Towards onions and shallots (*Allium cepa* L.) resistant to beet armyworm (*Spodoptera exigua* Hübner) by transgenesis and conventional breeding

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Bibliographic Abstract: This thesis describes the development of onions and shallots (*Allium cepa* L.) resistant to beet armyworm (*Spodoptera exigua*) via genetic transformation and via molecular marker-assisted breeding (MAB). Firstly, an *in vivo* and *in vitro* bio-assay for the *Spodoptera exigua* was developed. Because no high level of resistance was found in *A. cepa* and its wild relatives, a marker-assisted breeding approach was abandoned and emphasis was laid on the development of a genetic transformation system. First of all a reliable plant regeneration system from callus cultures and suspension cultures had to be established. It was found that plant regeneration capacity significantly decreased with time and that it was highly dependent upon the callus line used. On the basis of these results it was concluded that only relatively young callus (less 3 months) could be used for transformation. Eventually, an *Agrobacterium tumefaciens*-mediated transformation system both for onion and shallot was developed using three-week old callus derived from mature embryos with two different strains EHA105(pCAMBIA1301) and LBA4404(pTOK233) carrying a binary vector coding for *uidA* and *hpt*. This transformation system for onion and shallot can be used year-round. A total of 11 independent callus lines regenerated transgenic plants. These transformants were molecular genetically characterized by means of standard PCR, genomic DNA blot hybridization and FISH (fluorescence *in situ* hybridization). An adapator ligation PCR (AL-PCR) followed by sequencing of the genomic DNA flanking the T-DNA borders was developed. The AL-PCR patterns obtained were specific and reproducible for a given transgenic line. The results showed how T-DNA integration took place and also gave insight in the number of T-DNA copies present. After cloning and sequencing the AL-PCR products, the junctions between plant genomic DNA and the T-DNA insert were analysed in detail. We concluded that in case of the introduction of resistance to beet armyworm into onion and shallot genetic transformation is the most promising method.

谨以此书敬献给

我敬爱的父母

我亲爱的妻子

我可爱的儿女

WU8201,2891

Propositions

1. Adaptor ligation PCR (AL-PCR) is a very powerful technique for the molecular genetic characterization of transgenic plants especially in the case of large genome species such as *Allium cepa* (this thesis).

2. The monocotyledonous nature of species no longer prevents the application of *Agrobacterium*-mediated techniques to transfer genes to these species as soon as methodological parameters are optimized (Hiei et al., 1997. Plant Molecular Biology 35: 205-218; this thesis).

3. The number of truncated T-DNA copies in transgenic plants is probably highly underestimated because of the use of conventional genomic DNA blot (Southern) hybridization (this thesis).

4. Marker-assisted breeding is an attractive strategy for crop improvement, however, the obstacles have turned out to be much bigger than originally thought (Young, 1999. Molecular Breeding 5: 505-510; this thesis).

5. Fluorescence *in situ* hybridization (FISH) is a necessary, yet not fully appreciated, technique to study the effect of T-DNA insertion on the plant genome organization in addition to Southern and PCR analysis (Wolters et al., 1998. Plant Journal 13: 837-847; this thesis).

6. In onion and shallot, insect resistance can be more easily obtained by transgenesis than by conventional breeding (this thesis).

7. The beet armyworm *(Spodoptera exigua)* larva falls down easily during its climbing on the onion plant after inoculation just like an inexperienced child climbing a tree.

8. The best way in life (and research) is to follow your intuition.

9. Reading is learning, seeing is believing, doing is knowing *(Chinese proverb)*. All this is also true for carrying out research.

10. An onion a day keeps the doctor away.

11. The longer the road, the better you know the strength of the horse; the longer you get along with each other, the better you know the heart of each other *(Chinese proverb).*

12. **ABCD** is very important in the daily life of **Plant Research International.**
Agenda; **B**irthday celebration; **C**offee and tea break; **D**iscussion.

(Checking your agenda, you know who has a birthday party and when you have an appointment for a meeting. Attending a birthday party, you share friendship with your colleagues. During coffee and tea break, you can freely talk with your colleagues and have an informal discussion. During discussion, you know the research progress and can fix a date in your agenda for next meeting).

Propositions associated with the Ph.D. Thesis of Si-Jun Zheng:

Towards onions and shallots (*Allium cepa* L.) resistant to beet armyworm (*Spodoptera exigua* Hübner) by transgenesis and conventional breeding

Wageningen, The Netherlands

20 November, 2000

Preface

This thesis was carried out within the framework of the BIOBREES (Biotechnology, Plant Breeding and Seed Technology for Horticulture) programme and therefore I gratefully acknowledge the financial support of the Dutch Ministry of Agriculture, Nature Management and Fisheries and the Indonesian Ministry of Agriculture. The thesis contains the results that were obtained during the past four years of my study entitled "Towards onions and shallots (*Allium cepa* L.) resistant to beet armyworm (*Spodoptera exigua* Hübner) by transgenesis and conventional breeding". It would have been impossible for me to complete my PhD research without the help from many people in the Netherlands and in China. It is my great pleasure to take this opportunity to thank all of them.

I am very grateful to my promotor, Prof. Dr. Evert Jacobsen, for his scientific guidance. He always encouraged me and provided me with useful advice for my scientific research and for daily life. I appreciated very much his way of thinking and openness when I discussed with him.

I wish to express my sincerest thanks to my co-promotors Dr. Chris Kik and Dr. Frans A. Krens. Together with Prof. Dr. Evert Jacobsen they proved to be a strong supervision group. Frans gave me numerous valuable comments and suggestions when I encountered problems. I will always remember the pleasant weekly discussions with Frans. His profound knowledge of cell biology provided me a solid basis to carry out smoothly this research. I have always enjoyed showing him my latest unexpected results. His critical comments greatly helped in accomplishing this thesis.

Discussions with Chris on scientific topics or daily life were also of great value to me. His open mind and bright charisma encouraged me and was a constant source of inspiration. His strong logical thinking and sharpness helped greatly in accomplishing this thesis. We have had uncountable meetings and discussions. Also he gave me a lot of good suggestions, which made practical work and scientific writing much easier to me. His remarks on many draft manuscripts and his own style of efficiently "quick-doing" has been a major stimulant for me to produce the final manuscripts. Due to his help, my English writing has improved to a large extent,

particularly after I got used to his comments in his specific red handwriting. Chris also made the Dutch translation of the summary of this thesis. I met Chris and Evert in 1995 during the 25th International Course on Applied Plant Breeding. One year later, I was their student by 'arrangement of fate'. As my co-promotor, Chris approached me as a friend, providing me and my family valuable support during our stay in Wageningen. Without his kind help and his car, it is hard to imagine how I could have picked up my wife and daughter at Schiphol airport very early in the morning when no train was available. After our son was born in Wageningen, the visits and kind help from Chris and his family was most appreciated: we did not feel lonely anymore and could face difficulties and problems much better. We will never forget the friendship and hospitality from him and his wife Adrie.

I am greatly indebted to Betty Henken. Her hard work made research very efficient and fruitful. Her smart mind and skilful hands helped to avoid a lot of trouble. Also I highly appreciate her positive way of thinking. I am very pleased that we cooperated very well in practical research and also shared the great success of the results. Her patience and kind help in translating different Dutch letters, notes, forms and bills made daily life in Holland for my family and me much easier. Betty especially decorated the office for my birthday and thus helped me to enjoy the Dutch custom of birthday celebrations. I was very glad to share the same office with her during these four years, and appreciated the open, free and pleasant conversations. I am very happy to have such a kind colleague and friend.

I am also very grateful to Sjaak van Heusden, who picked me up at the Schiphol airport and let me choose the two most important things from his home immediately - a pillow and a lamp. This made it possible for me to stay in the student flat without any problem the first night. With the pillow, I could sleep quite well which prevented that I missed my family too much. The light from his lamp filled me with hope and warmth in my heart. Now his lamp, still kept in my home, continues to make my life easy: 'If there is a light, there will be a road'. Sjaak is a very kind person and colleague. His critical comments and valuable advice on my research were also highly beneficial especially for the molecular characterization of the transgenic onions and shallots.

Special thanks to Willem Wietsma, who provided me with a huge amount of onion and shallot seeds. Together with Jaap van de Berg he also provided me with good plant material. Thanks also to Dick Geurtsen for taking care of these transgenic onion and shallot plants. I was very glad to share the office with Willem, and to have the opportunity for open and free discussions. I am also very glad to have a chance working together with Jeroen Nannes.

Many thanks go to my colleagues of the Cell Biology Lab where I almost worked for three years. Greetje Kuiper, Bernadette Kronenburg, Herma Koehorst-van Putten, Iris Capel, Ingrid Maas, Annetta Noppert, Yvonne Nöllen, Marian van Harmelen, Lim Ki-Byung gave me all kinds of help. Greetje and Bernadette: I am sorry that our 'one Dutch sentence a day' did not result in much progress. It was a great pity that I never actually understood what my colleagues were talking about when they spoke Dutch. But, inadequate knowledge of the Dutch language gave me the opportunity to fully concentrate on the job.

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I am also grateful for the birthday presents from my colleagues of the Cell Biology Lab and Molecular Marker Lab for my son and myself.

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Martin Beek was the co-ordinator of the 25th International Course on Applied Plant Breeding and I was very glad to be one of participants of this course. I would like to thank Martin and his wife Ineke for their hospitality and friendship. I never forget the international dinner we prepared at their home. Because of this course I had the chance to meet my promotor Professor Evert Jacobsen and co-promotor Dr. Chris Kik. During the years we kept contact, whenever we met in the supermarket, openmarket or at home, we always had a lot to talk about.

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My children, my wife and myself will never forget Jos Kanne for giving us so many delicious and wonderful strawberries, apples and different kinds of other fruits. I am very grateful for Jos his kindness.

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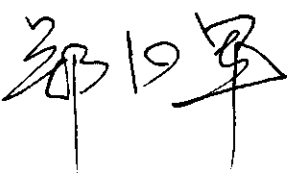
My parents, brothers and sisters in China have always been very much interested in my family life in the Netherlands and my research progress. They have always encouraged and supported me. My deep gratitude goes to my parents for bringing me up, giving me a firm background and emotional strength that have enabled me to do what I have been doing. My parents-in-law and family members-in-law also have given me a lot of moral support. I thank all of them.

I feel guilty that I have not spent much time at the weekends with my wife Ye Xiu-Hua (Hua) and my children Zheng Tian-Lun (BeiBei) and Zheng Tian-Yu (ShuangShuang) during these four years. The separation from my wife and daughter during the first seven months of my PhD study were also not easy for all of us. My wife's deep love for me made her give up her good job in China and join me in Wageningen. Reunion with my family and the companionship of my wife gave me a solid basis to work more efficiently. I am deeply indebted to my dear wife Hua, beautiful and lovely daughter BeiBei and son ShuangShuang for their patience, tolerance, understanding, love and support. I am very proud of my daughter because she is so excellent at school and also in music and ballet. The birth of my son in August 1998 in Wageningen brought us a lot of new colour and excitement in our daily life.

All of these people I have mentioned have contributed greatly to the success of this research project and the completion of this thesis.

Now the best thing I can give them is this book!



Si-Jun Zheng

Wageningen, 7 July, 2000

Contents

Chapter 1

General introduction

Onion (*Allium cepa* L. group Common Onion) and shallot (*A. cepa* L. group Aggregatum) are two subspecies of *A. cepa*. Both are members of the genus *Allium* (Family *Alliaceae*), a genus which comprises more than 600 species (Hanelt, 1990). *A. cepa* originates from central Asia and it is thought that *A. vavilovii* is its wild progenitor (Hanelt, 1990). *A. cepa* is one of the few vegetable species that was domesticated in the Old World at a very early stage. It has been in cultivation for more than 4000 years. The earliest records come from Egypt, where it was cultivated at the time of the Old Kingdom. Onions appear as carvings on pyramid walls and in tombs from the third and fourth dynasties (2700 BC).

Nowadays both onion and shallot are very important vegetable crops on a worldwide scale (Anonymous, 1999). Onion is cultivated mainly as a biennial but some types are treated as perennials. It is propagated by seeds, bulbs, or sets (small bulbs). Onion bulbs are quite variable with respect to shape, size, skin and flesh colour, pungency, skin retention, storage ability, hardiness, and dry matter content. Shallot differs from common onion primarily in bulb characteristics. The bulbs of common onion are large, normally single, and plants are grown from seeds or from seed-grown sets. The bulbs of shallot are smaller compared to the bulbs of common onion, they form an aggregated cluster of small bulbs as a result of the rapid formation of lateral bulbs or shoots. Reproduction of shallot is predominantly vegetative via daughter bulbs (sets) although seed production is possible.

General breeding

Onion is generally regarded as an outcrossing species. Onion populations are genetically heterogeneous with a high degree of heterozygosity maintained by substantial levels of outcrossing. Thus, individual populations or varieties provide a source of genetically plant-to-plant variation on which the breeder can impose

selection. Bulbs are the predominant yield component and their development is daylength dependent and hence there are different types of onion cultivars for specific latitudes. Long-daylength (16h) onions are adapted to the Northern and Southern hemispheres, while short-daylength (12 h) onions are suitable for the tropics. Both open-pollinated varieties and F_1 hybrids are grown in these areas (Kik et al., 1998). Open-pollinated (OP) varieties are defined as genetically variable populations, which are maintained and multiplied by mass pollination in isolation. A major contribution to onion breeding has been the development of F_1 hybrids since an early identification of CMS (cytoplasmic male sterility) in the cultivar Italian Red (Monosmith, 1926) and its later application for producing hybrids (Jones & Clarke, 1943). This male sterility resulted from an interaction between nucleus and cytoplasm. The genetic basis of the CMS is simple: a sterility-inducing cytoplasm (S) and a nuclear restorer locus with two alleles, *Ms* and *ms*. Male fertility is restored by the dominant allele. In the 1960s, another CMS cytoplasm, namely the T-cytoplasm, was discovered (Berninger, 1965; Schweisguth, 1973). This source of male sterility was found in the French cultivar Jaune Paille de Vertus. The T-cytoplasm is restored by two independently operating restorer systems. The first restorer system comprised a single locus, A, with two alleles, in which male fertility is restored by the dominant allele. The second restorer system is composed of two loci, B and C, with complementary gene action. Male fertility in this system is restored only when a dominant allele is present on both B and C loci. Onion breeding based on F_1 hybrids is now widespread. The main reasons why F_1 hybrids have become popular is probably the opportunity for breeders to protect their parental lines more efficiently, combined with the possibility to improve F_1 hybrids more quickly than OP cultivars.

Onion and shallot are vulnerable to a number of diseases and pests (Rabinowitch, 1997). In temperate zones *Botrytis* and *Fusarium* diseases, downy mildew (*Peronospora destructor*), white rot (*Sclerotium cepivorum*), thrips (*Thrips tabaci*) and onion fly (*Delia antiqua*) can cause substantial yield losses. In tropical zones purple blotch (*Alternaria porri*), anthracnose (*Colletotrichum gloeosporioides*) and beet armyworm (*Spodoptera exigua*) are threatening the onion and shallot cultivations. This thesis is focussed on the development of transgenics for the introduction of resistance to beet armyworm into *A. cepa*, as this pest represents one of the major problems nowadays in tropical onion and shallot cultivation. The thesis is carried out in the framework of the BIOBREES (BIOtechnology, BREEding and Seed technology for horticulture) programme. The programme is a joint initiative of the Dutch and Indonesian Ministry of Agriculture for the improvement of tropical crops.

Beet armyworm (*Spodoptera exigua*, Hübner)

Description

Beet armyworm has four life cycle stages namely adult, egg, larva and pupa. The moth has a wingspan of 25 to 32 mm. The ribbed egg is white to pink and is roughly spherical in shape and slightly peaked on top. Scales and hairs from the moth give the egg cluster a gray, fuzzy appearance. The larva of *S. exigua* is a green or black caterpillar with a dark head, five pairs of prolegs, and sometimes three lightly coloured stripes running over the length of the body. On the second segment behind the head, there is a small black spot on each side of the body. This spot usually becomes visible to the field observer when the caterpillar reaches a length of 7 - 8 mm. About 1 mm long when newly emerged, a larva may be 25 - 30 mm long when fully grown. The pupa of *S. exigua* is about 15 - 20 mm long, the pupa is light brown with dark brown margins along the abdominal segments.

Distribution, host plants and damage

The beet armyworm originates from Southern Asia and has spread throughout most temperate and tropic zones. It has an extremely wide host range including numerous weeds, trees, grasses, legumes, ornamental crops, and field crops such as onion and shallot. Beet armyworms weave foliage together with a web and feed within this shelter. They also bore into leaves and flowers. Early instars most frequently damage the young terminal growth of plants. Profuse silk webbing may give infested plants a shiny appearance. Old instars do not feed gregariously and the production of webbing is discontinued, they feed extensively on leaves, flowers and buds. In heavily infested fields total yield losses can amount up to 70 %.

Life history

The beet armyworm has several generations per year. Eggs are laid in masses of 15 - 150 mostly on the undersides of leaves and mostly within 10 cm of the soil surface. Females may cover the eggs with a mat of scales that protects them from parasites but also from insecticides. Females prefer younger plants to older plants. Two to nine days later the eggs hatch. First and second instar beet armyworms feed in groups, especially in the growing tips where they weave several leaves together with a web. Older larvae

do not restrict feeding to young leaves. The third and fourth instars last one to three days. The fifth instar feeds for two to six days. Beet armyworms are cannibalistic, particularly when feeding on plants with low nitrogen levels. Moths that develop from cannibalistic caterpillars lay more eggs than moths that developed from caterpillars that fed only on plant material. Mature beet armyworms tunnel into the soil 2.5 cm and form a cell in which they pupate. The pupal stage lasts four to eleven days. The moths are nocturnal and mating and egg laying occur at night. Females deposit up to 600 eggs during their seven or eight-day life at this stage. However, moths may live up to 30 days altogether.

Control

The beet armyworm is difficult to control with chemicals because the young larvae weave leaves together and feed under the webbing that protects them from insecticides. Older larvae are more tolerant to pesticides. In addition, some beet armyworm populations have acquired resistance to methomyl and other pesticides. Pheromone traps can help to monitor for moth flight activity.

Introduction of resistance to *S. exigua* has been carried out in tomatoes (Eigenbrode & Trunkle, 1993a; Eigenbrode & Trunkle, 1993b; Eigenbrode et al., 1996; Hartman & Clair, 1999). It was shown that *Lycopersicon hirsutum* harbours two types of resistance, namely a trichome-based and a lamellar factor-based. For onion and shallot such a study has never been performed. Introduction of resistance to *S. exigua* by means of genetic transformation is also possible (Fischoff et al., 1987; Bosch et al., 1994; Schuler et al., 1998). However, the introduction of alien genes into the genome of *A. cepa* was, until recently (see this thesis) not feasible, as no reliable transformation system was available.

Introduction of resistance to *S. exigua* into *A. cepa*

In this thesis a two-way strategy will be followed to develop onions and shallots which are resistant to *S. exigua*. Firstly, we will explore the possibility if there is natural variation present in *A. cepa* and its wild crossable relatives for resistance to the beet armyworm. Secondly, we will develop a regeneration and transformation system for onions and shallots to introduce gene sequences, which are expected to confer

resistance to beet armyworm, e.g. coding for *Bacillus thuringiensis* insecticidal proteins or proteinase inhibitors.

Marker-assisted breeding

Introduction of resistant onion and shallot cultivars is of significant importance to enhance the sustainability of (sub)tropical onion and shallot cultivations. Recent developments in DNA marker technology provide new solutions for selecting and maintaining desirable genotypes. Once molecular markers closely linked to desirable traits are identified, marker-assisted breeding can be performed in early segregating populations and at early stages of plant development. Marker-assisted breeding can be used to pyramid disease and pest resistance genes, with the ultimate goal of producing high quality varieties. The essential requirements for marker-assisted breeding are: marker(s) should be closely linked with the desired traits; an efficient means of screening large populations for the molecular marker(s) should be available, the screening technique should have high reproducibility, be economical to use and be user-friendly (Mohan et al., 1997).

Molecular marker studies in *A. cepa* are scarce because of the biennial nature of the crop, its severe inbreeding depression (Jones & Davis, 1944) and its huge genome size (Labani & Elkington, 1987). King et al. (1998) presented a low-density RFLP map based on an intraspecific onion cross. This study showed that the genome organization of onion is complex and involves many duplicated loci. Van Heusden et al. (2000) constructed a high density AFLP marker map based on an interspecific cross between onion and *A. roylei*. The alliinase gene involved in pungency and a SCAR marker linked to the downy mildew disease resistance gene are present on this map. Furthermore, Van Heusden and Shigyo et al. (2000) used a complete set of *A. fistulosum* - *A. cepa* monosomic addition lines (2n=2x+1=17) to assign the physical *A. cepa* chromosomes to their respective molecular marker linkage groups.

Genetic transformation

Regeneration system

The development of an efficient system for genetic transformation might be a valuable extension of the tools for further crop improvement. In order to establish a successful

onion genetic transformation system, two key factors should be taken into account. One is the development of sophisticated methods to recover intact plants, either from fully dedifferentiated tissue or from organized tissues that are easy to regenerate. The other is the refinement of methods for the introduction of exogenous DNA into *Allium* germplasm.

In *Allium*, various plant regeneration systems have been developed using different starting material. Eady (1995) reviewed the different source materials used for *in vitro* culture of *Allium* species. The most successful regeneration systems in *Allium* are using (im)mature embryos, root tips, flower buds, suspension cultures or protoplasts as starting materials (Table 1).

Transformation system

Transformation of recalcitrant monocots like rice, wheat, barley and maize has been achieved by using direct gene delivery systems: chemical methods, electroporation, particle bombardment and silicon carbide fibres. Recently, *Agrobacterium*-mediated transformation of monocots has gained favour and many transgenic plants have been obtained using specific *Agrobacterium* strains (Hiei et al., 1994; Rashid et al., 1996; Cheng et al., 1997; Tingay et al., 1997; Ishida et al., 1996; Arencibia et al., 1998). The progress of transformation research in *Allium* is summarized in Table 2. It is already more than ten years since Klein et al. (1987) first developed a high-velocity microprojectile method and demonstrated that epidermal tissue of onion could take up foreign DNA sequences. Wang (1996) obtained transgenic leek plants by particle bombardment with *barnase* gene and *barstar* gene, and it was shown that the genes were present in the leek genome. Dommisse et al. (1990) demonstrated that onion is also a host for *Agrobacterium* as evidenced by tumorigenic responses and opine production inside these tumours. Recently, successes have been reported in the transformation of immature embryos of *A. cepa* using *Agrobacterium tumefaciens* (Eady et al., 2000).

Molecular characterization of transgenic plants

Characterization of transgenic plants can be carried out by histochemical, molecular or genetic analysis. The histochemical assays, GUS or Green Fluorescent Protein (GFP) demonstrate expression of introduced DNA sequences and activity of the foreign gene

Table 1. Successful *in vitro* plant regeneration systems of *Allium* species

Initial material	Species	Type of regeneration	Reference
Mature embryo	*A. cepa*	Somatic embryogenesis	Van der Valk et al., 1992; Saker, 1998
Mature embryo	*A. cepa*	Organogenesis	Tanikawa et al., 1998
Mature embryo	*A. fistulosum*	Somatic embryogenesis	Shahin & Kaneko, 1986; Van der Valk et al., 1992
Mature embryo	*A. fistulosum* × *A. cepa*	Somatic embryogenesis	Shahin & Kaneko, 1986; Van der Valk et al., 1992
Mature embryo	*A. porrum*	Somatic embryogenesis	Van der Valk et al., 1992
Mature embryo	*A. ampeloprasum*	Somatic embryogenesis	Buiteveld et al., 1993; Schavemaker & Jacobsen, 1995
Mature embryo	*A. ampeloprasum*	Organogenesis and somatic embryogenesis	Silvertand et al., 1996
Immature embryo	*A. cepa*	Somatic embryogenesis	Eady et al., 1998a; 1998b
Immature embryo	*A. tuberosum*	Organogenesis	Xue et al., 1997
Shoot apex	*A. tuberosum*	Somatic embryogenesis	Mastsuda & Adachi, 1996
Shoot base	*A. tuberosum*	Organogenesis	Pandey et al., 1992
Shoot base	*A. porrum*	Somatic embryogenesis	Wang & Debergh, 1995
Shoot tip	*A. cepa*	Somatic embryogenesis	Phillips & Luteyn, 1983
Shoot tip	*A. cepa*	Organogenesis	Phillips & Hubstenberger, 1987
Shoot tip	*A. fistulosum*	Organogenesis	Phillips & Hubstenberger, 1987
Shoot tip	*A. fistulosum* × *A. cepa*	Organogenesis	Phillips & Hubstenberger, 1987
Shoot tip	*A. cepa* × *A. fistulosum*	Organogenesis	Phillips & Hubstenberger, 1987
Shoot sheath	*A. cepa*	Organogenesis	Kahane et al.,1992
Stem disc	*A. sativum*	Organogenesis	Ayabe & Sumi, 1998

Flower bud	*A. cepa*	Organogenesis	Jeong et al., 1998
Flower bud or ovary	*A. cepa*	Somatic organogenesis	Luthar & Bohanec, 1999
Flower stalk	*A. ampeloprasum*	Organogenesis	Silvertand et al., 1995
Inflorescence	*A. fistulosum* ×*A. cepa*	Somatic embryogenesis	Lu et al., 1989
Anther	*A. fistulosum*	Somatic embryogenesis	Saijn et al., 1994
Basal plate	*A. fistulosum* ×*A. cepa*	Somatic embryogenesis	Lu et al., 1989
Basal plate and receptacle	*A. sativum*	Somatic embryogenesis	Xue et al., 1991
Twin scales	*A. cepa*	Organogenesis	Hussey & Falavigna, 1980
Root tip	*A. sativum*	Organogenesis	Shuto et al., 1993; Haque et al., 1997; Myers & Simon, 1998; Barandiaran et al., 1999a; 1999b
Root tip	*A. sativum*	Somatic embryogenesis	Haque et al., 1998
Root tip	*A. tuberosum*	Organogenesis	Shuto et al., 1993
Suspension culture	*A. cepa*	Organogenesis	Tanikawa et al., 1998
Suspension culture	*A. fistulosum*	Organogenesis	Song & Peffley, 1994
Suspension culture	*A. fistulosum*	Somatic embryogenesis	Kim & Soh, 1996
Suspension culture	*A. fistulosum* ×*A. cepa*	Organogenesis	Song & Peffley, 1994
Suspension culture	*A. ampeloprasum*	Somatic embryogenesis	Buiteveld et al., 1994
Protoplast	*A. cepa*	Somatic embryogenesis	Hansen et al., 1995
Protoplast	*A. ampeloprasum*	Somatic embryogenesis	Buiteveld et al., 1994
Protoplast	*A. porrum*	Organogenesis	Schum et al., 1994
Protoplast	*A. sativum*	Organogenesis	Ayabe et al., 1995

Table 2. Genetic transformation research in different *Allium* species

Species	Target tissue	Transformation method	Result	Reference
A. cepa	Epidermal tissue	High-velocity microprojectiles	Transient expression of a foreign gene (*cat* gene)	Klein et al., 1987
A. cepa	Epidermal tissue	High-velocity microprojectiles	Transient expression of GFP	Scott et al., 1999
A. cepa	Bulb	*Agrobacterium tumefaciens*, *A. rhizogenes*, *A. rubi*	Tumorigenic response and opine production	Dommisse et al., 1990
A. cepa	Zygotic mature embryo after *in vitro* culturing for 12 days	*Agrobacterium tumefaciens*	Transient expression of *gusA*	Joubert et al., 1995
A. cepa	Microbulbs from germinating mature seeds, immature embryo after *in vitro* culturing for 14 days	Particle bombardment, *Agrobacterium tumefaciens*	Transient expression of *gusA*	Eady et al., 1996
A. cepa	Immature embryo	*Agrobacterium tumefaciens*	Stable expression of *nptII* and *m-gfp5-ER*	Eady et al., 2000
A. porrum	Embryogenic callus derived from shoot base	Particle bombardment	Stable expression of *gusA* and *bar*	Wang, 1996
A. sativum	Leaf, immature bulb, callus from basal plate	Particle bombardment	Transient expression of *gusA*	Barandiaran et al., 1998

products. Of the molecular methods, standard PCR only proves the evidence for the presence of a foreign gene sequences; Southern hybridization demonstrates the presence, integration and copy number. Genetic characterization entails checking of the progeny and gives additional information on presence, integration and genome organization (loci number). Progeny analysis is easy but time-consuming. Although Southern hybridization can be used to determine the presence of T-DNA and copy number, it becomes labour-intensive when many samples have to be assayed, and it consumes relatively large amounts of genomic DNA. This is especially true for large genome species, like lily, onion and tulip, where genome sizes are much more than a hundred times larger than *Arabidopsis* (Arumuganathan & Earle, 1991). Southern hybridization analysis for these large genome crops is very difficult. Recently, Watad et al. (1998) reported some success in analysing transgenic lily plants via Southern hybridization after microprojectile bombardment. However, the molecular characterization of transgenics via Southern hybridization remains difficult. Therefore, it is of importance to find an alternative technique for Southern hybridization to prove stable integration of alien DNA into the host genome in large genome species. Several approaches based on different PCR techniques to confirm stable integration have been put forward (Campisi et al., 1999; Devic, et al., 1997; Does et al., 1991; Liu et al., 1995; Mathur et al., 1998; McGarvey & Kaper, 1991; Padegimas & Reichert, 1998; Spertini et al., 1999; Trueba & Johnson, 1996; Zhou et al., 1997). By cloning and sequencing , foreign gene transfer and integration into the plant genome can be further analysed (Krizkova & Hrouda, 1998; Ponce et al., 1998). However, most of the aforementioned techniques have only been applied in *Arabidopsis* genome research. Therefore, it would be of value to test some of these techniques also in a large genome species like *A. cepa*.

