

**BIOTECHNOLOGICAL APPROACHES  
IN LILY (*LILIUM*) PRODUCTION**

**VELI-PEKKA  
PELKONEN**

Faculty of Science,  
Department of Biology,  
University of Oulu

OULU 2005





**VELI-PEKKA PELKONEN**

**BIOTECHNOLOGICAL APPROACHES  
IN LILY (*LILIUM*) PRODUCTION**

Academic Dissertation to be presented with the assent of  
the Faculty of Science, University of Oulu, for public  
discussion in Kuusamonsali (Auditorium YB210),  
Linnanmaa, on April 15th, 2005, at 12 noon

OULUN YLIOPISTO, OULU 2005

Copyright © 2005  
University of Oulu, 2005

Supervised by  
Professor Anja Hohtola  
Professor Hely Häggman

Reviewed by  
Professor Anna Bach  
Professor Risto Tahvonen

ISBN 951-42-7658-2 (nid.)  
ISBN 951-42-7659-0 (PDF) <http://herkules.oulu.fi/isbn9514276590/>  
ISSN 0355-3191 <http://herkules.oulu.fi/issn03553191/>

OULU UNIVERSITY PRESS  
OULU 2005

## **Pelkonen, Veli-Pekka, Biotechnological approaches in lily (*Lilium*) production**

Faculty of Science, Department of Biology, University of Oulu, P.O.Box 3000, FIN-90014

University of Oulu, Finland

2005

Oulu, Finland

### ***Abstract***

Biotechnology has become a necessity, not only in research, but also in the culture and breeding of lilies. Various methods in tissue culture and molecular breeding have been applied to the production of commercially important lily species and cultivars. However, scientific research data of such species and varieties that have potential in the northern climate is scarce.

In this work, different biotechnological methods were developed and used in the production and culture of a diversity of lily species belonging to different taxonomic groups. The aim was to test and develop further the existing methods in plant biotechnology for the developmental work and the production of novel hardy lily cultivars for northern climates.

Most of the plant material was started from seeds, which provided genetic variability and new material for breeding. Different features in seed structure were studied with light microscopy and SEM, and different parameters affecting germination were tested. Several tissue culture protocols were also compared with different species using both solid and liquid media. Molecular biological methods were used in assessing genetic background of traditionally grown lilies. Somatic embryogenesis in callus differentiation of callus cultures was studied, and gene expression behind differentiation processes was analyzed with various molecular biological methods. Particle bombardment system was used in genetic transformation. In addition, protoplast isolation methods from various tissues were tested.

The main results indicate that many tissue culture methods can be used in research and in mass production with all tested species. Especially in a large-scale production, temporary immersion system is promising. In addition to the conventional bulb scale material, seeds were found to be a suitable starting material for genetic variability required for production of new cultivars, and in the preservation of natural populations. RAPD techniques proved a suitable method for revealing phylogenetic relations of different lily species and cultivars. Methods in DNA and RNA isolation, cloning and analysis were optimized for lily material. In addition, particle bombardment system was successfully used for genetic transformation of lily callus.

In the future, more information is needed to understand better the germination and differentiation processes, focusing especially in the genes, their products and function. In addition, the large and still mostly unknown lily genome is a challenge for research in the future. However, the currently presented results provide good opportunities for further developmental work and research of hardy lily species.

*Keywords:* differentiation, genetic transformation, germination, molecular biology, morphology, somatic embryogenesis, tissue culture



*To my family*



## Acknowledgements

This work was done in the department of Biology and the Botanical Gardens in the University of Oulu. It was financially supported by the Finnish Academy, the Ministry of Agriculture of Finland, the Finnish Cultural Foundation and Nikolaj and Ljudmila Borisoff's foundation. In various parts of the work, there was co-operation with the Agrifood Research Finland, the Finnish Association of Landscape Industries, the Botanical Gardens of the University of Helsinki, and the University of Athens, Greece. I wish to express my gratitude to all my financiers and co-operation parties for their awarding support.

I am most grateful to my supervisors, prof. Anja Hohtola and prof. Hely Häggman, for their professional, accurate and supportive guidance. Special thanks to the official referees of this thesis, prof. Anna Bach (Agricultural University of Krakow, Poland) and prof. Risto Tahvonen (Agrifood Research Finland, Piikkiö), for their valuable comments on this work. Thanks also to Leena Id for her thorough work in revising the language in this work.

My greatest gratitude to docent Kari Laine, whose comments and encouragement have kept me going from the very beginning of my work. In Athens, I was privileged to work in the science group of prof. Mitrakos. His wisdom and attitude will remain my guideline in my life. I also give my thanks to Alexia Alexaki and Stelios Vaseiliadis for their kind companion and help during my stay in Greece. My special thanks to the chief of Agrifood Finland Research Laukaa station, Marjatta Uosukainen, of her valuable advice and inspiring co-operation.

I want to express my gratitude to my teachers in the university of Oulu, especially Dr. Anneli Kauppi, prof. Sirkka Kupila-Ahvenniemi, and prof. Pekka Lähdesmäki for their inspiration and intellectual contribution for my studies and my work. The staff of the Botanical Garden, especially intendants Mirja Siuruainen and Ritva Hiltunen, and Aino Hämäläinen, Pasi Paavola, Tuula Kangas, Leena Ridell, Elsa Rantakallio, Veikko Oinas, Jouni Lammela and Tuomas Kauppila have given their invaluable contribution to my work in its every stage, and to whom I owe my greatest gratitude. I also thank Tuulikki Pakonen, Hanna-Liisa Suvilampi, Minna Vanhatalo, and the ladies in the office of the department of biology for their patient assistance. My work mates in the department of biology deserve a great acknowledgement for their nice company, support, and keeping

the spirits high no matter the day, season or reason! Big hug to Terttu Kämäräinen, Laura Jaakola, Anna-Maria Mattila, Ulla Timonen, Minna Pakonen, Katja Karppinen, Soile Jokipii, Jaana Vuosku, Erja Taulavuori and Taina Uusitalo. Ladies: I just couldn't manage without you! I also want to thank my student mates Eija Kukkola, Tarja Ukkonen, Veli-Matti Rokka, Sari Kontunen-Soppela and Liisa Kytölä who have also been there to give their encouragement and enjoyable company.

Personal life reflects to anyone's work, and I am lucky to have guys like Reijo Prokkola, Juha Tapio, Ville Urponen, Timo Arbelius, Esa Hilli, Anne Kokko and Ulla Sohlo as my faithful friends giving their encouragement and a shoulder, when I most need it. Collective thanks also to the great fellow singers in the Oulu Chamber Choir for keeping me inspired with all the musical activities.

Finally, my greatest acknowledgement and gratitude goes to my beloved family. Mum and Dad, you gave me life, but you gave me also the best possible provisions for my life, better than anyone can ever get. You are the best parents in the world! My dear sisters, Tiina and Katri, and my dear brother Sauli have always stayed by my side, and never turn their back to me. Can anyone ask any better sisters or a brother, not only siblings but also best friends. I also give my thanks to my brother-in-law, Ford, for his fellowship, no matter how far geographically, but mentally always close. Last but not least, my dear nephews, Vesa, Juha, Tommi, Andreas and Alexander, and my dear niece, Tea, have always brought about some excitement and fun in my life assuring, that it never gets too boring out there!

*The longer the path, the more friends and dear people you meet along your way. As this work has been a long path, I have the privilege to owe so much gratitude to so many people.*

Oulu, February 2005

Veli-Pekka Pelkonen

## Abbreviations

ABA	abscissic acid
AGP	arabinogalactan protein
BA	6-benzyladenine
cDNA	complementary DNA
2,4-D	2,4-dichlorophenoxyacetic acid
FAA	formaldehyde:acetic acid fixative
GA	gibberellic acid
GUS	$\beta$ -glucuronidase
HSP	heat shock protein
IAA	indole-3-acetic acid
LEA	late embryogenesis abundant
LTP	lipid transfer protein
NAA	$\alpha$ -naphthaleneacetic acid
<i>npt</i>	nopaline synthase gene
PCR	polymerase chain reaction
PEM	proembryonic cell mass
PIC	(picloram) 4-amino-3,5,6-trichloropicolinic acid
RAPD	random amplified polymorphic DNA
RT	reverse transcriptase
se	somatic embryo
SEM	scanning electron microscopy
SERK	somatic embryogenesis related receptor-like kinase
TDZ	(thiadiazuron) N-(1,2,3-thiadiazol-5-yl)-N'-phenylurea
TIBA	2,3,5-triiodobenzoic acid
<i>uidA</i>	$\beta$ -glucuronidase gene



## **List of original papers**

THIS thesis is based on the following publications, which are referred to in the text by their Roman numerals:

Pelkonen V-P, Kauppi A (2004) The role of light, growth regulators and the seed structures on the germination of lily (*Lilium*) species in vitro. Submitted for publication.

Pelkonen V-P, Hohtola A, Häggman H (2004) Regal lily (*Lilium regale* Wil.) propagation, temporary immersion system RITA® versus conventional solid medium culture. Submitted for publication.

Pelkonen V-P, Niittyvuopio A, Pirttilä A-M, Laine K, Hohtola A (2004) Phylogenetic background of orange lily (*Lilium bulbiferum* s.l.) cultivars by RAPD analysis. Submitted for publication.

Pelkonen V-P, Kauppi A (1999) The effect of light and auxins on the regeneration of lily (*Lilium regale* Wil.) cells by somatic embryogenesis and organogenesis. *Int J Plant Sci* 160: 483-490.

Pelkonen V-P, Hohtola A, Häggman H (2004) Genetic transformation method for a trumpet lily. Manuscript.



# Contents

Abstract	
Acknowledgements	
Abbreviations	
List of original papers	
Contents	
1 Introduction	15
1.1 The genus of lilies	15
1.1.1 The organogenic structure of a lily	17
1.1.2 Germination types of lilies	18
1.1.3 Genome organization of lily species	19
1.2 Lilies as garden plants in Northern Europe	20
1.2.1 Traditionally grown lilies	20
1.2.2 New species and cultivars in Finland	20
1.3 <i>In vitro</i> propagation methods of lily species	21
1.3.1 Liquid cultures	22
1.4 Differentiation of <i>in vitro</i> propagated tissues	22
1.4.1 The effect of sugar on differentiation <i>in vitro</i>	23
1.4.2 The effect of growth regulators on differentiation <i>in vitro</i>	23
1.4.3 The effect of physical factors on differentiation <i>in vitro</i>	24
1.4.4 Changes in gene expression during somatic embryogenesis	25
1.5 Protoplast isolation and culture	26
1.6 Genetic transformation	27
1.6.1 Gene transfer via electroporation and somatic fusion	27
1.6.2 Particle bombardment	28
2 Aims of the research	29
3 Materials and methods	30
3.1 Plant material	30
3.1.1 Germination	30
3.1.2 Propagation, callus induction and differentiation	31
3.2 Histological and morphological analysis	31
3.3 DNA isolation and RAPD analysis	32

3.4 Biolistic transformation experiments.....	32
3.5 RNA isolation and gene expression studies (additional data).....	32
3.5.1 cDNA synthesis and PCR analysis (additional data).....	33
3.6 Protoplast isolation (additional data).....	33
3.7 Statistical analysis.....	33
4 Results .....	34
4.1 Germination in vitro .....	34
4.1.1 Seed coat and endosperm .....	34
4.1.2 The effect of light and temperature .....	35
4.1.3 The effect of GA, ABA and fluridon .....	36
4.2 Induction and growth of callus .....	37
4.2.1 Suspension and liquid cultures .....	38
4.3 Bulb regeneration and culture.....	39
4.4 Organogenesis and somatic embryogenesis in callus tissue .....	40
4.5 Protoplast isolation and maintenance (additional data) .....	41
4.6 Expression of differentiation related genes (additional data) .....	43
4.7 Gene transfer via particle bombardment.....	43
4.8 Phylogenetic analysis of the Orange lily .....	44
5 Discussion .....	45
5.1 Tissue culture of bulbous plants .....	45
5.2 Genetic diversity.....	46
5.3 Pathogen control and purification.....	47
5.4 Gene expression during differentiation.....	47
5.5 New methods for breeding from biotechnology .....	49
6 Conclusions .....	50
References	
Appendix	
Original papers	

# 1 Introduction

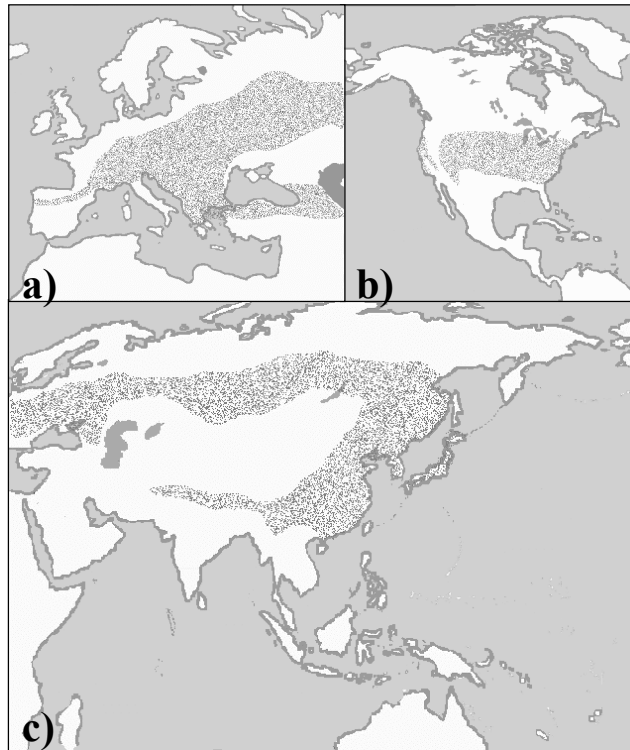
Lily species have been used as ornamental plants for centuries. Although the very first hybrids originate from the 19<sup>th</sup> century (Woodcock & Stearn 1950, Jefferson-Brown & Howland 1995), the systematic breeding of lily cultivars, the number of which exceeds several thousands nowadays, started only in the 1950's by Jan de Graaff.

Tissue culture has become an important method in research and mass production of various plants during the last decades. Combining the benefits of mass production and fast regeneration of uniform plant material in tissue culture is a necessity for the future breeding and culture of lilies. However, to make tissue culture a commercially relevant production system, production protocols need to be developed separately for each plant crop and cultivar.

Bulbous plants, like lilies, have proved to be ideal for tissue culture, as their regeneration potential is usually high. Furthermore, the compact structure of the shoot makes them easy to handle both in solid and in liquid cultures. Nowadays, lilies are one of the most important bulbous crops produced in tissue culture also in an industrial scale (Chu & Kurtz 1990).

## 1.1 The genus of lilies

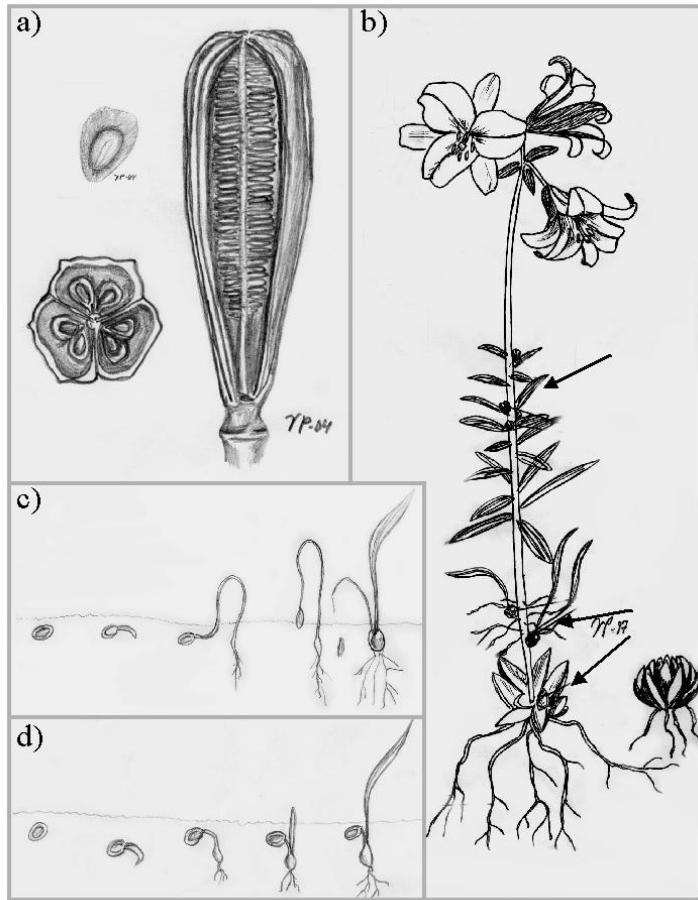
Genuine lilies belong to the *Lilium* family consisting of approximately 100 species in the Eurasia and North American continents (Fig. 1, Table 1). Most lily species originate from South-East Asia (China, Korean peninsula and Japan). Another large group of species comes from North America. The number of native European and Caucasian (Eurasian) species is approximately 10. (Woodcock & Stearn 1950, Baranova 1990.)



**Fig. 1. The geographical distribution of native lily species. a) Eurasian species range from the Atlantic through Mediterranean and Central Europe to the Caucasus and Ural mountains. b) Most American species are found in an area ranging from the Atlantic to the Midwest. The distribution of the Western species is limited by the Rocky Mountains and the Pacific. c) Most native lilies originate from the Eastern Asia. Their distribution ranges from the Pacific to Ural and Caucasus mountains, to the Northern India and Burma in the south and Siberia in the north.**

Natural habitats of lilies are mostly on high altitudes, reaching up to over 2000 meters from the sea level. Their adaptation to extreme annual changes in temperature and humidity makes them suitable garden plants in a large climatic area from temperate to subarctic latitudes.

Most lily species have been used as ornamental plants for centuries. Systematic breeding of lily cultivars, the number of which exceeding nowadays to several thousands, started only in the 1950's by Jan de Graaff. Though, the very first hybrids originate from the 19th century. (Woodcock & Stearn 1950, Jefferson-Brown & Howland 1995.) Today lilies are important plants that are grown in gardens and cultivated for cut flowers. In addition to a few traditionally grown species, dozens of imported species and cultivars are grown all over Finland.



