Regeneration and transformation by particle bombardment in leek (*Allium ampeloprasum* L.)

Promotoren: **dr. ir. E. Jacobsen**
Hoogleraar in de plantenveredeling
in het bijzonder in de genetische variatie en reproductie

dr. ir. R.G.F. Visser
Hoogleraar in de plantenveredeling
Persoonlijk hoogleraar bij het laboratorium voor Plantenveredeling

C.M. Schavemaker

Regeneration and transformation by Particle bombardment in leek (*Allium ampeloprasum* L.)

Proefschrift
Ter verkrijging van de graad van doctor
op het gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen
dr. ir. L.Speelman
in het openbaar te verdedigen
op dinsdag 3 oktober 2000
des middags te half twee in de Aula.

The investigation described in this thesis was carried out at the Department of Plant Breeding of Wageningen Agricultural University.

CIP-DATA KONINKLIJKE BIBLIOTHEEK DEN-HAAG

Schavemaker C.M.

Regeneration and transformation by particle bombardments in leek (*Allium ampeloprasum* L.)

Thesis Wageningen Agricultural University- With references- With summaries in English and Dutch. Department of Plant Breeding, P.O.Box 386, 6700 AJ Wageningen, The Netherlands.

ISBN 90-5808-234-2

Bibliographic abstract: This thesis describes the first steps of genetic modification of leek (*Allium ampeloprasum* L.). A cyclic somatic embryogenesis regeneration system was achieved and a flower stalk regeneration system was adjusted for transformation purposes. Particle gun bombardments were carried out and several factors influencing transformation have been analyzed and optimized. This finally resulted in chimeric leek plants.

Stellingen

1. Een positieve PCR van een potentiële transgene plant afkomstig van particle bombardment is mogelijk een valse positief (dit proefschrift)

2. De theorie over de licht reactie van luciferase zal moeten worden aangepast (dit proefschrift)

3. Hoge transiente genexpressie heeft geen directe correlatie met transformatie frequentie maar is wel een meetbare aanwijzing dat DNA in de cellen is terecht gekomen, maar (nog) niet in de kern (dit proefschrift)

4. Plastiden transformatie kan bijdragen aan de acceptatie van transgene voedselgewassen in verband met het niet verspreiden van transgenen via pollen

5. Plastiden transformatie is een goede manier om in een kruisbevruchter en transformant als prei dat via zaad vermeerderd wordt rassen te verkrijgen die zuivertelend zijn voor de transgene eigenschap (dit proefschrift)

6. Hybride prei is makkelijker te verwerken voor de boer maar verkleint de persoonlijke keuze voor de consument

7. Als celbioloog moet je bij het praten met moleculair biologen extra alert zijn op misverstanden

8. Een heilige oorlog bestaat niet

9. De tip op de verpakking van de aardappelen, om aardappelen zo dun mogelijk te schillen i.v.m. het behoud van vitamine C , is onjuist.

10. Ook met kleine kinderen is een goede buur beter dan een ver familielid

11. Als Albert Hein echt op de kleintjes let, moet hij de schappen verlagen

12. Van een gemakkelijke weg is alleen het begin geasfalteerd

Stellingen behorende bij het proefschrift "Regeneration and transformation by particle bombardment in leek (*Allium ampeloprasum* L.)" door C.M. Schavemaker, in het openbaar te verdedigen op dinsdag 3 oktober 2000, te Wageningen

Contents

Chapter 1

General introduction

The vegetable leek

Leek (*Allium ampeloprasum* L.) is an important field grown vegetable in the Netherlands (Fig 1.1). From 1974 to 1994 the production of leek increased in the Netherlands (Table 1.1). In 1994 the production area of leek was the largest of the last two decades. The low prices for arable crops made the farmers grow more market garden crops like leek. From 1995 until now the total area remained stable at 4100 ha. With these numbers is leek the third export vegetable of the Netherlands after potato and tomato. Most leek production is concentrated in the south of the Netherlands, roughly 40% is produced in the province of Limburg and 50% is produced in Brabant. Ten percent of the leek production is for industrial purposes such as for instant soup and 40% of the production is exported, mainly to Germany. New culture practices are being developed like seed-leek (Vorm, 1999) or hydro-culture (Stallen, 1999) to obtain higher yields and better quality.

Fig 1.1. Harvested leek (*Allium ampeloprasum* L.)

The primary center of origin of the present cultivated leek is believed to be the Near East and the Mediterranean region (Vavilov, 1926). In these areas still some wild species of the genus *Allium* can be found (DeWilde-Duyfjes 1976, Stearn 1978). Masefield *et al.* (1969) stated that A. *ampeloprasum* is also a native of the Atlantic islands of the Azores, Canaries, Cape Verdes and Madeira.

Table 1.1. Production of leek in the Netherlands from 1974 to 1994 (x 1000 kg) (Figures derived from Anonymous-a, Anonymous-b,)

Year	Area	Production	Import	Export cons.	Fresh Proc.	Industrial (ha)
1975	1 283	47 602	830	7123	33920	6806
1980	1 645	51 039	2536	7220	38989	6753
1985	2 856	57 602	4025	12117	43772	5464
1990	2 896	94 312	2353	40091	47333	8901
1991	3 799	98 793	4410	45099	47926	10035
1992	4 683	114 299	3101	55907	48638	11085
1993	4 495	109 200	4918	52544	43881	-[2]
1994	4 873	120 000	-[2]	50398	57587	-[2]

[2] not known

Taxonomy

Leek is a member of the genus *Allium* (family *Alliaceae*), which consists of over 600 species (Hanelt 1990, Jones 1991). One characteristic of this family is the basal plate. The basal plate contains both shoot and root meristems in a circular disc. Some disagreement exists about the taxonomic position of leek. Some taxonomists use the taxonomic classification *Allium porrum* L for leek (Mathews, 1996). Hanelt (1990) reviewed the *Allium* genera and distinguished 5 subgenera. Leek was placed in the *A. ampeloprasum* complex, section *Allium* of the subgenus *Allium*. In this thesis the name *A. ampeloprasum* will be adopted for leek, because of its crossability with the other members of the complex and the fertile offspring produced by these crossings (Silvertand, 1996).

The life cycle of leek

Leek is a biannual crop. In the first year the seeds germinate in spring and form an edible pseudostem, for which the leek is cultivated. After summer this pseudostem is harvested. For seed production the leek needs a vernalisation period and it is a winterhard crop. Next spring the leek will develop new leaves and in summer a tall flower stalk is produced (see Fig.1.2). A big flowerhead of approximately 300-600 small flowers is seen in the summer and in autumn the seed is harvested. Most seeds are produced after cross-pollination by insects. The use of artificial climate rooms can shorten the growing season to one year, but with losses of

seed production as the number of flowers and the size of the flowerhead is decreased (Silvertand 1996).

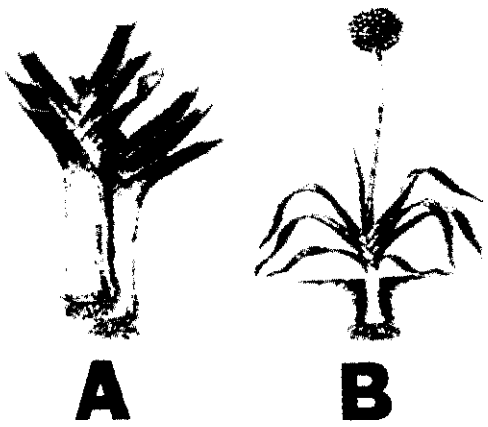

Fig.1.2. Leek (*Allium ampeloprasum* L) in the vegetative state (a) and in the reproductive state (b)

Breeding research

The cultivated leek is tetraploid (2n=4x=32) and a monocot. At present the leek cultivars are classified according to their harvest period, as summer, autumn and winter types. These cultivars show differences in length of the shaft, leaf color, earliness and winter hardiness. For summer varieties earliness and bolting resistance are the most important characteristics. Breeding emphasis for the autumn and winter cultivars is focussing on increased shaft length, absence of bulbing of the shaft and improvement of the winter hardiness. For all cultivars the general breeding aims are improvement of uniformity, higher yields and resistance against pests and diseases (Currah, 1986). Many pests and diseases attack leek of which the onion fly (*Delia antiqua*), trips (*Thrips tabaci*) and leek moths (*Acrolepiopsis assectella*) are the most important pests organisms. The most devastating diseases are: white tip (*Phytophthora*

porri), purple blotch (*Alternaria porri*), leaf blotch (*Cladosporium allii-porri*) and rust (*Puccinia allii*). The available breeding systems (mass and family selection) are unsuitable to solve the problems associated with these diseases or to introduce resistances, in all plants of a variety, to these diseases. The most suitable system able to cope with these problems seems to be hybrid leek breeding. Hybrid leek breeding is hampered by the lack of a suitable emasculation or male sterility system and the severe inbreeding depression. Efforts have been made to obtain male sterile leek plants and nuclear male sterility has been found in leek production fields. Probably, these male sterile plants arise as spontaneous mutants. A dutch breeding company (Nunhems seeds) used such a (nuclear) male sterile plant as female for producing hybrids seeds, maintaining this male sterile plant by vegetative propagation. Kaul (1988) stated that recessive genes mostly control the spontaneous male sterility in monocots. The offspring of crosses with nuclear male sterile plants was only for 50% male sterile when the male sterile plant was pollinated with a heterozygous fertile plant and the character was, therefore, monogenic. Half of the offspring should be removed during flowering, which is a great handicap of using nuclear male sterility. Even if the character is controlled by a single dominant gene, half the offspring should be male fertile and removed during flowering. When using cytoplasmic male sterility often restorer genes are found and this can make the possible production of leek hybrids even more complicated. Silvertand (1996) tried to obtain cytoplasmic male sterile (CMS) leek plants by means of chemical mutagenesis. The chemical *N*-nitroso-*N*-methyl-urea (NMU) gave a relatively high percentage of male sterile plants in the M_1. The NMU treatment resulted in poor seedling emergence, a high percentage of chlorophyll deficient seedlings, female sterility and a low percentage (16%) of the plants reached the flowering stage, which were derived from over 20.000 seeds. Twelve percent of the flowering M_1-plants were male sterile. Out of 412 selected male sterile M_1 plants only 59 progenies were obtained. The plants of these progenies were, as expected, mostly male fertile. Only 8 M_2 progenies from female fertile M_1 plants showed an increased percentage of male sterility and from this only one half-sib M_3 family exhibited a relatively higher number of male sterile plants. The results of that research are still being investigated. Buiteveld (1998) produced plants by means of protoplast fusion between CMS-onion protoplasts and those of leek. She described a tissue culture procedure to obtain regenerable friable callus from immature zygotic embryos. Already many researchers have been working on regeneration and *in vitro* multiplication of leek. The application of tissue culture in leek breeding could be very helpful in for example the maintenance of male sterile leek plants or inbred lines (Baumunk-Wende, 1989). *In vitro* multiplication via organogenesis and somatic embryogenesis has been described for leek (for a review see Novák *et al.* 1986). Stem

explants, basal plates, leaf discs, leaf base, flower heads, open flowers, single flower bud receptacles and anthers have been used as starting plant material for regeneration (Debergh and Standaert-de Metsenaere 1976; Dunstan and Short 1979; Doré and Sweisguth 1980; Novák and Havel 1981; Van Doorne *et al* 1988; Doré 1988; Rauber and Grunewald 1988; Baumunk-Wende 1989; Wang, 1996). Silvertand (1996) was one of the first who reported shoot regeneration from the flower stalk. Ziv *et al* (1983) mentioned briefly the possibility of shoot development on explants of young elongating inflorescence stalks of ornamental forms of *Allium ampeloprasum* L. Flower stalks cultured on 6-benzyladeninepurine (BAP) and - naphthaleneacetic acid (NAA) medium produced plantlets via organogenesis. Van der Valk *et al.* (1992), Silvertand (1996) and Buiteveld *et al.* (1998) have described somatic embryogenesis. According to Silvertand (1996) the culture of mature zygotic embryos on medium containing the auxin 2,4 di-chlorophenoxyacetic acid (2,4D) produced somatic embryos. The most important factor, reported by Buiteveld (1998), in obtaining the desirable, embryogenic friable callus was the size of the immature embryo from seed. Friable callus is described as soft, friable, less differentiated with aggregates of embryogenic cells (Buiteveld, 1998). Immature embryos of 0.5 to 2.5 mm gave the highest frequency of friable callus. From these friable callus cultures a suspension culture was successfully made and it stayed regenerable for 9 months. The next step was isolation of protoplasts. A plating density was 2 x 10^5 pp.ml^{-1} was necessary to obtain microcalli (plating efficiency of 0.219%). The protoplasts were cultured in Ca^{2+}- alginate to obtain this plating density and microcalli were visible after 6 to 8 weeks of culture. Protoplasts isolated from mesophyll cells of leaf bases did not divide. Symmetric protoplast fusion resulted into novel combinations of chloroplast- and mitochondrial DNA in two hybrids. In this thesis the systems of somatic embryogenesis and flower stalk regeneration of Silvertand (1996) have been used and further exploited for the purpose of genetic transformation.

Transformation studies

Isolated genes coding for the desired male sterility are known. In 1990 Mariani *et al* (1990) reported the establishment of nuclear male sterility by a genetic engineered gene, which can prevent pollen development through a RNA-ase gene with a tapetum specific promoter. This gene could solve a lot of problems in leek breeding if a suitable transformation method could be successfully employed in this crop. A successful transformation method depends on a highly efficient regeneration system, a gene transfer system and a selection method. Leek can

in this crop and to solve many problems in leek, like disease and pest resistance, higher and uniform yields and hybrid breeding, genetic transformation could be a solution. Several steps are necessary in order to develop a transformation procedure for leek. At first a suitable regeneration system or systems have to be developed. In Chapter 2 cyclic somatic embryogenesis for leek is described as a possible regeneration system that could be used in transformation. In Chapter 3 is shown which cell-layers are involved in regeneration during the cyclic embryogenesis regeneration system and in the flower stalk regeneration system. Studies on the prerequisites for transformation with the particle gun like distance, pressure, origin of explant, coating and selection are described in Chapter 4. In Chapter 5 the optimal conditions have been applied to flower stalk explants and the results of transformation experiments are discussed. A general discussion and final conclusions and recommendations for future research are presented in Chapter 6.

Literature

Anonymous-a (1996) Groententeelt in de vollegrond, 40e rassenlijst, CPRO-DLO, Wageningen

Anonymous-b (1995,1996,1997,1998) CBS tuinbouwcijfers LEI-DLO, 's Gravenhage, Voorburg, Heerlen.Tuinbouw statistiek PGF, 's Gravenhage.

Arokiaraj P, Jones H, Cheong KF, Coomber S, Charlwood BV (1994) gene insertion into *Hevea brasiliensis*, Plant Cell Reports 13: 425-431.

Baumunk-Wende E (1989) Application of tissue culture in leek breeding (*Allium porrum* L.) Gartenbauwissenschaft 54: 20-24.

Bommineni VR, Chibbar N, Datla RSS, Tsang EWT (1993) Transformation of white spruce (*Picea glauca*) by microprojectile bombardment, Plant Cell Reports 13: 17-23

Bower R, Birch G (1992) Transgenic sugarcane plant via microprojectile bombardment, The Plant Journal 2(3): 409-416.

Buiteveld J (1998) Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek. PhD-thesis Wageningen Agricultural University, The Netherlands.

Castillo AM, Vasil V, Vasil IK (1994) Rapid production of fertile transgenic plants of Rye (*Secale cereale* L.) Biotechnology 12: 1366-1371.

Christou P, Tameria L, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electrical discharge particle acceleration of exogenous DNA into immature zygotic embryos, Biotechnology 9: 957-962.

Currah L (1986) Leek breeding: a review, J. Hort. Sci 61(4): 407-415.

Debergh PC, Standaert-de Metsenaere S (1976) Neoformation of bulbils in *Allium porrum* L., Sci. Hort 5: 11-12

L., Sci. Hort 5: 11-12
Dewilde-Duyfjes BEE (1976) A revision of the genus *Allium* L. (*Liliaceae*) in Africa. Meded, Landbouw Hogeschool Wageningen, 73-311.
Dommisse EM, Leung DWM, Shaw ML, Conner AJ (1990) Onion is a host for *Agrobacterium*, Plant Science 69: 249-257.
Doré C, Sweisguth B (1980) application de la culture *in vitro* a l'amélioration des plantes potageres, Reunion EUCARPIA, Versailles INRA: 54-57.
Doré C (1988) Multiplication végétative et conservation *in vitro* chez le poireau (*Allium porrum* L.), Agronomie 8: 509-511.
Dunstan DI, Short KC (1979) Shoot production from cultured *Allium porrum* tissues, Sci Hort 11: 37-43.
Finer JJ, McMullen MD (1990) Transformation of cotton (*Gossypium hirsitum* L.) via particle bombardment, Plant Cell Reports 8: 586-589.
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojectile bombardment, Plant Cell Reports 9: 189-194.
Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Gray DJ, Finer JJ (Eds) (1993) Development and operation of five particle guns for introduction of DNA into plant cells, Plant Cell Tissue and Organ Culture 33: 220-257.
Gray DJ, Hiebert E, Lin CM, Compton ME, McColley DW, Harrison RJ, Gaba VP (1994) Simplified construction and performance of a device for particle bombardment, Plant Cell Tissue and Organ Culture 37: 179-184.
Hagio T, Blowers AD, Earle ED (1991) Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles, Plant Cell Reports 10: 260-264.
Hanelt P (1990) Taxonomy, evolution and history. In: Rabinowitch HD, Brewster JL. (Eds). Onion and allied crops, Vol. 1, CRC Press, Inc. Boca Raton, Florida, pp 1-26.
Jones RN (1991) Cytogenetics of *Allium*, In Tsuchiya T, Gupta PK (Eds) Chromosome engineering in plants: Genetics. Breeding, evolution, Part B, Elsevier, Oxford, New York, Tokyo, 215-227.
Kaul MLH (1988) Male sterility in higher plants, Springer Verlag, Berlin.
Klein TM, Wolf ED, WU R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells, Nature 325: 70-73.
Klein TM, Harper EC, Scab Z, Sanford JC, Fromm ME, Maliga P (1988) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process, Proc. Natl. Acad. Sci. USA 85: 8502-8505.
Li B, Wolyn DJ (1997) Recovery of transgenic asparagus plant by particle gun bombardment of somatic cells, Plant Science 126(1): 59-68.
Lin H. (1998) Development of two *in vitro* regeneration systems through leaf explant and callus culture and the application for genetic transformation in *Alstroemeria*, PhD-thesis, University of Wageningen, the Netherlands.
Mariani C, De Beuckeleer M, Truettner J, Leemans J, Goldberg B (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene, Nature 347: 737-739.
Masefield GB, Wallis M, Harrison SG, Nicholson BE (1969) The oxford book of food

plants, Oxford Univ Press, London, p.168.
Mathews B (1996) A review of *Allium* section *Allium*, Whitestable Litho Ltd. Whitestable Kent. UK.
McCabe DE, Swain WF, Martinell BJ, Christou P(1988) Stable transformation of soybean (*Glycine max*) by particle acceleration, Biotechnolgy 10: 286-291.
Murray FR, Latch GCM, Scott DB (1992) Surrogate transformation of perennial ryegrass, *Lolium perenne*, using genetically modified *Acremionium* endophyte, Mol. Gen. Genet. 233: 1-9.
Novák FJ, Havel L (1981). Shoot production from *in vitro* cultured flower heads of *Allium porrum* L., Biol Plantarum 23(4):266-269.
Novák FJ, Havel L, Dolezel (1986) *Allium*, In: Evans DA, Sharp WR, Ammirato PV (Eds) Handbook of plant Cell Culture vol IV Macmillan New York, pp 419-456.
Ozias-Akins P, Schnall JA, Anderson WF, Singit C, Clemente TE, Adang MJ, Weissinger AK (1993) regeneration of transgenic peanut plants from stable transformed embryogenic callus, Plant Science 93: 185-194.
Raemakers. CJJM, Sofiari E, Taylor N, Henshaw G, Jacobsen E, Visser RGF (1996) Production of transgenic cassava (*Manihot esculenta* Crantz) plants by particle bombardment using luciferase activity as selection marker, Molecular Breeding 2: 339-349.
Rauber M, Grunewald J (1988) *In vitro* regeneration in *Allium* species, Plant Cell Reports 7: 426-429.
Ritala A, Aspegren K, Kurtén U, Salmenkallio-Marttila M (1994) Fertile transgenic barley by particle bombardment of immature embryos, Plant Mol. Biol. 24:317-325.
Russel DR, Wallace KM, Bathe JH, Martinell BJ, McCabe DE (1993) Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle acceleration, Plant Cell Reports 12: 165-169.
Sagi L, Panis B, Remy , Schoofs H, Smet K-de, Swennen R, Cammue BPA (1995) Genetic transformation of banana and plantain (*Musa spp*) via particle bombardment, Biotechnology 13 (5): 481-485.
Silvertand B (1996) Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L), PhD thesis Agricultural University Wageningen, The Netherlands.
Songstad DD, Somers DA, Griesbach RJ (1995) Advances in alternative DNA delivery techniques, Plant Cell Tissue and Organ Culture 40: 1-15.
Stallen J (1999) Prei op water kan nu de kilo's nog, Volllegronds groenten, Groenten en fruit 1 oktober:4-5.
Stearn WT (1978) European species of *Allium* and allied genera of *Alliaceae*: a synonymic enumeration, Ann Musei Goulandris 4: 83-198.
Syun Zheng, pers. communication.
Van Doorne LE, Stolte AJ, Van Harten AM (1988) Application of *in vitro* techniques for multiplying and breeding leek (*Allium porrum* L.), 4th EUCARPIA *Allium* Symposium 1988.

Van der Valk P, Scholten OE, Verstappen RC, Dons JJ (1992) High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species, Plant Cell Tissue Org Cult 30: 181-191.

Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus, Biotechnolgy 10: 667-674.

