Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek

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Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek

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Abstract

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This thesis describes the development of a protoplast to plant system for leek and a method for somatic hybridization between two *Allium* species with the ultimate goal to transfer cytoplasmic male sterility to leek. An efficient plant regeneration system from zygotic embryo-derived compact callus was described. The system was applicable for a large range of cultivars, however, significant differences were found in plant regeneration capacity between and within leek cultivars. In addition to compact embryogenic callus, two other callus types could be distinguished in leek: a watery non-embryogenic callus and a friable, embryogenic callus. Friable, embryogenic callus is induced on immature embryos and is highly regenerative. This callus appeared a crucial factor for the development of finely-dispersed embryogenic suspension cultures, from which regeneration-competent protoplasts could be isolated. Symmetric somatic hybridization of leek and onion resulted in high numbers of hybrid plants. As determined by flow cytometry, all hybrids were aneuploid. Detailed analysis by genomic *in situ* hybridization of some hybrids revealed that their chromosome number varied from 41 to 45. The hybrids showed an intermediate leaf morphology. Southern analysis of the organelle DNA showed that the majority of the hybrids possessed leek chloroplasts and had a rearranged mitochondrial genome derived from both parents, but with a predominance of mtDNA fragments from leek.

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The research described in this thesis was carried out at the DLO-Center for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, 6700 AA Wageningen, The Netherlands.

The cover shows chromosomes of a somatic hybrid between leek and onion. The leek chromosomes fluoresce orange-red, the onion chromosomes fluoresce yellow.

NN08201, 2402.

Stellingen

1. Het ontwikkelen en instandhouden van stabiele, embryogene suspensiecultures in monocotyle gewassen, in het bijzonder in prei, blijft een beperkende factor voor het succesvol toepassen van fusie- en transformatietechnieken en zou vermeden moeten kunnen worden.

   Dit proefschrift

2. Het gebruik van 'friable', embryogeen callus is een cruciale stap in de ontwikkeling van een protoplast-tot-plant systeem voor prei.

   Dit proefschrift

3. De hoge mate van pessimisme over de regeneratiecapaciteit van mesofylprotoplasten van monocotylen is onterecht.

   Gupta HS and Pattanayak A (1993) Bio/Technology 11: 90-94

4. Het is mogelijk om via protoplastenfusie preiplanten te verkrijgen, die verrijkt zijn met de CMS-eigenschap van ui.

   Dit proefschrift

5. Het verdient aanbeveling om alternatieve methoden te ontwikkelen voor het overdragen van een CMS-factor, zoals transformatie van extra-chromosomaal DNA.

   Svab Z, Hajdukiewicz P and Maliga P (1990) Proc Natl Acad Sci USA 87: 8526-8530

   Dewey RE, Levings III CS and Timothy DH (1986) Cell 44: 439-449

6. Met de totstandkoming van somatische soorthybriden tussen prei en ui zullen de taxonomische problemen in het geslacht *Allium* er niet kleiner op worden.

7. De belangrijkste vaardigheid voor het moderne windows-georienteerde computergebruik wordt ons reeds op de kleuterschool bijgebracht: knippen en plakken.

8. Het winnen van de huidige oceaanraces op het hoogste niveau, zoals de 'The Whitebread around the world race', is voornamelijk een kwestie van veel geld.

9. De toenemende populariteit van biologische produkten betekent de ondergang van de kleine natuurvoedingswinkel.

10. De door de overheid gestimuleerde arbeidsparticipatie van vrouwen strookt vooralsnog niet met het beleid ten aanzien van kinderopvang.

11. Door de stijgende levensverwachting van de mens en de op jongere leeftijd optredende MIDlife crisis, staat deze niet meer in het midden.

Stellingen behorende bij het proefschrift 'Regeneration and interspecific somatic hybridization in *Allium* for transfer of cytoplasmic male sterility to leek' door Joukje Buiteveld, in het openbaar te verdedigen op woensdag 4 maart 1998, te Wageningen.

Voorwoord

In 1990 ben ik begonnen aan een tweejarig onderzoeksproject, gericht op het ontwikkelen van regeneratiemethoden bij prei. Dat deze twee jaar uiteindelijk zouden uitmonden in 6 jaar onderzoek, met als eindresultaat dit proefschrift, had ik destijds nooit kunnen vermoeden. Graag wil ik op deze plaats iedereen bedanken, die heeft bijgedragen aan de totstandkoming van het proefschrift.

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Tineke Creemers-Molenaar, mijn co-promotor, wil ik bedanken voor de inspirerende wijze waarop zij haar kennis over protoplastenfusie op mij heeft overgebracht. Bijzonder veel dank ben ik haar verschuldigd voor alle tijd die zij heeft uitgetrokken om de manuscripten te becommentariëren en met mij van gedachten te wisselen als het onderzoek in een moeilijke fase verkeerde of een nieuwe wending moest krijgen.

Chris Kik wil ik met name noemen, niet alleen omdat hij de initiator is van dit onderzoek, maar ook omdat hij zijn enorme kennis over Alliums altijd met mij wilde delen. Zijn commentaren op enkele van de hoofdstukken waren van onschatbare waarde.

Ik kijk met veel plezier terug op de tijd bij het CPRO. Van de medewerkers van de oude afdeling Celbiologie, heb ik de kunst van het regenereren van recalcitrante gewassen kunnen afkijken. De collega's van de afdeling Ontwikkelingsbiologie hebben mij altijd met raad en daad terzijde gestaan bij mijn eerste schreden op het moleculaire pad. Ik wil hen dan ook allen bedanken voor de goede samenwerking, de collegiale sfeer en natuurlijk niet in de laatste plaats voor de gezelligheid.

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I also would like to thank Suo Yuying (Shanxi Agricultural University, China) for participating in my project for one year. De studenten Els Meyer, Richard Geels en Wiesje Kassies hebben in het kader van een doctoraalonderzoek of stage hun steentje bij gedragen aan het onderzoek.

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Mijn ouders bedank ik voor de belangstelling die zij altijd voor mijn werk hebben getoond. Maar ook in de hoedanigheid van pake en beppe wil ik hen en oma Phil bedanken. Door op Ilse te passen hebben zij het schrijven tijdens het afgelopen jaar vele malen lichter gemaakt.

Olaf wil ik bedanken voor zijn altijd aanwezige steun en voor de hulp bij één van de laatste loodjes: de lay-out van het boekje. En natuurlijk wil ik Ilse bedanken voor de broodnodige afleiding gedurende het laatste anderhalf jaar.

Youkje

Contents

Chapter 1

General introduction

Leek

Leek (*A. ampeloprasum* L.) is a member of the genus *Allium* (Family *Alliaceae*), which comprises more than 600 species, distributed across the northern hemisphere (Jones 1991). In 1990 Hanelt reviewed earlier classifications of the genus (Traub 1968, Wendelbo 1971, Kamelin 1973, Stearn 1978) and distinguished 5 subgenera. According to this author leek is placed in the *A. ampeloprasum* complex, which belongs to the section *Allium* of the subgenus *Allium*. This classification was mainly made for a better understanding of the relationships of the cultivated species of *Allium*. The *A. ampeloprasum* complex is an extremely variable species and comprises several wild and cultivated forms with ploidy levels of 4x, 5x and 6x. The four cultivated forms are leek, kurrat, great-headed garlic and pearl onion (Van der Meer and Hanelt 1990). Leek probably originates from wild *A. ampeloprasum*. Wild forms of the species are found from Portugal in the west through the Mediterranean countries to west Iran in the east (de Wilde-Duyfjes 1976, Stearn 1978). There is still some disagreement about the nomenclaturally correct name for leek. According to Hanelt (1990) the correct name is *A. ampeloprasum* L. In the latest review of *Allium* section *Allium*, Mathews (1996) retained the name *A. porrum* for leek. Another synonym that is used is *A. ampeloprasum* var. *porrum* Gay.

Economical importance

Leek is grown for its edible pseudostem formed by the leaf sheaths. It is grown world-wide, but only in Europe it is an important vegetable crop (Pink 1993). Production by the 12 countries of the European Union (EU) is totally about 700,000 tonnes per year. France is the largest EU producer with nearly 9000 ha yielding about 200,000 tonnes. In the Netherlands the total area has increased enormously during the past years and comprised 3854 ha in 1995 (Anonymous 1996). The increase in acreage was not directly a result of profitability, but was due to low prices for arable crops, which made farmers switch to the cultivation of leek (de Kraker 1993). The increase in production has led to a substantial higher export. Approximately, half of the total production is exported. Germany is the most important consumer of Dutch leek, accounting for 50% of the total export.

Leek is mainly a fresh-market crop. In 1994 the market sales at the Dutch auctions valued Dfl. 83 million (Anonymous 1996). Most of the leek production is marketed via auctions, only a small amount (10%) is sold to the industry for processing. A large number of cultivars

is grown, which are all adapted to different cropping seasons. The cultivation of leek can be subdivided in very early, early, normal summer, early and late autumn, normal and late winter cultivation. Harvested leek of the winter cultivation is nowadays stored more often to fill up the gap between early summer and late winter, which makes year round supply possible.

Conventional breeding

Objectives for leek breeding include the improvement of winter-hardiness, a long shaft length, absence of bulbing, resistance to bolting, upright habitus and dark-green leaf blade colour (Pink and Innes 1984). In addition to these improvements in quality, breeders also aim for improvement in yield, uniformity and resistance to pests and diseases. Long shaft length is desirable, particularly for processing. Improvement in uniformity is also important, since leek plants vary considerably in size at harvest. Variability in size is a major factor in the marketability of the crop (Pink 1993). According to the harvest period, the seasonal types are selected for different characteristics. For spring and early summer production fast growth, earliness and resistance to bolting are important. For cultivation later in the season, hardy types able to stand outside throughout the winter are needed. Winter hardiness is usually associated with a short shaft length. Thus, for selection of winter types more attention is paid to winter hardiness, improvement of the shaft length and resistance to diseases (de Kraker 1993). Leek suffers severely from many pests and diseases. The need for disease and pest resistant cultivars has increased enormously, also because of the growing public and political pressure to minimize the use of fungicides and pesticides. Thrips (*Thrips tabaci*) is probably the most damaging insect pest in leek. Other insects that attack leek are onion fly (*Delia antiqua*) and leek moth (*Acrolepiopsis assectella*). White tip (*Phytophthora porri*), leek rust (*Puccinia allii*), purple blotch (*Alternaria porri*), black stripe (*Leptotrochila porri*), leek yellow stripe virus (LYSV) are diseases that occur in leek, of which white tip and leek rust are the most important ones (Smilde 1996).

Breeding problems

Several factors make leek a difficult crop to improve (for a review see Currah 1986). Leek is subject to severe inbreeding depression. The frequency of recessive lethal and deleterious genes is high. Already after one generation of selfing severe losses of vigour and seed

production can be observed (Schweisguth 1970, Gagnebin and Bonnett 1979). As a consequence of the inbreeding depression, positive mass selection under heavy stress conditions has been the chief means of improvement (McCollum 1976), usually within the seasonal types (Bonnet 1976). Nowadays more attention is paid to line or family selection (Van der Meer and Hanelt 1990). Leek is an outbreeding species, but self-pollination does occur and frequencies up to 20% have been found in commercial cultivars (Berninger and Buret 1967). The cultivars are highly heterogeneous. One of the factors contributing to this high heterogeneity is this high percentage of selfing, which results in some inbreeding depression. The presence of aneuploid seedlings, originating from meiotic irregularities, may be another factor responsible for the high variability within leek cultivars (Khazanehdari and Jones 1997). The cultivated leek is tetraploid (2n=4x=32) (Levan 1940), which also makes breeding complicated. There are some differences of opinion whether it is an autotetraploid or an allotetraploid. Most authors agree that leek is an autotetraploid, because for most of the genes leek shows tetrasomic inheritance (Levan 1940, Kadry and Kamel 1955, Berninger and Buret 1967, Schweisguth 1970). Koul and Gohil (1970) proposed that leek is a segmental allotetraploid with the genomic formula AAA'A", obtained from three closely related parental species. The fact that in leek the chiasmata are localized near to the centromeres has as the consequence that the amount of recombination is limited (Gohil 1984, Stack and Roelofs 1996).

Crossability

Because leek has a narrow gene pool, the use of closely-related wild species in breeding programmes would be interesting. They may provide a possible source of desirable traits, such as disease resistance or cytoplasmic male sterility.

Within the subgenus *Allium*, little research has been done on interspecific crosses, but there are a number of species that have been crossed with leek. Von Bothmer (1974) observed that hybrids probably occur between some members of the *A. ampeloprasum* group on Crete, namely *A. ampeloprasum*, *A. commutatum* and *A. bourgeaui*. Fertile hybrids have been obtained from reciprocal crosses between leek and *A. kurrat* (Kadry and Kamel 1955, Van der Meer and van Dam 1982) and also pearl onions (*A. ampeloprasum* L. var *sectivum* Lued.) can easily be crossed with leek (Van der Meer and Hanelt 1990). More recently, successful crosses between leek and some wild relatives of the *A. ampeloprasum* complex: *A. ampeloprasum*, *A. commutatum*, *A. bourgeaui* and *A. atroviolaceum* were carried out

(Verbeek *et al* 1995, Kik *et al* 1997).

Hybridization between species of different subgenera is much more difficult. In an earlier report of Dolezel *et al* (1980) attempts to cross leek with onion (*A. cepa*), which belongs to the subgenus *Rhizirideum* failed. However, recently, hybrids were sexually produced between these two species with the use of embryo rescue (Peterka *et al* 1997). Also *A. sphaercephalon* and *A. sativum*, both species belong to the same subgenus *Allium* as leek, have recently been sexually hybridized with *A. cepa* (Ohsumi *et al* 1993, Keller *et al* 1996). Unfortunately, in these cases the hybrids showed a high degree of sterility.

F_1 hybrid breeding

Nowadays the main objective of many breeding programmes is the development of F_1 hybrid cultivars, which have considerable benefits with regard to the present open-pollinated cultivars. Advantages of production of F_1 hybrids are improvement of uniformity of the crop, better exploitation of heterosis for several characteristics, i.e. yield and a faster fixation of desirable traits. In 1970, Schweisguth demonstrated heterosis in leek by producing experimental hybrids from I_1 x I_1 crosses. These hybrids gave mean yields equal or greater than the best commercial cultivars and were more uniform than the open-pollinated cultivars. Kampe (1980) found positive effects of heterosis for yield and shaft thickness. Similarly, Smith and Crowther (1995) produced experimental hybrids with a better performance for uniformity, yield and quality than the open-pollinated commercial cultivars.

The trait for male sterility in leek, described for the first time by Schweisguth (1970), is controlled by nuclear genes. The use of nuclear male sterility is not attractive, because the progeny of a cross will not be 100% male sterile. The male sterile parent needs to be maintained vegetatively, which is an expensive method. However, with the development of efficient propagation techniques for leek, for example via topsets or tissue culture (Silvertand 1995 *et al*), the F_1 hybrid approach has become economically feasible. In 1995 Nunhems Zaden introduced its first leek hybrid cultivars, which showed a significant increase in yield of 10 to 30%. An alternative for hybrid breeding in leek using vegetatively propagated nuclear male sterile plants can be a system using genetically engineered nuclear male sterility (Wang 1996). In this system plants are transformed with a chimeric gene, consisting of an anther tapetum specific gene (*TA29*) and a ribonuclease gene (*RNAse*). Expression of this gene destroys the anther tapetal cells and prevents pollen formation (Mariani *et al* 1990). The problem of not producing a 100% male sterile progeny can be solved by linking this gene to a

dominant herbicide resistance gene and treatment with the corresponding herbicide.

However, the most suitable system for large scale seed production of hybrid cultivars is a system based on cytoplasmic male sterility.

Cytoplasmic male sterility

Cytoplasmic male sterility (CMS) is the inability of plants to produce functional pollen. The phenomenon is generally not accompanied by changes in female fertility. It is an agronomically important trait for the production of hybrid seed, since it prevents self-fertilization. In hybrid seed production based on a system of CMS the maintenance of the male sterile parent line is not a problem, since CMS is maternally inherited and, in the absence of restorer genes, no segregation of the offspring in male sterile and fertile plants will occur. CMS has been widely used in the commercial production of hybrid seeds of several crops such as sorghum, sunflower, sugar beet, onion, carrot, maize and oilseed rape (Kaul 1988).