**Fig. 2. The reproduction and structure of a lily. a) A cross and longitudinal section of a mature seed pod containing seeds. b) The vegetative shoot and inflorescence of lily; arrows point to the adventitious or axillary buds (bulbils). c) Epigeal germination, d) hypogeal germination.**

### ***1.1.1 The organogenic structure of a lily***

The main organs of an adult lily plant are basal roots, the bulb, which is the underground part of the shoot, and the inflorescence or the flowering shoot that emerges above the ground and carries the flowers (Fig. 2). The size and form of these organs vary greatly within the genus. The bulb is always without any protective tunic or coat, and the growth type is sympodial. The bulb scales are morphologically specialised leaves containing nutrient and water reservoirs. The apical meristem produces the inflorescence whereas

the axillary meristems produce the side bulbs that continue the growth after the main shoot perishes. (Woodcock & Stearn 1950, Baranova 1990, McRae 1998.)

*Table 1. Classification of lily species*

Class	Species
1. Martagon group	<i>LL. distichum, hansonii, martagon, medeoloides, tsingtauense</i>
2. American group	a) <i>LL. bolander, columbianum, kelloggii, humboldtii, rubescens, washingtonianum</i> b) <i>LL. maritimum, nevadense, occidentale, pardalinum, parryi, parvum, roezlii</i> c) <i>LL. canadense, grayi, iridollae, michauxii, michiganense, superbum</i> d) <i>LL. catesbaei, philadelphicum</i>
3. Candidum group	<i>LL. bulbiferum, candidum, carniolicum, chalconicum, monadelphum, polyphyllum, pomponium, pyrenaicum</i>
4. Oriental group	<i>LL. auratum, brownii, japonicum, nobilissimum, rubellum, speciosum</i>
5. Asiatic group	a) <i>LL. davidii, duchartrei, henryi, lancifolium, lankongense, leichtlinii, papilliferum</i> b) <i>LL. amabile, callosum, cernuum, concolor, pumilum</i> c) <i>LL. bakerianum, mackliniae, nepalense, ochraceum, sempervivoideum, taliense, wardii</i>
6. Trumpet group	a) <i>LL. leucanthum, regale, sargentiae, sulphureum</i> b) <i>LL. formosanum, longiflorum, neilgherrense, philippinense, wallichianum</i>
7. Dauricum group	<i>LL. dauricum, maculatum</i>

The inflorescence bears the leaves that lie either in whorls or are scattered along the stem. The flowers are situated on top of the stem either individually, in racemes or in umbels. The flower is the model of the monocotyledon type of structure. It is symmetric consisting of two whorls of petals, two whorls of anthers and a single trilobed ovary. The flowers are large and showy, and they vary in size and colour. (Woodcock & Stearn 1950, McRae 1998.)

### **1.1.2 Germination types of lilies**

Lily species are divided into two main groups according to their germination type: epigeal and hypogeal. Epigeal seeds germinate usually immediately after sowing without showing any signs of dormancy. The cotyledon emerges from the seed at the same time as the radicle intrudes into the soil. The growth of the small seedling continues, and a small plant is formed without dormancy. Hypogeal germination is usually controlled by dormancy which breaks only after the seed has been exposed to cold treatment. During germination, the radicle grows out of the seed but the cotyledon stays within the endosperm conducting reserve nutrients to the forming primary bulblet (Fig. 2). Dormancy is often re-induced after the primary bulblet has formed, and another cold period is required for further development of the plant. (Woodcock & Stearn 1950, Baranova 1990.)

The germination type seems to have evolutionary significance since it is species-specific and thus genetically inherited. Most of the Eurasian and American species represent the delayed hypogeal type of germination whereas most East Asian species represent the immediate epigeal type (Table 2).

Of the several pathogens infecting lilies, such as lily symptomless virus (LSV), tulip breaking virus (TBV) or fungal diseases causing basal bulb, scale or root rots (*Fusarium*, *Cylindrocarpon*), none has proved to be transmitted by seeds. This makes seed propagation an invaluable method for disease control in cultivated stocks. (van Aartrijk *et al.* 1990.)

In this work, the germination of different lily species and the effect of physical factors and growth regulators were studied with in vitro germinated seeds (I).

*Table 2. Lily species with different germination type in various taxonomic groups*

Group	Epigeal		Hypogeal		Total
	Immediate	Delayed	Immediate	Delayed	
1	-	-	-	5	5
2	2	-	1	21	24
3	1	3	-	8	12
4	1	-	1	5	7
5	27	-	-	-	27
6	7	2	-	-	9
7	2	-	-	-	2
Total	40	5	2	39	86

### ***1.1.3 Genome organization of lily species***

The genome size of lilies is one of the biggest among all organisms. For example, the genomic DNA *L. henryi* consists of 32 billion base pairs, and with some species the number of base pairs can rise up to 100 billion (Bennet & Smith 1976, Sentry & Smyth 1989). Thus, there are differences in the genome size between species (Siljak-Yakovlev *et al.* 2003).

The lily genome is organized in large metacentric and subtelocentric chromosomes. The haploid number of chromosomes is 12, and it is very constant throughout the whole genus. Natural species are mostly diploid ( $2n = 24$ ), but with some species there are also triploid forms ( $3n = 36$ ) that are sterile. Some naturally occurring tetraploids have also been found, and these are usually fertile. It has been assumed that some interference in the meiosis of the forming reproducing cells can result in the spontaneous formation of polyploidy in lilies. There are also examples of spontaneous inter-specific hybrids that show a normal diploid chromosome number. Abnormal chromosome numbers have been found in some studies, but in general their existence is very rare. (Abraham 1939, Stewart & Bamford 1943, Stewart 1947, Sharma & Bhattacharyya 1957, Siljak-Yakovlev *et al.* 2003.)

As breeding techniques have developed, the production of tetraploid plants has been accomplished artificially by treating either seeds or bulb scales of diploid plants with colchicine. Tetraploid forms have proved to be more robust in their growth habit having a thicker texture of tissues and higher resistance to diseases. When crossed with diploid forms they produce triploid offspring. (McRae 1998.) Generally the genome size and

organisation is very stable in the genus, and the normal diploid chromosome number predominates both with species and with hybrids or cultivars.

The immense size of the lily genome is partly due to the substantial amount of repetitive sequences in the chromosomes. Considering that the genome organization of lilies is very conservative and that the repetitive sequences have remained in the genome through millions of years of evolution, their importance to lily species must have evolutionary significance.

## **1.2 Lilies as garden plants in Northern Europe**

In addition to a few traditionally grown species the diversity of the lilies has increased along with the popularity also in Scandinavia. However, the variety of lilies available in this area is limited compared to that in Central Europe and North America. Preliminary observations on the persistence of a large scale of lily species in Northern Finland are promising with regard to further breeding and developmental work. This is the prerequisite for the production of hardy lilies of high quality for the culture in the demanding climate.

### ***1.2.1 Traditionally grown lilies***

Lilies have had their place in European gardens for centuries. Various strains of the Orange lily (*L. bulbiferum s.l.*) are found all around the countryside in Nordic countries up to Lapland. Originally these lilies came from Central Europe where they have been grown in gardens since the 16th century (Bos 1993). The Turk's cap lily (*L. martagon* L.) and the Tiger lily (*L. lancifolium* Thunb..) are also old garden plants. Along with these more common species there are some examples of other, less known lilies of old origin. Some strains of the Caucasian lily (*L. monadelphum* Bieberstein), and the Marhan lily (*L. x Marhan* Van Tubergen), which is a *grex* (an inter-specific hybrid) between the Turk's cap lily and the Korean lily (*L. hansonii* Leichtlin), have been found.

### ***1.2.2 New species and cultivars in Finland***

Although the variety of lilies grown in gardens consists of dozens of natural species and cultivars, their number has remained limited in the Nordic countries. Most of the imported lily cultivars are Asiatic hybrids. Only a few Oriental and Trumpet hybrids have been available, and their persistence has not been very good in the northern climate. Of the species, only some strains of the Regal lily (*L. regale* Wil.) originating from South Western China, and the Speciosum lily (*L. speciosum* Thunb.) originating from Eastern Asia, have been provided, but their culture has not proved to be successful in Finland. Today, the main areas for commercial lily production are the Netherlands, Japan and the United States (van Aartrijk *et al.* 1990).

The Regal lily was selected as one of the new species for this work. It is one of the parent species that have been used for the breeding of the Trumpet lily hybrids, and it has many advantageous features. It is relatively fast growing and reaches the flowering size within approximately 1-2 years after germination. Its development can be accelerated further by tissue culture. It reproduces very effectively from seeds and from side-bulbs, and has a high ornamental value. Its blooming time in the Oulu region (65° N; 25° 30' E) is in July.

The Gold band lily and its white-flowered form *virginale* have proved hardy also in the north. The flowering time of this species is earlier than that of the Speciosum lily, the former blooming in the Oulu region at the end of July and the latter in August or September. They both belong to the Oriental species group originating from Eastern Asia.

Of the many American species, the most promising is the Canadian lily. It is hardy, and blooms in the Oulu region at the end of June or in the beginning of July. It also proliferates very easily both sexually and vegetatively.

Of the Eurasian species (Candidum group) the Caucasian lily has already been introduced to culture in Finland. However, its availability is very scarce and the present strains are physiologically old. Its production starting from seeds is easy, and it is completely hardy. It blooms quite early in the Oulu region, already in June.

In addition, there are various other lily species from all taxonomic groups that have potential as garden plants in Finland.

### 1.3 *In vitro* propagation methods of lily species

Tissue culture has been applied to the propagation of lilies since the late 1950's (Robb 1957). Soon after that, multitude of research articles on the development of tissue culture methods for lilies became available (Sheridan 1968, Simmonds & Cumming 1976, Stimart & Ascher 1978).

Lily tissues in general have a high regeneration potential (George 1993). Still, bulb scales have the best capacity to regenerate adventitious bulbs (Takayama & Misawa 1979). Hence, bulb scales are most commonly used as explants for vegetative propagation. Unfortunately, being under-ground parts, there is a high contamination risk with bulb scales. There are also reports on the use of other tissues. In some cases, contamination problems have been overcome by using shoot apices and internodes as a starting material (Sheridan 1968, Tanaka *et al.* 1991, Godo *et al.* 1996, Nhut 1998). Leaves have weaker regeneration capacity than shoots, but still they have also been successfully used as starting material (Niimi 1986, 1995). Somatic tissues from flowers, like pedicles, styles and stamens' filaments have been used for callus induction (Montezuma-de-Carvalho & Guimarães 1974, Tribulato *et al.* 1997).

Basic nutrient concentration does not seem to play any crucial role in the growth and differentiation, as far as the below mentioned standard culture media are concerned. Although MS (Murashige & Skoog 1962) medium with variations is the most commonly used culture medium, also others, like LS (Linsmaier & Skoog 1965) or White's medium (White 1963) have successfully been used with lilies (Robb 1957, Sheridan 1968, George 1993).

Generative tissues as starting material are suitable for different purposes. Anther and pollen, as well as parts of ovary, can be used for production of haploid or polyploid plants. Niimi and co-workers (2001) recently reported the use of anthers in the production of virus-free plants. Inter-specific hybrids have successfully been produced by growing fertilised ovaries on tissue culture medium (Okazaki *et al.* 1992). In addition, both intact seeds and excised embryos have been cultured *in vitro* (Maesato *et al.* 1994). Roots have not been reported to have any regeneration potential in lilies.

### **1.3.1 Liquid cultures**

Besides on solid medium, tissue culture is also possible in suspension cultures (Sheridan 1968). Several culture methods and techniques have been applied to the culture of lilies. These include, for example, mass production systems for suspension cultures in rotary shakers (Takayama & Misawa 1981, Niimi *et al.* 1997, Nakano *et al.* 2000). In addition, temporary immersion culture system has been developed for the mass production of many important crops, including some bulbous plants (Teisson & Alvard 1995). The advantage in the immersion system is that it is not necessary to change the medium as often as in shake cultures, and the application of fresh medium is also easier (Escalona *et al.* 1999). There is no risk of anoxia, because the tissue is only immersed to the culture medium temporarily in contrast to the conventional suspension cultures, where tissue is in the medium all the time. In this work, both rotary shaker and an immersion culture system were tested in the differentiation and culture of lily callus.

## **1.4 Differentiation of *in vitro* propagated tissues**

The differentiation potential of callus tissue depends on the cultivar or genotype and the age of the tissue. It has been demonstrated that juvenile tissues with less degree of differentiation, like embryos and meristems, have more organogenic and embryogenic potential than more differentiated or aged tissues. (Takayama & Misawa 1980, Tisserat 1987, Loiseau *et al.* 1998.) Prolonged culture of the callus results in decline in differentiation capacity although the rate of cell divisions may remain on the same level (Nakano *et al.* 2000).

Organogenesis is, at present, the predominant differentiation process in lilies (III). In bulb scale explants, small bulblets are formed on the basal part of the scale. This is the case also in 'scaling' which is the conventional way of vegetative reproduction. (George 1993.) Organogenesis takes place also in natural life cycle of certain lily species when bulbils are formed in the leaf axils of the stem. Adventitious shoots, or bulblets, are usually formed also in the underground part of the stem (Fig. 2). Somatic embryogenesis can be induced in tissue culture basically in any tissue, but embryogenic cell masses of the callus tissue have mostly been used for this purpose. Juvenile or reproductive tissues, like immature embryos, ovaries or young seedlings, are mostly used for inducing callus with high embryogenic potential. Primary somatic embryos may develop straight from the tissue, and they can be used, in turn, in the production of embryogenic cell masses or

secondary somatic embryos. The rate of somatic embryogenesis varies a lot depending on the species, genotype or tissue used. Direct somatic embryos are usually formed by juvenile or meristematic tissues, e.g., embryos. Somatic embryos typically arise from embryogenic cell clusters consisting of a few to several dozens of small cells with embryogenic potential. (Williams & Maheswaran 1986, Lo Schiavo 1995.)

Differentiation processes have been studied with a multitude of plant species from conifers to dicotyledons and monocotyledons. There are big differences both in protocols used as well as in the differentiation response between different plant species. There are also substantial differences in responses between genotypes, or even between different tissues or developmental stages within the same genotype. Differentiation is promoted by the adjusting phytohormone concentration and ratio, sugar content and quality and certain physical factors such as light intensity and temperature. Reducing the hormone concentration or reducing the auxin/cytokinin ratio can induce the differentiation of callus. Differentiation is also promoted by reducing the sugar concentration and increasing light intensity. (Tisserat 1987, Kyte 1990, George 1993.)

#### ***1.4.1 The effect of sugar on differentiation in vitro***

Sucrose concentration varies usually between 2-6% (w/v) depending on the species and the tissue used. Higher concentrations are used in callus induction, somatic embryogenesis and protoplast cultures whereas lower concentrations are used in the mass production of differentiated plants. The sugar content has a clear effect on morphogenesis because in higher concentrations the sprouting or formation of leaves decreases and the size of the bulb scales increases. The combination of sucrose and mannose has also been proved to promote the bulb growth (Yamagishi 1995). This tendency may be due to dormancy induction in the shoots caused by elevated osmolality of the medium, which, in turn, imposes a water stress on the tissue (Takayama & Misawa 1980, Aguetaz *et al.* 1990, Gerrits & De Klerk 1992).

#### ***1.4.2 The effect of growth regulators on differentiation in vitro***

Growth regulators have a very important role in both induction of differentiation and in growth. The role of different plant hormones and their concentrations on the morphogenesis of differentiating plants has been studied in various respects. Takayama and Misawa (1979) showed clearly the interaction of auxin,  $\alpha$ -naphthalene acetic acid (NAA) and cytokinin (kinetin) in the formation of bulblets and roots; higher auxin/cytokinin ratio increased root formation whereas lower ratio increased bulb formation. In studies on the effect of auxin/cytokinin ratio on the number and size of differentiating bulbs it has been noticed, that at higher cytokinin concentrations the number of differentiating bulblets per explant is higher (Takayama & Misawa 1982, Niimi 1995). When different cytokinins, such as 6-benzyladenine (BA), kinetin, 2iP and zeatin, were tested in combination with NAA, differences in regeneration response in general were found (Maesato *et al.* 1994). Besides NAA, also other auxins have been

used in culture of lilies. Caputo and co-workers (1990) reported that a combination of indole-3-acetic acid (IAA) and BA gives the best differentiation response. IAA has also been used in inducing direct regeneration of shoots from differentiated tissue (Montezuma-de-Carvalho & Guimarães 1974). 2,4-dichlorophenoxyacetic acid (2,4-D) has been used as an auxin prior to regeneration in callus cultures, and it has also been shown to be more effective than other auxins in dedifferentiation and callusing (Priyadarshi & Sen 1992). Transfer from hormone-containing propagation medium into hormone-free induction medium has proved to be most effective for the regeneration of callus (Tribulato *et al.* 1997).

The formation of dormancy strongly affects the morphogenesis of differentiating shoots in bulbous plants. Dormant plants typically form only bulb scales and no leaves. Dormancy can be induced or released by growth regulators. Applied abscissic acid (ABA) increases the number of bulb scales and reduces the number of leaves. On the other hand, induced dormancy could be overcome by applying the ABA synthesis inhibitor 1-methyl-3-phenyl-5-(3-[trifluoromethyl]-4(1H)-pyridone (fluridone) and gibberellic acid (GA). (Aguettaz *et al.* 1990, De Klerk 1992.)

In addition to the previously mentioned growth regulators, some other compounds, such as 24-epibrassinolide, 4-amino-3,5,6-trichloropicolinic acid (picloram or PIC), N-(1,2,3-thiadiazol-5yl)-N'-phenylurea (thidiazuron or TDZ) and 2,3,5-triodobenzoic acid (TIBA), have proved to have promotional effect on the differentiation of lilies (van Aartrijk & Blom-Barnhoorn 1983, Ohkawa *et al.* 1996, Nakano *et al.* 2000). TIBA has also proved to be active in restoring the regeneration potential of callus after prolonged cultures (Nakano *et al.* 2000).