Vavilov NI (1926) Studies on the origins of cultivated plants, Bull. Appl Bot and Plant Breed 16: 1-245.

Vorm van de J (1999) Prei van land tot klant, Oogst Landbouw, 15 oktober p.64-65.

Wang H (1996) Genetic engineering male sterility in leek (*Allium porrum* L.), PhD thesis Applied Biological Sciences, University of Gent, Gent, Belgium.

Wilmink A (1996) Genetic modification of tulip by means of particle bombardment, PhD thesis University of Nijmegen, The Netherlands.

Ziv M, Hertz N, Biran Y (1983) Vegetative reproduction of *Allium ampeloprasum* L. *in vivo* and *in vitro*, Isr J Bot 32: 1-9.

Zhong et al. submitted

Chapter 2

Development of a cyclic somatic embryogenesis regeneration system for leek (*Allium ampeloprasum* L.) using zygotic embryos.

Published in Plant Cell Reports 14: 227-231 (1995)

Abstract

In leek (*Allium ampeloprasum* L.) a cyclic system of somatic embryogenesis was developed. Somatic embryos used for cyclic embryogenesis were able to develop the same type of embryogenic callus as zygotic embryos in the primary cycle. For the first time a comparison of the efficiencies of both explants was made. Ten families were investigated for somatic embryogenesis. There was a genetic relationship with respect to somatic embryo production between the reciprocal crosses. From each family one genotype was selected for investigating cyclic somatic embryogenesis. Different levels of somatic embryo production were found between the explants of zygotic and somatic embryos. The two best genotypes, 92.001-03 and 92.002-33 produced twice as many somatic embryos as the overall average. On average, 56% of the somatic embryos finally developed into greenhouse plantlets.

Introduction

Leek (*Allium ampeloprasum*) is a vegetable grown for its edible (false) stem and belongs to the *Alliaceae*, together with onion (*Allium cepa*) and garlic (*Allium sativum*). The production of leek is mainly confined to Europe. In the last few years production has increased along with the consumers demand. It is propagated through seeds and gives rise to heterogeneous progeny. Problems in cultivation of leek are rust (*Puccinea allii, P. mixtu*), yellow stripe virus and the lack of uniformity. The feasibility to overcome these negative factors by classical breeding is hampered because of several problems such as the out- and inbreeding character which results in a high degree of heterogeneity, polyploidy and biannual flowering of leek. Therefore emphasis has been focused on the application of genetic modification in order to solve some of these problems. A prerequisite for this is the availability of an efficient regeneration system. A few *in vitro* regeneration systems have been described for *Allium* species. Regeneration has been described out of basal plates (Dunstan and Short, 1978), flowerheads, basal disc, ovules and zygotic embryos (Novák and Havel 1981, Novák *et al.*, 1986, Keller, 1990, Van der Valk *et al.*, 1992, Buiteveld *et al.*, 1993, Silvertand, 1996). The most promising regeneration system to integrated genetic modification, for example with particle bombardment, is indirect somatic embryogenesis. Somatic embryogenesis is characterized by the formation of a bipolar structure out of plant tissue without connection of the vascular system and can be a single cell event. The first report on somatic embryogenesis was published for garlic (*Allium sativum*) by Abo-El-Nil (1977) followed by several others

(Phillips and Luteyn, 1983; Havel and Novák, 1988; Lu *et al.*, 1989; Van der Valk *et al.*, 1992; Buiteveld *et al.*, 1993). All reports on somatic embryogenesis in *Allium ampeloprasum* describe the indirect form involving a callus phase. On explants, initiated mostly from mature or immature zygotic embryos, callus is induced and somatic embryos are developed on this callus. After the induction period the callus with pro-embryos is transferred to a germination medium to mature the somatic embryos. These somatic embryos regenerate into normal leek plantlets (fig 2.1). One problem is that long term callus cultures often lose their ability of regeneration (Dunstan and Short, 1978, Phillips and Luteyn, 1983). In this report we demonstrate the possibility of producing somatic embryos on a year round basis.

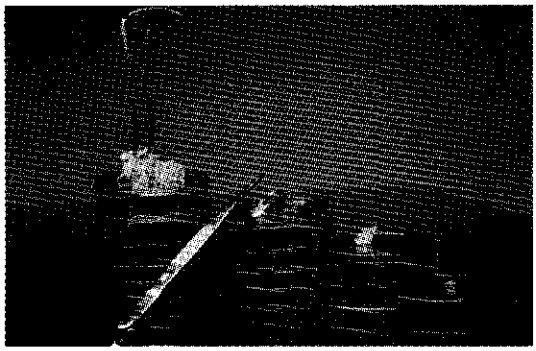

Fig 2.1. Somatic embryogenesis of leek

Materials and Methods

Ten families (full sibs) were selected from previous experiments based on their regeneration performance *in vitro* (Silvertand ,1996). These selections were derived from crosses between individual genotypes of different leek-cultivars that could be more easily regenerated. The genetic background of the crosses is presented in Table 2.1. Family 92.001 and 92.002, 92.003 and 92.004, 92.005 and 92.006 were reciprocal crosses of individual genotypes of two different cultivars. Family 92.007 and 92.008 were crosses between other individual plants of two cultivars. Family 92.009 and 92.010 were selfings.

Table 2.1. Genetic background of the ten full sib families.

	Castelstar	Dakota	Farinto	Gavia
cv.Castelstar	92.009	92.001	92.004	-
cv.Dakota	92.002/92.007	-	92.005	-
cv.Farinto	92.003/92.008	92.006	-	
cv Gavia	-	-	-	92.010

cv. Castelstar and Gavia; Enza-zaden, Enkhuizen

cv. Dakota; Royal Sluis, Enkhuizen

cv. Farinto; Nunhems zaden, Haelen

Primary somatic embryogenesis

Seeds of ten families were surface sterilized in 70% ethanol for 30 s followed by 20 min 1.5% sodium hypochlorite with a drop of Tween 20 and rinsed three times in sterilized water. The seeds were allowed to imbibe overnight and were sterilized again in 1.5% sodium hypochlorite and rinsed three times in sterilized water. After sterilization the zygotic embryos were excised from the seed and placed on a callus induction medium, MS medium (Murashige and Skoog, 1962) with 2 mg l^{-1} 2,4-D and 3% saccharose, solidified with 0.8% micro-agar (Daishin, Brunschwig). Tissues were placed in the dark at 21°C. The medium was refreshed at two week intervals. After six weeks of induction the calli with somatic embryos were placed on a germination medium, MS medium with 2 mg l^{-1} BA and 3% saccharose, solidified with 0.8% micro-agar. Tissues were placed in 12 hours light at 21°C.

Cyclic somatic embryogenesis

The experiment was set up as a complete randomized design. Each family was cultured in 4 replications (10 explants per replication). Mature green somatic embryos were isolated from the calli after four, six and eight weeks on germination medium. These somatic embryos were counted and forty of them belonging to one genotype were returned to callus induction medium for the second cycle of somatic embryogenesis.

Since every single seed of leek represented a different genotype, variation within families was observed as expected. To avoid this difficulty, the second cycle was initiated by using

somatic embryos selected from a promising genotype of one family from the first cycle. This genotype became a representative for the whole family of that cross.

Shoot development

From the second cycle till the fifth, twenty four embryos were placed on medium (MS medium with 3% saccharose) to allow shoot development. The number of shoots was counted after 3 months. A shoot was counted when a somatic embryo had a shoot length of about 1 cm and possessed at least one root. The entire process is schematically shown in Figure 2.2.

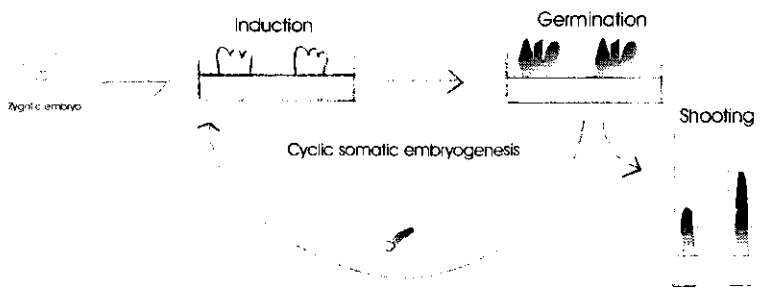

Fig. 2.2. Schematic representation of the cyclic somatic embryogenesis and shoot development of leek (*A. ampeloprasum* L.)

Results

Primary somatic embryogenesis

In the first cycle callus induction and somatic embryo production were determined for each family. The percentage of zygotic embryos with callus formation varied between 58% and 100% (Table 2.2). The average number of somatic embryos per initial, callus forming, zygotic embryo varied among investigated families between 8.0 and 39.4. The families with the highest somatic embryo production, 92.001, 92.002 and 92.007 were derived from crosses with individual plants of cv. Dakota and cv. Castelstar as parents. In family 92.005

and 92.006 cv. Dakota was also involved. Within the high producing family, 92.001, the number of somatic embryos varied between 25.0 and 60.8 and in the low producing family, 92.008, between 1.3 and 14.8 (data not shown). There was no correlation between the percentage of callus induction and the number of somatic embryos produced per callus. For the characters callus formation and somatic embryo formation, the reciprocals did not differ significantly from each other.

Table 2.2. Results of the first cycle; percentage of callus formation, average number of somatic embryos per initial, callus forming, zygotic embryo of leek (*A. ampeloprasum* L.)

Family	% callus	average
92.001	83	39.4
92.002	71	38.1
92.003	96	17.2
92.004	96	15.3
92.005	75	33.5
92.006	96	21.6
92.007	58	38.0
92.008	83	8.0
92.009	88	16.1
92.010	100	11.0
average	85	23.8

Cyclic somatic embryogenesis

Callus induction

After the first cycle, mature somatic embryos of one high yielding zygotic embryo per family were used for callus induction in the second cycle (fig 2.3).

Fig.2.3. Isolated somatic embryos of leek (bar = 250μm)

The percentage of somatic embryos with callus induction in the second cycle varied between 65% and 93% (Table 2.3). These percentages decreased in the successive cycles to 40% and 87% in the sixth cycle, respectively. On an average, the best callus forming genotypes were 92.002-33, 92.003-42 and 92.005-32 with 82%, 80% and 80% callus induction respectively. The lowest performing genotype was 92.008-07 with an average of only 56% callus induction. Comparison between callus induction on zygotic (Table 2.2) and on somatic embryo explants (Table 2.3) indicated that for seven of the ten genotypes zygotic embryos were more efficient. For the high yielding families 92.002, 92.005 and 92.007, selection of the best somatic embryo producing genotype did have a positive effect on callus formation of these somatic embryos. Not only in the second but also in the following cycles the percentage of callus formation in these genotypes was higher than in the first cycle. For the other families selection of the best genotype did not have a positive effect on callus formation.

Table 2.3. Percentage of initial somatic embryos reacting with callus formation, during five cycles, of ten leek genotypes (*A. ampeloprasum* L.)

	cycle					
Genotype	2	3	4	5	6	av.
92.001-03	77	70	93	77	65	76
92.002-33	93	68	85	78	87	82
92.003-42	83	80	83	88	65	80
92.004-37	70	75	83	67	65	72
92.005-32	83	83	83	78	73	80
92.006-36	65	83	65	43	68	65
92.007-27	80	75	88	70	70	77
92.008-07	80	50	63	30	--	56*
92.009-39	83	55	90	58	67	71
92.010-45	75	60	90	68	40	67
av.	79	70	82	66	67	73

* based on the average of four cycli

Comparison of the somatic embryo production between zygotic and somatic embryos indicates a reduced production when somatic embryos were used as source explants. This reduction was most clear with embryos of genotypes 92.005-32 and 92.007-27. The families 92.008 and 92.010 with the selected genotypes 92.008-07 and 92.010-45 had the lowest somatic embryo production when either zygotic or somatic embryos were used as source explants. Genotype 92.008-07 did not produce sufficient embryos to start the sixth cycle.

Table 2.4. Somatic embryo production in number of somatic embryos per initial, callus forming, somatic embryo of 10 leek genotypes (*A. ampeloprasum* L.), during five (2-6) cycles.

Genotype	cycle					
	2	3	4	5	6	av.
92.001-03	21.5	23.5	24.7	36.2	28.2	26.8
92.002-33	22.2	23.5	21.8	25.3	31.9	24.9
92.003-42	10.5	9.6	6.5	10.6	10.9	9.6
92.004-37	14.0	14.2	13.3	10.0	12.4	12.8
92.005-32	12.0	13.1	16.3	19.4	18.6	15.9
92.006-36	8.9	10.6	7.4	11.6	15.8	10.9
92.007-27	13.2	10.8	4.7	22.0	14.1	13.0
92.008-07	6.1	2.5	1.1	2.5	-	3.1
92.009-39	20.9	8.0	9.1	6.7	6.3	10.2
92.010-45	6.1	3.6	6.0	5.3	6.2	5.4
av.	13.5	11.9	11.1	15.0	16.0	13.3

Shoot development

Instead of starting a new cycle, a part of the mature somatic embryos was tested for shoot development (Fig. 2.2). The shooting frequencies in four successive cycles of ten genotypes are shown in Table 2.5. Although no special effort was made to optimize conditions for shoot development, an average of 56% of the somatic embryos regenerated into plantlets *in vitro*. In the second cycle the shooting percentage was relatively high (68%). In the successive cycles this declined to about 50%. A relatively high percentage of somatic embryos regenerated into multiple shoots.

Table 2.5. Shooting percentages of 24 germinated somatic embryos of 10 genotypes of leek (*A. ampeloprasum* L.) during four cycles.

Cycle	2				3				4				5				Av
				%				%				%				%	%
genotype	A	B	C	(B+C)	A	B	C	(B+C)	A	B	C	(B+C)	A	B	C	(B+C)	(B+C)
92.001-03	4	9	11	(83)	7	2	15	(71)	8	1	15	(67)	7	2	15	(71)	(73)
92.002-33	4	3	17	(83)	9	1	14	(63)	8	2	14	(67)	8	0	16	(67)	(70)
92.003-42	10	7	7	(58)	13	3	8	(46)	14	0	10	(42)	11	0	13	(54)	(50)
92.004-37	10	4	10	(58)	19	2	3	(21)	22	1	1	(8)	22	0	2	(8)	(24)
92.005-32	13	4	7	(46)	10	3	11	(57)	9	0	15	(63)	9	0	15	(63)	(57)
92.006-36	5	1	18	(79)	7	1	16	(70)	9	3	12	(63)	10	1	13	(58)	(68)
92.007-27	3	5	16	(88)	7	4	13	(71)	14	0	10	(42)	6	1	17	(57)	(70)
92.008-07	7	3	14	(71)	11	2	11	(54)	-	-	-	(--)	-	-	-	(--)	(63)
92.009-39	14	3	7	(42)	22	0	2	(8)	17	0	7	(29)	17	3	4	(29)	(27)
92.010-45	8	7	9	(67)	9	2	13	(61)	11	2	11	(54)	10	0	14	(58)	(60)
average	8	4	12	(68)	11	2	11	(52)	13	1	10	(46)	11	1	12	(52)	(56)

A: number of somatic embryos with no response
B: number of somatic embryos with one or two shoots
C: number of somatic embryos with multiple shoots (>2)
%: percentage regeneration
(A+B+C=24)

Up to 15 shoots per initial somatic embryo were observed. The percentage of somatic embryos regenerating into one or two shoots (B in Table 2.5) decreased in each successive cycle. The highest percentage of shoots was also found for the earlier mentioned two high yielding genotypes 92.001-03 and 92-002-33. The genotypes 92.004-37 and 92.009-39 had a low percentage of shoot development, although these genotypes produced a moderate number of somatic embryos. All rooted plantlets were successfully transferred to the greenhouse and were able to flower and set seed.

Discussion

Several authors described somatic embryogenesis in *Allium species* for only one cycle. This report shows that for all tested genotypes, selected from ten families, a longterm regeneration system, via cyclic somatic embryogenesis, could be developed.

Callus induction

Zygotic embryos are more effective in callus formation than somatic embryos. The decrease can be explained by the physiological stage of the embryos used. Zygotic embryos, isolated from mature seeds, have the same developmental stage. Somatic embryos differ more in development. This means that the procedure of cyclic somatic embryogenesis can be improved by selecting the optimal developmental stage.

A higher percentage of zygotic embryo with callus initiation does not necessarily imply that a family has a higher ability to produce somatic embryos. There was no correlation between these two factors. However no callus formation means no somatic embryo production. The morphology of the callus formed by zygotic embryos does not differ from that obtained by a somatic embryo. In some cases, for zygotic as well as somatic embryos initiated callus, embryogenic callus turned into non-embryogenic callus.

Somatic embryo production

Regeneration ability can be maintained for more than nine cycles of somatic embryogenesis (data not shown), depending on genotype. In one year over 10.000 *in vitro* plantlets (average) can be obtained out of one zygotic embryo. This number is much higher than the long-term multiplication of onion (*Allium cepa* L.) by cyclic shoot regeneration *in vitro*, used by Kahane *et al.* (1992).

The first cycle gave the highest somatic embryo production. Successive cycles could only produce equal or lower numbers of somatic embryos. It is remarkable that the average somatic embryo production of a family is a good prediction of the behavior of the somatic embryo production of one genotype selected from that family. Especially for genotype 92.008-07, the average somatic embryo production was low in the first cycle, eight somatic embryos per initial embryo, but still one zygotic embryo produced over forty. This level was not maintained in the second cycle, but dropped to a lower average production than in the first cycle. Selection of a family which yields large numbers of somatic embryos from initial

zygotic embryos is not a guarantee for the detection of superior genotypes in the successive cycles but it is an important first step in the procedure. The genotypic effect is evident in the reciprocal crosses. Within a reciprocal cross no difference was found. Between the reciprocal crosses, some differences appeared. The contributions of the cultivars Dakota and Castelstar is clearly shown in the families 92.001, 92.002 and 92.007 and in the genotypes 92.001-03 and 92.002-33. Both genotypes 92.008-07 and 92.010-45 produced the lowest number of somatic embryos. Family 92.010 is a selfing (Table 1), probably suffering of an inbreeding depression. Leek is known to have a strong inbreeding depression (Currah, 1986).

Morphological observations showed that in some cases pro-embryos were formed but they were not able to mature. This phenomenon was also found in somatic embryogenesis of rice (*Oryza sativa* L.) (Jones and Rost, 1989). Due to the clear definition of germinated embryos, being only mature and green, these white pro-embryos were not counted. Therefore, it could happen that three promising somatic embryo producing families had a positive selection for callus formation but lacked somatic embryo production in the successive cycles.

Shoot development

The development of somatic embryos into *in vitro* plantlets seems to be satisfactory (Table 5). Only two genotypes have low shooting percentages. This might be caused by insufficient maturation of the somatic embryos. If the somatic embryogenesis process is completed, there should be no difficulty of shooting on normal MS medium without growth regulators. However, if the process is disturbed or not completed, the somatic embryos may need some extra growth regulators for completing the process. Further research on improvement of shoot development has to be continued.

In leek the difference between the first and further cycles of embryogenesis is relatively small compared to other cyclic somatic embryogenesis systems. For example the cyclic embryogenesis of *Manihot esculenta* Cranz (Raemakers *et al.*, 1993) and *Picea abies* {L.} Karst (Mo *et al.*, 1989) starting with young leaves and immature zygotic embryos respectively has a low response in the first cycle. After this first step, somatic embryogenesis is highly efficient and stable over many cycles when somatic embryos are used as explants. Similar studies for monocots have, as far is known, never been published before.

In this study the first cycle was not performed under optimal conditions. Silvertand *et al.*, (submitted) have shown that a lower concentration of 2,4-D gave significantly higher somatic

embryo production. In this cyclic system a minimal concentration of 2 mg 2,4-D/l is necessary to guarantee the somatic embryo production in the successive cycles. Lower 2,4-D concentrations stimulate the somatic embryos in rooting rather than in callus initiation (data not shown). A concentration of 2 mg 2,4-D/l seems to be sufficient to maintain the ability of callus and somatic embryo formation.

This cyclic system can be universally applied to different genotypes and is a promissing method to obtain transgenic plants out of chimeric structures. Further research on the development and optimalization of secondary somatic embryogenesis has to be conducted. More detailed analysis of the morphology of greenhouse grown regenerants has to be intensified to obtain data on somaclonal variation.

Literature

Abo El-Nil MM (1977) Organogenesis and embryogenesis in callus cultures of garlic (*Allium sativum* L.) Plant Sci. Lett. 9: 259-264.

Buiteveld J, Van der Valk P, Jansen J, Creemers-Molenaar J, Colijn-Hooymans CM (1993) Callus induction and plant regeneration from explants of commercial cultivars of leek (*Allium ampeloprasum* var porrumL.) Plant Cell Reports 12: 431-434.

Currah L (1986) Leek breeding review. J. Hort. Sci. 61: 407-415.

Dunstan DI, Short KC (1978) Shoot production from onion callus tissue cultures Scienta. Hort. 9: 99-110.

Jones TJ, Rost TL (1989)The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Botanical Gazette 150: 41-49.

Havel L, Novák FJ (1988) Regulation of somatic embryogenesis and organogenesis in *Allium carinamtum* L.J. Plant Physiol. 132: 373-377.

Kahane R, Rancillac M, Teyssendier de la Serve B (1992) Long-term multiplication of onion (*Allium cepa* L.) by cyclic shoot regeneration in vitro. Plant Cell Tissue and Organ Culture. 28: 281-288.

Keller J (1990) Culture of unpollinated ovules, ovaries and flowerbuds in some species of the genus *Allium* and haploid induction via gynogenesis in onion (A*llium cepa* L.)Euphytica 47: 241-247.

Lu C, Currah L, Peffley EB (1989) Somatic embryogenesis and plant regeneration in diploid *Allium fistulosum* * *A. cepa* F1 hybrid onions. Plant Cell Reports. 7: 696-700.

Mo LH, Von Arnold S, Lagererantz U (1989) Morphogenic and genetic stability in longterm embryogenic cultures and somatic embryos of Norway spruce (*Picea abies* L. Karst)Plant Cell Reports 8: 375-378.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.

