FEC.

A. FEC used in transformation, B. GUS-positive FEC clump, C. Hygromycin selection D. Germination of kanamycin resistant somatic embryos, E. Transgenic *in vitro* plants F. GUS expression of somatic embryo, G. GUS expressions in leaf tissues, H. Transgenic *Alstroemeria* plants with well-developed roots, I. Transgenic *Alstroemeria* plant with flower

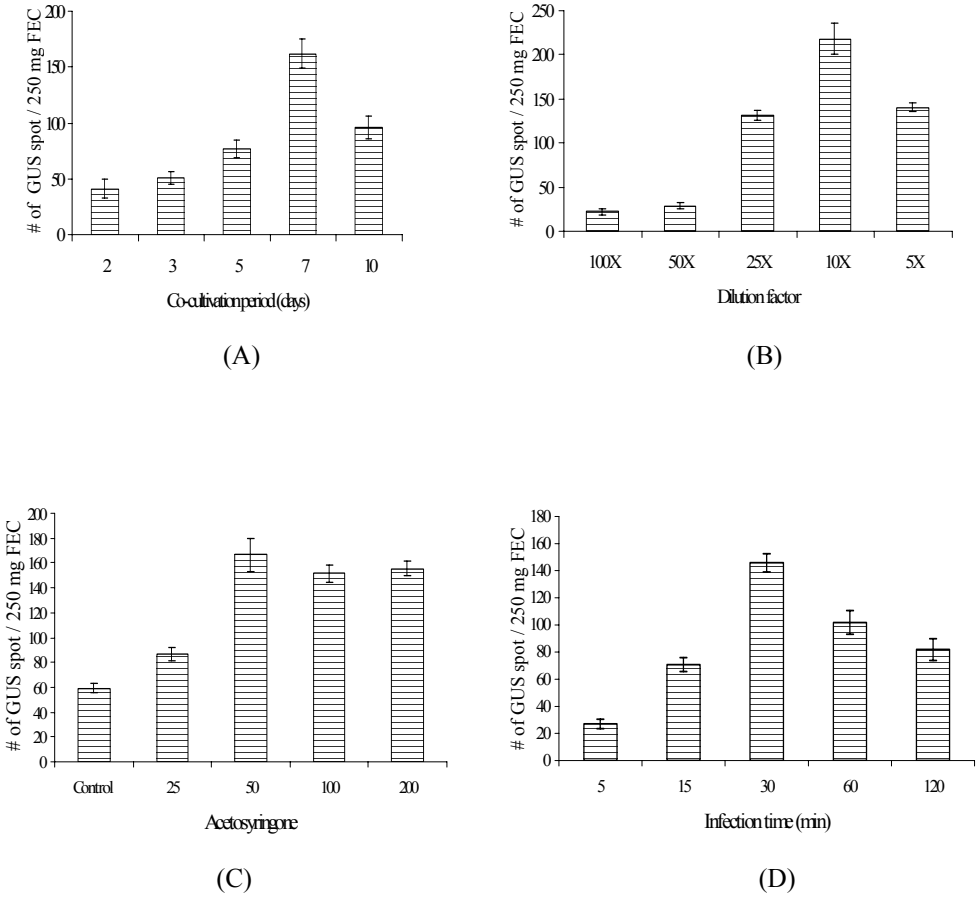
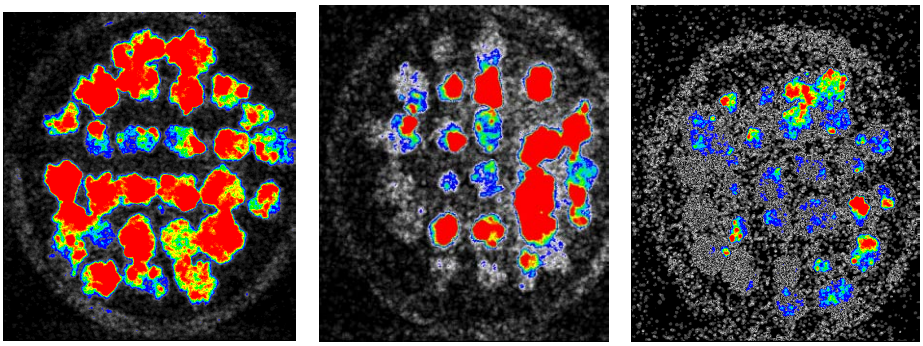


Figure 2. Effect of different transformation factors on the transient GUS gene expression in FEC lines of *Alstroemeria* VV024



A) B) C)

Figure 3. Transformation of *Alstroemeria* via *Agrobacterium tumefaciens*: AGL1 (PBS101)

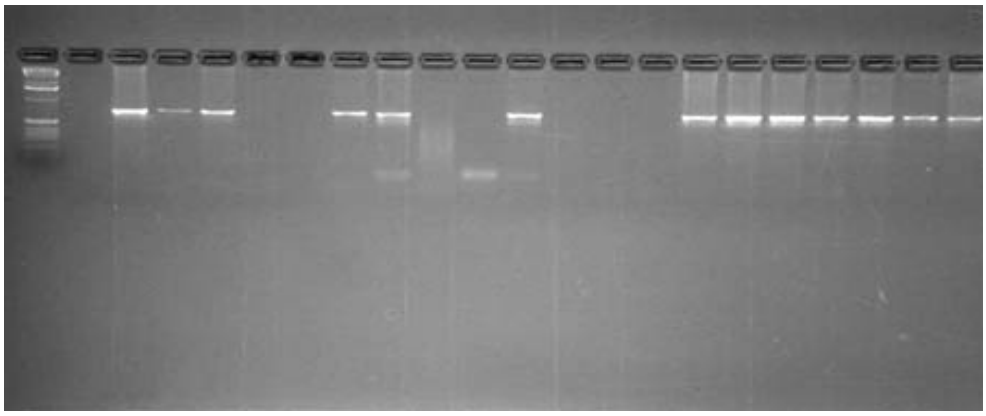
A. Transient luciferase expression 1 week after transformation

B. Luciferase expression 3 weeks after transformation

C. Stable luciferase expression 16 weeks after transformation

A) *NPTII* (722 bp)

M C PC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



B) *uidA* (710 bp)