CMS can be the result of spontaneous mutational changes in the mitochondrial genome (autoplasmic CMS). However, CMS can also be induced by interspecific or intergeneric crosses, interspecific protoplast fusion, by passage through tissue culture and by chemical mutagenesis. About 75% of the CMS types are thought to have arisen after interspecific or intergeneric crosses. CMS arising through interspecific crosses or protoplast fusion is called alloplasmic CMS. In these cases, the male sterility results from incompatibility between the nuclear genome of one species and the cytoplasm of another (Kaul 1988, Lasa and Bosemark 1993, Vedel *et al* 1994). Alloplasmic CMS plants can be deliberately created by successive backcrossing of an interspecific or intergeneric hybrid as female to the male parent whose nuclear genome is to be placed in the cytoplasm of the female parent. For example such backcrosses have given rise to CMS lines in sunflower and *Nicotiana* (Hanson 1991).

The mechanism that causes the pollen abortion in CMS plants differs among the plant species. The tapetum of higher plants is thought to play a vital nutritive role during and after microsporogenesis. In most cases of CMS an abnormal behaviour of this tissue is considered to cause male sterility. For instance, in cms-T maize, mitochondria in the tapetal cell layer become disorganized soon after meiosis, resulting in early vacuolization and degeneration of the tapetal cell layer (Warmke and Lee 1977). In other CMS types like cms-S maize,

abnormalities occur only in the individual developing microspores and do not involve the tapetal cells (Lee *et al* 1980). In some cases, male sterility is the result of abnormal development or feminization of the male reproductive structures (Kofer *et al* 1991). Microscopic evidence suggests that mitochondrial activities are important in the tapetal cell layer. Warmke and Lee (1978) observed that during early development of normal maize anthers, mitochondria increase in number about 20 times in sporogeneous tissue and 40 times in tapetal cells. An increased respiratory demand of maize tapetal cells during pollen development may make them particularly susceptible to mitochondrial dysfunction, which might result in pollen abortion (Williams and Levings III 1992).

In many plant species the CMS phenotype is thought to be associated with changes in the organization and expression of the mitochondrial genome. These changes in the mitochondrial genome may involve DNA deletion, insertion or aberrant intra- or intermolecular DNA recombination events resulting in the creation of new genes or alteration of existing genes, which are specific to the male sterile cytoplasm. The two best characterised mitochondrial genome rearrangements associated with CMS are the Texas (T) cytoplasm of maize and the CMS cytoplasm of *Petunia*. The two CMS-associated loci in these species both contain one chimeric gene and one or more co-transcribed standard mitochondrial genes with apparently normal coding regions. The chimeric maize gene, called T-*urf13*, has been generated by at least seven recombination events (Dewey *et al* 1986).

In both plant species, a strong correlation exists between CMS and a unique polypeptide, encoded by these new mitochondrial genes. For instance, in cms-T maize, the mitochondrial gene T-*urf13* encodes a 13 kDa polypeptide, designated URF13. URF13, that is a component of the inner mitochondrial membrane, is responsible both for disease susceptibility (T-toxin sensitivity) and CMS. Toxin-URF13 interaction results in permeability of the inner mitochondrial membrane, leading to dissipation of the membrane potential and loss of mitochondrial function, which eventually causes cell death and colonization by pathogens. It is possible that a tapetum-specific compound causes pollen sterility in cms-T maize by interacting with URF13 to form pores in the inner mitochondrial membrane. Another hypothesis is that pollen production is an unique event which requires an increase in mitochondrial function and that cells expressing URF13 are unable to cope with the need for such an increased mitochondrial function. (Williams and Levings III 1992, Vedel *et al* 1994, Ward 1995).

Restoration of CMS

The male sterility effect of the CMS cytoplasm can be reversed by specific nuclear restorer genes (*Rf* genes). All CMS cytoplasms identified so far have their own unique set of nuclear restorer genes. This indicates that different mechanisms are responsible for restoration of the various CMS types. These nuclear restorer genes probably suppress CMS by regulating the CMS genes, for instance by acting in mitochondrial transcript processing, alternation of relative transcript levels, or possibly mitochondrial translation (Brown 1993). In cms-T maize, the nuclear gene *Rf1* reduces the accumulation of URF13, probably by altering the T-*urf13* transcripts. In one unusual fertility restorer system in the common bean the fertility restoration is associated with the complete loss of the mitochondrial sterility-associated sequence in the genome (Mackenzie and Chase 1990).

Isolation and characterization of these *Rf* genes would be helpful for a better understanding how a CMS-associated gene product interferes with pollen development to cause male sterility. Recently, the *Rf2* gene of cms-T maize has been cloned and characterized. This gene is together with *Rf1* responsible for fertility restoration, but does not affect URF13 expression. *Rf2* encodes an aldehyde dehydrogenase, but it is unclear how it participates in pollen restoration. It is hypothesized that this protein may play a role in energy metabolism or that it interacts with URF13 and thereby diminishes its deleterious effects (Cui *et al* 1996).

Approaches to introduce CMS in leek

As mentioned before, the most desirable way to produce hybrid seeds in leek would be a system based on CMS. Unfortunately, no source of CMS is currently available in leek. The search for spontaneously occurring cytoplasmic male sterile leek plants in large seed production fields has not been successful up till now (Silvertand 1996). It is possible that CMS is present in leek, but due to the action of restorer genes not phenotypically expressed. Therefore, other approaches to introduce or create CMS in leek have been investigated. Silvertand (1996) investigated the possibility of inducing CMS in leek by using chemical mutagens. In other crops the use of chemical mutagens has proven to be effective in inducing mitochondrial mutations (Kaul 1988). It was possible to induce male sterility in leek by this method, but this was not proven to be of a cytoplasmic origin. Attempts to transfer CMS from related *Allium* species via interspecific crosses, whereby the CMS donor is used as a female parent have failed, due to serious difficulties in obtaining fertile hybrids. A possibility that has not been explored by leek breeders and researchers yet, is the introduction of alien cytoplasms

in leek by intraspecific crosses with members of the *A. ampeloprasum* complex, in order to create an alloplasmic type of CMS. The members of the *A. ampeloprasum* complex, which are interfertile with leek, provide a large pool of cytoplasmic genome variation (Kik *et al* 1997). A more intensive search within these close relatives of leek might even result in the identification of a sterile cytoplasm.

Transfer of CMS via somatic hybridization

An attractive alternative to the introduction of CMS by conventional breeding methods is the transfer of CMS to leek via somatic hybridization with an appropriate *Allium* species. The approach of somatic hybridization via protoplast fusion provides a way to circumvent the existing sexual crossing barriers between leek and related species. In addition, protoplast fusion makes it possible to combine the cytoplasms of both parents. As the initial heteroplasmic state of a fusion product is usually not stable, it is possible to produce plants with new combinations of nuclei, chloroplasts and mitochondria (Kumar and Cocking 1987). Mitochondria and chloroplasts normally segregate independently. The chloroplasts, generally, sort out during plant regeneration, so that the resulting hybrid plants possess only one of the parental chloroplast types. Recombination of chloroplast DNA is a rare event (Medgyesy *et al* 1985). Segregation of mitochondria is similar to chloroplasts, but occurs in a more complicated way. The mitochondrial genomes usually recombine after protoplast fusion so that the mitochondrial genome of fusion products often consists of DNA fragments of both parents as well as novel fragments (Belliard *et al* 1979, Rothenberg *et al* 1985). Because of the creation of novel mixtures of cell organelles and the spontaneous processes of segregation and intergenomic mitochondrial recombination, protoplast fusion offers the possibility to achieve maximum cytoplasmic heterogeneity in contrast to sexual hybridization.

Protoplast fusion has proven to be an attractive tool to transfer traits encoded by the mitochondrial DNA such as CMS to a desired cultivar or species (Kumar and Cocking 1987). The most frequently used method is the donor-recipient method, in which recipient protoplasts are fused with X- or γ-irradiated donor protoplasts. By this way, transfer of the CMS trait has been established in several plant species such as *Nicotiana* (Zelcer *et al* 1978), *Brassica* (Pelletier *et al* 1983, Barsby *et al* 1987), carrot (Tanno-Suenaga *et al* 1988) and rice (Kyozuka *et al* 1989). The major advantage of this method is that an existing CMS cytoplasm can be transferred in a single step, avoiding many years of backcrossing which are required after sexual hybridization. In addition to transfer of an existing CMS cytoplasm, protoplast

fusion can be used for the generation of a new alloplasmic type of CMS. Here, the transfer of a cytoplasm to an alien nuclear background can lead to incompatibility between the nuclear and mitochondrial genome which results in pollen sterility. By this way, CMS tomato plants have been obtained by fusion of tomato protoplasts and *Solanum* protoplasts, while both parental lines have fertile cytoplasms (Melchers *et al* 1992). When a new type of CMS is induced after protoplast fusion between different species or genera it is difficult to determine if CMS expression is due to alloplasmic disruptions of nuclear-mitochondrial interactions. It may also be possible that the fusion process itself gives rise to a new chimeric gene that induces a new type of male sterility (Earle 1995).

CMS sources within the genus Allium

In order to transfer CMS by protoplast fusion, an appropriate cytoplasmic donor has to be chosen. Within the large *Allium* genus, cytoplasmic male sterility is only documented for *A. cepa*, *A. fistulosum*, *A. schoenoprasum* and five interspecific hybrids (Table 1). The CMS-systems in *A. cepa* and *A. schoenoprasum* are well defined. In this paragraph some characteristics of the CMS systems in these two species are described.

In *A. cepa* two types of sterile cytoplasm are known, designated S-cytoplasm and T-cytoplasm. Monosmith (1926) detected for the first time CMS in a plant from the onion cultivar 'Italian Red'. Sterility in this plant was cytoplasmically inherited (S-type) with fertility restored by a single nuclear dominant gene (Jones and Clark 1943). The second source of CMS (T-type) was discovered by Berninger (1965) in the French onion cultivar 'Jeanne Paille des Vertus'. Schweisguth (1973) characterized this type of CMS and identified three dominant nuclear genes *A*, *B* and *C* that can restore fertility. Gene *A* acts independent of *B* and *C*, while *B* and *C* are complementary in their action. The complex inheritance and common occurrence of the restorer alleles make the T-cytoplasm difficult to use for hybrid

Table 1. Cytoplasmic male sterility in *Allium* spp. (Adapted from Kaul 1988).

Species/species crosses	Reference
A. cepa	Jones and Emsweller (1937), Schweisguth (1973)
A. fistulosum	Virnich (1967)
A. schoenoprasum	Tatlioglu (1982)
A. cepa x *A. ascalonicum*	Little *et al* (1944)
A. cepa x *A. drobovii*	Saini and Davis (1969)
A. cepa x *A. galanthum*	Saini and Davis (1969)
A. cepa x *A. pskemense*	Saini and Davis (1969)
A. roylei x *A. drobovii*	Saini and Davis (1969)

seed production (Havey 1993). Apart from these two CMS types, male sterility has been observed in other onion cultivars, but these have not yet been characterized (de Courcel *et al* 1989). The expression of male sterility in onion is influenced by temperature. Fertility can be restored in some onion populations when the temperature rises. The response to temperature is considered to be affected by the genetic background (Van der Meer and van Bennekom 1978).

Male sterile onion plants with S- or T-cytoplasm can be distinguished by their different anther phenotype. The anthers of S-cytoplasm plants are normal, but at anthesis the thecae are green, small and indehiscent, whereas in T-cytoplasm plants the anther morphology is disturbed (Kaul 1988). The male sterility of onion plants, containing the S-cytoplasm is probably caused by abnormal tapetal development. Different types of abnormal development of the tapetum are observed in these onions. For instance, in a Japanese onion the tapetal degeneration is precocious (Tatebe 1952). In an American male sterile onion the tapetum enlarges drastically, apressing the microspores (Monosmith 1926, Peterson and Foskett 1953). A third type, in which the tapetum remains intact for an abnormally long period of time has been found in a German male sterile onion (Kobabe 1958). All three types of abnormal tapetal behaviour can also exist in a single CMS onion population (Holford *et al* 1991a). In these plants meiosis is normal, but the microspores degenerate after release from the tetrads or during pollen mitosis. In T-cytoplasm plants pollen abortion is caused by an abnormal meiosis, which leads to the formation of diads and triads. In these plants, intensive vacuolization of the tapetum occurs, followed by microspore and tapetal degeneration (Dyke 1973).

There is little knowledge about the molecular basis of CMS in onion with respect to species like *Petunia* (Hanson 1991) and maize (Williams and Levings III 1992). So far, the mitochondrial DNAs of normal, fertile and male sterile onion plants have only been examined by using restriction fragment length polymorphism (RFLP) analysis. De Courcel *et al* (1989) identified two cytoplasmic groups, a M group and a group that contains the S-cytoplasm. The mitochondrial DNA patterns of the M group could be subdivided in four groups, M_1 and M_2 corresponding to normal N-cytoplasm and M_3 and M_4 probably corresponding to T-cytoplasms. Holford *et al* (1991b) could not distinguish the T-cytoplasm from the N-cytoplasm using RFLP profiles of mtDNA. Based on these results they proposed that two different origins for the sterility-conferring cytoplasms exist. The male sterility in the T-cytoplasm plants is probably caused by a mutation within the N-cytoplasm, suggesting an autoplasmic origin. In the S-cytoplasm onions CMS was correlated with differences in RFLP

profiles of both mtDNA and cpDNA, which suggests that the male sterility is alloplasmic and a result from an interspecific cross. The viviparous interspecific hybrid 'Pran' may be the donor of the S-cytoplasm (Havey 1993). Satoh and Mikami (1994) characterized the transcriptional patterns of several mitochondrial genes for N-, S-cytoplasm plants and nuclear restored plants. They found differences between N- and S-cytoplasm plants for the mt genes *cob* and *coxI*. Because the transcriptional pattern of *cob* was altered by nuclear restorer genes, they suggested a possible relationship between the *cob* gene and CMS in onion.

CMS in *A. schoenoprasum* is controlled by a cytoplasmic factor (S) and three nuclear restorer genes. The dominant nuclear gene *X* restores the fertility independent of the environmental conditions (Tatlioglu 1982). The function of the other two genes *T* and *a* is dependent on environmental factors. The dominant nuclear gene *T* is temperature sensitive and restores fertility at a constant temperature of 24 °C (Tatlioglu 1987). The recessive gene *a* is responsible for tetracycline sensitivity of the CMS. Plants, which are sensitive to tetracycline have a reversible fertility after treatment with tetracycline (Tatlioglu and Wricke 1988). Anthers of the male sterile chives are shrunken, indehiscent and dry up at anthesis. Microsporogenesis is similar to that observed in fertile plants up to the tetrad stage. Although in contrast to fertile plants, the tapetum is highly enlarged and does not degenerate after the tetrad stage. Microspore degeneration occurs after release from the tetrads (Ruge *et al* 1993). The mtDNA of the S- and fertile N-cytoplasms of chives differ considerably from each other. It is suggested that the CMS phenotype in chives is associated with the appearance of an additional mitochondrial 18 kDa polypeptide (Potz and Tatlioglu 1993). The synthesis of this polypeptide is inhibited by the restorer gene *X*. Besides the N- and S-cytoplasm other cytoplasms are found in chives, which differ from each other in mt genome. A relation of these cytoplasms with CMS is not clear (Rauber *et al* 1993).

Protoplast technology in the genus *Allium*

For the utilization of a technique as somatic hybridization, the availability of a protoplast to plant regeneration system is an essential prerequisite. In general, substantial progress has been made in *in vitro* culture techniques for *Allium* species (reviewed by Novák *et al* 1986), but the number of reports on successful regeneration of protoplasts is limited. A brief summary of the studies on protoplast culture and regeneration of *Allium* species during the last two decades is

Table 2. Culture and regeneration of protoplasts of *Allium* spp.