Novák FJ, Havel L (1981) Shoot production from *in vitro* cultured flower heads of *Allium*

porrum L. Biol. Plant. 23: 266-269.

Novák FJ, Havel L, Dolezel J (1986) *Alliums* In: Evans DA, Sharp WR, Ammirato PV (Eds). Handbook of Plant Cell Culture, vol 4. Macmillan, New York, pp 419-456.

Phillips GC, Luteyn KJ (1983) Effect of picloram and other auxins on onion tissue cultures. J. Amer. Soc. Hort. Sci. 108: 948-953.

Raemakers CJJM, Amati M, Staritsky G, Jacobsen E, Visser RGF (1993) Cyclic somatic embryogenesis and plant regeneration in cassava. Annals of Botany. 71: 289-294.

Silvertand B (1996) Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L). PhD thesis Agricultural University Wageningen, The Netherlands.

Van der Valk P, Scholten OE, Verstappen F, Jansen RC, Dons JJM (1992) High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species. Plant Cell Tissue and Organ Culture 30: 181-191.

Chapter 3

A histological and morphological study of the leek cyclic somatic embryogenesis system to assess suitability for transformation

Abstract

A cyclic somatic embryogenesis system of leek (*Allium ampeloprasum* L.) was developed for multiplication, availability of season independent explant source and transformation purposes. Theoretically a somatic embryo of leek only partially transgenic could be transferred to the next cycles in order to obtain complete non-chimeric transformants of the transgenic part of the first somatic embryo. To establish an efficient transformation method for leek a selection procedure had to be applied, the stringency of which would determine the outcome of transformation experiments. In this report three selective agents, kanamycin, hygromycin and phosphinothricin, were tested on two leek cultivars, at various concentrations, on callus growth, regeneration and survival rates. Phosphinothricin was the only agent that really could inhibit callus growth. All of the selective agents were capable of inhibiting regeneration. However, none of the three selective agents were able to completely inhibit growth of leek tissue. The best performing selective agent was phosphinothricin at a high concentration (>10 mg l^{-1}). After several attempts of transforming somatic embryos, bombarded by particle bombardment with the firefly luciferase gene, without any success, a histological study of the cyclic somatic embryogenesis system was made to investigate the reasons for lack of success in this transformation system. Somatic embryos of the third cycle were followed in time and fixed at 3 days intervals from the embryo induction period until the end of the embryo germination period. Detailed studies showed that the origin of the new somatic embryos came from the center of the somatic embryo, in the same way that leaves are produced by a mature leek plant. This indicated that somatic embryos are unsuitable targets for transformation at this time and that other tissue types have to be found for transformation purposes.

Introduction

The totipotency of a cell was stated in 1838 by a theory of Schwan and Schleiden (in: Pierik, 1985). It took another 100 years to obtain some proof of this theory as Nobécourt, Gautheret and White practiced the first real tissue culture of plants by a callus culture (in: Street 1973). In 1971 the first plants were derived from tobacco protoplasts (Takebe, 1971). Unfortunately, it took a lot of effort to prove the totipotency of a plant cell and it is still not clearly known whether all cells can be triggered to be totipotent. A lot of effort is necessary in different plant species to identify for the right explant for propagation, regeneration and transformation. Plant regeneration *in vitro* occurs via organogenesis or somatic

embryogenesis. Stem explants, basal plates, leaf discs, flower heads, open flowers, single bud receptacles and flower stalks have been described as suitable explant material for *in vitro* propagation of leek by organogenesis (Debergh & Standaert-De Mestenaere 1976; Dunstan & Short, 1979; Novák & Havel, 1981; Van Doorne *et al.* 1988; Doré 1988; Rauber & Grunewaldt, 1988; Bauwmunk-Wende, 1989; Silvertand, 1996). Van der Valk *et al.* (1992), Silvertand (1996) and Buiteveld (1998) have described somatic embryogenesis of leek. The totipotent cells of leek capable of forming new somatic embryos should preferably also be competent cells for successful transformation in order to be able to produce transgenic plants. To be able to monitor the transformation process a reporter gene was used. A powerful tool in particle gun transformation studies is the use of luminescence genes, such as the luciferase gene. Luminescence genes are regularly used to monitor plant transformation systems. A number of luminescence genes has been identified and cloned from different sources like the bacteria *Vibrio harveyi* and *V. fishery*, the marine ostracod crustacean *Vargula hilgendorfii*, the jellyfish *Aequorea victoria*, the soft coral *Renilla reniformis* and the fire fly *Photinus pyralis* (Belas *et al.*1982; De Wet *et al.* 1985; Thompson *et al.*, 1990; Mayerhofer *et al.* 1995). The firefly luciferase gene is one of the most commonly used luminescence reporter genes in transient gene expression assays. The enzymatic reaction catalyzed by the luciferase enzyme was reported by McElroy *et al.* (1969). In Fig 3.1 an overview of the reaction is given.

1. LH_2 + ATP +E ----> E LH_2-AMP + PPi
2. E LH_2-AMP + O_2 ----> E + product + CO_2 + AMP + light
3. L + ATP + E <-----> E L-AMP + PPi

Fig. 3.1 Firefly luciferase catalyzed reaction; Abbreviations used: LH_2, luciferin; L, dehydroluciferin; E, Enzyme, PPi, Inorganic pyrophosphate.

The initial activation step involves the formation of an enzyme-bound luciferyladenylate and inorganic pyrophosphate (1). This complex reacts with oxygen and light is one of the reaction products (2). One quantum of light is emitted for each luciferin molecule oxidized. There is also a third reaction that can take place. Luciferase catalyzes the activation of dehydroluciferin to form an enzyme bound dehydroluciferyladenylate and inorganic pyrophosphate (3). This reaction gives no light and forms an equilibrium. The presence of Mg^{2+} and Coenzyme-A are also important. Mg^{2+} as catalyzer of the first and third reaction.

Different methods can be used to analyze luciferase expression; in most reports a cell lysis method was used (Ow *et al.*, 1986; Gupta *et al.*, 1988; Schneider *et al.*, 1990; Grasser *et al.*, 1993; Martinussen *et al.*, 1994; Manzara *et al.*, 1994). The transfected cells were buffered, lysed and centrifuged. Total analysis kits can be purchased from some companies like Promega and Boehringer. The reaction is started by injection of the substrate. For the detection of the emitted light several luminometers are available like the Packard Pico-lite luminometer Analyzer, Turner Design Model 20 Luminometer, Labsystem Luminoscan and Lumat LB 9501. Several factors, like buffer concentration, luciferin concentration and pH can influence the counted photon emission (Manzara *et al.*, 1994; Sherf and Wood, 1993). The latter reports made more use of visualization of the emitted photons by x-ray photos (Ow *et al.*, 1986), Agfa Pan 400 or Kodak T max 400 films or by image intensifier/video camera unit in series with an Argus 50 image processor (Hamamatsu Photonics; Millar *et al.*, 1992; Howell *et al*, 1989).

Firefly luciferase has a number of advantages over, more convential reporter genes like CAT and GUS. The enzyme assay is sensitive, can be done in a non-destructive way to the plant tissue, non-toxic to plant and human, fast and makes a whole plant survey possible, for following gene expression in time. Qualitative and quantitative measurements can be used as a vital selection marker as well. It was shown in cassava and *Dendrobium* that selection of transformed tissue by luciferase activity led to a reproducible transformation method (Chia *et al.*, 1994, Raemakers *et al.*, 1996). An important disadvantage is that the substrate needed for the light reaction is expensive and the reaction in living plant cells is not yet fully understood. Also the use of advanced video equipment to perform the non-destructive assay requires an expensive investment in equipment.

Selection in transformation procedures is a necessity. In only a few reports transgenic plants were obtained without using selective substances in the media (Christou *et al.*, 1991, Bommineni *et al.*, 1993, Zhong *et al.*, 1993, Ritala *et al.*, 1994; Raemakers *et al.*, 1996,) while many other reports stated the need of a severe selection pressure (Fitch *et al.*, 1990, Gordon-Kamm *et al.*, 1990, Hagio *et al.*, 1991, Fitch *et al.*, 1992, Hébert *et al.*, 1993, Hensgens *et al.*, 1993, Hartman *et al.*, 1994 and Wang, 1996). A selection marker gives the transgenic cell within a group of non-transgenic cells the possibility and advantage to survive or to multiply more frequently. Transformation of monocotyledon species has been hampered by the insensitivity of monocots to the most widely used selective agent kanamycin due to natural resistance, cross-protection or the presence of detoxifying enzymes (Potrykus *et al.*, 1988, Fromm *et al.*, 1990, Christou *et al.*, 1991,Wilmink and Dons, 1993).

In this report, data have been obtained about callus growth, regeneration and survival rates under various selection concentrations using zygotic embryos of leek. Attempts to transform by particle gun were made in the cyclic somatic embryogenesis system of leek. When this proved to be unsuccessful, a histological and morphological study on the cyclic somatic embryogenesis was undertaken in order to identify the underlying mechanism for the observed recalcitrancy.

Materials and methods

Plant material

Seeds of Dutch leek cultivars Strata (Royal Sluis, Enkhuizen), Farinto and Porino (Nunhems Zaden, Haelen), Castelstar and Gavia (Enza Zaden, Enkhuizen) were sterilized according to the method described in chapter 2 of this thesis. After sterilization, the zygotic embryos were excised from the seed and placed on a callus induction medium, MS medium (Murashige and Skoog, 1962) with 2 mg l^{-1} 2,4-D and 3% saccharose, solidified with 0.8% micro-agar. The excised zygotic embryos were placed in the dark at 21°C. The medium was refreshed at two-week intervals. After six weeks of induction the calli with somatic embryos were placed on a germination medium, MS medium with 2 mg l^{-1} BA and 3% saccharose, solidified with 0.8% micro-agar(Daishin, Brunschwig). Tissues were placed in 12 hours light at 21°C. Mature green somatic embryos were isolated from the calli after four weeks on germination medium. These somatic embryos were replaced on callus induction medium for the second cycle of somatic embryogenesis. This procedure was repeated for the later cycli.

Selection

Seeds of two leek cultivars, Porino and Strata were sterilized according to the method described in chapter 2. After sterilization the zygotic embryos were excised from the seed and 10 zygotic embryos were placed per dish on callus induction medium, MS medium (Murashige and Skoog, 1962) with 2 mg l^{-1} 2,4 D and 3% sucrose, solidified with 0.8% micro-agar. Tissues were placed in the dark at 21°C. One day after isolation the 10 zygotic embryos per petridish were weighed together and placed on selection medium. Selective agents, kanamycin, hygromycin and phosphinothricin were filter sterilized and added to the induction medium at various concentrations and in 3 or 4 replications. The medium was refreshed at two-week intervals. Three measurements of growth and survival by weighing and visual observations were done. After 6 weeks on callus induction medium the calli were

transferred to germination medium, MS medium with 2 mg l^{-1} BA, 3% sucrose and the selective agent, solidified with 0.8% micro-agar, 12 hours light at 21°C.

Bombardments

Zygotic and somatic embryos, *in vitro* roots and basal plates of different age and in different stages of development were submitted to bombardments of different pressures ranging from 450 to 1800 psi (pounds per square inch). One day before bombardment, the explants were placed in the center of the petridish on fresh medium. Gold particles, median size 1.0 µm, coated with DNA were prepared essentially according to the protocol of the Biorad® PDS-1000/He particle gun. The vacuum was kept on 27 inch Hg. After bombardment the explants were placed on selection medium, except for some controls.

Plasmids

pJIT100 and pJIT65 were kindly provided by Dr. J.F. Guerinaeu, John Innes Institute, UK (Guerineau & Mullineaux 1993). pACH18 was kindly provided by Prof. P.H. Quail (Christensen and Quail, 1996, Christensen *et* al, 1993). pDC2 was obtained from Plant Genetic Systems, Belgium (now; Aventis Crop Science).

Expression vector	gene construct	references/sources
pJIT100	p35S:LUC:t35S/p35S:BAR:t35S	Guerineau & Mullineaux 1993
pJIT65	p2x35S:GUS:t35S	Guerineau & Mullineaux 1993
pACH18	pUbi:LUC:tnos	Christensen & Quail 1996
pDC2	pTR2`:GUS:t35S/pTR1`:NPTII:tnos	Plant Genetic systems

LUC	= Luciferase	p35S	=35S promoter
BAR	=phoshinothricin-N-acetyltransferase	p2z35S	=double 35S promoter
GUS	=ß-glucuronidase	pUbi	=ubiquitin promoter
NPTII	=neomycin phoshotransferase II	pTR1` or 2`	=TR1` or 2` promoter
tnos	=nos terminator	t35S	=35S terminator

Plasmid DNA was isolated using the Wizard™ Maxiprep DNA purification system of Promega according to the instructions of the supplier.

Luciferin substrate

In order to start a chemical reaction with light as a reaction product a substrate has to be

provided to the plant cells. In our experiments the beetle luciferin-salt (Promega) dissolved in sterile water was used at a concentration of 0.15 mg/ml. Filtersterile beetle luciferin was sprayed (fine mist by pump action of a cosmetic spraybottle) over the explants (150 µl per petridish). After addition of the substrate the plant material was placed under the luminometer to measure the excitation of light.

Handling the luminometer

For visualization and measurements of the luciferase activity in the plant material a luminometer was used. The luminometer consisted of a very sensitive camera (Nikon) with an intensifier, a dark room, a computer with software, two system units of ARGUS-50 and a monitor with build-in printer (Argus 100/v/M1 system, Hamamatsu Phototonic System, Japan). First, a black and white photograph was made of the plant material. The petridish had to be properly placed and focused in order to obtain a sharp luminescence image. After focusing, the picture was stored in one of the computer memories. Photon counting could start after the room was made completely dark. Factors like time of photon counting, discrimination and resolution could be chosen from the Argus software menu. After the luminescence picture was build up the life picture and the luminescence picture were superimposed. In this way the exact location of positive spots could be determined.

Morphology and histology

After visual examination of the external structures of the explants, the explants of different ages were fixed in 5% glutaraldehyde in 0.1 M phosphatebuffer, dehydrated and embedded in Technovit for morphological and histological studies. Sections of 7 µm thickness were made using a microtome. The sections were stained with 1% Toluidine Blue and observed using a light microscope.

Results

Selection of leek

Kanamycin selection

At day 1 the 10 zygotic embryos per petridish were weighed and placed on callus induction medium supplemented with 0, 100, 200 and 400 mg l^{-1} kanamycin in four replications. At day 20 the first growth observation was made by determining the increase in weight, survival rates and callus formation of the explants. At day 40 the second measurement was performed. At the end of the germination period (day 75) the number of somatic embryos per petridish were counted. The results are shown in Table 3.1. In the first 20 days of callus induction, the kanamycin levels in the medium do not influence callus growth. Both cultivars had even higher growth rates at 100 mg l^{-1} kanamycin than the control. For kanamycin callus formation was normal at all treatments compared to the control. In the second time period, from day 20 to 40, kanamycin did have an influence on the growth rate. The growth rates were reduced at a kanamycin concentration of 200 mg l^{-1}. Cultivar Strata had a more vigorous growth than cultivar Porino, even with the antibiotic added to the medium. Here, the overall growth rates were already influenced by the kanamycin at 100 mg l^{-1}. Complete growth inhibition was not found at these concentrations. Complete regeneration inhibition was found at a kanamycin concentration of 200 mg l^{-1}. Although cultivar Strata had a higher growth rate than cultivar Porino, no differences in regeneration were observed.

The color of the calli on kanamycin was white compared to the more yellowish color of the calli on medium without kanamycin. No necroses or browning of dying cells was observed.

Hygromycin selection

In the first 20 days hygromycin caused a decrease of growth of at least 17% with a maximum of 53%. Callus formation was affected by hygromycin, especially in the cultivar Porino. In the second time period differences became more clear. Porino seemed more sensitive to hygromycin than Strata. At the highest concentration of hygromycin growth was reduced to 1.9 and 2.8 mg/embryo in the second period of 20 days for the 2 cultivars. This was a reduction of resp. 65% and 75% compared to the control. None of the used concentrations could inhibit growth completely. Regeneration was also affected by hygromycin. Although at all concentrations regeneration occurred, 80 mg l^{-1} hygromycin seemed the best concentration to inhibit regeneration.

Table 3.1. Average growth-rate per explant, callus formation for two time periods and regeneration of 2 cultivars of leek on different concentrations of kanamycin ([km]), hygromycin ([hygro]) and phosphinothricin ([ppt]).

Selective agent Concentration mg l^{-1}	Growth rate Day 1-Day 20		Growth rate Day 20-Day 40		Growth rate Overall	Number of somatic embryos		
	Porino mg/embryo (% callus)	Strata mg/embryo (% callus)	Porino mg/embryo	Strata mg/embryo	Porino	Strata	Porino	Strata
[km]								
0	7.7 (85)	8.5 (90)	7.9	15.2	15.6	23.7	102	99
100	8.3 (88)	10.1 (83)	6.1	6.2	14.3	16.3	1	2
200	7.7 (93)	7.8 (95)	2.7	4.3	10.4	12.1	0	0
400	6.0 (88)	7.6 (93)	1.4	2.2	7.4	9.8	0	0
[hygro]								
0	5.9 (83)	7.8 (88)	5.4	11.2	11.3	18.9	67	76
20	4.9 (73)	4.8 (75)	5.6	6.7	10.4	11.5	13	19
40	4.3 (53)	4.8 (73)	2.3	3.7	6.6	8.4	5	9
80	4.3 (45)	3.7 (83)	1.9	2.8	6.2	6.6	2	0
[ppt]								
0	11.1 (90)	9.7 (90)	13.2	22.9	24.2	32.6	69	98
5	7.3 (38)	5.7 (70)	1.1	4.6	8.4	10.3	0	4
10	4.3 (55)	5.8 (55)	1.0	2.3	5.3	8.1	0	1
25	3.9 (50)	3.4 (60)	0.4	0.6	4.3	4.0	0	0

The effect of hygromycin is comparable to kanamycin with respect to growth inhibition. The differences lie in callus formation, which is partly affected by hygromycin and not by kanamycin, and regeneration which is more affected by kanamycin than by hygromycin. The color of the calli on hygromycin containing medium was more brownish and pale compared to the control calli. Necrotic or brown embryos were observed.

Phosphinothricin selection

At day 3 of the callus induction period the 10 zygotic embryos per petridish were weighed and placed on callus induction medium complemented with 0, 5, 10, and 25 mg l^{-1} phosphinothricin. At day 20 the weight measurement of the 10 zygotic embryos was taken and the number of zygotic embryos with callus formation was counted. At day 40 the second measurement was performed. After this measurement the calli were placed in the light. At

the end of the germination period (day 75) the number of somatic embryos per petridish was counted. The results of the data of selection are presented in Table 3.1. The growth reduction of phosphinothricin in the first 20 days was comparable with kanamycin and hygromycin. No differences in growth appeared between the light or dark callus induction treatment. Phosphinothricin did affect the callus formation percentages just like hygromycin. In the second period of 20 days the growth reduction by phosphinothricin was considerably larger than with the other two selective agents. The concentration of 25 mg l^{-1} caused only a small increase in weight during the second period. Regeneration was strongly reduced by phosphinothricin.

Transformation

In several independent experiments somatic embryos of the five cultivars were bombarded with a lot of different bombardment conditions like different concentrations of DNA, different stages of development of the somatic embryo, different distances and pressures to the plant material, different plasmids with different promoters etc. In total more than 30 experiments were performed using somatic embryos or callus derived from somatic embryos. The results of the particle gun bombardments were a few tiny transient luciferase spots. These spots were only detectable one week after bombardment. The only responding promoter was a 35S promoter or a doubled 35S promoter. Other promoters like the typical monocotyledon promoter ubiquitin or TR1'2' showed hardly any transient expression. The experiments were performed with at least 20 explants per treatment and with three replications. The only positive results came from one-week-old callus. In one replication of the treatment 1800 psi, three luciferase spots were visible after one month (fig. 3.2). However, no further growth of the fluorescing spots were seen and after two months the luciferase activity had vanished. Repeated experiments showed similar results. Additional treatments like changing the osmotic stage, efforts to synchronize cells, cold treatments, using linear DNA, other plasmids and many other treatments had no clear positive effect on transient or stable expression of luciferase. No transgenic or chimeric leek plants were obtained from the bombarded, non selected controls. Initially over 15000 zygotic embryos have been used, as zygotic embryo, callus or later as multiple somatic embryos in transformation experiments. It seems that the compentence of the treated cells for particle gun, even from callus cells, is low. An important question is whether the initial cells for the cyclic somatic embryogenesis are found at the surface, near the surface or inside the embryo. This information is crucial for successful transformation by the particle gun.

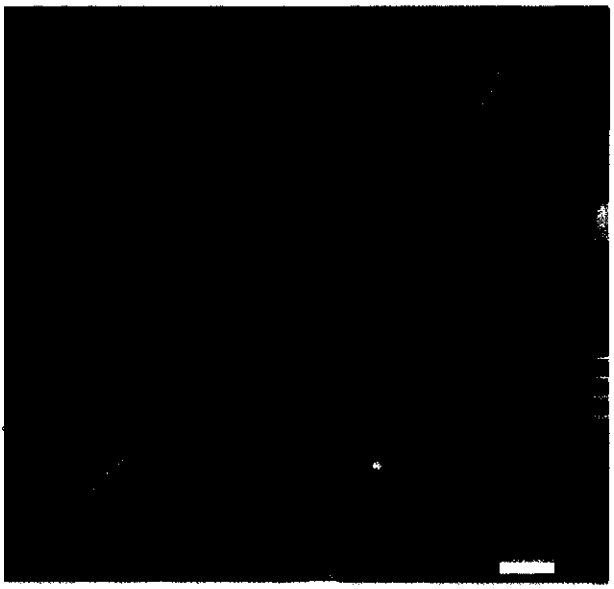

Fig. 3.2 Three luciferase spots on leek callus, one month after bombardment. White bar represent 5 mm, colored bar represent the photon excitement on one place; every color stands for one photon hit in the camera, red being a high number of photons and blue a low number of photons.