M NC 5 6 9 13 14 15 16 17 18 19 PC

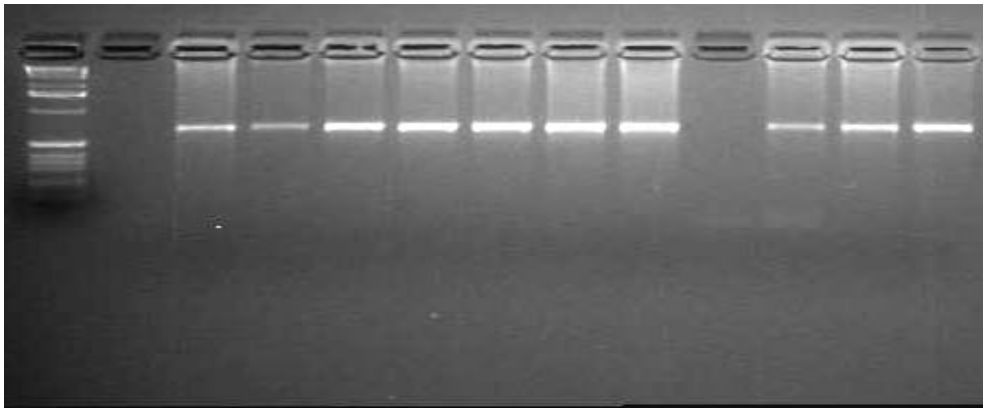


Figure 4. PCR analysis of *nptII* and *uid*A gene in the transformed *Alstroemeria*.
(NC: negative control – non-transformed plant, PC: positive control (pTOK233 plasmid, M: 1 kb ladder)

A: lanes 1-19: hygromycin resistant transformed lines

B: lanes 5, 6, 9, 13-19: selected hygromycin resistant / *nptII* positive transformed lines

Table 1. Effect of different explants transformed with LBA4404 (pTOK233) in *Alstroemeria* VV024.

Lines	# *gus* spot[1]	# *gus* spot [2]	# of explants with *gus* (+)[2]	# of transgenic plants (%)[3]
CEC	199±6.1a[4]	89±18.5b	17/96	47/168 (27.9)
FEC	247±15.5a	197±9.9a	23/96	64/182 (35.2)
Leaves	12±2.5b	7 ±1.4c	4/96	2/190 (1.1)

[1]: GUS spots were counted 3 days after transformation from 96 infected explants selected at random
[2]: GUS spots were counted 6 weeks after transformation from 96 infected explants selected at random
[3]: Data were collected 6 weeks after transformation and they were all resistant to hygromycin and kanamycin with *gus* positive
[4]: Different letters denote a significantly difference at $P < 0.05$, as determined by one-way ANOVA test.

Table 2. Effect of different temperatures on transgene expression and production of transgenic *Alstroemeria* plants using FEC infected with *Agrobacterium tumefaciens* LBA4404 (pTOK233).

	Temperature (˚C)		
	18	24	30
# of *gus* spot[1]	161.3±9.3b[3]	291.8±23.5a	79.8±3.7c
# of explants with *gus* spot[1]	28.5±4.5b	45.9±5.1a	7.5±3.3c
# of stable transgenic lines[2]	344	408	22

[1]: Data were obtained by assaying 200 units of FEC 1 week after transformation
[2]: Data were collected 8 weeks after transformation and were based on transforming 250 mg of FEC. Four experiments were conducted.
[3]: Different letters denote a significant difference at $P < 0.05$, as determined by one-way ANOVA test.

Table 3. The effect of different selectable agents for an efficient selection system of *Agrobacterium*-mediated transformation in *Alstroemeria*.

Antibiotics[1]	# of resistant lines[1]	# of resistant lines with *gus*+ and hyg+/kan+[2]	# of germinated SE[3]	% of rooting of transgenic plants
Kanamycin	239	112	49	23.2±4.1a[4]
Hygromycin	167	145	42	18.5±5.3a

[1]: For this experiment, 200 mg/l of kanamycin and 20 mg/l of hygromycin were added to the selection medium. Data were collected after 6 weeks of culture on the selection medium with two subcultures and based on 250 mg of FEC.
[2]: Data were collected 12 weeks after transformation by counting both *gus* and *hyg*/*kan* positive lines
[3]: Data were collected 24 weeks after transformation from 96 somatic embryos.
[4]: The mean value ± standard errors were obtained from three independent experiments. 48 callus clumps were used for each experiment.

Table 4. A comparison of luciferase-based and PPT-based selection regime on the transformation efficiency and recovery of transgenic tissue of *Alstroemeria* plants. Transformation was conducted with the AGL1 strain.

Selection regime	# of positive clumps[1]	% of escapes[2]	% of albino somatic embryo[2]	% of FEC with browning[2]	Transformation efficiency (%)[1]
Luciferase	24	25.5±6.1b[3]	0.0±0.0b	1.6±0.2b	18.6±3.4b
PPT	8	8.3±0.9a	6.7±1.1a	12.5±3.7a	7.6±1.5a

[1]: Each selection regime started with 96 clumps. Data were collected 4 weeks after transformation. Transformation efficiency was calculated as (*luc* positive with PPT positive clumps) / 96 FEC clumps
[2]: Data were collected 12 weeks after transformation. Escapes was calculated as (# of luc negative clumps/ # of PPT positive clumps) or (# of PPT negative clumps / # of luc positive clumps)
[3]: The mean value ± standard errors were obtained from three independent experiments. Different letters denote a significantly difference at $P < 0.05$, as determined by one-way ANOVA test.

CHAPTER 6. GENERAL DISCUSSION

General discussion

The first introduction of foreign genes into plants was achieved in the early 1980s. Since then, there have been many revolutionary events in plant genetic engineering and thus transformation is now a routine procedure for over 100 different plant species (Primrose et al., 2001), including commercially valuable crops. In 2004, transgenic crops were cultivated on nearly 81 million hectare in several countries including Argentina, Canada, China, and the U.S.A (James, 2004). Representative transgenic crops are soybean, corn, oilseed rape (canola) and cotton.