Plant species	Donor tissue	Culture medium	Culture technique	Regeneration	Reference
A. sativum	Leaves	MS[a] mod, 1 mg/L 2,4-D and 1 mg/L Kin	Droplets or thin-layer agar	First cell division	Opatrny and Havranek (1977)
A. cepa	Leaves	MS mod, 2 mg/L 2,4-D and 0.5 mg/L BA	Liquid	Plantlets	Wang *et al* (1986)
A. cepa	Leaves	BDS[b] mod, 10 μM Kin + 10μM IAA + 5 μM 2,4-D	Liquid	No cell division	Novák *et al* (1986)
A. cepa	Callus	BDS salts + KM organics, various hormones	Agarose	Microcalli	Balakrishnamurthy *et al* (1990)
A. longicuspis	Basal leaf parts and callus	MS mod, 9.1 μM 2,4-D + 2.2 μM BA or BDS mod, 1.0 μM 2,4-D + 5.0 μM Kin	Liquid	Small cell colonies	Fellner and Havranek (1994)
A. sativum	Basal leaf parts and callus	MS mod, 9.1 μM 2,4-D + 2.2 μM BA or BDS mod, 1.0 μM 2,4-D + 5.0 μM Kin	Liquid	First cell divisions	Fellner and Havranek (1994)
A. fistulosum	Suspension cells	BDS mod, 2 μM 2,4-D + 1 μM BA	Liquid	Shoots	Shimonaka (1994)
A. ampeloprasum	Suspension cells	KM[c] mod, 1 mg/L 2,4-D	Alginate plates	Plants	Buiteveld and Creemers-Molenaar (1994)
A. porrum	Suspension cells	Pelletier salts[d] + KM organics mod, various hormones	Alginate plates	Plants	Schum *et al* (1994)
A. sativum	Shoot primordia	B_5[e] mod, 1 mg/l NAA + 1 mg/l BA, 0.1% casein hydrolysate	Agarose	Plants	Ayabe *et al* (1995)
A. cepa	Suspension cells	BDS salts + KM organics mod, 1 mg/L NAA + 0.5 mg/L Zeatin	Liquid	Shoots	Hansen *et al* (1995)

[a] Murashige and Skoog (1962).
[b] Dunstan and Short (1977).
[c] Kao and Michayluk (1975).
[d] Pelletier *et al* (1983).
[e] Gamborg *et al* (1968).

given in Table 2. The first successful attempt on regeneration of protoplasts of *Allium* was reported by Wang *et al* in 1986. In this study, young leaves derived from bulb material were used for the isolation of protoplasts. These results are remarkable, because in these experiments leaf mesophyll as a donor source for protoplast regeneration was used and it is generally known that for monocotyledonous species it is very difficult to obtain totipotent protoplasts isolated from differentiated tissue. This system has not yet been reproduced in *Allium*. Other explants used for protoplast culture of *Allium* species are callus, basal leaf parts, suspension cells and shoot primordia.

It is evident that during the last four years important progress has been made in the development of protoplast to plant systems for *Allium* species. To date, regeneration procedures for four economical important *Allium* species; leek (*A. ampeloprasum*, syn. *A. porrum*), onion (*A. cepa*), Japanese bunching onion (*A. fistulosum*) and garlic (*A. sativum*) are available (For ref. see Table 2). A major break through in the development of such procedures was the source of starting material used for the isolation of protoplasts. In leek, onion and Japanese bunching onion cell suspensions were used as a donor source for the isolation of protoplasts. The suspension cultures were initiated from callus cultures, which were selected for their high friability and regenerability. The development of the suspension cultures appeared to be affected by the genotype. For the development of a protoplast system in garlic, the use of shoot primordia as donor source was a crucial step.

Furthermore, in the garlic protoplast system specific medium requirements were needed, like addition of casein hydrolysate, supplementation of adenine and coconut milk for the formation of calli and regeneration of plants. For leek protoplast systems alginate embedding was essential. Buiteveld and Creemers-Molenaar (1994) mentioned a 5 fold increase of the plating efficiency, while Schum *et al* (1994) exclusively could regenerate protoplasts when cultured in alginate. Hansen *et al* (1995) mentioned that in their onion protoplast system the age of the suspension culture was important to obtain sustained cell division of protoplasts and demonstrated that 4 - 5 months old suspension cultures were most suitable.

Aim and outline of this thesis

The aim of the research, described in this thesis, was to develop a method for somatic hybridization between leek and a related *Allium* species, with the ultimate goal to transfer

CMS to leek. In order to apply a technique as somatic hybridization, a procedure to regenerate plants from protoplasts is required. At the start of the research, no such a regeneration procedure was available for leek. For this reason, the research was for a substantial part focused on the development of regeneration techniques for leek.

Monocotyledonous species are known for their recalcitrance to protoplast regeneration. To obtain plant regeneration in these species, the use of embryogenic callus cultures and cell suspensions as donor tissue for protoplast isolation is essential. Leek is a member of the monocotyledons and, therefore, such a strategy was also applied to develop a protoplast regeneration system for leek. To obtain protoplasts competent for regeneration three successive stages were passed through: the initiation of embryogenic callus cultures, the development of embryogenic suspension cultures and the regeneration of protoplasts derived from these suspension cultures. In chapter 2, the first stage in this procedure, the development of embryogenic callus cultures, is described. Different explant types were used to initiate embryogenic callus cultures. Beside explant type, genotype was an important factor that influenced callus induction and regeneration efficiency. Therefore, we compared 12 different cultivars and accessions with respect to their callus induction capacity and regeneration ability. These compact callus cultures did not appear to be a suitable starting material for the initiation of suspension cultures. A second type of embryogenic callus, first reported in maize by Green and Rhodes (1982) proved to be very emenable to form suspension cultures, because of high friability and high regeneration capacity. In analogy with maize, the initiation of such a friable type of callus for leek and its amenability to form suspension cultures was investigated, which is described in chapter 3. In addition, a light microscopic comparison between compact and friable callus was made. Subsequently, high responding suspension lines were selected, which were used for isolation of protoplasts. The development of a procedure for isolation, culture and regeneration of protoplasts is decribed in chapter 4. Using this protoplast regeneration procedure, it was possible to perform protoplast fusion experiments and to recover somatic hybrid plants. Subsequently, an appropriate cytoplasmic donor had to be chosen. There are several good candidates for CMS introduction in leek. As mentioned before, it is possible to induce an alloplasmic type of CMS by fusing two distant related species with both fertile cytoplasm. However, we have chosen for a cytoplasmic donor with a strong sterilizing plasma. Within the genus *Allium*, there are two species, onion and chives, that can serve as a putative CMS donor for leek. The system in onion is best defined and commercially used for hybrid seed production, whereas the CMS system in chives has a

rather complicated nuclear control. For these reasons, onion was preferred as a CMS donor in the fusion experiments. Two onion cultivars 'Hyton' and 'Alamo', possessing different cytoplasms leading to male sterility, were used. In some fusion experiments the onion protoplasts were gamma-irradiated prior to fusion with leek. In chapter 5, results of these fusion experiments and the characterization of some somatic hybrid plants are described. Since the ultimate goal of this research was the transfer of CMS to leek, the organelle composition of these somatic hybrids is of particular interest. In chapter 6 the analysis of mitochondrial and chloroplast DNA of some hybrid plants is described. In chapter 7 a general discussion is given.

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Chapter 2

Callus induction and plant regeneration from explants of commercial cultivars of leek (*Allium ampeloprasum* L.)

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Summary

Plant regeneration capacity was studied for 8 cultivars and 4 accessions of leek (*A. ampeloprasum* L.). Compact callus was induced on embryo and leaf explants on three different media. The highest frequency of compact callus formation (up to 90%) was obtained when mature, zygotic embryos were cultured on MS medium, containing 30 g/l sucrose and 1 mg/l 2,4-D. Regeneration occurred through somatic embryogenesis on MS medium, supplemented with 1 mg/l kinetin. Plants could be regenerated from all cultivars and accessions tested. These cultivars and accessions could be classified into three groups with respect to shoot formation frequency. The results suggest a distinct influence of the genotype on the morphogenic response of leek embryo explants *in vitro*.

Introduction

There is considerable interest in plant tissue culture techniques, such as somatic hybridization, genetic transformation and *in vitro* selection for mutants as means to complement the leek breeding program. For the successful application of most of these techniques the availability of an efficient procedure for plant regeneration from protoplasts or suspension cultures is a prerequisite.

In monocots, including *Allium*, it has proven difficult to culture and regenerate protoplasts that have been directly isolated from the plant (Novák 1990, Vasil 1983). Indeed, plant regeneration has only been successful when morphogenic suspension cultures were used as the starting material for isolation of protoplasts (Vasil 1988). Therefore, in our research, emphasis has been placed firstly on the initiation of callus cultures that are suitable for the establishment of morphogenic suspension cultures. Thereafter, a reliable system for protoplast regeneration can be established and subsequently a method for somatic hybridization or cybridization.

Plant regeneration from callus cultures derived from different explants has been studied using several *Allium* species (Fridborg 1971, Dunstan and Short 1977, 1978, 1979a, Phillips and Hubstenberger 1987, Seo and Kim 1988, Van der Valk *et al* 1992). In leek, callus cultures have been successfully established from different explants, i.e., basal plates (Debergh and Standaert-De Metsenaere 1976, Dunstan and Short 1979b), floral heads (Novák and Havel 1981) and zygotic embryos (Van der Valk *et al* 1992). Shoots have also been regenerated from such callus cultures. However, whether regeneration occurs through organogenesis or somatic embryogenesis has not been investigated in detail for leek.

To our knowledge only few data have been reported on the regeneration frequency from callus cultures of leek (Van der Valk *et al* 1992). In this study we describe callus induction and plant regeneration from callus cultures of different cultivars and accessions of *A. ampeloprasum*. The regeneration frequency, as well as the mode of regeneration, were determined. Different callus induction media were used and embryo and leaf explants from different cultivars were tested for their amenability to form morphogenic callus, in order to define the optimal conditions for callus induction and plant regeneration.

Materials and methods

Plant material

Seeds of 8 *Allium ampeloprasum* cultivars and 4 accessions of *Allium ampeloprasum* from Israel, as listed in Table 3, were supplied by the Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen, The Netherlands.

Explants

Mature seeds were sterilized according to the method of Van der Valk *et al* (1992). The embryos were aseptically excised using a stereo microscope. That part of the embryo containing the radicle, part of the cotyledon, and including the shoot apex was cut off and used for culture. For callus induction from mature embryos, 8 cultivars and 4 accessions were used. Leaf explants were obtained from 20 to 30 mm plantlets that had previously been regenerated from mature embryo-derived callus cultures. Shoots were sliced into approximately 2 mm long sections, starting at the base of the leaf, and were referred to as explant number 1, 2 and 3, respectively. Explant 1 contained meristematic tissue. Ten genotypes of both 'Porino' and 'Tilina' were used for callus induction on leaf explants and, on average, 6 explants per genotype were cultured.

Media and culture conditions

For callus induction, mature embryos were cultured on MS medium (Murashige and Skoog 1962), supplemented with 1 mg/l 2,4-D, 30 g/l sucrose and 0.3% (w/v) phytagel (Sigma). The medium was autoclaved and the pH was adjusted to 5.8. Per cultivar, 225 embryos were cultured. Leek cultivars are open-pollinated populations, thus each embryo represents an individual genotype. The cultures were incubated in the dark at 25 °C for 6 weeks, after which

the formation of compact callus was assessed. Compact calli were then transferred to fresh MS medium and subcultured at 3-weekly intervals. To compare the tissue culture response of the embryo and leaf explants, the explants and calli were cultured under the same conditions.

For regeneration, 3 month old compact calli (5 x 5 mm) derived from 20 embryos of each cultivar were transferred to MS medium, supplemented with 1 mg/l kinetin (MSK) and cultured at 25 °C with a 16h photoperiod (ca. 3000 lux white fluorescent light). Generally, 6 calli were cultured per embryo, but when an embryo did not produce enough callus, fewer were cultured. The morphogenic response was determined on the basis of the formation of somatic embryos, which was assessed after 3 weeks of culture and on the basis of the regeneration frequency (the mean number of plantlets produced per callus), which was assessed after 8 weeks. Regenerated plantlets were transferred to glass culture jars with half strength MS, 20 g/l sucrose and without hormones. The plants were transferred to the greenhouse after 4 weeks.

In one experiment, embryos of the cultivar 'Tilina' were compared for callus induction on three basal media: MS, BDS (B_5 medium (Gamborg *et al* 1968), modified by Dunstan and Short (1977)) and N_6 medium (Chu *et al* 1975). As indicated, the media were supplemented with 2 or 3% sucrose. In this experiment 1 mg/l 2,4-D, 200 mg/l casein hydrolysate and 2.3 g/l L-proline were added to all media. Per medium, 20 explants were cultured.

Data analysis

Counts of embryos and shoots were analyzed according to a generalized linear model for count data (McCullagh and Nelder 1989). Calculations were carried out by means of the Genstat computer program. To test the equality of the frequency of compact callus formation, Pearson's χ^2 test was used.

Measurement of ploidy level

The ploidy levels of 30 shoots of 7 cultivars regenerated from both 3 and 10 month old calli were measured by flow cytometry. Nuclear suspensions were prepared by chopping leaf material in a nuclear isolation/staining buffer containing DAPI, as described by Verhoeven *et al* (1990). The mixture was then filtered through a 55 µm filter to remove debris. The nuclei were measured for DNA content with a Partec PAS-II flow cytometer (Verhoeven *et al* 1990).

Results

Effect of explant type on compact callus formation

Independent of the explant type cultured, two morphologically different callus types could be distinguished: (1) a compact, white and nodular type with pro-embryogenic structures (Fig. 1A); and (2) a friable, watery and non-embryogenic type. Callus formation from embryos and leaf explants could be observed after 6 - 8 d culture. Callus was initiated between the radicle and cotyledon of the embryo, at the position where the basal plate would be formed. Data in Table 1 show that up to 100% of the embryos formed callus and 80% of the embryos formed compact callus. Compared to callus induction on mature embryos, the frequency of callus induction on leaf explants was much lower. A distinct difference in the callus induction response between explants from different positions of the leaf was observed. The upper and middle explants (numbers 2 and 3) exhibited poor callus growth at low frequency (up to 8%). The majority of these explants showed only swelling along the cut surfaces and after a number of weeks became necrotic. In contrast, up to 75% of the explants containing the meristem, formed callus. Compact callus formation in leaf explants ranged from 0 to 55%. The compact callus had the same appearance as the compact callus cultures derived from mature embryos.

Effect of medium composition on callus formation

Results in Table 1 showed that embryos gave a high callus response and therefore embryos of the cultivar 'Tilina' were used for the comparison of different callus initiation media. Statistical analysis showed that no significant effect of the media tested was found with respect to number of explants that produced compact callus. The percentage of explants that formed compact callus

Table 1. Callus initation from different explants of *A. ampeloprasum* on MS medium containing 1 mg/l 2,4-D.

Explant	Cultivar	Explants cultured (N)	Explants with callus (%)	Explants with compact callus (%)[a]
Embryo	Porino	225	92	70
	Tilina	225	100	80
Leaf 1	Porino	60	75	55
2	Porino	60	3	2
3	Porino	60	2	2
Leaf 1	Tilina	60	68	22
2	Tilina	60	8	2
3	Tilina	60	0	0

[a] Percentage of explants producing compact callus was assessed after 6 weeks of culture.

was lowest on N_6 medium with 2% sucrose (Table 2). Maximum frequency of explants producing compact callus could be achieved when embryos were cultured on MS medium with 3% sucrose.

Effect of different cultivars and accessions on compact callus induction and regeneration

All cultivars produced compact callus, although the percentage of embryos doing so varied considerably (from 21% to 80%; Table 3). The four accessions had the lowest percentage of embryos forming compact callus (21% to 35%). The genotypes, within cultivars and accessions, showed differences for the amount of compact callus that was formed (results not presented).

The calli turned green and showed protuberances within a week after transfer of compact calli to regeneration medium. After a further culture period of 2 weeks, the green areas differentiated into somatic embryos. These showed the characteristic bipolar appearance even during the early stages of development. The somatic embryos usually formed in clusters on the surface of the callus but also appeared singly (Fig. 1B). Detaching the embryos at this early stage inhibited further growth. When leaving them in contact with the callus the somatic embryos subsequently germinated and developed into plantlets (Fig. 1C, 1D). The data presented in Table 3 show that somatic embryos were formed in all cultivars, but at varied frequencies.

There were significant differences in regeneration frequency between the cultivars (Table 3.) Statistical analysis showed that a classification of the cultivars and accessions into three groups accounted for 88% of the variation in shoot regeneration found between the cultivars and accessions. The first group was comprised of the highest regenerative cultivars 'Elbeuf', 'JGPC', 'Tilina' and 'VVG'. Within this group the regeneration frequency was 6.7 shoots per callus. The second group, presenting 7 cultivars, showed a lower regeneration frequency (2.8 to

Table 2. Effect of callus induction medium on compact callus formation from zygotic embryos of *A. ampeloprasum* cv. 'Tilina'.

Callus induction medium[a]	Explants with callus (%)	Explants with compact callus (%)[b]
MS3	100	90
MS2	100	70
BDS3	100	64
BDS2	100	65
$N_6$3	100	80
$N_6$2	100	55

[a] MS3 = MS + 3% sucrose, MS2 = MS + 2% sucrose, BDS3 = BDS + 3% sucrose, BDS2 = BDS + 2% sucrose, $N_6$3 = N_6 + 3% sucrose, $N_6$2 = N_6 + 2% sucrose.

[b] Percentage of explants producing compact callus was accessed after 6 weeks of culture.

Table 3. Callus initiation and regeneration from embryo-derived compact callus cultures of *A. ampeloprasum*.