Morphology and histological observations

The histology and morphology of the somatic embryogenesis of leek during the first cycle was similar to that described by Van der Valk *et al.*, (1992), Silvertand, (1996) and Buiteveld (1998). Callus development started a few days after culture of the isolated zygotic embryo on callus induction medium. Histological observations showed that callus was produced by meristems situated just under the germination pore of the mature zygotic embryo. Cell division and callus formation started at the shoot meristem, which is situated just beneath the germination pore. Different callus types have been described ranging from friable to compact, regenerable to non-regenerable in color ranging from yellow to opaque. The most

frequently seen callus was the compact callus type. This type of callus formed meristematic zones. These zones differentiated into clusters of somatic embryos with a distinct root and shoot meristem. The green leaf shelves and the white end with the basal plate were clearly visible on the callus (Fig 3.3).

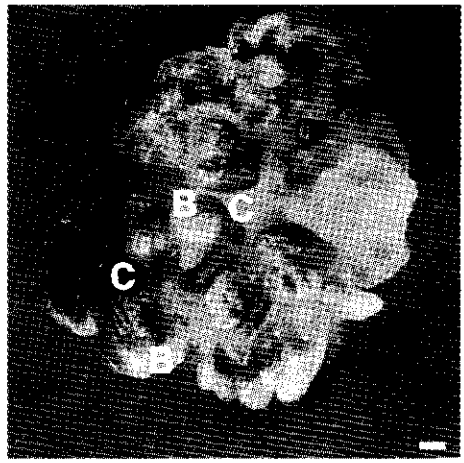

Fig 3.3.Mature somatic leek embryos growing on the callus;
C=cotyledon, B= basal plate (bar represent 300μm)

After transferring these calli to germination medium and light, clusters of somatic embryos matured. Not all somatic embryos were able to mature, they remained white. At the basal plate of some mature, green somatic embryos sometimes even a 'germination-pore like structure' was observed. Green somatic embryos were excised from the callus with a needle and placed on callus induction medium for the next cycle (Fig 3.4). Sometimes the somatic embryos were difficult to separate as their cotyledons and basal plates were attached to each other, than a knife was used for separation.

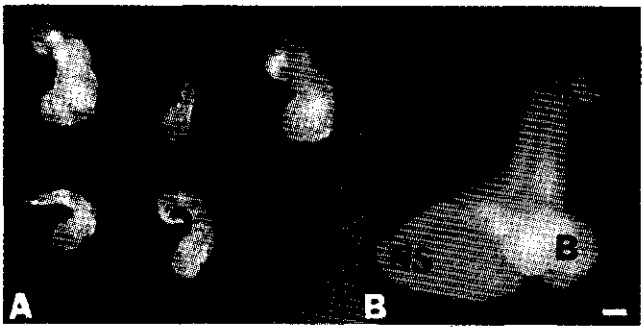

Fig 3.4. A: Separated somatic embryos of leek T=9; B: individual somatic embryo with callus T=18 days after initiation of secondary somatic embryogenesis. c=cotyledons, ns= new somatic embryos, b=basal plate (bar represent 50µm)

Separated individual somatic embryos, placed on induction medium started to form compact callus at the basal plate side of the somatic embryo (Fig 3.5). The shoot and root meristems started with a lot of cell divisions under the influence of the phythohormone 2,4D.

Fig 3.5 Histological view of a somatic embryo of leek after 9 days on induction medium; R=root meristem, S=shoot meristem

After looking in detail to a somatic embryo several meristem and vascular tissue like structures could be found. The first meristems were observed 6 to 7 cell layers under the

epidermis. On 2,4D containing medium these shoot meristems produced callus. In a somatic embryo the circular structure of the basal plate can still be seen. The cell divisions, leading to new somatic embryos, were located in the center of the embryo placing the cells to the outside. Most likely more than one cell is involved in the somatic embryogenesis process. As the callus developed it grows though the side of the somatic embryo. On this callus new somatic embryos could be formed. This callus was similar to the compact callus derived from the zygotic embryo on induction medium. The secondary and later somatic embryos developed in the same way as the primary somatic embryos. After 4 to 6 weeks clusters of secondary somatic embryos were visible leaving only small pieces of the primary somatic embryo visible (Fig. 3.6). To obtain a true transgenic somatic embryo the particles should reach the inner part of the somatic embryo. In theory the DNA coated particle could get 6 to 7 cell layers deep into the somatic embryo reaching the shoot meristem. However, it only reached the outer part of the meristem and as the initiation sites of new somatic embryos formation are located at the inner part of the somatic embryo the chance of a successful transformation or even chimeric leek tissue is very small.

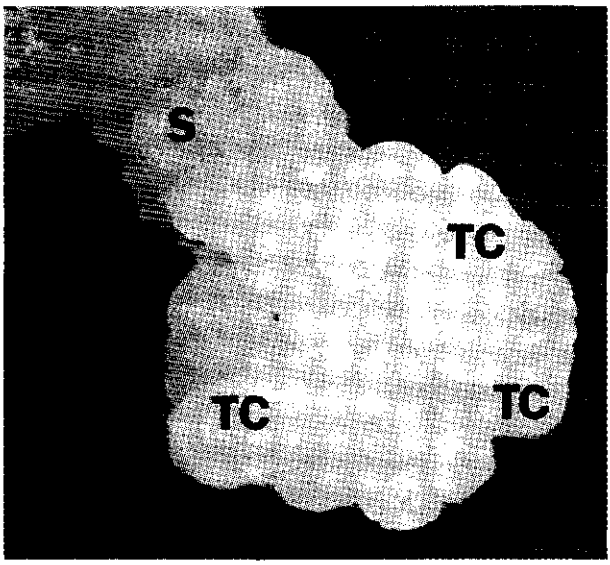

Fig 3.6. Tertiary somatic embryos growing in clusters on the callus of secondary somatic embryos; S= secondary somatic embryo, TC= tertiary clusters of somatic embryos.

Discussion

Selection

Severe selection is necessary to inhibit leek callus growth. The herbicide PPT was the best agent for growth inhibition as the growth rate decreased to 12%-20% of the control. Wang (1996) also found that callus growth of leek was more difficult to inhibit than inhibition of regeneration. He also found that 10 mg PPT/l inhibited formation of leek callus; plant regeneration and plant elongation were inhibited at 5 and 2 mg PPT/l respectively. The antibiotics kanamycin and hygromycin were less effective. Only 35%-50% callus growth inhibition was achieved. The natural tolerance to antibiotics is well known and typical for a monocot (Wilmink and Dons, 1993). For onion (*Allium* cepa L.) immature embryos and immature embryo-derived cultures kanamycin was unsuitable as a selective agent , where a light dose of hygromycin (10-30mg/l) reduced callus growth but enhanced the regeneration frequency (Eady and Lister, 1998). Phosphinotricin was an effective growth reducer for onion in absence of casein (Eady and Lister,1998). Leek seems more persistent than onion. In this report total callus growth inhibition was not observed even at 25 mg l^{-1}PPT selection. Only growth rate reduction was achieved. Leek calli showed to be very insensitive to selective agents. The best growth inhibition was achieved after 20 days of culture. Cultivar Porino was more sensitive to the antibiotics and herbicide than cultivar Strata. To prevent somatic embryos of leek to develop into a mature, green stage only a relatively low dose of any of the selective agents, 100 mg l^{-1}Kanamycin, 40 mg l^{-1}Hygromycin and 5 mg l^{-1}PPT. was sufficient. Leek plant regeneration was inhibited by 5 mg l^{-1} PPT. To prevent callus from growing is more difficult. Selection of callus should take place with at least 10 mg/l phosphinothricin.

Transformation experiments

A lot of different conditions have been applied to the leek tissue. No transgenic somatic embryo or even a chimeric structure was found after bombardment and selection. This indicates that at this moment somatic embryos are unsuitable for transformation by particle gun bombardments. Wang (1996) described a nodular, embryogenic callus originated from basal plates of *in vitro* sown seeds. The regeneration process was described as somatic embryogenesis. In Wang's transformation study this nodular callus was successfully used for transformation with selection of 10 – 20 mg/l phosphinothricin. However, since then no reports have been published, which makes it difficult to asses the usefulness of this method to obtain stable transformants.

Morphological and histological observations

There is a strong resemblance in growth between a small somatic embryo and a mature leek plant. Both induce new cells in the inner part of the basal plate and arrange the cells periclinal. The morphological and histological study made it clear that the favorite target cells for transformation are in the center of the somatic embryo and therefore are hard or impossible to reach by particle bombardment at this stage. Instead of using somatic embryos for transformation, other explant types of leek have to be found for transformation purposes.

Literature

Baumunk-Wende E (1989) Application of tissue culture in leek breeding (*Allium porrum* L.), Gartenbauwissenschaft 54: 20-24.

Belas R, Mileham A, Cohn D, Hilmen M, Simon M, Silverman M (1982) Bacterial bioluminescence: Isolation and expression of luciferase genes from *Vibrio harveyi*, Science 218: 791-793.

Bommineni VR, Chibbar N, Datla RSS, Tsang EWT (1993) Transformation of white spruce (*Picea glauca*) by microprojectile bombardment, Plant Cell Reports 13: 17-23.

Buiteveld J (1998) Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek, PhD-thesis Wageningen Agricultural University, The Netherlands.

Chia T, Chan Y, Chua N (1994) The firefly luciferase gene a non-invasive reporter for *Dendrobium* transformation, The Plant Journal 6(3): 441-446.

Christensen, AH, Sharrock RA and Quail P (1992) Maize polyubiquitin genes; structure, terminal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation, Plant Molecular Biology 18: 675-689.

Christensen AH and Quail PH (1996) Ubiquitin promoter based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants, Trans.res. 5: 213-218.

Christou. P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electrical discharge particle acceleration of exogenous DNA into immature zygotic embryos, Bio/Technology 9: 958-961.

Debergh PC, Standaert-de Metsenaere S (1976) Neoformation of bulbils in *Allium porrum* L., Sci. Hort 5: 11-12

De Wet JR, Wood KV, Helinski DR, DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferas in *Escherichia coli,* Proc. Natl. Acad. Sci. USA. 82:7870-7873.

Doré C (1988) Multiplication végétative et conservation *in vitro* chez le poireau (*Allium porrum* L.), Agronomie 8: 509-511.

Dunstan DI, Short KC (1979) Shoot production from cultured *Allium porrum* tissues,

Sci Hort 11: 37-43.
Eady CC, Lister CE (1998) A comparison of four selective agents for the use with *Allium cepa* L. immature embryos and immature embryo-derived cultures, Plant Cell Reports 18:117-121.
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojectile bombardment, Plant Cell ReportS 9: 189-194.
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992) Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus, Biotechnology 10:1466-1472.
Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants, Biotechnology 8: 833-839.
Gorden-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG. (1990) Transformation of maize cells and regeneration of fertile transgenic plants, The Plant Cell 2:603-618.
Grasser KD, Hetz W, Griess EA, Feix G (1993) Stimulatory effect of the maize HMGa protein on reporter gene expression in maize protoplasts, Federation of European Biochemical Societies 327 (2):141-144.
Guerineau F and Mullineaux PM (1993) Plant transformation and expression vectors, In: Croy, RRD (Eds), Plant Molecular Biology, labfax, BIOS Scientific publishers, Oxford, UK. pp. 121-148.
Gupta PK, Dandekar AM, Durzan DJ (1988) Somatic pro-embryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts, Plant Science 58: 85-92.
Hagio T, Blowers AD, Earle ED (1991) Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles, Plant Cell Reports 10: 260-264.
Hartman CL, Lee L, Day PR, Turner NE (1994) herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation, Biotechnology 12: 919-923.
Hébert D, Kikkert JR, Smith FD, Reisch B (1993) Optimization of biolistic transformation of embryogenic grape cell suspensions, Plant Cell Reports 12 :585-589.
Hensgens LAM, Bakker EPHM de, Os-Ruygrok EP van, Rueb S, Mark F van der, Maas HM van der, Veen S,van der, Kooman-Gersmann M, 't Hart L, Schilperoort RA (1993) Transient and stable expression of GUS-A fusions with rice genes in rice, barley and perennial ryegrass, Plant Molecular Biology 22: 1101-1127.
Howell SH, Ow DW, Schneider M (1989) Use of firefly luciferase gene as a reporter of gene expression in plants. Plant Molecular Biology Manual B8:1-11.
Manzara T, Tarchevskaya S, Narita JO (1994) Optimization of luciferase activity in a tomato transient assay system, Plant Molecular Biology Reporter 12 (3): 221-226.
Martinussen I, Junttila O, Twell D (1994) Optimization of transient gene expression in pollen of Norway spruce (*Picea abies*) by particle acceleration, Physiologia Plantarum 92: 412-416.
Mayerhofer R, Langridge WHR, Cormier MJ, Szalay AA (1995) Expression of recombinant

Renilla luciferase in transgenic plants results in high levels of light emission, The Plant Journal 7(6): 1031-1038.

McElroy WD., Seliger HH., White EH(1969) Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin, Photochemistry Photobiology 10: 153-170.

Millar AJ, Short SR, Hiratsuka K, Chua N, Kay SA (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants, Plant Molecular Biology Reporter 10 (4): 324-337.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assay with tobacco tissue cultures, Physiol. Plant 15: 473-497.

Novák FJ, Havel L (1981). Shoot production from *in vitro* cultured flower heads of *Allium porrum* L., Biol Plantarum 23(4):266-269.

Ow DW, Wood KV, DeLuca M, De Wet JR, Helinsky DR, Howell SH (1986) Transient and stable expression of the fire fly luciferase gene in plant cells and transgenic plants, Science 234: 856-859.

Pierik RLM (1985) Plantenteelt in kweekbuizen, 2e druk Ponsen en Looijen, Wageningen The Netherlands, 202p.

Potrykus I (1988) Direct gene transfer to plants, Application of Plant Cell and Tissue Culture. Chistester, Sussex, UK, Wiley p.144-162.

Raemakers. C.J.J.M., Sofiari E, Taylor N, Henshaw G, Jacobsen E , Visser RGF, Molecular Breeding (1996) Production of transgenic cassava (Manihot esculenta Crantz) plants by particle bombardment using luciferase activity as selection marker, Molecular breeding 2: 339-349.

Rauber M, Grunewald J (1988) *In vitro* regeneration in *Allium* species, Plant Cell Reports 7: 426-429.

Ritala A, Aspegren K, Kurtén U, Salmenkallio-Marttila M, Mannonen L, Hannus R, Kauppinen V, Teeri TH, Enari TM (1994) Fertile transgenic barley by particle bombardment of immature embryos, Plant Molecular Biology 24: 317-325.

Sherf BA, Wood KV (1993) Luciferase; Firefly luciferase engineered for improved genetic reporting, Promega Notes 49: 14-21.

Silvertand B (1996) Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L), Ph.D. thesis Agricultural University Wageningen, The Netherlands.

Schneider M, Ow DW, Howell SH (1990) The *in vivo* pattern of firefly luciferase expression in transgenic plants, Plant Molecular Biology 14: 935-947.

Street HE (1973) Plant tissue and cell culture, Botanical monographs v11. Berkley, University of California press 503p.

Takebe I, Labib G, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco, Naturwissenschaften 58(6): 318-320.

Thompson EM, Nagata S, Tsuji FI (1990) *Vargula hilgendorfii* luciferase; a secret reporter gene for monitoring gene expression in mammalian cells, Gene 96: 257-262.

Van der Valk P, Scholten OE, Verstappen RC, Dons JJ (1992) High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species, Plant Cell Tissue Org Cult 30: 181-191.

Van Doorne LE, Stolte AJ, van Harten AM (1988) Application of *in vitro* techniques for

multiplying and breeding leek (*Allium porrum*), 4th EUCARPIA *Allium* symposium 1988.

Wang. H., (1996) Genetic engineering male sterility in leek (*Allium porrum* L.), Ph.D. thesis Applied Biological Sciences, University of Gent, Gent, Belgium.

Wilmink A, Dons JJM (1993) Selective agents and marker genes for use in transformation of monocotyledonous plants, Plant Molecular Biology Reporter 11: 165-185.

Zhong H, Bolyard MG, Srinivasan C, Sticklen MB (1993) Transgenic plants of turfgrass (*Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus, Plant Cell Reports 13: 1-6.

Chapter 4

Factors affecting transformation in leek (*Allium ampeloprasum* L.) monitored by transient luciferase expression of flower stalk explants.

Abstract

Transient expression of luciferase has been studied in flower stalk explants of *Allium ampeloprasum* L. following particle gun bombardment with the Dupont PDS-1000/He gun. Several factors known to be important in particle gun bombardment have been investigated in order to understand and improve the process. Expression of the luciferase gene could be easily quantified by a luminometer, enabling the investigation of the different factors. Distance between macrocarrier and plant material, using flower stalk strips instead of flower stalk discs, the origin and pre-culture period of the explants were investigated qualitatively and quantitatively by *in vivo* luminometer assays. The coating of the DNA onto the particles proved to be extremely important. Gold particles bind the DNA better than tungsten particles. Addition of more calcium chloride caused the binding of more DNA to the particles. Without loss of transient expression the amount of DNA could be reduced to 50%, but an increase in DNA gave higher transient expression levels. From all these experiments the optimal conditions for transient expression could be established in leek. Flower stalk strips of leek as explants, cut from the basal part of the flower stalk, pre-cultured for 2 days, are bombarded at a distance of 5 cm, with a pressure of 1800 psi. Gold particles must be used and coated following the procedure of Christou *et al.*, (1991) adding more DNA. After bombardment the explants should be transferred to selection medium.

Introduction

The production and breeding of leek (*Allium ampeloprasum* L.) is increasing in Europe due to the greater access to the East European market, where leek is a commonly eaten vegetable. Leek is a monocot, autotetraploid (2n=4x=32) and mainly outbreeding. In most leek breeding programmes positive mass selection is used. Therefore the cultivars are heterogeneous. Research efforts in leek are directed towards creating an efficient method to obtain male sterile leek plants that are resistant to diseases like white tip (*Acrolepiopsis assectella)* and purple blotch (*Alternaria porri*). Next to the normal selection methods, the application of transformation and mutation techniques are desirable. Success of a transformation method depends on the availability of an efficient and reliable regeneration system. Several regeneration systems for leek have been described in literature. *In vitro* regeneration has been reported by organogenesis as well as by somatic embryogenesis from different explant sources (for a review see Novák *et al.*, 1986). Stem explants, basal plates, leaf discs, leaf base, flower stalks, flower heads, open flowers, single flower bud receptacles, anthers and

zygotic embryos have been used as starting plant material for regeneration (Debergh and Standaert-de Metsenaere 1976; Dunstan and Short 1979; Doré and Sweisguth 1980; Wang, 1996; Novák and Havel 1981; van Doorne *et al.,* 1988; Rauber and Grunewald 1988; Doré 1988; Baumunk-Wende 1989; Buiteveld *et al*, 1993; Schavemaker & Jacobsen, 1995; Silvertand, 1996). The flower stalks seemed to have several favorable characteristics for transformation. Flower stalks have a good regeneration ability, regeneration starts subepidermal and ,probably, regeneration is a single cell event. Competence of these cells for transient expression and transformation is important but not known. The aim of this study was to identify the optimal conditions for biolistic transformation of flower stalks. Regeneration of flower stalks occurs just beneath the epidermis, which can be easily reached by particles. The regeneration protocol described by Silvertand (1996) was adjusted for particle gun experiments. Silvertand (1996) found that from the basal parts of the flower stalk more plantlets regenerated than from the apical part of the flower stalk. The firefly (*Photinus pyralis*) luciferase gene was used as a marker gene in the experiments for determining and optimizing conditions important for transformation of leek. The firefly luciferase gene is a reporter gene, which can be used to monitor gene expression patterns without destroying the tissue investigated. The first use of the firefly luciferase gene in a plant system was reported by Ow *et al.*(1986). The light emitted can be detected by methods varying from simple x-ray film (Ow *et al.*, 1986) to an advanced video equipment (Millar *et al.*, 1992). The first reports of advanced video equipment only showed qualitative data and used protein extracts to obtain quantitative data (Millar *et al.*, 1992, Chia *et al.*, 1994, Mayhofer *et al.*, 1995). In this report a quantitative analysis has been successfully made, using a luminometer, showing clear differences in transient expression levels after different treatments. In transformation studies little attention is given to the efficiency of the coating of the DNA onto the particles. In the review of Gray and Finer (1993), several coating procedures for five different particle gun devices are described. Klein *et al.*, (1988), Oard (1991), Sanford *et al.*, (1993), Ratnayaka and Oard(1995) are some of the few authors who investigated different ways of coating and factors involved. In the BioRad manual provided with the particle gun (PDS-1000-He), the coating is briefly described. No explanations about critical steps in the procedure are given. Here, two different coating procedures were compared and two factors, calcium chloride and DNA concentration, were investigated. Although the conversion of transient expression to stable introgression is often reported to be very low, most transgenic plants obtained by particle gun bombardment are made by using conditions that are optimal for transient expression. High transient expression levels are therefore thought to be necessary to determine the optimal conditions for inducing stable introgression and as a first step in obtaining stable transformed cells and plants.

Materials and methods

Plant material

Individual genotypes of four cultivars Porino, Poriade (Nunhems seeds, Haelen) and Gavia, Castelstar (Enza Zaden, Enkhuizen) were artificially vernalized for 10 weeks in climate room at 6°C, 16 hrs light, and transferred to the greenhouse to allow flowering. Young flower stalks of 10-20 cm, still inside the plant, were harvested and used for the different experiments. The flower stalks were surface sterilized with a 1.5% sodiumhypochloride solution for 30 min. and rinsed three times with sterile water. The flower stalks were cut in discs, like described by Silvertand *et al* (1996) or stripped. The strips were subsequently cut into 10mm by 5mm explants. These explants were cultured on a MS-medium (Murasige and Skoog, 1962) supplemented with 3% sucrose, 1 mg l^{-1} NAA, 1 mg l^{-1} BAP and solidified with 8 g l^{-1} micro-agar (Daishin, Brunschwig), sterilized by autoclaving (121°C, 1.2 Bar, 20 min). Filter-sterile cefotaxime, was added after autoclaving at a final concentration of 200 mg l^{-1}. Regeneration was performed essentially as described by Silvertand (1996).