In addition, transgenic genotypes of important crops such as wheat, rice, barley, and potato are grown. Besides these edible crops, plant genetic engineering technologies including genetic modification systems are now also actively used in ornamental species. To date, only a small number of reports on regeneration in *Alstroemeria* have been published. These reported systems could not be readily used for genetic modification. To this end, several approaches were investigated and described in this thesis to try to obtain a more efficient regeneration as well as transformation system in *Alstroemeria*. The main aim of this research program was to develop a transformation system to produce resistant *Alstroemeria* plants against *Alstroemeria Mosaic Virus* (AlMV), which is one of the most dangerous and endemic viruses in *Alstroemeria*. Before commencing the genetic modification experiments, the development of a regeneration system more efficient than the existing one was demanded.

Somatic embryogenesis from leaves with axil tissue

In Chapter 2, leaves with axil tissue were chosen from the preliminary experiments and chosen for both somatic embryogenesis and further transformation work. Generally, *Alstroemeria* has been categorized as a recalcitrant plant species because of its low multiplication rate and difficulty in the production of good quality callus. Rhizome division was the only reliable propagation method in *Alstroemeria*. However, this method has shown limitations such as an extremely low multiplication rate and was proven to be inappropriate for use in genetic modification. To develop an efficient somatic embryogenesis system, which can be applied to genetic transformation in *Alstroemeria*, explant screening is essential. In this study, leaves with axil tissues were chosen through the comparison with other

explants. Leaf axil tissue was shown to better performance in inducing compact embryogenic callus (CEC), which acts as a precursor for friable embryogenic callus (FEC), and somatic embryos in general. Before using leaves with axil tissues, zygotic embryos or ovules were used to induce callus in *Alstroemeria* (Hutchinson et al., 1994; 1997) and Van Schaik et al. (1996), Buitendijk et al. (1995)). However, plants regenerated from these generative parts could produce undesired genetic variation as well as non-true to type plants. Therefore, genetic modification based on somatic embryogenesis using leaves with axil tissue could be a good alternative in *Alstroemeria* breeding programs in the future. In the somatic embryogenesis system of the *Alstroemeria* tetraploid cultivar, VV024 used in this thesis, two types of embryogenic calluses were observed: CEC and FEC (described in Chapter 2 and Lin et al. (2000a)).

In order to generate plants via somatic embryogenesis in other crops, CEC has been used in other crops such as *Allium* (Valk et al., 1992), rice (Suprasanna et al., 1997) and wheat (Anshulika et al., 1999). Using FEC culture, plants have regenerated in cassava (Raemakers et al., 1997), garlic (Myers and Simon, 1998), gladiolus (Stefaniek, 1994), and marigold (Bespalhok and Hattori, 1998). In spite of these successful reports on somatic embryogenesis in a number of crops, only Lin et al. (2000a) used FEC as an explant for somatic embryogenesis in *Alstroemeria*. However, the time required to develop in s complete plant from an explant was long. It took 11 months from explant to plant due to the inefficiency of the regeneration system in the stem-derived compact callus culture of seedling plants, as Akutsu et al. (2002) indicated as well.

In monocot ornamental flowers, somatic embryogenesis was developed in freesia (Wang et al., 1990), gladiolus (Kamo et al., 1990; Stefaniak, 1994), iris (Jehan et al., 1994; Shimizu et al., 1996), lily (Haensch, 1996), narcissus (Sage et al., 2000), and tulip (Van den Bulk et al., 1994). Although several somatic embryogenesis systems have been reported in *Alstroemeria* from vegetative tissue (Lin et al., 2000a) or generative tissues (Hutchinson et al., 1994, 1997; Van Schaik, 1996; Akutsu et al., 2002), the frequency of regeneration is still low. Very recently, Akutsu et al. (2002) developed an efficient regeneration system through ovule-derived somatic embryogenesis. They compared organogenesis and somatic embryogenesis for efficiency of regeneration. Their protocol produced 450 regenerants from 1g of FEC in 3 months. This regeneration efficiency seems to be comparable to ours (350 regenerants from 1g FEC in 3 months by using the

protocol described in Chapter 2). However, as stated earlier, regenerants produced from ovule tissue (Akutsu et al., 2002) show more genetic variation than our regenerants. Furthermore, *Alstroemeria* is a vegetatively propagated crop in which use of clonal tissue is needed for somatic embryogenesis and genetic transformation.

FEC is induced directly from the surface of an explant and indirectly via CEC that is induced from the surface of an explant. In this thesis, FEC induction by the indirect pathway was used because directly induced FEC showed severe browning. However, to reduce the somaclonal variation because of relatively long tissue culture periods, use of directly induced FEC will be needed in the future. For maintenance of CEC and FEC cultures, PCA medium (Sofiari et al., 1998) was essential. This medium showed a significantly faster and more active growth of CEC and FEC cultures in *Alstroemeria*. Although Lin et al. (1998) claimed that liquid cultures could not be used efficiently for regeneration it was shown in preliminary studies that there was no significant difference in growth rates of FEC and CEC cultures on PCA-based liquid medium compared with a PCA-based solid medium.

Chapter 3 shows that more than 60 plants from 15 independent lines were obtained from protoplast-derived friable embryogenic callus (FEC). This research was performed to verify if plants could be obtained from protoplast cultures, which could be applied for somatic hybridization. A reliable regeneration system can offer an alternative way to transfer genes of interest from either wild species or current cultivars to target parent plants. Until now, there was only one report on protoplast isolation in *Alstroemeria* (Blom et al., 1995). However, this report described a protoplast isolation protocol from leaf tissue but failed to show further cell division needed for plant regeneration. The protoplast culture protocol described here can be used as an alternative regeneration system, although the plants regenerated from protoplasts in showed somaclonal variation, protoplast-derived plants from 1-month-old FEC showed much less variation than did protoplast-derived plants from 6-month-old FEC. Based on the protocol described in Chapter 3, protoplasts isolated from *Alstroemeria* FEC cultures could be fused with inter/intraspecific hybrids, or non-related ornamental species, which can not be crossed and carry new desirable traits such as novel colors or resistance against viruses.