Cultivar and accession[a]	Embryos with compact callus (%)[b]	Calli with somatic embryos (%)[c]	Mean no. of somatic embryos per callus[d]	Calli with shoots (%)	Mean no. of shoots per callus[e]
JGPC	63	99 (120)	5.6 (0.51)	94	6.7 (0.70)
Tilina	80	91 (119)	4.0 (0.43)	96	6.6 (0.70)
VVG	70	98 (120)	5.7 (0.51)	94	6.6 (0.70)
Elbeuf	52	97 (96)	5.6 (0.56)	92	6.5 (0.77)
Arcona	66	96 (120)	4.6 (0.46)	88	5.2 (0.62)
Arkansas	65	92 (119)	4.3 (0.45)	84	4.7 (0.59)
90002	35	99 (84)	5.2 (0.57)	92	4.3 (0.67)
Varna	38	91 (110)	3.9 (0.44)	90	3.7 (0.54)
90003	21	46 (54)	3.0 (0.55)	59	3.1 (0.70)
Porino	70	83 (120)	3.0 (0.37)	73	3.0 (0.47)
90004	25	79 (89)	4.0 (0.50)	70	2.8 (0.52)
90001	26	20 (51)	0.5 (0.22)	20	0.3 (0.22)

[a] JGPC = 'Jaune Gros du Poitou Clause', VVG = 'Violet de St. Victor Gautier'.
[b] Percentage of embryos producing compact callus was assessed after 6 weeks of culture on initiation medium (MS + 1 mg/l 2,4-D).
[c] Within brackets the number of calli cultured is given.
[d] Mean number of somatic embryos was assessed after 3 weeks of culture on MSK medium, within brackets the standard error is given.
[e] Mean number of shoots was assessed after 8 weeks of culture on MSK medium, within brackets the standard error is given.

5.2 shoots per callus). The third group consisted of only one accession (90001) that showed a very low regeneration capacity. On average, only 0.3 shoots were formed per callus and the percentage of responsive calli was low (20%). The accession 90003 could not be classified, because of its heterogeneous nature and the large number of missing values, due to the fact that not all embryos produced enough callus. In most cases, cultivars that formed a high number of somatic embryos also exhibited a high shoot production. However, for the accessions 90004 and 90002 and the cultivar 'Varna' only part of the somatic embryos that were formed after 3 weeks germinated and developed into shoots. On the other hand, the number of somatic embryos formed by the cultivar 'Tilina' was much lower than the number of shoots which eventually formed.

The plantlets developed into phenotypically normal and well-rooted plants that flowered and set seed. As determined by flow cytometric analysis, plants regenerated from young (3 months) and old (10 months) callus cultures had the normal tetraploid DNA content.

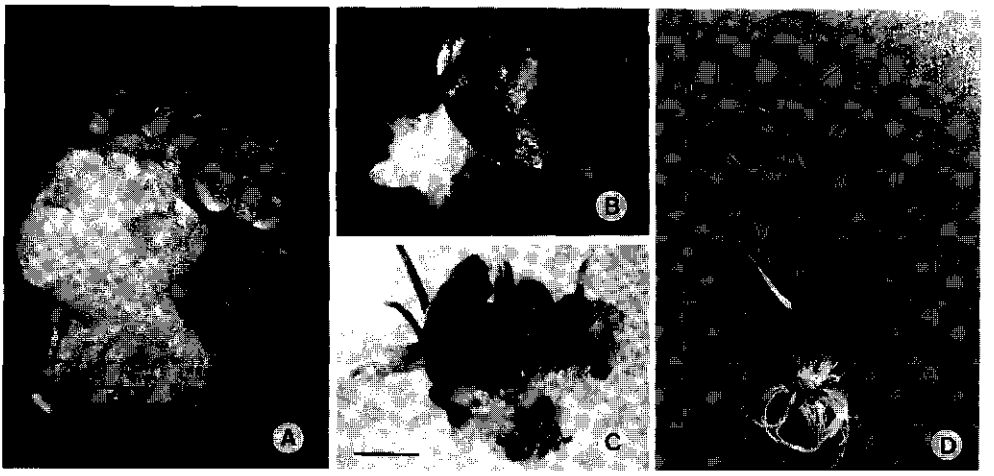

Fig. 1. Somatic embryogenesis and plant regeneration from compact callus cultures of *A. ampeloprasum*. (A) Compact and embryogenic callus (Bar = 1 mm). (B) Early stage of a single somatic embryo (Bar = 1 mm). (C) A cluster of germinating somatic embryos attached to callus derived 3 weeks after transfer to MS + 1 mg/l kinetin (Bar = 2 mm). (D) Regenerated plantlets (Bar = 1 cm).

Discussion

In leek, mature zygotic embryos are highly responding explants for the initiation of embryogenic callus cultures. The Murashige and Skoog (MS) medium, supplemented with 2,4-D, proved to be a suitable medium for the induction of embryogenic callus on these zygotic embryos. The embryogenic callus cultures were compact and nodular and similar in appearance to those obtained for gramineous species (Vasil 1985) and other *Allium* species (Phillips and Luteyn 1983, Van der Valk *et al* 1992). The desired callus type was often surrounded by a friable and non-embryogenic callus, thus making continued selection for the compact, regeneration-competent callus type necessary. Mature and immature embryos have been successfully used to initiate embryogenic callus cultures for the major species within the Gramineae (Vasil 1985) and within the genus *Allium* (Phillips and Luteyn 1983, Van der Valk *et al* 1992).

While for most Allium species, regeneration has been reported to occur through organogenesis, in a few species somatic embryogenesis has been reported, i.e., *A. sativum* (Abo El-Nil 1977), *A. cepa* (Dunstan and Short 1978, Phillips and Luteyn 1983, Van der Valk *et al* 1992), *A. carinatum* (Havel and Novák 1988), *A. fistulosum* (Shahin and Kaneko 1986) and

A. fistulosum x *A. cepa* (Lu *et al* 1989). In this study, plant regeneration appeared to occur mainly through somatic embryogenesis. However, plant regeneration via organogenesis could not be excluded. Due to the fact that on regeneration medium somatic embryos germinated very easily, all calli with embryogenic sectors gave rise to plantlets, independent of the original explant type. The embryogenic calli, which so far have been maintained for about a year are still capable of regenerating plants.

A genotype-dependent response was observed for the formation of shoots. Variation in shoot regeneration within cultivars from *Allium* callus cultures has also been reported by other authors (Phillips and Hubstenberger 1987, Phillips and Luteyn 1983). It is therefore recommended to choose the cultivars with the highest regeneration capacity and to select within these cultivars for the high responding genotypes for future experiments.

This study has shown that it is possible to develop a very simple and efficient callus induction and plant regeneration system for leek. Provided that compact calli were cultured, a high frequency of calli with shoots could be obtained for almost all cultivars. The procedure is generally applicable as shown by the fact that we were able to regenerate plants from all cultivars and accessions tested.

The compact and embryogenic callus cultures derived in this study were used to establish a cell suspension. Transfer of the compact and embryogenic callus to liquid medium did not lead to a well-dispersed suspension culture, although the cultures in liquid medium kept the ability to form somatic embryos and shoots for about 6 months. In further experiments we have been able to distinguish new callus types that are morphologically different from those observed in this study. These callus types will be characterized by histological examination and the amenability for the establishment of cell suspension cultures and the subsequent isolation and culture of protoplast will be tested.

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Chapter 3

Induction and characterization of embryogenic callus types for the initiation of suspension cultures of leek (*Allium ampeloprasum* L.)

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Summary

In this chapter the development and characterization of a friable, embryogenic callus culture of leek is described. This callus type was initiated on immature embryos and differed in appearance from formerly induced compact, embryogenic callus (Buiteveld *et al* 1993). The friable callus was comprised of numerous globular embryoids, embedded in a mucilaginous substance. The genotype of the donor plant and the embryo size were important parameters in the initiation of this callus type. Embryos of 0.5 - 2.5 mm gave the highest frequency of friable callus production. The basal media and inclusion of L-proline into the media did not influence the friable callus production. Light microscopic comparison of compact and friable callus showed striking differences. Compact callus consisted of a meristematic zone and contained vascular elements. Friable callus was less differentiated and contained aggregates of embryogenic cells, separated by intercellular spaces, and somatic embryos. Ten independently induced friable callus cultures were tested for their amenability to form suspension cultures. From one of these, two highly embryogenic suspension cultures were selected.

Introduction

In many graminaceous species, fast growing and finely, dispersed suspension cultures have successfully been established from embryogenic callus cultures (Vasil and Vasil 1992). For the initiation of such callus cultures immature embryos are often used (Vasil 1985). In leek, embryogenic callus cultures have been initiated from zygotic embryos from mature seeds. This callus is described as white, compact and nodular with pro-embryogenic structures and is mostly surrounded by a watery, friable and non-embryogenic callus (Van der Valk *et al* 1992, Buiteveld *et al* 1993). Attempts to initiate suspension cultures from this compact callus have so far not been successful, because the callus clumps fail to disperse into fine cell aggregates.

In maize, a second type of embryogenic callus, the so-called type II callus, is distinguished (Green 1982, Green and Rhodes 1982). Type II callus is soft and friable and has a high regeneration capacity over a long period of time. From this callus, embryogenic cell suspensions could be established, which proved to be a suitable source for the production of regenerable protoplasts (Green *et al* 1983, Kamo *et al* 1987, Rhodes *et al* 1988, Shillito *et al* 1989, Vasil and Vasil 1986).

The aim of the present study was to induce friable, embryogenic callus of leek and the subsequent establishment of suspension cultures from this callus. Effects of embryo size, genotype, basal culture medium and L-proline on the induction of friable, embryogenic callus

was investigated. In addition, the morphology of compact and friable callus was compared by light microscopy.

Materials and methods

Explant material

Greenhouse grown and open-pollinated plants of *Allium ampeloprasum* L. cultivar 'Porino' were used as donor material for immature embryos. The plants were regenerated from compact callus cultures, representing 3 individual genotypes (R7, M2, M10). Ovaries were harvested from these plants and surface-sterilized in 70% (v/v) ethanol (30 s), followed by 1% (v/v) NaOCl (10 min) and rinsed three times in sterile water. Immature embryos were dissected from the ovaries under a stereo microscope. Based on their length (stage 1: 0.5 - 1.5 mm, stage 2: 1.5 - 2.5 mm and stage 3: 2.5 - 3.3 mm), the immature embryos were classified into 3 developmental stages and cultured on medium.

Media and culture conditions

For callus induction the basal media BDS (Dunstan and Short 1977) and N6 (Chu *et al* 1975) were used. Both media were supplemented with 30 g/l sucrose, 300 mg/l casamino acids, 2 mg/l 2,4-D and 3 g/l phytagel (Sigma). The media were adjusted to pH 5.6 and filter-sterilized. L-proline was added to BDS and N_6 medium to final concentrations of 0, 10 and 25 mM. The cultures were incubated in the dark at 25 °C. Friable, embryogenic calli were selected and subcultured at 2-weeks intervals on BDS medium, supplemented with 2 mg/l 2,4-D. To test the regeneration capacity of friable callus, 50 mg fresh weight callus was plated on MS medium (Murashige and Skoog 1962), supplemented with 1 mg/l kinetin and 3 g/l phytagel (Sigma). Cultures were incubated at 25 °C in the light (± 3000 lux). The number of shoots developing was counted after 8 weeks.

Light microscopy

For light microscopy, pieces of callus were fixed in 2.5% (v/v) glutaraldehyde for 1 h at room temperature. The fixed samples were rinsed in 20 mM sodium phosphate buffer (pH 7.2). Subsequently, they were gradually dehydrated in an ethanol series (70 - 100%) and embedded

in Technovit 7100 (Kulzer, Wehrheim, Germany). Sections of 5 mm thick were cut using a microtome (Jung) and stained with 1% toluidine blue.

Initiation of suspension cultures

The amenability to form suspension cultures was tested for 10 friable callus cultures derived from 10 individual immature embryos. Between 40 - 50 mg callus from each culture was transferred to one well of a 6-well macroplate (∅ 3 cm, Greiner), containing 2.5 ml BDS medium as used for callus induction, but without agar. The cultures were kept on an orbital shaker (100 rev/min, Gallenkamp) at 25 °C in the dark. During the first 3 weeks of culture initiation, the medium was refreshed twice or three times a week to prevent mucus of the suspensions. The development of the suspension cultures was followed under an inverted-microscope and cell debris, large cell clumps and vacuolated or plasmolyzed cells were removed by pipetting. When proliferated, the cultures were diluted with up to 4 ml fresh BDS medium and divided over different wells. From this stage on, the smaller cell clumps were selected and transferred to new culture wells. Well-growing suspension cultures were maintained by weekly subculturing half the cell mass in 4 ml fresh medium. Regeneration capacity of well-growing suspension cultures was measured by plating suspension callus onto solid MS medium, supplemented with 1 mg/l kinetin, incubated in the light (3000 lux) at 25 °C. After one week, the suspension callus was transferred to fresh medium, to prevent browning of the callus. The number of shoots was counted after 8 weeks. Regeneration capacity was tested 4, 6, 7, 8, 9 and 14 months after initiation of the suspension culture.

Statistical analysis

A generalized linear model based on a binomial distribution and with a logit as link function was used to analyze the effect of medium composition, genotype and embryo size on the frequency of friable, embryogenic callus formation (McCullagh and Nelder 1989).

Results

Induction of friable, embryogenic callus

Within two weeks of culture on callus induction medium, some embryos became almost translucent, and turned into friable callus. In most cases, the friable callus arose on a small

section of the embryo after 3 - 4 weeks, simultaneously with compact and non-embryogenic callus types. The friable type of callus was characterized by a soft and white, friable appearance, and contained numerous globular embryoids, embedded in a mucilaginous substance.

The results in Table 1 show that there was no difference between BDS and N6 basal medium with respect to the percentage of friable callus formation. On the contrary, substantial effect of the embryo size on the percentage of friable callus formation was observed (Table 1). Embryos shorter than 2.5 mm exhibited a significant higher frequency of friable callus, than embryos between 2.5 and 3.3 mm ($P < 0.05$). Embryos of 0.5 - 2.5 mm produced up to 24% friable callus, while only up to 9% of the embryos of the older developmental stage (2.5 - 3.3 mm) produced this callus. No significant differences were found between embryos of the size classes 0.5 - 1.5 mm and 1.5 - 2.5 mm. When mature embryos (± 4 mm) were cultured, friable callus was never observed and compact callus proliferated exclusively (results not shown). The friable callus formation was not stimulated by the addition of L-proline in either BDS medium or N_6 medium (Table 1). Increasing the L-proline concentration to 25 mM even gave a significant decline in the formation of friable callus ($P < 0.05$).

Table 1. Effect of embryo size and medium composition on friable, embryogenic callus formation of leek (*Allium ampeloprasum* L.)

Embryo size class (mm)	Medium	L-proline (mM)	Embryos cultured (N)	Embryos with friable callus (%)
0.5 - 1.5	BDS	0	75	13
	BDS	10	84	14
	BDS	25	71	1
	N_6	0	61	20
	N_6	10	63	13
	N_6	25	68	6
1.5 - 2.5	BDS	0	41	22
	BDS	10	33	12
	BDS	25	34	15
	N_6	0	42	24
	N_6	10	37	22
	N_6	25	33	6
2.5 - 3.3	BDS	0	71	6
	BDS	10	69	3
	BDS	25	74	1
	N_6	0	81	9
	N_6	10	69	4
	N_6	25	77	3

Genotype effect on friable callus induction

The cultured embryos were derived from donor plants of three different genotypes and were divided randomly over the callus induction media. In Table 2, the response is given of the embryos in relation to the genotype of the donor plant for the combined data of the media treatments. Statistical analysis showed that donor plants of genotype R7 generated a significant higher percentage of embryos with friable callus than M10 and M2 ($P < 0.05$).

After 2 months, pronounced differences in morphology between the friable, embryogenic callus cultures derived from different embryos were noticed. Some cultures showed a high friability, with many globular sectors and loose cells in a mucilaginous substance (Fig. 1A). However, 50% of the friable callus cultures showed a strong tendency to differentiation and these callus cultures transformed into compact callus or developed into somatic embryos (Fig. 1C). Therefore, selective subculture at 2-week intervals of the more undifferentiated and globular sectors of the callus was necessary to maintain the friable character for more than a year.

Plant regeneration

Eight friable callus cultures were tested for regeneration capacity. Somatic embryogenesis was observed in all cultures, but plants could be regenerated from only six cultures. In 5 cultures, the regeneration capacity was high, ranging from 48 to 84 shoots per Petri dish (Fig. 1D), while one culture only produced 7 shoots. In the other two cultures, numerous green and white somatic embryos were formed, but these didn't germinate into normal shoots.

Table 2. Effect of the genotype of donor plant on friable, embryogenic callus formation of immature embryos, irrespective of the callus induction media used.