Plasmid

The vector used in the bombardment experiments was the plasmid pJIT100. pJIT100 contained the firefly luciferase and the phosphinotricin acetyl transferase gene both under control of the CaMV 35S promoter and the CaMV polyadenylation region (Guerineau and Mullineaux, 1993). Plasmid DNA was isolated using the Wizard™ Maxipreps DNA purification system of Promega.

Bombardments

Gold particles, median size 1.0 µm, coated with DNA were prepared essentially according to the protocol described by Christou *et al.*, (1991) and applied to the Biolistic® PDS-1000/He particle gun. The pressure of bombardments was 450 or 1800 psi (pounds per square inch), vacuum was kept at 27 inch Hg. For the coating experiment the particle gun device was installed at the shortest distance possible (5 cm) at 650 psi and 27 Hg vacuum.

Luciferase assay

The quantitative luciferase assay was performed 2 days after bombardment. Filter-sterile beetle luciferine substrate (0.15 mg/ml) was sprayed (fine mist by pump action of a cosmetic spray bottle) over the bombarded explants (± 150 µl per petridish). The freshly sprayed explants were placed under the luminometer (Hamamatsu), in a darkroom. Excited photons

were counted for 2 min. at 9.8 intensity. This time interval was enough to make a clearly visible difference with the negative control. Non-bombarded explants also excited a very low basal level of photons. Therefore, all data were corrected for this (negative) control. The data were analyzed by the ARGUS software program.

Coating experiment

For the binding experiment the BioRad coating method was used; from a stock of particles 3 mg of particles were placed in 50 µl sterile water, 5 µl DNA [1 µg/µl] was added and mixed well. Then 50 µl of 2.5 M $CaCl_2$ was added and mixed, 20 µl of 0.1M free-base spermidine was added and this mixture was vortexed. After vortexing the mixure was left on ice for 5 min. The supernatant was removed. The particle mixture was rinsed with 250 µl 100% ethanol and pulse centrifuged. This mixture was resuspended in 60µl of 100% ethanol. The particle-DNA mixture had to be used within 4 hours after the preparation was made. For one bombardment 7 µl of the mixture was used. In this experiment the amount of 2.5M $CaCl_2$ was varied from 0 to 60 µl and also an 5M NaCl stock was tested, replacing the $CaCl_2$. After removal, the supernatant was kept separate and the amount of DNA in 10µl sample of the supernatant was determined by spectrophotometry in duplo. The ethanol of the particle-DNA mixture was allowed to evaporate and 10 µl of the particle-DNA mixture was placed on an electrophoresis-gel (1%), subjecting the DNA to an electric field. Tungsten (M17) and gold particles were coated in two ways; according to BioRad (BioRad manual) (described above) and according to Christou *et al.* (1991). The particle coating of Christou constited of 10 mg of gold particles with a solution of the DNA (20µg) in 100µl of buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) and votexted gently for 5-10 seconds. Spermidine (100µl of 0.1 M solution) and 100 µl of a 25% PEG solution (MW 1300-1600) were added whith vortexing, followed by dropwise addition of 100µl $CaCl_2$ (2.5M). The mixture was allowed to stand at roomtemperature for 10 minutes and then spun in a microfuge. The supernatant was removed and the precipitated gold with DNA complex was resuspend in 10 ml of 100% ethanol. Per bombardment 163 µl was used on a macrocarrier. Calculations of DNA/bombardment showed that the coating of Christou *et al.* (1991) resulted in two times less DNA. One bombardment according to BioRad contained 0.625 µg DNA and one bombardment according to Christou contained 0.326 µg DNA. For comparison the amount of DNA was doubled for both coatings. Six weeks old leek calli were used to perform bombardments with construct pJIT100. After two days the leek calli were analyzed by photon counting.

Results

Regeneration of plantlets from flower stalk explants

Silvertand (1996) established a simple, efficient and rapid method for mass clonal propagation for leek, using small (5mm) flower stalk (peduncle) explants. An average of 300 plants per flower stalk was obtained. First, the leek plants had to be vernalized artifically or naturally to induce bolting. Flower stalks of 10 to 20 cm were isolated from the leek plants. After sterilization the flower stalk is divided into discs of 5 mm and transferred to the regeneration medium. Histological observations made it clear that regeneration started in the sub-epidermal cells of the flower stalk (Silvertand, 1996). Most of the regeneration occurred on the part of the explant that was in contact with the medium. For bombardment this type of explants was not suitable. Only a low percentage of the sub-epidermal cells will be the target. Instead of using discs explants regeneration can also occur from strips of the flower stalk (Fig 4.1.).

Fig 4.1. Regeneration of plantlets from a flower stalk strip of leek

The flower stalk was stripped for only a few cell layers in the longitudial direction and placed with the epidermis up to prevent drying out of the explant. For bombardment only one cell layer, the epidermis, had to be passed by the microprojectiles to reach the target tissue, sub-epidermal cells. The regenerants were cut from the explant together with a part of the explant because the basal plate of the leek plantlet was hidden in the explant.

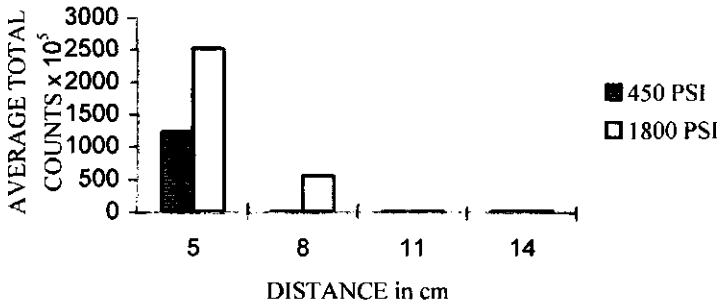

Fig.4.2. Effect of shot distance and pressure on transient expression of luciferase on leek flower stalk strips of all varieties used

From the sides where plantlets were cut from the flowerstalk explant new plantlets arose until no explant tissue was left. The regeneration of plantlets from flower stalk strips was determined. The flower stalk strips used as control in several bombardment experiments, with different genotypes, had an average regeneration percentage of 80%, meaning that the bombardments had no effect on the regeneration. The average number of plants regenerating from flower stalk strips with and without bombardment were resp. 4.2 and 5.0 plants per explant.

Effect of shot distance on transient luciferase expression

The PDS-1000/He gun has the possibility to vary the distance between plant material and the stopperplate from 5 to 14 cm (with steps of 3 cm). In Fig 4.2 the influence of the distance of the stopperplate to the plant material on the transient luciferase expression in the flower stalk strips is shown. There was an exponential increase in photons as the distance became shorter. The shortest distance technically possible (5 cm) was the most optimal position for transient expression for all the genotypes tested. The highest number of total counts in these experiments was 4.5. 10^6 compared to the negative control of 9.10^3 total counts. The other 3 distances were much less effective. The pressure also had a considerable influence. The highest pressure 1800 PSI gave a much higher luciferase activity than 450 PSI. At 450 PSI no gene expression was found at the distance of 8 cm and further, while at 1800 PSI still luciferase activity was found at a distance of 14 cm although much lower than at shorter distances.

Effect of origin of flower stalk strip and pressure on transient luciferase activity

Silvertand (1996) found differences in regeneration between basal and apical disc explants from the flower stalk. The basal discs gave more plantlets than the apical ones. This appeared also true for flower stalk strips as explant. For optimization for transient expression a flower stalk was cut in half, one apical part and one basal part. In Fig 4.3 the results are shown of a representative experiment in which two pressures, 450 and 1800 PSI were used. Lower pressures were not available and higher pressure rupture discs were not capable of bursting at the stated pressure (2000 and 2200 PSI). The basal explants had a much higher number of photons, up to 11 times than the apical explants. The transient luciferase activity was highest at 1800 PSI pressure for both the apical and basal explants.

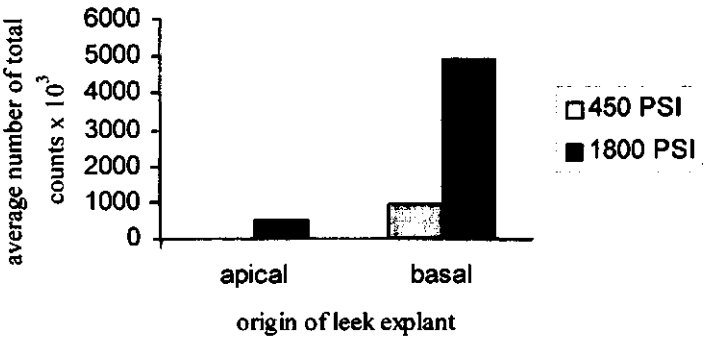

Fig 4.3. Differences in transient luciferase expression caused by the origin of the leek strip explant and the pressure used, during particle bombardment

Effect of pre-culturing of strip explants on transient luciferase expression

The regeneration of plantlets from the flower stalk strip explant is a relatively rapid process. The first shoots could be seen after 4 weeks of culture. The optimal physiological stages for transient expression were determined by pre-culturing the strip explants. Explants were cultured for 29, 24, 21, 16, 3, 2 and 1 day on regeneration medium prior to bombardment. The pressure used was 1800 PSI and only explants of the basal side of the flower stalk, with highest transient expression were used. The results are shown in Figure 4.4. It is clear that a pre-culture period of the flower stalk explant of 2 days is optimal for a high transient expression. If the flower stalk strip explants were older then 3 days, transient luciferase expression dropped dramatically.

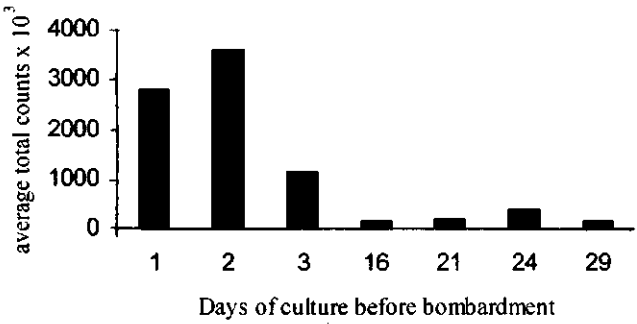

Fig.4.4. Influence of the pre-culture period of leek strip explants on transient luciferase expression after particle bombardment

DNA Binding experiment

For particle gun bombardments two kinds of particles are available, tungsten and gold. To compare the coating for both kinds of particles, coated particles, according to the BioRad manual, were subjected to electrophoresis to determine the strenght of the coating. The results of the electrophoresis, shown in Fig. 4.5., of the particle-DNA mixture showed clearly that the binding of DNA to gold particles is stronger than that of the DNA to the tungsten particles. The DNA of the tungsten particles had made a smear in the gel (T), while the DNA of the gold particles was still located in the slots (A). No DNA is visible when $CaCl_2$ was replaced by NaCl (N). If more $CaCl_2$ or NaCl was used the amount of DNA bound to the particles increased. This was confirmed by the analysis of the supernatant by spectophotometer. If no DNA was precipitated onto the particles, no $CaCl_2$, no spermidine, only DNA and particles, the amount of DNA in the supernatant was 5µg. In the controls (0 µl salts added) already a lot of DNA was bound to the particles(90%). In the supernatant of the particles coated with NaCl more DNA was seen then when $CaCl_2$ was used. Only 120 or 160 µl 5M NaCl gave lower values of DNA in the supernatant. A strange phenomenon was the reaction of 10 µl $CaCl_2$ and tungsten particles, no DNA was found in the supernatant. In the supernatant of the smaller tungsten particles more DNA was found than at the bigger particles (data not shown).

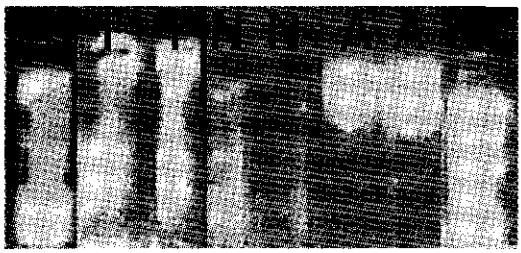

Fig.4.5. Gel-electroforese of DNA-particle mixture with different kinds of particles;
M = marker
T = tungsten and $CaCl_2$
N = tungsten and NaCl
A = Gold and $CaCl_2$

Comparison of two coatings

The standard BioRad coating contained, in theory, 0.667 µg of DNA per bombardment. The standard coating of Christou *et al.*, (1991) contained 0.326 µg per bombardment. While two times more DNA is used with the BioRad coating (Bio5 (BioRad coating with 5µg DNA) compared to CH20 (Christou coating with 20µg DNA); Fig 4.6) only the same expression levels were obtained on the 2 days-old flower strips cut from the basal side of the flower stalk.

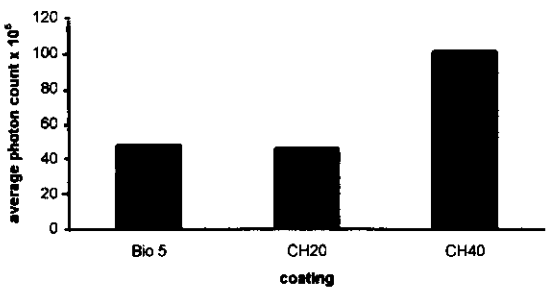

Figure 4.6. Comparison of transient luciferase expression of 2-days old leek basal flower strips bombarded with three different coatings; Bio5 is the BioRad coating with 5 µg DNA, CH20 is the Christou *et al.*, (1991) coating with 20 µg DNA and CH40 is the Christou *et al.*, (1991) coating with 40µg DNA.

The coating of Christou *et al.*, (1991) had more transient expression when the same amount of DNA per bombardment was used as at the standard BioRad coating (Bio5 compared to

CH40(Christou coating with 40µg DNA)). More DNA used in the BioRad coating did not lead to higher levels of expression (data not shown). In Fig. 4.6 the results are shown of the luciferase assay two days after bombardment.

Discussion

Regeneration of plantlets from flower stalks

A comparison was made between the regeneration procedure according to the method of Silvertand (1996) with dissc explants and the adjusted protocol with strip explants used for the bombardment experiments (described in Material and Methods). Both explant types had an average regeneration percentage of 80%. The average number of plants regenerating from flower discs was 18 plants per explant (Silvertand, 1996). The average number of plants regenerating from flower stalk strips was 4.6 plants per explant. The differences in average number of shoots between strips and discs seemed to be large. However, one must consider that one disc of 5 mm is the basis of four strip explants. Compared to the flower stalk disc method of Silvertand (1996) the regeneration efficiency of flower stalk strips is of a similar order of magnitude. The use of strips instead of discs of the flower stalks gives more potential for transformation because of the improved exposure of the regeneration-competent cells to the incoming particles. The plant regeneration on these strips occured mostly at the side of the explant that was in contact with the medium at the edges. Regeneration from the middle of the explant was seldomly observed. Therefore, the explant type does not have an effect on the regeneration. When the epidermis was removed or physically damaged (by carbonpowder or needle) no regeneration was observed at all. The bombardments itself and the vacuum during bombardment had no negative effect on the regeneration. Although they can result in quite some damage to the explant.

Physical factors

Effect of distance between plant material and macrocarrier on transient expression.

Comparison of some factors like distance, pressure and preculture time for a number of different plant species and explant types showed that no general conclusions can be drawn for plant cell transformation. Each plant species or explant type had other optimal conditions for transient expression. Also the particle gun device used could influence the optimal distance. The optimal distance in several particle gun systems ranged between 5 and 12 cm (Iida *et al.*, 1990, Taylor and Vasil, 1991, Hébert *et al.*, 1993 and Ritala et. al., 1994). Differences can also occur by different settings of the stopperplate and macro-carrier holders. Not all particle gun devices can alter distances, although together with the factors pressure and vacuum,

distance is an important factor for obtaining transgenic plants. These three factors influence the momentum of the particles entering the plant cell and determine which cell layer the particles will affect (Klein *et al.*, 1992). In this report transient expression was greatly enhanced by a short distance between macrocarrier and the plant material, the shortest distance (5cm) used was the best.

Coating

The coating process of binding DNA onto the particles is not yet fully understood and often neglected. Spermidine is an important factor in the DNA-binding process. With no salts added over 90% of the DNA was coated. The results showed that more DNA was bound by increasing amounts of $CaCl_2$. $CaCl_2$ cannot be replaced by twice the amount of the single positive NaCl. NaCl binds the DNA only at very large amounts and it probably salts the DNA onto the particles. According to these experiments it is believed that negative DNA binds to the spermidine and the positive $CaCl_2$ and formed a complex spermidine-DNA-+CaCl+-DNA-spermidine etc. These results were confirmed by the analysis of the supernatant of the particle-DNA mixture. A bigger tungsten particle binds more DNA, as the surface becomes larger. Gold particles bind the DNA stronger than tungsten particles suggesting a binding process between the particles and the DNA- $CaCl_2$ mixture. Russell *et al.*, (1992) found that tungsten was toxic to plant material. It inhibited the cell growth of a tobacco cell suspension when tungsten particles were mixed in the medium. Gold particles did not inhibit cell growth. How the DNA is released and how it integrates into the host genome is still not understood. Also the fact that gold particles bind the DNA stronger, does not influence the transient expression. Often other chemicals, like EDTA, are used to preserve the DNA against deterioration. In recent research Uzé *et al.* (1999) stated that also the form of the DNA is important for integration. Linear DNA gave the highest frequency of transformation both single and double stranded.

The coating of Christou *et al.*, (1991) was used with the electrical discharge gun, which has other macrocarriers than the Dupont Biolistic gun. With some little adjustments, this coating was better than the standard BioRad coating which is included in the users manual. A bigger volume (10 ml vs 60µl) makes the particle distribution more even as the BioRad coating often forms clusters of particles. The clusters of particles often lead to cell death (Russell and Oard, 1995) Half the amount of DNA of the Christou *et al.*, (1991) coating almost evens out the transient expression on leek calli of the BioRad coating. More DNA onto the particles enhances transient expression in leek calli. This was confirmed in rice and white spruce by the research of Christou *et al.*, (1991), Ying-Hong *et al.* (1994) and Ratnayaka and Oard

(1995). Increased transient expression of GUS spots of bombarded rice cells was obtained if more DNA was added to the particle mixture. They proved that also more DNA was coated onto the particles by a fast fluorescence assay. From their research it was concluded that there was a maximum to the DNA amount. More DNA lead to toxic DNA levels for the plant cell.

Plant factors

Effect of the origin of the explant and pressure on transient luciferase activity

In Silvertand (1996) differences in regeneration occurred which were related to the part of the flowerstalk from which the explants were derived. The basal explants, cut from the basal part of the flower stalk, gave more regenerants per explant than the apical ones. The basal parts of the flower stalk also had a higher transient expression. These characters together give good prospects for further research. Many reports do not give information of the pressure used. Some particle gun devices do not have a possibility of altering the pressure. Pressure is an important factor with regard to transient expression. The optimal pressure is determined by the constitution of the plant material, cell wall characteristics, cell layer(s) where regeneration takes place, particles and vacuum used. The flower stalks of leek have a relatively hard and thick epidermis as the plants were cultivated in the green house. At 1800 psi more particles will enter the (sub) epidermis than at 450 psi. In softer tissue, like suspension cultures, the most commonly used pressure is about 1200 psi. However, the role of pressure for stable integration is still under discussion (Hébert et. al., 1993).

Effect of pre-culturing of explants on transient luciferase expression

In at least three reports a clear pre-culture effect is mentioned (Goldfarb *et al.*, 1991, Seki *et al.*, 1991, Brown *et al.*, 1994). The reason for improved transient expression is found in increasing cell activity and division. Goldfarb *et al.*(1991) stated that for transformation the nuclear membrane is a great barrier for DNA uptake. During cell divisions the nuclear membrane is absent and DNA uptake should be easier. Seki *et al.*, (1991) found different optimal pre-culture durations for leaves and roots of *Arabidopsis thaliana*. Activation of the cellular metabolism could be responsible for the optimum. The decrease of transient expression after 3 or 4 days is due mostly due to the start of callus formation. Only the research of Hébert *et al.*, (1993) on suspension cultures of grape, did not show any effect of a pre-culture period of 15 or 24 days. In spite of the high level of transient expression, the 2 time points chosen seemed very long compared to the pre-culture period ranging from 1 to 7

days used in the other reports. The data for flower stalk explants of leek showed a transient expression optimum in the first few days after culturing the explants on the medium. After 2 days of culture no cell divisions can be observed histologically so an optimum due to the absence of the nuclear membrane can be eliminated (Silvertand *et al.*, 1995). Silvertand (1996) found active cell divisions after 5 days. The best theory at this moment is the activation of the cellular metabolism according to Seki (1991). This is also confirmed by the results from the experiments of the origin of the flower stalk explant. The basal explants have a higher regeneration potential and gave a higher transient expression.

Conclusions

In this report different factors have been varied to obtain the optimal conditions for particle gun bombardment in leek. The optimal conditions have to be determined for each plant species, its specific culture system and particle gun device used. Many other factors, like particle size, DNA, constructs and selection are also important for transient expression (Klein *et al.*, 1988, Kartha *et al.*, 1989, Hébert *et al.*, 1993, Nishihara *et al.*, 1993, Ritala *et al.*, 1993, Brown *et al.*, 1994). All these factors have to be investigated in order to develop the most optimal transient conditions for particle gun bombardment. However, these experiments are hampered by a large variation within and between experiments. Transient expression is a tool to improve DNA transfer to the cell. The relation between transient expression and stable integration is not yet understood. However, in many cases of stable integration the optimal settings for transient expression were used (Christou et al., 1991, Ritala *et al.*, 1993,). The results obtained from the experiments presented here, will contribute to the efforts of succesful genetic engineering of leek. The conclusion is that leek flowerstalk explants should be bombarded at 1800 psi, with gold particles, at 5 cm distance from the stopperplate, the explant should be taken from the basal part of the flowerstalk and have a preculture period of 2 days. Particles should be coated with the Christou *et al.*, (1991) method with twice as much DNA as described. Applying this procedure should lead to a successful stable transformation system for leek.