Outline of the thesis

This thesis starts with the development of an *in vivo* and *in vitro* bio-assay to test whether sources of resistance are present in *A. cepa* and its crossable wild relatives (Chapter 2). Unfortunately, no high level of resistance was found and, therefore, the marker-assisted breeding approach to introduce beet armyworm resistance in *A. cepa* was abandoned. Consequently, the rest of the study was focussed on introduction of resistance via genetic transformation. Firstly, a reliable regeneration system (Chapter 3

and 4) was developed and secondly a reliable transformation system was established based on *Agrobacterium tumefaciens* as a vector (Chapter 5). A number of stably transformed onion and shallot plants were obtained and these transformants were molecularly characterized by means of standard PCR, Southern hybridization and FISH (Chapter 5). Because of the large genome size of *Allium* species, an alternative method was developed for the molecular genetic characterization of the transgenic plants obtained. A so-called adaptor ligation PCR (AL-PCR) technique followed by sequencing of the genomic DNA flanking the T-DNA borders was set up (Chapter 6). Chapter 7 provides a general discussion, and in this chapter aspects of the introduction of resistance to *S. exigua* in onions and shallots via molecular marker-assisted breeding and genetic transformation are discussed. Furthermore, the results obtained in this study are placed in perspective of future breeding activities in onions and shallots.

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Chapter 2

Development of bio-assays and screening for resistance to beet armyworm (*Spodoptera exigua* Hübner) in *Allium cepa* L. and its wild relatives

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Abstract

The beet armyworm (*Spodoptera exigua* Hübner) is the most important pest in tropical *Allium* cultivations. All shallot (*Allium cepa* L. group Aggregatum) cultivars are susceptible to this pest. Therefore accessions from three wild *Allium* species, namely *A. galanthum* Kar. et Kir., *A. fistulosum* L. and *A. roylei* Stearn, next to *A. cepa* L. were used to screen for resistance. First of all, a reliable bio-assay had to be developed. To this end transparent plastic cages with in total 5 plants of one accession per cage were placed on perlite in a heated greenhouse. Five 3-day old larvae were inoculated on each plant. Eight days after inoculation the number of surviving larvae per cage and the mean fresh weight per larva was determined. The lowest larval survival (36 %) was found on *A. roylei*. This was not, however, significantly different from other *Allium* accessions. Significant differences were found in the fresh weight per larva fed on different *Allium* accessions. The larvae survived on *A. roylei* had a very low fresh weight (10.3 mg per larva), while those on an accession of *A. fistulosum* had the highest fresh weight (45.1 mg per larva). The larval fresh weight on *A. roylei* was lower than all the other accessions except from the tropical shallot cultivar Bawang Bali.

To check whether or not a toxic compound was involved in the resistance present in *A. roylei*, ten accessions from four *Allium* species were screened. Five 3-day old larvae were inoculated on regularly replaced leaf material of each accession of *Allium* species. No significant differences were found in mean fresh weight per larva and mean survival of larvae among different accessions. There were also no significant differences in pupal weight and developmental time. All larvae became pupae 10 days after inoculation. The data indicate that there is no toxic compound present in *A. roylei*. These results are underlined by the observation in the greenhouse bio-assay that *A. roylei* plants were equally damaged by the beet armyworm compared to other *Allium* species.

The results obtained so far therefore suggest that introduction of resistance to *S. exigua* via the exploitation of variation for resistance to the beet armyworm in *A. roylei* is unclear and that genetic engineering using *Cry* sequences could provide a way forward.

Introduction

Both onion (*Allium cepa* L. group Common Onion) and shallot (*A. cepa* L. group Aggregatum) are very important vegetable crops on a worldwide scale. The cultivation of these crops is sometimes severely limited due to the occurrence of pests. The most important pest in *A. cepa* cultivation for (sub)tropical zones is the beet armyworm (*Spodoptera exigua* Hübner). The beet armyworm is an extremely polyphagous insect (Goh et al., 1991). Current methods for controlling *S. exigua* are the use of sex pheromone, pesticides and cultural measures. The success of sex pheromone in *Allium* cultivation until now is limited (Mitchell et al., 1983; Park & Goh, 1992; Takai & Wakamura, 1990; 1996; Wakamura et al., 1989; 1990; Yoo et al., 1995). Consequently, farmers are heavily dependent upon chemical control or cultural measures like handpicking. These practices provide some protection but still far from adequate. A problem with spraying chemical agents is that the caterpillars in the hollow leaves of onion and shallot are protected against contact insecticides. Furthermore, chemical control is expensive, causes environmental pollution, and is a threat to the health of farmers and consumers. Moreover insecticide resistance is reported to occur in *Spodoptera* (Brewer et al., 1990; Brewer & Trumble, 1991a; 1991b; Cheng & Kao, 1993). Sterile insect technique (SIT) has been successfully used to control the onion fly (*Delia antiqua* Meigen; Tichelaar et al., 1974; Theunissen et al., 1974; 1975). However, SIT is very laborious to apply and only effective when used by all farmers in an *Allium* cultivation area.

The introduction of resistance to *S. exigua* via genetic methods has not yet been carried out in an *A. cepa* breeding programme. Two strategies are open to introduce resistance to *S. exigua* into *A. cepa*, i.e. via conventional methods if reliable sources of resistance are available or via genetic engineering if a transformation system and gene constructs are present which confer resistance to *S. exigua*. The latter strategy has been applied in other crops (Adamczyk et al., 1998; Hardee & Bryan, 1997; Harris et al., 1996; Motoyama, 1998; Schuler et al., 1998; Stapel et al., 1998), but it is not yet an option for *Allium* because no transformation system is present for *Allium* (Eady, 1995; Eady et al., 1996). The focus in this paper is on the development of reliable bio-assays and screening strategy for resistance to *S. exigua* in *A. cepa* and its wild species.

Materials and methods

Plant material

Seeds of thirteen accessions of four *Allium* species, namely *A. cepa* L., *A. galanthum* Kar. et Kir, *A. fistulosum* L. and A. *roylei* Stearn (Table 1), were sown on in a greenhouse at 16 / 20°C (night / day) with additional light for 16 hours (approx. 100 $\mu E\ s^{-1}m^{-2}$; lamps used: SON-T 400 Watt). Several weeks later, seedlings were transplanted into pots ($8 \times 8 \times 8\ cm^3$). There was one plant in each pot and no insecticide was applied. Plants with 5-6 leaves were used in greenhouse screening and for toxic compound tests.

Table 1. *Allium cepa* and its wild relatives used to screen for resistance to *S. exigua*. Accessions 96181, 96179 and 97208 were not used in the toxic compound test. The number column is used in combination with Figure 3.

Species	CPRO-DLO Accession number	Number	Origin
A. cepa cv. Sturon	96209	1	Novartis Seeds, The Netherlands
A. cepa cv. Hyton	96211	2	Bejo Zaden, The Netherlands
A. cepa cv. Tropix	96181	3	Bejo Zaden, The Netherlands
A. cepa cv. Atlas	96179	4	Bejo Zaden, The Netherlands
A. cepa cv. Bawang Bali	97208	5	Indonesia
A. galanthum	97171	6	All 256/84, Gatersleben, Germany
A. galanthum	97200	7	Bot. Garden, Berlin, Germany
A. galanthum	97205	8	H.B. Alma-Ata, Kazahkstan
A. fistulosum	96201	9	Leningrad, Russia
A. fistulosum	96202	10	Japan
A. fistulosum	96203	11	Japan
A. fistulosum	96217	12	H.B. Odessa, Ukraine
A. roylei	97175	13	PI 243009 (C502), Beltsville USA

Spodoptera exigua

The Laboratory of Entomology, Wageningen University and Research Center, the Netherlands, kindly supplied the eggs of *S. exigua*. The eggs were maintained in the dark at 28°C in a continuous culture on an artificial diet (Poitout & Bues, 1974). Three days after incubation, the larvae were used for plant inoculation.

Figure 1. The physical set-up of greenhouse test for screening for resistance to *S. exigua*. Plant material was covered with transparent hard plastic cages and perlite was put around the cages and pots in order to prevent larval escape and to allow the larvae to climb back on a plant after falling off.

Greenhouse bio-assay

The experimental set-up underlying the greenhouse-screening test (Figure 1) was a randomized complete block design with 4 blocks. Each block contained one replication per accession with 5 plants (1 plant / pot) , which was put into one transparent hard plastic cage. Perlite was spread around the pots and cages to prevent larval escape and to allow the larvae to climb back on a plant after falling off. Five 3-day old larvae were inoculated with a paintbrush on the base of each plant. There were

a total of 25 larvae in each cage. Eight days after inoculation the number of surviving larvae per cage and the fresh weight of all larvae present per cage were determined. The screening for resistance was carried out in the greenhouse with ambient temperatures varying between 26-35°C.

Toxic compound bio-assay

The mean fresh weight of a larva after 8 days feeding on *Allium* leaves in a Petri dish (Figure 2) was determined in order to test whether a toxic compound was involved in the partial resistance. Ten of the thirteen accessions from four *Allium* species were tested. Three accessions were not tested because the leaves had died due to advanced developmental stage (Table 1). Four replications per accession were used, with each 3-4 gram fresh *Allium* leaves on 0.8 % sterile water agar in a Petri dish. Five 3-day old larvae were inoculated on each replication. Leaves and Petri dish with water agar were replaced every two days before day 5 and every day after day 5. The Petri dishes were covered under a layer of white paper to avoid larval exposure to bright light. The experiment was carried out at 25°C with a photoperiod of 16 h (lamps used: Philips TLD 50W/840HF, Electronic NG). The light intensity measured under the layer of white paper was approx. 20 $\mu E\ s^{-1}m^{-2}$. Eight days after inoculation the percentage of surviving larvae per Petri dish and their fresh weight of all surviving larvae per Petri dish were determined.

In addition, one accession from each *Allium* species was used in another experiment with 4 replications (Petri dishes) for testing the fresh weight of pupae and the number of days to pupation. Conditions were the same as described as above, but after day 5 *Allium* leaves and sterile Petri dish with water agar were replaced every day until pupation.

Statistical analyses

The untransformed data were analysed by means of analysis of variance (ANOVA). Multiple comparison testing took place via the Student-Newman-Keuls test. The statistical package $SPSS^X$ (SPSS Inc, 1986) was used for the analyses of the data of both bio-assays.

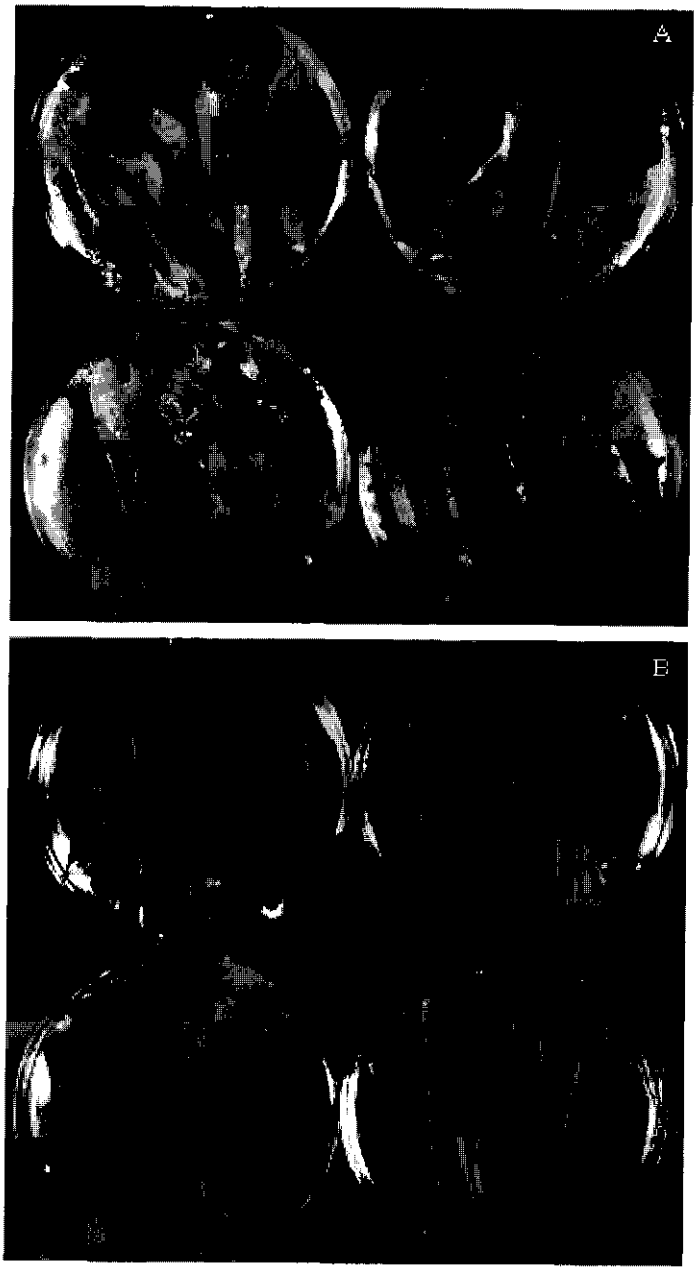

Figure 2. Toxic compound test. (A). Eight days after inoculation, from left to right, upper case: *A. cepa* and *A. galanthum;* lower case: *A. fistulosum* and *A. roylei.* (B). Ten days after inoculation, from left to right, upper case: *A. cepa* and *A. galanthum;* lower case: *A. fistulosum* and *A. roylei.*

Results

Greenhouse bio-assay

Three days after incubation on artificial diet, the length and fresh weight of an individual larva were about 3 mm and 0.38 mg, respectively. After inoculation the larvae started to climb to the top of the leaf. Often larvae let themselves down from the leaves to the ground via a thread, but they returned mostly quite quickly to the plant. In general, the tops of the leaves were first damaged (Figure 3A). All thirteen accessions of the four *Allium* species were equally damaged eight days after the start of the experiment (Figure 3B). The mean number of surviving larvae and the mean fresh weight per larva on the various *Allium* accessions are plotted in Figure 4. The lowest mean number of surviving larvae (9.0 per cage) was on *A. roylei* and the highest mean number of surviving larvae (19.0 per cage) on A. *fistulosum* (accession 96201), however, no significant differences were found (Figure 4). The mean fresh weight of a larva differed significantly among the various *Allium* accessions (ANOVA: $F_{[12,\ 39]} = 9.08$, $P < 0.001$). On *A. roylei* the surviving larvae had statistically significantly the lowest mean fresh weight (10.3 mg per larva). Only *A. cepa* cv. Bawang Bali did not differ from *A. roylei* with respect to larval fresh weight (Table 2).

Toxic compound bio-assay

At first, the larvae ate few leaves, so the leaves were replaced every second day. From the fifth day larvae developed faster and therefore, the leaves had to be replaced every day. No significant differences were found in mean fresh weight of larvae and survival of larvae among the 10 different accessions of the four *Allium* species tested (Table 3 & Figure 2A). Ten days after inoculation pupae appeared in all tested accessions of *A. cepa, A. galanthum, A. fistulosum* and *A. roylei* (Figure 2B). No significant differences among the various *Allium* species tested for pupal fresh weight were observed (data not shown).

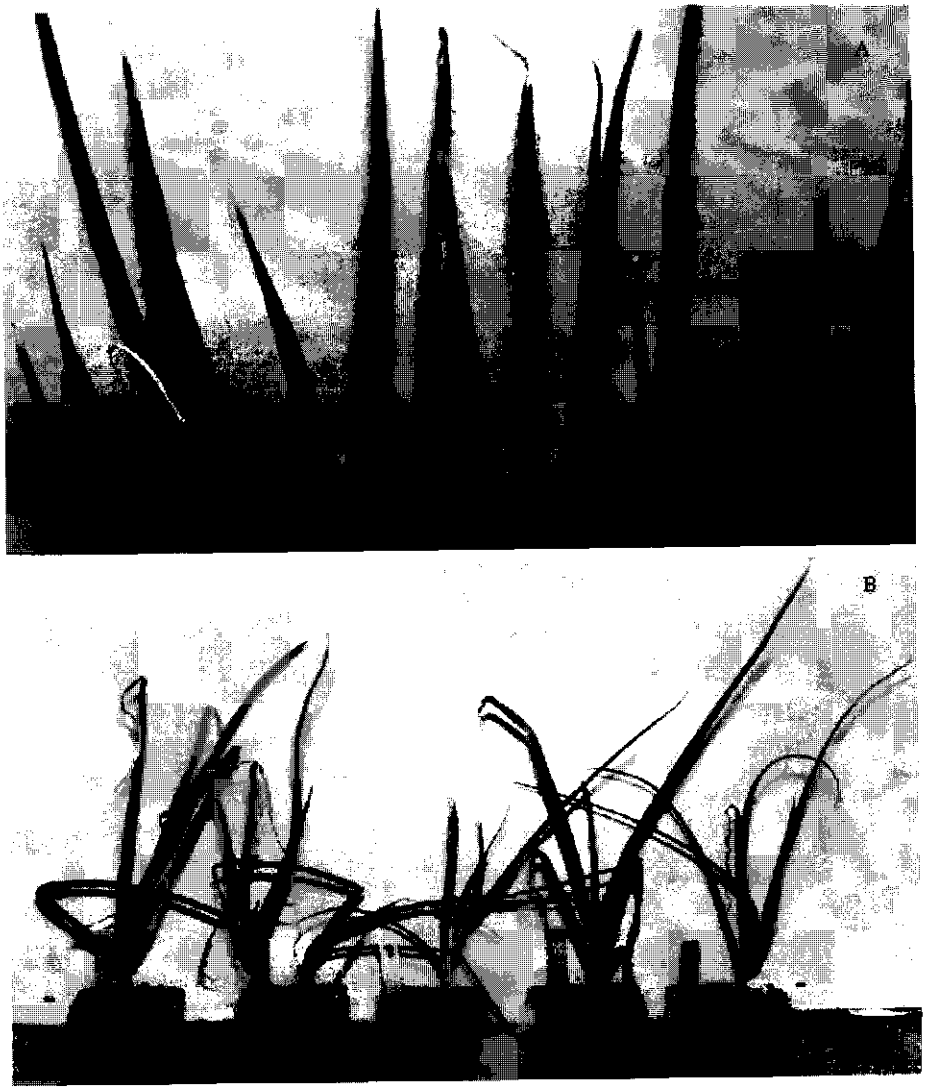

Figure 3. Greenhouse test for screening for resistance to *S. exigua*. (A). Damage of the tops of the leaves of 5 plants by *S. exigua* larvae. (B). Damaged plants, eight days after inoculation, from left to right, *A. cepa* (onion), *A. cepa* (shallot), *A. galanthum, A. fistulosum* and *A. roylei*.

Table 2. Student-Newman-Keuls (SNK) multiple range test (P = 0.05) on the mean larval fresh weight (mg) of *Spodoptera exigua* larvae when fed for 8 days on different accessions of *A. cepa* and its wild relatives in greenhouse screening test. Identical letters indicate that there is no significant difference between accessions.

Species	CPRO-DLO Accession No.	Mean fresh weight (mg)	SNK multiple range				
A. roylei	97175	10.3	A				
A. cepa cv Bawang Bali	97208	13.7	A	B			
A. cepa cv Atlas	96179	22.0		B	C		
A. cepa cv Tropix	96181	25.8			C	D	
A. fistulosum	96203	26.5			C	D	E
A. fistulosum	96202	27.8			C	D	E
A. galanthum	97205	29.0			C	D	E
A. cepa cv. Hyton	96211	30.4			C	D	E
A. galanthum	97200	30.5			C	D	E
A. galanthum	97171	30.7			C	D	E
A. cepa cv. Sturon	96209	36.4			C	D	E
A. fistulosum	96201	38.1				D	E
A. fistulosum	96217	45.1					E

Table 3. Mean fresh weight (mg) and survival (%) of *S. exigua* larvae on fresh leaves from various *Allium* accessions 8 days after inoculation in toxic compound test.

Species	CPRO-DLO Accession number	Mean fresh weight (mg)	Mean Survival (%)
A. cepa cv. Sturon	96209	155.7	90
A. cepa cv. Hyton	96211	153.9	100
A. galanthum	97171	126.3	70
A. galanthum	97200	140.6	90
A. galanthum	97205	119.7	85
A. fistulosum	96201	112.5	90
A. fistulosum	96202	135.2	65
A. fistulosum	96203	131.1	95
A. fistulosum	96217	110.2	70
A. roylei	97175	143.0	95

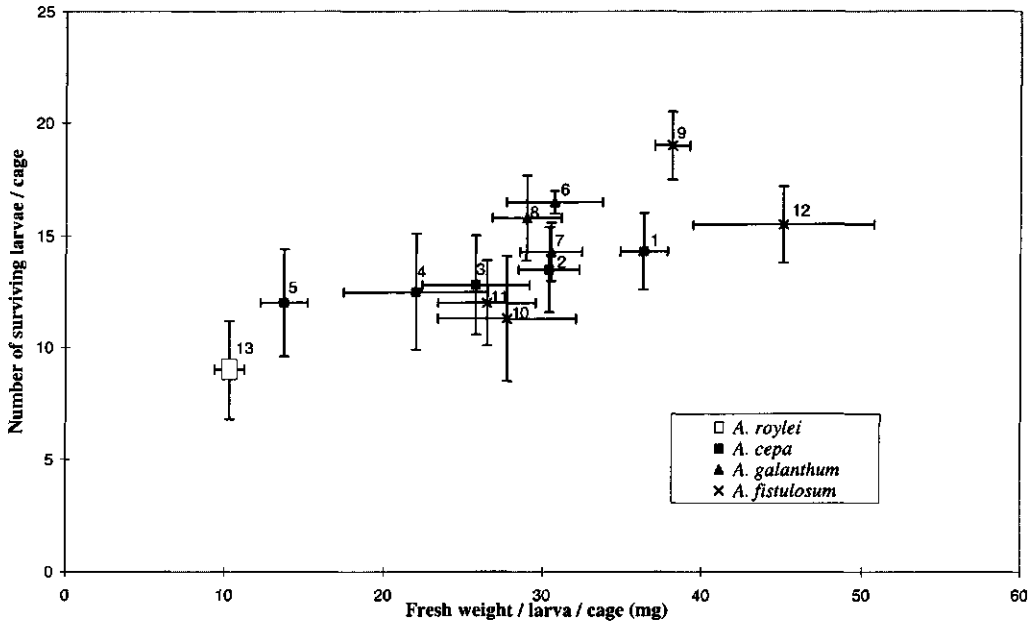

Figure 4. The mean larval fresh weight (mg; X-axis) plotted against the mean number of surviving larvae (Y-axis) in *Allium cepa* and its wild relatives. The standard error of the mean is indicated.

Discussion

This is the first report on screening for resistance to *Spodoptera exigua* in *Allium* species. First of all bio-assays had to be developed. The greenhouse bio-assay proved to be most valuable because it mimicked the natural circumstances experienced by the beet armyworm to a large extent. Moreover this bio-assay with its transparent plastic cages allowed us to observe the behaviour of the beet armyworm on *Allium* plants.
The test was aimed at the identification of resistance to *S. exigua* in *A. cepa* and its wild relatives. A number of factors were important for the set-up of a reliable and quick biotest. First of all, the age of the larvae is critical. When larvae were used immediately after hatching, they were very fragile and could be easily damaged or killed during inoculation with a paintbrush. Three days old larvae were most suitable for inoculation. A second factor is the age of the *Allium* plant: young plants (< 3 leaves) or fully-grown plants (> 15 leaves) were difficult to use. The young plants were totally destroyed by the larvae. Damage to fully-grown plants caused by the larvae was difficult to compare among the *Allium* species. Therefore the plants used in this study had 5-6 leaves. A third factor is the physical set-up of the experiment. Beet armyworm larvae can easily fall off the leaves of the *Allium* plants after inoculation. Therefore they must be prevented from escaping and must be able to return to the plants present in the cage after falling off. At the end of the experiment some of the larvae were on the ground, so for detecting them it is necessary that the soil substrate contrasts with the colour of the larvae. Perlite (white) was an adequate soil substrate for detecting the green caterpillars and also for helping larvae to return to the plants when falling off.

In the greenhouse screening test in which *S. exigua* larvae were fed on 5-6 leaf stage *Allium* plants, it was shown that no differences among the *Allium* species were present with respect to the survival of the larvae, however the lowest survival was found on *A. roylei*. Larval growth proved to be significantly slower on *A. roylei* compared to all the other *Allium* species, except the tropical shallot cultivar Bawang Bali. Furthermore in the toxic compound bio-assay in which larvae of *S. exigua* were fed with *Allium* leaves, no significant differences were found in larval growth and survival and also not in pupal weight and development time to the pupal stage. Thus this test suggests that the resistance present in *A. roylei* was probably not based on a toxic compound. Also the resistance present in the shallot cultivar Bawang Bali was not based on a toxic compound since we observed in the greenhouse test that all

Allium accessions were equally damaged (data not shown). The mechanism of the resistance in *A. roylei* may be due to its deviant leaf morphology when compared with the other *Allium* accessions: *A. roylei*, has solid leaves with a small diameter, whereas the other three *Allium* species have hollow leaves with a large diameter. The absence of the leaf cavity in *A. roylei* might disturb the feeding behaviour of the beet armyworm. The beet armyworm normally moves towards the top of the leaf when placed on the base of an onion or shallot plant and feeds on the outside of the leaves. The beet armyworm makes holes in the leaf, enters the leaf cavity and continues feeding inside the leaf. In *A. roylei* this behaviour is not possible due to the absence of the leaf cavity and consequently the insect can only feed on the outside of the leaf. We also hypothesize that the slender leaf type of *A. roylei* might be another factor contributing to the resistance, because caterpillars could fall off more easily from slender leaves compared to the large onion and shallot leaves. The larvae on *A. roylei* leaves apparently have to spend more energy for climbing and consequently spend less time for feeding. This hypothesis, however, proved to be incorrect because the time spent by the larvae on the leaves of both *A. roylei* and *A. cepa* species was the same (data not shown).