Genotype of donor plant	Embryo size class (mm)	Embryos cultured (N)	Embryos with friable callus (%)
R7	0.5 - 1.5	142	18
	1.5 - 2.5	66	33
	2.5 - 3.3	189	6
M10	0.5 - 1.5	232	8
	1.5 - 2.5	134	11
	2.5 - 3.3	237	2
M2	0.5 - 1.5	48	4
	1.5 - 2.5	20	5
	2.5 - 3.3	15	13

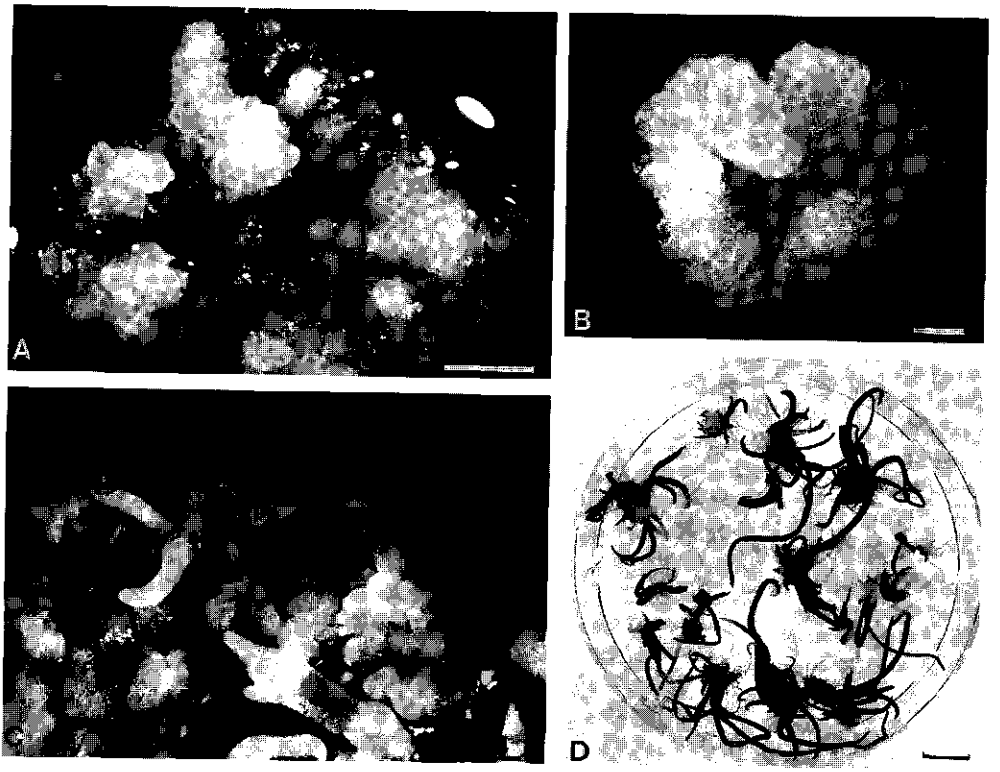

Fig. 1. (A) Friable, embryogenic callus with a high friable appearance, after subculture on BDS medium with 2 mg/l 2,4-D. Note the globular embryoids (arrows) (bar = 1 mm). (B) Compact, embryogenic callus (bar = 1 mm). (C) Friable, embryogenic callus that is differentiated into compact callus and somatic embryos (bar = 1 mm). (D) Numerous green plants developing from friable, embryogenic callus after culture of 8 weeks on MS medium containing 1 mg/l kinetin (bar = 1 cm).

Histological examination of callus types

Light microscopic observations revealed clear differences between the compact and friable callus types. Sections of the compact callus showed a broad meristematic zone on the surface of the callus, whereas in the center of the callus, large, vacuolated parenchyma cells were found (Fig. 2A). The meristematic zone consisted of small, cytoplasm-dense cells with small, fragmentated vacuoles and showed a high mitotic activity. We found vascular elements in the deeper regions of the callus (Fig. 2B). Figure 2C shows a section through friable callus. This callus consisted of small meristematic clumps, containing two cell types: small cytoplasm-rich cells and large vacuolated cells and contained organized, embryogenic units resembling

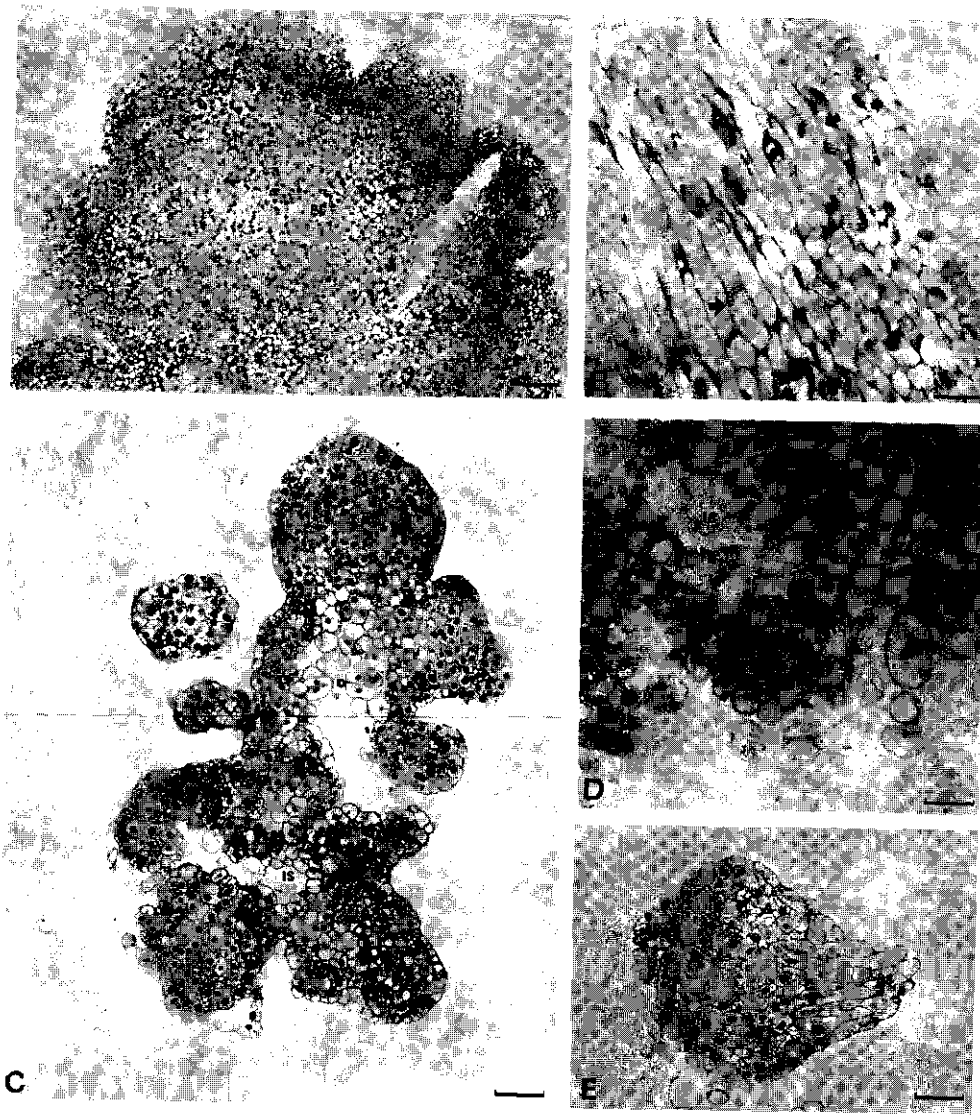

Fig. 2. (A) Light micrograph of compact callus tissue, showing a region with meristematic cells (mc) and parenchyma cells (pc) (bar = 200 μm). (B) Detail of compact callus tissue with parenchyma cells and vessel elements (arrows) (bar = 50 μm). (C) Light micrograph of friable callus tissue, showing meristematic clumps (mc) and intercellular spaces (is) (bar = 100 μm). (D) Detail of friable, embryogenic callus with cytoplasm-rich cells (cc), vacuolated cells (vc) and intercellular spaces (is) (bar = 50 μm). (E) Section through a somatic embryo in friable callus, showing cells with dense cytoplasma and high mitotic activity at the apical side (ap) and at the basal side (bs) long elongated cells (bar = 100 μm).

somatic pro-embryos. Friable callus contained no vascular elements and intercellular spaces were present at low frequency. Figure 2E shows a longitudinal section through a somatic pro-embryo in the friable callus. This pro-embryo shows the typical polarity with small isodiametric and mitotically active cells at the apical side and large, elongated cells towards the basal side.

Establishment of suspension cultures

The friable, embryogenic callus type described above was used for initiation of suspension cultures. Approximately 50 suspension cultures from individual, friable callus cultures, representing 10 genotypes were initiated. All suspension cultures were highly heterogeneous and consisted of cell aggregates and single cells, vacuolated as well as cytoplasm-rich. After 4 to 5 weeks striking differences for dispersion, growth rate and browning could be observed between the suspension cultures. Most of the suspension cultures were well-dispersed with a moderate to good growth rate, but turned brown within 2 months. Suspensions of two callus cultures failed to dissociate in liquid medium, instead increased in size and released only large, vacuolated single cells. These cultures turned into slow-growing suspension cultures which consisted only of large compact callus clumps. Suspensions of only three callus cultures showed the release of small groups of cytoplasm-rich cells. Finally, we obtained two well-growing, highly embryogenic suspension cultures from one callus culture (Fig. 3). These

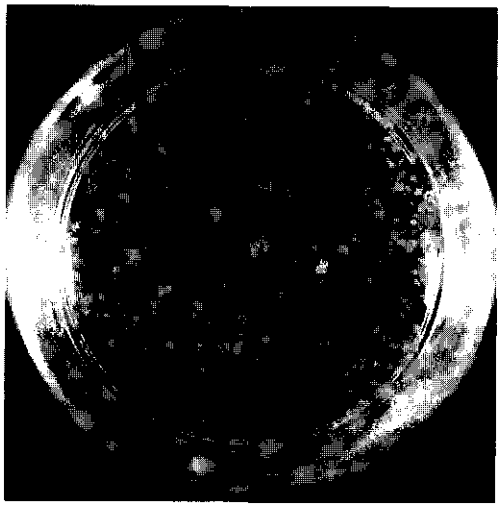

Fig. 3. Suspension culture derived from friable, embryogenic callus (bar = 1.3 cm).

cultures did not become fully established, but consisted of various cell types. These suspension cultures retained a high capacity for plant regeneration for about 8 months (250 shoots/g FW callus). After that, the regeneration capacity decreased with time and after 14 months only 30 shoots per gram FW callus could be regenerated.

Discussion

In this study a friable, embryogenic callus has been induced on immature embryos of leek, and this callus type appeared to be crucial for the initiation of finely dispersed embryogenic suspension cultures. The friable, embryogenic callus could be easily distinguished by its morphology from previously described compact callus cultures of leek (Van der Valk *et al* 1992, Buiteveld *et al* 1993). It was composed of globular structures and somatic embryos that developed from undifferentiated friable callus and was similar in appearance to friable callus cultures of maize (Green and Rhodes 1982). Our results show that for the initiation of this friable, embryogenic callus, the developmental stage of the immature embryo was the most important factor. Immature embryos of 0.5 to 2.5 mm gave the highest frequency of friable embryogenic callus and was not correlated to the genotype of the donor plant. In previous experiments (Van der Valk *et al* 1992, Buiteveld *et al* 1993), when fully matured embryos ($\pm$ 4 mm) were cultured, this type of callus was never observed and exclusively compact, embryogenic callus was produced.

Armstrong & Green (1985) found that addition of L-proline caused a linear increase in the formation of friable embryogenic callus from embryos of the maize inbred line A188. They also observed that this effect was dependent on the organic nitrogen components of the medium and that the frequency of friable callus formation was only increased when L-proline was added to N_6 medium, but not to MS medium. In leek, L-proline had a slight negative effect when added in a concentration of 25 mM to the medium. An even stronger negative effect was reported for oat, where addition to the callus initiation medium appeared to be toxic (Bregitzer *et al* 1989).

Friable embryogenic callus in leek could only be established as a primary callus from immature embryos. In maize, friable, embryogenic callus arises only at a very low frequency and originates directly from the scutellum of cultured immature embryos or spontaneously in established callus cultures (Lu *et al* 1982). Similar results were reported for oat (Bregitzer *et*

al 1989). In maize, it was further shown that lowering of the sucrose concentration in the medium induced the formation of friable callus from compact, embryogenic callus (Rhodes *et al* 1988, Vasil and Vasil 1984). In leek no such effect of sucrose was observed (unpublished results). The type II callus of maize seems more friable than the friable callus we obtained for leek, regarding the long strands of embryogenic cells and larger intercellular spaces, as observed in maize callus (Fransz and Schel 1991), while the compact callus types of both species appear similar in morphology and structure.

Whether friable or compact callus develops on immature embryos or simultaneously remains unclear untill now. In further callus initiation experiments, conducted with different cultivars, we also used seedling-derived donor plants in stead of regenerated plants from callus cultures. We observed that immature embryos derived from seedling-derived donor plants formed only compact callus or responded poorly (up to 3%) with respect to friable callus induction. This suggests that other factors, e.g. the physiology of the explant tissue, may affect the callus type that develops.

In accordance with results obtained for maize (Vasil and Vasil 1986), results from our work showed that for the initiation of embryogenic suspension cultures it is crucial to select a friable type of callus with a high regeneration capacity. In previous experiments when compact callus was used to initiate suspension cultures, we only obtained slow growing large callus clumps in liquid medium. Even after several months, these callus clumps failed to release cytoplasm-rich cells but dispersed only vacuolated single cells. The fact that friable callus is more suitable for initiating suspension cultures than compact callus is also supported by our microscopic observations. In contrast to friable callus, the compact callus is firm and organized, which is due to the fact that it contains vascular elements in the deeper regions of the callus and no intercellular spaces are found. The microscopic observations and morphology were helpful to select between the callus types for their amenability to culture in liquid medium, however, factors such as genotype and culture conditions have important effects on the further development of the initiated suspension cultures.

The embryogenic suspension cultures described in this study will be used for isolation and culture of protoplasts as a first step to somatic hybridization of leek.

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Chapter 4

Plant regeneration from protoplasts isolated from suspension cultures of leek (*Allium ampeloprasum* L.)

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Plant Science (1994) 100: 203-210

Summary

A procedure is described for the regeneration of plants from protoplasts of tetraploid leek (*Allium ampeloprasum* L.), 2n=4x=32. Regeneration-competent protoplasts could only be obtained from an embryogenic suspension culture, that was initiated with friable, embryogenic callus derived from immature embryos. The generally low plating efficiency could be increased by embedding the protoplasts in Ca-alginate, compared to culturing the protoplasts in liquid or agarose-solidified medium. A minimum plating density of 2 x 10^5 pps/ml was required to obtain microcalli. Upon transfer of the protoplast-derived calli on agarose-solidified BDS medium, morphologically different callus types proliferated. After transfer to regeneration medium, compact or friable calli with an embryogenic appearance produced somatic embryos and plantlets at a frequency of up to 80%. Calli that had been classified as heterogeneous also regenerated shoots, but mainly via organogenesis, at a frequency of 46%. After transfer of shoots to half strength MS medium, healthy, well-rooted plants were obtained, that were successfully transferred to soil. All plants contained the tetraploid DNA level.

Introduction

In leek (*Allium ampeloprasum* L.), F_1 hybrid breeding for improving uniformity is of great economical importance. For the production of hybrid seed, the availability of male sterile lines is advantageous. In 1970 Schweisguth reported on genic male sterility (GMS) in leek. However, the propagation of GMS lines appeared to be very difficult, thereby prohibiting the application of these lines for breeding purposes. F_1 hybrid breeding based on a system with cytoplasmic male sterility (CMS) would be preferable, but unfortunately, no source of CMS is known in leek up to now. Introduction of this trait from related species, as onion (Schweisguth 1973) or chive (Tatlioglu 1982), through interspecific hybridization has not yet been successful, because of hybrid sterility and crossing barriers. However, transfer of CMS via somatic cybridization may overcome these problems. For successful application of somatic cybridization, the regeneration of plants from protoplasts is a prerequisite.

During the last two decades substantial progress has been made in tissue culture techniques for *Allium* species. Regeneration of shoots and plantlets from callus cultures has been achieved for many *Allium* species and can either occur via organogenesis or embryogenesis (Novák *et al* 1986, Novák 1990). Recently, embryogenic callus cultures have been described for leek (Van der Valk *et al* 1992, Buiteveld *et al* 1993). There are a few reports on the isolation of *Allium* protoplasts, aimed at enucleation of protoplasts or the development of

fusion procedures (Opatrny and Havránek 1977, Bradley 1978, Bracha and Sher 1981, Kim *et al* 1986). However, none of these authors studied the culture of protoplasts in detail or described sustained cell division of cultured protoplasts. Balakrishnamurthy *et al* (1990) obtained microcalli from callus-derived protoplasts, but subsequent development was arrested.