Acknowledgment
We thank to Dr. P. Mullineaux from the John Innes Institute, Norwich, UK, who kindly provided the plasmid pJIT100 for bombardment.

Literature

Baumunk-Wende E (1989) Application of tissue culture in leek breeding (*Allium porrum* L.) Gartenbauwissenschaft 54: 20-24.

Brown DCW, Tian L, Buckley DJ, Lefebre M, McGrath A, Webb J (1994) Development of a simple particle bombardment device for gene transfer into plant cells. Plant Cell, Tissue and Organ Culture 37:47-53

Buiteveld J, Van der Valk P, Jansen J, Creemers-Molenaar J, Colijn-Hooymans CM (1993) Callus induction and plant regeneration from explants of commercial cultivars of leek (*Allium ampeloprasum* var. *Porrum*L.)Plant Cell Reports 12: 431-434.

Chia T, Chan Y, Chua N (1994) The firefly luciferase gene a non-invasive reporter for *Dendrobium* transformation.The Plant Journal 6(3): 441-446

Christou. P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieteis via electrical discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9: 958-961

Debergh PC, Standaert-de Metsenaere S (1976) Neoformation of bulbils in *Allium porrum* L.. Sci. Hort 5: 11-12

Doré C, Sweisguth B (1980) application de la culture *in vitro* a l'amélioration des plantes potageres. Reunion EUCARPIA, Versailles INRA: 54-57.

Doré C (1988) Multiplication végétative et conservation *in vitro* chez le poireau (*Allium porrum* L.). Agronomie 8: 509-511.

Dunstan DI, Short KC (1979) Shoot production from cultured *Allium porrum* tissues. Sci Hort 11: 37-43.

Gray DJ, Finer JJ (Eds)(1993) Development and operation of five particle guns for introduction of DNA into plant cells. Plant Cell Tissue and Organ Culture 33: 220-257.

Goldfarb B, Strauss SH, Howe GT, Zaerr JB (1991) Transient gene expression of microprojectile-introduced DNA in Douglas-fir cotyledons, Plant Cell Reports 10: 517-521.

Guerineau F and Mullineaux PM (1993) Plant transformation and expression vectors In: Croy, RRD (Eds), Plant Molecular Biology, labfax, BIOS Scientific publishers, Oxford, UK. pp. 121-148

Hébert D, Kikkert JR, Smith FD, Reisch Bi (1993) Optimization of biolistic transformation of embryogenic grape cell suspensions. Plant Cell Reports 12 :585-589.

Iida A, Seki M, Kamada M, Yamada Y, Morikawa H (1990) Gene delivery into cultured plant cells by DNA-coated gold particles accelerated by a pneumatic particle gun. Theor. Appl. Genet 80: 817-825.

Kartha KK, Chibbar RN, George F, Leung N, Caswell K, Kendall E, Qureshi J (1989) Transient expression of chloroamphenicol acetyltransferase (CAT) gene in barley cell cultures and immature embryos through microprojectile bombardment. Plant Cell Reports 8: 429-432.

Klein TM, Fromm M, Weissinger A, Tomes D, Schaaf S, Sletten M, Sanford J (1988) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc. Natl. Acad. Sci. USA 85: 4305-4309.

Klein TM, Arentzen R, Lewis PA, Fitzpatrick-McElligot S (1992) Transformation of microbes, plants and animals by particle bombardment. Bio/Technology 10: 286-291.

Mayerhofer R, Langridge WHR, Cormier MJ, Szalay AA, (1995) Expression of recombinat renilla luciferase in transgenic plants results in high levels of light emission. The Plant Journal 7(6): 1031-1038.

Millar AJ, Short SR, Chau N, S.A. Kay, A novel circadian phenotype based on fire fly luciferase expression in transgenic plants.The Plant Cell (1992) 4: 1072-1087.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assay with tobacco tissue cultures. Physiol. Plant 15: 473-497.

Nishihara M, Ito M, Tanaka I, Kyo M, Ono K, Irifune K, Morikawa H (1993) Expression of the β-Glucuronidase gene in pollen of lily (*Lilium longiflorum*), Tobacco (*Nicotiana tabacum*), *nicotiana rustica*, and Peony (*Paenia lactiflora*) by particle bombardment, Plant Physiol. 102: 357-361.

Novák FJ, Havel L (1981). Shoot production from *in vitro* cultured flower heads of *Allium porrum* L. Biol plantarum 23(4):266-269.

Novák FJ, Havel L, Dolezel (1986) *Allium*. In: Evans DA, Sharp WR, Ammirato PV (Eds) Handbook of plant Cell Culture vol IV Macmillan New York, pp 419-456.

Oard, J.H. (1991) Physical methods for the transformation of plant cells. Biotechology Advance 9:1-11.

Ow DW, Wood KV, DeLuca M, De Wet JR, Helinsky DR, Howell SH (1986) Transient and stable expression of the fire fly luciferase gene in plant cells and transgenic plants, Science 234: 856-859.

Ratnayaka, I.J.S. and J.H. Oard (1995)A rapid method to monitor DNA precipitation onto microcarriers before particle bombardment. Plant Cell Reports 14: 794-798.

Ritala A, Mannonen L, Aspegren K, Salmenkallio-Marttila M, Kurtén U, Hannus R, Mendez Lozano J, Teeri TH, Kauppinen V (1993) Stable transformation of barley tissue culture by particle bombardment, Plant Cell Reports 12: 435-440.

Ritala A, Aspegren K, Kurtén U, Salmenkallio-Marttila M, Mannonen L, Hannus R, Kauppinen V, Teeri TH, Enari TM (1994) Fertile transgenic barley by particle bombardment of immature embryos, Plant Molecular Biology 24: 317-325.

Rauber M, Grunewald J (1988) *In vitro* regeneration in *Allium* species. Plant Cell Reports 7: 426-429.

Russell JA, Roy MK, Sanford JC (1992) Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol. 98: 1050-1056.

Sanford JC, Smith FD, Russel JA (1993) Optimizimng the biolistic process for different biological applications Meth. Enzymolology. 217:483-509.

Schavemaker CM, Jacobsen E (1995) Development of a cyclic somatic embryogenesis regeneration system for leek (*Allium ampeloprasum* L.) using zygotic embryos, Plant Cell Reports 14: 227-231.

Seki M, Komeda Y, Iida A, Yamada Y, Morikawa H (1991) transient expression of β-glucuronidase in *aribidopsis thaliana* leaves and roots and *Brassica napus* stems using a pneumatic particle gun, Plant Molecular Biology 17:259-263.

Silvertand B (1996) Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L). PhD thesis Agricultural University Wageningen, The Netherlands.

Taylor MG, Vasil IK (1991) Histology of, and physical factors affecting, transient GUS expression in pearl millet (*pennisetum glaucum* (L.) R. BR.) embryos following microprojectile bombardment, Plant Cell Reports 10: 120-125.

Uzé M, Potrykus I, Sautter C. Single-stranded DNA in the genetic transformation of wheat (*Triticum aestivum* L.); transformation frequency and integration pattern. Theor. Appl. Genet 99:487-495.

Van Doorne LE, Stolte AJ, Van Harten AM (1988) Application of *in vitro* techniques for multiplying

and breeding leek (*Allium porrum* L.), 4th EUCARPIA *Allium* Symposium 1988.

Wang. H., (1996) Genetic engineering male sterility in leek (*Allium porrum* L.), PhD thesis Applied Biological Sciences, University of Gent, Gent, Belgium.

Ying-hong L. Tremblay FM, Séguin A (1994) Transient transformation of pollen and embryogenic tissues of white spruce (*Picea glauca* (Moench.) Voss) resulting from microprojectile bombardment, Plant Cell Reports, 13:661-665.

Chapter 5

Chimeric transgenic leek (*Allium ampeloprasum* L.) and the simultaneous expression of the luciferase-and GUS-gene after particle bombardment

Abstract

Nowadays, in order to be able to obtain putative transformants the use of non-destructive gene expression assays is becoming more and more important. The latest addition is the use of the luciferase gene in combination with a luminometer, which can be used as a qualitative, but also as a quantitative assay. The optimized conditions for particle bombardment, determined from previous experiments, were applied to leek flower stalk strip explants. Efforts were made to obtain genetically transformed leek plants. Luciferase and GUS expression were compared and monitored in time after bombardment and both showed, as expected, a decrease in expression levels. The correlation between the site of a luciferase spot and a GUS spot was 100%. Bombarded explants were compared in expression of the luciferase gene by applying the substrate luciferin in two ways. Explants, which were sprayed with substrate and monitored by the luminometer weekly were compared to explants which were sprayed only once just before monitoring the luciferase expression. Leek explants, which were transferred to selective medium after bombardment, regenerated shoots. Shoots, chimeric for GUS and/or LUC activity, were found, stating that the regeneration from flower stalk explants is not a single cell event or the bombardments are too late.

Introduction

Recent progress in molecular and cell biology makes it possible to add genetically well-defined traits to the gene pools in many dicot and monocot crops. Prerequisites for genetic transformation are a reproducible and efficient protocol for plant regeneration, an efficient gene transfer system and a good selection method. Reproducible and efficient plant regeneration has been established in leek by means of somatic embryogenesis, organogenesis or suspension culture (Schavemaker and Jacobsen, 1995, Silvertand, 1996, Buiteveld, 1998). Transformation by particle gun bombardment has been described for all these regeneration systems in a number of recalcitrant crops. Stable transformation of somatic embryos of white spruce (*Picea glauca*) has been described by Bommineni *et al.* (1993). Regeneration of transgenic plants derived from suspension culture was described for rice by Cao *et al.*, (1992) and for cotton by Finer and McMullen (1990). McCabe and Martinell (1993) described the transformation of cotton meristems followed by organogenesis. Transformation by *Agrobacterium tumefaciens* is not often effective in these recalcitrant crops, although rice was transformed by an improved, very virulent, so called 'super-binary vector' *Agrobacterium* strain (Hiei *et al.*, 1994). Most of the transformation research published until

now is done with the use of reporter genes, such as GUS. Some practically and economically interesting particle gun transformations are the introduction of the coat protein of the papaya ringspot virus in papaya (Fitch *et al.*, 1992), herbicide resistant rice plants (Christou *et al.*, 1991), insect-resistant rice (Alam *et al*, 1999), virus resistance to bean yellow mosaic virus in Gladiolus plants (Kamo and Altman, 1997), antifungal proteins production in banana plants (Remy *et al*, 1998) and pod-borer resistance in chickpea (Kar *et al*, 1997). Although more and more crops can be added to the list of successful transformations, still a lot is unknown about the process of transformation. In this report flower stalk strip explants of leek were bombarded under optimized conditions described in Chapter 4. Monitoring of luciferase and GUS expression in time gave more information about the transformation process. Transformed chimeric leek plants were the result.

Materials and methods

Plant material

Two days before bombardment flower stalks of individual genotypes of cultivar Poriade (Nunhems Seeds, Haelen) were harvested from the field. The flower stalks were surface sterilized with a 1.5% sodiumhypochloride solution for 30 min. and rinsed three times with sterile water. The flower stalks were then stripped and cut into 10mm by 5mm explants. The explants of the individual genotypes were mixed and placed on a culture medium with MS-micro-, macrosalts and vitamins (Murasige and Skoog, 1962) supplemented with 3% sucrose, 1 mg l^{-1} NAA, 1 mg l^{-1} BAP and solidified with 0.8% micro-agar (Daishin, Brunschwig), sterilized by autoclaving (121°C, 1.2 Bar, 20 min). Filter-sterile cefotaxime was added after autoclaving to a final concentration of 200 mg l^{-1}. This culture method is described by Silvertand (1996) and adjusted for strip explants by Schavemaker (chapter 4, this thesis). Before bombardment, the next day, the explants were transferred to fresh medium and organized in the middle of the petridish. The total area covered with explants was equal for all petridishes, the number of explants per petridish was variable. After bombardment the explants were spread opver the entire surface of the medium in the same petridish. After 2 days the explants bombarded with plasmid DNA were transferred to selection medium (culture-medium supplemented with 10 mg l^{-1} L-phosphinothricin). Plantlets regenerating from the explants were isolated and placed on culture medium supplemented with 5 mg l^{-1} L-phosphinothricin for further growth.

Plasmid

Plasmids pJIT126 and pJIT100 were used (kindly provided by J.F. Guerineau, John Innes

Institue, UK). pJIT126 included the luciferase gene and the GUS gene both under the control of a CaMV-35S promoter and terminated by a CaMV-polyA-sequence and a neomycin phosphotransferase II-gene. pJIT100 contained the firefly luciferase and the phosphinotricin acetyl transferase gene both under control of a CaMV-35S promoter and the CaMV polyadenylation region (Guerineau and Mullineaux, 1993). Plasmid DNA was isolated using the Wizard™ Maxiprep DNA purification system of Promega according to the instructions of the supplier.

Bombardments

Gold particles, median size 1.0 µm, coated with DNA were prepared essentially according to the protocol described by Christou et al. (1991) and applied to the Biolistic® PDS-1000/He particle gun. The pressure of bombardments was 1800 psi; vacuum was kept on 27 inch Hg.

Luciferin assay

In order to start a chemical reaction where light is a reaction product, substrate has to be provided to the plant cells. In our experiments the beetle luciferin-salt (Promega) dissolved in sterile water was used at a concentration of 0.15 mg/ml. Filtersterile beetle luciferin was sprayed (fine mist by pump action of a cosmetic spraybottle) over the explants (150 µl per petridish). After addition of the substrate the plant material was placed directly under the luminometer as soon as possible to measure the excitation of light.

GUS-assay

For comparison of luciferase and GUS expression, the explants were bombarded 1 day before the weekly measurements of luciferase activity and GUS were started (in threefold) for a period of 7 weeks. The GUS-assay was performed after finishing the luciferase assay. Individual explants were isolated and put in a solution of x-Gluc in a buffer (50 mg X-gluc substrate, 0.06M Na_2HPO_4, 0.04M NAH_2PO_4, 1% Triton, 0.2% hydrogenperoxide, 10mM Na_2EDTA, 0.5M K^+ferrocyanide), The explants were left overnight at 37°C. Using Na_2CO_3 stopped the reaction. The chlorophyll was extracted from the explants by rinsing several times with 70% ethanol. The individual blue GUS spots were counted by using the binocular microscope.

Handling the luminometer

For visualization and measurements of the luciferase activity in the plant material a luminometer was used. The luminometer consisted of a very sensitive camera (Nikon) with an intensifier, a dark room, a computer with software, two system units of ARGUS-50 and a monitor with build-in printer (Argus 100/v/M1 system, Hamamatsu Phototonic System,

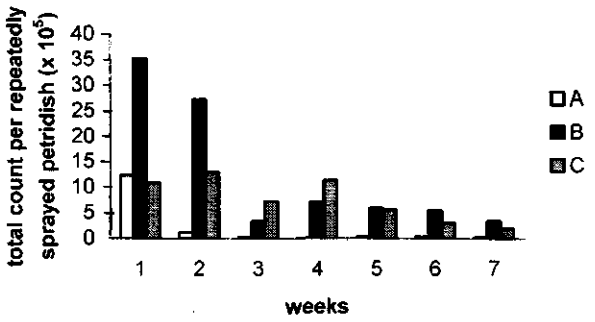

Fig 5.3 A; Petridishes A,B and C continuously sprayed with substrate during the 7 weeks monitored and luciferase expression as total counts per petridish

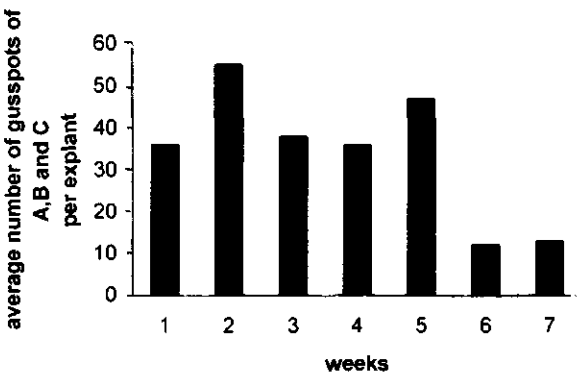

Fig 5.3 B; Average number of GUS spots of flower stalk explants of leek measured at a weekly interval

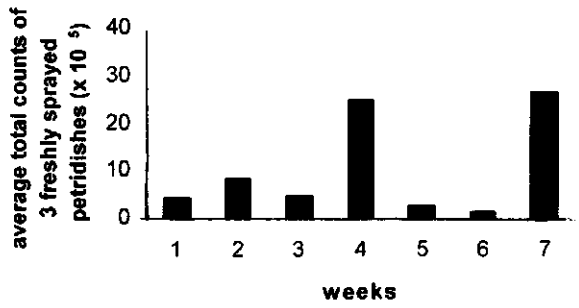

Fig 5.3 C; Luciferase activity of freshly sprayed flower stalk explants of leek with luciferin measured at a weekly interval

In the second week luciferase activity dropped, especially in petridish A (Fig. 5.3 A). From the 25 luciferase-active explants only 3 were still expressing luciferase. The freshly sprayed explants were comparable in luciferase expression to the explants, which had been sprayed two times since the start of the experiment (Fig 5.3C). The repeatedly sprayed petridishes did not give a lower luciferase expression. The explants of petridish B gave the highest luciferase activity of the 6 petridishes tested (Fig 5.3 A and C). The number of GUS spots increased due to a small number of explants totally covered with GUS spots (Fig 5.3B). In the second week the first shoots emerged from the explants. In week 3, 4, 5 and 6 gene expression dropped further to lower levels. The process described above is visualized by the weekly pictures of petridish B for luciferase (Fig 5.4). The luciferase expression decreased in the third week and remained more or less at the same level in the weeks thereafter. The freshly sprayed explants also had a lower gene expression, except in week 4 and 7, where one petridish with high luciferase expressing explants, increased the average (Fig 5.3C). The number of GUS spots remained at the same level between 38 and 47 spots per explant. In weeks 6 and 7 the number of GUS spots decreased to an average of 12 spots per explant.

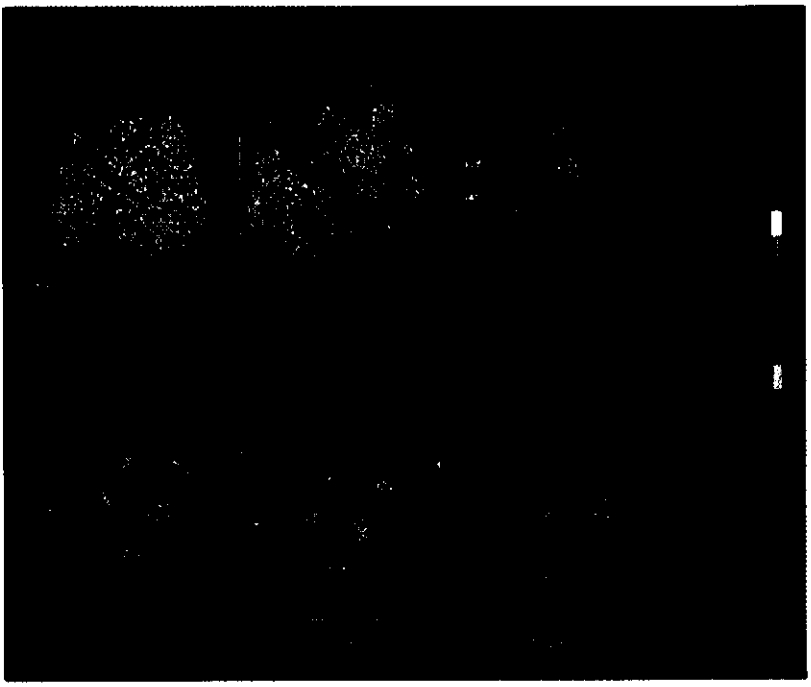

Fig. 5.4 Weekly overview of petridish B (9 cm) with 10 bombarded flower stalk explants, luciferase activity checked at weekly intervals, colored bar representing the photon excitation on one place; every color stands for one photon hit in the camera, pink being a high number of photons and blue being a low number of photons.

Most of the explants bombarded with pJ1T100 or PJIT126 for transformation experiments were expressing the luciferase gene at high levels. After the luciferase assay the explants were placed on selective medium. Luciferase activity was never found on the regenerated plants of the bombarded, non-selected controls and in the few plants of the non-bombarded, selected controls. Luciferase activity was found in some shoots regenerating from explants cultured on selective medium. From 200 explants bombarded with pJIT100, 122 plants regenerated on selection medium. Most of the regenerated plantlets were small and in bad condition. After 3 months, on selective medium, these plantlets were again analyzed for luciferase activity. Three plantlets gave poor luciferase expression at the basal part of the plantlets. From about 170 explants bombarded with pJIT126, 117 independent plantlets regenerated on selective medium. From these 117 plantlets, 13 plantlets showed some

luciferase activity. After cutting the leaves of all the plantlets it showed that all luciferase positive plantlets had also blue GUS spots, sometimes even a vertical chimeric sector was found. In Fig. 5.5 three independent plantlets with GUS expressing (blue) cell lines are shown. After the leaves were cut, the plantlets were unable to recover new leaves from the basal plate, even not when transferred to non-selective medium.