Detection and analysis of somaclonal variation in *Alstroemeria*

Tissue culture techniques have been commonly used in many plant species. Amongst several *in vitro* techniques, somatic embryogenesis has been already applied as an alternative propagation method as well as regeneration model for genetic transformation. However, during the tissue culture and transformation processes, genetic variability can be induced which is called somaclonal variation (Larkin and Scowcroft, 1981) and which occurs in many crops. Since Larkin and Scowcroft developed the concept of somaclonal variation (SV), we know that most of this variation is undesirable. In fact, SV is an obstacle for the production of true-to-type plants via somatic embryogenesis or organogenesis as well as after transformation events. SV has been observed in many crops and has been reviewed in detail (Phillips et al., 1994; Scowcroft and Larkin, 1988). It is now generally accepted that somaclonal variation is caused by several factors such as chromosome breakage, DNA methylation patterns, single base change, changes in repeated sequence, etc (Larkin and Scowcroft, 1981; Munthali et al., 1996).

Until now, to detect and analyze induced genetic variation, ploidy level determinations and molecular techniques such as RAPD and AFLP were used. SV has been detected and analyzed by using RAPD in beet (Munthali et al., 1996), begonia (Bouman and De Klerk, 2001), chrysanthemum (Martin et al., 2002), garlic (Al-Zahim et al., 1999), and rice (Pong et al., 2001). AFLP has been used to detect SV in Arabidopsis (Polanco and Ruiz, 2002), pecan (Vendrame et al., 1999), and sugarcane (Arencibia et al., 1999). In Chapter 3, somaclonal variation was detected and analyzed by using ploidy level determination and morphological description.

Virus resistant plants production using particle bombardment

Alstroemeria Mosaic Virus (AlMV) belongs to the potyviruses, which have flexuous rod-shaped particles and are transmitted by aphids in a non-persistent manner. It causes severe damage to *Alstroemeria*. The biggest problem is that the symptoms in the plants are difficult to assess. In spite of the development of meristem tissue culture to generate virus-free stocks, new breeding lines, which carry a virus-resistance, are still needed. Because conventional breeding has shown

its limitations to extend genetic variation, genetic transformation could be used for the development of virus-resistant *Alstroemeria* plants.

In general, most methods of transformation are restricted to a limited range of explant types (Southgate et al., 1995). For the production of AlMV resistant *Alstroemeria* plants by genetic modification, therefore, particle gun-mediated transformation system was used. FEC was transformed using particle bombardment in the beginning of the project, because particle bombardment was the only available transformation method in *Alstroemeria* at that time (Chapter 5). A DNA vector containing the coat protein gene and the 3'-non translated region sequence was constructed to confer virus-resistance against AlMV. Since there was no ATG codon in the construct, and thus a protein would not be produced, it was established that resistance would occur at the transcript level by so called RNA-mediated resistance. *Alstroemeria* FEC cultures were transformed both by *A.tumefaciens* and particle bombardment. Finally, virus-resistant *Alstroemeria* plants were obtained. However, regeneration from FEC cultures occurs still at a lower efficiency (ca 20%), in comparison with the regeneration rate of CEC (>65%). Because FEC is the tissue preferred for genetic transformation, more research should be directed to the improvement of plant regeneration from FEC cultures in the future. It was possible to obtain transient and stable transgene expression in *Alstroemeria* by using either *Agrobacterium*-mediated or particle bombardment. However, considerable variations in transgene expression were observed. In addition, as about 20% of the transgenic *Alstroemeria* plants that were obtained through *Agrobacterium*-mediated transformation were escapes, experiments on the elimination of escapes and chimeric plants will be needed. Further, we observed that some of the transgenic lines, which were GUS and/or *luc* positive and were resistant to the herbicide or antibiotic during the selection process, lost their transgene expression in the greenhouse after several weeks or months. Further, although virus-resistant transgenic *Alstroemeria* plants were produced, it is questionable whether the levels of resistance are sufficient for practical use. Therefore, much more work should be focused on stacking of virus genes by co-transformation or combining with other strategies such as coat protein-mediated resistance.

Finally, because somaclonal variation can be an issue in *Alstroemeria* more research should be conducted in this field so that it can be prevented or minimized. Due to the occurrence of somaclonal variation, obtained transgenic *Alstroemeria*

plants from CEC, FEC or protoplast cultures can be underrated by either breeder or grower. Consequently, efforts on reducing and detecting, in time the occurrence and level of somaclonal variation should be high on the research list.

Genetic transformation of *Alstroemeria*

When this project was set out early 1999, there was no efficient transformation protocol available. The first transformation protocol using particle bombardment was reported by Lin and co-workers in 2000 (Lin et al., 2000b). However, this system was not very user friendly, mainly because the regeneration protocol was not efficient enough. Therefore, in the beginning of the project, a lot of time and effort was devoted to develop an efficient regeneration system, which could be used in combination with genetic transformation. This system, based on regeneration system via somatic embryogenesis, made it possible to initiate transformation experiments via *Agrobacterium tumefaciens* and particle bombardment. In Chapter 5, FEC cultures were inoculated with *Agrobacterium tumefaciens* LBA4404 (pTOK233) and transgenic *Alstroemeria* plants were produced. Before this, CEC, FEC and leaves with axil tissues were compared to determine the best explant type for *Agrobacterium*-mediated transformation. Transgenic plants were obtained from all tissues compared. However, FEC was chosen as the best explant for transformation because FEC demonstrated a high number of GUS spots, compared with CEC and leaves with axils 3 days and 6 weeks after transformation. Almost 24% of the FEC colonies infected with LB4404 (pTOK233) had intensive blue spots, while this frequency was 18% for CEC and 4.2% for leaves with axil tissue. Transgene expression in the leaves with axil tissues was too weak, with only small spots and there were no completely blue explants obtained. Most of the transgenic plants regenerated from CEC and FEC had complete blue sections of explant, while no complete transgenic plants were produced from leaves with axil.