In this chapter we describe the development of a procedure for the regeneration of plants from protoplasts of leek. Successful plant regeneration from protoplasts was obtained from an embryogenic suspension culture.

Materials and methods

Establishment of embryogenic cell suspensions

Friable, embryogenic callus cultures were induced from immature embryos, obtained from *in vitro* plants of *A. ampeloprasum* L., cultivar 'Porino', as described previously (Buiteveld *et al* 1994). After 6 months, friable calli derived from individual immature embryos were transferred separately to 6-well macroplates (3 cm ∅, Greiner). Per well, 50 mg callus was transferred into 2.5 ml liquid BDS medium (Dunstan and Short 1977), containing 2 mg/l 2,4-D, 30 g/l sucrose and 200 mg/l casein hydrolysate. The suspension cultures were kept on an orbital shaker (100 rpm, Gallenkamp) in the dark at 25 °C. The suspension cultures were subcultured weekly by pipetting the finer cell aggregates into 4 ml fresh medium. After 2 months, a well-growing embryogenic suspension culture was selected, transferred to a 6-cm culture jar (Greiner) and subcultured weekly by pipetting half of the cell mass with 5 ml medium into 15 ml fresh medium. This suspension culture was used for protoplast isolation. Regeneration capacity of the suspension culture was measured by plating suspension callus of the 4-day-old culture onto solid MS medium (Murashige and Skoog 1962), supplemented with 1 mg/l kinetin. Plates were incubated in the light (3000 lx) at 25 °C. After one week, the suspension callus was transferred to fresh medium to prevent browning of the calli. After 8 weeks the number of regenerated shoots was counted. If necessary, an additional subculture of the calli and differentiated somatic embryos was carried out.

Isolation of protoplasts

Protoplasts were isolated from a 4 - 9 month-old suspension culture, at the 3rd or 4th day after subculture. About 1 g FW of suspension cells was incubated in 10 ml enzyme solution that

contained 1% (w/v) Cellulase "Onozuka" RS (Yakult Honsha CO., Tokyo, Japan), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical CO., Tokyo, Japan), 0.6 M mannitol, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 5 mM $MgCl_2.6H_2O$, at pH 5.6. The cells were digested for 6 h on an orbital shaker (60 rpm) in the dark at 25 °C. The mixture was filtered through nylon sieves of mesh sizes 55 µm and 35 µm respectively, washed with an equal volume of washing solution, i.e. an aqueous solution of 0.65 M mannitol and 1 mM $CaCl_2$. After centrifugation at 30 x g for 3 min, the supernatant was removed and the washing/ centrifugation step was repeated 3 times. Protoplasts were resuspended in washing solution or in culture medium and counted using a haemocytometer.

Protoplast culture and regeneration

Protoplasts were cultured in KM basal medium (Kao and Michayluk 1975), supplemented with KM vitamins, organic acids, 0.6 M glucose, 100 mg/l myo-inositol, 250 mg/l casein hydrolysate, 2% (v/v) coconut milk and 1 mg/l 2,4-D at pH 5.6. The osmolality of the medium was adjusted to 750 mOsmol/kg with glucose. Protoplasts were cultured at densities ranging between 5 x 10^4 and 8 x 10^5 pps/ml.

Protoplasts were plated in 1% (w/v) alginate (Sigma), 0.3% (w/v) agarose (Sea plaque) or liquid medium. The alginate embedding protocol was as detailed in Damm *et al* (1989) and modified by Hall *et al* (1993). After the washing steps protoplasts were resuspended in washing solution and the density/ml was adjusted at twice the final density. The suspension was then mixed thoroughly with an equal volume of 2% (w/v) alginate (made up in 0.65 M mannitol and 1 mM $CaCl_2$). Aliquots of 0.3 ml were spread onto calcium/agarose plates, containing 0.55 M mannitol, 50 mM $CaCl_2$ and 0.8% (w/v) agarose (3 cm Petri dishes) and left for 1 h at room temperature. The solidified alginate discs were transferred to 1 ml 50 mM $CaCl_2$/0.55 M mannitol solution in 3 cm Petri dishes (Greiner, TC Quality). After storage for at least 1 h at 4 °C, the calcium/mannitol solution was removed and replaced with 1 ml protoplast culture medium. Protoplasts in agarose and liquid medium were cultured in 1 ml aliquots in 3 cm Petri dishes (Greiner, TC Quality). All protoplast cultures were kept in the dark at 25 °C.

After 10 and 28 days of culture, 1 ml KM medium, containing 0.3 M glucose was added to each Petri dish. The plating efficiency, expressed as the percentage of plated protoplasts which had divided to form a colony of at least 10 cells, was measured after 4 weeks. Six to 8 weeks after plating, macroscopically visible microcalli were transferred and spread onto 0.8%

(w/v) agarose medium, containing BDS salts and vitamins, 3% (w/v) glucose, 1 mg/l 2,4-D and 200 mg/l casein hydrolysate (pH 5.6). The microcalli were subcultured every 2 weeks for an additional period of 4 - 6 weeks.

For plant regeneration, protoplast-derived calli of approximately 3 - 5 mm were transferred to MS medium, supplemented with 1 mg/l kinetin, 3% (w/v) sucrose and 0.3% (w/v) phytagel (Sigma) and cultured in the light (3000 lx). The effect of different light intensities (1200, 3000 and 5000 lx) and various concentrations of BA (1, 2 mg/l) and NAA (0.25, 0.5 mg/l) in the medium on the regeneration frequency were tested. After 3 months on regeneration medium, shoots were transferred to glass jars (450 ml), containing hormone-free, half strength MS medium and 2% sucrose. Well-developed plantlets were transferred to soil.

All media were filter-sterilized, except the media used for alginate embedding and the regeneration media, which were autoclaved for 15 min. at 120 °C.

Measurement of ploidy level

The DNA content of interphase nuclei isolated from leaf cells of regenerants was determined by flow cytometry according to Verhoeven *et al* (1990). Nuclei were stained with DAPI (1 mg/l) and measured using a Partec Pas-II flow cytometer.

Statistical analysis

A generalized linear model based on a Poison distribution (McCullagh and Nelder 1989) was used to analyze the effect of culture method and protoplast density on the plating efficiency.

Results

Protoplast isolation and culture

The experiments were carried out over a period of 5 months, during which period the regeneration capacity of the suspension culture was constant. On average, 250 shoots could be regenerated from 1 g FW suspension cells. After 9 months, the number of regenerated shoots decreased to 78 shoots. Protoplasts isolated from the suspension culture when it was younger than 4 months contained large starch granules. These protoplasts were quite fragile and divided rarely. Generally, 50% of these protoplasts died after 1 d of culture. The number of

Table 1. The effect of embedding agent on the plating efficiency of *Allium ampeloprasum* L. protoplasts. Protoplasts were cultured at a density of 2×10^5 pps/ml.

Culture method	Plating efficiency (%)[a]
Liquid	0.035 ± 0.005 a[b]
Agarose (Sea Plaque)	0.030 ± 0.004 a
Alginate	0.150 ± 0.009 b

[a] Plating efficiency was determined as the percentage of plated protoplasts that formed microcalli after 4 weeks of culture. Values are means with standard error, obtained from 3 independent experiments, 4 - 5 replicates per treatment.

[b] Means followed by a common letter are not significantly different at 0.1% level.

Table 2. The effect of protoplast density on the plating efficiency of *Allium ampeloprasum* L. protoplasts after 4 weeks of culture. Protoplasts were embedded in alginate.

Protoplast density (pps/ml)	Plating efficiency (%)[a]
8×10^5	0.278 ± 0.019 a[b]
6×10^5	0.281 ± 0.019 a
4×10^5	0.248 ± 0.018 a
2×10^5	0.219 ± 0.017 a
1×10^5	0.078 ± 0.009 b
5×10^4	0.038 ± 0.007 c

[a] Plating efficiency was determined as the percentage of plated protoplasts that formed microcalli after 4 weeks of culture. Values are means with standard error, obtained from 3 independent experiments, 3 - 5 replicates per treatment.

[b] Means followed by a common letter are not significantly different at 0.1% level.

protoplasts with starch granules decreased, when the suspension culture aged. For this reason, protoplast isolation and regeneration experiments were started, when the suspension culture was at least 4-months old (Fig. 1A). Protoplast yields ranged from 2.1 - 4.3 x 10^6 protoplasts per gram FW suspension culture. Freshly isolated protoplasts appeared highly cytoplasmic (Fig. 1B). More than 85% of these protoplasts had a viable appearance, as measured by microscopic examination using FDA immediately after isolation. The first cell divisions occurred after 7 days and small colonies were observed microscopically after 2 weeks of culture (Figs. 1C, 1D). Already one day after plating, substantial differences were observed between the three different methods of culture. In alginate cultures, more protoplasts remained viable and no budding or browning occurred. In agarose and liquid cultures initial divisions occurred less frequently and were arrested. This resulted in a significantly higher plating efficiency in the Ca-alginate cultures, compared to the agarose and liquid cultures (Table 1). No differences in plating efficiency were observed between cultures in agarose-solidified medium and liquid medium. Another important factor that affected the plating efficiency was

the plating density. Data in table 2 show that at least a plating density of 2 x 10^5 pps/ml was necessary to obtain an optimum plating efficiency. When plating densities of 1 x 10^5 pps/ml or lower were used, the plating efficiency decreased significantly.

Plant regeneration

Between 50 and 90% of the cell colonies developed into calli after transfer to agarose-solidified BDS medium. The calli grew vigorously on this medium and developed into morphologically different callus types. Both compact and friable embryogenic calli developed from the individual cell colonies. In addition to these two embryogenic callus types a third group of calli was distinguished, which was characterized by a heterogeneous morphology. This group consisted of calli which were non-compact, watery, or semi-compact with a rough surface and with deformed somatic embryos. Table 3 summarises the frequency of plant regeneration of the various callus types, derived from one protoplast culture experiment. The results show that upon transfer of the calli to regeneration medium about 80% of embryogenic calli formed shoots. Of the heterogeneous calli, 46% appeared competent for shoot regeneration. The number of shoots per regenerating callus was much lower, compared to the compact and friable calli. Calli obtained from 2 other experiments showed similar regeneration frequencies (compact, 69 and 71%, respectively; friable, 100%; heterogeneous, 40 and 26%, respectively). Compact and friable callus regenerated mainly through somatic embryogenesis (Fig. 1F). Regeneration of the heterogeneous calli progressed slowly and after successive subcultures the general mode of regeneration was through organogenesis.

Various regeneration media with different combinations of BA and NAA were tested to improve the regeneration frequency of the protoplast-derived calli. However, none of these media gave a substantial increase in the number of regenerating calli (ranging from 32 to

Table 3. Frequency of plant regeneration of various callus types, obtained from *Allium ampeloprasum* L. protoplasts.

Callus type	Number of calli[a]	Number of calli transferred to regeneration medium[b]	Number of regenerating calli (%)[c]	Mean number of shoots per regenerating callus
Compact	75	75	79	7.8
Friable	16	16	81	10.7
Heterogeneous	122	48	46	5.1

[a] Calli were obtained from one experiment. Plating efficiency was ± 0.05 and on average 50% of the cell colonies developed into calli.

[b] Calli were cultured on MS medium, supplemented with 1 mg/l kinetin for 3 months.

[c] Percentage of calli transferred to regeneration medium, that produced shoots.

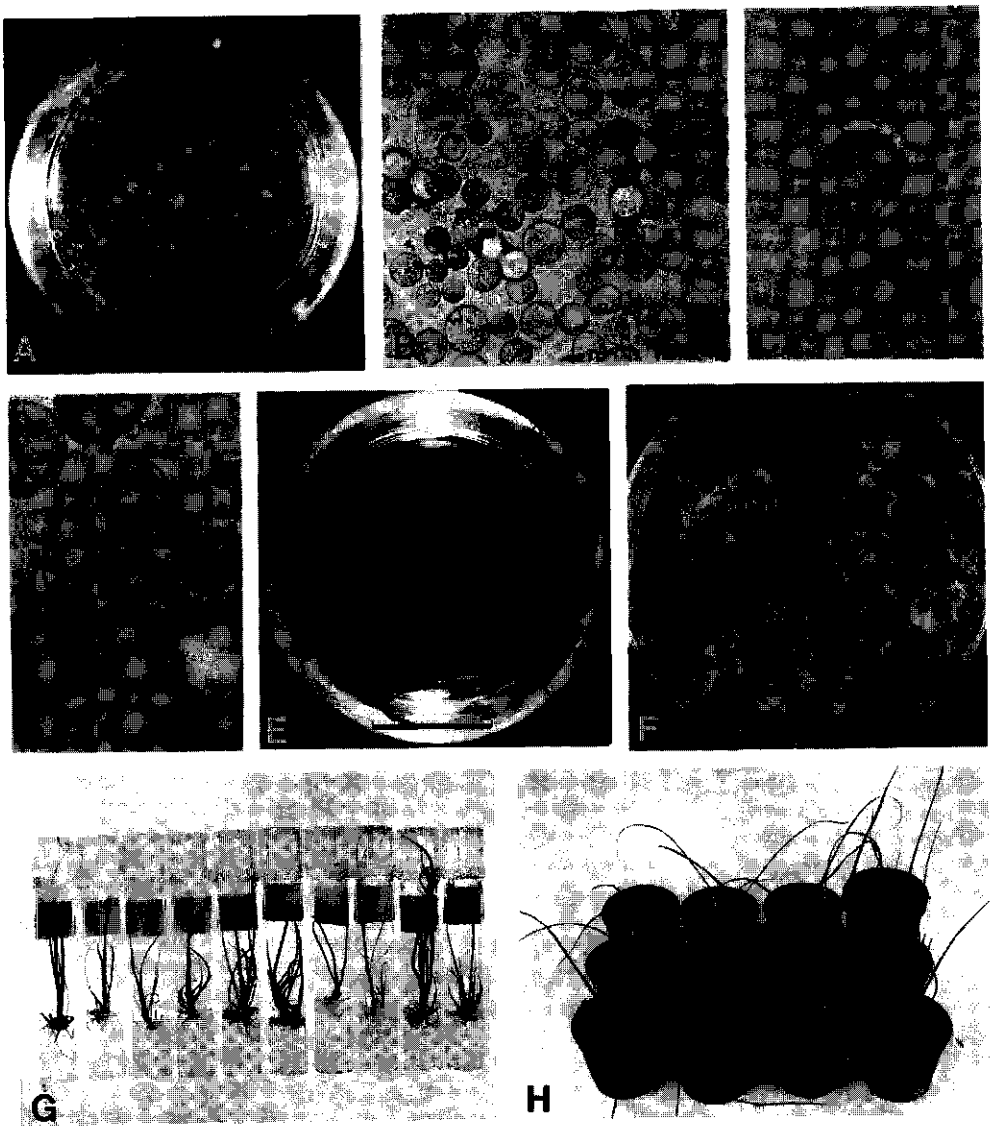

Fig. 1. Regeneration from *Allium ampeloprasum* L. protoplasts. (A) Embryogenic suspension culture used for protoplast isolation (bar = 1 cm). (B) Freshly isolated suspension culture protoplasts (bar = 40 μm). (C) First division of protoplasts embedded in alginate (bar = 25 μm). (D) Protoplast-derived micro colony (bar = 25 μm). (E) Microcalli derived from protoplasts embedded in alginate after 7 weeks of culture (bar = 1 cm). (F) Regeneration of plantlets from protoplast-derived calli on MS medium, containing 1 mg/l kinetin, after two months of culture (bar = 1 cm). (G) Plantlets on hormone-free medium. (H) Plants in soil.

49%) compared to medium, containing only kinetin (52% regenerating calli). In addition, the effect of three light intensities on the regeneration from protoplast-derived calli was tested. It was found that the light intensity did not influence the regeneration frequency of the calli (between 45 and 54% regenerating calli), although the number of shoots per regenerating callus was highest at 3000 lux (6.5 shoots) compared to 1200 lux (4.2 shoots) and 5000 lux (3.4 shoots).

With the procedure described, plantlets were obtained approximately 6 months after isolation. They were transferred to hormone-free medium, where they developed a normal root system. Subsequently, they were transferred to the greenhouse (Figs. 1G, 1H).

Ploidy of the regenerants

The DNA content of interphase nuclei from 75 regenerants, obtained from 75 independent calli, was determined by flow cytometry. This analysis showed that all regenerants were tetraploid. The DNA distribution patterns of 9 regenerants were aberrant, in that a substantial number of cells were endopolyploid, with a nuclear DNA content up to 16C (Fig. 2).