The value of the *A. roylei* source of resistance is unclear at present. On the one hand we have shown under greenhouse conditions that larval fresh weight and survival on *A. roylei* is not statistically significantly different from the tropical shallot cultivar Bawang Bali. On the other hand we expect that in the field an *A. roylei* type of leaf could be beneficial for preventing glutton because predators can more easily locate caterpillars on the outside than on the inside of a leaf and secondly contact insecticides have more effect. Therefore to analyse the resistance present in *A. roylei* more precisely, the next step in this research has to be the evaluation of *A. roylei* under tropical field conditions. However given the fact that the resistance present in *A. roylei* is only partial we also will try to develop transgenic shallots, which are completely resistant to the beet armyworm. To this end a reliable regeneration protocol has already been developed (Zheng et al., 1998; 1999) and currently we are developing a transformation protocol.

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Chapter 3

Factors influencing induction, propagation and regeneration of mature zygotic embryo-derived callus from *Allium cepa* L.

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Keywords: Callus production, line selection, onion, plant regeneration, shallot

Abstract

A systematic study on the effects of subspecies, cultivar, basal medium, sucrose concentration and 2,4-dichlorophenoxyacetic acid concentration on callus induction, propagation and subsequent plant regeneration in *Allium cepa* has been carried out. Mature zygotic embryos from two onion (cvs. Sturon and Hyton) and two shallot (cvs. Tropix and Atlas) varieties were used as explants. After callus initiation and growth on both Murashige and Skoog (MS) and Gamborg's B5 modified by Dunstan & Short (BDS) basal media with different 2,4-dichlorophenoxyacetic acid and sucrose concentrations for eight weeks, lines were identified on which compact or friable callus was induced. Callus induction and propagation were largely determined by the concentration of 2,4-dichlorophenoxyacetic acid whereas subspecies, cultivar, sucrose concentration and basal media were of less importance. After callus propagation for twelve weeks, 315 lines from a total of 3348 embryo's initially subcultured were selected to test their regeneration capacity on growth regulator-free medium. It was found that shallot formed more shoots and roots than onion. The MS basal medium proved to be more beneficial for shoot regeneration and root formation than the BDS basal medium. There were no differences in plant regeneration among selected calli which had been previously subcultured on different concentrations of 2,4-dichlorophenoxyacetic acid and sucrose. The results show that plant regeneration strongly depended on the line: 45.4 % from 315 tested lines could produce shoots while 93.0 % formed roots.

Abbreviations: BDS - Gamborg's B5 medium modified by Dunstan & Short (1977); 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog (1962) medium; MS30 - a growth regulator-free MS basal medium with 30 g/l sucrose

Introduction

An efficient genetic transformation system is vital for the further improvement of modern crops. In onion (*Allium cepa* var. *cepa*) and shallot (*A. cepa* var. *aggregatum*) such a system has not yet been developed, although both crops are considered very important vegetables on a worldwide scale. A prerequisite for the development of a genetic transformation system is the availability of an efficient callus induction and plant regeneration protocol.

Somatic embryogenesis and plant regeneration have been observed in callus cultures of *A. cepa* (Dunstan & Short, 1977; 1978; Phillips & Luteyn, 1983; Van der Valk et al., 1992), *A. fistulosum* (Phillips & Hubstenberger, 1987; Van der Valk et al., 1992), species hybrids between *A. cepa* and *A. fistulosum* (Shahin & Kaneko, 1986; Phillips & Hubstenberger, 1987; Lu et al., 1989; Peffley, 1992; Van der Valk et al., 1992) and *A. porrum* (Van der Valk et al., 1992; Buiteveld et al., 1993; Wang & DeBergh, 1995; Silvertand et al., 1996). Regeneration of plants from suspension cultures has been reported for *A. fistulosum* (Song & Peffley, 1994; Kim & Soh, 1996) and for the hybrid between *A. fistulosum* and *A. cepa* (Song & Peffley, 1994). Plant regeneration has been observed at high frequency using embryogenic suspension-derived protoplasts of *A. porrum*; however, the plating efficiency was extremely low (Buiteveld et al., 1994). Hansen et al. (1995) obtained regeneration of shoots from cell suspension-derived protoplasts of *A. cepa*.

To date, no systematic study has been reported in *A. cepa* on the effects of subspecies (onion and shallot), cultivar, basal medium, sucrose concentration and 2,4-D concentration on callus induction, callus propagation and subsequent plant regeneration. The outcome of such a study is presented here and will form the basis for further research on the development of a reliable transformation protocol for *Allium*.

Material and methods

Explant material

Two onion cultivars (Sturon and Hyton) and two shallot cultivars (Tropix and Atlas) were supplied by Bejo-de Groot en Slot, Noord-Scharwoude, The Netherlands. Mature

seeds were sterilized according to the method of Van der Valk et al. (1992). The embryos were aseptically excised using a stereo microscope. The part of the embryo containing the radicle, the shoot apex and the lower part of the cotyledon (Van der Valk et al., 1992) was separated from the main part of the cotyledon and cultured in a 6-cm Petri dish containing 10 ml callus induction medium. It is known that changes on the DNA can occur during *in vitro* culture (Cecchini et al., 1992; Zheng, 1992; Puolimatka & Karp, 1993). Therefore we consider callus cultures which are derived from a single embryo as a line.

Media and culture conditions

For callus induction and growth on solidified media, both MS and BDS basal media with different 2,4-D and sucrose concentrations were used. A total of 18 treatments, from MS10-1 to BDS30-5 (Table 1), were tested for each cultivar. Callus was induced and propagated at 25°C in the dark. Propagation of individual lines was performed on the same medium on which the callus was originally induced.

For testing the plant regeneration potential of each line, MS medium with 30 g/l sucrose (MS30) was used without any plant growth regulator, at 25°C with a 16h photoperiod (ca. 45 TE m^{-2} s^{-1} ; lamps used: Philips, TLD 50W/840HF, Electronic NG). To allow for a quantitative determination of the regeneration capacity, calli from each line were carefully divided into equal parts (5 × 5 mm) and 10 units of callus (about 100 mg per unit) were placed on regeneration medium. Shoot regeneration and root formation of each line were recorded after two months. In all media, 0.4 % phytagel (Sigma) was used as the solidifying agent. Media were adjusted to pH 5.8 prior to autoclaving (103 kPa, 121°C; 20 min).

Statistical analysis

The experimental set-up used to analyse this single unreplicated experiment on callus induction and propagation was a five factorial design with subspecies, cultivar, basal medium, sucrose and 2,4-D as the main parameters (See also Table 1). The subspecies, cultivar, basal medium, sucrose and 2,4-D factors had 2 (onion and shallot), 4 (Sturon, Hyton, Tropix and Atlas), 2 (BDS and MS), 3 (10, 20 and 30 g/l) and 3 [4.52 µM (1 mg/l), 9.05 µM (2 mg/l), 22.62 µM (5 mg/l)] levels, respectively. Five embryo's were cultured per dish for callus induction. At least 10 dishes were used for callus induction per treatment. A total of 3348 embryo's from four cultivars

were inoculated on different media. The statistical package SPSS^X (subprograms ANOVA and NPAR TESTS; SPSS Inc, 1986) was used for the analyses of the data.

Table 1. Effect of basal medium, sucrose and 2,4-D on callus induction and growth for the onion cultivar Sturon. Scoring took place after eight weeks. MS10-1 treatment represents MS basal medium supplemented with 10 g/l sucrose and 4.52 μM (1 mg/l) 2,4-D, while e. g. BDS20-5 treatment represents BDS basal medium supplemented with 20 g/l sucrose and 22.62 μM (5 mg/l) 2,4-D. The other treatments are coded in a similar way. The percentage is indicated in parentheses.

Treatment	Number of inoculated embryos	Number of lines which produced compact or friable callus
MS10-1	50	12 (24.0)
MS10-2	45	5 (11.1)
MS10-5	50	0 (0.0)
MS20-1	50	15 (30.0)
MS20-2	48	5 (10.4)
MS20-5	50	0 (0.0)
MS30-1	99	21 (21.2)
MS30-2	50	2 (4.0)
MS30-5	60	0 (0.0)
BDS10-1	50	22 (44.0)
BDS10-2	50	16 (32.0)
BDS10-5	49	0 (0.0)
BDS20-1	50	15 (30.0)
BDS20-2	50	9 (18.0)
BDS20-5	50	0 (0.0)
BDS30-1	50	29 (58.0)
BDS30-2	50	14 (28.0)
BDS30-5	44	1 (2.3)

The effect of the concentration of 2,4-D on callus induction and growth was highly significant (ANOVA, $P < 0.001$).

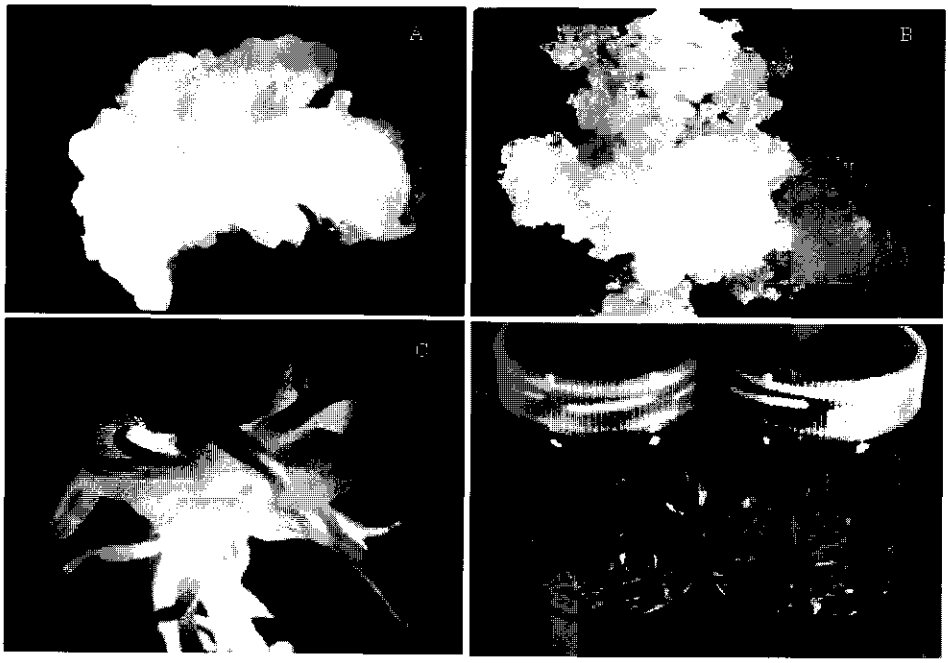

Figure 1. Callus type and plant regeneration from onion and shallot. (A). A compact, white and nodular type of callus. (B). A friable type of callus with no apparent structure. (C). Regenerated shoots obtained from shallot. (D). Numerous green plants from onion and shallot (the left onion and the right shallot).

Results

Callus production and selection

A total of 3348 embryo's from four cultivars were subjected to 18 different treatments (Table 1). Callus formation from embryos could be observed after four to five days of culture. Three morphologically different callus types could be easily distinguished: (1) a compact, white and nodular type (Figure 1A); (2) a friable type with no apparent structure (Figure 1B); and (3) a watery, transparent type. Compact callus was prominent at the site of the shoot apex whereas friable callus was abundantly

produced by the cotyledon part. During callus initiation and growth, a mucilaginous substance was sometimes produced. After eight weeks of callus induction, lines which had formed compact or friable callus were selected and subcultured on the same fresh media. In this paper, we only present the callus production results of cultivar Sturon because the other cultivars (Hyton, Tropix and Atlas) showed similar responses. The data listed in Table 1 demonstrate the effects of the basal media, sucrose and 2,4-D concentrations on callus formation of the cultivar Sturon. The effect of the concentration of 2,4-D on callus induction and growth was highly significant: 4.52 µM (1 mg/l) 2,4-D proved to be the best, 9.05 µM (2 mg/l) gave an intermediate response and subculture on 22.62 µM (5 mg/l) was generally unsuccessful. On average, 33.3 %, 14.0 % and 0.3 % of the lines from the four cultivars used had good callus production at 4.52 µM , 9.05 µM and 22.62 µM 2,4 -D, respectively. Lowering the concentration of 2,4-D to 2.26 µM (0.5 mg/l) gave a similar response as 4.52 µM 2,4-D, but 0.45 µM (0.1 mg/l) 2,4-D significantly reduced callus induction and growth (data not shown). Cultivar, basal medium composition and sucrose concentration effects proved to be of minor importance and interactions between the different factors were also not significant. Furthermore, onion and shallot did not differ in callus induction and growth.

Compared with other monocots such as rice (Mikami & Kinoshita, 1988), callus growth in *Allium* was very slow. Based on the amount of callus produced by each line (at least doubling of the volume in 4 weeks), the colour (white) and the quality of the callus (absence of the mucilaginous substance), 526 lines were selected after callus growth for eight weeks. After another four weeks of subculture on the same medium, there was a second selection step with the same criteria as mentioned above and 315 lines were chosen for further experiments. At this stage, all calli had become of the nodular and compact type. The calli from each selected line were divided into two parts: one part of the callus was propagated in the same medium and the other part of the callus after having been subdivided as described in 'Material & Methods' was placed on a growth-regulator-free medium (MS30) to test the regeneration potential.

Plant regeneration

From the 315 selected lines of the four cultivars, 143 lines (45.4 %) produced shoots while 293 lines (93.0 %) formed roots (Table 2). Regenerated plants with normal leaves and roots have been obtained via somatic embryogenesis and organogenesis. However, some lines did not respond. There were significant differences in shoot regeneration potential among the four cultivars. The shallot cultivars Tropix and Atlas

proved to have a significantly higher shoot and root regeneration capacity compared to the onion cultivars Sturon and Hyton (Figure 1C; Table 2). Of the shallot cultivars, 48.8-87.5 % lines gave shoots as compared to 5.3-44.4 % of the lines of the onion cultivars. 81.3-94.4 % lines from onion lines showed root formation, but for the shallot cultivars, all lines produced roots. Shoot regeneration and root formation from callus which was induced and subsequently subcultured on the MS basal medium were significantly higher than that of callus which was induced and subcultured on the BDS basal medium (Table 3).

Callus derived from different 2,4-D and sucrose levels showed no differences with respect to plant regeneration. After two months of subculture on full-strength regeneration medium, the plantlets were transferred to a half-strength regeneration medium for further maintenance and root development. The plantlets quickly produced new roots after 3-5 days and subsequently developed into whole normal plants (Figure 1D). The regenerated plants were successfully transferred (100 %) to a greenhouse with an ambient temperature of 22 °C.

Table 2. Onion and shallot lines with shoot and root formation on growth regulator-free MS30 medium after two months. The percentages of lines with shoot and root formation are indicated in parentheses.

Cultivar	Callus derived from original medium	Number of lines with shoot formation (%)	Number of lines with root formation (%)
Sturon	BDS	3 (5.3)	48 (84.2)
	MS	16 (44.4)	34 (94.4)
Hyton	BDS	7 (21.9)	26 (81.3)
	MS	11 (30.6)	31 (86.1)
Tropix	BDS	21 (48.8)	43 (100.0)
	MS	37 (71.2)	52 (100.0)
Atlas	BDS	13 (68.4)	19 (100.0)
	MS	35 (87.5)	40 (100.0)
Total		143 (45.4)	293 (93.0)

The shallot cultivars (Tropix and Atlas) proved to have a significantly higher shoot and root regeneration capacity compared to the onion cultivars. Both Mann-Whitney tests: $n_1 = n_2 = 4$, U=0, P = 0.014, respectively.

Table 3. Effect of basal medium used for callus induction and subculture on onion and shallot shoot and root formation on growth regulator-free MS30 medium. The percentages are in parentheses.

Basal medium	Total number of lines tested	Number of lines with shoot formation (%)	Number of lines with roots (%)
BDS	151	44 (29.1)	136 (90.1)
MS	164	99 (60.4)	157 (95.7)

Shoot regeneration and root formation from callus which was induced and subsequently subcultured on the MS basal medium were significantly higher than that of callus which was induced and subcultured on the BDS basal medium. $\chi_1^2 = 30.80$, $P < 0.001$; $\chi_1^2 = 3.97$, $P < 0.05$, respectively.

Discussion

This is the first study in *Allium* tissue culture which systematically compares the effects of subspecies, cultivar, basal medium, sucrose and 2,4-D levels on callus induction, callus propagation and subsequent plant regeneration. The results demonstrate clearly that the 2,4-D concentration is the most important determining factor for callus production and later plant regeneration in *A. cepa*. We observed that for callus induction a 2,4-D concentration between 2.26 - 4.52 µM was most effective. Similar result had also been found by Silvertand et al. (1996) who showed that for leek (*A. porrum*) low 2,4-D levels 1.13 - 2.26 µM (0.25 - 0.5 mg/l) were required for callus induction and subsequent regeneration of shoots. Contrary to the expectations (Dunstan & Short, 1977; Hansen et al., 1995; Phillips & Luteyn, 1983; Phillips & Hubstenberger, 1987), no significant differences between the MS and BDS basal media were found for *A. cepa* tissue culture. In future, the MS basal medium will be used because less mucilaginous substance was produced on this medium compared to the BDS medium. This type of mucilaginous material is known for its negative effect on callus growth and somatic embryo induction: a phenomenon which was also observed in leek immature embryo cultures (Buiteveld et al., 1994). Furthermore, callus which had been induced and subcultured on the MS medium produced significantly more shoots and roots than callus which originated from a subculture on the BDS medium during plant regeneration on the MS30 medium.

The present study also shows that different concentrations of sucrose (10, 20 and 30 g/l) had no significant effect on the tissue culture response and plant regeneration of onion and shallot. However, Buiteveld et al. (1993) observed that the maximum frequency of explants producing compact callus was obtained when embryos were cultured on the MS medium with 30 g/l sucrose. On the other hand, in maize the formation of friable callus was increased when the sucrose concentration in the medium was decreased from 12% to 2% (Shillito et al., 1989; Vasil & Vasil, 1984). The callus types that we identified in this study were sometimes heterogeneous and consisted of a mixture of different types. In our experiments three callus types were identified at the first four weeks after callus initiation; namely a compact, white and nodular type; a friable type with no apparent structure; and a watery, transparent type. In this study only compact and friable callus types were selected. After eight to twelve weeks, all callus turned into the compact type which was similar in appearance to that obtained for gramineous species (Vasil, 1985) and other *Allium* species (Buiteveld et al., 1994; Phillips & Luteyn, 1983; Van der Valk et al., 1992; Wang & Debergh, 1995).

In *Allium* tissue culture research, the common practice for regeneration induction is to use MS or BDS medium supplemented with cytokinins (Buiteveld et al., 1993; Lu et al., 1989; Shahin & Kaneko, 1986; Silvertand et al., 1996; Van der Valk et al., 1992; Wang & Debergh, 1995). However, in our experiments with onion and shallot it was found that the growth regulator-free medium (MS30) proved to be suitable for identifying lines which had a good plant regeneration capacity. It was possible to identify 143 lines with a superior regeneration potential among the 315 lines tested. Similar to results presented by other authors (Buiteveld et al., 1993; Phillips & Hubstenberger, 1987; Van der Valk et al., 1992; Wang & Debergh, 1995), we found that callus induction and plant regeneration in *Allium* species are strongly embryo dependent. Genetic factors as well as physiological factors during seed development might play a role.

In conclusion, we have established an efficient procedure for callus induction, propagation and plant regeneration for mature zygotic embryos of onion and shallot, and we have shown that we can obtain regeneration in 45.4 % of the lines using the best combination of factors influencing tissue culture response in *Allium*. The development of this protocol is thought to be of vital importance for the next step in our research, which is the development of a reliable transformation protocol.

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Chapter 4

Effect of cytokinins and lines on plant regeneration from long-term callus and suspension cultures of *Allium cepa* L.

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Key words: Line, onion, regeneration, shallot; suspension culture

Abstract

Suspension cultures were initiated from callus cultures of *Allium cepa*, which had been precultured on a solidified medium for 7 months. For another 3 months the 83 callus lines were kept in suspension culture. Each line is derived from a single zygotic embryo, 24 lines from onion and 59 lines from shallot. Of these, 20 suspension lines showed adequate growth and were used to test the effect of cytokinins on plant regeneration. On average, 1.25 % of the calli produced shoots on a growth regulator-free medium (MS30: MS basal medium supplemented with 30 g/l sucrose). Thus plant regeneration decreases significantly with time because the overall average plant regeneration frequency was 35.5 % after 3 months of culture on solidified medium. Plant regeneration after 3 months proved not to be a reliable predictor of plant regeneration after 10 months *in vitro* culture. Overall only 0.25-0.88 % of the calli produced shoots when the regeneration medium was supplemented with different types and concentrations of cytokinins. Contrary to expectations, the type and concentration of cytokinins could not increase the shoot regeneration capacity. However as expected, the increasing cytokinin concentration (especially TDZ at the range of 1-5 mg/l) led to a decrease in root formation. Plant regeneration proved to be highly dependent upon the line used. The best line was atm24 with an overall shoot regeneration capacity of 4.62 % (among the 13 treatments); it had its highest shoot regeneration on MS30 treatment with 12.5 %. The results obtained show that for the development of a reliable transformation protocol only young callus material (< 3 months), which has still a high regeneration potential, can be used.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; BDS, Gamborg's B5 medium modified by Dunstan & Short (1977); MS, Murashige & Skoog (1962) medium; MS30, a growth regulator-free MS basal medium with 30 g/l sucrose; TDZ, thidiazuron (N-phenyl-N-1,2,3-thidiazol-5-yl urea); ZR, zeatin riboside; ZT, zeatin.

Introduction

Plant growth regulators play an essential role in *in vitro* culture. In monocots such as cereal crops, the addition of cytokinin can be significant for plant regeneration (Bhaskaran & Smith, 1990). According to their chemical structure, cytokinins can be classified into at least two broad groups, namely adenine derivatives and phenylurea derivatives (Mok et al., 1987). Kinetin, 6-benzylaminopurine, N^6 -(2-isopentenyl) adenine, zeatin and zeatin riboside belong to adenine-type cytokinins while thidiazuron is a representative of the phenylurea derivative group. These cytokinins are all known to stimulate plant regeneration (Bhaskaran & Smith, 1990; Böhmer et al., 1995; Fellman et al., 1987; Lin et al., 1997; Mok et al., 1987; Sarwar & Skirvin, 1997). In *Allium* tissue culture, MS (Murashige & Skoog, 1962) or BDS (Gamborg's B5 modified by Dunstan & Short, 1977) medium supplemented with a cytokinin is normally used for plant regeneration, but auxins are not so important as cytokinins for regeneration (Buiteveld et al., 1993; Hansen et al., 1995; Phillips & Luteyn, 1983; Shahin & Kaneko, 1986; Song & Peffley, 1994; Van der Valk et al., 1992; Wang & Debergh, 1995). However until present, no systematic study has yet been reported on *A. cepa* suspension cultures with different types and concentrations of cytokinins to stimulate plant regeneration.

A prerequisite for the success of gene transformation techniques is the presence of an effective tissue culture system. Suspension cultures are generally more preferable than solid support systems due to the fact that callus growth, especially in *Allium*, is very slow on solidified medium. For onion, only Hansen et al. (1995) obtained plant regeneration using protoplasts derived from suspension cultures, however the regeneration capacity was low. The other two studies on this subject in onion namely those of Song & Peffley (1994) and Karim & Adachi (1997) did not result in any plant regeneration from suspension cultures. For *A. fistulosum*, a related species of onion, Song & Peffley (1994), Hansen et al. (1995) and Kim & Soh (1996) reported on the regeneration of plants from suspension cultures. Buiteveld et al. (1994) and Schum et al. (1994) achieved plant regeneration in leek (*A. porrum*) using embryogenic suspension-derived protoplasts. For garlic (*A. sativum*), there are no reports on successful regeneration from suspension cultures (Nagasawa & Finer, 1988).

We have established an effective procedure for callus induction, callus propagation and plant regeneration starting from mature zygotic embryos of onion and shallot (Zheng et al., 1998). Our main objective was to identify suitable lines with sustained high regenerating ability from long-term callus cultures and suspension cultures for

genetic transformation research. Here, we present a systematic study for *A. cepa* on the establishment of suspension cultures of onion and shallot from callus lines and the effect of different types and concentrations of cytokinins on regeneration capacity.

Material and methods

The establishment of suspension cultures

Callus induction, callus propagation and callus line selection procedure from mature zygotic embryos of two cultivars of onion (Sturon and Hyton) and two cultivars of shallot (Tropix and Atlas) have been described earlier (Zheng et al., 1998). Each callus line is derived from a zygotic embryo. It is known that changes on the DNA can occur during *in vitro* culture (Cecchini et al., 1992; Zheng, 1992; Puolimatka & Karp, 1993). Therefore we consider callus cultures which are derived from a single embryo as a line. These lines showed different regeneration capacity which was determined earlier. After 7 months subculture on a solidified medium 83 lines from 4 cultivars were identified for the initiation of suspension cultures. About 2 g of callus were inoculated to a 68 mm × 66 mm plastic jar (Greiner) containing 20 ml of MS (Murashige & Skoog, 1962) or BDS (Gamborg's B5 modified by Dunstan & Short, 1977) liquid medium supplemented with 1 mg/l (4.52 μM) 2,4-D and 30 g/l sucrose. The cultures were kept on an orbital shaker (100 rev./min) at 25°C in the dark. During the first 3 weeks of culture initiation, the medium was refreshed weekly. During the refreshment of the medium, cell debris and very large cell clumps were removed. Once a suspension culture was established, the medium was refreshed every two weeks. Eventually, 20 lines were selected from suspension cultures on the basis of their growth in liquid medium and on their original regeneration capacity determined earlier to test whether cytokinins had a stimulatory effect on the regeneration capacity of suspensions (Table 1).

Plant regeneration

For testing the effect of cytokinins on the regeneration capacity of each line, cell clumps taken from suspension cultures were placed on 13 different regeneration media (Table 3). MS medium was supplemented with 30 g/l sucrose as a basic regeneration medium (MS30). In all media, 0.4 % phytagel (Sigma) was used as a solidifying agent. Media were adjusted to pH 5.8 prior to autoclaving (103 kPa, 121°C; 20 min.). BA and TDZ (Duchefa) were added to media before autoclaving,

Table 1. Shoot and root regeneration (expressed as the percentage of calli that produced shoots or roots) of 20 lines in *A. cepa* for the 13 treatments used. The lines had a preculture of 7 months on solidified medium and 3 months on liquid medium. The original shoot and root regeneration of 3 months old callus cultures and the quality of the suspension are given. Line identification: the first two letters indicate the cultivar used, st: onion cultivar Sturon, hy: onion cultivar Hyton, at: shallot cultivar Atlas, tr: shallot cultivar Tropix. The third letter indicates the basal medium in which the lines were precultured namely m: MS and b: BDS. The numbers indicate the number of the line. Means and their standard errors (SE) are indicated.