Using the FEC, important factors such as infection time, co-cultivation period, temperature, and concentration of bacterial suspension and acetosyringone were optimized. Transformation with LBA 4404 (pTOK233; Hiei et al., 1994) showed a high frequency of transformation (>500 plantlets from 1g FEC in 7-8 months) and stable transgene expression (over 1-1.5 yr). However, this strain has a drawback, in that it cannot be easily combined with genes of interests, because of its huge size

and lack of unique restriction sites. Owing to this reason, another strain, AGL-1 (pSB101; Lazo et al., 1991) that contains the *luc* gene as a reporter gene and the *bar* gene as a selectable marker and strain C58C1 were used in further experiments. Although transgenic *Alstroemeria* plants were obtained from the C58C1 strain, the transformation efficiency was too low, and this strain generated chimeric transgenic *Alstroemeria* plants. Therefore, AGL-1 (pSB101) will be used for future transformation system in *Alstroemeria* because of its relatively high transformation efficiency and stable gene expression. During the regeneration process of transgenic plants, root formation was severely inhibited. Only 20-25% of transgenic plants produced roots, as compared to the rooting production in control plants (>90%). This rooting problem occurred for transgenic plants obtained both after *Agrobacterium tumefaciens* and particle bombardment transformation. It seemed that hygromycin (*A.tumefaciens*) or PPT (particle bombardment) used for selection negatively affected the root development. In order to investigate this, luciferase-based selection (non-chemical selection) was compared with PPT-based selection (chemical selection strategy) as well (Chapter 5). PPT-based selection had proven to be effective earlier (Lin et al., 2000b). He described the PPT-based selection as a labor-saving and cheap selection system. Later, however, transgenic *Alstroemeria* plants from Lin's report showed severe inhibition of growth and no flowering were observed in the greenhouse compared to the control plants**.** In addition, the establishment of transgenic *Alstroemeria* plants in the greenhouse was hindered by the absence of root formation. Therefore, a new strategy based on the use of non-chemical selection was proposed. In our study, luciferase was introduced as a reporter gene and used for selection. The successful use luciferase as a selectable marker was shown before in dendrobium (Chia et al., 1994), cassava (Raemakers et al., 1997), and tobacco (Ow et al., 1986). As Lin et al., (2000b) mentioned, selection by luciferase was labor intensive and expensive. However, these disadvantages were compensated by the recovery of a relatively high number of transgenic plants and by the high survival rate of transgenic plants. By introducing one time a high concentration (15-20 mg) of PPT in the selection medium, around 3-4 months after transformation, the occurrence of escapes was decreased and, thereby, transformation efficiency increased. Further, this one time selection step during luciferase-based selection showed that there was no harmful effect on plant regeneration and development, including rooting. With this result, it can be concluded that luciferase-based selection (either alone or in combination)

can be applied efficiently in future transformation systems via both *A.tumefaciens* and particle bombardment in *Alstroemeria*. Three transgenic lines produced from *A. tumefaciens* LBA4404 (pTOK233) in this study showed the presence of *NPT*II gene by PCR analysis, however, these three lines died in selection medium containing 200 mg/l kanamycin. Moreover, most lines transformed with pAHC25 showed also the presence of the *bar* gene by PCR. Nevertheless, 5% of the PCR-positive lines for the *bar* gene died in selection medium with 10 mg/l PPT. In addition, there was also variation in GUS expression of transient and stable gene expression. All these phenomena might be related to variability in expression levels or stability of expression. The reasons for these fluctuations are not clear.

Although somaclonal variation was observed in FEC or CEC-derived plants, future transformation should be continued with somatic embryogenesis via FEC or CEC. To avoid or minimize SV, FEC or CEC lines younger than 3 months should be used for experiments to offer the possibility to produce transgenic plants with less variation in the greenhouse.

In general it is believed that *Agrobacterium*-mediated transformation is to be preferred over particle bombardment. Particle bombardment was the only transformation system, which produced transgenic plants so far (Lin et al., 2000b). In this thesis, comparison of the transformation efficiency with both systems showed that *Agrobacterium*-mediated transformation has a two times higher transformation efficiency (19% at maximum) than did the particle bombardment (8.5% for the pAHC25 and 11.5% for the PBL9780 constructs). However, the recovery rate of the transgenic plants from transgenic somatic embryos was 55% for particle bombardment using the pHAC25 plasmid and only 22.5% for *Agrobacterium*-mediated transformation using the LBA4404 (pTOK233) strain. A low recovery rate of the transgenic plants in *Agrobacterium*-mediated transformation could be due to the use of a high concentration of hygromycin during the selection periods.

Conclusion

In *Alstroemeria*, friable embryogenic callus (FEC) induced from leaves with axil tissue has become a great potential source in the application of genetic modification using both *Agrobacterium tumefaciens* and particle bombardment. Besides, FEC was also used as explant source for protoplast culture as an

alternative regeneration system with successful results. Finally, the first generation of virus-resistant *Alstroemeria* plants containing viral sequences was produced by particle bombardment. This holds clearly promises for the future but more research is needed to obtain virus-resistant plants, which also might be of practical value.

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Summary

The monocotyledonous, *Alstroemeria* is an important cut flower in the Netherlands, and many other countries. During the last decades, *Alstroemeria* has shown its potential as a pot plant as well as a cut flower. The main reasons for its popularity are a variety of flower colors, a long vase life and the low energy requirement during the cultivation period.

Until now, interspecific hybridization and mutation breeding combined with both efficient selection breeding and excellent culture practices have been the keys to success in producing new *Alstroemeria* cultivars. A lot of information is available on the cultivation, multiplication by rhizome division and mutation breeding. However, for a number of traits, which should be improved, such as virus resistance and a delayed post-harvest leaf yellowing, no target genes have been identified yet in the *Alstroemeria* gene pool. Particularly, virus diseases cause severe constraints on the production and quality of *Alstroemeria* plants and flowers. Indeed, few practical measures to control virus disease are available in *Alstroemeria* at this moment. This thesis describes the development of an efficient regeneration and transformation system in order to produce virus-resistant *Alstroemeria* plants.

In **Chapter 1**, a general overview is given of *Alstroemeria* cultivation, breeding history, virus problems, and the available protocols for regeneration and transformation. The crucial prerequisite for genetic transformation is an efficient plant regeneration system. In general, two pathways for plant regeneration are available. Organogenesis whereby shoots develop directly from the explants and somatic embryogenesis whereby somatic embryos generate from the explant via a callus phase.