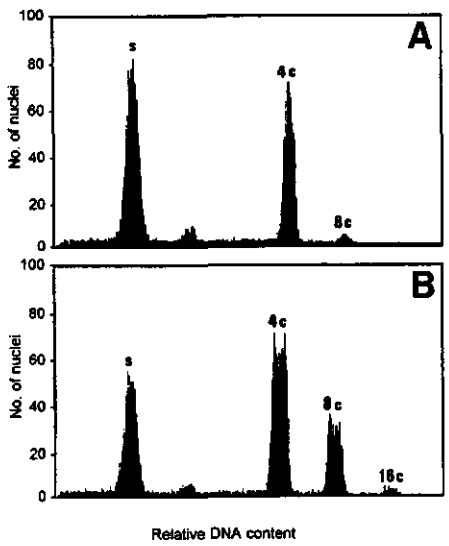

Fig. 2. Flow cytometric analysis of the relative nuclear DNA content of *Allium ampeloprasum* L. cells. (A) normal tetraploid regenerant. (B) regenerant with aberrant DNA distribution pattern. Nuclei of *Nicotiana plumbaginofolia* were added as internal standard (S).

Discussion

This is the first report on plant regeneration from protoplasts of leek (*A. ampeloprasum* L.). The availability of an embryogenic cell suspension appeared a crucial step. Wang *et al* (1986) mentioned plant regeneration from mesophyll protoplasts of *A. cepa*. In our case, protoplasts isolated from the leaf base of seedlings of leek did not divide (unpublished results). For the establishment of an embryogenic cell suspension of leek, a friable and embryogenic callus culture had to be used. In many graminaceous species compact, embryogenic callus has been successfully used to establish stable, embryogenic cell suspensions (Vasil and Vasil 1992). For leek, compact, embryogenic callus was not suitable for suspension initiation; only non-regenerable suspension cultures were obtained, which yielded protoplasts that divided only scarcely. These results are consistent with those reported for maize, where friable, embryogenic callus has also proven to be essential to establish suspension cultures capable of producing regenerable protoplasts (Rhodes *et al* 1988, Shillito *et al* 1989).

The suspension culture remained regenerative for at least 9 months, during this period also regenerable protoplasts could be isolated. In wheat, Vasil *et al* (1990) could regenerate plants from 1-year-old suspension cultures, but in many other cereal and grass species, e.g. rice, barley and *Lolium*, the long-term maintenance of morphogenic suspension cultures was a problem (Göbel *et al* 1985, Lührs and Lörz 1988, Creemers-Molenaar *et al* 1989). On the other hand, a minimum age of the suspension culture was often needed, for successful isolation of regenerable protoplasts as was reported for maize (Shillito *et al* 1989) and rice (Abullah *et al* 1986). During the period in which the protoplast experiments were carried out (4 - 9 months), the plating efficiency increased from 0.05 to 0.3. During the same period, the age of the suspension culture also affected the regeneration frequency of the protoplast-derived embryogenic calli, which decreased from 80% to ± 50%.

A beneficial effect on the plating efficiency was observed when protoplasts were embedded in alginate. This stimulatory effect of alginate embedding has also been described for other recalcitrant protoplast systems such as sugarbeet (Hall *et al* 1993) and oat mesophyll protoplasts (Hahne *et al* 1991). For leek protoplasts we observed a 5-fold increase of the plating efficiency, which is comparable with the results of sugarbeet (Hall *et al* 1993). For oat mesophyll protoplasts, which rarely divide, plating efficiencies of up to 80% could be obtained (Hahne *et al* 1991).

Our results indicate that the regeneration frequency of protoplast-derived calli depends

primarily on the type of callus that is formed and to a minor extent on external culture conditions. To improve this regeneration system, it will be interesting to increase the percentage of cell colonies that proliferate into embryogenic calli.

All tested regenerants appeared tetraploid, as was the original plant material. Evidently, prolonged *in vitro* culture of leek does not lead to a high frequency of DNA instability. The occurrence of cells with polyploid DNA content in one and the same tissue of 9 plants (polysomaty) is a well-known phenomenon in *Allium* species (Novák *et al* 1986). The endopolyploidization might be caused by the aging of the leafs. In flow cytometric analyses of regenerants of callus cultures we also found that the older leafs showed more endopolyploid cells (unpublished results).

The results presented here, open up good perspectives for further research aimed at the transfer of CMS through somatic cybridization.

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Chapter 5

Production and characterization of somatic hybrid plants between leek (*Allium ampeloprasum* L.) and onion (*Allium cepa* L.)

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Summary

Results are reported on the production and characterization of somatic hybrids between *Allium ampeloprasum* and *Allium cepa*. Both symmetric and asymmetric protoplast fusions were carried out using a polyethylene-based mass fusion protocol. Asymmetric fusions were performed using gamma ray-treated donor protoplasts of *A. cepa* and iodoacetamide-treated *A. ampeloprasum* protoplasts. However, the use of gamma irradiation to eliminate or inactivate the donor DNA of *A. cepa* proved to be detrimental to the development of fusion calli and thus it was not possible to obtain hybrids from asymmetric fusions. The symmetric fusions yielded a high number of hybrid calli and regenerated plants. The analysis of the nuclear DNA composition using interspecific variation of rDNA revealed that most of the regenerated plants were hybrids. Flow cytometric analysis of nuclear DNA showed that these hybrid plants contained lower DNA content than the sum of the DNA amounts of the parental species, suggesting that they were aneuploid. Shortage of chromosomes in the hybrids was confirmed by genomic *in situ* hybridization. Chromosome counts in metaphase cells of six hybrids revealed that these plants lacked 2 - 7 leek chromosomes. One hybrid showed also the loss of onion chromosomes. The hybrids had an intermediate phenotype in leaf morphology. The application of these somatic hybrids in breeding is discussed.

Introduction

Leek (*A. ampeloprasum*) is an important vegetable crop in Europe. There is great interest in improving the quality of the existing leek cultivars, because they are heterogeneous, unstable and are highly susceptible to pests and diseases. The transfer of desirable traits from related species by interspecific crossing has been hampered by sexual incompatibility within the genus *Allium*. Successful crosses between species within the *A. ampeloprasum* complex have been achieved by Kik *et al* (1997). However, successful gene transfer between species of different sections or subgenera is not possible or very difficult (Gonzalez and Ford-Lloyd 1987, Ohsumi *et al* 1993). In this regard, somatic hybridization may be a viable alternative to combine the genomes of incompatible species and to transfer nuclear or cytoplasmic traits from one species to another (Gleba and Sytnik 1984, Waara and Glimelius 1995).

The number of reports on successful realization of somatic hybridization and cybridization of monocotyledons, to which the genus *Allium* belongs, is very limited, in comparison to dicotyledonous species. This is due to recalcitrance for regeneration from protoplasts. Until now, successful regeneration of somatic hybrid and cybrid plants has been restricted to members of the *Gramineae*. In rice, several authors reported on the production of somatic

cybrids and hybrids and the successful transfer of mitochondrial genomes from CMS lines (Akagi *et al* 1989, 1995, Hayashi *et al* 1988, Kyozuka *et al* 1989, Terada *et al* 1987, Yang *et al* 1989). Also, in *Festuca* and *Lolium* asymmetric and symmetric hybrid plants were obtained by protoplast fusion (Spangenberg *et al* 1994, 1995, Takamizo *et al* 1991, Takamizo and Spangenberg 1994).

The aim of our research was to see whether it is possible to transfer traits from *A. cepa* to *A. ampeloprasum* by means of somatic hybridization. Onion was chosen as a fusion partner because it possesses several agronomically useful traits for the breeding of leek and cannot be hybridized sexually with leek. For the application of somatic hybridization first a regeneration procedure for leek was developed. In this regard, embryogenic suspension cultures were established from friable callus cultures of leek and a method was developed for plant regeneration from protoplasts isolated from these suspension cultures (Buiteveld *et al* 1994, Buiteveld and Creemers-Molenaar 1994). Symmetric protoplasts fusions, as well as asymmetric protoplasts fusions using gamma irradiation, were performed. Selection of the somatic hybrids was based on metabolic complementation of iodoacetamide-treated *A. ampeloprasum* protoplasts by *A. cepa* protoplasts which alone are unable to divide.

Here we report the results from our analyses of the calli and plants obtained from the fusion experiments. For identification, on a molecular basis, of the hybrid nature of the calli and plants obtained, a simple and convenient method was needed. There are no species-specific probes available for leek and onion and consequently we developed a method based on PCR and restriction-site variation of rDNA to detect the nuclear hybrids. In addition, the nuclear composition of the hybrid plants was analyzed by flow cytometry and *in situ* hybridization.

Materials and Methods

Plant material and culture conditions

Tetraploid (2n=4x=32) leek (*A. ampeloprasum*) and diploid (2n=2x=16) onion (*A. cepa*) were used as fusion partners. Suspension cultures of *A. ampeloprasum* cv. 'Porino' were derived from an embryogenic callus line (3992), as described previously (Buiteveld *et al* 1994). Seeds of *A. cepa* cv. 'Alamo' and 'Hyton' were provided by S&G Seeds (Enkhuizen, The Netherlands) and Bejo Zaden BV (Warmenhuizen, The Netherlands) respectively. The onion

seeds were sown aseptically on ½ MS medium (Murashige and Skoog 1962), supplemented with 2% (w/v) sucrose and 0.7% (w/v) agar (Oxoid) and the seedlings were cultured in the light (3000 lux, 16 h) at 25 °C.

Isolation and fusion of protoplasts

Protoplasts were isolated from an embryogenic cell suspension of *A. ampeloprasum* cv. 'Porino' (3992), as described previously (Buiteveld and Creemers-Molenaar 1994). However, a modified enzyme mixture was used; (0.5% (w/v) Cellulase 'Onozuka' RS (Yakult Honsha Co., Tokyo, Japan) and 0.05% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan)). The cell walls were digested for 16 h on a rotary shaker at 40 rpm. To identify the fusion products, the leek protoplasts were stained with Fluorescein diacetate (FDA) (20 µg/ml) during the enzyme incubation. Leek protoplasts were resuspended for metabolic inactivation in wash medium (CPW salts (Frearson *et al* 1973), 12% mannitol, pH 5.7) containing varying concentrations of iodoacetamide (4, 5, 6 or 7 mM IOA) and incubated at 4 °C for 15 min. After IOA treatment, leek protoplasts were washed twice by centrifugation and were finally resuspended in wash medium. For isolation of leaf mesophyll protoplasts of onion, 4 week-old seedlings were used. Leaf material (± 0.5 g FW) was cut into small pieces and incubated for 1 h in the dark in CPW salts, 9% mannitol, 3 mM 2-morpholino-ethansulfonic acid (MES) and 3 mM ascorbic acid, pH 5.7. This medium was then replaced by 15 ml of the same medium containing 0.75% Cellulase 'Onozuka' R-10 and 0.15% Macerozyme R-10 (both from Yakult Honsha Co., Tokyo, Japan). The leaves were incubated for 16 h stationary in the dark at 25 °C. Protoplasts were separated from cell debris by filtration through 88 µm and 55 µm nylon filters and centrifuged for 5 min at 600 rpm (30 x g). Subsequently, they were resuspended in CPW salts, 15% (w/v) sucrose and 3 mM MES (pH 5.7) with an upper layer of 1 ml wash medium (CPW salts, 9% mannitol and 3 mM ascorbic acid, pH 5.7) and centrifuged for 10 min at 700 rpm (40 x g). The floating protoplasts were collected in the upper layer and washed twice in wash medium. In some of the experiments the onion protoplasts were irradiated with 50, 150 or 300 Gy of gamma-rays from a ^{60}Co source at a dose rate of approximately 500 Gy/h. During irradiation, protoplasts were kept on ice. Leek protoplasts and onion protoplasts were mixed in a 1:1 ratio. Fusion was performed as described by Gilmour *et al* (1989) with the following modifications: 0.3 ml PEG solution (30% (w/v) PEG 6000, 4% (w/v) sucrose and 0.147% (w/v) $CaCl_2.2H_2O$) was added dropwise without mixing to 2 x 10^6 protoplasts resuspended in 0.3 ml wash medium. The

protoplasts were incubated for 30 min. at room temperature. After incubation, 0.8 ml of high pH/Ca^{2+} solution (Keller and Melchers 1973) was added and the protoplasts were incubated for a further 15 min.

Culture of protoplasts

After fusion, the protoplasts were counted and cultured according to Buiteveld and Creemers-Molenaar (1994). Instead of KM medium, the fused protoplasts were cultured in KM medium supplemented with 25% conditioned medium (KM25CM). KM25CM medium was prepared by adding 25% conditioned culture medium of a suspension culture, harvested 7 days after subculture in KM medium. At weekly intervals the culture medium was replaced by fresh KM25CM medium. The osmolality was reduced by steps of 100 mOsm/kg (by reducing the glucose concentration) to 450 mOsm/kg. After 4 to 6 weeks, when microcalli had formed, the alginate plates were transferred to solid KM medium, containing 3% (w/v) glucose, 1 mg/L 2,4-D and 0.8% (w/v) agarose (pH 5.6). Plant regeneration from calli was carried out on a culture medium as described earlier (Buiteveld and Creemers-Molenaar 1994). Well-rooted plants were transferred to the greenhouse.

Nuclear DNA analysis by PCR and restriction-site variation

To distinguish between the nuclear genomes of the two fusion parents, the variability in the internal transcribed spacer (ITS) region of rDNA was used. For this purpose, total DNA was isolated from both callus cultures and *in vitro* plants according to Cheung *et al* (1993). The precipitated DNA was resuspended in 50 µl TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). For PCR 3 µl of a 10x-diluted DNA solution was used. Two primers 'ITS4' (5'-TCCTCCGCTTATTGATATGC-3') and 'ITS5' (5'-GGAAGTAAAAGTCGTAACAAGG-3'), developed by White *et al* (1990) were used for amplification of the nuclear rDNA of the ITS region. The primers were synthesized by Isogen Bioscience BV. The PCR was carried out in a 50 µl reaction mix, containing 100 ng of each primer, 3 µl template DNA, 0.4 mM dNTPs, 1x-Supertaq PCR buffer (HT Biotechnology) and 0.2 units Supertaq (HT Biotechnology). Amplification was carried out under the following conditions: 1 cycle of 94 °C, 1 min, 35 cycles of 94 °C, 1 min, 50 °C, 1 min, 72 °C, 2 min ending with 1 cycle of 72 °C, 5 min. Approximately 0.25 µg of PCR-amplified DNA was digested with *Dde*I under conditions recommended by the manufacturer. The DNA restriction fragments were separated

by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transillumination.

Flow cytometric analysis of nuclear DNA content

The nuclear DNA content of parental plants and plants regenerated from fusions was measured by flow cytometry as described earlier (Buiteveld and Creemers-Molenaar 1994). Nuclei of leek leaf material were used as an internal standard.

Isozyme analysis

Isozyme analysis was performed for two enzymes: glutamate oxaloacetate transaminase (*Got*; EC 2.6.1.1) and shikimic dehydrogenase (*Skd*; EC1.1.1.25). Leaf tissue (± 50 mg) was ground in 45 µl extraction buffer on ice. The extraction buffer contained 0.1 M Tris-HCl (pH 8.0), 1.4% (w/v) dithiothreitol, 1.4% (w/v) polyvinylpyrrolidon, 6% (w/v) sucrose, 0.5% (w/v) bromo cresol green and 30% (v/v) dimethylsulfoxide. Samples were centrifuged in an Eppendorf centrifuge for 10 min at maximum speed, after which 15 µl of the supernatant was used for electrophoresis. Samples were run on a 6% (w/v) stacking gel and 10% (w/v) separating gel using a Desaphor VA (Desaga) vertical gel system, at 800 V for 90 min. The electrophoresis buffer consisted of 0.03 M Tris-HCl and 0.09 M Boric acid. The gels were stained for *Skd* according to Wendel and Weeden (1989) and for *Got* according to Rick *et al* (1977). For *Got*, the gel was incubated for 30 min at room temperature, for *Skd* 2 h at 37 °C, both in the dark.