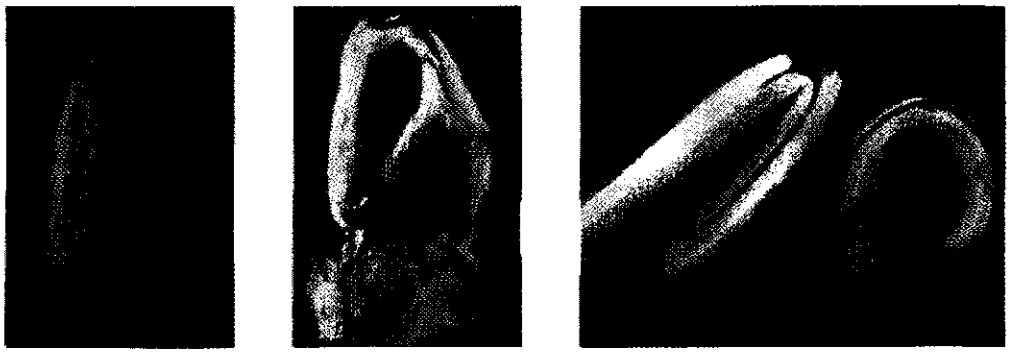

Fig 5.5 Blue GUS sectors in a leaf of three regenerated plantlets of leek on selective medium.

Luciferase positive plants or suspected GUS positive plants (growing relatively well on selective medium) recovered from several experiments were transplanted to the greenhouse and were allowed to flower. Controlled crossings were made and the seeds were harvested. After a short storage, the seeds were sterilized and allowed to germinate. After germination the plantlets were analyzed for GUS or luciferase activity. Among the 2715 seedling derived from 130 potentially chimeric plants no GUS or luciferase activity was found.

Discussion and conclusions

In the regeneration system of leek flower stalk explants it is possible to transfer genes into the leek genome, which is proven by the visible luciferase and GUS expression in the tissues of regenerated shoots. Silvertand (1996) concluded that regeneration was a single cell event, thus making this system suitable for transformation. In our experiments no complete transgenic plants were obtained. Only chimeric plants were obtained despite a selective step was used. This observation suggests a multiple cell origin of the regenerated plants via the flower stalk strips explant method or that the bombardments were done too late. The histological observations of Silvertand (1996) do not exclude multiple cell origin, probably not one but a few cells are responsible for shoot regeneration. Based on the conclusions of

Silvertand (1996) flower stalk segment were potentially good explants for transformation experiments. After all experiments performed with this kind of explant the conclusion must be drawn that until now only chimeric plants have been regenerated. In a highly comparable regeneration system of tulip a multiple cell origin of regeneration was earlier found (Wilmink *et al.*, 1995). Evaluation of transformation of tulip with particle gun bombardment did not exclude a chimeric nature of the transgenic plants. The problem now is how to recover stable transgenic plants from these chimeric sectors. All plantlets recovered had poor vigor. Efforts have to be made to keep the plantlets in good condition. It is possible by cutting the basal plate of a healthy looking plantlet to enlarge the transgenic sector or even excise the transgenic sector from the basal plate. Efforts also have to be made to find a better selection method for the transgenic cells of leek. An other possibility is to harvest the flowerstalk in a much earlier stage. The problem with this is the recognition of the leek plants which are about to flower. To obtain transgenic leek plants by seed from chimeric plants is still an option. An alternative explant source for transformation is a suspension culture. However, the procedure for obtaining such a culture is very laborious and genotype dependent, but it seems to be a suitable explant type for transformation (Buiteveld, 1998).
The gene expression in time is slowly decreasing. Luciferase activity in week 6 was only a fraction of the first 2 weeks but still present. Local increases in gene expression should indicate the growth of transgenic plant sectors. The comparison between the two reporter genes made clear that luciferase can be compared to GUS. High numbers of photons were correlated with high numbers of GUS spots, although no fixed number of counts for 1 GUS spot could be calculated. This is probably due to differences between cells for transcription rates or amount of transcript present in the cells. Kamo (1999) detected also different levels of GUS expression between leaves in one transgenic plant of Gladiolus. The number of GUS spots dramatically dropped in the 6th week of culture, although differences between petridishes were large. The difference between explants that were repeatedly sprayed and explants that were sprayed only once were negligible. In theory, the transient luciferase gene expression should decrease after addition of luciferin. Luciferase is a relatively new reporter gene in plants studies. The addition of substrate to plant material causes first a light reaction and after that reaction the luciferase molecule is no longer available for a light reaction. The chemical reaction leads to a complex of the luciferine and the luciferase (McElroy *et al.*, 1969). This complex of substrate and enzyme should eliminate the transient expression. Stable expression should be seen at the same place and with increasing activity after the transient transcript is used in the light reaction. Luciferase is believed to be relatively unstable *in vivo* with a half-life of about 3 hours in animal cells (Nguyen *et al.*, 1989; Thompson *et al.*, 1991). This report showed that transient luciferase gene expression is still present after repeated application of substrate and that the expression is essentially at the

same level as in newly sprayed explants. This has certain implications and restrictions in the use of luciferase for optimizing transformation experiments. The GUS-gene product is very stable in vivo. A better comparison could be made if GUS could also be followed in time by using a non-destructive fluorescence assay. Unfortunately the fluorescence assay for GUS is in most cases lethal to the plant tissues. Only applied in very low concentrations and handled with care sometimes plants can be rescued from the MUG-assay (Martin *et al.*, 1992). Other non-destructive assays, like GFP (green fluorescent protein), could be an alternative, but all new reporter genes have to be checked for their suitability in this system. The only proof for stable transformation events is the occurrence of blue sectors in leaves of regenerants. The flower stalk explants of leek were insensitive for selection with phosphinothricin. Non-transgenic plantlets regenerated on ppt-containing medium although the plant vigor was poor. The growth force to regenerate from the flower stalk explants is so large that selective medium did not entirely stop the regeneration process. On the other hand, most plantlets had a bad vigor *in vitro* and *in vivo* after a period on a selective medium. This report shows that although a lot of important transformation factors are optimized, showing high transient expression levels, still some prerequisites, like a good selection system, is still limiting the transformation process. The use of luciferase is a step forwards to a quick, non destructive gene expression detection, but the exact behavior of the luciferase – luciferin reaction in the plant cells has to be further analyzed. Furthermore, in order to obtain completely transgenic plants other regeneration systems will have to be combined with transformation or seeds from chimeric plants have to be sown for selection of a transgenic seedling originating from a transgenic sector.

Literature

Alam MF, Datta K, Abrogo E, Oliva N, Tu J, Virmani SS, Datta SK (1999) Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice Plant Cell Reports 18: 572-575.

Bommineni VR, Chibbar N, Datla RSS, Tsang EWT (1993) Transformation of white spruce (*Picea glauca*) by microprojectile bombardment, Plant Cell Reports 13: 17-23

Buiteveld J (1998) Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek. PhD-thesis Wageningen Agricultural University, The Netherlands.

Cao J, Duan X, McElroy D, Wu R. (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension cells. Plant Cell Reports 11: 586-591.

Christou P, Tameria L, Kofron M (1991) production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electrical discharge particle acceleration of exogenous DNA into immature zygotic embryos, Biotechnolgy 9: 957-962.

Finer JJ, McMullen MD (1990) Transformation of cotton (*Gossypium hirsitum* L.) via particle bombardment, Plant Cell Reports 8: 586-589.

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992) Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus,Biotechnolgy 10:1466-1472.

Guerineau F and Mullineaux PM (1993) Plant transformation and expression vectors In: Croy, RRD (Eds), Plant Molecular Biology, labfax, BIOS Scientific publishers, Oxford, UK. pp. 121-148

Hiei Y, Ohta S, komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA, Plant J. 6: 271-282.

Kamo K Altman A, (1997)Bean yellow mosaic virus coat protein and GUSA gene expression in transgenic Gladiolus plants, Acta Horticulturae 447: 393-399.

Kar S, Basu D, Das S, Ramkrishnan NA, Mukherjee P, Nayak P, Sen SK (1997) Expression of crylA© gene of Bacillus thuriensis in transgenic chickpea plants inhibits development of pod-borer (Heliothis armigera) larvae, Transgenic Research 6(2): 177-185.

Martin T, Schmidt R, Altmann T, Frommer WB (1992) Non-destructive assay systems for detection of β-glucuronidase activity in higher plants, Plant Molecular Biology reporter 10(1): 37-46.

McCabe DE, Martinell BJ. (1993) Transformation of elite cotton cultivars via particle bombardment of meristems. Bio/tech 11:596-598.

McElroy WD., Seliger HH., White EH(1969) Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin. Photochemistry Photobiology 10: 153-170.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assay with tobacco tissue cultures, Physiol. Plant 15: 473-497.

Nguyyen VT, Morange M, Bensaude O (1989) Protein denaturation during heat shock and related stress. Journal of Biochemical Chemistry 264: 10487-10492.

Remy s, Buyens A, Cammue BPA, Swennen R, Sagi L (1998) Production of transgenic banana plants expressing antifungal proteins Acta Horticulturae 490: 433-436.

Schavemaker CM, Jacobsen J (1995) Development of a cyclic somatic embryogenesis regeneration system for leek (*Allium ampeloprasum* L.) using zygotic embryos. Plant Cell Reports 14:227-231.

Silvertand B (1996) Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L). Ph.D. thesis Agricultural University Wageningen, The Netherlands.

Thompson JF, Hayes LS, Lloyd DB (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation Gene 103: 171-177.

Wilmink A, Van de Ven B, Custers J, Nöllen Y, Dons H (1995) Adventitious shoot formation in tulip: Histological analysis and response to selective agents, Plant Science 110: 155-164.

Chapter 6

General discussion

Breeding

Leek has some characteristics that make conventional breeding more difficult. Leek is a tetraploid crop, an outbreeder suffering from a severe inbreeding depression and has a biannual life cycle. Therefore, most of the present cultivars are bred by positive mass selection or family selection methods and thus are not very stable or of high quality. F1 hybrids can be produced in different ways. One is the use of male sterile plants. Two different forms of genetically based male sterility are known. Nuclear male sterility (NMS) is mostly controlled by one or more (recessive) genes. In most cases NMS occurs as a spontaneous mutation. The other form is cytoplasmic male sterility in which the male sterility is a result of a malfunction in the interaction between the nucleus and the organellar genes or of a malfunction of the organellar genes only. F1 hybrids are more uniform, give a higher production, have a better vigor and make it easier to make a cultivar more disease resistant. Also the registration of a cultivar will be easier by the improved uniformity. To accomplish hybrid breeding in leek a form of male sterility is needed. In nature sometimes male sterile leek plants are found (Silvertand, 1996). In most cases it turns out to be based on a recessive gene and it is very difficult to maintain for breeding purposes. In other *Alliums*, for example onion (*Allium cepa* L.), cytoplasmic male sterility (CMS) is known and used for hybrid breeding. Research efforts have been made to integrate the CMS from onion to leek (Buiteveld, 1998) or to obtain male sterility by mutation breeding (Silvertand, 1996). In this thesis efforts are made to introduce genes in the leek genome by means of transformation. This approach will not only give the possibility of making leek male sterile, but also solve other problems like disease or pest resistance. It will also allow the introducion of other interesting traits. For the development of a transformation method it is necessary to have an efficient regeneration and multiplication system available.

Regeneration

The most preferable explant sources, the young vegetative parts of leek, like easily obtainable leaves or roots, are until now, not capable to undergo any kind of regeneration.
Different groups have studied *in vitro* regeneration of *Allium* species. Most of the research was performed on onion (*Allium cepa* L.) and garlic (*Allium sativum* L.), because of their greater economic importance. Leek is a monocotyledon but in its regeneration behavior it seemed not to be very recalcitrant like some other monocotyledons. Many kinds of explants of the leek plant are suitable for regeneration.

In leek two systems, somatic embryogenesis from zygotic embryos (Chapter 2) and adventitious shoot formation from flower stalk explants (Chapter 4) have good regeneration and multiplication characteristics. Furthermore, both systems can be applied in a wide range of genotypes. Somatic embryogenesis is a relatively efficient way of *in vitro* multiplication. The use of mature zygotic embryos isolated from seed as starting material is an efficient way of setting up this system, as the isolation of immature zygotic embryos, used in the system of Buiteveld (1998), is very laborious, delicate work which has to be done in the right stage of the seed ripening process. As opposed to callus cultures the cyclic regeneration system guaranteed the continuous production of somatic embryos of one genotype as a normal callus culture rapidly loses its regeneration ability. Here, somatic embryos were produced over more than 9 cycli using the cyclic somatic embryogenesis system. Although genotypic differences were found, all chosen genotypes were capable of producing somatic embryos for several cycli. Plants derived from these experiments were allowed to flower and had normal seed set (Chapter 2). Cyclic somatic embryogenesis starts by cell division in the most inner part of the basal plate of the somatic embryo or zygotic embryo. In addition, probably more than one cell is involved in the somatic embryogenesis process (Chapter 3). Wang (1996) stated in his PhD thesis that, using somatic embryogenesis, transformants of leek could be obtained. However, no further results were published until now. Wang (1996) used different steps in the somatic embryogenesis procedure, involving induction of callus from leaf bases and the use of different media and growth regulators. Silvertand (1996) developed a novel method of shoot regeneration by means of flower stalk explants. This shoot regeneration method seems not to be genotype dependent and can efficiently be used for *in vitro* propagation of selected genotypes. Silvertand (1996) stated that regeneration from flower stalk explants was a single cell event, based on histological observations. The same kind of regeneration system was used by Wilmink *et al* (1995) on tulip (*Tulipa gesneriana* L.) in a transformation study. Wilmink concluded from histological studies that the adventitious shoots originated from multicellular regions. The regeneration potential of the flower stalk explant is fairly high. Silvertand (1996) calculated that an average of 300 shoots per flower stalk could be produced. The disadvantage of this system is the fact that leek is a biannual crop and it takes a vernalisation period necessary for inducing flowering. In nature this process takes about 1.5 years, under artificial climate conditions it still takes about 10 months to obtain a flower stalk. Once the flower stalk is harvested and taken *in vitro* culture, it takes just 4 weeks before the first shoots emerge. For the transformation experiments the flower stalk regeneration method was slightly adjusted, leaving the regeneration potential and efficiency intact. Instead of flowers stalk discs, strips were used for particle bombardment. The advantage of using strips instead of discs was that more sub-epidermal

target cells could be hit in a particle bombardment (Chapter 4). The most desirable regeneration systems for transformation experiments of leek are embryogenic suspension cultures and protoplasts capable of regeneration. In such a system regeneration starts from a single cell and selection is more efficient due to the smaller explants. At the time of this research no such system was available with sufficient efficiency for transformation.

Transformation

An efficient regeneration system is a prerequisite for genetic modification. For leek it seemed that regeneration was no problem. Compared to other monocotyledons, leek had a relatively high regeneration frequency with minor genotypic effects. The primary research plan was to set up a transformation system using a disarmed *Agrobacterium tumefaciens* and promotor studies. *Agrobacterium* turned out to be a real non-host for leek. This line of research was terminated as never having observed a single GUS spot in leek. The next step was optimizing the particle gun procedure in transformation experiments. Due to some technical failure of the particle gun transient expression was very low in the first period of this research. Therefore, all experiments to optimize the gun parameters and promoters failed due to the low transient expression results. After adjustment of the particle gun, results with transformation studies performed on somatic embryos, were still rather poor. The selective agent phosphinothricin, (a component of the herbicide BASTA) was used, because antibiotics, like kanamycin and hygromycin, had a inhibitory effect on callus growth and regeneration. Selection systems using kanamycin, hygromycin or phosphinothricin showed a qualitative effect on shoot regeneration. A promoter study showed that the constitutive 35S promoter (or double 35S) of the cauliflower mosaic virus gave the highest transient expression. Other promoters like ubiquitin or TR1' gave hardly any transient expression. This was also shown in the research of Wilmink (1995), comparing promoters in tulip and leek. A histological and morphological study of the cyclic somatic embryogenesis system was performed which made it clear that the cells responsible for regeneration were buried deep inside the tissue and could hardly be reached by particle bombardment. The cyclic system has been used as a tool to obtain true transformants out of chimeric somatic embryos. Unfortunately, this was not achieved in this thesis due to the low level of transient expression found after particle bombardment (starting problems) during the first period of this research and to the characteristics of the regeneration determined by histological analysis (Chapter 3). The main attention became focused on flower stalk explants (Chapter 4 and 5). The protocol of flower stalk regeneration of Silvertand (1996), primarily developed for *in vitro*

multiplication, was adjusted for transformation purposes. Instead of flower stalk discs, flower stalk strips were used as explants for the particle gun, no loss of the regeneration potential of 300 shoots per flower stalk was observed. In this way, more target cells are exposed in a single bombardment. The optimal conditions for the particle gun were determined on flower stalk strip explants. The shortest distance possible (5 cm), at 1800 psi and a preculture period of 2 days were optimal parameters for transient expression. Application of the optimal bombardment conditions resulted into 16 chimeric plants from 370 explants bombarded. Based on the transformation results of this thesis (Chapter 5) and the histological pictures of Silvertand (1996) and Wilmink (1995) it cannot be excluded that regeneration from the leek flower stalk explants is a multicellular event. A multicellular regeneration process is not preferred in transformation experiments, because of the chance of chimeric plants.

A study of the coating process showed differences in the DNA binding between gold and tungsten particles. Most researchers will perform experiments in order to find the most optimal conditions for transient expression with the idea that this might also lead to the best stable expression after DNA integration into the genome. However, several reports show that there is no clear correlation between transient and stable expression. Still, it is the only way of making some kind of standard protocol. Factors ranging from 10% to smaller than 1% have been mentioned as calculation factor of how many cells with transient expression turn into stably transformed cells (Christou et al. 1988; Finer and McMullen, 1990; Bower and Birch, 1992, Christou 1992, Bommineni et al., 1993). In tissue culture leek can be described as a relatively high regenerative crop. A lot of plant cells are capable of regeneration. The transient expression after particle bombardment reached very high levels. However, only scarce transformation events were observed leading to chimeric transgenics. The cause of low transformation events could be due to the damage caused by the particle gun, improper cell stage of the explant types or lack of good selective agents to stimulate preferential regeneration of transformed cells or other unknown reasons. One improvement in optimization of transformation proved to be the LUC-gene that makes it possible to investigate transient expression, *in situ*, *in vivo* and in time. Surprisingly, transient expression in the flower stalk explants did not decrease as fast as expected, but could be seen over a long period of time (Chapter 4).

Applying these optimized conditions to flower stalk strip explants and placing them subsequently on selective medium yielded some chimeric plants (Chapter 5). This confirmed the indications that the origin of shoot regeneration in the flower stalk strip explants is more

likely to be multicellular than unicllular. To obtain a homogeneous transformant of a chimeric plant a small part of the basal plate had to be transgenic.

Prospects for future research

For transformation of leek by particle bombardment it is essential to establish an efficient regeneration system in which the newly formed structures are formed out of single cells located in exposed cell layers. The described system of cyclic somatic embryogenesis in this thesis is not yet suitable for transformation. Adjustments of the protocol of the cyclic somatic embryogenesis system can be helpful in making this system suitable for transformation purposes. Different basic media, other ways of isolation of the somatic embryos, better recognition of the right callus and liquid media could be important factors to improve this protocol for transformation. Comparable cyclic somatic embryogenesis systems have been set up for cassava (*Manihot esculenta* Crantz) and *Alstroemeria* (*Alstroemeria spp*) and with some adjustments these systems are now suitable for transformation (Raemakers *et al*, 1996, Lin, 1998). Flower stalk strips are useful for transformation. The high regeneration potential and the location of the cells, responsible for regeneration, make the flower stalk strips a good source explant. However, the risks of chimeric sectors is high as regeneration is shown not to be a single cell event. The next step is to develop solid transgenics from these chimeric sectors or to circumvent chimerism by more stringent selection. In mutation breeding, where a mutation is also caused in a single cell, chimaeras have been successfully transformed into so-called "homo-histons" by means of selection (Donini and Sonnino, 1998). In vegetatively propagated crops selection and sub-culturing of the chimeric sector frequently lead to a completely uniform mutant. In seed crops the M_2 or later generations are used to select uniform mutants. The use of the luciferase gene in combination with a selective agent is very important. An alternative source for transformation could be suspension or protoplast culture. At time of this thesis research no such system for leek was developed. Buiteveld (1998) described a regenerable suspension and protoplasts culture method. To overcome the problems of multicellular regeneration (Chapter 3 and 5) an embryogenic suspension culture can be used. The application of successful selection at a suspension of friable callus or protoplast level of leek might also be more direct than at somatic embryo or flower stalk explant level as the contact and target area is enlarged.

Chloroplast transformation

Further research should be concentrated on adaptation of the existing methods. Also attention should be given to other selectable markers. For hybrid production homozygous transgenic plants are necessary. This is time-consuming in a tetraploid crop and even more difficult in leek (biannual, outbreeder, inbreeding depression). In such crops plastid transformation could be used as an alternative. The process of biolistic transformation is a random process. With a violent force DNA is brought into the cell, subsequently has to find its way to the nucleus and is randomly integrated. Written in this way it seems that using biolistics is a very rough and hardly-controlled method. Efforts are made to make this process less random. For example the target DNA can be surrounded with sequences which might integrate less randomly in the genome or even in plastid DNA. For obtaining male sterile leek the approach of plastid transformation seems to have good possibilities at this moment. Leek is a tetraploid cross-fertilizing crop, and, therefore a complicated object for creating true breeding homozygous transgenic plants. The creation of plastid transformed transgenic plants could be an alternative, because harvested seeds on mother plants are expected to provide 100% transgenic offspring. An important requisite is the creation of plants with all chloroplasts or mitochondria transgenic. Plant cells contain a number of photosynthetic types such as chloroplasts and non-photosynthetic plastid types, such as amyloplasts and chromoplasts. Amyloplasts and chromoplasts are usually found in specific tissues. In the last 2 decades more detailed plastid information has been obtained with respect to the organization and coding capacity (Dyer, 1985: Mullet, 1988). The chloroplast genome generally contains 120-170 kbp and codes for around 70 proteins next to tRNAs and rRNAs. The plastome of higher plants contains about two inverted repeats of 10-75 kbp. The majority of proteins are associated with the structure or the functioning of chloroplasts. The number of plastomes in a plastid and the number of plastids in a cell vary greatly between plant species and even within one plant species and between individual cells. In most plant species, the plastids are of uniparental-maternal origin. Transformation of plastids involves integration of the transforming DNA by homologous recombination. Replication of the transplastome and sorting of the transformed genome multiplies under selection pressure eventually will yield homoplasmic lines. Transformation of only one plastid in a cell can evolve in a 100% transgenic plastid population in a cell after many plastid replications especially after selective pressure. In contrast with the convential particle bombardment that aims for nuclear genomic DNA, the process of plastid transformation is much faster and efficient. In figure 6.1 the differences between the two methods are visualized. For a 100% transgenic offspring with the convential particle bombardment method at least two sexual

fases had to be passed, taking at least 2,5 years of culturing. The results of the two crosses are only partly of the favorable type. Problems like the recognition of a transgenic duplex form and other specific, earlier mentioned leek problems seems to overcome with plastid transformation.