Chapter 2 describes somatic embryogenesis from clonal tissue (leaves with axil tissue). Two different types of embryogenic callus are formed; friable embryogenic callus (FEC), and compact embryogenic callus (CEC). CEC is produced first. It is initiated directly from the surface of the region between leaf base and node tissue. FEC is formed from CEC after 4-5 weeks of culture. CEC and FEC cultures were maintained on PCA medium (modified MS medium with a variety of vitamins) containing 5 mg/l picloram for 6 months without significant loss of regeneration capacity by a four-week subculture regime. Plants regenerated from FEC and CEC by culturing them on regeneration medium (RM) containing 0.5 mg/l BA. Somatic embryos were generated from the surfaces of CEC and FEC, and were able to

develop into complete plants. In general, CEC showed a regeneration rate of 60% of the cultured individual clumps on Murashige and Skoog (MS) medium supplemented with 0.5 mg/l BA, while for FEC, it was 22%.

Chapter 3 shows that FEC can be used as source for protoplasts, which are able to regenerate into plants. Protoplasts were isolated when FEC clumps were treated with enzyme mixtures containing cell degrading enzymes under dark conditions for 16 hours at 24˚C. In this study, a high frequency of cell division and the development of micro-calli colonies were observed. Protoplasts isolated from FEC developed micro-calli within 12 weeks. Moreover, 59% of the protoplast-derived micro-calli generated somatic embryos and half of these somatic embryos developed into plants. The *Alstroemeria* plants showed somaclonal variation in morphological traits (leaves and flowers) compared to vegetatively propagated control plants. This was most probably caused by the use of 6 month-old FEC for the isolation of the protoplasts.

In **Chapter 4**, the production of AlMV-resistant plants is described. For the construction of pathogen-derived resistance (RNA-mediated resistance), a construct was assembled to carry the viral gene [coat protein gene (CP), and a 3'-non translated region sequence (3'-NTR)], and containing the *bar* gene as a selectable marker. FEC was transformed using particle bombardment.

Although critical parameters influencing the transformation efficiency through particle bombardment were optimized previously, two additional parameters such as the effect of an additional bombardment and the osmotic treatment were studied by evaluating transient/stable transgene expressions. Two bombardments of FEC showed a better result than did a single bombardment. Additionally, particle bombardment with FEC cultures placed on a medium containing 0.2M mannitol and 0.2M sorbitol with a culture period of 8 hours before and 16 hours after bombardment, resulted in high transformation efficiency and positively affected the recovery of transgenic plants.

Using this established protocol, the transformation of *Alstroemeria* FEC with the viral gene (CP and 3'-NTR) from AlMV was carried out. Putative transgenic plants showed variation in resistance; from high resistance, to moderate resistance, and to a delay of infection up to susceptibility. The presence of the *bar* gene and viral genes was confirmed by PCR analysis. About 70% of the transgenic virus-resistant *Alstroemeria* plants did not survive in the greenhouse after virus inoculation, showing yellowing of plants and necrosis. It could not be determined

whether death or necrosis of transformed *Alstroemeria* plants in the greenhouse was caused either by the virus infection or by physiological stress. In addition, the initial resistance observed in the plants described in this chapter should be characterized further by using Southern and Northern blot analysis in order to exactly determine the relationship between the number of inserted copies and the level of resistance. Results in Chapter 4 suggest that RNA-mediated resistance was obtained, although malformation of plants and severe damage by the virus challenge were observed in a number of transgenic lines.

The development of an efficient transformation protocol via *Agrobacterium tumefaciens* is described in **Chapter 5**. FEC of genotype VV024 was transformed with LBA4404 (pTOK233) harboring the *hpt*, *nptII* and *gus* genes. Several parameters including infection time, co-cultivation period, effect of acetosyringone, different dilutions of the bacterial suspension and temperature during the co-cultivation were evaluated and optimized for further transformation experiments. A high frequency of transient and stable GUS expression was observed when FEC calli were infected with LBA4404 (pTOK233) for 30 min with 50 μM acetosyringone in 1:10 dilution of bacterial solution and co-cultivated at 24˚C for 7 days. The GUS expressions were stable and detectable over a period of 1.5 years. The presence and expression of the transgenes in the transformed *Alstroemeria* plants were confirmed by using PCR analysis and GUS assay.

Selection systems based on hygromycin and kanamycin were compared. The frequency of escapes with hygromycin selection was lower than with kanamycin selection, although the number of transgenic lines produced using kanamycin selection was higher than using hygromycin-based selection. Furthermore, a comparison was made between luciferase-based selection and phosphinotricin (PPT)-based selection. Luciferase-based selection resulted in a significant increase (two-fold) in transformation efficiency with low frequencies of browning and the production of albino plants, compared to the PPT-based selection. The transformation described in this chapter with FEC cultures of *Alstroemeria* can be applied year-round due to the use of clonal tissue as starting material. In the future, experiments should be performed using the viral gene construct and *Agrobacterium tumefaciens* to obtain transgenic *Alstroemeria* plants. In addition, more attention should be allocated to exploit somaclonal variation, which occurs with prolonged tissue culture coupled to genetic transformation in *Alstroemeria*.

In **Chapter 6**, general aspects of somatic embryogenesis, occurrence of somaclonal variation, and the transformation system are discussed. Also, information on non-chemical selection, co-transformation and somaclonal variation is given.

In conclusion, we believe that the present study is a contribution to the establishment of a more efficient regeneration and genetic transformation system in *Alstroemeria* and allows the introduction of molecular breeding techniques for the further development of resistant plants through transformation. In the near future, regeneration from FEC should be improved with regard to the somaclonal variation.

Samenvatting

Alstroemeria is een belangrijk sierteelt gewas in Nederland en veel andere landen. Het wordt verhandeld als potplant en als snijbloem in (gemengde) boeketten. De belangrijkste redenen voor zijn populariteit zijn de grote variatie in bloemkleuren, een lange houdbaarheid en relatief lage energiebehoefte tijdens de teelt.