### ***1.4.3 The effect of physical factors on differentiation in vitro***

Light is the most important physical factor in promoting differentiation. Differentiation of shoots is usually promoted in the light whereas for root formation darkness is often more favourable. (Tisserat 1987, Read 1990.) Lily tissue cultures are performed both in the dark and in the light depending on the purpose. In general, differentiation is promoted by transferring the cultures from dark to light (Montezuma-de-Carvalho & Guimarães 1974). For the propagation of callus or bulbs darkness is favoured whereas leafy shoots are cultured in the light (Maesato *et al.* 1994, Niimi *et al.* 1997). It has been demonstrated that in the dark fewer bulbs per explant are formed, but their size is larger than when grown in continuous light (Gerrits & De Klerk 1992). Continuous light or photoperiod (16 h) are the most common illumination conditions used in tissue culture of lilies (Takayama & Misawa 1983, Priyadarshi & Sen 1992). The quality of light has been studied with many plant species including some bulbous plants in respect to its affect on differentiation in tissue culture (Bach & Swiderski 2000). However, there are no reports concerning the effect of light quality on lilies.

Although temperature affects more on the morphogenesis of differentiating shoots, it also seems to have an effect on the differentiation process itself, as the number of differentiating shoots has been shown to increase with increasing temperature (van Aartrijk & Blom-Barnhoorn 1983). Still, the most important temperature-mediated

mechanism in tissue culture is the control of dormancy. As mentioned before, dormancy and its release can be observed in the changes in the overall growth habit of bulblets. The sprouting or formation of leaves is a sign of a non-dormant state, and is arrested when dormancy is induced (Aguettaz *et al.* 1990). Temperatures over +15 °C have proved to be favourable for the formation of dormancy, and respectively temperatures below this threshold are effective in breaking it (De Klerk 1992). Furthermore, it has been demonstrated that the dormancy-inducing effect is relative to the temperature being strongest at +25 °C (Delavallée *et al.* 1990). Consequently, the average bulb size is relative to the incubation temperature. Hence, temperatures between +20 and +25 °C are used in the propagation of shoots. (Takayama & Misawa 1979, van Aartrijk & Blom-Barnhoorn, Gerrits & De Klerk 1992.) It is known that physiological processes related to the induction of differentiation have common features and mechanisms of general stress responses.

#### ***1.4.4 Changes in gene expression during somatic embryogenesis***

Gene expression in differentiation has been studied with a variety of plants. The transition from proembryonic cell masses (PEM) to somatic embryos (SE) requires a high auxin concentration followed by hormone-free treatment. After the auxin treatment, a set of embryogenesis-related genes are switched on and off during the morphological developmental stages (Kawahara & Komamine 1995). Transfer to an auxin-free medium induces the polarization of embryogenic cells, which results also cytoskeletal rearrangements within the cells (Samaj *et al.* 2003). In sunflower, somatic embryogenesis can be induced by cytokinin application without any exogenous auxin in the medium (Thomas *et al.* 2002). The accumulation of endogenous auxin resembles the response achieved with an exogenously applied auxin pulse which is the first signal for the somatic embryogenesis (Kitamiya *et al.* 2000). Many regeneration-related genes are induced by various stress conditions or by ABA and ethylene (Rojas-Herrera *et al.* 2002). Late embryogenesis abundant genes (LEA) are induced by drought and they are also expressed during the maturation of zygotic or somatic embryos (Zimmerman 1993; Fowler *et al.* 1998). Also pathogen-induced genes are expressed during somatic embryogenesis. In Norway spruce, the expression of chitinases, related to somatic embryogenesis induction and programmed cell death (PCD), is induced after auxin pre-treatment (Wiweger *et al.* 2003). In carrot, extracellular endochitinase is expressed during zygotic and somatic embryogenesis (van Hengel *et al.* 1998). In coffee, several chitinases have been isolated during the induction of somatic embryogenesis, and their expression is also induced by wounding the leaves (Rojas-Herrera & Loyola-Vargas 2002). Peroxidases are assumed to have a role in signal transduction and in gene expression activation during somatic embryogenesis (Kairong *et al.* 1999). There is also evidence on their plausible role in the control of cell division, lignification of cell walls and in tissue differentiation (Kay & Basile 1987). Genes, encoding proteins with characteristic domains, like MADS and HOMEO box genes, are also expressed during various stages of differentiation. They possibly participate in the regulation of programs active during the early stages of embryo development in plants (Perry *et al.* 1999, Heuer *et al.* 2001, Blanckaert *et al.*

2002). They are found in various plant species including conifers, as well as monocotyledons and dicotyledons (Ingouff *et al.* 2003). Other sequence-specific DNA-binding proteins are found in WRKY superfamily which are transcriptional regulators involved in senescence and the signal transduction pathway from elicitor perception to the activation of pathogenesis-related (PR) genes (Alexandrova & Conger 2002). In parsley, *WRKY* gene is activated in the leaf tissue around fungal infection sites, and besides to regulating the transcription of early defence-resoponse genes, it also regulates its own transcription (Eulgem *et al.* 1999).

Somatic embryogenesis receptor-like kinases (SERK) are another superfamily of proteins with specific leucine zipper (ZIP) after the kinase domain (Hecht *et al.* 2001). They are involved in cellular signaling pathways of different organisms (Becraft 1998). In maize, SERK gene expression has been found in both embryogenic and non-embryogenic tissues (Baudino *et al.* 2001). In *Arabidopsis*, SERK gene expression increases in the early stages of somatic embryogenesis and organogenesis (Thomas *et al.* 2004).

Lipid transfer proteins (LTP) have the ability to facilitate the exchange of lipid molecules between membranes. They are involved in membrane biogenesis by importing newly synthesized phospholipids to the endoplasmic reticulum. They have been suggested to be involved in defence reactions against bacterial and fungal pathogens or in the adaptation of plants to various environmental conditions. LTPs have been reported to bind precursors of cutin monomers and thus play a key role in cuticle formation (Kader 1996).

Calnexin-like proteins are molecular chaperons in quality control in endoplasmic reticulum, and they have been found to accumulate during somatic embryogenesis. (Fowler *et al.* 1998). In maize, during the induction stage with 2,4-D, cells secrete arabinogalactan proteins (AGP) into outer cell walls of embryogenic cells during the induction stage with 2,4-D when switching from non-polar to polar structures in embryogenic development. Embryogenic cells have been reported to have loose contacts and contain AGPs with putative signalling properties (Samaj *et al.* 2003).

Heat shock proteins (HSP) are heat sensitive proteins that are expressed during several stresses and differentiation. In carrot, *HSPi* genes are induced both by 2,4-D and stress, and their are possibly involved in the initiation of somatic embryogenesis after the initiation of unequal cell divisions (Kitayima *et al.* 2000).

## 1.5 Protoplast isolation and culture

Protoplasts are a special material in plant biotechnology. They are ideal for investigating the plant cell functions or cell wall formation without interference from the cell wall. They have also been successfully used as targets of genetic transformation especially to study promoter strengths, and in cell fusions to produce somatic hybrids. There exist a few reports of successful protoplast isolation and regeneration of the cells in lilies. Pollen cells have also been isolated for fusion studies (Tanaka *et al.* 1987, Ueda *et al.* 1990). In addition, callus cultures and meristematic cells have been used successfully as the starting material for protoplasts with some lily species and cultivars (Sugiura 1993, Mii *et*

*al.* 1994, Godo 1996). There are a few reports on some species of successful cell divisions and differentiation of protoplasts through microcallus stage into fully-grown plants (Horita *et al.* 2002, 2003). Leaves are often used as the starting material for protoplast isolation with species other than lilies (Nagy & Maliga 1976, Binet *et al.* 1991). The regeneration potential of protoplasts from lily leaves is not very high, which may explain the lack of interest in using them for this purpose. Therefore, only a few reports are available on the successful regeneration of leaf-derived protoplasts in lilies (Ishioka & Tanimoto 1994, Tanimoto & Ishioka 1994). However, the advantage of leaf tissue is that it is readily available and easy to handle.

The maintenance and regeneration of isolated protoplasts requires special methods. Nurse-cell cultures have been successfully used for regenerating protoplasts derived from suspension-cultured callus (Horita *et al.* 2002). Another method is to use a special gelling agent with low melting temperature, such as gellan gum or phytigel, and mount the cells inside the medium (Mii *et al.* 1994). With this protocol, the medium provides physical support and oxygen for the cells.

Different factors have been studied in respect to their significance in the viability and regeneration of protoplasts with many lily species. Nutritional composition of the media, growth regulators, pH, sugar type, osmotic potential and cell density have proved to be crucial for the persistence and cell division capacity of protoplasts. In addition, different enzyme combinations have been applied for digesting the cell wall. (Ishioka & Tanimoto 1994, Tanimoto & Ishioka 1994, Godo *et al.* 1996.)

## **1.6 Genetic transformation**

Genetic transformation has become an important tool in plant breeding for producing novel plant cultivars. Genetic transformation methods also serve as an important research tool in molecular biology, especially in the investigation of genetic regulation and gene or genome structure. (Birch 1997.) So far, genetic transformation of lilies has not been applied to molecular breeding of the species. On the other hand, for research purposes several methods have been used. Especially in pollen and fertilisation studies, both electroporation and chemically-mediated gene transfer have proved to be applicable although transformation protocols have not been developed yet for the use in the molecular research or breeding of lily species. Thus, somatic and generative tissues have been transformed in some species by particle bombardment, but on a larger scale, gene transfer methods have not been applied to the research and culture of lilies as yet.

### ***1.6.1 Gene transfer via electroporation and somatic fusion***

Electroporation usually requires a protoplast isolation method although there are some reports on electroporation of intact tissues. With lilies, the most commonly used target tissue is pollen cells, or microsporocytes. There are still only a few reports on regeneration of electroporated protoplasts of lilies (Tabata *et al.* 1993). In addition to gene constructs, also parts of genome can be transferred to protoplasts fusing

microprotoplasts, i.e., cells containing only a few intact chromosomes, with normal protoplasts using electroporation (Saito & Nakano 2002).

Apart from genetic material, larger particles, like organelles, can be introduced to the cells. (Power & Chapman 1987.) However, the production of genetically modified (GM) lilies or other plants via protoplast transformation is difficult due to the problems arising from the high fragility of the cells. Without the support of the cell wall, cells are very susceptible to the changes in osmolality of the surrounding medium. In addition, their viability is not very high, which means that a very high number of cells is required for the successful regeneration of protoplast (Buc-Dang-Ha & Mackenzie 1973, Kao & Michayluk 1975, Nagy & Maliga 1976). In many cases, the major problems protoplast techniques arise from the difficulties in regenerating individual cells into plants. On the other hand, regenerated tissues are derived from one transformed cell, which diminishes the risk of chimeras.

Another method for the production of genetically modified plants from protoplasts is fusion of protoplast cells of different genetic origins. Recently, there is a report on the production of a somatic lily hybrid between two different species groups with the aid of electrofusion of protoplasts (Horita *et al.* 2003).

### ***1.6.2 Particle bombardment***

Particle bombardment is another method that can be used to produce genetically modified plant material ranging from trees to herbaceous plants. Generally this method is used to transform non-host *Agrobacterium* species, such as monocots or conifers, but it is applicable also to other species. (Binet *et al.* 1991, Takumi *et al.* 1994, Aronen *et al.* 1994.) This method can be applied to many different tissues, but as with other transformation methods, the competence of the used genotype strongly affects the transformation efficiency (Aronen *et al.* 1995, Häggman & Aronen 1998). Technically, gene constructs are introduced to the plant material by shooting with high pressure. After transformation, the tissues can be cultured normally in tissue culture media. To improve the efficiency of transformation, a suitable selection procedure is needed. As a selective marker, antibiotic or herbicide resistance genes are commonly used. In addition, some reporter genes, such as  $\beta$ -glucuronidase gene (GUS), can be used to confirm successful transformation (Jefferson *et al.* 1987). This method has also been applied to some lily species to achieve transient and stable expression of reporter gene in pollen, bulb scales, callus and immature embryos (Nishihara *et al.* 1993, Tsuchiya *et al.* 1996, Watad *et al.* 1998).

## **2 Aims of the research**

The objective of this work was to test and develop biotechnological methods to be applied in the production, research and breeding of a variety of lily species. Main aims were to develop

- i biotechnological methods for the culture of various lily species,
- ii molecular methods for the analysis of the genetic background of lily strains,
- iii and a genetic transformation method for gene expression studies and for molecular breeding.

## **3 Materials and methods**

### **3.1 Plant material**

Seeds from several lily species of different origin were used as the starting material for all subsequent cultures. The seeds were obtained through the international seed exchange of the Botanical Gardens of Oulu and from seed exchange programmes of the North American Lily Society and Royal Horticultural Society. Some seeds were provided also by individual lily growers. See Appendix 1 for the complete list of seed lots and origins used in different experiments. The seeds were stored refrigerated until use. They were surface-sterilised in 1.2% NaOCl for 5-10 minutes before sowing. The sterilisation time depended on species. After 3 rinses in sterile, distilled water the seeds were placed on Petri dishes containing either N (Nitsch 1969) or MS (Murashige & Skoog 1962) germination medium. The media contained macro and micro nutrients and vitamins without hormones. The sucrose concentration of the germination medium was 10 g/l in all experiments, and 0.8% (w/v) agar (Ph.Eur. TAMRO) was used as the gelling agent. The plates were kept in darkness until germination occurred.

In some experiments, tissue culture and analysis were started from adult plants (III). Bulb scales or bulbils were used as the starting material. The explants were washed with a detergent and water, rinsed, sterilized in 2.5% NaOCl, and finally rinsed 3 times with sterile distilled water. The explants were placed on Petri dishes containing MS macro and micro nutrients, vitamins and sucrose 30 g/l. For bulblet differentiation NAA and BA were added in concentrations of 3.0  $\mu\text{M}$  and 0.1  $\mu\text{M}$ , respectively.

#### ***3.1.1 Germination***

In germination studies, the seeds were sown on germination media with or without 0.5 or 5.0  $\mu\text{M}$  gibberellic acid (GA). For the abscisic acid (ABA) antagonist tests, some seeds were exposed to 2.4  $\mu\text{M}$  fluridon prior to germination. For some experiments, also peeling and embryo rescue were performed after the sterilization. In those cases, the seed

coat was removed with a scalpel. The embryo was excised from the endosperm by gently squeezing the seed with the aid of a scalpel and forceps. Cultures were kept in the darkness or in the light, depending on the experiment. Germination was observed in certain time intervals.

### ***3.1.2 Propagation, callus induction and differentiation***

In callus experiments, germinated small seedlings were placed either on the callus induction medium, propagation medium or differentiation medium. (II, III, IV and V). The composition of the medium was MS macro and micro nutrients with vitamins. For growth regulators, 2,4-D, indolyl butyric acid (IBA) and naphthalene acetic acid (NAA) were used as auxins, benzylamine (BA) as cytokinin. In the callus induction medium, the concentration of auxins was 6.0  $\mu\text{M}$  and in the propagation medium it was 3.0  $\mu\text{M}$ . The concentration of BA was 1.0  $\mu\text{M}$  in the callus induction medium and 0.1  $\mu\text{M}$  in the propagation medium. In the differentiation medium, no growth regulators were added. In the callus induction medium, the sucrose content was 60 g/l. In the propagation and differentiation media, the sucrose content was 30 g/l. For differentiation, cultures were kept in the light whereas callus induction plates were kept in the dark. Differentiated plantlets were grown in the differentiation or propagation medium until the bulblets reached the size of approximately 2-5 cm in diameter. In case the plants were planted outdoors, the culture vessels were kept in a cool temperature (+5°C) for 6 weeks prior to transplantation into soil.

In growth experiments, fresh weight of the tissue explants was measured in the initiation of the experiment (FW1) and at the end of the experiment (FW2). These measurements were used for the calculation of the relative growth as the fresh weight growth ratio (FW2/FW1).

The liquid culture media were the same as the solid media except that no agar was added. The cultures were maintained either in a rotary shaker or in RITA® culture vessels (Vitropic S.A. CIRAD) in the temporary immersion system (II).

## **3.2 Histological and morphological analysis**

Light microscopy and scanning electron microscopy (SEM) samples were fixed in FAA fixative (85:5:10; 100% acetic acid: 100% formaldehyde: 96% ethanol). After fixation, the samples were run in an alcohol series for dehydration starting with 70% ethanol and ending up with absolute ethanol. After dehydration, the light microscope samples were mounted in plastic (Historesin, Leica Instruments GmbH), and cut in sections (ca. 5 $\mu\text{M}$ ). The tissue slices were placed on glass, stained with methylene blue for 5 min and rinsed with distilled water. The samples were analysed and photographed with a light microscope (I, IV).

The SEM samples were dehydrated with a critical point drying system (Balzers CPD 030) and covered with 8 nm of platinum (Polaron E 1500). The samples were analysed and photographed with a scanning electron microscope (Jeol JSM-6400) (I).

### 3.3 DNA isolation and RAPD analysis

For the RAPD analysis, a slightly modified DNA isolation protocol of Doyle and Doyle (1990) was used. After ethanol precipitation, the pellet was dried as in the RNA isolation protocol, and dissolved in sterile distilled water. The amount and purity of the DNA was measured with spectrophotometer.

For the random amplified polymorphic DNA analysis (RAPD), approximately 50 ng of DNA was used for each 50  $\mu$ l reaction mixture. Commercially provided random and semi-specific primers were used (Operon Technologies Inc., Amersham Pharmacia Biotech, Sigma-Genosys Ltd.) in the polymerase chain reaction (PCR). The samples were run in an agarose gel electrophoresis (1% TBE), and the generated bands were analysed using several clustering methods to reveal the phylogenetic relations of the genotypes (III).