Line	Original regeneration of 3 months old callus on MS30		Colour and state of suspension cultures after 3 months in liquid culture	Overall shoot regeneration Mean ± SE	Overall root regeneration Mean ± SE
	Shoot	Root			
atm24	50	100	yellow-green	4.62 ± 0.91	80.19 ± 1.22
atm37	80	100	yellow-green	1.54 ± 0.53	86.35 ± 1.26
atm7	10	100	yellow-green, small amount of root-like structures	1.35 ± 0.50	70.00 ± 1.30
trm31	0	100	yellow-green	1.35 ± 0.50	67.69 ± 1.01
hym19	10	40	a little brown	1.15 ± 0.46	60.00 ± 1.63
trm42	90	100	yellow-green	1.15 ± 0.45	81.73 ± 1.16
trm55	50	100	yellow-green	0.77 ± 0.38	69.04 ± 1.08
atm38	70	100	yellow-green	0.38 ± 0.27	73.08 ± 0.94
stm4	10	100	yellow, large amount of root-like structures	0.19 ± 0.19	82.69 ± 1.30
stm17	10	100	yellow, some root-like structures	0.19 ± 0.19	74.04 ± 1.22
trm56	50	100	yellow	0.19 ± 0.19	67.88 ± 1.14
stm18	20	100	yellow	0	43.84 ± 1.72
stm19	30	100	yellow, large amount of root-like structures	0	81.54 ± 0.91
stm31	0	100	yellow, some root-like structures	0	88.08 ± 1.05
hyb12	10	70	yellow	0	65.38 ± 1.25
hyb13	10	100	yellow	0	66.73 ± 0.81
trm29	70	100	yellow-green	0	76.54 ± 1.06
trm59	40	100	yellow	0	81.92 ± 1.20
atm35	40	100	yellow-green	0	66.92 ± 0.79
atm39	60	100	yellow-green	0	55.96 ± 1.47

while ZT (Duchefa) and ZR (Sigma) were added to media after autoclaving through filter sterilization. The BA1 treatment represents MS30 medium supplemented with 1 mg/l (4.44 μM) BA; the ZT5 treatment represents MS30 medium supplemented with 5 mg/l (22.81 μM) ZT; the other treatments, as listed in Table 3, are coded in a similar way. To allow for a quantitative determination of the regeneration capacity, cell clumps from each treatment were carefully divided into equal parts (5 × 5 mm) and 10 units of cell clumps (about 100 mg per unit) were placed on a regeneration medium. All treatments were carried out at an ambient temperature of 25°C with a 16h photoperiod (ca. 60 μ E s^{-1} m^{-2}; lamps used: Philips, TLD 50W/840HF, Electronic NG). After one month, all materials were subcultured. Shoot and root regeneration, expressed as the percentage of calli that regenerated per Petri dish, were recorded after two months.

In order to test the restoration potential of polyamines with lines that had lost their regeneration capacity, the regeneration medium was supplemented with either spermidine at 0.1, 1 and 5 mM or with putrescine at 1 and 5 mM.

Statistical analysis

The experimental set-up used to analyse shoot and root regeneration from suspension cultures was a two factorial design. The main effects were lines (20 lines used) and treatments (MS30 and the cytokinins BA, TDZ, ZR and ZT each at 1, 2, 5 mg/l). Each treatment was replicated four times and per replicate 10 calli were used. A generalized linear model (McCullagh & Nelder, 1990) based on a binomial distribution and a logit as link function was used for the analysis.

Results

Initiation of suspension cultures

Eighty-three lines were chosen for starting suspension cultures based on earlier studies (Zheng et al., 1998) in which differences in regeneration capacity and quality of callus among lines were determined. We selected more lines from shallot (59 lines from Tropix and Atlas) than from onion (24 lines from Sturon and Hyton). All 83 initiated suspensions were highly heterogeneous in the beginning and consisted of cell

aggregates. Mucilaginous substances were also produced by some lines. In the first three weeks, differences in dispersion, browning and growth rate could be observed among the lines used. In this period, the liquid medium was refreshed weekly and cell debris and white cell clumps were removed. Very large yellow-green cell clumps were chopped into small pieces. Once the suspension cultures were established, most lines were well-dispersed with a moderate to good growth rate with yellow or yellow-green colour (Figure 1A). Suspensions were subcultured in the same medium every two weeks. During subculturing for 3 months, browning of 15 suspension lines occurred. On the basis of a good growth in suspension and differences in original regeneration capacity, 20 lines were selected to test whether the type and concentrations of cytokinins had stimulatory effects on regeneration capacity of the suspension cultures (Table 1).

Shoot regeneration

In this study, we only test whether cytokinins had a stimulatory effect on the regeneration capacity of suspensions because auxins are of less importance. After transfer of the cell clumps of the 20 selected lines to the 13 treatments with different types and concentrations of cytokinins, the occurrence of green shoots and roots was recorded after 2 months (Figure 1B). There was a highly significant effect of the line on shoot regeneration (Table 2). Different types and concentrations of cytokinins proved to be of minor importance and the interaction between cytokinins and lines was not significant (Table 2). On average, 1.25 % of the cell clumps among 20 lines tested showed shoot regeneration on regulator-free medium (MS30) while 0.25-0.88 % of the cell clumps regenerated shoots in different types and concentrations of cytokinin treatments (Table 3). Regenerated shoots easily developed into whole plants when transferred to half strength MS30 (Figure 1C).

Because there were no significant differences with respect to shoot regeneration among different types and concentrations of cytokinins, the 20 lines tested could be divided into 3 groups. In the first group, which consisted only of one line (atm24), an overall shoot regeneration average of 4.62 % was found among the 13 treatments used (Table 1). This was significantly higher compared to the other lines. The second group, which consisted of atm37, atm7, trm31, hym19, trm42 and trm55, had an average shoot regeneration in the range from 0.77 % to 1.54 %. The remaining 13 lines were in the third group. Although in this group 4 lines (atm38, stm4, stm17 and trm56) had some shoot regeneration, there was no significant difference with the other 9 lines which showed no regeneration response at all. From the 11 lines which

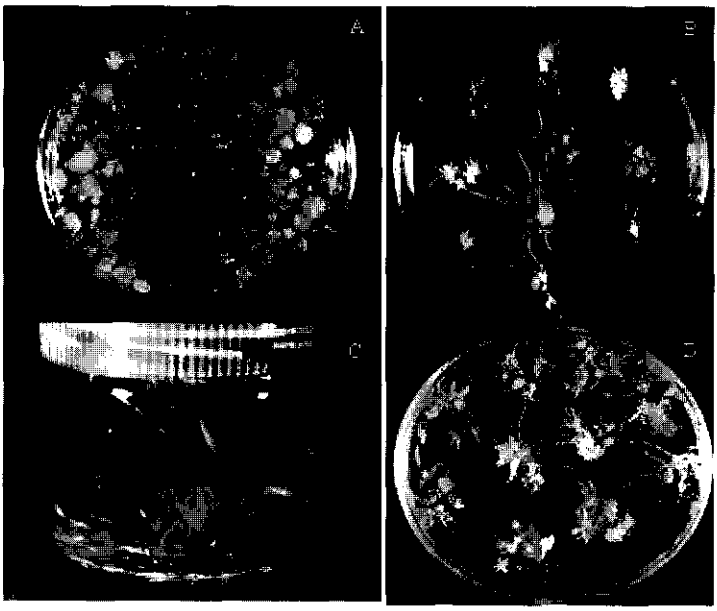

Figure 1. (A). Cell suspension of *Allium cepa* after 3 months of suspension culture. (B). Regenerated green shoots and roots after 2 months on regeneration medium. (C). Regenerated shoots easily developed into whole plants when transferred to half strength MS30. (D). A line with only root formation.

Table 2. Analysis of deviance of the effect of cytokinins and lines on shoot and root regeneration (expressed as the percentage of calli that produced shoots or roots) of mature embryo-derived suspension cultures of *A. cepa*. Ratios are based on a dispersion parameter with value 1. d.f.: degree of freedom, M.D.: mean deviance. ***: $P \leq 0.001$, NS: not significant.

Source of variation	d.f.	Shoot M.D.	Root M.D.
Treatment (T)	12	0.9203^{NS}	469.779^{***}
Line (L)	19	7.1869^{***}	71.342^{***}
T × L	228	0.5450^{NS}	3.613^{***}
Residual	780	0.1732	1.088
Total	1039		

regenerated shoots, 2 lines originated from Sturon, 1 from Hyton, 4 from Tropix and 4 from Atlas. Plant regeneration proved to be higher in shallot compared to onion. The best line (atm24) had its highest shoot regeneration capacity (12.5 %) on the growth regulator-free MS30 medium (Table 3), however, this figure did not differ statistically significantly from the other treatments and not even from the treatments where no regeneration occurred.

Of the 19 lines which lost their regeneration capacity and were tested for restoration with polyamines, only one line produced a few shoots (data not shown).

Table 3. The effect of various cytokinins on overall shoot and root formation (expressed as the percentage of calli that produced shoots and roots) in *A. cepa* among the 20 lines tested and the best line atm24. The lines used had been precultured on solidified medium for 7 months and for 3 months on a liquid medium. Means and their standard errors (SE) are indicated.

Treatment	Shoot Mean ± SE		Root Mean ± SE	
	20 lines	atm24	20 lines	atm24
MS30	1.25 ± 0.38	12.50 ± 5.23	97.88 ± 0.50	100.00 ± 0.02
ZR1	0.63 ± 0.27	7.50 ± 4.16	97.38 ± 0.57	100.00 ± 0.02
ZR2	0.25 ± 0.18	0.00 ± 0.02	96.88 ± 0.56	100.00 ± 0.02
ZR5	0.38 ± 0.21	0.00 ± 0.02	96.00 ± 0.68	97.50 ± 2.33
ZT1	0.50 ± 0.25	2.50 ± 2.47	94.00 ± 0.78	97.50 ± 2.33
ZT2	0.50 ± 0.24	5.00 ± 3.45	94.50 ± 0.71	100.00 ± 0.02
ZT5	0.88 ± 0.32	7.50 ± 4.16	88.63 ± 0.93	92.50 ± 3.93
BA1	0.63 ± 0.27	5.00 ± 3.45	89.75 ± 0.98	95.00 ± 3.25
BA2	0.75 ± 0.30	5.00 ± 3.45	81.00 ± 1.23	100.00 ± 0.02
BA5	0.88 ± 0.32	0.00 ± 0.02	47.38 ± 1.43	70.00 ± 6.84
TDZ1	0.88 ± 0.33	5.00 ± 3.45	25.25 ± 1.34	50.00 ± 7.64
TDZ2	0.50 ± 0.24	5.00 ± 3.45	16.13 ± 1.20	30.00 ± 6.84
TDZ5	0.38 ± 0.21	5.00 ± 3.45	11.00 ± 1.04	10.00 ± 4.48

Root formation

After transferring the cell clumps from suspension culture to media with different types and concentrations of cytokinins, root formation was visible in some treatments after two weeks. After subculturing for one month, extensive root formation on the surface of calli was present. In total there were 9 lines which had only root formation (Figure 1D). It was observed that cytokinins as well as lines had highly significant effects on the level of root formation (Table 2). The interaction between cytokinins and lines was also highly significant (Table 2), but this was of much less importance than the two main effects.

For root formation, it was found that there were highly significant differences among the cytokinins supplemented (Table 3). Root formation was highest on the growth regulator-free MS30 medium with an average of 97.88 % among the 20 lines used. In the ZR treatments, root formation was similar to MS30. In the other treatments, it was shown that there was a significant reduction of the root formation compared to MS30. At the highest concentration of 5 mg/l (22.19 μM) BA and all concentrations used for 1, 2 and 5 mg/l (4.54, 9.08, 22.71μM) TDZ, the percentage of root formation was reduced to 47.38 %, 25.25 %, 16.13 % and 11.00 %, respectively. In those treatments, most calli had a green colour. Significant differences were also observed for root-like structures among the individual lines (Table 1).

Discussion

Effect of callus age on plant regeneration

On average, 1.25 % of the calli from 20 lines showed shoot regeneration on the growth regulator-free medium MS30 after a preculture on solidified callus induction medium (Zheng et al., 1998) for 7 months and on liquid medium for suspension cultures for 3 months. From the Table 1, it can be deduced that on average after 3 months culture on solidified callus induction medium 35.50 % shoot regeneration capacity was observed. As we found in an earlier study (Zheng et al., 1998) the line effect had an overriding effect on plant regeneration. It is not expected that there will be large differences in plant regeneration in liquid compared to solid medium. So, it is clear that shoot regeneration in *A. cepa* decreases very quickly after a prolonged period of *in vitro* culture. Van der Valk et al.(1992) mentioned that green plants could

be regenerated from compact callus of selected embryogenic onion lines after regular transfer for up to 15 months after culture initiation, however no data were presented on this issue. In leek, embryogenic calli from mature embryos which were maintained for about one year are still capable of regenerating plants although with a low frequency (Buiteveld et al., 1993). For friable and embryogenic suspension cultures, regeneration capacity also decreased with time (Buiteveld et al., 1994). Silvertand et al. (1996) suggested that a callus growth period of 6 weeks is the best for shoot regeneration in leek. We attempted to restore regeneration potential of these long-term *A. cepa* suspension lines with applying polyamines such as spermidine and putrescine to the regeneration medium. From rice it is known that these polyamines have a positive effect on plant regeneration (Bajaj & Rajam, 1996). However, these polyamines did not show a significant stimulation of shoot regeneration for *Allium* suspensions (data not shown). The results obtained underline the recalcitrant nature of *Allium* species to tissue culture.

Effect of plant growth regulators on plant regeneration

The common practice for plant regeneration in *Allium* tissue culture is to use MS or BDS medium supplemented with different types and concentrations of cytokinins such as benzyladenine, kinetin and N^6 -(2-isopentenyl) adenine (Buiteveld et al., 1993; 1994; Lu et al., 1989; Phillips & Hubstenberger, 1987; Shahin & Kaneko, 1986; Silvertand et al., 1996; Song & Peffley, 1994; Van der Valk et al., 1992). Wang & Debergh (1995) observed that plant regeneration in *A. porrum* was clearly dependent on the presence of cytokinins while on growth regulator-free medium shoot regeneration was hardly ever seen. Hansen et al. (1995) obtained plant regeneration for suspension cultures of *A. cepa* on BDS with 0.5 mg/l BA and 0.025 mg/l picloram or 0.35 mg/l BA and 0.03 mg/l picloram. Song & Peffley (1994) used BDS with 2 mg/l BA and 0.03 mg/l picloram as regeneration medium using cell suspensions of *A. fistulosum* and the interspecific hybrid between *A. fistulosum* and *A. cepa*. Kim & Soh (1996) observed for *A. fistulosum* that MS basal liquid medium without growth regulators or with the addition of 0.1 mg/l zeatin and 0.1 mg/l ABA gave the highest frequency of mature somatic embryos. They also found that the balance between ABA and zeatin could effectively control somatic embryo development. All in all, there is no clear indication which combination of plant growth regulators is most beneficial for *A. cepa* plant regeneration. In our experiments, the different types and concentrations of cytokinins (BA, TDZ, ZR and ZT) did not enhance shoot regeneration of *A. cepa* as compared to growth regulator-free medium. A similar situation was observed in other monocots such as in rice, where callus initiated on 2,4-

D regenerated into plants when moved to a growth regulator-free medium (Bhaskaran & Smith, 1990). Saker (1998) also observed that somatic embryos were formed on the surface of callus cultures and plantlets regenerated after the removal of 2,4-D in *in vitro* culture of onion. On the basis of these results we will use a growth regulator-free medium for plant regeneration in *A. cepa* in the future.

Effect of subspecies and line on plant regeneration

Out of the 20 lines tested, 11 lines showed shoot regeneration (Table 1). For onion 3 out of 8 lines produced shoots, whereas for shallot 8 out of 12 lines produced shoots. Onion and shallot are different subspecies of *A. cepa*. Although onion and shallot do not differ from each other with respect to shoot regeneration, the trend is visible that shallot has a better shoot regeneration than onion.

To set up cell suspension cultures of *A. cepa,* it is known from literature that a vast number of callus lines must be initiated because only a few lines can be successfully established (Hansen et al., 1995; Song & Peffley, 1994). We also observed a similar trend in our material: from the 83 callus lines that were brought into suspension culture 20 lines showed good growth. The results obtained once again show that the line effect in *in vitro* culture studies is a very important variable (Buiteveld et al., 1993; Phillips & Luteyn, 1983; Phillips & Hubstenberger, 1987; Van der Valk et al., 1992; Wang & Debergh, 1995).

In view of the results obtained we will not focus anymore for transformation of *A. cepa* on the obtainment of lines which maintain a high regeneration capacity for a long time (> 2 years). Instead we will use highly regenerating young callus material (< 3 months old) originating from mature and immature embryos for the development of several gene transfer methods in *Allium* species including particle bombardment and *Agrobacterium tumefaciens*.

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Chapter 5

Agrobacterium tumefaciens-mediated transformation of *Allium cepa* L.: the production of transgenic onions and shallots

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Key words: *Agrobacterium tumefaciens*; *Allium cepa;* FISH (fluorescence *in situ* hybridization); transformation, transgenic plant characterization.

Abstract

This paper describes the development of a reliable transformation protocol for onion and shallot (*Allium cepa* L.) which can be used year-round. It is based on *Agrobacterium tumefaciens* as a vector, using three-week old callus, induced from mature zygotic embryos, as target tissue. For the development of the protocol a large number of parameters were studied. The expression of the *uidA* gene coding for β-glucuronidase was used as an indicator in the optimization of the protocol. Subspecies (onion and shallot) and cultivar were important factors for successful transformations: shallot was better than onion and with shallot cv. Kuning the best results were obtained. Also, it was found that constantly reducing the size of the calli during subculturing and selection by chopping, thus enhancing exposure to the selective agent hygromycin, improved the selection efficiency significantly. Furthermore, callus induction medium and co-cultivation period showed a significant effect on successful stable transformation. The usage of different *Agrobacterium* strains, callus ages, callus sources and osmotic treatments during co-cultivation did not improve transformation efficiency. The highest transformation frequency (1.95 %), was obtained using shallot cv. Kuning.

A total of 11 independent transformed callus lines derived from zygotic embryos were obtained: seven lines from shallot and four lines from onion. Large differences in plantlet production were observed among these lines. The best line produced over 90 plantlets. Via PCR the presence of the *uidA* and *hpt* (hygromycin phosphotransferase) genes could be demonstrated in these putative transformed plants. Southern hybridization showed that most lines originated from one transformation event. However, in one line plants were obtained indicating the occurrence and rescue of at least three independent transformation events. This suggested that T-DNA integration occurred in different cells within the callus. Most transgenic plants only had one copy of T-DNA integrated into their genomes. FISH performed on 12 plants from two different lines representing two integration events, showed that original T-DNA integration had taken place on the distal end of chromosome 1 or 5. A total of 83 transgenic plants were transferred to the greenhouse and these plants appeared to be diploid and normal in morphology.

Introduction

The presence of efficient methods for genetic transformation is of considerable importance for the improvement of modern crops. The most commonly used vectors for transformation are *Agrobacterium tumefaciens* and particle bombardment (Songstad et al., 1995). *Agrobacterium tumefaciens* is routinely utilized in gene transfer to dicotyledonous plants. Monocotyledonous plants were thought to be recalcitrant to this technology as they were outside the host range of the bacterium. However recently transgenic plants have been obtained in monocotyledonous using specific *Agrobacterium* strains (Arencibia et al., 1998; Cheng et al., 1997; Enriquez et al., 1998; Gonzalez et al., 1998; Hiei et al., 1994, 1997; Ishida et al., 1996; Khanna & Raina, 1999; Li et al., 1996; Liu et al., 1998; Park et al., 1996; Rashid et al., 1996; Tingay et al., 1997; Toki, S., 1997). Therefore, the monocotyledonous nature of species no longer prevents the application of *Agrobacterium*-mediated techniques to the transfer of genes to these species as soon as methodological parameters are optimized (Hiei et al., 1997).

Onion and shallot (*Allium cepa* L.) are among the most important vegetables in the world and have proven to be recalcitrant to regeneration and genetic transformation (Barandiaran et al., 1998; Eady, 1995; Eady et al., 1996). It is already more than ten years since Klein et al. (1987) developed a high-velocity microprojectile method and demonstrated that epidermal tissue of onion could be transformed. Dommisse et al. (1990) demonstrated that onion was a host for *Agrobacterium* as evidenced by tumorigenic responses and production of opines by these tumours. Joubert et al. (1995) showed that *Agrobacterium vir* gene induction and subsequent gene transfer in onion was influenced by phenolic compounds. Eady et al. (1996) used both particle bombardment and *Agrobacterium*-mediated DNA delivery systems to transform *in vitro* onion cultures with the *uidA* reporter gene. Barandiaran et al. (1998) used a particle bombardment approach to transform garlic. They showed that transient expression of *uidA* in garlic (*A. sativum* L.) was only detected when a nuclease inhibitor was included in the transformation procedure. Scott et al. (1999) studied transient expression of GFP using onion epidermal peels. Recently, a successful transformation of one onion cultivar mediated by *Agrobacterium tumefaciens* was reported using immature embryos as inoculated explants (Eady et al., 2000).

We have developed a reliable plant regeneration system from mature zygotic embryo-derived callus from *Allium cepa* (Zheng et al., 1998). However we also showed that the plant regeneration capacity of selected lines with a high regeneration potential significantly decreased with time. Plant regeneration also proved to be highly dependent upon the line used (Zheng et al., 1999). On the basis of these results we came to the conclusion that only relatively young callus can be used for transformation. In this paper we have developed a

reproducible *Agrobacterium tumefaciens*-mediated transformation system both for onion and shallot using young callus derived from mature embryos with two different *Agrobacterium* strains.

Materials and methods

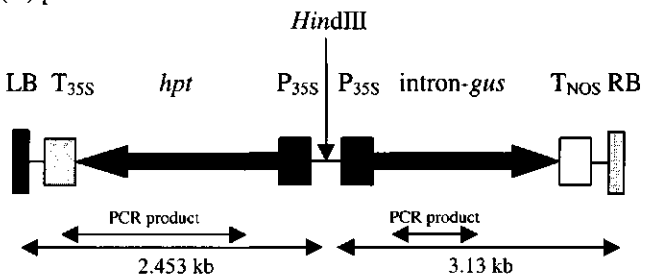
Callus induction

Callus was induced on both mature and immature embryos. For callus induction on mature zygotic embryos, three shallot cultivars (cvs. Atlas, Bawang Bali and Kuning) and two onion cultivars (Sturon and Hyton) were used. The procedure of isolation of mature zygotic embryos has been described earlier (Zheng et al., 1998). In general, callus induction and callus propagation from mature embryos were carried out on solidified MS medium with 1 mg/l 2,4-D and 30 g/l sucrose (MS30-1) in the dark at 25°C. For immature embryos, twenty-five days after flowering, immature seeds from Atlas and Bawang Bali were surface-sterilized by immersion in 70 % ethanol for 30 seconds, and subsequently in 1 % (w/v) Na-hypochlorite (containing two drops of Tween-20 per 100 ml) for 12 minutes under continuous agitation. Immature seeds were rinsed three times in sterile MQ water prior to the isolation of immature embryos. The procedure of callus induction and callus propagation followed the same procedure as with mature embryos.

Agrobacterium strains and plasmids

Agrobacterium strains can be classified as octopine, nopaline and L, L-succinamopine types, depending on which opine synthesis is encoded by the T-DNA. Two strains of *A. tumefaciens,* i.e. EHA105 and LBA4404, were used in this study. EHA105 is an L, L-succinamopine strain with a C58 chromosome background. It contains as virulence helper plasmid pEHA105, originally derived from supervirulent pTiBo542 (Hood et al., 1986; 1993). The plasmid pCAMBIA1301 is a normal binary vector original from the Center for Application of Molecular Biology to International Agriculture, Canberra, Australia (CAMBIA) having *hpt* and intron-*uidA* genes in the T-DNA region (Figure 1A). LBA4404 is an octopine strain with Ach5 chromosomal background carrying pAL4404 as virulence plasmid (Hoekema et al., 1983). Plasmid pTOK233 (Hiei et al., 1994) is a superbinary vector carrying the *vir*B, *vir*C and *vir*G genes of pTiBo542 and having the *nptII*, *hpt* and intron-*uidA* genes in the T-DNA region (Figure 1B). Because both pCAMBIA1301 and pTOK233 have an intron-interrupted *uidA* gene, the expression of *uidA* only occurs in transformed calli and plants rather than in *A. tumefaciens* itself (Ohta et al., 1990).

(A) pCAMBIA1301



(B) pTOK233

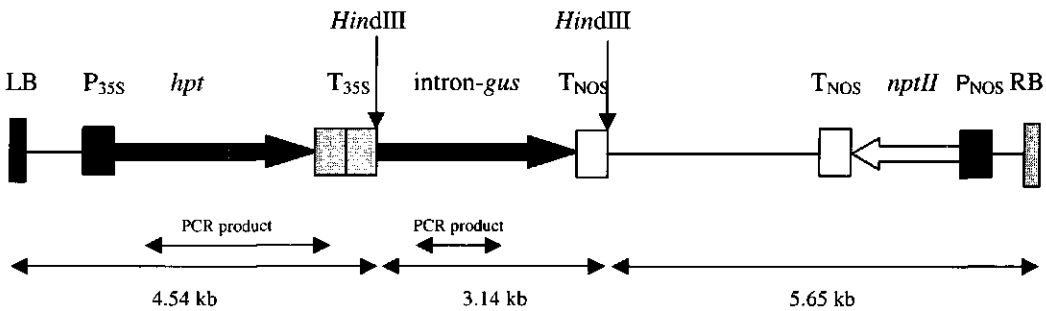


Figure 1. T-DNA region of (A) binary vector pCAMBIA1301 and (B) super-binary vector pTOK233, showing the size of T-DNA, the region for PCR amplification, PCR products used as a probe and *Hind*III restriction sites used for Southern hybridization. Abbreviations: RB: right border; LB: left border; P_{35S} and T_{35S}: CaMV 35S promoter and terminator; P_{NOS} and T_{NOS}: nopaline synthase promoter and terminator; *hpt*: hygromycin phosphotransferase; intron-*gus*: intron interrupted β-glucuronidase; *nptII*: neomycin phosphotransferase.

Co-cultivation, selection and regeneration

Both EHA105 (pCAMBIA1301) and LBA4404 (pTOK233) were streaked out on LB solidified medium with appropriate antibiotics and grown at 28 °C for three days for colonies to appear. EHA105 (pCAMBIA 1301) was grown on LB medium with 25 mg/l rifampicin and 50 mg/l kanamycin, while LBA4404 (pTOK233) was grown on LB medium with 50 mg/l rifampicin and 50 mg/l hygromycin. A single bacterial colony was collected from a plate and suspended for further culture in LB liquid medium for two days. Suspensions were centrifuged at 3,000 rpm for 10 minutes and the *Agrobacterium* pellet was resuspended in MS30-1 liquid medium with acetosyringone 100 µM at an optical density of 0.5 to 1 (OD_{600}).

The first series of experiments were carried out to test whether callus age and callus source had an effect on transient and stable expression of the *uidA* gene. Shallot cultivars, namely Atlas and Bawang Bali were used for callus induction. The resuspended *Agrobacterium* cultures were transferred to small jars for inoculation. Per treatment about 100 - 200 calli from different ages, i.e. 1, 2, 3, 4, and 6 weeks old, were immersed in the bacterial suspension for about 10 min. After 10 min., excess bacterial suspension was removed from the explants by placing them on a dry, sterilized filter paper in a Petri-dish. Subsequently, the calli were put on solidified co-cultivation medium. The co-cultivation medium contained MS30-1 with glucose 10 g/l and acetosyringone 100 µM. About 20 - 40 of calli were examined immediately after the co-cultivation period of 4 days for GUS transient expression. The remaining calli were put on a pre-selection medium MS30-1 supplemented with cefotaxime 400 mg/l and vancomycin 100 mg/l for one week. After pre-selection, selection was carried out at two levels: MS30-1 with cefotaxime 400 mg/l, vancomycin 100 mg/l and hygromycin either at 50 mg/l or at 100 mg/l. After continuous selection for at least two months and subculturing the calli every two weeks, putative transformed calli were put on regeneration medium: MS solidified with sucrose 30 g/l without any phytohormones (MS30). Regeneration was carried out at an ambient temperature of 25°C with a 16 h photoperiod (ca. 60 µE m^{-2} s^{-1} ; lamps used: Philips, TLD 50W/840HF, and Electronic NG). After one month, all material was subcultured. Shoot regeneration was recorded after two months.