Tot op de dag van vandaag zijn interspecifieke hybridisatie en mutatieveredeling gekoppeld aan efficiënte selectie en goede teeltpraktijken de belangrijkste sleutels voor succes geweest in het produceren van nieuwe Alstroemeria cultivars. Voor veel eigenschappen die terug te vinden zijn in de nieuwe variëteiten is informatie en genetische variatie voorhanden, maar voor een aantal eigenschappen zoals resistentie tegen virussen en afwezigheid van blad vergeling na het oogsten zijn (nog) geen genen geïdentificeerd in de Alstroemeria genenpool. Vooral ziekten veroorzaakt door virussen trekken een zware wissel op een gezonde en economische productie van Alstroemeria planten en snijbloemen. Er zijn daarnaast relatief weinig praktische methoden voorhanden die een goede controle van de ziekte mogelijk maken. In dit proefschrift worden efficiënte regeneratie en transformatie protocollen beschreven die kunnen leiden tot de productie van virusresistente Alstroemeria planten.

In Hoofdstuk 1 wordt een algemeen overzicht gegeven over de historie van Alstroemeria veredeling, teelt en de beschikbare regeneratie en transformatie methoden. Cruciaal voor genetische transformatie is het beschikbaar hebben van een efficiënt regeneratie systeem. Er zijn in principe twee manieren voor plant regeneratie beschikbaar. Organogenese waarbij scheuten direct vanuit explantaten ontstaan en somatische embryogenese waarbij somatische embryos zich ontwikkelen vanuit explantaten al dan niet via een callus fase.

Hoofdstuk 2 beschrijft een procedure om vanuit vegetatief weefsel (blad met stengel aanhechting) somatische embryos te laten ontstaan. Twee verschillende typen embryogeen weefsel werden gevormd: ‘friable’ embryogeen callus (FEC) en compact embryogeen callus (CEC). CEC wordt het eerst gevormd. FEC wordt uit CEC gevormd 4 á 5 weken na cultivatie op PCA medium (een aangepast medium rijk aan vitaminen) met 5 mg/l picloram. Op dit medium konden CEC en FEC gedurende zes maanden in stand worden gehouden zonder verlies van regeneratie capaciteit. Planten konden geregenereerd worden uit CEC en FEC na plaatsing op regeneratie medium met 0.5 mg/l BA. Somatische embryos ontstonden uit CEC en

FEC en ontwikkelden zich verder tot planten. De regeneratie frequentie van CEC bedroeg ca 60%, terwijl die van FEC 22% was.

Hoofdstuk 3 laat zien dat FEC een uitstekend uitgangsmateriaal is voor de isolatie van protoplasten die zich kunnen ontwikkelen tot planten. Protoplasten werden geïsoleerd door FEC weefsel te behandelen met mengsels van enzymen die de celwand afbreken gedurende 26 uur bij 24 ^{o}C. In deze studie werd een hoge frequentie van celdelingen en micro-calli vorming verkregen. Protoplasten ontwikkelden zich tot micro-calli binnen 12 weken. Hiervan waren 59% in staat om somatische embryos te genereren waarvan de helft zich ontwikkelde tot planten. De op deze manier verkregen Alstroemeria planten vertoonden somaclonale afwijkingen in blad en bloem morfologie vergeleken met vegetatief vermeerderde controle planten. Dit werd hoogst waarschijnlijk veroorzaakt doordat relatief oud FEC weefsel als uitgangsmateriaal voor de protoplasten isolatie was gebruikt.

In Hoofdstuk 4 wordt de productie van genetische gemodificeerde planten beschreven met als doel om Alstroemeria Mozaïek Virus (AIMV) resistentie te verkrijgen. Dit gebeurde door een DNA construct te maken dat via zogenaamde RNA verkregen resistentie moest werken en waarbij via 'particle bombardment' FEC werd beschoten met dit construct dat gebaseerd was op het mantel eiwit gen (CP-gen) en een 3'- onvertaalde sequentie van het virus. Het *bar* gen, dat resistentie geeft tegen het herbicide basta, was in dit construct aanwezig als selecteerbare merker. Twee parameters van belang voor een betere efficiëntie van de beschieting, te weten het effect van een extra beschieting en de osmotische voorbehandeling van het FEC weefsel dat de beschieting onderging, werden beproefd door de transiente en stabiele trans-gen expressie te meten. Twee beschietingen van FEC gaf betere resultaten dan een enkelvoudige beschieting. Verder bleek dat FEC cultures die gedurende 8 uur vóór en 16 uur na de beschieting op een medium met 0.2 M mannitol en 0.2 M sorbitol waren geplaatst een hogere transformatie efficiëntie gaven en een groter aantal transgene planten opleverden. Met dit verbeterde protocol werd FEC weefsel getransformeerd met het virusgen construct. De hieruit verkregen potentieel getransformeerde planten vertoonden variatie in hun resistentie tegen het AIMV virus. De aanwezigheid van het *bar* gen en het virale genconstruct werd gedemonstreerd door middel van PCR analyse. Alhoewel een groot aantal planten de transfer naar de kas om onduidelijke redenen niet overleefden konden inocculatie experimenten met het virus worden uitgevoerd. Twee weken na toediening bleek bleken er een aantal planten te zijn

die duidelijk minder vatbaar waren voor het virus dan de ongetransformeerde controle planten. Na hertoetsing bleek dat niet alle planten hetzelfde niveau van resistentie hadden. Het was echter duidelijk dat in ieder geval de snelheid van aantasting door het virus in een aantal (PCR positieve) planten aanmerkelijk lager lag dan in de controle. Deze resultaten zijn bemoedigend voor verder onderzoek naar deze methode van het verkrijgen van virus resistentie in Alstroemeria, alhoewel er veel verkregen planten waren die last hadden van verminderde groei of zelfs van een ander (afwijkend) groeigedrag.