### 3.4 Biolistic transformation experiments

Callus tissues from different genotypes were selected for particle bombardment experiments. The calli were bombarded with a pCGU $\Delta$ 0 plasmid, consisting of nopaline synthase transferase gene (*npt*) as the selective marker and  $\beta$ -glucuronidase (*uidA*) as the reporter gene under the cauliflower mosaic virus promoter (35 SCaMV). In the experiment, constant vacuum of 27 Hg was used, and the effect of 650, 900 and 1100 psi bombardment pressures were tested. The expression of the *uidA* gene was assayed 2, 7, 14 and 21 days after the bombardment using histochemical X-Gluc assay originally described by Jefferson (1987), and further modified by Aronen and co-workers (1994). The number of gene expression sites was calculated (V).

### 3.5 RNA isolation and gene expression studies (additional data)

Tissue samples were collected from several stages of differentiating callus, and they were immediately frozen in liquid nitrogen and stored in  $-70$  °C until use. Frozen samples of approximately 100 mg were ground in a mortar with liquid nitrogen. Different methods were tested for the isolation, among others several commercially provided kits: RNeasy (Qiagen), automatic isolation devise (KingFisher®, Thermo LabSystems) and magnetic bead system (Novagen). In addition, different manual procedures and lyse buffers were tested. For expression analysis, modified RNA isolation method of Bachem *et al.* (1998) was selected.

The dried samples were dissolved in sterile distilled water. The purity and the amount of RNA was assayed with spectrophotometer, and with agarose gel electrophoresis.

### **3.5.1 cDNA synthesis and PCR analysis (additional data)**

For reverse transcriptase (RT) synthesis, 0.5 µg of isolated RNA was used. Complementary DNA (cDNA) was synthesised using a routine protocol. The amount and purity of the cDNA was assayed with spectrophotometer, and 1 µg of cDNA was used for 20 µl reaction volume. Semi-specific primers were constructed using the sequence data provided by a gene-bank database (BLAST). In addition, commercial random primers were used. Various PCR programmes were used in the synthesis of gene products. After PCR, the samples were separated with agarose gel electrophoresis and the gels were analysed with an image analyser (Fluor-S MultiImager, Bio-Rad). Fragments of interest were dissected from the gel and sequenced. The obtained sequences were compared with the reference material (BLAST), and new primers were constructed for more precise PCR analysis.

### **3.6 Protoplast isolation (additional data)**

Protoplasts were isolated using leaf and callus tissues as the starting material. The tissues were grown *in vitro* until the protoplasts were isolated. The tissues were incubated in the digestion medium overnight before the addition of the digesting enzymes cellulase (1.2% w/v), pectinase (0.4% w/v), driselase (0.5% w/v) and hemicellulase (0.5% w/v). The composition of the digestion medium varied in its sugar content; in addition to sucrose, glucose, mannose and xylose were used. The tissues were incubated overnight (approximately 8 hours) in the enzyme solution before isolation. The protoplast suspensions were either plated onto a solid growing medium with sucrose or mixed with gelrite. Before plating, the cell density was measured with a hemocytometer. The viability and growth of the plated cells was observed during several weeks.

### **3.7 Statistical analysis**

Variance analysis (ANOVA) was applied to the germination and growth results wherever indicated. Population size varied between experiments, and the size is indicated together with the experiment results.

## 4 Results

### 4.1 Germination in vitro

Seed germination was accelerated in tissue culture in all tested species. However, there were differences between species of different germination types. Delayed hypogeal type species germinated slower than immediate epigeal type species. In addition, some species did not germinate at all in the course of several weeks although they remained alive. This could be demonstrated when the rescued embryos of these seeds started to grow immediately after excision. Germination was slowest or non-existing from certain seed lots of American and Candidum species (Table 1, I). The average seed weight did not correlate with the germination type (Table 3).

Cotyledons followed the same kind of development pattern as in normal germination. With epigeal species, the cotyledon emerged from the seed and the empty seed coat detached soon after germination. In contrast, with hypogeal species, the distal part of the cotyledon stayed inside the seed for a prolonged time and small bulblet was formed on the basal part of the small seedling. In some cases, further development of the seedling was arrested soon after the germination indicating the induction of shoot dormancy in the shoot (I).

#### *4.1.1 Seed coat and endosperm*

An inhibiting effect of the seed coat and endosperm on germination was clearly demonstrated. Especially with hypogeal germination, both removing the seed coat and excising the embryo out of the seed significantly accelerated the germination. It can be assumed that, besides as a physical barrier, these seed structures may possess a physiological control system that maintains dormancy in the seeds (I). The average seed weight differed greatly between species and it was dependant on the taxonomic species group. However, it did not have any correlation on the germination rate or type (Table 4).

Table 3. Germination rates of in vitro grown lily species after 2 months incubation. (n=10-150 seeds)

Group	Species	Germination %
Martagon	<i>L. martagon</i>	66
	<i>L. martagon album</i>	67
	<i>L. medeoloides</i>	13
American	<i>L. canadense</i>	60
	<i>L. columbianum</i>	70
	<i>L. humboldtii</i>	90
	<i>L. pardalinum</i>	67
	<i>L. parvum</i>	100
Candidum	<i>L. philadelphicum</i>	75
	<i>L. candidum var. salonikae</i>	41
	<i>L. carniolicum</i>	9
Oriental	<i>L. monadelphum</i>	48
	<i>L. japonicum</i>	76
	<i>L. nobilissimum</i>	39
Asiatic	<i>L. rubellum</i>	16
	<i>L. amabile var. luteum</i>	93
	<i>L. callosum</i>	80
	<i>L. cernuum</i>	83
	<i>L. concolor</i>	100
	<i>L. davidii var. willmottiae</i>	93
	<i>L. duchartrei</i>	100
	<i>L. henryi</i>	52
	<i>L. leichtlinii var. maximowiczii</i>	94
	<i>L. mackliniae</i>	100
Trumpet	<i>L. nanum</i>	71
	<i>L. pumilum</i>	78
	<i>L. formosanum</i>	12
	<i>L. regale</i>	100

#### 4.1.2 The effect of light and temperature

Germination was faster in the dark, and after two weeks the difference became significant. Furthermore, the final germination rate after four weeks was almost 95% in the dark and 87% in the light. Also, the temperature had a significant effect on germination. Within the four weeks' period, no germination was observed in the cold-incubated seeds (+5 °C), whereas seeds kept in +20 °C started to germinate after one week, and reached a final germination rate of 95% (I).

Table 4. Seed weights and germination types of various lily species.

Species	Type	Weight (g) of 1000 seeds
<i>L. tsingtauense</i>	dh	14.2
<i>L. canadense</i>	dh	5.3
<i>L. columbianum</i>	dh	3.4
<i>L. humboldtii</i>	dh	5.6
<i>L. michiganense</i>	dh	2.8
<i>L. pardalinum</i>	dh	7.7
<i>L. parryi</i>	ih	3.5
<i>L. parvum</i>	dh	5.8
<i>L. kelloggii</i>	dh	3.6
<i>L. kelleyanum</i>	dh	9.5
<i>L. superbum</i>	dh	4.2
<i>L. vollmeri</i>	dh	3.9
<i>L. bulbiferum</i>	ih	4.7
<i>L. monadelphum</i>	dh	12.2
<i>L. pyrenaicum</i>	de	7.4
<i>L. szovitsianum</i>	dh	3.7
<i>L. japonicum</i>	dh	6.4
<i>L. cernuum</i>	ie	5.7
<i>L. duchartrei</i>	ie	4.4
<i>L. henryi</i>	ie	4.2
<i>L. lancifolium</i>	ie	4.5
<i>L. nanum</i>	ie	1.3
<i>L. formosanum</i>	ie	7.7
<i>L. regale</i>	ie	6.6
<i>L. sargentiae</i>	ie	1.9

d = delayed, i = immediate, h = hypogeal, e = epigeal.

### 4.1.3 The effect of GA, ABA and fluridon

The results clearly showed that GA does not have any effect on the germination of lilies. The germination rate in GA-treated seeds remained slightly lower than in control treatments, although the difference was not significant. The final germination rate in GA treatment was 70%, whereas in control treatments it was 87%. On the contrary, ABA-treated seeds germinated slower than those in other treatments, and the final germination rate was 63%. When fluridon - an ABA antagonist - was added, the germination rate was significantly higher than with other treatments during the whole experiment. Germination took place almost immediately and the germination percentage reached 100% after two weeks (I).

## 4.2 Induction and growth of callus

High auxin concentration combined with an elevated cytokinin concentration proved effective in inducing callus formation. After transplanting on callus induction media, small seedlings and excised embryos started to form callus within a few weeks. Callus was formed practically on any part of the small seedlings, but mostly on the basal parts. Similarly, when leaves were used as explants, pedicles and the basal parts of the leaf blades were most likely to form callus. In some tissues, shoots and roots started to regenerate simultaneously with callus induction, but some calli did not form any differentiated shoots. The sugar content also affected callus formation. It was promoted when the sucrose concentration was increased up to 60 g/l. Induction and growth of callus was fastest in the dark (Table 5, Fig.3).

Table 5. Callus induction in certain lily species with different phytohormone contents. (n=10 explants/treatment).

Species	Explant	Auxin, $\mu\text{M}$	Cytokinin, $\mu\text{M}$	Sucrose (g/l)	Callus formation (%)
<i>L. regale</i>	bulb scale	NAA or IBA 5.0	BA or K 0.5	30	100
	pedicle	2,4-D 5.0	BA 0.5	30	43
	leaf blade	2,4-D or IBA 5.0	K or BA 0.5	30	69
<i>L. martagon</i>	bulb scale	IBA 5.0	K 0.5	30	66
	pedicle	IBA 5.0	K 0.5	30	30
	leaf blade	IBA 5.0	K 0.5	30	26
<i>L. bulbiferum</i>	bulb scale	2,4-D or NAA 100	BA 10	100	10-20

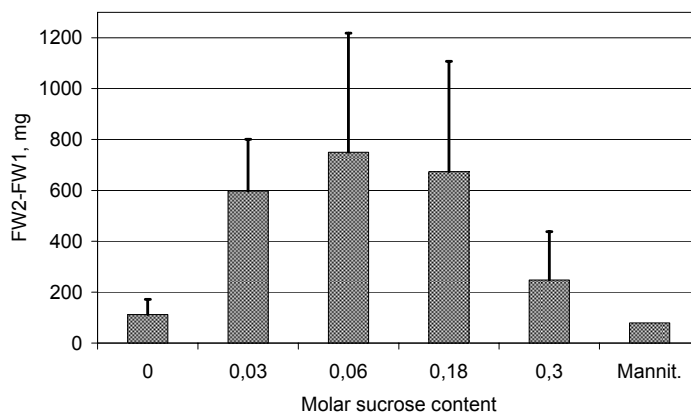
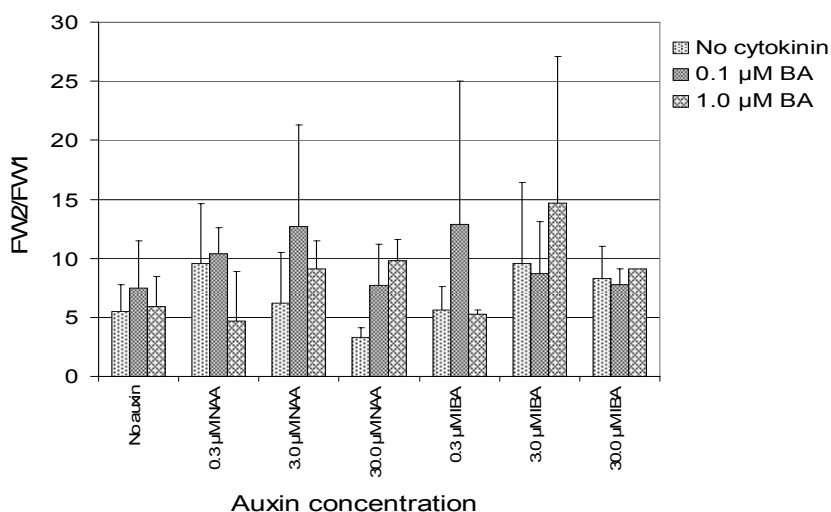


Fig. 3. The effect of sugar on callus growth in lily (*Lilium regale* Wil.) measured as FW2-FW1. Mannit. refers to mannitol control, where sucrose was replaced with 0.15 M mannitol. FW1 – fresh weight in the initiation, FW2 – fresh weight in the end of culture. The bars indicate the standard deviation. (n=15 explants / treatment).

Callus proliferated readily in the propagation medium. The composition and phytohormone content of the medium were suitable for all species. NAA proved more

suitable for callus culture than 2,4-D because the calli tended to get brownish and necrotic in prolonged culture when 2,4-D was present. In addition, calli cultured on 2,4-D containing medium produced malformed shoots whereas calli cultured with NAA produced mostly normally formed shoots (IV). When IBA and NAA were compared with various concentrations of BA (0, 0.1 or 1.0  $\mu\text{M}$ ), the best growth rate of callus was achieved either with 3.0  $\mu\text{M}$  NAA or 0.3  $\mu\text{M}$  IBA with 0.1  $\mu\text{M}$  BA, or 3.0  $\mu\text{M}$  IBA with 1.0  $\mu\text{M}$  BA (Fig. 4).



**Fig. 4.** The effect of the auxin type and concentration on the relative fresh weight (FW) growth ratio in lily (*Lilium regale* Wil.) callus. FW1 – the initial FW of the callus explants, FW2 – the final FW of the callus explants after eight weeks in culture. The bars indicate the standard error of the mean. (n=15 explants / treatment).

### 4.2.1 Suspension and liquid cultures

The culture method clearly affected the growth rate and differentiation of all tested species. With the Regal lily, callus proliferation was faster in suspensions than on solid medium. Cultures either four or eight weeks in RITA® system were compared with the solid cultures. Differences in growth rate and differentiation were greatest after eight weeks in culture, whereas differences after four weeks were not as profound between liquid and solid cultures. The dry weight was higher in RITA® cultures than in solid cultures. The contamination risk was higher in liquid cultures than in solid cultures (II). In shake cultures, the growth rate of callus increased as the culture period increased being at its highest after three weeks in culture (Fig. 5).

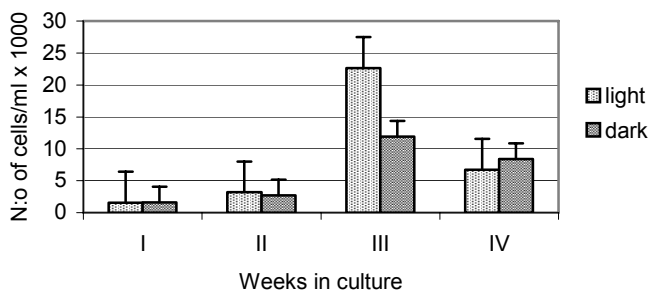


Fig. 5. Callus growth in shake cultures measured as number of cells per ml ( $\pm$ SD). The culture medium consisted of MS salts, 3.0  $\mu$ M NAA and 40 g/l sucrose. The bars indicate the standard deviation. (n= 5 sampels / treatment).

### 4.3 Bulb regeneration and culture

The regeneration of shoots could be induced by transplanting the callus onto a differentiation medium without any hormones. Also, transfer from dark to light increased the number of differentiating shoots.

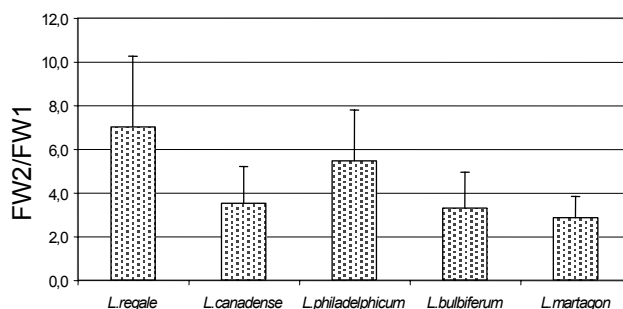
The regenerated bulblets were transferred onto a propagation medium when they reached the minimum size of approximately 2-3 mm in diameter. The growth rate varied between different species. The culture of bulblets was carried out in the light. When the effect of different growth regulators on the growth of bulb-scale explants was tested, slight differences in the dormancy and leaf emergence, as well as in the number of differentiating bulb scales was observed (Table 6).

Table 6. The effect of GA and paclobutrazol on the organogenesis and dormancy induction of bulb-scale explants in different lily species grown on MS medium. The figures show the average number of leaves, roots and bulblets formed per bulb-scale explant. (n = 5 explants /treatment).

Species	Leaves formed			Roots formed			Bulblets formed		
	0	GA	Paclob.	0	GA	Paclob.	0	GA	Paclob.
<i>L. regale</i>	4.8	6.1	0.3	1.2	1.5	0.9	3.4	3.8	1.0
<i>L. martagon</i>	1.1	0.5	0.4	0.9	1.1	1.6	1.7	1.0	1.3
<i>L. bulbiferum</i>	5.0	6.0	0	4.1	4.5	0.9	3.0	4.0	1.0
<i>L. canadense</i>	2.8	2.1	0	1.8	0.6	0.5	1.8	1.2	0.8
<i>L. philadelphicum</i>	90.5	87.3	11.0	90.1	86.8	3.9	4.7	4.6	3.4

There were differences in the growth rate of differentiated bulblets between treatments. Of the tested species, *L. regale* had the fastest growth rate as the fresh weight increased approx. 7-fold compared with that of the starting material. *L. martagon* and *L. bulbiferum*

had the slowest growth rates as their fresh weights increased only to approximately 2.5-fold compared with that of the starting material (Fig. 6).