The second series of experiments were carried out to test whether callus induction medium composition (MS30-1 supplemented with 0.2 g/l casein or 0.5 mg/l kinetin, or both), co-cultivation time (2 or 4 days), osmotic treatment (3 % (w/v) sucrose or 10 % (w/v) sucrose) and cultivar had an effect on transient and stable expression of the *uidA* gene. LBA4404 (pTOK233) was used in this series of experiments. Three-week old callus from mature embryos originating from cvs. Atlas and Kuning was used for transformation (Figure 2A). This time, callus was chopped into small pieces before putting it on co-cultivation medium. The calli were chopped either directly after retrieving them from the *Agrobacterium* suspension or after drying on filter paper first. Transient GUS expression detection, resistant

callus selection and transformed callus regeneration has been described above. However, the regeneration medium contained MS30 with 1 mg/l kinetin and 50 mg/l hygromycin. Shoot regeneration and rooting conditions were the same as the first series of experiments. Transgenic plantlets were obtained after resistant calli were put on regeneration medium for at least 2 months. These plantlets were transplanted into pots ($8 \times 8 \times 8$ cm^3) in the greenhouse. The plantlets were first covered with a plastic trap for one week in order to recover growth, then they were grown in the greenhouse at 16 / 20°C (night / day) with additional light for 16 hours (approx. 100 $\mu E\ s^{-1}m^{-2}$; lamps used: SON-T 400 Watt).

The third series of experiments were identical to the second series, only LBA4404 (pTOK233) was replaced by EHA105 (pCAMBIA1301). Three-week old calli from mature embryos of only cv. Kuning were used for transformation. Transient GUS expression detection, resistant callus selection, transformed callus regeneration and transferring plantlets to the greenhouse were identical to the second series of experiments.

The fourth series of experiments were carried out to test onion callus as opposed to shallot callus for transient and stable GUS expression. LBA4404 (pTOK233) and EHA105 (pCAMBIA1301) were both used. Three-week old callus from mature embryos of the onion cvs. Sturon and Hyton was used for transformation. Transient GUS expression detection, resistant callus selection, transformed callus regeneration and transferring plantlets to the greenhouse were identical to the second and third series of experiments.

Histochemical GUS-assay

Expression of GUS in onion and shallot callus, and transformed leaf and root was assayed as described by Jefferson et al. (1987) with some modifications. 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) was used as a substrate. Calli and leaf, root materials were stained overnight at 37 °C with 1 mM X-Gluc in 50 mM phosphate buffer (pH 7.5), supplemented with 10 mM EDTA and 0.1% Triton X-100 . The GUS staining was stopped by washing with 70 % ethanol until destaining was complete and the ethanol remained colourless.

Data analysis

An explant showing one or more GUS blue patches was recorded as positive. In the first series of experiments, the experimental set-up used to analyse this single unreplicated experiment on transient expression of GUS was a four factorial design with *Agrobacterium* strain, cultivar, callus age and callus source (from mature embryo or immature embryo; see also Table 1). In

the second series of experiments, the experimental set-up was a six factorial design with cultivar, callus induction medium, co-cultivation medium, co-cultivation period, the time of chopping callus and the presence or absence of a pre-selection period. In the third series of experiments, a five factorial design with callus induction medium, co-cultivation medium, co-cultivation period, the way of chopping callus and the presence or absence of a pre-selection period. A generalized linear model (McCullagh & Nelder, 1990) based on a binomial distribution and a logit as link function was used for the analysis of these data.

PCR analysis

Approximately 0.25 g fresh leaf samples from putative transgenic plants were collected from the greenhouse. DNA was isolated from these plants via a miniprep protocol (van Heusden et al., 2000). DNA concentration was estimated fluorometrically using the Hoechst 33258 dye. Optimal conditions, such as temperature, DNA concentration and suitable primers for PCR were checked in primary experiments. Successful PCR was performed using specific primers for *uidA* (forward: 5'-GGAATTGATCAGCGTTGGTG-3' and reverse: 5'-TAGATATCAC ACTCTGTCTG-3') and for *hpt* (forward: 5'-ATGAAAAAGCCTGAACTCA-3', and reverse: 5'-ACTGGATTTTGGTTTTAGG-3'). The PCR cycle was 94°C for 2 min (1 cycle); 94°C for 1 min, 56°C for 1 min, 72°C for 2 min (35 cycles); 72°C annealing extension for 10 min. The reactions were carried out in a Perkin Elmer Cetus Thermocycler. Primers for *uidA* amplify a sequence of 710 bp (Figure 1A), while *hpt* primers amplify a sequence of 1.2 kb (Figure 1B).

Southern blot analysis

Putative transgenic onion and shallot genomic DNA was isolated from 1-1.5 g frozen leaf tissue collected from the greenhouse with a midiprep DNA-isolation method as also described by van Heusden et al. (2000). The DNA concentration was estimated fluorometrically using the Hoechst 33258 dye. From each sample 25 μg DNA was digested overnight with 100 units of *Hin*dIII. After digestion, the DNA was loaded on a 1.0 % agrose gel with TAE at 25 V for overnight electrophoresis (running time about 17 hours). DNA was transferred to a nylon Hybond^{TM}-N+ membrane (Amersham Lifescience, UK) by vacuum blotting. 100 ng DNA of PCR products from *uidA* and *hpt* genes were used for random prime labeling.

Detection of T-DNA integration by FISH

Fluorescence *in situ* hybridization (FISH) with prophase and metaphase preparations was used to localize T-DNA insertion in transgenic plants. A 13 kb T-DNA fragment was used as a probe using the protocol developed by Khrustaleva & Kik (2000).

Results

Factors affecting the efficiency of T-DNA delivery

In the first series of experiments, calli derived from both mature and immature embryos of two shallot cultivars, were used to test for transient GUS expression after co-cultivation with two different *Agrobacterium* strains. Calli of different ages (1, 2, 3, 4 and 6 weeks) were inoculated with *Agrobacterium tumefaciens* without chopping the callus into small pieces. After a co-cultivation period of four days using calli of cvs. Atlas and Bawang Bali, GUS assays showed that in most cases blue spots are located around the shoot apex area of both mature and immature embryos (Figure 2B and 2C). The number of blue spots varied from a few isolated ones (1-2) to whole areas. The root and elongated cotyledon part did not show any GUS expression. No blue spots were observed in untransformed calli. The percentage of calli showing transient expression of the *uid*A gene could amount up to 100 % (Table 1). The analysis of deviance showed that only cultivar type had a significant effect on transient GUS expression: Indonesian shallot cv. Bawang Bali had a much higher transient GUS expression than shallot cv. Atlas (Table 2). No significant effect on transient GUS expression was observed for callus age, different sources of embryos (mature and immature) and *Agrobacterium* strains. For practical reasons, we continued to work in future experiments with three-week old callus induced from mature embryos as these can be obtained year-round.
In order to study the effect of callus induction medium, co-cultivation time and osmotic treatment during co-cultivation on transient expression and stable transformation, a new series of experiments were carried out. First of all, the calli were chopped into small pieces just after inoculation with *Agrobacterium* and at each subculture to allow for a better contact with the selective agent hygromycin. The elongated part from the cotyledon was also removed (Figure 2D). The second series of experiments were carried out using *Agrobacterium* LBA4404 (pTOK233). Transient expression of GUS was analysed immediately after co-cultivation with *Agrobacterium.* Twenty-five callus lines per treatment were used for the GUS assay. Transient expression of GUS after four days of co-cultivation was significantly higher compared to two days co-cultivation (Table 2). There were also highly significant differences

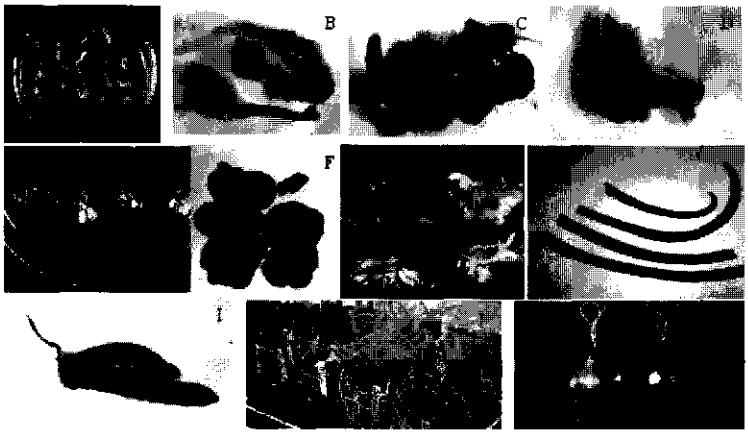


Figure 2. An overview of the transformation procedure developed for onion and shallot (*A. cepa* L.)

(A). Three-week old callus derived from mature embryos.

(B). Transient expression of GUS in two-week old callus derived from mature embryos of Bawang Bali; infection with LBA4404 (pTOK233) after four days of co-cultivation.

(C). Transient expression of GUS in two-week old callus derived from immature embryos of Bawang Bali; infection with LBA4404 (pTOK233) after four days of co-cultivation.

(D). Transient expression of GUS in three-week old callus derived from mature embryo of Kuning; infection with EHA105(pCAMBIA1301) after four days of co-cultivation. Callus was chopped and the elongated part of the cotyledon was removed.

(E). Hygromycin-resistant callus of Kuning after two months growth on selective medium.

(F). Stable and uniform expression of GUS in hygromycin-resistant callus of Kuning .

(G).Plant regeneration of Kuning. The photograph was taken four weeks after the hygromycin-resistant callus had been transferred to regeneration medium with hygromycin.

(H).Expression of GUS in the leaves of a transformant.

(I). Expression of GUS in the root of a transformant.

(J). Transgenic onion and shallot plants in the greenhouse.

(K). Bulbs from transgenic shallot plants.

Table 1. Effect of shallot cultivar, callus age and bacterial strain on transient expression of GUS in mature and immature zygotic embryo-derived calli after co-cultivation with *Agrobacterium tumefaciens* EHA105 (pCAMBIA1301) and LBA4404 (pTOK233) for 4 days. Calli used in exp. 1-4 are from mature embryos, while calli in experiment 5 are from immature embryos.

Cultivar	Exp.	Calli that produced GUS$^+$ cells / Calli inoculated (%)		
		Callus age (week)	EHA105 (pCAMBIA1301)	LBA4404 (pTOK233)
Atlas	1	2	1/20 (5.0)	
	1	4	10/40 (25.0)	
	1	6	5/40 (12.5)	
	2	1	21/40 (52.5)	19/40 (47.5)
	2	3	15/40 (37.5)	10/40 (25.0)
	3	2		22/40 (55.0)
	3	4		3/40 (7.5)
	3	6		7/40 (17.5)
	5	2	35/40 (87.5)	33/40 (82.5)
Bawang Bali	1	2	39/40 (97.5)	
	1	4	24/40 (60.0)	
	1	6	25/40 (62.5)	
	2	1	28/40 (70.0)	37/40 (92.5)
	2	3	40/40(100.0)	32/40 (80.0)
	3	2		30/40 (75.0)
	3	4		26/40 (65.0)
	3	6		25/40 (62.5)
	4	1		27/40 (67.5)
	5	2	33/40 (82.5)	34/40 (85.0)

Table 2. Analysis of deviance of the effect of cultivar, callus induction medium, co-cultivation medium, co-cultivation period, the way of chopping callus and the presence or absence of pre-selection on transient expression of GUS. Ratios are based on a dispersion parameter with value 1. d.f.: degree of freedom, M.D.: mean deviance. ***: $P \leq 0.001$, *: $P \leq 0.05$, NS: not significant.

Source of variation	First series of experiments		Second series of experiments		Third series of experiments	
	d.f.	M.D.	d.f.	M.D.	d.f.	M.D.
Strain	1	0.881^{NS}				
Cultivar	1	146.103^{*}	1	7.057^{NS}		
Callus age	4	24.766^{NS}				
Callus source	1	20.302^{NS}				
Callus induction medium			3	29.268***	3	9.4811*
Callus osmotic treatment			1	8.818^{NS}	1	0.1580^{NS}
Co-cultivation period			1	14.820*	1	3.6964^{NS}
The way of chopping callus •			1	9.383^{NS}	1	0.3299^{NS}
Pre-selection or without pre-selection			1	5.214^{NS}	1	19.9939*
Residual	8	7.877	10	2.146	4	5.0924
Total	24		18		11	

• Please note that chopping itself has a marked effect, but that the way of chopping (see M & M section, co-cultivation, selection and regeneration) is not significant.

among callus induction treatments with respect to transient expression of GUS. The addition of casein, or kinetin or both to the callus induction medium proved to be very beneficial for high GUS expression (Table 2). Other factors did not show any significant effects on transient GUS expression.

The third series of experiments were carried out with *Agrobacterium* EHA105 (pCAMBIA1301). Transient expression of GUS was analysed immediately after co-cultivation with *Agrobacterium*. Twenty-five callus lines per treatment were used for the GUS assay. There were significant differences in transient expression of GUS among the callus induction treatments (Table 2). Again, the addition of casein, kinetin or both to the callus induction medium proved to be beneficial for high GUS expression. Furthermore,

transient expression of GUS was also found to differ significantly between pre-selection for one week versus no pre-selection; without pre-selection gave a better GUS staining.

The fourth series of experiments were carried out with two *Agrobacterium* strains, EHA105 (pCAMBIA1301) and LBA4404 (pTOK233), and two onion cultivars (Sturon and Hyton). On the basis of the three series of previous experiments, the transformation protocol involved a. callus induction medium with MS30 and 0.2 g/l casein; b. the chopping of callus into small pieces; c. a co-cultivation time of four days and d. a co-cultivation medium composed of MS-1, 3 % sucrose, 1 % glucose and acetosyringone 100 µM. Transient expression of GUS was analysed after the co-cultivation period. Twenty-five callus lines per treatment were used for the GUS assay. Transient expression of GUS in cultivar Sturon (96.0 %) was higher compared to cultivar Hyton (60.0 %) using LBA4404 (pTOK233). A similar situation was found in case of transformation by EHA105 (pCAMBIA1301). Transient expression of GUS in cultivar Sturon (64.0 %) was higher compared to cultivar Hyton (44.0 %). For both cultivars, transient expression of GUS after inoculation with LBA4404 (pTOK233) was better than after inoculation with EHA105 (pCAMBIA1301).

Regeneration of transgenic onion and shallot plants

In the first series of five experiments, a total of 1832 callus lines derived from cvs. Atlas and Bawang Bali, were put on MS30 regeneration medium for two months after selection on two different hygromycin concentrations, namely 50 mg/l and 100 mg/l. It was observed that regeneration from cv. Bawang Bali generally performed much better than from cv. Atlas (data not shown). There were some stable transformed organs obtained from the two cultivars in these experiments, which originated from EHA105 (pCAMBIA1301) and LBA4404 (pTOK233) (data not shown). At this stage the selection efficiency was not optimal, because also in the control treatment without exposure to *Agrobacterium*, plants regenerated on medium with 50 mg/l hygromycin.

In order to reduce the number of escaped untransformed calli and improve the selection efficiency, a second series of experiments was carried out. First of all, the callus was chopped into small pieces to allow for a better contact with the selective agent hygromycin during subculture in different selective media. Transformation took place with LBA4404 (pTOK233) and a total of 2249 callus lines from both cvs. Atlas and Kuning were transferred to the different selective media for a minimum of two months. Resistant callus lines were kept separately during subculture. Resistant calli were selected for further propagation (Figure 2E). Small pieces of callus from some callus lines were taken for GUS staining. A large number of proliferating regions showed a uniform expression of GUS (Figure 2F). Forty-six putative transformed callus lines were identified. Transgenic plants were regenerated after more than

Table 3. Effect of shallot cultivar, callus induction medium, co-cultivation time and osmotic treatment on selection and stable transformation efficiency in mature zygotic embryo-derived calli after co-cultivation with *Agrobacterium* LBA4404 (pTOK233).

Cultivar	Exp.	Callus induction medium	Co-cultivation medium	Co-cultivation days	Selective medium	Number of callus lines on selective medium	Number of surviving callus lines put on regeneration medium (R)	Number of callus lines regenerating transgenic plants
Atlas	6-1	A	B	4	S	54	1	0
	7-1	A	B	4	S	60	2	0
	7-1	A + 0.2 g/l casein	B	4	S + 0.2 g/l casein	66	3	0
	7-1	A + 0.5 mg/l kinetin	B	4	S + 0.5 mg/l kinetin	69	1	0
	7-1	A + 0.2 g/l casein + 0.5 mg/l kinetin	B	4	S+ 0.2 g/l casein + 0.5 mg/l kinetin	65	0	0
	8-1	A	B	2	S	75	3	0
	8-1	A	C	2	S	76	7	0
	8-1	A	B	4	S	59	0	0
	8-1	A	C	4	S	59	1	0
Kuning	6-1	A	B	4	S	151	2	0
	6-1	One week pre-selection after co-cultivation			S	151	1	0
	7-1	A	B	4	S	162	4	0
	7-1	A + 0.2 g/l casein	B	4	S + 0.2 g/l casein	154	7	3
	7-1	A + 0.5 mg/l kinetin	B	4	S + 0.5 mg/l kinetin	167	0	0
	7-1	A + 0.2 g/l casein + 0.5 mg/l kinetin	B	4	S + 0.2 g/l casein + 0.5 mg/l kinetin	209	1	0
	8-1	A	B	2	S	167	3	1
	8-1	A	C	2	S	167	3	0
	8-1	A	B	4	S	169	4	0
	8-1	A	C	4	S	169	3	0
Total						2249	46	4

Denote: A: MS30-1 callus induction medium

B: Co-cultivation medium MS-1 + 3 % sucrose + 1 % glucose + 100 µM acetosyringone

C: Co-cultivation medium MS-1 + 10 % sucrose + 100 µM acetosyringone

S: MS30-1+ 50 mg/l hygromycin.

R: MS30 + 1 mg/l kinetin + 50 mg/l hygromycin

Table 4. Summary of the onion and shallot transformation experiments with *Agrobacterium tumefaciens* EHA105 (pCAMBIA1301) and LBA4404 (pTOK233)

Strain/plasmid	Cultivar	Series of experiments	Number of callus lines on selective medium (A)	Number of surviving callus lines put on regeneration medium	Number of callus lines regenerating transgenic plants (B)	Transformation efficiency (B/A, %)
LBA4404 (pTOK233)	Kuning (shallot)	2	154	7	3	1.95 (3.25*)
LBA4404 (pTOK233)	Kuning (shallot)	2	167	3	1	0.60
EHA105 (pCAMBIA1301)	Kuning (shallot)	3	127	2	1	0.79
EHA105 (pCAMBIA1301)	Kuning (shallot)	3	125	3	1	0.80
EHA105 (pCAMBIA1301)	Kuning (shallot)	3	125	2	1	0.80
LBA4404 (pTOK233)	Hyton (onion)	4	675	75	1	0.15
EHA105 (pCAMBIA1301)	Sturon (onion)	4	561	50	3	0.53
Total			1934	142	11	0.57

Denote: * One line had at least 3 transgenic events. Therefore, there were a total of 5 events in this experiments. If transformation efficiency was calculated as number of transgenic events/ number of callus lines used, then transformation efficiency was 3.25 %.

two months on a regeneration medium, which contained 50 mg/l hygromycin (Figure 2G). Some lines had a very high regeneration capacity. Finally, a total of four individual callus lines from cv. Kuning regenerated several putative transgenic plants (Table 3). The best line originated from experiment 7-1 and produced 90 individual plantlets of which 40 survived after three months cultivation in the greenhouse.

In the third series of experiments, a total of 1394 callus lines from cv. Kuning in different treatments transformed with EHA105 (pCAMBIA1301) were transferred to the different selective media for a minimum of two months (data not shown). Callus lines were kept separately during subculture. Resistant callus lines were selected for further propagation. Twenty-five putative transformed callus lines were identified and put on regeneration medium (data not shown). Finally, three callus lines from cv. Kuning regenerated transgenic plants (Table 4).

In the fourth series of experiments, a total of 2571 callus lines from onion cvs. Hyton and Sturon were transferred to the selective medium MS30-1 with 50 mg/l hygromycin and 0.2 g/l casein (data not shown). Two hundred and seventeen putative transformed callus lines were identified after selection for more than two months and they were put on a regeneration medium containing MS30 with 50 mg/l hygromycin and 1 mg/l kinetin (data not shown). Finally, a total of four callus lines from Sturon and Hyton regenerated transgenic plants (Table 4).

Characterization of transgenic plants and transformation efficiency

Transgenic plants were identified by the GUS assay on leaf and root tissues while the plantlets grew on the regeneration medium containing hygromycin. Before the transgenic plants were transferred to the greenhouse, leaf and root tissues were collected for an additional histochemical GUS assay. Most plantlets had visible GUS activity in the entire tissues (Figure 2H and 2 I), but dot-like expression was also observed especially in leaf tissue. Sometimes GUS activity was different from leaf to leaf even within the same plantlet. In most cases, root tissue had a higher activity than leaf tissue while in the bulb part it was extremely difficult to detect any GUS expression. All of the plantlets showing GUS expression also had *hpt* activity as demonstrated by their ability to grow on medium containing hygromycin. However, plantlets from one line of onion cv. Hyton transformed with LBA4404 (pTOK233) did not show any GUS expression in leaf and root tissue while these plantlets could survive on a regeneration medium containing hygromycin. The transgenic plants produced from all experiments are summarized in Table 4. Eventually 11 independent callus lines produced transgenic plants both from onion and shallot. In one of these experiments (experiment 7-1; Table 3) transgenic plants were recovered from three independent callus lines, which

originated from 154 embryos, giving a maximum transformation frequency of 1.95 %. The best line produced 90 transgenic plantlets.

All transgenic plants were analysed by PCR to check whether the *uid*A and the *hpt* gene sequences were present. To this end genomic DNA from the putative transformants was amplified with the *uid*A and the *hpt* gene primers. Both genes were present in all transformed plants and PCR products were generated of 710 bp and 1.2 kb respectively (Figure 3A and 3B).

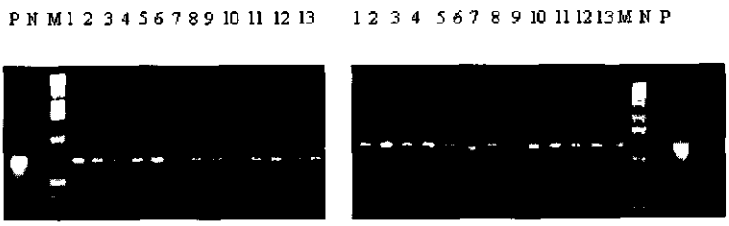


Figure 3. PCR amplification of genomic DNA from transformants. Lane P: plasmid pTOK233 as positive control; Lane N: untransformed plant as negative control; Lane M: 1kb DNA ladder marker; Lane 1-7: individual transgenic plants with LBA4404 (pTOK233). Lane 8-13: individual transgenic plants with EHA105 (pCAMBIA1301).
(A). DNA amplified with *uidA* primers resulting in a 710 bp fragment.
(B). DNA amplified with *hpt* primers resulting in a 1.2 kb fragment.

In order to determine whether T-DNA integration had taken place and how many copies of the T-DNA were present in the plant's genome Southern hybridization was carried out. DNA was extracted from young leaf tissue of individual transgenic plants, digested with *Hin*dIII and hybridized with probes from the PCR products of the *uid*A and *hpt* genes. DNA from non-transformed plants was used as a negative control and showed no hybridization to both probes. Because the T-DNA of pCAMBIA1301 had only one *Hin*dIII site, located between the *hpt* and *uidA* gene (Figure 1A), the second *Hin*dIII site must be located on the plant genome. Hence, probing with either gene sequence gives an indication of the number of T-DNA copies integrated. The eight plants which were transformed with EHA105 (pCAMBIA1301) did have fragments larger than 3.13 kb, which is the minimum size of a hybridizing fragment expected from the map of pCAMBIA1301 (Figure 1A and Figure 4A), probing with the *uidA* fragment. Four individual transgenic plants isolated from one line (lane 1-4, Figure 4A) had identical bands with a fragment size between 4.36-6.56 kb. This finding suggests that these four plants originated from one transformed callus cell. Two plants isolated from another two lines had two different hybridization patterns. Since the T-DNA of pTOK233 had two *Hin*dIII sites, the 3.14-kb band represents the internal fragment harbouring the *uid*A gene when the *uid*A gene PCR product was used as probe (Figure 1B). Forty transgenic plants out of 45 plants

transformed with LBA4404 (pTOK233) had the internal fragment of 3.14 kb (data not shown). Interestingly, the line which had produced 90 plantlets, comprised three groups of plants showing different hybridization patterns:

a. a group of nine plants which only had the internal fragment harbouring the *uid*A gene (lane 1, 2, 3, 6, 7, 8, 9, 11, 14; Figure 4B),
b. a group of two plants, which had the internal fragment and also an extra band with a fragment size of 4.36-6.56 kb (lane 4 and 12; Figure 4B) and
c. a group of three plants which had three bands with fragment sizes of 4.36-6.56 kb, 9.42-23.13 kb and one fragment close to 23.13 kb; however, the internal 3.14 kb fragment was missing (lane 5, 10 and 13; Figure 4B).

Consequently, in the original callus at least three, independent transformation events took place which could be isolated separately after selection and regeneration. In this line with different plants there were one, two and three copies of T-DNA insertion, respectively. Analyses of the eight plants obtained from transformations with pCAMBIA1301 using the *hpt* gene as probe and *Hin*dIII as restriction enzyme showed that most of the detected bands had a size more than 2.453 kb as expected (Figure 1A and Figure 5A). One plant had a fragment close to 2.03 kb (data not shown). This finding suggests that this plant contained a truncated T-DNA copy. Four individual transgenic plants from the same line (lane 1-4, Figure 5A) had identical bands with a fragment size between 2.32-4.36 kb. Most probably, these plants originated from one transformed callus cell. This result completely corresponded to Figure 4A (lane 1-4). This transgenic plants carried one T-DNA copy as suggested by this Southern analysis. For transgenic plants originating from pTOK233 transformations, most hybridizing fragments were of a size larger than 4.54 kb (Figure 1B and Figure 5B) when using *hpt* as a probe and *Hin*dIII as a restriction enzyme. One line which comprised seven individual plants (lane 2-8, Figure 5B), had one band estimated to be slightly smaller than 4.54 kb, which is the minimal fragment length in case of a normal integration event with the proper T-DNA borders being used. Therefore, in this line a truncated T-DNA integration might have taken place.

A total of 12 transgenic plants from two different lines were performed using FISH to demonstrate that T-DNA was integrated into the *Allium* genome. In 11 transgenic plants originating from one embryo, a clearly visible signal was present at the distal end of chromosome 1 both in the prophase and metaphase stage (Figure 6A and 6B). From another transgenic plant originating from a different embryo, it was observed that T-DNA was integrated into the distal end of chromosome 5. Furthermore transgenic plants had 16 mitotic metaphase chromosomes, indicating a normal ploidy level. Finally, a total number of 83 individual plants were transferred to the greenhouse and they appeared normal in morphology and produced bulbs (Figure 2J and 2K).