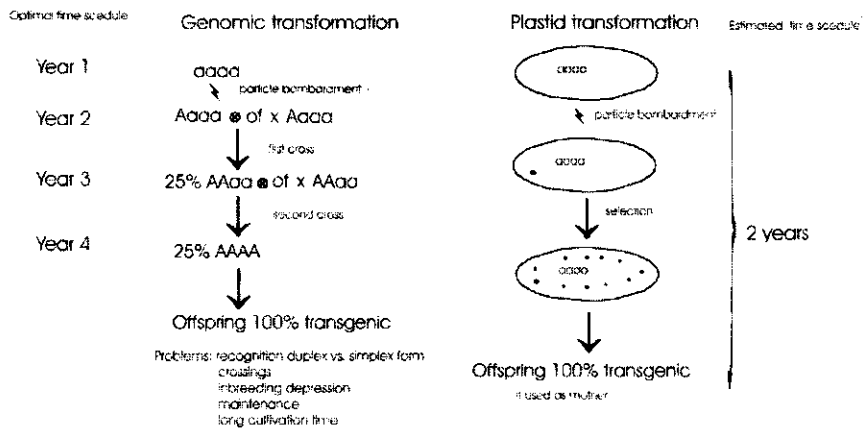

Fig.6.1. Schematic view of genomic transformation versus plastid Transformation.
[1] Estimated from Buiteveld, 1998.

The approaches of plastid transformation have been taken into practice. The first reports on plastid transformation of *Nicotiana tobacco* came from the laboratory of P. Maliga (Svab et al, 1990; Svab & Maliga, 1993, Staub & Maliga 1992, 1993, 1994, Kanevksi & Maliga, 1994; Bock et al, 1994). Zou *et al* (1998) described stable inheritance of a spectinomycin resistance of transplastome tobacco. Recently *Arabidopsis thaliana* has been added to the list of plastid transformations (Sikdar et al, 1998). All these experiments are in a fundamental phase; new selective or marker genes or flanking ptDNA borders are being discovered or engineered or the functions of the plastid proteins are investigated (McBride *et al*, 1994, Dix *et al*, 1995, Chaudhuri and Maliga, 1997). This technique applied to higher plants and plant breeding is promising. The plastome transformation could be an attractive site for genes concerned, proving gene amplification up to 10.000 times (Maliga, 1993), maternal inheritance and high levels of gene products in the leaves (Staub & Maliga, 1993).

Introducing cytoplasmic male sterility in leek by means of plastid transformation overcomes the problem of maintenance of the transgenic plants.

Transformation of the plastids provides an elegant and alternative way to transformation of the nucleus. It will be a matter of time and research efforts before all higher plants can be transformed in one way or another.

The public acceptance of the use of transformation techniques for food and non-food products is still a hot discussion item. The outcome of this discussion will also determine the importance of this technique in the future.

Literature

Bock R, Kössel H, Maliga P (1994) Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype. The EMBO Journal 13(19) 4623-4628.

Bommineni VR, Chibbar RN, Datla RSS, Tsang EWT (1993) Transformation of white *spruce (Picea glauca)* somatic embryos by microprojectile bombardment. Plant Cell Reports 13: 17-23.

Bower R, Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. The Plant Journal 2(3): 409-416.

Buiteveld J (1998) Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek. PhD-thesis Wageningen Agricultural University, The Netherlands.

Chaudhuri S, Maliga P (1997) New insights into plastid RNA editing. Trends in Plant Science 2(1): 5-6.

Christou P, McCabe DE, Swain FS (1988) Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiology 87: 671-674.

Christou P (1992) Mini review; Genetic transformation of crop plants using microprojectile bombardment. The Plant Journal 2(3): 275-281

Dix PJ, Kavanagh TA, Cassels AC (ed.) Jones PW (1995) Transforming the plastome: genetic markers and DNA delivery systems. Euphytica 85:1-3.

Donini P & Sonnino A (1998) Induced Mutation in Plant Breeding: current status and future outlook. In: Somaclonal variation and induced mutations in crop improvement ,in: Jain SM, Brar DS, Ahloowalia BS (eds); Kluwer academic publisher, London, Great Britain. p.255-291.

Dyer TA (1985) The chloroplast genome and its products. Oxford surveys of Plant Molecular and Cell Biology 2: 147-177.

Finer JJ, McMullen MD (1990) Transformation of cotton (*Gossypium hisutum* L.) via particle bombardment. Plant Cell Reports 8: 586-589.

Kavevski I & Maliga P (1994) Relocation of the plast *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco plastids. Proc. Natl Acad Sci USA 91: 1969-1973.

Lin HS (1998) Development of two *in vitro* regeneration systems through leaf explant and callus culture and the application for genetic transformation in *Alstroemeria*. PhD thesis, Agricultural University Wageningen, The Netherlands.

Maliga P (1993) Towards plastid transformation in flowering plants. Trends in Biotechnology 11: 101-106.

McBride KE, Schaaf DJ, Daley M, Stalker DM (1994) Controlled expression of plastid transgenes in plants based on nuclear DNA-encoded and plastid targeted T7 RNA polymerase. Proceedings of the national Academy of Sciences of the United States of America 91(15) 7301-7305.

Mullet JE (1988) Chloroplast development and gene expression. Ann. Rev. Plant Physiol. Plant Mol. Biol 39:475-502.

Raemakers ÇJJM, Sofiari E, Taylor N, Henshaw G, Jacobsen E, Visser RGF (1996) Production of transgenic cassava (Manihot esculenta Crantz) plants by particle bombardment using luciferase activity as selection marker. Molecular Breeding 2(4): 339-349.

Sikdar SR, Serino G, Chaudhuri S, Maliga P (1998) Plastid transformation in *Arabidopsis thaliana*. Plant Cell Reports 18: 20-24.

Silvertand B (1996) Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L). Ph.D. thesis Agricultural University Wageningen, The Netherlands.

Staub JM & Maliga P (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation, The Plant Cell 4: 39-45.

Staub JM & Maliga P (1993) Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the *psb*A mRNA. The EMBO Journal 12 (2) 601-606.

Staub JM & Maliga P (1994) Extrachromosomal elements in tobacco plastids. Proc. Natl. Acad. Sci. USA 91: 7468-7472.

Svab Z, Hajdukiewitz P, Maliga P (1990) Stable chloroplast transformation in higher plants. Proc. Natl. Acad. Sci. USA 87: 8526-8530.

Svab Z and Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aad*A gene. Proc. Natl. Acad. Sci. USA 90: 913-917.

Wilmink A, Van de Ven B, Custers J, Nöllen Y, Dons H (1995) Adventitious shoot formation in tulip: Histological analysis and response to selective agents, Plant Science 110: 155-164.

Wilmink A (1996) Genetic modification of tulip by means of particle bombardment, PhD-thesis, CPRO, Wageningen , The Netherlands.

Wang H (1996) Genetic engineering male sterility in leek (*Allium porrum* L.), Ph.D. thesis Applied Biological Sciences, University of Gent, Gent, Belgium.

Zou ZR, Zhang ZL, Shan S, Ni PC, Shen GF (1998) Construction of vector for tobacco plastid transformation and obtainment of tobacco transgenic plants. Acta Agronomica Sinica 24(4): 410-415.

Summary

In this thesis the results are presented of experiments aiming at the genetic modification of leek (*Allium ampeloprasum* L.). Leek is a vegetable grown for its edible (false) stem and belongs to the Alliaceae, together with onion (*Allium cepa*) and garlic (*Allium sativum*). The production of leek is mainly confined to Europe. In the last few years production has increased along with consumer demands. It is propagated through seeds and gives rise to heterogeneous progeny. Problems in cultivation of leek are rust (*Puccinea allii, P.mixtu*), yellow stripe virus and the lack of uniformity. The most suitable system able to cope with these problems seems to be hybrid breeding. However, hybrid breeding is hampered by the lack of a suitable emasculation system or male sterility system and the severe inbreeding depression. Therefore, emphasis has been put on the application of genetic modification in order to solve some of these problems. This relatively new technique opens the possibility to add or alter traits which cannot easily be achieved with conventional breeding methods. The most important prerequisites for genetic modification were investigated during this thesis research. The first prerequisite is an efficient regeneration system. Starting from seed, a cyclic somatic embryogenesis regeneration system was developed with long term regeneration potential, providing a regeneration system where it should be possible to obtain true transformants from chimeric structures (Chapter 2). A comparison was made between the first cycle, starting from zygotic embryos, and latter cycli, starting with somatic embryos. All genotypes tested were able to produce somatic embryos although genotypic differences in somatic embryo production occurred. The first cycle, using zygotic embryos, was the most effective in somatic embryo production compared to the later cycli using somatic embryos. On average the first cycle produced 23.8 somatic embryos per zygotic embryo and the later cycli ranged from 11.1 to 16.0 somatic embryos per initial somatic embryo. Shoot regeneration from somatic embryos was satisfactory, obtaining normal looking greenhouse plantlets. In Chapter 3 this cyclic somatic embryogenesis system was analyzed for its suitability in a genetic transformation system, like particle bombardment. The relatively new reporter gene luciferase was used in the transformation experiments. Leek, like most monocotyledons, seemed to be very persistent to selective media. Neither the selective agent kanamycin nor hygromycin could prevent leek cells from growing. The selective agent phosphinothricin had a better inhibitory effect on cell growth. A histological and morphological study showed that regeneration occurred from the deeper cell layers inside the somatic embryo, in the same way that leaves are produced on a mature leek plant. These cell layers are hardly reached by particles, explaining the poor results of the transformation

experiments using somatic embryos. In Chapter 4 a newly developed regeneration system from flower stalk strip explants was used to determine the optimal conditions for particle bombardment with the luciferase reporter gene. After a vernalisation period leek starts to develop a flower stalk. This flower stalk, still inside the plant, is harvested and stripped. Regeneration from these strips occurs just beneath the epidermis, which is easy to reach for the particles of the particle gun. The regeneration frequency was neither influenced by the stripping nor by the bombardments. Over 300 plantlets could be obtained from 1 flower stalk. Important factors for transformation experiments like pre-culture time, pressure, distance and coating of the particles were analyzed and optimized for these highly regenerative explants. Flower stalk strips of leek as explants, cut from the basal part of the flower stalk, pre-cultured for 2 days, should be bombarded at a distance of 5 cm, with a pressure of 1800 psi to obtain a high transient expression. Gold particles should be used and coated following the procedure of Christou (1991). After bombardment the explants should be transferred to selection medium. In Chapter 5 these optimal conditions were applied to the flower stalk strip explants. A comparison of the use of the reporter genes GUS and luciferase was made. Both genes, combined in one plasmid, showed similar expression patterns. Expression of both genes was still present 7 weeks after bombardment, but local increases in gene activity were not observed. The reporter gene luciferase facilitates the investigations in the genetic modification research as the chemical reaction with this reporter gene is non-lethal to the plant tissue whereas the reaction with GUS-reporter gene is. The non-detrimental effect of the luciferin treatment made it possible to investigate gene expression in time. Still, using a novel reporter gene means also unexpected results like the long transient expression time of the luciferase gene product even after application of the substrate luciferin. Eventually, 16 chimeric plantlets were obtained. Probably, the regeneration from flower stalk strip explants is a multicellular event. Seeds harvested from potentially chimeric plants did not show any GUS or luciferase activity after germination.
In the near future leek transformation research has to focus on the development of embryogenic cell suspension or protoplast cultures, to obtain true transformants of the chimeric plants and an efficient selection system to select and favor the transgenic cells. To facilitate success in transformation research, efforts could also be directed at plastid transformation, in order to come to true breeding transgenic lines

Samenvatting

In dit proefschrift worden de resultaten van het onderzoek naar genetische modificatie van het gewas prei (*Allium ampeloprasum* L.) beschreven. Prei is een groente die gekweekt wordt voor zijn schijnstam. Prei behoort tot de familie van de *Alliaceae* samen met de ui (*Allium cepa* L) en knoflook (*Allium sativum* L). De preiproductie beperkt zich in grote mate tot Europa. In de laatste jaren is de vraag naar prei toegenomen tezamen met de productie. Prei wordt vermeerderd via zaad dat een heterogeen nakomelingschap oplevert. Problemem bij de teelt van prei zijn schimmels, preiroest (*Puccinea allii, P. mixtu*), gele streep virus en een geringe uniformiteit. De huidige veredelingstechnieken zijn niet of nauwelijks in staat deze problemen snel en simpel te verhelpen. Daarbij is het heterogene gewas prei voor de veredeling een "lastige" plant. Met hybriden-veredeling zouden deze problemen eenvoudiger aan te pakken zijn, maar tot nu toe ontbreekt in prei een bruikbare vorm van mannelijks steriliteit en is prei gevoelig voor inteelt. Een relatieve nieuwe techniek, genetische modificatie, zou in de huidige veredelingsprogramma's van prei een welkome aanvulling zijn. Het veranderen van eigenschappen of nieuwe eigenschappen toevoegen aan prei behoort dan tot de mogelijkheden. Om de techniek van genetische modificatie toe te passen zijn er enkele voorwaarden waaraan voldaan moet worden. In dit proefschrift zijn enkele voorwaarden nader bestudeerd. De eerste voorwaarde waaraan voldaan moet worden is een efficiënt regeneratie systeem. Uitgaande van zaad, werd een cyclisch somatisch embryogenese regeneratie systeem voor prei opgezet om over een lange periode een continue regeneratie te verkrijgen en tevens de mogelijkheid te krijgen om vanuit een chimere sector in een somatisch embryo een complete transgene plant te verkrijgen (Hoofdstuk 2). Een vergelijking werd gemaakt tussen de eerste cyclus, uitgaande van rijpe zygotische embryo's en de volgende cycli, uitgaande van somatische embryo's. Alle geteste genotypen waren in staat somatische embryo's te produceren, alhoewel verschillen tussen genotypen optraden. De eerste cyclus leverde meer somatische embryo's dan de latere cycli. Gemiddeld werden er 23.8 somatische embryo's gevormd in de eerste cyclus t.o.v. 16.0 tot 11.1 somatische embryo's in de latere cycli. De uitgroei van somatische embryo's tot bewortelde scheuten vormde geen probleem en gaven normaal lijkende kasplanten. In hoofdstuk 3 werd dit cyclische regeneratie systeem nader bekeken en getest op geschiktheid voor transformatie m.b.v. "particle bombardment". Prei , net als andere monocotylen, bleek erg resistent tegen de selectiemiddelen kanamycine en hygromycine. Deze antibiotica konden het preiweefsel niet afdoden hetgeen voor selectie van transgene cellen noodzakelijk is. Het selectiemiddel phosphinotricine was hier beter toe in staat. Uit een histologische en morfologische studie

van de somatische embryogenese van prei bleek dat de oorsprong van de nieuwe cycli somatische embryo's uit de dieper gelegen cellagen afkomstig waren. Deze manier van groeien is vergelijkbaar met het uitgroeien van een blad uit een gewone preiplant. Deze cellagen zijn voor de kogeltjes van de particle gun niet te bereiken. Dit verklaart een groot gedeelte van het uitblijven van resultaten van transformatie experimenten. In hoofdstuk 4 werd een nieuwe regeneratie methode voor prei gebruikt, bloemsteel strips, om de optimale condities voor de particle gun te verkrijgen. Na een vernalisatieperiode vormt een preiplant een bloemsteel. Deze bloemsteel wordt geoogst als deze nog in de plant zit en vervolgens gestript. Regeneratie van deze bloemsteelstrips komt vanuit de cellaag direct onder de epidermis en deze is goed bereikbaar voor de kogeltjes. De regeneratie-frequentie werd niet beïnvloed door het gebruik van strips en door de beschietingen. Meer dan 300 planten kunnen vanuit 1 bloemsteel van prei regenereren tot plant. Belangrijke factoren in transformatie experimenten zoals, precultuur tijd, druk, afstand en coating zijn geanalyseerd. Bloemsteel strips van het basale deel van de bloemsteel, precultuur tijd van 2 dagen moeten beschoten worden op 5 cm afstand met een druk van 1800 PSI om zo een hoog mogelijke transiënte expressie te verkrijgen. De kogeltjes moeten van goud zijn en gecoat met DNA volgens de procedure van Christou (1991). Na de beschieting moeten de explantaten overgebracht worden naar selectiemedium. In hoofdstuk 5 zijn deze optimale condities toegepast op bloemsteel strips. Een vergelijking van 2 reporter genen GUS en luciferase werd gemaakt. Beide genen, in één plasmide, vertoonden vergelijkbare expressie patronen. Zeven weken na het beschieten vertoonden beide genen nog expressie in het preiweefsel, maar en uitbreiding van de getransformeerde regio's, voortkomend uit actieve delingen van transgene cellen werden niet gevonden. Het reporter gen luciferase vergemakkelijkt het onderzoek van genetische modificatie omdat de chemische reactie met dit reportergen niet destructief voor het plantmateriaal is, zoals de chemische reactie om de blauwe GUS spots zichtbaar te maken. Toch blijkt uit het werken met een nieuw reportergen dat ook onverwachte resultaten verkregen kunnen worden, zoals de lange transiënte expressie van het luciferasegen. Uiteindelijk werden 16 chimere planten verkregen. Waarschijnlijk is de regeneratie vanuit deze bloemsteelstrips van multicellulaire oorsprong. Kiemende zaden van mogelijke chimere planten hadden geen van alle enige mate van genexpressie. In de toekomst zal het onderzoek van het genetisch modificeren van prei zich moeten richten op de ontwikkeling van een celsuspensie, een protoplastenculture of een methode om vanuit chimere planten een gehele transgene preiplant te verkrijgen en een efficiënt selectiesysteem. Plastiden transformatie zou bij dit prei-onderzoek mogelijk van waarde kunnen zijn.

Nawoord

Met veel geduld hebben een aantal mensen op dit boekje moeten wachten, maar hier is dan eindelijk het resultaat van 4 jaar onderzoek gedurende de periode 1992-1996 op de vakgroep plantenveredeling te Wageningen. In deze periode zijn veel mensen betrokken geweest bij dit onderzoek die mij veel geleerd en verteld hebben. Zonder hen zou dit boekje niet zijn verschenen. De volgende personen wil ik hier bedanken en met name noemen . Allereerst mijn jongste zoon Simon, die zelfs voor zijn geboorte, een zo grote stok achter de deur heeft gezet dat ik eindelijk besefte dat het nu of nooit was. Verder de begeleiding op de vakgroep plantenveredeling; mijn promotor Prof. E. Jacobsen en mijn co-promotor Prof. R.Visser en dr. T van Harten. Mijn waardering is groot voor het geduld en tijdrovende werk van het corrigeren van mijn onregelmatige aanvoer van teksten. Mijn mede-collega's Carla van Schaik, Krit Raemakers, Marjan Bergervoet, Caroline van de Toorn, Petra van de Berg, Joska Buitedijk, Ben Silvertand en vele anderen. De leuke discussies en experimenten, de gezamelijke dipjes en taarten bij succesjes vormden een prima werksfeer. In het kader van hun docteraal onderzoek hebben ook vele studenten een steentje aan dit onderzoek bijgedragen; Trinette van Selling, Jaap Mazereeuw, Pedro Constantino Molinas, Pieta Bunt en Jan-Dick Lighthart. Het is leuker en leerzamer om met z'n tweeën aan een onderzoek te werken dan alleen. Voor de verzorging van mijn planten bedank ik Sjaak Jansen en Jan van Schaik, zonder bijna bloeiende preiplanten geen experimenten! Ook alle anderen die mij met raad en daad hebben geholpen wil ik hiervoor bedanken. Als laatste het thuisfront. Ook zij hebben veel geduld met mij gehad. Mijn ouders die wel wilden weten of ik ooit nog ging promoveren maar het niet wilden vragen; de boodschap kwam toch af en toe wel over. Orlando, voor zijn altijd aanwezige steun en Thomas die zo mooi tekende op alle verkeerde printjes, die nodig waren om dit boekje te schrijven.

Curriculum vitae

De auteur van dit proefschrift, Christina Maria Schavemaker, werd geboren op 25 maart 1967 te Amsterdam. In 1984 begon zij aan haar studie Tropische plantenteelt aan de Internationale Agrarische Hogeschool te Deventer. De verplicht buitenlandse stage werd in Thailand volbracht. In 1989 behaalde zij haar ingenieurs diploma (ing.). Dat zelfde jaar begon zij aan de verkorte studie Plantenveredeling aan de Landbouw Universiteit te Wageningen. In 1991 verkreeg zij hier haar bul. In 1992 startte zij met haar promotieonderzoek op de vakgroep plantenveredeling aan de Landbouw Universiteit te Wageningen . De resultaten van dit onderzoek staan beschreven in dit proefschrift.

The author of this thesis, Christina Maria Schavemaker, was born in Amsterdam on 25 March 1967. In 1984 she started her study Tropical Plant Science at the International Agricultural College in Deventer, Netherlands. The training period was performed in Thailand. In 1989 she graduated and got her Bachelor Degree. The same year she started the study Plant Breeding at the Wageningse Agricultural University. In 1991 she graduated. In 1992 she worked on the Department of Plant Breeding at the Wageningse Agricultural University. The results from this research are described in this thesis.