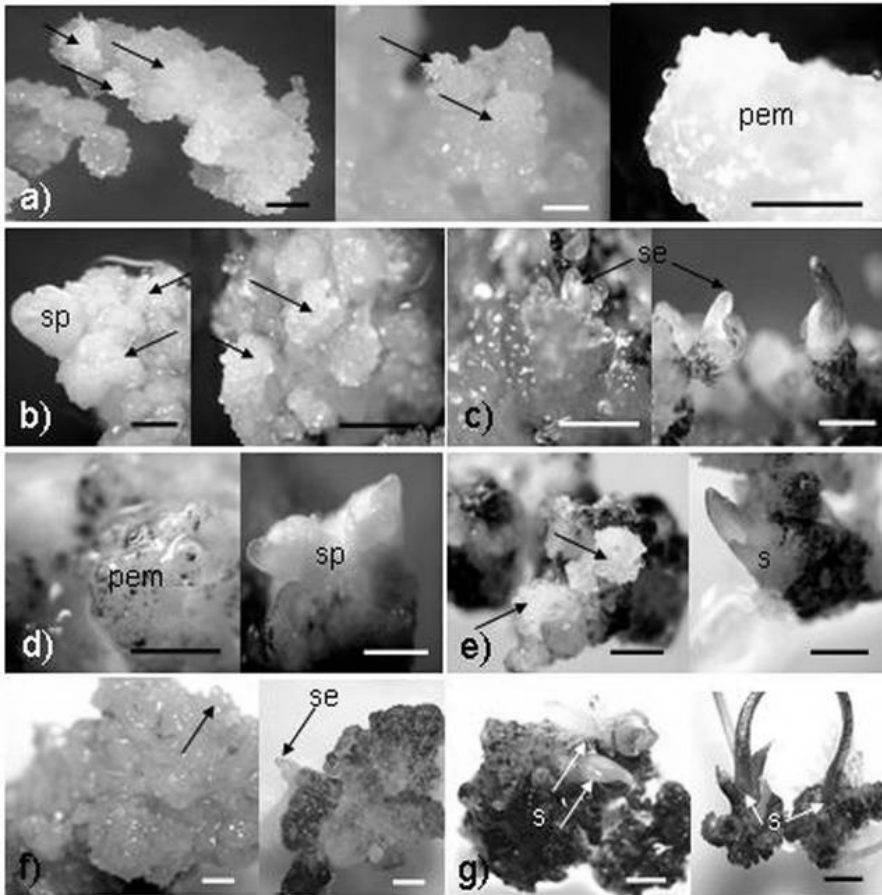


**Fig. 6. The growth ratio of the bulb in various lily species grown *in vitro*. FW1 – the initial fresh weight of the bulbs, FW2 – the fresh weight of the bulbs after 12 weeks in culture. The bars indicate the standard deviation. (n= 15 explants / treatment).**

#### 4.4 Organogenesis and somatic embryogenesis in callus tissue

Organogenesis was the most common differentiation process in the callus of the Regal lily calli, but somatic embryogenesis was also involved to some extent (Fig. 7). Embryogenic callus formed proembryonic cell masses (PEM) on the tissue surface which in auxin free medium developed into shoot primordia or somatic embryos. In the developing somatic embryos, cotyledon-like structure was observed although it was significantly shorter than in zygotic embryos (Fig. 7 c). In suspension cultures, small embryonic cell masses could be revealed in the light microscopy samples. These cell clumps contained cell clusters with a cell organisation resembling a typical proembryo formation (IV). Furthermore, in differentiating calli, shoots derived from somatic embryos had a clear simultaneous formation of a root and shoot system without any clear attachment of the tissue to the callus tissue. However, in most cases the shoots developed first and the adventitious or secondary roots differentiated later. These shoots were defined as organogenic. The final proportion of embryogenic shoots of all shoots formed was estimated to be 8-10% (IV).

Differentiation occurred in both callus proliferation and differentiation media, but was slightly higher in the latter. Light was also shown to promote shoot differentiation (II, IV).

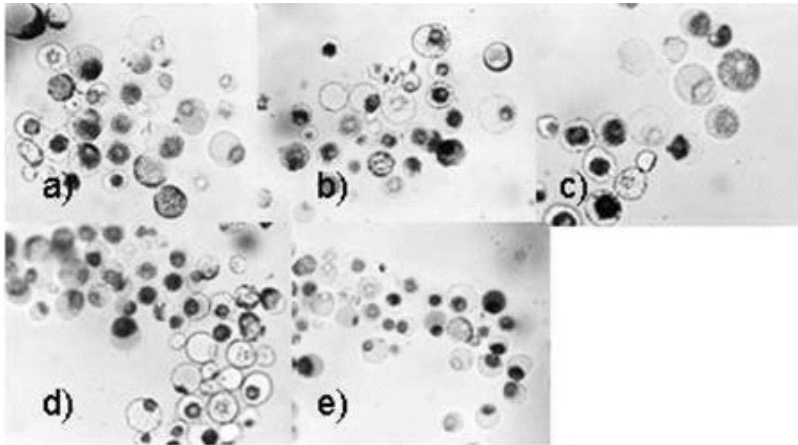


**Fig. 7. Embryogenic callus in various differentiation stages. a) Callus in the proliferation medium with auxin (5  $\mu$ M NAA). Callus after one week in the differentiation induction medium without auxin b) in the dark and c) in the light. The arrows point to the proembryonic cell masses (pem) and somatic proembryos (se). Differentiating callus after two weeks in the induction medium without auxin d) in the dark and e) in the light. Proembryonic cell masses have developed into shoot primordia (sp) or shoots (s). Callus after three weeks in the induction medium without auxin f) in the dark and g) in the light. Some PEMs are still forming simultaneously with differentiating somatic embryos and shoots, especially in the dark grown. In the light, some shoots are fully developed and the callus is substantially more pigmented than in the dark-grown callus. However, antocyanins (red pigment) and chlorophyll are formed also in the dark. Bar 1 mm.**

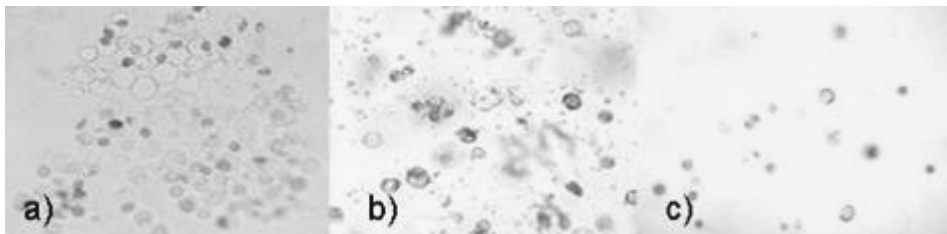
#### **4.5 Protoplast isolation and maintenance (additional data)**

Viable protoplasts could be isolated from various tissues, but leaf material gave the best result in terms of number of cells (Fig. 8). Callus tissue and bulb scales were more

problematic because the substantial amount of starch released from the cells during the isolation procedure (Fig. 9 b and c).



**Fig. 8. Protoplasts isolated from different species and tissues. a) *L. regale*, b) *L. martagon* and c) *L. lancifolium* leaves, d) leaf blade and e) leaf pedicle protoplasts from *L. regale*.**

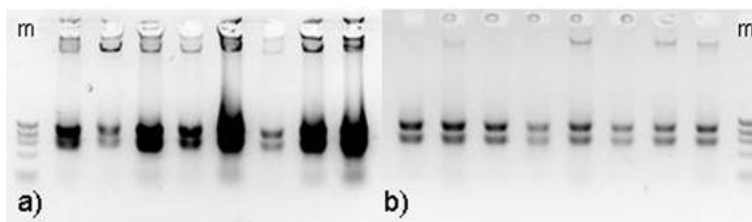


**Fig. 9. Protoplasts from *L. regale* a) leaves, b) callus and c) bulb scales.**

Although alive, the divisions and subsequent growth of the plated protoplasts were scarce. The type of sugar used did not have any significant effect on the plating efficiency or cell growth (Figures 8 and 9). The best plating efficiency was achieved with a digestion medium containing cellulase (1.4%) and pectinase (0.4%) as the only enzymes. The number of cells ranged between  $200-300 \times 10^3$  in ml/mg tissue fresh weight. The basal medium consisted of MS salts, and its osmolality was adjusted to approximately 0.6 osmol/kg with sucrose. When other sugar combinations in the digestion and growth medium were tested, the cells seemed to be more fragile, and isolation resulted in almost merely broken cell debris (data not shown).

## 4.6 Expression of differentiation related genes (additional data)

When different isolation methods were tested, manual protocols proved to give better results in RNA analysis. High DNA content interfered with the purification of RNA, which could be overcome with DNAase treatment. The removal of excess RNA was necessary for the reverse transcriptase synthesis of cDNA (Fig. 10). In genomic DNA isolation, automated KingFisher® system gave slightly better PCR fragments than the manual system. However, with the both protocols, PCR was successful with some specific housekeeping genes (Fig. 11).



**Fig. 10. Manually isolated and purified total RNA before and after DNAase treatment. The substantial amount of DNA interferes with the agarose gel run resulting smear and obscure bands (a). DNAase treatment of the same RNA samples as in a) was necessary for distinct RNA bands in the gel (b).**

The semi-specific primers did not generate any fragments in PCR of cDNA. This may have been caused by their insufficient homology with lily's genes or they were not expressed in the tissue. Random primers generated clear fragments, with differences between different genotypes and different treatments (Fig. 12). After isolation and sequencing these specific fragments, some homology with known gene sequences was found. Similarities with maize specific peroxidase and zinc-finger protein were found when the nucleotide sequences were compared with the corresponding polypeptide sequences of known genes. However, the results of gene expression need further analysis to specify more accurately the differentiation related gene activity in lilies.

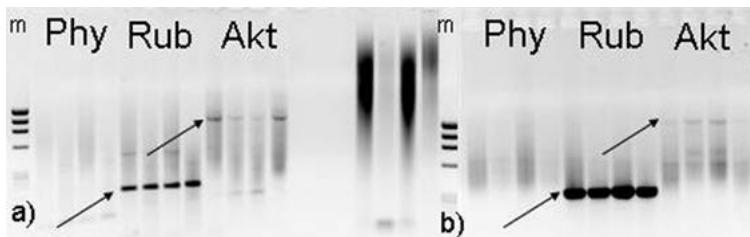
## 4.7 Gene transfer via particle bombardment

Biolistic transformation proved an effective method for the genetic transformation of lily callus. Of the different pressures tested, 900 psi was shown to be most efficient both in terms of the number of transformed cell clusters and the persistence of the transgene expression. Although the preliminary tests on the kanamycin resistance of different callus strains indicated that the best concentration of antibiotic would be 50 mg/l, after transformation it did not prove efficient enough for the selection of transgenic cell lines. After increasing the antibiotic concentration in the selective media to 100 mg/l, the selection became more efficient. Transgenic cell lines were cultured, and transgenic bulblets could be regenerated from the callus (V).

**Fig. 11. PCR of cDNA with specific primers of ribosomal gene (Rib) and with random**



**primers (OPU1, 2, 10 and 20). The samples for random PCR represented a single lily genotype collected at different time points. a) day 0, b) day 3, c) day 7, d) day 14 and e) day 21 after the transfer to hormone free medium for differentiation induction.**



**Fig. 12. PCR of genomic DNA isolated either manually (a) or with automated KingFisher® system (b) with specific phytocrome (Phy), rubisco (Rub) and actin (Akt) primers. Samples from left to right: genotype 1 leaf and bulb, genotype 2 leaf and bulb.**

#### 4.8 Phylogenetic analysis of the Orange lily

The phylogenetic tree based on the RAPD analysis of traditional cultivars and genuine species of the Orange lily clearly indicated that there is a genetic relation between these populations. The cultivars fell into two subgroups that, in turn, were closely situated in the two subgroups of the genuine species and its variety *croceum*. The reference species used were clearly distinct from each other and from the Orange lily populations, which indicated that variation between cultivars remained normal within a given species. Thus, the results did not provide any evidence of genetic crossing between the Orange lily and the reference species (III).

## 5 Discussion

### 5.1 Tissue culture of bulbous plants

Since the early work of Robb (1957), there has been substantial development in tissue culture methods for *Lilium* and other bulbous plants. Mass production and conservation of endangered and endemic lily species using biotechnological applications has also become reality during the last decade (Priyadarshi & Sen 1992, Wickremesinhe *et al.* 1994, Wawrosch *et al.* 2001, Mao *et al.* 2002). Several parameters and culture conditions have been studied with a great variety of species and cultivars, and commercially adoptable mass-production protocols have been developed (Aguettaz *et al.* 1990, Maesato *et al.* 1992, Yamagishi 1995).

For the culture medium in the present work, MS medium (Murashige & Skoog 1962) was selected. In early works, also LS medium (Linsmaier & Skoog 1965) was used in lily cultures (Sheridan 1968). Although, according to the published data, the different tissue culture media have not been compared in terms of growth of lilies, the MS medium is mostly used. In some cases, Nitsch's (Nitsch & Nitsch 1969), White's (1963) and Kao's (Kao & Michayluk 1975) media have been used for germination, embryo and protoplast cultures due to their low ammonium concentration, which may be harmful for susceptible tissues or cells. Also the strengths of the media have been altered for these purposes. Increasing the medium strength has been proved to have an enhancing effect on bulblet formation (Takayama & Misawa 1979). Similar results were achieved with higher sucrose concentration (Gerrits & de Klerk 1992). Sucrose is mainly used in tissue culture of lilies as a carbon source, although the structural and reserve carbon hydrates in lilies are starch and glucomannan consisting of glucose and mannose (Langens-Gerrits *et al.* 2003). In some cases, monosaccharides, like fructose or mannose, have been used in combination with sucrose. Sucrose was used also in the present work in varying concentrations depending on the purpose. Higher sucrose concentration clearly improved callus induction with all the tested species. It also had a positive correlation with the increment of the tissue dry weight, which indicates that besides in metabolic energy resource, sucrose is readily metabolised and used for the biosynthesis of new compounds.

Growth regulators in the initiation of culture, callus induction, differentiation and proliferation has been tested with a variety of lily species. In addition to the commonly used growth regulators, some special compounds have also been tested in the culture of lilies (Ohkawa *et al.* 1996).

In the culture of bulbous plants, different developmental and physiological stages are distinguished by their morphological traits. The growth habit changes during dormancy induction and the leaf primordia differentiate into bulb scales instead of leaves (Delvallée *et al.* 1990). Both culture conditions and growth regulators affect the dormancy status.

The significance of the starting material in successful tissue culture has been demonstrated. Bulb scale explants are superior in their availability, handling and regeneration capacity (Takayama & Misawa 1979). However, alternative explants may be needed, e.g., for the propagation of new genotypes and for such special techniques as protoplasts and transformation protocols. Pathogens may also hamper the successful use of bulb material. Therefore, the use of floral parts, like ovaries, pistils and anthers, is many times preferred (Arzate-Fernández *et al.* 1997). Juvenile parts, e.g., young seedlings, have the best differentiation potential. Also younger parts of the shoots, like apical meristems and axillary buds, have been used (Tanaka *et al.* 1991, Nhut 1998). In contrast, the more differentiated parts, such as leaves, are weaker in callus production and differentiation. However, also in leaves the basal parts like pedicels have more differentiation capacity than the distal parts like leaf blades (Niimi 1986). In general, all the species used in current work produced callus, proliferated efficiently and differentiated into fully formed plants. The profound differences in initiation and differentiation between different lily species confirmed the significance of the genotype when selecting the explant and method for tissue culture. Furthermore, the differences between species were evident throughout the developmental stages from germination until the flowering stage of development.

## 5.2 Genetic diversity

Classification of the genus *Lilium* has been well established during the last few decades. DNA techniques have brought more accuracy to the earlier taxonomic definitions and have, for its part, untangled the confusion of diverse species and varieties (Persson *et al.* 1998, Wen & Hsiao 2001, Horning *et al.* 2003). Approximately 100 species in the lily family possess a great genetic diversity that is manifested in forms, sizes, growth habits and persistence. Seedlings of genuine species obtained by inbreeding or backcrossing with the same species are usually very uniform in phenotype. Species provide an invaluable material for breeding, and they have been used for centuries as ornamentals (McRae 1998). In addition to new species, also traditionally cultivated strains and genotypes that have adapted to demanding climatic conditions, provide a precious genetic source for the production of hardy and healthy cultivars. The availability of these lily types has been limited although their popularity has increased especially in the north.

Traditionally grown old cultivars have been challenging for taxonomists because of their heterogeneous inheritance. The genetic background and origin of the traditionally grown Orange lily strains have not been studied before. It has been assumed that they

have been imported from Central Europe through Sweden or Russia, and they have since spread with people all over Finland. The RAPD fingerprint analysis has been applied to assess the genetic background of a great variety of cultivated plants (Galderisi *et al.* 1999, Palombi & Damiano 2002, Al-Khalifah & Askari 2003). In this work, this technique was selected as a simple analysis method to avoid the possible problems arising from the substantial size of lily genome. On the other hand, as there is no previous genomic map information concerning lilies, the RAPD method can still be applied because known sequence data is not required for this method. The present RAPD results indicate that, according to their genetic background, the traditional Orange lily strains are closer to the native species from Central Europe than to the reference species from other taxonomic groups (III).

### 5.3 Pathogen control and purification

Spreading of pathogens, in particular viruses, is a constant problem when somatic tissues are used as the starting material. The use of heat treatments and antibiotics are time consuming, costly and laborious methods to overcome the problem. Seeds have not been used in tissue culture of lilies before. One reason is that with most applications, a certain cultivar or hybrid, i.e., selected, specific elite genotype with known features, need to be mass-produced. In these cases, somatic tissues, especially bulb scales are ideal starting material.

To reveal the processes in seed dormancy, a well-controlled germination system is required. *In vitro* germination provides an excellent tool for testing different parameters and the role of different compartments in seeds in germination without any interference of microbes or other unwanted agents. In the present work, the seed coat tissues and endosperm had a significant role in arrested, especially delayed type germination. For production of pathogen free plants, seeds make an ideal material, as they have not been reported to carry any viruses or endophytes in lilies. According to the present results, *in vitro* techniques can be used to accelerate the germination of any lily species. Furthermore, induced dormancy of the seedling, or epicotyl dormancy, can be overcome by *in vitro* germination (I).