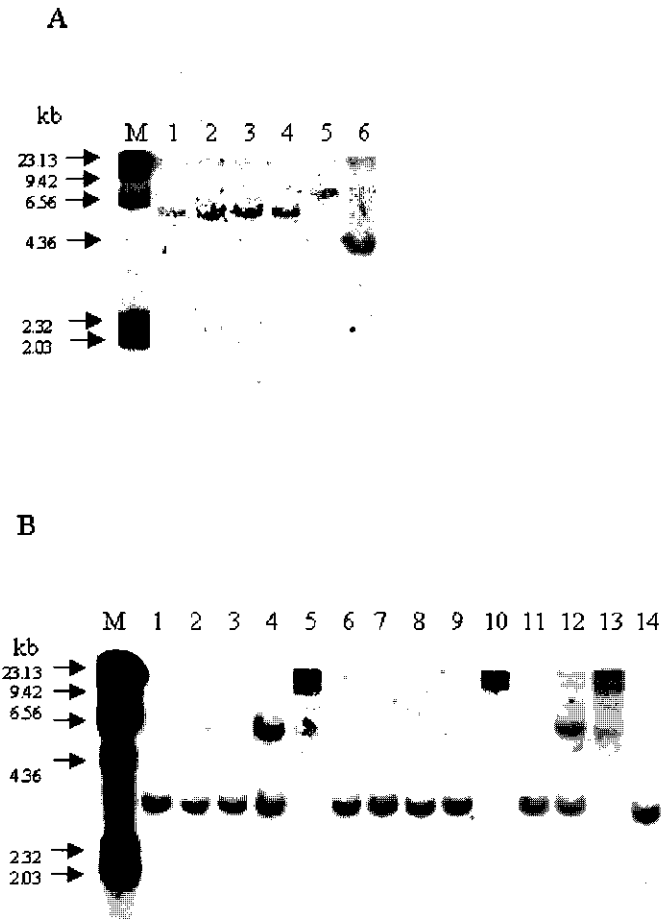


Figure 4. Southern blot analysis of individual shallot transformants. DNA from transformants, which were hygromycin-resistant and GUS-positive, was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to a probe. DNA from a PCR amplification using *uidA* primers generated a 710 bp fragment. This 710 bp fragment was used a probe. Lane M: λDNA digested with *Hin*dIII.
(A). Plants transformed with EHA105 (pCAMBIA1301). Lane 1-4: plants originated from one line; lane 5 and lane 6: plants from two other lines.
(B). Plants transformed with LBA4404 (pTOK233); lane 1-14: plants from the same line.

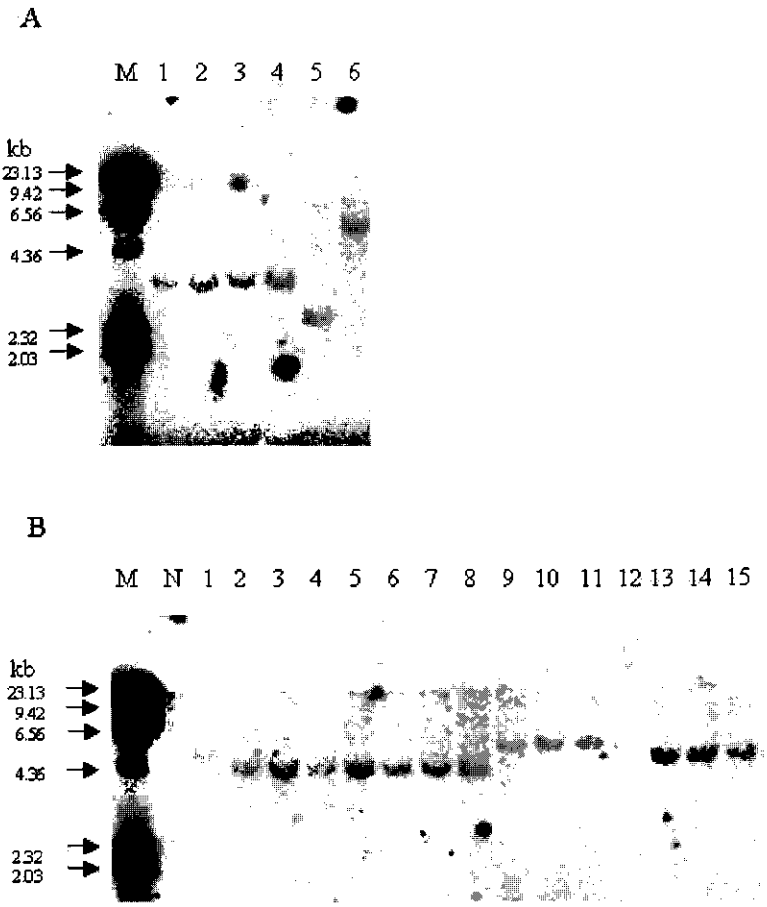


Figure 5. Southern blot analysis of individual onion and shallot transformants. DNA from transformants, which were hygromycin-resistant, was digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to a probe. DNA from a PCR amplification using *hpt* primers generated a 1.2 kb fragment. This 1.2 kb fragment was used as a probe. Lane M: λDNA digested with *Hin*dIII; Lane N: untransformed plant as negative control.

(A). Plants transformed with EHA105 (pCAMBIA1301). Lane 1-4: plants originated from one line; lane 5 and lane 6: plants from two other lines.

(B). Plants transformed with LBA4404 (pTOK233). Lane 1: plant from one onion transformant; lane 2-8: plants from the one shallot line; lane 9-12; plants from a second shallot line; lane 13-15: plants from a third shallot line.

Figure 6. FISH with a 13 kb probe visualizing the integration of a T-DNA from pTOK233 insert in the shallot genome; (A). Prophase, (B). Metaphase.

Discussion

A reproducible and stable transformation system both for onion and shallot has been developed. The transformation procedure is based on *Agrobacterium tumefaciens* using as a target tissue for transformation three week old callus induced on mature zygotic embryos. The possibility to use embryos from mature seeds is a significant step forward as it is now feasible to carry out onion and shallot transformation experiments year-round.

Various factors influencing *Agrobacterium*-mediated transient expression of *uid*A were intensively studied in rice and wheat by Li et al. (1992) and Guo et al. (1998). Optimization of the co-cultivation period, co-cultivation medium and selection medium proved to be of considerable importance. Activation of the *A. tumefaciens* virulence (*vir*) genes is also a critical step in the whole procedure because they have to be induced by specific compounds (Hooykaas, 1989). Phenolic compounds such as acetosyringone are vital in this respect (Guo et al., 1998; Godwin et al., 1991). Therefore we added 100 μM acetosyringone

both in the *Agrobacterium* liquid culture medium and in the co-cultivation medium. The choice of the target tissue for transformation is also critical. A common feature of almost all cases of *Agrobacterium*-mediated transformation of cereal crops is the use of tissues consisting of actively dividing, embryonic cells, such as immature embryos and calli induced from scutella. Generally, immature embryos are the preferred target explants due to their excellent morphogenetic competence (Aldemita and Hodges, 1996; Cheng et al., 1997; Dong et al., 1996; Eady et al., 2000; Hiei et al., 1994; Isida et al., 1996). However also mature embryos have been used successfully (Rashid et al., 1996; Cheng et al., 1998). We prefer to use mature embryos as starting material for genetic transformation as this explant source can be obtained year-round and because its regeneration potential was comparable, in our hands, to immature embryos.

Another important factor for transformation success of onions and shallots was the chopping of the callus just before the co-cultivation with *Agrobacterium* and the subsequent selection phase with hygromycin. In the first series of experiments, intact callus was used during these phases and no transgenic plants were recovered: only some transformed organs were obtained. Furthermore, a number of non-transformed plants escaped selection. This indicated that the selection on hygromycin resistance was not effective enough. Chopping the callus to ensure a more efficient exposure to the selective agent improved the selection process considerably. Other treatments such as co-cultivation medium with casein and kinetin gave a high transient expression of GUS, however, this treatment did not always result in high stable transformation. So there is not always a clear-cut correlation between transient expression and stable transformation. Uze et al. (1997) showed that plasmolysis of precultured immature embryos by osmotic treatment could improve *Agrobacterium*-mediated gene transfer in rice. We also applied an osmotic treatment in the co-cultivation period, however this did not significantly improve our protocol.

The effect of specific *Agrobacterium* strains and vectors in the transformation process is well-known. The performance of so-called 'super-virulent' strains of *A. tumefaciens* has been emphasized in previous reports (Arencibia et al., 1998; Chan et al., 1993; Gould et al., 1991; Hiei et al., 1994; Ishida et al., 1996; Khanna & Raina, 1999; Li et al., 1996; Liu et al., 1998; Raineri et al., 1990; Rashid et al., 1996). The strains we used were an ordinary LBA4404 strain (Hoekema et al., 1983) and a super-virulent EHA105 strain (Hood et al., 1993). The vectors we used were pCAMBIA1301, a normal binary vector, and pTOK233, a derivative of a super-binary vector pTOK162 (Komari, 1990), equipped with additional copies of *vir* genes derived from the supervirulent plasmid pTiBo542. In our hands, both LBA4404 (pTOK233) and EHA105 (pCAMBIA1301) were equally successful. Transgenic onions and shallots were obtained in different independent experiments. A total of 11 independent callus lines produced transgenic plants. On a subspecies basis: seven lines from shallot and four lines

from onion and on a vector basis: five lines from pTOK233 and six lines from pCAMBIA1301.

The use of different subspecies proved to be a very important factor for regeneration in our previous studies: shallots had a higher regeneration capacity than onions (Zheng et al., 1998; 1999). Also in the present transformation study, it was clear that shallot performed better than onion (Table 4). Among shallot cultivars, cv. Kuning was much better than cv. Atlas. Successful transformation of onion and shallot was also dependent on the lines used. Some lines produced a lot of rapidly growing callus material and a lot of transgenic plants: the best line from cv. Kuning even generated 90 individual plantlets. Interestingly, in the aforementioned line, which was derived from a single embryo, at least three independent transformation events occurred and were isolated. One event had normal T-DNA integration pattern, while in the other two events a truncated T-DNA insertion took place (Figure 4B). This suggested that T-DNA integration occurred in at least three different totipotent cells (Figure 2B-2D). From this line, 11 transgenic plants were used to demonstrate via FISH that T-DNA integration took place into the *Allium* genome. A clearly visible signal was present at the distal end of chromosome 1 (Figure 6A and 6B). A total of 53 individual plants derived from ten independent lines have been used for Southern blot analyses to confirm stable integration. Southern hybridization results showed that different patterns of bands were present among these transgenic plants (Figure 4 and 5). These plants were normal in morphology and also in cytology (Figure 2J and Figure 6B). In one plant transformed with pCAMBIA1301, Southern analysis showed that one band was present when probing with the *uidA* fragment. But there were two bands when probing with the *hpt* fragment. It indicated that truncated T-DNA insertion also took place in this plant.

Gene silencing was also found in one transgenic onion line. Plants from this transformant had no any GUS expression in the histochemical GUS-assay, but it had a clear 3.14 kb internal band when probing with the *uidA* gene in the Southern hybridization experiments (lane 1, Figure 5B). The phenomenon of gene silencing is well documented in transgenic plants (Baulcombe & English, 1996; Meyer & Saedler, 1996; Wassenegger & Pelissier, 1998). In some transgenic plants, dot-like patterns of GUS expression in leaves or hardly any expression in the bulb might also point at this expression problem. It also could be that 35S promoter has not a constitutive nature in onion, however in rice it is generally accepted as a constitutive promoter (Tyagi et al., 1999).

Selective agents and the concentration in which they are applied are also quite critical for successful transformation. In our previous experiments, kanamycin and geneticin were not successful as selective agents. Eady et al. (1998) also demonstrated that kanamycin was not suitable selective agent and they used geneticin as selective agent to develop their transformation protocol with LBA4404 (pBIN m-gfp-ER) using immature embryos as target tissue for transformation (Eady et al., 2000). We chose hygromycin as a selective agent and

used a concentration of 50 mg/l. Furthermore, we chopped the callus into small pieces in order to reduce survival of non-transgenic tissue.

All in all, we have developed a system for the production of stable transformants both for onion and shallot using young callus derived from mature embryos inoculation using *A. tumefaciens*. A large number of individual transgenic plants have been produced which grow currently in the greenhouse. As a rule, the T-DNA is integrated in the *Allium* genome as one intact copy, hence a simple integration pattern. Our transformation method takes about six months to generate transgenic onion and shallot plants from callus derived from mature zygotic embryos. Together with Eady et al. (2000) we have shown that an *Agrobacterium*-mediated gene transfer is now available as a straightforward and routine method for the future genetic modification of onion and shallot.

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Chapter 6

Molecular characterization of transgenic shallots (*Allium cepa* L.) by adaptor ligation PCR (AL-PCR) and sequencing of genomic DNA flanking T-DNA borders

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***Key words*:** Adaptor ligation PCR; *Allium cepa*; copy number, genomic target site; T-DNA integration.

Abstract

Genomic DNA blot hybridization is traditionally used to demonstrate that via genetic transformation foreign genes are integrated into host genomes. However in large genome species, such as *Allium cepa* L., the use of genomic DNA blot hybridization is pushed towards its limits, because a considerable quantity of DNA is needed to obtain enough genome copies for a clear hybridization pattern. Furthermore, genomic DNA blot hybridization is a time-consuming method. Adaptor ligation PCR (AL-PCR) of genomic DNA flanking T-DNA borders does not have these drawbacks and seems to be an adequate alternative to genomic DNA blot hybridization.

Using AL-PCR we proved that T-DNA was integrated into the *A. cepa* genome of three transgenic lines transformed with *Agrobacterium tumefaciens* EHA105 (pCAMBIA 1301). The AL-PCR patterns obtained were specific and reproducible for a given transgenic line. The results showed that T-DNA integration took place and gave insight in the number of T-DNA copies present. Comparison of AL-PCR and previously obtained genomic DNA blot hybridization results pointed towards complex T-DNA integration patterns in some of the transgenic plants. After cloning and sequencing the AL-PCR products, the junctions between plant genomic DNA and the T-DNA insert could be analysed in great detail. For example it was shown that upon T-DNA integration a 66 bp genomic sequence was deleted, and no filler DNA was inserted. Primers located within the left and right flanking genomic DNA in transgenic shallot plants were used to recover the target site of T-DNA integration.

Abbreviations: AL-PCR, adaptor ligation PCR; AP1, adaptor primer; AP2, nested adaptor primer; LB1, left-border (LB)-specific primer; LB2, nested left-border (LB)-specific primer; RB1, right-border (RB)-specific primer; RB2, nested right-border (RB)-specific primer.

Introduction

Integration of transferred genes into host genomes is traditionally analysed by genomic DNA blot hybridization (Southern, 1975). Although genomic DNA blot hybridization can be used to determine the integration of T-DNA and the copy number, it becomes labour-intensive when many samples have to be assayed, and it requires relatively large amounts of genomic DNA. For species with large genome species, e.g. onion (15290 Mbp/1C) and tulip (24704 Mbp/1C), which have genome sizes 105 and 170 times larger than *Arabidopsis* (Arumuganathan & Earle, 1991), genomic DNA blot hybridization analysis is pushed towards its limits. Therefore, there is a clear need to have an alternative for genomic DNA blot hybridization for large genome species. Several approaches can be envisaged. Direct polymerase chain reaction (PCR) methods using transgenes as PCR targets allow a rapid detection of the presence of T-DNA (McGarvey & Kaper, 1991). Other techniques e.g. plasmid rescue (Grant et al., 1990; Mandal et al., 1993; Mathur et al., 1998), inverse-PCR (Does et al., 1991; Mathur et al., 1998; Ochman et al., 1988; Triglia et al., 1988), random primed PCR (Trueba & Johnson, 1996; Swensen, 1996), supported PCR (Rudenko et al., 1993), thermal asymmetric interlaced (TAIL-) PCR (Campisi et al., 1999; Liu et al., 1995), vector ligation PCR (Zhou et al., 1997), adaptor ligation PCR (Choi et al., 1999; Padegimas & Reichert, 1998; Spertini et al., 1999; Willems, 1998) and PCR walking (Devic, et al., 1997) have been used for characterizing the plant genomic DNA flanking T-DNA. By cloning and sequencing of junction regions, T-DNA integration into the plant genome can be further characterized (Cormack & Somssich, 1997; Graaff et al., 1996; Krizkova & Hrouda, 1998; Ponce et al., 1998). However, most of the aforementioned techniques have only been routinely used in *Arabidopsis* or human genomic research.

Allium crops are some of the most important vegetables in the world and have proven to be recalcitrant to genetic transformation (Barandiaran et al., 1998; Eady, 1995). Recently, genetic transformation of *A. cepa* mediated by *Agrobacterium* has made a significant step forward and transgenic plants can currently be made (Eady et al., 2000; Zheng et al., 2000). However, the molecular genetic characterization of the transgenic *A. cepa* plants via genomic DNA blot hybridization is fraught with difficulties. Therefore, we have developed an alternative protocol that a.) can prove that T-DNA is present and integrated, b.) allows the determination of the copy number and c.) allows the detailed characterization of the integration event. The method is derived from Siebert et al. (1995) in human genomics and Spertini et al. (1999) in *Arabidopsis* genomics. It combines the ligation of specific adaptors to restriction fragments obtained from genomic DNA, followed by two successive PCR amplifications.

Materials and methods

Plant material

Shallot (*A. cepa* var. *aggregatum* cv. Kuning) was transformed by *Agrobacterium tumefaciens* EHA105 (pCAMBIA 1301) using the co-cultivation method with young callus. Tissue culture media, transformed cell selection and growth conditions were described by Zheng et al. (2000). Transgenic plants, which were GUS positive and resistant to the selection agent hygromycin, were grown in the greenhouse. In the present study, seven plants coming from three transgenic shallot lines were analysed. They were chosen because they represent different, independent transformation events.

Plant genomic DNA isolation

Young, fresh leaves of transgenic shallots were collected from the greenhouse. Plant genomic DNA was isolated from 1-1.5 g frozen leaf tissue with the midi prep DNA-isolation method described by Van Heusden et al. (2000). DNA concentration was estimated fluorometrically using the Hoechst 33258 dye.

Construction of uncloned genomic DNA libraries

Uncloned genomic DNA library construction was performed as described by Siebert et al. (1995) with some modifications. Seven libraries were prepared using seven different restriction enzymes: *AluI, DraI, Eco*RV, *HpaI, RsaI, ScaI* and *SspI*. The enzymes used were selected because they generate blunt-end fragments, which are easily ligated to adaptors. The sequence of the T-DNA was used for right-border-specific or left-border-specific primer design. For each library, 1 μg of genomic DNA was digested in a 40 μl final reaction volume of One-Phor-All^{TM} buffer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with 5 U of the appropriate restriction enzyme for 1.5 h at 37 °C. The aforementioned restriction enzymes, except *HpaI*, have specific endonuclease sites on plasmid pCAMBIA 1301, but do not cut in the right or left border region of the T-DNA.

Adaptor preparation and ligation

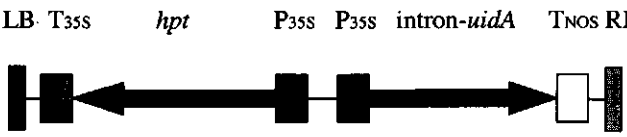
The adaptors were prepared by annealing the following complementary oligonucleotides: upper strand (48 nucleotides), 5'-GTAATACGACTCACTATAGGGCACGCGTGGTC GACGGCCCGGGCAGGT-3'; lower strand (8 nucleotides), 5'-PO_4-ACCTGCCC-NH_2-3'

(Isogen, Maarssen, the Netherlands). The adaptor molecule comprises a long strand and a short complementary strand. In the long strand, sequences homologous to the adaptor primers are present. The use of these specific adaptors in combination with a high annealing temperature reduce nonspecific amplification. The adaptor annealing reaction was performed in water with a final concentration of 30 μM for the upper and the lower strand oligonucleotide. The reaction mixture was heated at 37 °C for 10 min, then cooled down to room temperature over a period of 1 h before use. Ligation of the adaptor to the restricted genomic DNA was performed in One-Phor-All^{TM} buffer with ATP in a final volume of 50 μl. One Weiss unit of T4 DNA ligase was used as indicated by the manufacturers (Amersham Pharmacia Biotech, Piscataway, USA). After overnight ligation at 15 °C, five microliters of each ligation reaction mixture was loaded onto a 1.5 % agarose gel to determine ligation efficiency.

PCR amplification

PCRs were optimized for amplifying fragments with PTC-200 (DNA Engine, MJ Research, INC, USA). The target for the PCR amplifications were fragments with on both sides a specific restriction site plus adaptor that contains the adaptor primer sequence and carries the T-DNA with the RB or LB sequences. Specific T-DNA primers were designed to be homologous to sequences near the RB or LB region of the T-DNA (Figure 1). Primary PCRs were conducted in a 25 μl volume. We designed nested primers of 27 base pairs in length with an optimal melting temperature of 67°C for the right and the left borders of the T-DNA. With respect to the right border PCR amplification, the PCR reaction mixture contained 5 μl of the 10 times diluted ligation reaction mixture, 2.5 μl of the 10 times concentrated PCR buffer (with no $MgCl_2$), 0.5 μl 10mM dNTP, 1.5 μl 25 mM $MgCl_2$, 1 μl 10 μM adaptor primer AP1 (5'-GTAATACGACTCACTATAGGGC-3'), 1 μl 10 μM right-border (RB)-specific primer RB1 (5' -GGGTTTTTATGATTAGAGTCCCGCAAT-3') (Figure 1) and 1 U of Taq DNA Polymerase (Promega, Wisconsin, USA). Primer RB1 was homologous to the region located between +5425 to +5451 from the first nucleotide of the LB (Figure 2). PCR was done after a hot start at 94°C for 3 min. Three-step cycling conditions were used: the denaturing step at 94°C for 30 s was followed by an annealing and extension step. The first five cycles were done with an annealing temperature starting at 65°C for 30 s with a decline at the rate of 1°C every cycle. The extension step was done at 72°C for 1 min at each cycle. Additional 40 cycles were done with a denaturing step at 94°C for 30 s, an annealing step at 60°C for 30 s and an extension step at 72°C for 1 min. PCR was terminated with a 15-min extension step at 72°C. Secondary PCRs were conducted with 5 μl of a 50-fold dilution of the primary PCR using nested adaptor primer AP2 (5'-ACTATAGGGCACGCGTGGT-3') and the nested right-border (RB)-specific primer RB2 (5'-AAACAAAATATAGCGCGCAAACTAGGA-3')

(A) pCAMBIA1301



(B)

TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACA
Left border

CATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAATTA**ATTCGGGGGATCT**

LB2

GGATTTTAGTACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTA

TTTTACAAATACAAATACATACTAAGGGTTTCTTATATGCT**CAACACATGAGCGA**

LB1

AACCCTATAGGAA

(C)

RB1 **RB2**

GGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGA**AAAC**

AAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTA

Right border

CTAGATCGGGAATTAAACTATCAGTGTTT**GACAGGATATATTGGCGGGTAAAC**

Figure 1. Left and right border sequences of binary vector pCAMBIA1301. (A). General overview of the T-DNA region of pCAMBIA1301. Abbreviation: RB: right border; LB: left border; P_{35S} and T_{35S}: CaMV 35S promoter and terminator; T_{NOS}: nopaline synthase terminator; *hpt*: hygromycin phosphotransferase; intron-*uidA*: intron interrupted β-glucuronidase. (B). T-DNA sequence close to the left border. Solid arrows indicate the positions used for primer design. (C). T-DNA sequence close to the right border. Solid arrows indicate the positions used for primer design.

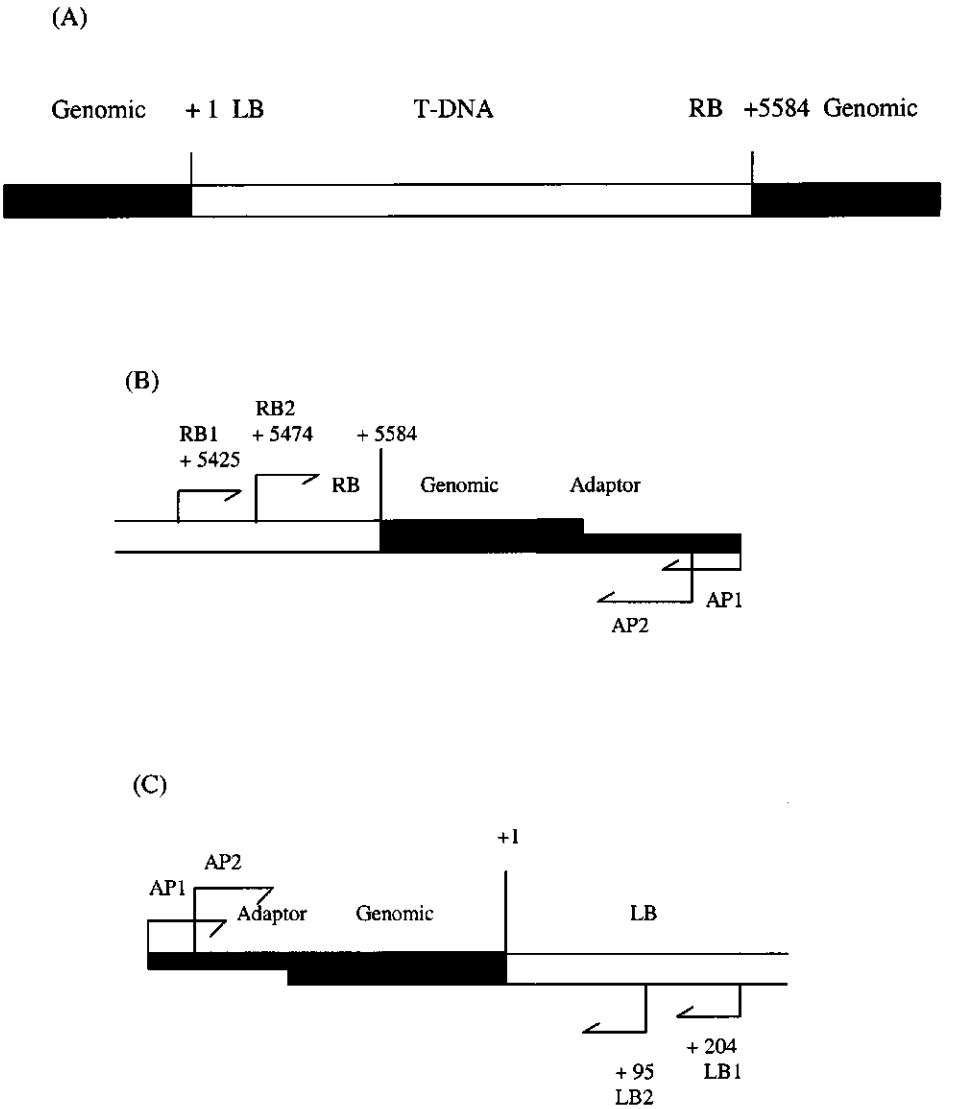


Figure 2. Schematic representation of the T-DNA border regions. (A). The T-DNA region of the pCAMBIA1301 insert in the *Allium* genome. Open box: full region of the T-DNA insert; RB: right border; LB: left border; black box: flanking plant DNA. (B). After digestion with a specific restriction enzyme, adaptors were ligated to the genomic DNA, AL-PCR was performed on the right border using AP1 and AP2 (adaptor primers) and RB1 and RB2 (right border specific PCR primers). (C). After digestion with a specific restriction enzyme, adaptors were ligated to the genomic DNA, AL-PCR was performed on the left border using AP1 and AP2 (adaptor primers) and LB1 and LB2 (left border specific PCR primers).

(Figure 1). Primer RB2 was homologous to the region located between +5474 to +5580 from the first nucleotide of the LB (Figure 2). The same PCR reagent mixture conditions were used and 25 additional cycles were performed. A similar amplification could be done with a set of primers specific to the left border. Primer LB1 was homologous to the region located between +204 to +230 from the first nucleotide of the LB, while primer LB2 was homologous to the region located between +95 to +121 from the first nucleotide of the LB, respectively (Figure 1 and 2). Ten microliter of the second PCR product was analysed via electrophoresis in 1.5 % agarose gel.

Cloning PCR products

Unpurified secondary PCR products were directly used for cloning in the vector pGEM® -T Easy (Promega, Wisconsin, USA). One microliter PCR product was used for each ligation to the pGEM® -T Easy vector. After overnight ligation at 4°C, supercompetent cells from *Escherichia coli* Epicurian Coli® XL1-Blue MRF' (Stratagene, California, USA) were transformed with the ligation mixture by a heat shock as suggested by the supplier. Ampicillin-resistant colonies were identified and plasmid DNA was isolated by the Bio Robot 9600 (Qiagen, USA). Plasmids were selected which showed inserts of the expected size after digestion with *Eco*RI. Some of the inserts were used for sequencing.