De ontwikkeling van een efficiënt transformatie systeem gebruik makend van *Agrobacterium tumefaciens* is beschreven in Hoofdstuk 5. FEC van genotype VV024 werd getransformeerd met Agrobacterium stam LBA 4404 die het plasmide pTOK233 bevatte. Het plasmide pTOK233 bevat verschillende genen waaronder het hygromycine phosphotransferase (*hpt*) gen dat resistentie geeft tegen het antibioticum hygromycine, het neomycine phosphotransferase (*nptII*) gen dat resistentie geeft tegen het antibiticum kanamycine, en het beta-glucuronidase (*gus*) gen dat een substraat, dat van nature niet voorkomt in plantencellen, kan omzetten in een blauw precipitaat. Verschillende parameters waaronder, infectie tijd, co-cultivatie periode, effect van acetosyringon, verschillende verdunningen van de bacteriesuspensie en temperatuur gedurende de co-cultivatie periode werden beproefd en geoptimaliseerd. Een hoge frequentie van transiente en stabiele GUS expressie werd verkregen wanneer FEC callus werd geïnfecteerd met LBA 4404(pTOK 233) gedurende 30 minuten met 50 uM acetosyringon in een 1:10 verdunning van de bacterie cultuur en werd gecocultiveerd bij 24 ^{o}C gedurende zeven dagen. Aanwezigheid en expressie van de transgenen in geregenereerde planten werd aangetoond door middel van PCR en GUS analyse. Selectie gebaseerd op hygromycine en kanamycine resistentie werden met elkaar vergeleken. De frequentie van 'ontsnappers' (planten die getransformeerd lijken maar het niet zijn) was lager bij hygromycine selectie, daarentegen was het aantal transgene lijnen die met kanamycine selectie werd verkregen veel hoger. Naast deze vergelijking werd ook luciferase selectie versus phosphinotricine (PPT) selectie vergeleken. Luciferase gebaseerde selectie resulteerde in een twee maal zo hoge transformatie frequentie als PPT gebaseerde selectie. Ook het aantal cultures die bruin werden en geen groei meer vertoonden alsmede het aantal albino planten dat uiteindelijk verkregen werden was aanmerkelijk lager met luciferase selectie. Het transformatie protocol beschreven in dit hoofdstuk heeft als groot voordeel dat

het jaarrond kan worden toegepast vanwege het gebruik van vegetatief weefsel als start materiaal. In de toekomst zouden experimenten moeten worden uitgevoerd waarbij het virale construct middels Agrobacterium in het FEC weefsel wordt overgebracht. Hierbij moet voldoende aandacht geschonken worden aan het fenomeen somaclonal variatie.

In Hoofdstuk 6 tenslotte worden enkele algemene aspecten van somatische embryogenese, het voorkomen van somaclonale variatie en de verschillende transformatie systemen beschreven.

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Gunpo, Korea

Curriculum Vitae

Jong-Bo Kim was born in Seoul, Korea on 18, January 1971. He entered the department of Horticulture, Kon-Kuk University, Seoul, Korea in 1989 and obtained a bachelor degree in horticulture in 1998 from the same university. During this period, he finished a military service from July 1991 to December 1992. From March 1996, he continued to start MSc course, majoring in floriculture in the same department, Kon-Kuk University and obtained his MSc degree in February, 1998. The title of MSc thesis was: “In Vitro study on callus induction and regeneration with stolon and caryopsis in Zoysiagrass”. After successful completion of MSc course, he decided to prepare study in abroad to extend his knowledge and experience. In the end, he passed national competition examination and awarded a three-year scholarship from the Ministry of Education, Korea. Thus, he started the Ph.D. research project at the Laboratory of Plant Breeding, Wageningen University in the Netherlands in February 1999. This thesis contained the results of research for the past four years. The project was conducted at the Laboratory of Plant Breeding, Wageningen University under supervision of Prof. Dr. Richard. G. F. Visser, Prof. Dr. Ir. E Jacobsen, Dr. Ir. Marjo de Jeu and Dr. Ir. Krit Raemakers. Since 1 March of 2003, he has been working at Turfgrass and Environmental Institute, Samsung Everland Inc. as an associate researcher.

Education Statement of the Graduate School Experimental Plant Sciences

1) Start-up phase	date
► First presentation of your project	
► Writing a project proposal	
► Writing a review or book chapter	
► MSc courses	
Genetic variation and modification, code F750-210	1999-2000
Selection Methods, code F750-219	1999-2000
Botanical Somatic Cell Genetics, code F700-207	1999-2000
► Laboratory use of isotopes	

Subtotal Start-up phase 7.0 credits *

1) Scientific Exposure	date
► EPS PhD student days	
Wageningen	December 9, 1999
Wageningen	January 25, 2001
Wageningen	January 24, 2002
► EPS theme symposia	
Theme symposium 4, Wageningen	October 20, 2000
Theme symposium 4, Nijmegen	December 13, 2001
Theme symposium 4, Wageningen	December 20, 2002
► Regular research discussion	
Attendance internal research discussions of the group	1999 – 2002
► Seminars (series), workshops and symposia	
"Control of cell division in the yeast Saccheromyces cerevisiae", Weinert (Seminar)	February 12, 1999
"The use of microarray techniques in Arabidopsis", Wisman (Seminar)	December 21, 1999
"Combining genetics and genomic analysis to understand disease resistance in plants", Glazebro (Flying seminar)	April 24, 2001
► International symposia and conferences	
"In vitro culture and Horticulture Breeding Symposium", Tampere, Finland	July 2-7, 2000
"Eucarpia Breeding, Ornametal section", Melle, Belgium	July 3-6, 2001

► Presentations	
Theme symposium 4, Wageningen (Oral)	October 20, 2000
► Excursion	
Visiting of Breeding Companies (Konst Alstroemeria, Floricultura B.V., Hilverda B.V, Freesman BV)	1999 - 2002

Subtotal Scientific Exposure 8.9 credits*

3) In-Depth Studies	**date**
► EPS courses or other PhD course	
Autumn school " Interactions between plants and attacking organisms: mechanisms, genetics, ecology and evolution"	October 16-18, 2000
► Individual research tranining	
Virus inoculation techniques at Plant Research International	2001

Subtotal In-Depth Studies 1.6 credits*

4) Personal development	**date**
► Skill training courses	
Written English Course (Centa)	March-May, 2002
Scientific writing course (Centa)	Setember-November 2002
Endnote course (Library)	September, 2001
► Other courses	
Plant Virology, code G150-203	1999-2000
Biotechnology in crop production, code G250-209	1999-2000
Recominant DNA technology, code 450-203	1999-2000
► Membership pf Board, Committee and PhD council	

Subtotal Personal development 9.1 crredits*

TOTAL NUMBER OF CREDIT POINTS*	**26.6**

* A credit represents a normative study load of 40 hours of study

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