### 5.4 Gene expression during differentiation

In addition to seed dormancy and germination, the physiological control of differentiation is another key event in tissue culture. To understand the processes and the genetic machinery involved in the whole concept need to be investigated further. It has been demonstrated with many plant species that the induction of somatic embryogenesis triggers the expression of a variety of genes (Hjortswang *et al.* 2002, Baldan *et al.* 2003). Whether the specifically expressing gene groups have relevance in the physiological processes themselves or are merely a consequence of the inductive stimuli, like stress, is a case-specific issue. Some genes are induced in general stress responses, and thus may coincidentally be activated simultaneously with the essential genes for somatic

embryogenesis (Schrader *et al.* 1997, Kairong *et al.* 1999, Duncan *et al.* 2003). However, there is strong evidence of the significance of certain gene families in differentiation processes. Certain genes are involved in signal transduction during the initiation stage whereas others have a role in physiological processes such as altered cell divisions, desiccation and determination of the fate of undifferentiated cells into differentiated tissues (Hecht *et al.* 2001, Mogami *et al.* 2002, Footitt *et al.* 2003). The role of pathogene-induced gene expression in the differentiation processes is an interesting issue. The role of chitinases, for instance, in control of the cell cycle and apoptosis suggests that they are required both in embryo and organ maturation, and for defense mechanisms (van Hengel *et al.* 1998). It is known that apoptosis plays a role both as a defense mechanism in lesions around pathogene infection site and in massive programmed cell death (PCD) that takes place simultaneously with the transition phase from proembryonic cell masses to somatic embryos (Wiweger *et al.* 2003). Interestingly, in coffee, salicylic acid, which is a systemic defense mechanism inducer, has a promotive effect on both cell proliferation and somatic embryogenesis (Quiroz-Figueroa *et al.* 2002).

The role of gene expression during the induction of differentiation is currently not very well known, and with lilies, the whole process has not been studied so far. Many specifically activated genes produce specific transcription factors that, in turn, activate other structural genes needed for the differentiation processes. The MADS domain functions as a dimerisation domain. Homeobox genes encoding homeodomain (HD) transcription regulators are found in all eukaryotics (Münster *et al.* 1997). The availability of deoxyribonucleotides is a prerequisite for active DNA synthesis during growth and development of embryos. Conversion of nucleotides into nucleic acids has been found to be very active at all stages of embryo development (Stasolla *et al.* 2003). DNA methylation has been found to be more abundant in non-embryogenic than embryogenic tissues, which may indicate that the expression of genes related to somatic embryogenesis is controlled by silencing in non-embryogenic tissues (Chakrabarty *et al.* 2003). In addition to growth regulators, such as ABA and ethylene, and stress-inducing treatments, such as drought or low temperature, light has proved to be one key stimulus that triggers signal transduction in differentiation related physiological processes down to the gene expression level (D'Onofrio *et al.* 1998, Bach & Swiderski 2000). In *Arabidopsis*, an embryo-specific gene required for the transition from the globular to the heart stage is expressed in embryos, and its function is related to embryonic photomorphogenesis (Li & Thomas 1998). In the present work, it was found that differentiation in general is promoted when callus cultures are transferred from dark to light (I). However, the route of the signal transduction is still poorly known.

Expression analysis with a variety of methods gives tools to uncover the genetic control behind the physiological processes gene by gene. Still, the final sequence data does not give any information of the functions of the genes *per se* in different tissues or cells. Genetic transformation with sense or anti-sense sequences and *in situ* techniques provide more precise data on the function of the genetic machinery.

## 5.5 New methods for breeding from biotechnology

Although there are a few examples of adapting gene transfer methods to plant breeding, its use in the production of new, transgenic lily cultivars has not been introduced, yet. However, biotechnology enables the use of special methods to overcome problems that arise from the incompatibility or decreased viability of inter-specific crossings. Instead, special techniques in sexual reproduction, like cut-style pollination, ovary slice culture and embryo rescue systems, combined with tissue culture have been used, especially in the production of inter-specific and inter-sectional hybrids (Okazaki *et al.* 1992, Buitendijk *et al.* 1995, Obata *et al.* 2000). In addition, polyploidy and aneuploidy have been proved to give new perspectives also into lily breeding for the future.

Protoplast isolation and culture methods have been developed for a great variety of plants. However, the use of this special material in plant production or research is still not always possible. In pollen biology and fertilization studies, protoplasts are routinely used but not for the production of transgenic or polyploid plants. Using protoplast in the research of plasma membranes, vacuole and other vesicle dynamics, as well as organelle dynamics and cell wall build-up and structure is a promising option also with lilies (Fowke & Wang 1992). Besides electroporation of protoplasts, various other gene transfer methods are available also for lilies. In the current work, the successful use of particle bombardment of lily callus was reported (V). This method has been applied in the transformation of lily microsporocytes (Tabata *et al.* 1993), bulb scales (Tsuchiya *et al.* 1996) and callus (Watad *et al.* 1998). Also, an *Agrobacterium*-mediated system has successfully been used with lilies (Hoshi *et al.* 2004).

Molecular biology and gene technology provide new prospects for breeding and selection of genotypes. To improve the efficiency of selection of desired characteristics, more specific marker genes with known transcription products are better than 'anonymous' marker genes. To identify and characterize of these gene candidates, a functional gene transfer system is necessary for any given species. Northern blot analysis, *in situ* hybridisation and quantitative PCR are powerful methods for the identification and characterization of unknown genes. To understand their role in different processes, the 'final expression', i.e., protein structure and activity, has to be revealed by various protein analysis methods.

## 6 Conclusions

The positive results in induction, growth and different analysis methods, tested with a great variety of lily species, in this work, indicate that there are no obstacles in applying these methods in the culture, breeding and research of lilies possessing horticultural potential for the culture also in the northern climate. *In vitro* methods, starting from germination, are excellent in biotechnology, and they are becoming more essential also in the commercial mass production of plant crops. Of the different methods used in this work, *in vitro* germination, expression studies, phylogenetic analysis and particle bombardment have been used only with none or only a few lily species so far. Also, different regeneration processes in callus and the proportion of somatic embryogenesis in differentiating callus were reported in this work. The results showed the significance of the starting material, as well as the growth medium and the physical factors in the regeneration in general and in somatic embryogenesis in particular. *In vitro* germination results indicated that seed coat and endosperm are the main structural obstacles for germination. In addition, some physiological germination inhibition system, like ABA, is involved in the delayed germination type.

Information concerning the control of differentiation is invaluable for the development of optimal procedure for mass production of lilies. In this work, somatic embryogenesis could be induced in lily callus cultures by a high auxin pretreatment prior to the culture in an auxin-free medium. Along with somatic embryogenesis, organogenesis was also induced, and it predominated in every treatment. In addition to the quantitative production of bulblets, the culture method either in liquid or solid medium has also a great importance for the quality and the applicability of the method in large-scale plant production. In the current work, the temporary immersion culture system proved a potential alternative culture system for the propagation of lily callus.

Transformation methods have not been applied in large scale to the production of novel lily cultivars. In this work, a functional gene transfer system with particle bombardment was introduced, and efficient transformation of a transgene was assessed. In addition to the genome and expression studies, genetic transformation has potential to become another method in the breeding of new lily cultivars with special new characteristics. These include, for instance, the introduction of new colour forms, improved climatic hardiness and pest or pathogen resistance. The desired features can be

screened from the wild populations when there is adequately information about the genes involved in these traits. For this purpose, candidate genes for selection with known expression products have to be characterized from the vast genome of lily.

With the ever-increasing production of new cultivars, the importance of accurate tools for assessing the genetic background and species or cultivar genuineness has become more important. In this work, RAPD analysis combined with the construction of phylogenetic tree according to the DNA fragment lengths was used to reveal the genetic relationships between the traditionally grown Orange lily cultivars and certain natural lily populations. The results showed a closer relationship between native and cultured strains when compared to the other reference species. The various DNA fingerprinting techniques, including RAPD analysis, are easily adopted to the basic research work, as well as routine commercial testing.

## References

- van Aartrijk J & Blom-Barnhoorn GJ (1983) Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Effects of wounding, TIBA, and temperature. *Z Pflanzenphysiol* 110: 355-363.
- van Aartrijk J, Blom-Barnhoorn J & van der Linde PCG (1990) Lilies. In: Ammirato PV, Evans DA, Sharp WR, Bajaj YPS (eds) *Handbook of Plant Cell Culture Vol. 5*. McGraw-Hill Publishing Co, New York, 535-575.
- Abraham A (1939) Chromosome structure and the mechanics of mitosis and meiosis. I. Mitosis in *Lilium*. *Ann Bot* 3: 545-568.
- Aguettaz P, Paffen A, Delvallée I, van der Linde P & De Klerk G-J (1990) The development of dormancy in bulblets of *Lilium speciosum* generated *in vitro*. *Plant Cell Tiss Org Cult* 22: 167-172.
- Alexandrova KS & Conger BV 2002. Isolation of two somatic embryogenesis-related genes from orchardgrass (*Dactylis glomerata*). *Plant Sci* 162: 301-307.
- Al-Khalifah NS & Askari E (2003) Molecular phylogeny of date palm (*Phoenix dactylifera* L.) cultivars from Saudi Arabia by DNA fingerprinting. *Theor Appl Genet* 107: 1266-1270.
- Aronen T, Häggman H & Hohtola A (1994) Transient  $\beta$ -glucuronidase expression in Scots pine tissues derived from mature trees. *Can J Forest Res* 24: 2006-2011.
- Aronen T, Hohtola A, Laukkanen H & Häggman H (1995) Seasonal changes in the transient expression of a 35S CaMV-GUS gene construct introduced into Scots pine buds. *Tree Physiol* 15: 65-70.
- Arzate-Fernández A-M, Nakazaki T, Okumoto Y & Tanisaka T (1997) Efficient callus induction and plant regeneration from filaments with anther in lily (*Lilium longiflorum* Thunb.) *Plant Cell Rep* 16: 836-840.
- Bach A & Swiderski A (2000) The effect of light quality on organogenesis of *Hyacinthus orientalis* L. *in vitro*. *Acta Biologica Cracoviensia* 42: 115-120.
- Bachem CWB, Oomen RFJF & Visser RGF (1998) Transcript imaging with cDNA-AFLP: a step-by-step protocol. *Plant Mol Biol Rep* 16: 157-173.
- Baldan B, Bertoldo A, Navazio L & Mariani P (2003) Oligonucleotide-induced changes in the developmental pattern of *Daucus carota* L. somatic embryos. *Plant Sci* 165: 337-348.
- Baranova MB (1990) *Lilij*. Jaroslavskej, poligrafkombinat, Jaroslavl.
- Baudino S, Hansen S, Bretschneider R, Hecht VFG, Dresselhaus T, Lörz H, Dumas C & Rogowsky PM (2001) Molecular characterisation of two novel maize LRR receptor-like kinases, which belong to the *SERK* gene family. *Planta* 213: 1-10.
- Baskin CC (2003) Breaking physical dormancy in seeds - focussing on the lens. *New Phytol* 158: 227-238.
- Becraft PW (1998) Receptor kinases in plant development. *Trends in Plant Sci* 3: 384-388.

- Bennet MD & Smith JB (1976) Nuclear DNA amounts in angiosperms. *Philos Trans R Soc London Ser B* 274: 227-274.
- Binet M-N, Lepetit M, Weil J-H & Tessier L-H (1991) Analysis of a sunflower polyubiquitin promoter by transient expression. *Plant Sci* 79: 87-94.
- Birch RG (1997) Plant transformation: Problems and strategies for practical application. *Annu Rev Plant Physiol Plant Mol Biol* 48: 297-326.
- Blanckaert A, Belingheri L, Sautiere P-E, Vasseur J & Hilbert J-L (2002) 9-kDa acidic and basic nsLTP-like proteins are secreted in the culture-medium conditioned by somatic embryogenesis in *Cichorium*. *Plant Physiol Biochem* 40: 339-345.
- Bos F (1993) *Lilium bulbiferum subsp. croceum* in the Netherlands and Northern Germany. *The Kew Magazine* 10: 190-197.
- Buc-Dang-Ha D & Mackenzie IA (1973) The division of protoplasts from *Asparagus officinalis* L. and their growth and differentiation. *Protoplasma* 78: 215-221.
- Buitendijk JH, Pinsonneaux N, van Donk AC, Ramanna MS & van Lammeren AAM (1995) Embryo rescue by half-ovule culture for the production of interspecific hybrids in *Alstroemeria*. *Sci Hortic* 64: 65-75.
- Chakrabarty D, Yu KW & Paek KY (2003) Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (*Eleutherococcus senticosus*). *Plant Sci* 165: 61-68.
- Chu YE & Kurtz SL (1990) Commercialization of Plant Micropropagation. In: Ammirato PV, Evans DA, Sharp WR, Bajaj YPS (eds) *Handbook of Plant Cell Culture Vol. 5*. McGraw-Hill Publishing Co, New York, 126-164.
- Delvallée I, Paffen A & De Klerk G-J (1990) The development of dormancy in bulblets of *Lilium speciosum* generated in vitro. II. The effect of temperature. *Physiol Plant* 80: 431-436.
- D'Onofrio CD, Morini S & Bellocchi G (1998) Effect of light quality on somatic embryogenesis of quince leaves. *Plant Cell Tissue Org Cult* 53: 91-98.
- Doyle JJ & Doyle JL (1990) Isolation of plant DNA from fresh tissue. *BRL Focus* 12: 13-15.
- Dubouzet JG & Shinoda K (1999a) ITS DNA sequence relationships between *Lilium concolor* Salisb., *L. dauricum* Ker-Gawl. and their putative hybrid, *L. maculatum* Thunb. *Theor Appl Genet* 98: 213-218.
- Dubouzet JG & Shinoda K (1999b) Phylogenetic analysis of the internal transcribed spacer region of Japanese *Lilium* species. *Theor Appl Genet* 98: 954-960.
- Duncan DR, Kriz AL, Paiva R & Widholm JM (2003) Globulin-1 gene expression in regenerable *Zea mays* (maize) callus. *Plant Cell Rep* 21: 684-689.
- Escalona M, Lorenzo JC, González B, Daquinta M, González JL, Desjardins Y & Borroto CG (1999) Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Rep* 18: 743-748.
- Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K & Somssich IE (1999) Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. *EMBO J* 18: 4698-4699.
- Footitt S, Ingouff M, Clapham D & von Arnold S (2003) Expression of the viviparous 1 (*Pavp1*) and p34<sup>cdc2</sup> protein kinase (*cdc2Pa*) genes during somatic embryogenesis in Norway spruce (*Picea abies* [L.] Kars). *J Exp Bot* 54: 1711-1719.
- Fowke LC & Wang H (1992) Protoplasts as tools in cell biology. *Physiol Plant* 85: 391-395.
- Fowler MR, Ong LM, Russinova E, Atanasov AI, Scott NW, Slater A & Elliot MC (1998) Early changes in gene expression during direct somatic embryogenesis in alfalfa revealed by RAP-PCR. *J Exp Bot* 49: 249-253.
- Fox D (1982) The propagation of lilies. *The Plantsman* 4: 16-28.
- Fox D (1987) Lilies from seeds. *Growing from Seeds* 2: 27-29.
- Galderisi U, Cipollaro M, Di Bernardo G, De Masi L, Galano G & Cascino A (1999) Identification of hazelnut (*Corylus avellana*) cultivars by RAPD analysis. *Plant Cell Rep* 18: 652-655.
- George EF (1993) *Plant Propagation by Tissue Culture*. Part 2. Exegetics Ltd., Eversley, 889-892.
- Gerrits MM & De Klerk G-J (1992) Dry-matter partitioning between bulbs and leaves in plantlets of *Lilium speciosum* regenerated in vitro. *Acta Bot Neerl* 41(4): 461-468.

- Godo T, Matsui K, Kida T & Mii M (1996) Effect of sugar type on the efficiency of plant regeneration from protoplasts isolated from shoot tip-derived meristematic nodular cell clumps of *Lilium x formolongi* hort. Plant Cell Rep 15: 401-404.
- Godo T, Kobayashi K, Tagami T, Matsui K & Kida T (1998) *In vitro* propagation utilizing suspension cultures of meristematic nodular cell clumps and chromosome stability of *Lilium x formolongi* hort. Sci Hortic 72: 193-202.
- Haensch K-T (1996) Plant regeneration through somatic embryogenesis in different genotypes of *Lilium*-hybrids. Gartenbauwissenschaft 61: 214-218.
- Hecht V, Vielle-Calzada J, Hartog MV, Schmidt EDL, Boutilier K, Grossniklaus U, de Vries SC (2001) The *Arabidopsis* somatic embryogenesis receptor kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127: 803-816.
- van Hengel AJ, Guzzo F, van Kammen A & de Vries SC (1998) Expression pattern of the carrot *EP3* endochitinase genes in suspension cultures and in developing seeds. Plant Physiol 117: 43-53.
- Heuer S, Hansen S, Bantin J, Bretschneider R, Kranz E, Lörz H & Dresselhaus T (2001) The maize MADS box gene *ZmMADS3* affects node number and spikelet development and is co-expressed with *ZmMADS1* during flower development, in egg cells and early embryogenesis. Plant Physiol 127: 33-45.
- Hjortswang HI, Larsson AS, Bharathan G, Bozhkov PV, von Arnold S & Vahala T (2002) *KNOTTED1*-like homeobox genes of a gymnosperm, Norway spruce, expressed during somatic embryogenesis. Plant Physiol 40: 837-843.
- Horita M, Morohashi H & Komai F (2002) Regeneration of flowering plants from difficile lily protoplasts by means of a nurse culture. Planta 215: 880-884.
- Horita M, Morohashi H & Komai F (2003) Production of fertile somatic hybrid plants between Oriental hybrid lily and *Lilium x formolongi*. Planta 217: 597-601.
- Horning ME, Maloney SC & Webster MS (2003) Isolation and characterization of variable microsatellite loci in *Lilium philadelphicum* (Liliaceae). Mol Ecol Notes 3: 412-413.
- Hoshi Y, Kondo M, Mori S, Adach Y, Nakano M & Kobayashi H (2004) Production of transgenic lily plants by *Agrobacterium*-mediated transformation. Plant Cell Rep 22: 359-364.
- Häggman H & Aronen T (1998) Transgene expression in regenerating cotyledons and embryogenic cultures of Scots pine. J Exp Bot 49: 1147-1156.
- Ingouff M, Farbos I, Wiweger M & von Arnold S (2003) The molecular characterization of *PaHB2*, a homeobox gene of the HD-GL2 family expressed during embryo development in Norway spruce. J Exp Bot 54: 1343-1350.
- Ishioka N & Tanimoto S (1994) Plant regeneration from mesophyll protoplasts in *Lilium longiflorum*. I. Conditions for isolation and culture. Bull Fac Agr Saga Univ 78: 23-32.
- Jefferson AR, Kavanagh TA & Bevan MW (1987) GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Embo J 6: 3901-3907.
- Jefferson-Brown M & Howland H (1995) The Gardener's Guide to Growing Lilies. David & Charles, Devon.
- Kader J-C (1996) Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 627-54.
- Kairong C, Gengsheng X, Xinmin L, Gengmei X & Yafu W (1999) Effect of hydrogen peroxide on somatic embryogenesis of *Lycium barbarum* L. Plant Sci 146: 9-16.
- Kao KN & Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126: 105-110.
- Kay LE & Basile DV (1987) Specific peroxidase isozymes are correlated with organogenesis. Plant Physiol 84: 99-105.
- Kawahara & Komamine (1995) Molecular Basis of Somatic Embryogenesis. In: YPS Bajaj (ed.) Biotechnology in Agriculture and Forestry 30. Somatic Embryogenesis and Synthetic Seed I, Springer-Verlag, Berlin, 30-40.
- Kitamiya E, Suzuki S, Sano T & Nagata T (2000) Isolation of two genes that were induced upon the initiation of somatic embryogenesis on carrot hypocotyls by high concentrations of 2,4-D. Plant Cell Rep 19: 551-557.