Sequencing of genomic DNA flanking T-DNA borders

PCR for sequencing was conducted in a 20 µl volume. The reaction mixture contained 5 µl of plasmid DNA, 5 µl sequencing mixture, 1 µl T7 or SP6 primer (100 ng/µl). PCR was done in a denaturing step at 96°C for 10 s, followed by an annealing step for 5 s and an extension step for 4 min. In total 26 cycles were used. After PCR, products were purified by AutoSeq™G-50 (Amersham Pharmacia Biotech, Wisconsin, USA) to remove the excess of dye-labeled dideoxynucleotides of the sequencing reactions. After purification, DNA was precipitated by addition of 0.1 volume 3.0 M sodium acetate (pH 4.6), and 2.5 volume ethanol following by 15 min incubation at -20 °C and 30 min centrifugation at full speed. The DNA pellet was washed with 70 % ethanol and vacuum dried. Before sequencing, 15 µl TSR (Template Suppression Reagent, ABI Prism Perkin Elmer) was added and shaken for 1200 rev./min for at least 2 h. Sequencing was performed in an ABI PRISM 310 automated DNA sequencer (Perkin Elmer, USA). The resulting sequence information was used to amplify the wild-type genomic target sites.

Results

Amplification of genomic DNA flanking T-DNA borders

Standard PCR was used to check whether the *uid*A and the *hpt* gene sequences were present in putative transgenic plants (Zheng et al., 2000). In order to determine whether T-DNA integration had taken place and how many copies of the T-DNA were present in the plant's genome, adaptor ligation PCR (AL-PCR) was carried out. The main procedure of amplification of T-DNA flanking genomic DNA sequences consisted of three steps: a) construction of DNA libraries by digestion of genomic DNA with several restriction enzymes, b) ligation of adaptors to the fragments in all libraries and c) two successive PCR amplifications using primary and nested primer pairs consecutively (Figure 2). In this way using a combination of adaptor and T-DNA border primers PCR products will only be obtained when T-DNA is integrated in the *Allium* genomic DNA and the maximum number of amplified fragments identified in the individual libraries represents a minimum estimation of the number of integrated copies. Fragments, too long for amplification will escape detection, therefore the use of several libraries generated by separate restriction enzymes will reduce the chance of missing inserted copies. Different blunt-end cleavage restriction enzymes were chosen because they yield different pools of fragments, to which blunt-end adaptors could be easily ligated.

Restriction with *Alu*I and *Ssp*I followed by the adaptor ligation PCR (AL-PCR) of the three transgenic lines gave good results, whereas the other five restriction enzymes did not give reproducibly amplified fragments. Figure 3A shows the results obtained from the secondary PCR amplification of the right border of the three transgenic lines analysed. All lines tested gave clear PCR products indicating T-DNA insertion in the shallot genome. To confirm that the length of the PCR products obtained by our procedure reflected the physical, contiguous distance between the right border and the nearest genomic *Ssp*I site as a result of the integration event, the entire procedure (*Ssp*I digestion and PCR amplification) was performed on a mixture of untransformed onion genomic DNA and pCAMBIA 1301 plasmid DNA. Lane C from Figure 3A shows that a mixture of untransformed onion genomic DNA and pCAMBIA 1301 plasmid DNA did not give any product. This is due to the fact that the nearest *Ssp*I restriction site present on the plasmid itself is too far away (5210 bp) to allow for a proper amplification of the sequences in between. Lanes 1-5 represent five individual transgenic plants from line 1. All plants from line 1 have two very clear bands. Lane 6 shows the amplification of three fragments of one transgenic plant from line 2. In lane 7 the

amplification of a single fragment from one transgenic plant of line 3 can be observed (Figure 3A). The PCR results obtained suggested that transgenic line 1 had at least two copies of T-DNA inserted and that line 2 and line 3 had at least three and one copy of the T-DNA inserted, respectively. Figure 3B shows the products obtained from the secondary PCR amplification from the left border of the same three transgenic lines, which had been restricted with *AluI*. The different lines gave clear PCR products. Lanes 1-5 representing five individual transgenic plants of line 1 had one, identical band. Lane 6 from one transgenic plant of line 2 had three bands and lane 7 that represents one transgenic plant of line 3 had one single band (Figure 3B). In lane C a 173 bp fragment was generated from a mixture of untransformed genomic DNA and pCAMBIA1301 plasmid DNA. This fragment was expected because on the pCAMBIA plasmid there are two *AluI* restriction sites.

Genomic DNA sequences of flanking T-DNA

The secondary PCR products from all transgenic lines as shown in Figure 3A and 3B were cloned and some of them were sequenced (Figure 4). All clones studied contained the sequence of the pCAMBIA1301 vector specific T-DNA primer at one end and the adaptor primer at the other end. This provided evidence that the AL-PCR products contained genomic sequences that flank the T-DNA insert. For the right border side: the upper band of lane 1-5 from transgenic line 1 (Figure 3A) consisted of a 89 bp T-DNA sequence with the 3-bp (TGA) residue of the original 25 bp right border sequence, a 135 bp genomic DNA sequence with the *Ssp*I cleavage site (aat) and a 36 bp adaptor sequence. The lower band of lane 1-5 from transgenic line 1 (Figure 3A) contained a 80 bp T-DNA sequence, no remainder of the right border sequence and a 82 bp genomic DNA sequence with restriction enzyme *Ssp*I cleavage recognition site (aat) and a 36 bp adaptor sequence. The band of lane 7 from transgenic line 3 (Figure 3A) consisted of a 88 bp T-DNA sequence with the 2-bp (TG) right border sequence, a 206 bp genomic DNA sequence with restriction enzyme *Ssp*I cleavage recognition site (aat) and a 36 bp adaptor sequence. For the left border side: the band of lane 1-5 from transgenic line 1 (Figure 3B) consisted of a 77 bp T-DNA sequence, a 129 bp genomic DNA sequence with restriction enzyme *Alu*I cleavage recognition site (ag) and a 36 bp adaptor sequence. The upper band of lane 6 from transgenic line 2 (Figure 3B) contained a 67 bp T-DNA sequence, a 155 bp genomic DNA sequence with restriction enzyme *Alu*I cleavage recognition site (AG) and a 36 bp adaptor sequence. No left border residual base pairs were found in both bands.

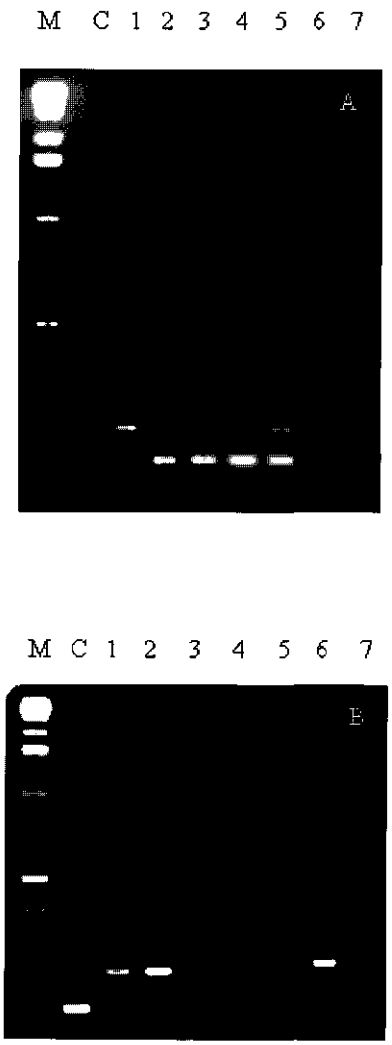


Figure 3. PCR amplification of genomic DNA flanking the T-DNA borders. Lane M: 1kb DNA ladder marker; lane C: mixture of untransformed onion DNA and pCAMBIA1301 plasmid; lane 1-5: five individual plants originating from transgenic line 1; lane 6: one plant originating from transgenic line 2; lane 7: one plant originating from transgenic line 3. (A). Genomic DNA flanking the T-DNA right border was digested by *Ssp*I. (B). Genomic DNA flanking the T-DNA left border was digested by *Alu*I.

Right boundary sequences

Upper band of lane 1-5 in Figure 3A:

AAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTAT
GTTACTAGATCGGGAATTAAACTATCAGTGTTTGAtatagcgtttttatttttctactatcgcgtttttgattct
attatagcgtttttgatgcataaatcttacatctcatatagagttgcgatagctttttatgaatcaatagcgtttttcaaacgctattctatataatA
CCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGT

Lower band of lane 1-5 in Figure 3A:

AAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTAT
GTTACTAGATCGGGAATTAAACTATCtatgttacaaaatcctatacaattttcaaacaaaactttaaccaattatctt
taaaataaatccattttataaaatataaat*ACCTGCCCGGGCCGTCGACCACGCGTGCCCTATAGT*

Band of lane 7 in Figure 3A:

AAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTAT
GTTACTAGATCGGGAATTAAACTATCAGTGTTTGttcttccacaacatccacaatttctttttacgaagtac
tataatttataataatggtgtgtgtgtatgtgtatatatatatatatattatatgaaaaaaattaaatcaaatataaatatatccaaaaaaataaaa
caatttttaaagctgaaaataatatatactatgtaactaatttttttgatatatatatatacacacacaat*ACCTGCCCGGGCCGTC*
GACCACGCGTGCCCTATAGT

Left boundary sequences

Band of lane 1-5 in Figure 3B:

CAGTACTAAAATCCAGATCCCCCGAATTAATTCGGCGTTAATTCAGTACATTAA
AAACGTCCGCAATGTGTTATTAAtaaaatgtgctcgaaaacgctatagaagttaaatgaaggattatattctctatag
catttaagtttaataacagagaaaaaacgctatggtaggttgcaaacagtatgccctttgtaaatgctattag*ACCTGCCCGGGC*
CGTCGACCACGCGTGCCCTATAGT

Upper band of lane 6 in Figure 3B:

CAGTACTAAAATCCAGATCCCCCGAATTAATTCGGCGTTAATTCAGTACATTAA
AAACGTCCGCAATtgttggcgaccttgtataattggtggtgtttgagccatctgggaaatgaataaacgtagacgtcaattttgt
tatcgacaaaggaaataggagaagacaaaagacgtgctgtctttggaacgagaagctttcgtaacagacaaatttgtagttag*ACCTG*
CCCGGGCCGTCGACCACGCGTGCCCTATAGT

Figure 4. Sequence analysis of T-DNA/plant DNA junctions. Sequences originating from the T-DNA border of pCAMBIA1301 are shown in upper case letters; shallot genomic DNA is shown in lower case letters; sequences from the adaptor are shown in upper case italic letters. AP2 and RB2 or LB2 primers are shown in bold letters. Restriction enzyme *Ssp*I and *Alu*I recognition sites aat and ag in genomic DNA are shown in blue colour; the typical 2-bp (TG) or 3-bp (TGA) right border sequences are shown in red colour. Genomic DNA sequences underlined are used for designing primers in subsequent PCR amplification to recover the target site of T-DNA integration.

T-DNA integration

On the basis of the flanking DNA sequences (Figure 4) of the integrated T-DNA copy represented by the RB upper band of line 1 and the LB band of line 1, 24 bp sequence primers were designed and used in PCR on untransformed *A. cepa* plants. Based on the previously established sequences of the junction location of the two 24 bp primers within the genomic left and right flanking DNA a PCR product of 216 bp was expected. A fragment was recovered close to this size (data not shown). This fragment was cloned and sequenced following the procedure as mentioned above and proved to be 282 bp long. Comparison of the original target site sequence with the right and left flanking plant genomic DNA sequences of the T-DNA insert revealed that a 66 bp fragment of plant DNA had been deleted upon T-DNA integration.

Discussion

In this paper we have demonstrated for the first time that AL-PCR represents an alternative to genomic DNA blot hybridization in the molecular characterization of transgenic plants in large genome species such as *Allium cepa*. Together with the sequencing data AL-PCR provided evidence for the true integration of T-DNA derived sequences into the *Allium* genome. Secondly, it allowed the determination of the T-DNA copy number and their integrity. It was also possible to isolate and analyse nucleotide sequence of the genomic area (target site) in which the T-DNA was inserted. At present it is not possible to predict upon transformation of plant cells where T-DNA sequences will integrate in the genome, nor is it possible, yet, to target them to specific sites in a controlled way. This means that it is unknown what restriction enzymes will have cutting sites so close to the integration site that PCR-amplifiable fragments will be generated. Therefore, several independent libraries using different restriction enzymes were made. Criteria for selecting enzymes were that their recognition sites were not too rare, i.e. four or six basepairs, that they yielded blunt-ends and preferably did not cut within the T-DNA RB or LB border areas. Furthermore, T-DNA specific primers were designed as close to the border sequences as possible. This set-up is such that only integration will give rise to PCR fragments. We chose to make seven libraries and it was found that five of them did not yield bands reproducibly. Two libraries proved useful and were taken for further characterization of three transgenic lines.

Table 1. Comparison of T-DNA copy number between genomic DNA blot hybridization and AL-PCR in *Allium cepa*

Transgenic line	Genomic DNA blot hybridization	AL-PCR	
		RB	LB
1	1	2	1
2	1	3	3
3	1	1	1

Comparison of the AL-PCR amplification results for the left and right border (Figure 3A and 3B) of the different transgenic shallot lines showed no differences in fragment numbers for line 2 and line 3, i.e. three copies and one copy respectively. This could indicate that the integrated copies were full-length. Genomic DNA blot analysis, using the *hpt* and *uid*A genes as probes for line 3 supported this conclusion (Table 1; Zheng et al., 2000). Here, one full-length copy was integrated. However, for line 2 the genomic DNA blot analysis suggested one copy. The difference between genomic DNA blot and AL-PCR could be explained by assuming that this line contains one full-length copy next to very small T-DNA fragments or next to a rather complex integration event with severe rearrangements. For line 1 a discrepancy was observed in AL-PCR, i.e. RB AL-PCR resulted in two fragments and LB AL-PCR resulted in a single fragment. The results obtained for line 1 could also be explained by assuming that next to a full-length T-DNA insert a truncated T-DNA insert is present. Genomic DNA blot analysis results for this line obtained previously (Zheng et al., 2000) indicated merely the presence of one, full-length copy. This line presumably contains one intact T-DNA copy together with one truncated copy that is too small to allow detection by annealing based techniques such as genomic DNA blot hybridization. Further cloning and sequencing, as well as fluorescence *in situ* hybridization (FISH) may be used to further characterize integration patterns and genome organization of line 1 and 2. For example Wolters et al. (1998) showed via FISH on extended fibers that T-DNA integration in potato was highly complex. In addition to plants harbouring single copy T-DNA insert, plants with a number of T-DNA loci were observed. Furthermore, they showed that some of the T-DNA loci were highly complex due to extensive deletion, inversion and recombination.

Three (TGA) and two (TG) base pairs of the original RB sequences were found in the upper band of transgenic line 1 and line 3, respectively. No left border sequences were found in all three transgenic lines analysed. The results obtained fit the general pattern in literature

upon T-DNA integration: partial loss or even complete loss of border sequences (Devic et al., 1997; Fladung, 1999; Meyerhofer et al., 1991), truncation or even the occurrence of binary plasmid sequences exceeding the T-DNA domain between the borders is known to occur upon T-DNA integration (Graaff et al., 1996; Iglesias et al., 1997; Ramanathan et al., 1995). So far, no reports have shown the presence of small T-DNA inserts. This is probably due to the fact that most previous studies were limited to genomic DNA blot analysis in which small inserts easily escape detection. However, AL-PCR is able to track these small integrated T-DNA stretches of only a few hundred basepairs long.

The site on the genome of the host in which the T-DNA has landed can be studied in closer detail when AL-PCR is combined with subsequent cloning and sequencing of the amplified fragments carrying flanking genomic DNA. In the one example presented here no rearrangements occurred, nor were filler sequences (De Buck et al., 1999; Fladung, 1999; Iglesias et al., 1997) found, however, a stretch of 66 bp of the *Allium* recipient genome was deleted upon integration of the T-DNA. The original landing site used in this particular transgenic plant was isolated by PCR from untransformed shallot DNA. Using databases searches with BLASTX or BLASTN on this sequence of 330 bp we did not find any homologous sequences in the public databases. Most probably the sequence found is *Allium*-specific as in *Lilium* and *Tulipa*, two closely related genera of *Allium* in the family of the Liliaceae, no amplification took place using the primers based on the target site sequences of the transgenic *Allium* plants.

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Chapter 7

General discussion

Both onion (*Allium cepa* L. group Common Onion) and shallot (*A. cepa* L. group Aggregatum) are very important vegetable crops on a worldwide scale. The cultivation of these crops is sometimes severely limited due to the occurrence of pests and diseases. One of the most important pests in *A. cepa* cultivation for (sub)tropical zones is the beet armyworm (*Spodoptera exigua* Hübner). The introduction of resistance to *S. exigua* via genetic methods has not yet been achieved in an *A. cepa* breeding programme (Wietsma et al., 1998). In this study within a framework of research programme, we investigated the potential of two strategies to introduce resistance to the beet armyworm in onion and shallot, namely via marker-assisted breeding (MAB) and via genetic transformation.

Marker assisted breeding

Marker-assisted breeding now plays a prominent role in the field of plant breeding. Once molecular markers closely linked to desirable traits are identified marker-assisted selection can be performed in early segregating populations and at early stages of plant development. MAB can be used to pyramid major genes coding for resistance, with the ultimate goal of producing varieties with more desirable characters. The basic requirements for marker-assisted breeding are: an assay for the traits studied; the genetic basis (and the mechanism of resistance) needs to be unraveled; markers should co-segregate or be closely linked with the desired traits; an efficient means of screening large populations for molecular markers should be available and the screening technique should have high reproducibility.

For carrying out MAB research, firstly, a reliable *in vivo* and *in vitro* bio-assay was developed for screening germplasm for sources of high level (partial) resistance to beet armyworm in *A. cepa* and its wild relatives (Chapter 2). In the *in vivo* bio-assay the lowest larval survival (36 %) and the lowest larval fresh weight (10.3 mg per larva) were found on *A. roylei*. This was, however not, significantly different from the tropical shallot cultivar Bawang Bali. Several different mechanisms can be responsible for the observed pest resistance, either physical, e.g. hairs or trichomes or chemical, e.g. toxic compounds of which the synthesis is induced by wounding. In *A. roylei* it was observed in the *in vivo* assays that the larvae fell down easily from the plants and could not settle within the leaves as they do e.g. in *A. cepa*. This is related to leaf shape. Furthermore, in the *in vitro* bio-assay no toxic compound appeared to be present in *A. roylei* because no significant differences were found in mean fresh weight per larva and mean survival of larvae among different accessions and species. There were also no significant differences in pupal weight and developmental time. All larvae became pupae 10 days after inoculation. In view of the results obtained we were forced to abandon the MAB approach to introduce beet armyworm resistance into *A. cepa*. However, it has to be realised that only a small sample of the total *Allium* germplasm was analysed in this study. Therefore, it is feasible that a more extensive survey will uncover sources with a higher level of resistance because the introduction of resistance to *S. exigua* has been carried out in tomatoes (Eigenbrode & Trunkle, 1993a; Eigenbrode & Trunkle, 1993b; Eigenbrode et al., 1996; Hartman & Clair, 1999). MAB can be extremely powerful as shown in soybean breeding for nematode resistance (Yong, 1999). Recently, Yencho et al. (2000) reviewed the literature on MAB for insect resistance and they showed convincingly the effectiveness of MAB in the development of insect resistant crops.

Genetic transformation

Regeneration system and regeneration potential

A reliable regeneration system is essential for the development of a reproducible transformation system. Both mature and immature embryos are most widely used as starting explants to set up a regeneration system for different *Allium* species (Buiteveld et al., 1993, 1994; Eady et al., 1998; Saker, 1998; Schavemaker & Jacobsen, 1995; Shahin & Kaneko, 1986; Silvertand et al., 1996; Tanikawa et al.,

1998; Van der Valk et al., 1992; Xue et al., 1997). In this thesis, an efficient plant regeneration system was developed using mature zygotic embryo-derived callus from both onion and shallot (Chapter 3). It was shown that regeneration in 45.4 % of the callus lines can be obtained. Results from Buiteveld et al. (1994) showed that friable embryogenic suspension cultures of leek retained a high capacity for plant regeneration for about eight months and after that the regeneration capacity decreased with time. Therefore, our next aim was to identify callus lines with a high regeneration potential and to set-up a suspension culture system for later transformation studies. Suspension cultures were initiated from callus cultures of *Allium cepa*, which had been precultured on a solidified medium for seven months. For another three months 83 callus lines were kept in suspension culture. Of these, 20 suspension lines showed adequate growth and were used to test the effect of cytokinins on plant regeneration. On average, 1.25 % of the calli produced shoots on a growth regulator-free medium (MS30). Thus, plant regeneration decreases significantly with time because the overall average plant regeneration frequency had been 35.5 % after 3 months of culture on solidified medium. Overall only 0.25-0.88 % of the calli produced shoots when the regeneration medium was supplemented with different types and concentrations of cytokinins. Contrary to expectations, the type and concentration of cytokinins could not increase shoot regeneration capacity. Plant regeneration also proved to be highly dependent upon the line used (Chapter 4). On the basis of these results the conclusion was made that only relatively young callus (less 3 months) could be used for transformation research.

Genetic transformation mediated by *Agrobacterium tumefaciens*

When this study was initiated four years ago, particle bombardment was the preferred transformation system for monocots (Songstad et al., 1995). However, during this study reports were published which showed that *Agrobacterium*-mediated transformation of monocots was also possible, primarily in the Gramineae (Arencibia et al., 1998; Cheng et al., 1997; Hiei et al., 1994, 1997; Ishida et al., 1996; Khanna & Raina, 1999; Li et al., 1996; Liu et al., 1998; Rashid et al., 1996; Tingay et al., 1997). Therefore, we also decided to test an *Agrobacterium*-mediated transformation approach in *Allium*.

For *Allium* transformation, Dommisse et al. (1990) demonstrated that onion was a host for *Agrobacterium* as evidenced by tumorigenic responses and production of opines by these tumours. Eady et al. (1996) also showed that *in vitro* onion cultures could be transformed using *Agrobacterium tumefaciens*. However, neither Dommisse

et al. (1990) nor Eady et al. (1996) obtained transgenic plants. We succeeded in obtaining transgenic plants using EHA105 (pCAMBIA1301) and LBA4404 (pTOK233) as vectors. A total of 11 independent transformed callus lines derived from mature zygotic embryos from two onion cvs. Sturon and Hyton, and one shallot cv. Kuning were obtained. On a subspecies basis: seven lines from shallot and four lines from onion and on a vector basis: five lines from pTOK233 and six lines from pCAMBIA1301. Large differences in plantlet production were observed among these lines. The best line produced over 90 plantlets. The transformants have been molecularly characterized by means of standard PCR, Southern hybridization and FISH. Most lines originated from one transformation event. However, in one line plants were found representing at least three independent transformation events. This suggested that T-DNA integration could take place in different cells within one callus line. Most transgenic plants only had one copy of T-DNA integrated into their genomes. FISH performed on 12 plants from two different callus lines representing two separate integration events, showed that T-DNA integration had taken place on the distal end of chromosome 1 or 5 (Chapter 5). In the meantime, a successful transformation of one onion cultivar using *Agrobacterium tumefaciens* was reported by Eady et al. (2000) using immature embryos as inoculated explants. The advantage of our transformation system compared to the system of Eady is that in our system the starting material for transformation is available year-round.

Gene silencing was also found in one transgenic onion line. Plants from this transformant had no GUS expression in the histochemical assay, but did show a clear hybridization signal for this gene in the Southern analysis. The phenomenon of gene silencing is well documented in transgenic plants (Baulcombe & English, 1996; Meyer & Saedler, 1996; Wassenegger & Pelissier, 1998). In some transgenic *A. cepa* plants, dot-like patterns of GUS expression in leaves were found and in others no expression in the bulb. This might also point at problems at the expression level. It could be that the 35S promoter does not have a true constitutive nature in onion, although in rice it is generally accepted as a constitutive promoter (Tyagi et al., 1999). It is known, however, that the 35S promoter sequence contains several domains for expression in a specific way and altogether these domains yield the constitutive expression pattern that is mostly observed (Benfey et al., 1990). Clearly more research is needed to analyse this phenomenon.

Molecular characterization of transgenic plants

Stable genomic integration of transferred genes is traditionally analysed by genomic DNA blot hybridization (Southern, 1975) or progeny analysis. Although genomic DNA blot hybridization can be used to determine the presence and integration of T-DNA sequences as well as the copy number, it becomes labour-intensive when many samples have to be assayed, and it consumes relatively large amounts of genomic DNA. This is especially true for large genome crops, like onion (15290 Mbp/1C) and tulip (24704 Mbp/1C), with genome sizes 105 and 170 times larger than that of *Arabidopsis* (Arumuganathan & Earle, 1991). Because of its large genome size, Wilmink (1996) was not able to prove conclusively via genomic DNA blot hybridization that foreign DNA was stably integrated into the tulip genome. Also in this study we experienced that Southern hybridization is pushed towards its limits. Therefore, we looked for a new technique that could be used as an alternative to Southern hybridization. Adaptor ligation PCR (AL-PCR) proved to be such an alternative technique. The results obtained using AL-PCR for our transformed onion and shallot plants showed that T-DNA integration took place and that the number of T-DNA copies present could be determined. After cloning and sequencing of the AL-PCR products, the junctions between plant genomic DNA and T-DNA inserts could be analysed in detail. Primers located within the left and right flanking T-DNA regions of the transgenic plants could be used to recover the particular target site for this transgenic line from the genome of untransformed plants. This genome sequence could be isolated as an identically-sized PCR amplification product also from other *Allium* species (Chapter 6). Comparison of the AL-PCR amplification results for the left and right border of the different transgenic shallot lines showed no differences in fragment numbers for line 2 and line 3, i.e. three copies and one copy respectively. This could indicate that the integrated copies were full-length. Southern data for line 3 supported this conclusion. Here, one full-length copy was integrated. However, for line 2 the Southern analysis suggested one copy only. The difference between Southern and AL-PCR could be explained by assuming that this line contained one full-length copy next to small debris or next to a rather complex integration event with severe rearrangements. For line 1 a discrepancy was observed in AL-PCR, i.e. RB AL-PCR resulted in two fragments and LB AL-PCR resulted in a single fragment. The AL-PCR and Southern results obtained for line 1 could also be explained by assuming that next to a full-length T-DNA insert a very small truncated T-DNA insert of a few hundred basepairs is present. The results obtained in our study fit the general pattern observed in a number of other transformed plant species. Partial loss of border

sequences (Devic et al., 1997; Fladung, 1999; Meyerhofer et al., 1991), truncation or even full length binary plasmid insertion (Graaff et al., 1996; Iglesias et al., 1997; Ramanathan et al., 1995) are known to take place upon T-DNA integration. Clear cut from our study was the observation that AL-PCR is particular useful in determining small T-DNA inserts. Most probably these small inserts were also present in many of the other transgenic individuals from other species which have been generated and characterized in the past years, however, they remained undetected because only Southern hybridization was used in the molecular characterization.

Future prospects

Nowadays, marker-assisted breeding (MAB) and genetic transformation are considered as powerful, modern methods to improve crops. There has been a lot of excitement about MAB in plant breeding over the past fifteen years when RFLP and PCR-based markers proved to be very beneficial in the construction of genetic linkage maps (Paterson, 1996; Young, 1999). Also in *Allium* genetic maps have been constructed (King et al., 1998; Van Heusden et al., 2000; Van Heusden and Shigyo et al., 2000). MAB is currently applied in the development of onion cultivars resistant to downy mildew (Kik et al., 1997) and MAB is a very promising application in soybean breeding for cyst nematode resistance. But, there are still a very few examples describing the successful use of MAB leading to released germplasm or varieties. MAB as an attractive strategy for crop improvement is still true, but the obstacles have turned out to be much bigger than originally thought.