- de Klerk G-J (1992) Hormonal control of dormancy and apical dominance in tissue-cultured plants. *Acta Bot Neerl* 41(4): 443-451.
- Kyte L (1990) Plants from Test Tubes. Timber Press, Portland.
- Langens-Gerrits M, Kuijpers A-M & de Klerk G-J (2003) Contribution of explant carbohydrate reserves and sucrose in the medium to bulb growth of lily regenerated on scale segments *in vitro*. *Physiol Plant* 117: 245-255.
- Lease K, Ingham E & Walker JC (1998) Challenges in understanding RLK function. *Curr Opin Plant Biol* 1: 388-392.
- Li Z & Thomas TL (1998) PEI1, an embryo-specific zinc finger protein gene required for heart-stage embryo formation in *Arabidopsis*. *The Plant Cell* 10: 383-398.
- Lim K-B, Ramanna MS & Jacobsen E (2003) Evaluation of BC<sub>2</sub> progenies derived from 3x-2x and 3x-4x crosses of *Lilium* hybrids: a GISH analysis. *Theor Appl Genet* 106: 568-574.
- Linsmaier EM & Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol Plant* 18: 100-127.
- Lo Schiavo F (1995) Early events in embryogenesis. In: Bajaj YPS (ed) *Biotechnology in Agriculture and Forestry 30: Somatic Embryogenesis and Synthetic Seed I*. Springer-Verlag, Berlin, 20-29.
- Loiseau J, Michaux-Ferrière N, Le Deunff Y (1998) Histology of somatic embryogenesis in pea. *Plant Physiol* 36: 638-687.
- Maesato K, Sharada K, Fukui H, Hara T & Sarma KS (1994) *In vitro* bulblet regeneration from bulb scale explants of *Lilium japonicum* Thunb. Effect of plant growth regulators and culture environment. *J Hort Sci* 69(2): 289-297.
- Martínez-Gómez P & Dicenta F (2001) Mechanisms of dormancy in seeds of peach (*Prunus persica* (L.) Batsch) cv. GF305. *Sci Hort* 91: 51-58.
- McRae EA (1998) *Lilies: A Guide for Growers and Collectors*. Timber Press, Oregon.
- Mii M, Yuzawa Y, Suetomi H, Motegi T & Godo T (1994) Fertile plant regeneration from protoplasts of a seed-propagated cultivar of *Lilium x formolongi* by utilizing meristematic nodular cell clumps. *Plant Sci* 100: 221-226.
- Mogami N, Shiota H & Tanaka I (2002) LP28, a lily pollen-specific LEA-like protein, is located in the callosic cell wall during male gametogenesis. *Sex Plant Reprod* 15: 57-63.
- Montezuma-de-Carvalho J & Guimarães MLL (1974) Production of buds and plantlets from the stamen's filament of *Lilium regale* cultivated *in vitro*. *Biologia Plantarum* 16(6): 472-473.
- Morris EC, Tieu A & Dixon K (2000) Seed coat dormancy in two species of *Grevillea* (Proteaceae). *Ann Bot* 86: 771-775.
- Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473-497.
- Münster T, Pahnke J, Di Rosa A, Kim JT, Marti W, Saedler H & Theissen G (1997) Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. *Proc Natl Acad Sci USA* 94: 2415-2420.
- Nagy JI & Maliga P (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. *Z Pflanzenphysiol* 78: 453-455.
- Nakano M, Sakakibara T, Suzuki S & Saito H (2000) Decrease in the regeneration potential of long-term cell suspension cultures of *Lilium formosanum* Wallace and its restoration by the auxin transport inhibitor, 2,3,5-triiodobenzoic acid. *Plant Sci* 158: 129-137.
- Nhut DT (1998) Micropropagation of lily (*Lilium longiflorum*) via *in vitro* stem node and pseudo-bulblet culture. *Plant cell rep* 17: 913-916.
- Niimi Y (1986) Application of leaf-segment cultures to *in vitro* bulblet production of six *Lilium* species. *Acta Bot Neerl* 35(3): 189-194.
- Niimi Y (1995) *In vitro* propagation and *post-in-vitro* establishment of bulblets of *Lilium japonicum* Thunb. *J Jpn Soc Hort Sci* 63(4): 843-852.
- Niimi Y, Nakano M & Saito S (1997) Production of commercial *Lilium rubellum* Baker bulbs: Effects of volume and renewal of liquid medium on *in vitro* growth, bulb rot infection during cold treatment, and *post-in-vitro* growth of bulblets. *J Jpn Soc Hort Sci* 66(1): 113-119.
- Niimi Y, Han D-S & Makoto F (2001) Production of virus-free plantlets by anther culture of *Lilium x 'Enchantment'*. *Scientia Hort* 90: 325-334.

- Nishihara M, Ito M, Tanaka I, Kyo M, Ono K, Irifune K & Morikawa H (1993) Expression of the  $\beta$ -glucuronidase gene in pollen of lily (*Lilium longiflorum*), tobacco (*Nicotiana tabacum*), *Nicotiana rustica*, and peony (*Paeonia lactiflora*) by particle bombardment. *Plant Physiol* 102: 357-361.
- Nitsch JP & Nitsch C (1969) Haploid plants from pollen grains. *Science* 163: 85-87.
- Obata Y, Niimi Y, Nakano M, Okazaki K & Miyajima I (2000) Interspecific hybrids between *Lilium nobilissimum* and *L. regale* produced via ovules-with-placental-tissue culture. *Sci Hortic* 84: 191-204.
- Ohkawa M, Ohshiro T & Ikekawa T (1996) Effects of 24-epibrassinolide and NAA on the formation of regenerated bulblet of *Lilium japonicum* Thunb. by scale culture *in vitro*. *Environ Control in Biol* 34: 15-19.
- Okazaki K, Umada Y, Urashima O, Kawada J, Kunishige M & Murakami K (1992) Interspecific hybrids of *Lilium longiflorum* and *L. x formolongi* with *L. rubellum* and *L. japonicum* through embryo culture. *J Jpn Soc Hortic Sci* 60: 997-1002.
- Olvera-Carrillo Y, Márquez-Guzmán J, Barradas VL, Sánchez-Coronado ME & Orozco-Segovia A (2003) Germination of the hard coated *Opuntia tomentosa* S.D., a cacti from the México valley. *J Arid Environ* 55: 29-42.
- Palombi MA & Damiano C (2002) Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). *Plant Cell Rep* 20: 1061-1066.
- Perry SE, Lehti MD & Fernandez DE (1999) The MADS-domain protein AGAMOUS-like 15 accumulates in embryonic tissues with diverse origins. *Plant Physiol* 120: 121-129.
- Persson HA, Lundquist K & Nybom H (1998) RAPD analysis of genetic variation within and among populations of Turk's-cap lily (*Lilium martagon* L.) *Hereditas* 128: 213-220.
- Power JB & Chapman JV (1987) Isolation, Culture and Genetic Manipulation of Plant Protoplasts. In: Dixon RA (ed) *Plant Cell Culture; a Practical Approach*. IRL Press Ltd, Oxford, 37-66.
- Priyadarshi S & Sen S (1992) A revised scheme for mass propagation of Easter lily. *Plant Cell Tiss Org Cult* 30: 193-197.
- Quiroz-Figueroa F, Méndez-Zeel M, Larqué-Saavedra A & Loyola-Vargas VM (2001) Picomolar concentrations of salicylates induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture. *Plant Cell Rep* 20: 679-684.
- Ranwala AP & Miller WB (1998) sucrose-cleaving enzymes and carbohydrate pools in *Lilium longiflorum* floral organs. *Physiol Plant* 103: 541-550.
- Ranwala AP, Baird V & Miller WB (1998) Organ-specific localization and molecular properties of three soluble invertases from *Lilium longiflorum* flower buds. *Physiol Plant* 103: 551-559.
- Read PE (1990) Environmental Effects in Microporpagation. In: Ammirato PV, Evans DA, Sharp WR & Bajaj YPS (eds) *Handbook of Plant Cell Culture Vol. 5*. McGraw-Hill Publishing Co, New York.
- Robb S (1957) The culture of excised tissue from bulb scales of *Lilium speciosum* Thunb. *J Exp Bot* 8: 348-352.
- Rojas-Herrera R & Loyola-Vargas VM (2002) Induction of a class III acidic chitinase in foliar explants of *Coffea arabica* L. during somatic embryogenesis and wounding. *Plant Sci* 163: 705-711.
- Rojas-Herrera R, Quiroz-Figueroa F, Monforte-González M, Sánchez-Teyer L & Loyola-Vargas VM (2002) Differential gene expression during somatic embryogenesis in *Coffea arabica* L., revealed by RT-PCR differential display. *Molecular Biotechnology* 21: 43-50.
- Saito H & Nakano M (2002) Isolation and characterization of gametic microprotoplasts from developing microspores of *Lilium longiflorum* for partial genome transfer in Liliaceous ornamentals. *Sex Plant Reprod* 15: 179-185.
- Samaj J, Baluska F, Pretová A & Volkmann D (2003) Auxin deprivation induces a developmental switch in maize somatic embryogenesis involving redistribution of microtubules and actin filaments from endoplasmic to cortical cytoskeletal arrays. *Plant Cell Rep* 21: 940-945.
- Schrader S, Kaldenhoff R & Richter G (1997) Expression of novel genes during somatic embryogenesis of suspension-cultured carrot cells (*Daucus carota*). *J Plant Physiol* 150: 63-68.

- Scheres B & Benfey PN (1999) Asymmetric cell division in plants. *Annu Rev Plant Physiol Plant Mol Biol* 50: 505-537.
- Sentry JW & Smyth DR (1989) An element with long terminal repeats and its variant arrangements in the genome of *Lilium henryi*. *Mol Gen Genet* 215: 349-354.
- Sharma AK & Bhattacharyya B (1957) Cytology of six species of *Asparagus* and *Lilium*. *Fyton* 8: 1-12.
- Sheridan WF (1968) Tissue culture of the monocot *Lilium*. *Planta* 82: 189-192.
- Simmonds JA & Cumming BG (1976) Propagation of *Lilium* hybrids. II. Production of plantlets from bulb scale callus cultures for increased propagation rates. *Sci Hortic* 5: 161-170.
- Siljak-Yakovlev S, Peccenini S, Muratovic E, Zoldos V, Robin O & Vallès J (2003) Chromosomal differentiation and genome size in three European mountain *Lilium* species. *Plant Syst Evol* 236: 165-173.
- Singh MG, Xu H & Bhalla PL (2002) Developmental expression of polyubiquitin genes and distribution of ubiquitinated proteins in generative and sperm cells. *Sex Plant Reprod* 14: 325-329.
- Stewart RN (1947) The morphology of somatic chromosomes in *Lilium*. *Am J Bot* 34: 9-26.
- Stewart NS & Bamford R (1943) The nature of polyploidy in *Lilium tigrinum*. *Am J Bot* 34: 1-7.
- Stasolla C, Loukanina N, Ashihara H, Yeung E & Thorpe TA (2003) Changes in deoxyribonucleotide biosynthesis during carrot somatic embryogenesis. *Plant Physiol Biochem* 41: 779-785.
- Stimart DP & Ascher PD (1978) Tissue culture of bulb scale sections for asexual propagation of *Lilium longiflorum* Thunb. *J Am Soc Hortic Sci* 103: 182-184.
- Sugiura H (1993) Plant regeneration from protoplast of *Lilium speciosum* and *L. x elegans*. *Japan J Breed* 43: 429-437.
- Tabata S, Sato S, Watanabe Y, Yamamoto M & Hotta Y (1993) Evidence of meiosis-specific regulation of gene expression in lily microsporocyte. *Plant Sci* 89: 31-41.
- Takayama S & Misawa M (1979) Differentiation in *Lilium* bulb scales grown *in vitro*. Effect of various cultural conditions. *Physiol Plant* 46: 184-190.
- Takayama S & Misawa M (1980) Differentiation in *Lilium* bulb scales grown *in vitro*. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation *in vitro*. *Physiol Plant* 48: 121-125.
- Takayama S & Misawa M (1981) The mass propagation of *Lilium in vitro* by stimulation of multiple adventitious bulb-scale formation and by shake culture. *Can J Bot* 61: 224-228.
- Takayama S & Misawa M (1982) Regulation of organ formation by cytokinin and auxin in *Lilium* bulb scales grown *in vitro*. *Plant & Cell Physiol* 23: 67-74.
- Takumi S, Otani M & Shimada T (1994) Effect of six promoter-intron combinations on transient reporter gene expression in einkorn, emmer and common wheat cells by particle bombardment. *Plant Sci* 103: 161-166.
- Tanaka A, Hoshi Y, Kondo K & Taniguchi K (1991) Induction and rapid shoot primordia from shoot apices of *Lilium japonicum*. *Plant Tissue Cult Lett* 8: 206-208.
- Tanaka I, Kitazume C & Ito M (1987) The isolation and culture of lily pollen protoplasts. *Plant Sci* 50: 205-211.
- Tanimoto S & Ishioka N (1994) Plant regeneration from mesophyll protoplasts in *Lilium longiflorum*. II. Conditions for division. *Bull Fac Agr Saga Univ* 78: 33-40.
- Teisson C & Alvard D (1995) A new concept of plant *in vitro* cultivation in liquid medium: temporary immersion. In: Terzi M, Cell R & Falavigna A (eds) *Current Issues in Plant Molecular and Cellular Biology*. Kluwer Academic Publ, Dordrecht, 105-109.
- Thomas C, Bronner R, Molinier J, Prinsen E, van Onckelen H & Hahne G (2002) Immunocytochemical localization of indole-3-acetic acid during induction of somatic embryogenesis in cultured sunflower embryos. *Planta* 215: 577-583.
- Thomas C, Meyer D, Himber C & Steinmetz A (2004) Spatial expression of a sunflower *SERK* gene during induction of somatic embryogenesis and shoot organogenesis. *Plant Physiol Biochem* 42: 35-42.
- Tillman-Sutela E & Kauppi A (2000) Structures contributing to the completion of conifer seed germination. *Trees* 14: 191-197.

- Tisserat B (1987) Embryogenesis, organogenesis and plant regeneration. In: Dixon RA (ed) Plant Cell Culture; a Practical Approach. IRL Press Ltd, Oxford, 79-105.
- Tribulato A, Remotti PC, Löffler HJM, van Tuyl JM (1997) Somatic embryogenesis and plant regeneration in *Lilium longiflorum* Thunb. Plant cell rep 17: 113-118.
- Tsuchiya T, Takumi S & Shimada T (1996) Transient expression of a reporter gene in bulb scales and immature embryos of three *Lilium* species is affected by 5' upstream sequences and culture conditions. Physiol Plant 98: 699-704.
- Tzeng T-Y, Chen H-Y & Yang C-H (2002) Ectopic expression of carpel-specific MADS box genes from lily and lisianthus causes similar homeotic conversion of sepal and petal in *Arabidopsis*. Plant Physiol 130: 1827-1836.
- Ueda K, Yoshihisa M & Tanaka I (1990) Fusion studies of pollen protoplasts and generative cell protoplasts in *Lilium longiflorum*. Plant Sci 72: 259-266.
- Wang H & Ng TB (2002) Isolation of liliin, a novel arginine- and glutamate-rich protein with potent antifungal and mitogenic activities from lily bulbs. Life Sci 70: 1075-1084.
- Watad AA, Yun D-J, Matsumoto T, Niu X, Wu Y, Kononowicz AK, Bressan RA and Hasegawa PM (1998) Microprojectile bombardment-mediated transformation of *Lilium longiflorum*. Plant Cell Rep 17: 262-267.
- Wawrosch C, Malla PR & Kopp B (2001) Clonal propagation of *Lilium nepalense* D. Don, a threatened medicinal plant of Nepal. Plant cell rep 20: 285-288.
- Wen CS & Hsiao JY (2001) Altitudinal genetic differentiation and diversity of Taiwan lily (*Lilium longiflorum* var. *formosanum*; Liliaceae) using RAPD markers and morphological characters. Int J Plant Sci 162: 287-297.
- Werker E (1997) Seed Anatomy. Gebrüder Borntraeger, Berlin.
- White PR (1963) The Cultivation of Animal and Plant Cells, Ronald Press, New York.
- Wickremesinhe ERM, Holcomb EJ & Artega RN (1994) A practical method for the production of flowering Easter lilies from callus cultures. Sci Hortic 60: 143-152.
- Williams EG & Maheswaran G (1986) Somatic embryogenesis: Factors influencing coordinated behaviour of cells as an embryogenic group. Ann Bot 57: 443-462.
- Wilson DPM, Sullivan JA, Marsolais AA, Tsujita MJ & Senaratna T (1996) Improvement of somatic embryogenesis in zonal geranium. Plant Cell Tiss Org Cult 47: 27-32.
- Wiweger M, Farbos I, Ingouff M, Lagercrantz U & von Arnold S (2003) Expression of *Chia4-Pa* chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): similarities and differences between gymnosperm and angiosperm class IV chitinases. J Exp Bot 54: 2691-2699.
- Wolter KE & Gordon JC (1975) Peroxidases as indicators of growth and differentiation in aspen callus cultures. Physiol Plant 33: 219-223.
- Woodcock HBD & Stearn WT (1950) Lilies of the World. Country Life Ltd, London
- Wu F-S & Feng T-Y (1999) Delivery of plasmid DNA into intact plant cells by electroporation of plasmolyzed cells. Plant Cell Rep 18: 381-386.
- Yamagishi M (1995) Effects of mannose on enlargement of *in vitro* bulblets of *Lilium japonicum* Thunb. Bull RIAR Agr Coll 4: 86-89.
- Yamaguchi S & Kamiya Y (2002) Gibberellins and light-stimulated seed germination. J Plant Growth Regul 20: 369-376.
- Yanagisawa H, Hamasima N & Kato T (1996) Polyamine oxidase from leaves of *Lilium longiflorum*: purification and properties. J Plant Physiol 149: 657-662.
- Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5: 1411-1423.

## Appendix 1. The origins of the plant material

Table 1. The origins of the plant material

Species	Donor	Experiments
<i>Lilium amabile</i> Palibin	Mr. B. Strohmaan The Lily Nook, Manitoba, Canada	<i>ig, ic, Fc</i>
<i>Lilium auratum</i> Lindley	Tohoku Botanical Garden Tohoku University, Sendai, Japan Jardin Alpin Meyrin, Switzerland Botanical Garden, University of Oulu, Finland	<i>ig, ic, Fc</i> <i>ig, ic, Fc</i>
<i>Lilium auratum</i> var. <i>platyphyllum</i> Baker	Mr. P. Howard, San Fransisco, California, USA	<i>ig, ic, Fc</i>
<i>Lilium bulbiferum</i> Linnaeus	Mr. P. Hanhela, Kempele, Finland Mrs. T. Anttila, Liminka, Finland Mrs. L. Mononen, Puukari, Finland Mrs. P. Makkonen, Hukkala, Finland Mrs. S. Tanskanen, Puukari, Finland Mrs. A. Degerman-Fyrstén, Oulu, Finland Mrs. T. Tuorila, Puutteenkylän Puutarha, Kuusamo, Finland Mrs. A. Peltoniemi, Haukipudas, Finland Giardino Alpino 'Rezia', Bormio, Italy Botanischer Garten, Tübingen, Germany	<i>Fc</i> III <i>ig, ic, Fc, III</i>
<i>Lilium bulbiferum</i> var. <i>croceum</i> (Chaix) Persson	Valdisotto, Verres, Italy Istituto de Orto Botanico 'Hanbury', Genova, Italy Giardino Alpino 'Rezia', Bormio, Italy Jardin Botanique de la Ville, parc de la Tête-d'Or, Lyon, France	<i>ig, ic, Fc</i> III <i>ig, ic, Fc</i> III <i>ig, ic, Fc</i> III
<i>Lilium callosum</i> Siebold & Zuccarini	Mr. D.W. Humphrey, Falls Church, Virginia, USA	<i>ig, ic</i>
<i>Lilium canadense</i> Linnaeus	Mr. G. Hughes, Overland, St. Lois, USA	<i>ig, ic, Fc, III</i>
<i>Lilium candidum</i> Linnaeus	Mr. P. Waister, Invergowrie, Dundee, Scotland	<i>ig, ic, Fc</i>

Species	Donor	Experiments
<i>Lilium candidum</i> var. <i>salonikae</i> Stoker	University of Marie-Curie Sklodowska, Lublin, Poland	<i>ig, ic, Fc</i>
<i>Lilium carniolicum</i> Bernhardt ex Koch (syn. <i>L. pyrenaicum</i> subsp. <i>carniolicum</i> Koch)	Botaniska Trädgården, Göteborg, Sweden	<i>ig, ic, Fc</i>
<i>Lilium carniolicum</i> var. <i>albanicum</i> (Griesb.) Baker (syn. <i>L. pyrenaicum</i> var. <i>albanicum</i> Hayek)	Mrs. D. Fiserova, Prague, Czech Republic Botaniska Trädgården, Göteborg, Sweden	<i>ig, ic, Fc</i>
<i>Lilium cernuum</i> Komarov	Mr. B. Strohmman, The Lily Nook, Manitoba, Canada Mrs. J.C. Ericksen, Wauchope, Saskatchewan, Canada Mr. P. Waister, Invergowrie, Dundee, Scotland	<i>ig, ic, Fc</i>
<i>Lilium columbianum</i> Hanson ex Baker	Mr. L. Riddle, Easter Lily Research Foundation, Brookings, Oregon, USA Botaniska Trädgården, Göteborg, Sweden Vancouver Botanic Garden, University of British Columbia, Vancouver, Canada University of Oslo, Norway	<i>ig, ic, Fc</i>
<i>Lilium concolor</i> var. <i>coridion</i> Baker	Mrs. J.C. Ericksen, Wauchope, Saskatchewan, Canada University of Oslo, Norway	<i>ig, ic, Fc</i>
<i>Lilium concolor</i> var. <i>pulchellum</i> Skinner	Mrs. J.C. Ericksen, Wauchope, Saskatchewan, Canada	<i>ig, ic, Fc</i>
<i>Lilium concolor</i> var. <i>stictum</i> Hooker	Mr. S. Bertrand, Ionia, Iowa, USA	<i>ig, ic, Fc</i>
<i>Lilium dauricum</i> Ker-Gawler	Mrs. J.C. Ericksen, Wauchope, Saskatchewan, Canada	<i>ig, ic, Fc, III</i>
<i>Lilium davidii</i> var. <i>willmottiae</i> (Wilson) Raffill	Mr. D. Meijles, Alphen, Rijn, the Netherlands Jardin Alpin Commune de Meyrin, Switzerland	<i>ig, ic, Fc</i>
<i>Lilium disthicum</i> Nakai	Botanisches Institut V.L. Komarova, St. Peterburg, Russia	<i>ig, ic, Fc</i>
<i>Lilium duchartrei</i> Franchet	Mr. F. Tarlton, Edmonton, Alberta, Canada University of Oslo, Norway	<i>ig, ic, Fc</i>
<i>Lilium formosanum</i> Wallace	Mr. J. Hafer, Blackwood, South Australia, Australia Royal Horticultural Society, London, England	<i>ig, ic, Fc</i>
<i>Lilium grayi</i> Watson	Mrs. D. Bishop, England Mr. D.C. Wilson, England	<i>ig, ic, Fc</i>
<i>Lilium hansonii</i> D.T.Moore	Botaniska Trädgården, Göteborg, Sweden	<i>ig, ic, Fc</i>
<i>Lilium henryi</i> Baker	Dr. S. Kiss, Debreceni, Hungary Botanischer Garten der Justus-Liebig Universität, Germany Jardin Alpin Meyrin, Commune de Meyrin, Switzerland	<i>ig, ic, Fc</i>
<i>Lilium humboldtii</i> Duchartre	Mr. R. Livingston, Placerville, California, USA Royal Horticultural Society, London, England	<i>ig, ic</i>
<i>Lilium kelleyanum</i> Lemmon	Mr. C. Hughes, Overland, St. Louis, USA	<i>ig, ic</i>
<i>Lilium kelloggii</i> Purdy	Mr. C. Hughes, Overland, St. Louis, USA	<i>ig, ic</i>
<i>Lilium lancifolium</i> Thunberg	Mr. O. Tarvainen, Viherlandia, Jyväskylä, Finland Mrs. T. Anttila, Liminka, Finland	<i>Fc</i> <i>Fc</i>

Species	Donor	Experiments
	Mrs. L. Mononen, Puukari, Finland	<i>Fc</i>
	Bratislava Hortus Plantarum Medicarum, Bratislava, Slovakia	<i>ig, ic, Fc</i>
	Department of Medicinal Plant, Ogród Botaniczny Uniwersytetu A. Mickiewicza, Poznań, Poland	<i>ig, ic, Fc</i>
<i>Lilium lankongense</i> Franchet	Mr. D.C. Wilson, England	<i>ig, ic</i>
<i>Lilium leichtlinii</i> var. <i>maximowiczii</i> (Regel) Baker	Mr. J. Zeabart, Terre Haute, Indiana, USA	<i>ig, ic, Fc</i>
	Botanischer Garten und Botanische Museum Berlin-Dahlem, Berlin, Germany	<i>ig, ic, Fc</i>
<i>Lilium mackliniae</i> Sealy	Mr. C. Hughes, Overland, St. Louis, USA	<i>ig, ic, Fc</i>
<i>Lilium maculatum</i> Thunberg	Mr. K. Arakawa, Japan	<i>ig, ic, Fc</i>
<i>Lilium martagon</i> Linnaeus	Metropolitan Medicinal Plants Garden, Tokyo, Japan	<i>ig, ic, Fc</i>
	Botanical Garden, University of Oulu, Finland	<i>ig, ic, Fc, I</i>
	Hortus Botanicus Arcto-Alpinus, Kirovsk, Russia	<i>ig, ic, Fc, I</i>
	Botanischer Garten und Arboretum der Stadt Linz, Austria	<i>ig, ic, Fc, I</i>
	Botanischer Garten der Universität Zürich, Switzerland	<i>ig, ic, Fc</i>
	Giardino Alpino 'Rezia', Bormio, Italy	<i>ig, ic, Fc</i>
	Botanischer Garten der Universität Innsbruck, Austria	<i>ig, ic, Fc</i>
	Hortus Botanicus Forminianus, Kiev, Ukraine	<i>ig, ic, Fc</i>
	Hortus Botanicus Centralis, Minsk, Belarus	<i>ig, ic, Fc</i>
	Botanischer Garten der Johan-Wolfgang-Goethe, Frankfurt, Germany	<i>ig, ic, Fc, I</i>
	Instituto de orto botanico 'Hanbury', Genova, Italy	<i>ig, ic, Fc</i>
	Conservatoire et Jardin Botaniques, Geneva, Switzerland	<i>ig, ic, Fc</i>
	Jardin Alpin Florealpe, Fondation J.-M. Aubert, Champex, Valais, Switzerland	<i>ig, ic, Fc, I</i>
<i>Lilium martagon</i> var. <i>album</i> Weston	Mrs. E. Majoinen, Tohmajärvi, Finland	<i>Fc</i>
	Botanical Garden, University of Oulu, Finland	<i>Fc</i>
	Botanicheskij Institut V.L. Komorova, St. Peterburg, Russia	<i>ig, ic</i>
<i>Lilium medeoloides</i> A. Gray	Mr. K. Arakawa, Japan	<i>ig, ic, Fc</i>
<i>Lilium michiganense</i> Farwell	Mr. D. Drews, Elk River, Minnesota, USA	<i>ig, ic</i>
<i>Lilium monadelphum</i> Bieberstein	Mrs. A. Balode, Riga, Latvia	<i>ig, ic, Fc</i>
	Mrs. I. Hirvonen, Rääkkylä, Finland	<i>Fc</i>
<i>Lilium nanum</i> Klotzsch & Garcke	Mr. J. Lee, England	<i>ig, ic</i>
	Mr. D.B. Nilsen, Norway	<i>ig, ic</i>
	Mrs. D. Smith, England	<i>ig, ic</i>
<i>Lilium nobilissimum</i> T. Makino	Dr. I. Watanabe, Kanum, Tochigi, Japan	<i>ig, ic</i>
	Mr. K. Arakawa, Japan	
<i>Lilium oxypetalum</i> Baker	University of Oslo, Norway	<i>ig, ic</i>
<i>Lilium pardalinum</i> Kellogg	Mr. I.A. Aird, England	<i>ig, ic, Fc</i>
	Mr. B. Baumer, USA	<i>ig, ic, Fc</i>
	Mr. N. Martschnike, Germany	<i>ig, ic, Fc</i>
	Mrs. B. Small, Fair Oaks, California, USA	<i>ig, ic, Fc</i>

Species	Donor	Experiments
<i>Lilium parryi</i> S. Watson	Mr. C. Hughes, Overland, St. Louis, USA	<i>ig, ic, Fc</i>
	Royal Horticultural Society, London, England	<i>ig, ic, Fc</i>
<i>Lilium parvum</i> Kellogg	Mr. K. Arakawa, Japan	<i>ig, ic</i>
	Mr. D. Wilson, England	
<i>Lilium philadelphicum</i> Linnaeus	Mr. B. Strohmman, The Lily Nook, Manitoba, Canada	<i>ig, ic, Fc</i>
	Mr. C. Hughes, Overland, St. Louis, USA	<i>ig, ic, Fc</i>
	Golden Rocky Mountain Seed Service, Canada	<i>ig, ic, Fc</i>
<i>Lilium pomponium</i> Linnaeus	Instituto de orto botanico 'Hanbury', Genova, Italy	<i>ig, ic</i>
	Botaniska Trädgården, Göteborg, Sweden	<i>ig, ic</i>
<i>Lilium pumilum</i> de Candolle	Mrs. J.C. Ericksen, Wauchope, Saskatchewan, Canada	<i>ig, ic, Fc</i>
	Mr. B. Strohmman, The Lily Nook, Neepawa, Manitoba, Canada	<i>ig, ic, Fc</i>
	Csapödy Növénybarat Kör, Vörös Zoltan, Budapest, Hungary	<i>ig, ic, Fc</i>
	Mr. D. Sierzega, Vancouver, British Columbia, Canada Botanical Garden of the Polish Academy of Science, Warsaw, Poland	<i>ig, ic, Fc</i> <i>ig, ic, Fc</i>
<i>Lilium pyrenaicum</i> Gouan	Botanischer Garten der Justus-Liebig Universität, Giessen, Germany	<i>ig, ic, Fc</i>
	Botanischer Garten der Universität Regensburg, Germany	<i>ig, ic, Fc</i>
	Jardin Botanique de la Ville Lyon, France	<i>ig, ic, Fc</i>
	University of Marie-Curie Sklodowska, Lublin, Poland	<i>ig, ic, Fc</i>
	Jardins Botaniques Villers-des-Nancy, France Jardin Alpin Florealpe, Fondation J.-M. Aubert, Champex, Valais, Switzerland	<i>ig, ic, Fc</i> <i>ig, ic, Fc</i>
<i>Lilium regale</i> Wilson	Botanischer Garten der Universität Düsseldorf, Germany	<i>ig, ic, Fc, I, IV</i>
	Botanischer Garten der Technische Universität, Duisburg, Germany	<i>ig, ic, Fc</i>
	University of Marie-Curie Sklodowska, Lublin, Poland	<i>ig, ic, Fc</i>
	Department of Medicinal Plant, Ogrod Botaniczny Uniwersytety A. Mickiewicza, Poznan, Poland	<i>ig, ic, Fc</i> <i>ig, ic, Fc</i>
	Jardin Alpin Meyrin Commune de Meyrin, Switzerland	<i>ig, ic, Fc</i>
<i>Lilium sargentiae</i> Wilson	Mr. K. Arakawa, Japan	<i>ig, ic, Fc</i>
	Mr. H. Skeen, England	
<i>Lilium speciosum</i> Thunberg	Mr. R. Brown, Papa Toe Toe, New Zealand	<i>ig, ic, Fc</i>
<i>Lilium superbum</i> Linnaeus	Mr. L. Lutz, Boalsburg, Pennsylvania, USA	<i>ig, ic, Fc</i>
	Northampton, Massachusetts, USA	<i>ig, ic</i>
	University of Oslo, Norway	<i>ig, ic</i>
<i>Lilium szovitsianum</i>	Mr. J. Vasarietis, Bauskas Raj, Latvia	<i>ig, ic, Fc</i>
Fischer & Avé-Lallemant (syn. <i>L. monadelphum</i> var. <i>armenum</i> Eremin)	Mrs. D.A. Martyn Simmons, England Mr. J.W.M. Graham, England	<i>ig, ic</i> <i>ig, ic</i>

Species	Donor	Experiments
	St. Andrews University Botanic Garden, Scotland	<i>ig, ic</i>
	Stavanger Botanic Garden, Norway	<i>ig, ic</i>
<i>Lilium tsingtauense</i> Gilg	Royal Horticultural Society, London, England	<i>ig, ic, Fc</i>
<i>Lilium wallichianum</i> var. <i>neilgherrense</i> Wight	Mr. Visaraghaven, India	<i>ig</i>
<i>Lilium washingtonianum</i> Kellogg	Mr. J. Robinett, Sebastopol, California, USA	<i>ig</i>
<i>Lilium washingtonianum</i> var. <i>purpurascens</i> Stearn	Mr. C. Hughes, Overland, St. Louis, USA	<i>ig, ic, Fc</i>
<i>Lilium vollmeri</i> Eastwood	Mr. I.A. Aird, England	<i>ig, ic</i>
	Stavanger Botanic Garden, Norway	
<i>Lilium</i> x 'Marhan'	Mrs. I. Hirvonen, Rääkkylä, Finland	<i>ic, Fc</i>
van Tubergen	Runnin Terveyskylpylä, Runni, Finland	

*ig* - *in vitro* germination; *ic* - *in vitro* culture

*Fc* - field culture

Roman numbers refer to the original papers.