Genetic engineering has been applied in many crops (Adamczyk et al., 1998; Hardee & Bryan, 1997; Harris et al., 1996; Hilder & Boulter, 1999; Motoyama, 1998; Schuler et al., 1998; Stapel et al., 1998) and there have been field trials of transgenics from at least 52 species including all the major field crops, vegetables, and several herbaceous and woody species (Dunwell, 2000). Several transgenic crops e.g. maize, soybean, cotton, and potatoes are now grown commercially.

The results obtained from Chapter 2 suggest that introduction of resistance to *S. exigua* via the exploitation of variation for resistance to the beet armyworm in *A. roylei* is not possible. Fortunately, a reliable transformation protocol mediated by *Agrobacterium tumefaciens* as a vector for both onion and shallot (*A. cepa* L.) which can be used year-round has been developed (Chapter 5). Together with Eady et al.

(2000) we have shown that an *Agrobacterium*-mediated gene transfer protocol is available as a straightforward and routine method for genetic modification of onion and shallot. Therefore, genetic engineering using *B. thuringiensis cry* or other gene sequences could provide a way forward to introduce resistance to beet armyworm in onion and shallot.

Adaptor ligation PCR (AL-PCR) followed by sequencing of the genomic DNA flanking the T-DNA borders for a large genome crop like *Allium* has been developed (Chapter 6). It can be used to isolate specific promoters (Mudge & Birch, 1998), as well as full gene sequences, based on limited specific sequence information (Mahadeva et al., 1998; Mathur et al., 1998). This is extremely important because there is still no specific promoter known for bulb crops. Combined with FISH, AL-PCR can also be used to investigate more detailed the localization of the T-DNA insert in the *Allium* genome. Furthermore, the transformation protocol developed in this thesis can also provide a starting point for setting up protocols in other *Allium* crops, e.g. garlic (*A. sativum*) or leek (*A. porrum*) for which still no transformation system is available.

All in all, it will be clear that also in *Allium* modern plant breeding using advanced techniques like MAB and genetic transformation is possible. It can be envisaged therefore that the application of both techniques will undoubtedly lead to the development of new high quality onion and shallot cultivars.

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Summary

Onion (*Allium cepa* L. group Common Onion) and shallot (*A. cepa* L. group Aggregatum) are two subspecies of *A. cepa*. Both onion and shallot together with other *Allium* species like garlic (*A. sativum*), leek (*A. porrum*) and bunching onion (*A. fistulosum*) are very important vegetable crops on a worldwide scale. *A. cepa* is propagated by seeds, bulbs, or sets (small bulbs). The bulbs of common onion are large, normally single, and plants are reproduced from seeds or from seed-grown sets. By contrast the bulbs of shallot are smaller than common onions, they form aggregated clusters as a result of the rapid formation of lateral bulbs or shoots. Reproduction of shallot is almost only vegetative via daughter bulbs although seed production is possible.

The cultivation of onion and shallot is sometimes severely limited due to the occurrence of diseases and pests. The most important pest in *A. cepa* cultivation for (sub)tropical zones is the beet armyworm (*Spodoptera exigua* Hübner). The beet armyworm is an extremely polyphagous insect. In this research programme, two different strategies were followed to develop plant material, which is resistant to *S. exigua*, namely via marker-assisted breeding (MAB) and via genetic transformation. For marker-assisted breeding, first of all a reliable bio-assay needs to be developed. Secondly, suitable sources of resistance must be identified. Thirdly, the genetic basis (and the mechanism of resistance) needs to be uncovered and fourthly, molecular markers must be linked to the resistance gene(s), and finally an efficient means of screening large populations for the molecular markers should be available and the screening technique should have high reproducibility.

In marker-assisted breeding, we succeeded to develop an *in vivo* and *in vitro* bio-assay for the identification of resistance to beet armyworm in *A. cepa* and its wild relatives (Chapter 2). In the *in vivo* bio-assay the lowest larval survival (36 %) and the lowest fresh weight (10.3 mg per larva) were found on *A. roylei*, a wild relative of *Allium cepa* L.. This was not, however, significantly different from the resistance in the tropical shallot cultivar Bawang Bali. Furthermore, in the *in vitro* bio-assay no insecticidal compound was present in *A. roylei* because no significant differences were found in mean fresh weight per larva and mean survival of larvae among different accessions. There were also no significant differences in pupal weight and developmental time. All larvae became pupae 10 days after inoculation. Therefore, a

marker-assisted breeding approach to introduce beet armyworm resistance in *A. cepa* was abandoned.

For genetic transformation, first of all a reliable plant regeneration system from callus cultures and suspension cultures must be established. Secondly, an efficient and stable transformation system should be developed. Thirdly, molecular characterization techniques of transgenic plants should be available and fourthly, a construct carrying specific *B. thuringiensis cry* or other gene sequences effective against *S. exigua* should be available. We started with a systematic study on the effects of subspecies, cultivar, basal medium, sucrose concentration and 2,4-dichlorophenoxyacetic acid concentration on callus induction, propagation and subsequent plant regeneration in *Allium cepa*. A reliable regeneration system was developed based on mature zygotic embryo-derived callus. It was shown that regeneration in 45.4% of the callus lines using an optimal combination of factors influencing tissue culture response in *Allium* could be obtained (Chapter 3).

The development of a reliable regeneration system is thought to be of vital importance for the next step in our research, which is the development of a reliable transformation system. The aim was to identify callus lines with a high regeneration potential and to set up a suspension culture system for later transformation studies by means of particle bombardment. Suspension cultures were initiated from callus cultures of *Allium cepa*, which had been precultured on a solidified medium for seven months. For another three months the 83 callus lines were kept in suspension culture. Of these, 20 suspension lines showed adequate growth and were used to test the effect of cytokinins on plant regeneration. However, it was found that the plant regeneration capacity of selected lines significantly decreased in a relatively short time-period. Plant regeneration also proved to be highly dependent upon the line used. Contrary to expectations, the type and concentration of cytokinins could not increase the shoot regeneration capacity (Chapter 4). On the basis of these results it was concluded that only relatively young callus (less 3 months) could be used for *Allium* transformation.

In Chapter 5 the development of a reliable transformation system is described using *Agrobacterium tumefaciens* as a vector. A number of parameters, such as callus age, callus induction medium, co-cultivation period, osmotic treatment, cultivars, selection method and *Agrobacterium* strains were used to evaluate transient expression of the *uidA* reporter gene in a series of experiments followed by evaluating stable expression conditions. Eventually, an *Agrobacterium tumefaciens*-mediated transformation system both for onion and shallot was developed using three-week old callus derived from mature embryos using two different strains EHA105(pCAMBIA 1301) and LBA4404(pTOK233) carrying a binary vector coding for *uidA* and *hpt*. This transformation protocol for onion and shallot can be used year-round because

mature zygotic embryos are used as starting material. A total of 11 independent callus lines regenerating transgenic plants have been produced in several independent experiments. In one of these experiments, starting with 154 embryos, transgenic plants were recovered from three independent callus lines, giving a transformation frequency of 1.9 %. The best line produced 90 transgenic plants. Subspecies (onion and shallot) and cultivar were important factors for successful transformation: shallot was better than onion and for shallot with cv. Kuning the best results were obtained. Also, it was found that constantly reducing the size of the calli during subculturing and selection by chopping, thus enhancing exposure to the selective agent hygromycin, improved the selection efficiency significantly. The transformants were genetically characterized by means of standard PCR, genomic DNA blot hybridization and FISH (fluorescence *in situ* hybridization).

Genomic DNA blot hybridization is traditionally used to analyse integration of foreign genes into host genomes. Although genomic DNA blot hybridization can be used to determine the presence of T-DNA and copy number, it becomes labour-intensive when many samples have to be assayed, and it consumes relatively large amounts of genomic DNA. Especially for large genome species crops, e.g. onion (15290 Mbp/1C), with a genome size which is 105 times larger than that of *Arabidopsis*. Adaptor ligation PCR (AL-PCR) followed by the sequencing of genomic DNA flanking the T-DNA borders was developed (Chapter 6). The AL-PCR patterns obtained were specific and reproducible for a given transgenic line. The results showed how T-DNA integration took place and it also provided insight into the number of T-DNA copies present. Comparison of AL-PCR and previously obtained Southern hybridization results pointed into the direction of rather complex T-DNA integration patterns in some of the transgenic plants. After cloning and sequencing of the AL-PCR products, the junctions between plant genomic DNA and the T-DNA inserts were analysed into great detail. For example, it was shown in one of the transgenic lines that upon T-DNA integration a 66 bp sequence was deleted, and no filler DNA was inserted. Primers located within the left and right flanking genomic DNA in transgenic shallot plants were used to recover this particular target site for T-DNA integration. The target site sequence proved to be *Allium* specific because a similarly-sized PCR fragment was obtained in all *Allium* species tested while it was not detected in closely related genera such as *Lilium* and *Tulipa*.

In Chapter 7, the general discussion, the possibilities for the development of onion and shallot resistant to *Spodoptera exigua* are discussed. It is argued that both MAB and genetic transformation are potentially very powerful but that in case of the introduction of resistance to beet armyworm into onion and shallot genetic transformation is the most promising method.

Samenvatting

Allium cepa maakt deel uit van het genus *Allium*: een genus dat bestaat uit ongeveer 600 soorten, die voornamelijk zijn verspreid over het Noordelijk halfrond. Ui (*A. cepa* L. groep 'Common onion') en sjalot (*A. cepa* groep 'Aggregatum') zijn de twee bekendste ondersoorten van *A. cepa* en zijn wereldwijd gezien zeer belangrijke groentegewassen. Andere veel geteelde groentegewassen uit het genus *Allium* zijn knoflook (*A. sativum*), prei (*A. porrum*) en stengelui (*A. fistulosum*). *A. cepa* is een tweejarige soort, die in het eerste jaar een bol vormt en in het tweede jaar zaad produceert. Ook zijn er varieteiten bekend die overblijvend zijn. *A. cepa* kan worden vermeerderd via zaad (ui), bollen of sets (sjalot). Bij de ui wordt er doorgaans één grote bol per plant gevormd terwijl bij de sjalot meerdere kleine bolletjes (sets) ontstaan. Uien worden meest geteeld in gematigde zones, terwijl sjalotten meer worden geteeld in tropische gebieden.

De teelt van ui en sjalot kan sterk worden beïnvloed door het voorkomen van ziekten en plagen. De belangrijkste plaag voor *Allium*-teelten in (sub)tropische gebieden is de rups van de floridamot (*Spodoptera exigua* Hübner). In dit onderzoeksproject zijn twee verschillende strategieën gevolgd voor de ontwikkeling van sjalotten en uien die resistent zijn tegen de rups van de floridamot, namelijk merker-gestuurde veredeling en genetische transformatie. Voor een successvol merker-gestuurd veredelingsprogramma dient allereerst een betrouwbare toetsmethode te worden ontwikkeld. Ten tweede zal een goede resistentiebron moeten worden geïdentificeerd, ten derde zal de genetische basis van de resistentie moeten worden opgehelderd en ten vierde dienen de genen die de resistentie bepalen gekoppeld te worden aan moleculaire merkers. Wij slaagden er in om betrouwbare *in vivo* en *in vitro* toetsmethoden te ontwikkelen voor de identificatie van bronnen van resistentie in *A. cepa* en zijn wilde verwanten (Hoofdstuk 2). De door ons ontwikkelde *in vivo* toets toonde aan dat de laagste larvale overleving (36 %) en het laagste larvale versgewicht (10.3 mg per larve) werd gevonden voor larven die waren geïnoculeerd op *A. roylei*. Echter deze waarden waren niet significant verschillend van larven die geïnoculeerd waren op de Indonesische sjalottencultivar Bawang Bali. Uit de door ons ontwikkelde *in vitro* toets bleek dat het mechanisme van de resistentie niet berustte op het voorkomen van een toxische stof. Gegeven het feit dat we geen significant betere resistentiebron konden vinden dan het reeds in

Indonesie geteelde ras Bawang Bali deed ons besluiten om niet verder te gaan met dit deel van het onderzoek.

Voor het succesvol toepassen van genetische transformatie in een gewas dient allereerst een betrouwbaar plant regeneratiesysteem vanuit explantaten, callus en/of suspensiecultures te worden ontwikkeld. Verder is de ontwikkeling van een efficient transformatiesysteem van cruciaal belang. Bovendien dienen constructen aanwezig te zijn voor de eigenschap die men wil verbeteren in een gewas. In deze studie zijn we gestart met een systematisch onderzoek naar het effect van achtereenvolgens ondersoort, cultivar, basaal medium, sucrose concentratie en 2,4-D concentratie op callusinductie, callusvermeerdering en plantregeneratie van ui en sjalot. Een betrouwbaar regeneratiesysteem werd ontwikkeld dat gebaseerd is op callus geïnduceerd aan volwassen embryo's uit zaad. We vonden in onze studie dat 45.4% van de calluslijnen konden worden geregenereerd onder optimale omstandigheden (Hoofdstuk 3). Het bleek dat de plantregeneratie-capaciteit sterk achteruit liep bij kweek onder *in vitro* condities en reeds een paar maanden na callusinductie bijna tot nul was gereduceerd. Het gebruik van cytokinines zoals bijvoorbeeld zeatine en thidiazuron kon dit niet voorkomen. Op basis van deze resultaten is dan ook gekozen voor het gebruik van relatief jong callus (jonger dan drie maanden) als startmateriaal voor transformatie-experimenten (Hoofdstuk 4).

In hoofdstuk 5 wordt de ontwikkeling van een efficient transformatie-systeem beschreven. In dit onderzoek is gebruik gemaakt van *Agrobacterium tumefaciens* als vector voor het overbrengen van genen. Een groot aantal parameters zijn getoetst gedurende de ontwikkeling van het transformatie-protocol, zoals leeftijd van het callus, callusinductie medium, co-cultivatie periode, osmotische behandeling, ui/sjalot cultivar, selectiemethode, *Agrobacterium* stam. De transiënte expressie van het *uid*A reporter gen werd gebruikt als maatstaf in de optimalisatie-procedure. Uiteindelijk kwam een protocol tot stand voor ui en sjalot dat gebaseerd is op het inoculeren van drie weken oud callus, afkomstig van volwassen zygotische embryo's, met *Agrobacterium tumefaciens*. Twee *Agrobacterium* stammen zijn hiervoor gebruikt namelijk EHA105 (pCAMBIA 1301) en LBA4404 (pTOK233). In beide stammen bevond zich een binaïre vector die codeerde voor het reporter gen *uid*A en het selectiegen hpt. Het door ons ontwikkelde transformatie-protocol heeft als groot voordeel dat het gedurende het hele jaar kan worden gebruikt omdat het uitgangsmateriaal voor transformatie, embryo's uit zaad, het gehele jaar beschikbaar is. Van elf onafhankelijke calluslijnen zijn uiteindelijk getransformeerde planten verkregen. De hoogst verkregen transformatie-frequentie binnen één experiment bedroeg 1.9 %. De beste calluslijn produceerde 90 transgene planten. Ondersoort en cultivar bleken belangrijke factoren te zijn voor een succesvolle transformatie: sjalot bleek beter te zijn dan ui en met de sjalotten-cultivar Kuning werden de beste resultaten verkregen. Ook bleek het constant reduceren van de grootte van de callusstukjes gedurende de weefselkweekfase na transformatie zeer

belangrijk. Dit vanwege het feit dat in grote callusstukken veel cellen niet voldoende werden blootgesteld aan het selectieve agens hygromycine en zodoende konden ontsnappen aan de selectieprocedure wat weer aanleiding gaf tot een hoog percentage ongetransformeerde regeneranten. De transformanten werden genetisch gekarakteriseerd met behulp van normale PCR, Southern hybridisatie en FISH (fluorescentie *in situ* hybridisatie).

De genetische analyse van de integratie van genen in een genoom geschiedt meestal via Southern hybridisatie. Alhoewel Southern hybridisatie kan worden gebruikt om de aanwezigheid en integratie van T-DNA aan te tonen en het aantal T-DNA kopieën te bepalen, is het een arbeidsintensieve techniek, vooral wanneer veel monsters moeten worden geanalyseerd. Bovendien is voor het uitvoeren van een Southern hybridisatie relatief veel DNA nodig. Dit is met name het geval voor 'grote genoom' soorten zoals ui met een genoom dat 105 maal groter is dan *Arabidopsis*. Om aan bovengenoemde bezwaren tegemoet te komen is gezocht naar een andere techniek om de integratie van vreemde genen vast te stellen na transformatie. Een adaptor ligatie PCR (AL-PCR) gevolgd door het sequencen van het plant - T-DNA grensgebied bleek in deze uitkomst te bieden (Hoofdstuk 6). De verkregen resultaten toonden aan dat T-DNA integratie plaats vond en gaf ook inzicht in het aantal T-DNA kopieen dat aanwezig was in het recipiënte genoom. Zowel uit de resultaten van de AL-PCR als de Southern hybridisatie kwam naar voren dat in sommige planten het T-DNA op complexe wijze was geïntegreerd in het *A. cepa* genoom. Na het kloneren en sequencen van de AL-PCR producten bleek dat als resultaat van het T-DNA integratieproces in één van de transgene lijnen een 66 baseparen lang fragment was geëlimineerd in vergelijking met de originele gastheersequentie van de doellocatie. Verder vonden wij geen zogenaamd 'filler DNA' in de door ons bestudeerde transgene planten. De sequentie van de doellocatie van het T-DNA bleek *Allium* specifiek te zijn, daar na PCR met de voor deze doellocatie specifiek ontworpen primers een fragment van dezelfde grootte werd aangetoond in alle geteste *Allium* soorten, maar niet in evolutionair verwante soorten, zoals lelie en tulp.

In hoofdstuk 7, de algemene discussie, zijn de mogelijkheden voor de ontwikkeling van uien en sjalotten die resistent zijn tegen *Spodoptera exigua* op een rijtje gezet. Beargumenteerd wordt dat merker-gestuurde veredeling en genetische transformatie krachtige technieken zijn voor de introductie van resistentie's, maar dat in geval van het verkrijgen van uien en sjalotten met een resistentie tegen *Spodoptera exigua*, genetische transformatie momenteel de meeste kansen op succes biedt.

摘要

普通洋葱(Allium cepa L. group Common Onion)和火葱(A. cepa L. group Aggregatum)属于同一洋葱种(A. cepa L.)的两个亚种. 它们与大蒜(A. sativum L.), 大葱(A. fistulosum L.) 和韭葱(A. porrum L.)都是在全世界范围内非常重要的蔬菜作物. 洋葱多数是两年生的, 但也有多年生的. 它们主要是通过种子, 鳞茎和小鳞片进行繁殖. 普通洋葱通常是较大的单一鳞茎. 与普通洋葱所不同的火葱是它的鳞茎由多个小磷茎组成. 火葱的繁殖主要是通过无性繁殖, 尽管通过种子繁育也有可能.

普通洋葱和火葱的生产由于受到病虫害的侵害而收到极其严重的影响. 甜菜夜蛾(Spodoptera exigua Hubner)是亚热带地区洋葱栽培最重要的虫害之一. 甜菜夜蛾是一种杂食性极其强的昆虫. 目前尚未产生有效的抗性品种来防治这一害虫. 在这个研究项目中我们采取两种不同的手段来培育植物的抗虫性:一种是通过分子标记辅助育种手段;另一种是通过遗传转化手段. 对于分子标记辅助育种来说, 首先要建立可靠的生物学鉴定方法; 其次要有可靠的抗源; 第三遗传机理必须明确; 第四分子标记与抗性基因必须紧密连锁. 我们已经成功地在洋葱和它的近缘野生种中建立了甜菜夜蛾活体和离体的抗性鉴定方法(第二章). 在洋葱的野生种 A. roylei 中发现甜菜夜蛾的幼虫成活率最低 (36 %)和存活幼虫的平均鲜重最低(10.3 毫克/幼虫), 然而这与热带火葱栽培品种 Bawang Bali 并没有显著的差异. 同时在 A. roylei 中也没有发现类似杀虫剂似的毒性化合物. 因此, 通过分子标记辅助育种来培育洋葱抗甜菜夜蛾的手段就放弃了.

对于遗传转化手段, 首先必须建立通过愈伤组织培养和悬浮培养体系获得可靠的植株再生体系. 其次必须建立有效和稳定的转化体系. 第三必须具备鉴定转基因植株的分子鉴定技术. 第四必须具备抗甜菜夜蛾的基因. 我们系统和深入地研究了洋葱亚种, 栽培品种, 基本培养基, 蔗糖浓度和激素对于愈伤组织诱导和植株再生的影响.采用洋葱组织培养最优化的组合, 45.4 % 的愈伤组织系能获得植株再生 (第三章).

稳定的植株再生体系对于建立可靠的遗传转化体系是至关重要的. 我们的最初目标是筛选出具有高度再生潜力的愈伤组织系和建立用于基因枪转化的细胞悬浮培养体系. 从固体培养基中培养了7个月的愈伤组织系中建立了细胞悬浮系. 在液体培养基中培养3个月后从83个细胞悬浮系中选择了生长合适的20个悬浮系用于测试不同细胞分裂素种类和浓度对于植株再生的影响. 然而我们很快就发现植株的再生能力随着时间的推移而很快地降低. 植株的再生能力高度地取决于所用的细胞悬浮系. 与预期的结果相反, 细胞分裂素的种类和浓度不能提高植株的再生能力(第四章). 基于上述结果我们认为只有相对幼嫩的愈伤组织(小于3个月)能用于洋葱的遗传转化.

第五章详细描述了利用根癌农杆菌(Agrobacterium tumefaciens)为载体建立稳定的洋葱遗传转化体系. 在一系列试验中, 愈伤组织诱导的年龄, 诱导愈伤组织的培养基, 与根癌农杆菌共培养的时间, 不同渗透压处理, 洋葱不同亚种, 不同栽培品种, 不同选择方法和农杆菌菌株等多项参数用于评估 GUS 基因的瞬间表达. 最后利用两个根癌农杆菌菌株

EHA105(pCAMBIA1301) 和 LBA4404(pTOK233)携带 GUS 报告基因和潮霉素选择抗性基因(hpt)的双元载体建立了洋葱和火葱稳定的遗传转化体系．这个体系利用从成熟胚中诱导 3 周的新鲜愈伤组织为外植体．这个体系的优点在于外植体不收季节的限制并能够整年使用．在几个独立的试验中，一共有 11 个不同的愈伤组织系 (line) 获得了转基因植株．在其中的一个试验中，起始外植体为 154 个胚，一共有 3 个愈伤组织系获得了转基因植株，转化频率达到 1.9 %．最好的一个愈伤组织系产生了 90 个转基因植株．亚种和品种间的差异对于遗传转化的成功是非常重要的因素．火葱优于洋葱，在火葱中栽培品种 Kuning 获得了最好的结果．同时我们也发现在继代培养和选择过程中经常减小愈伤组织的大小以增大潮霉素选择剂与愈伤组织的接触，能明显提高选择效率．这些转基因植株已通过常规的 PCR，Genomic DNA (Southern) 杂交和 FISH (Fluorescence in situ hybridization) 进行了分子生物学和分子细胞遗传鉴定．在多数转基因植株中，外源基因整合到第一对或第五对染色体上．

外源基因整合到受体基因组通常采用 Southern 杂交来进行分析．尽管 Southern 杂交能用于确定 T-DNA 的整合和拷贝数目，但它特别费时和麻烦．另外它消费相对大量的基因组DNA．尤其像对于洋葱 (15290 Mbp/1C)这样的大基因组作物，它的基因组是拟南芥菜的 105倍．Southern 杂交就相对比较困难．因此我们建立了 Adaptor Ligation PCR (AL-PCR)技术并对相邻 T-DNA 插入位点的基因组 DNA 进行了测序 (第六章)．AL-PCR获得的谱带对每一个转基因植株都是专一的和可靠的．通过 AL-PCR 所获得的结果能验证 T-DNA的整合和拷贝数．另外，AL-PCR与 Southern 杂交结果相比较后表明一些转基因植株 T-DNA的整合是非常复杂的．在对 AL-PCR 产物进行克隆和测序后，对植物基因组 DNA 序列与 T-DNA插入的连接部分可进行细致的分析．例如在一转基因植株中，T-DNA 整合到植物基因组 DNA 后，植物基因组的 66 个碱基序列就缺失了，并且没有填充的 DNA 插入．采用转基因火葱植株邻近 T-DNA 整合位点的分别位于左边和右边的植物基因组序列设计了引物并用于 PCR 分析，能够有特异性的 PCR 产物出现．我们发现 T-DNA 的整合到洋葱中有专一位点．因为在葱属其它种中也有非常相似的 PCR 产物，这证明 T-DNA 的整合位点是葱属专一的.因为在近缘的百合属和郁金香属中没有检测到类似的 PCR 产物．

第七章对建立普通洋葱和火葱抗甜菜夜蛾的抗性品种的可能性进行了总讨论．通过分子标记辅助育种和遗传转化方法都是非常强有力的收段．但是通过遗传转化手段导入抗甜菜夜蛾的性状到普通洋葱和火葱中则更有前景．

Curriculum Vitae

Si-Jun Zheng was born in Songyang County, Zhejiang Province, China on November 9th, 1963. After finishing high school in Songyang County and passing his national entrance exam, he started his study at the Department of Agronomy, Zhejiang Agricultural University (now Zhejiang University) in Hangzhou City. He received his Bachelor Degree of Science in Plant Genetics and Breeding in July 1984. He continued with his MSc studies at the same university and obtained his Master of Science Degree in Plant Genetics and Breeding in July 1987. The title of his dissertation was: "A study on characterisation of cotton wild races (*Gossypium hirsutum* L.) and their utilisation".

From 1987 to 1995 he worked as a teaching assistant (from 1987 to 1991) and as an assistant professor (from 1991 to 1995) at the same university. He was mainly involved in teaching students and in research on cotton disease resistance breeding, cytogenetics and tissue culture. He gave courses on "Cytogenetics" and "Plant Cell and Tissue Culture", both for graduate and undergraduate students, and on "Genetics", both for Chinese and African undergraduate students.

He wrote a textbook together with his colleagues on "Experiments in Genetics", published by China Agricultural Press in 1992 which is used by all agricultural universities in China. He was project leader of two research programs. One was supported by The Zhejiang Provincial Natural Science Foundation on "Investigation of characteristics of cotton wild races and their utilisation" from 1988-1991. The second was supported by The International Foundation for Science of Sweden on "Somatic embryogenesis and somaclonal variations in cotton" from 1990-1994. He participated at the International Congress on "DNA Bank-Net" at the Royal Botanic Gardens, Kew, England in 1991 and XIIIth EUCARPIA Congress in Angers, France in 1992.

Si-Jun Zheng obtained fellowships to attend two training courses outside of China. These were "Exploration of Wild Genetic Resources: Principles of Collection and Sampling" at the University of Jerusalem, Rehovot, Israel, April 10-22, 1994 and "Applied Plant Breeding" at the International Agricultural Center, Wageningen, The Netherlands, March 5- July 15, 1995.

Because of his outstanding performance both in teaching and research, he was promoted to associate professor at the Former Zhejiang Agricultural University (now Zhejiang University) in October, 1995 and was also appointed as the Director of the Plant Breeding and Genetics Unit.

In September 1996, he began a four-year PhD research project, the results of which are described in this thesis. The project was performed at the Business Unit Genetics and Breeding of Plant Research International and the Laboratory of Plant Breeding under supervision of the graduate school EPS, Wageningen University and Research Center in Wageningen, the Netherlands.

From September 2000 onwards he will continue his research on *Allium* transformation at Plant Research International.

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