Chromosome preparation and in situ hybridization

Root tips were collected from both greenhouse-grown regenerants and their parents, pre-treated in an aqueous solution of 2mM 8-hydroxyquinoline for 5h at 4 °C and fixed in cold Carnoy's solution (ethanol: glacial acetic acid, 3:1) for at least 24 h at -20 °C. The root tips were rinsed in water and incubated in an enzyme mixture containing 0.1% (w/v) Cellulase 'Onozuka' RS (Yakult Honsha Co., Tokyo, Japan), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) and 0.1% (w/v) Cytohelicase (Sigma) in a 10 mM citrate buffer, pH 4.5, for 45 min at 37 °C. The root tips were transferred to a grease-free slide and the cells were spread according to Pijnacker and Ferwerda (1984). DNA denaturation, *in situ* hybridization and detection were carried out using the protocol of Schwarzacher and Heslop-Harrison (1993) as modified by Ramulu *et al* (1996a). Total genomic DNA isolated from

leaves of *A. cepa* was used as a probe. The leaf DNA from *A. ampeloprasum* was used as a blocking DNA. The blocking DNA was 50 times the amount of the probe DNA. The *A. cepa* DNA was labelled with Fluorescein-High Prime kit Fluorescein-12 dUTP (Boehringer-Mannheim). The hybridization mix (100 µl per slide) consisted of 50% deionized formamide, 10% sodium dextran sulphate (Sigma), 2 x SSC, 0.25% (w/v) sodium dodecyl sulphate (Sigma), 200 ng of *A. cepa* probe DNA and 10 µg of *A. ampeloprasum* blocking DNA.

Results

Production of hybrids

In the symmetric protoplast fusions, two different *A. cepa* cultivars were used. One fusion experiment was performed with the combination *A. ampeloprasum* (+) *A. cepa* cv. 'Hyton' and 5 experiments with the combination *A. ampeloprasum* (+) *A. cepa* cv. 'Alamo' (Table 1). It was possible to identify the heterokaryons on basis of dual fluorescence, because suspension protoplasts stained with FDA were yellow and mesophyll protoplasts of *A. cepa* gave a red auto-fluorescence. On average, the fusion frequency was 2% as observed directly after protoplast fusion.

In the combination of *A. ampeloprasum* (+) *A. cepa* cv. 'Hyton' no selection by IOA treatment of *A. ampeloprasum* protoplasts was applied. As *A. cepa* mesophyll protoplasts are not able to divide in the culture medium used, cell division and callus formation was possible only from leek protoplasts or from hybrid protoplasts. As shown in Table 1 this experiment resulted in numerous calli of which 2000 were transferred to MS + 1 mg/l kinetin for plant regeneration. After 4 months, 23 of these calli gave rise to plants.

In the fusion experiments with the cultivar 'Alamo', the leek protoplasts were treated with IOA. Dose response experiments had shown that leek suspension protoplasts were very sensitive to IOA and division was completely inhibited by a treatment of 4 mM IOA for 15 min. However, control experiments in which IOA-inactivated protoplasts were treated with PEG and then cocultivated with *A. cepa* mesophyll protoplasts showed that colony formation was sometimes observed at 4 mM IOA and that higher concentrations may be needed to completely inhibit cell division. Therefore fusions were conducted with 4 - 7 mM IOA-treated protoplasts. Because of metabolic complementation, mainly the heterokaryons were expected to divide and to form colonies and hence, all calli obtained were putative hybrids. In these

experiments, 1244 calli were obtained of which eventually 119 regenerated plants.

Asymmetric fusions were performed involving 3 different doses of irradiation. The dose response effect of irradiation could only be tested on subsequent cell viability and not on the plating efficiency as mesophyll protoplasts of *A. cepa* are incapable of division. Results from these experiments showed that gamma irradiation had no effect on the viability, except that the viability of the 300 Gy-treated protoplasts declined to 50% compared to the unirradiated protoplasts as measured after 2 weeks (data not shown). However, after fusion we did see an effect of the irradiation and observed only half as many heterokaryons as in the unirradiated fusion experiments. In total only two plants were recovered from the 50 and 150 Gy fusions. From the 300 Gy treated series no calli could be regenerated to plants (Table 1).

Analysis of nuclear composition of calli and regenerated plants

The nuclear DNA of the putative hybrids was analysed by sequence variation in the rDNA of the parent species. First PCR was carried out with the primers ITS5 and ITS4 to amplify the ITS-region of the nuclear rDNA for *A. ampeloprasum* and *A. cepa* parent. The amplified DNA showed a single band of approximately 0.7 kb for both species. After digestion of this DNA

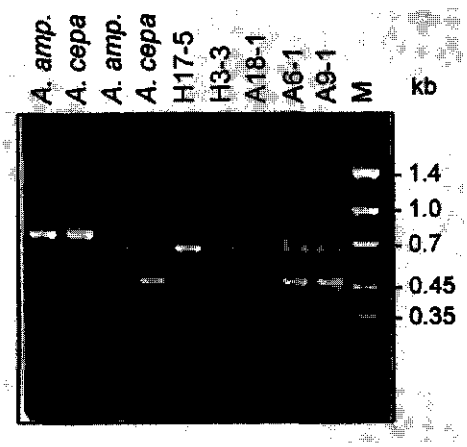

Fig. 1. Nuclear DNA analysis by PCR amplification and restriction site variation of putative hybrids. PCR product of ITS region of nuclear ribosomal DNA of parental line *A. ampeloprasum* 3992 (lane 1) and *A. cepa* cv. 'Hyton' (lane 2). Restriction fragment pattern of PCR product after digestion with *Dde*I, showing a polymorphism between *A. ampeloprasum* 3992 (lane 3) and *A. cepa* (lane 4) and restriction site pattern of examples of some regenerants, one non-hybrid regenerant (lane 5) and 4 hybrids (lane 6 - 9). M: pEMBL18 digested with *Taq*I as DNA size marker (lane 10).

Table 1. Details of fusion combinations between *A. ampeloprasum* and *A. cepa* using γ-irradiation of the donor parent (*A. cepa*) and/or IOA treatment of the recipient (*A. ampeloprasum*).

Fusion combination	Dose of gamma irradiation (Gy)	IOA treatment[a]	Total no. of pps[b]	No. of calli obtained[c]	No. of calli transferred to regeneration medium[d]	No. of calli regenerated to plants	Total no. of plants
A. ampeloprasum (+) *A. cepa* cv. 'Hyton'	0	–	1.2×10^7	>2000	2000	23	127
A. ampeloprasum (+) *A. cepa* cv. 'Alamo'	0	+	5.9×10^6	1244	1059	119	539
	50	+	4.4×10^6	264	258	1	1
	150	+	3.8×10^6	110	107	1	1
	300	+	2.0×10^6	40	40	0	0

[a] 4 - 7 mM IOA.
[b] Ratio of *A. ampeloprasum* : *A. cepa* protoplasts is 1:1 during fusion.
[c] Calli obtained 3 months after fusion.
[d] Regeneration medium contained 1 mg/l kinetin.

Table 2. Nuclear DNA composition determined by PCR analysis of calli and regenerants from two different fusion experiments between *A. ampeloprasum* and *A. cepa*.

Exp.	Fusion combination	IOA treatment	No. of calli transferred to regeneration medium[a]	No. of hybrid calli (%)	No. of calli regenerated to plants		Total no. of calli regenerated to plants (%)	% of hybrid plants
					hybrid	leek		
1	*A. amp.* (+) *A. cepa* cv. 'Hyton'	–	2000	n.d.[b]	16	7	23 (1)	70
2	*A. amp.* (+) *A. cepa* cv. 'Alamo'	+	177	101 (57)	39	3	42 (24)	93

[a] Regeneration medium contained 1 mg/l kinetin.
[b] n.d.: not determined.

fragment with *Dde*I, *A. ampeloprasum* and *A. cepa* were distinguishable by their different restriction fragment patterns (Fig. 1). *A. ampeloprasum* showed one band, slightly smaller than the 0.7 kb band (approx. 0.6 kb) and a very small band, which was not visible on the gel. In some cases, a faint second band was visible of nearly the same size, probably caused by an internal polymorphism within the leek genome. *A. cepa* showed two bands of approximately 0.5 kb and 0.2 kb.

To determine the frequency of hybrids that was obtained, calli and plants from two experiments were analysed for the composition of their nuclear genome based on this ITS polymorphism. For the fusion combination *A. ampeloprasum* (+) *A. cepa* cv. 'Alamo' 177 randomly chosen calli (Table 2, exp. 2) were examined. Of these calli, 101 showed a combination of the restriction fragment patterns of both parents and were thus hybrid clones. As expected, the remaining 76 calli appeared to be calli of leek. Not all calli were able to regenerate plants; 42 of the 177 calli produced plants, of which 39 were hybrids. In experiment 1 (*A. ampeloprasum* (+) *A. cepa* cv. 'Hyton') 2000 calli chosen randomly were transferred to regeneration medium. While the nuclear composition of these calli was not determined, analysis of the plants revealed that 16 out of 23 were hybrids. Thus, in both experiments the percentage of plants with a hybrid nature was high, resp. 70 and 93%. The IOA treatment was not water-tight, but did lead to an enhancement of the number of hybrids. This was indicated by the fact that in the first experiment, where no IOA treatment was applied to leek protoplasts 16 hybrids were obtained from a total of about 2000 calli (0.8%). On the other hand, in the second experiment, when the leek protoplasts were treated with IOA, 39 of the 177 calli (22%) gave rise to hybrid plants.

The two regenerants derived from the asymmetric fusions, contained exclusively leek nuclear DNA and were escapes from the IOA treatment.

Table 3. Analysis of isozymes *Got* and *Skd* in 28 plants regenerated from independent calli (data from 2 experiments).

Exp.	Fusion combination	No. of plants analysed	*Got-1*	*Got-2*	*Skd*
1	*A. ampeloprasum* (+) *A. cepa* cv. 'Hyton'	13	H	H	H
		2	L	L	L
2	*A. ampeloprasum* (+) *A. cepa* cv. 'Alamo'	11	H	H	H
		1	H	L	H
		1	L	L	L

H = Hybrid pattern, L = Leek pattern.

Isozyme analysis

Further evidence on the hybridity of the regenerated plants was obtained by isozyme analysis. In Figure 2 *Got* and *Skd* isozyme patterns of the parents and some of the hybrids are presented. Of 28 plants investigated for *Skd* isozyme pattern 25 showed all the bands from both parents. All plants that were identified as hybrids based on *Skd* patterns also showed the expected isozyme pattern of *Got* (Table 3). *Got* is a dimeric enzyme, so the hybrid plants expressed the parental bands as well as new hybrid bands (heterodimers). As expected, these extra bands were not formed when leaf extracts of both parents were mixed (data not shown). It clearly indicates that these plants were hybrids. The results for *Got* and *Skd* were in agreement with those from the PCR analysis.

In addition, the *Got* results provided some extra information about the nuclear composition of the hybrids. The *Got* system comprises two loci: *Got-1* and *Got-2* and each locus carries two alleles. For the *Got-1* locus, the leek parent displayed a three-banded phenotype, i.e. it contained two alleles coding for subunits with different electrophoretic mobilities. Between the two homodimeric bands, an intermediate heterodimeric band is formed. Theoretically three genome constitutions for the leek parent are possible, i.e. PPPp, PPpp and Pppp with,

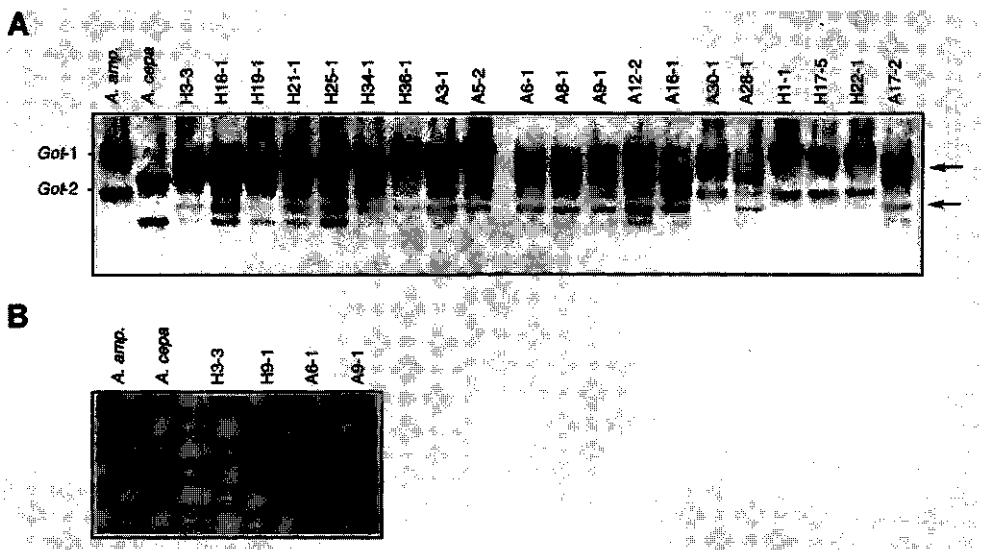

Fig. 2. *Got* (A) and *Skd* (B) isozyme patterns of *A. ampeloprasum* 3992 and *A. cepa* cv. 'Hyton', examples of some hybrids and non-hybrid regenerants (H1-1, H17-5, H22-1). Zymograms of hybrids show an extra heterodimeric band for *Got*, indicated by arrows.

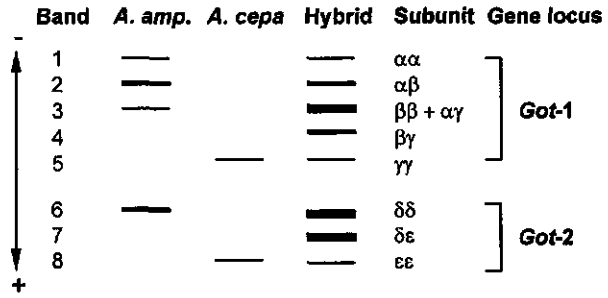

Fig. 3. Schematic illustration of Glutamate oxaloacetate transaminase zymograms of fusion parents and expected hybrid. From left to right, lane 1: *A. ampeloprasum* 3992, PPpp (*Got-1*) and PPPP (*Got-2*), lane 2: *A. cepa*, CC, lane 3: expected hybrid, PPppCC (*Got-1*) and PPPPCC (*Got-2*).

respectively, the following ratios of intensity of the three bands: 9:6:1, 1:2:1 and 1:6:9. Concerning the ratio of intensity of the bands in Figure 2A, which is more or less 1:2:1 the genome constitution is probably PPpp. The onion parent displayed only one allele, coding for one homodimeric band (CC). This means that the expected genome constitution of the hybrid is PPppCC. For the *Got-2* locus, both parents displayed one allele and the expected genome constitution of the hybrid is PPPPCC. The *Got* zymogram of the hybrid is schematically drawn in Figure 3. For a hybrid, two additional bands are expected, namely bands 4 and 7, while band 3 is more intensely stained because the heterodimeric protein αγ migrates at the same position as the homodimeric protein ββ.

From Figure 2A it can be seen that one hybrid did not show the expected zymogram. Plant A30-1 was identified as a hybrid for *Got-1*, while for *Got-2* it contained only the bands corresponding to the leek parent. Figure 2A also shows that the ratio of intensity of the bands is for all hybrids not that which would be expected (Fig. 3). For 8 out of 25 hybrids bands 4 and 5 were more intensely stained, while for *Got-2*, bands 7 and 8 in 10 out of 24 hybrids were more intense than expected.

Nuclear DNA measurements

Flow cytometric analyses were carried out to determine the amount of nuclear DNA in the hybrids. For estimation of the nuclear DNA amounts of the hybrids, the nuclear DNA value of 50.3 pg for *A. ampeloprasum* reported by Arumaganathan and Earle (1991) was taken as reference. For *A. cepa*, a DNA content of 33.2 pg DNA per nucleus was estimated on the basis of FCM measurement (Fig. 4A), which is similar to the published DNA value

Table 4. DNA content in leaf nuclei of parents, regenerants and somatic hybrids of *A. ampeloprasum* (+) *A. cepa*.

Plants	DNA content per nucleus (pg)
Standard	
A. ampeloprasum cv. 'Porino'	50.3
Parents	
A. cepa cv. 'Hyton'	33.2
A. ampeloprasum 3992[a]	36.2
No. of somatic hybrids	
1	50.3
6	62 - 64
1	66.4
8	68 - 69
6	70 - 71
4	72 - 73
No. of non-hybrid regenerants[b]	
4	36 - 40
1	65.4
1	109.6
1	123.2
1	137.2

[a] Protoplast regenerants from parental suspension line *A. ampeloprasum* 3992.
[b] Regenerants containing leek nuclear genome derived from fusion experiments.

(Arumaganathan and Earle, 1991). The DNA value of nuclei of the parental suspension line *A. ampeloprasum* 3992 was not estimated. However, flow cytometry measurements at the time of fusion showed that the suspension culture consisted of a mixture of aneuploid (i.e. hypotetraploid) and normal tetraploid cells (data not shown). Regenerants from protoplasts derived from this suspension culture also showed a lower nuclear DNA value (36.2 pg) than the standard tetraploid *A. ampeloprasum* (Fig. 4B). The amount of DNA per nucleus of the hybrids ranged from 50.3 to 73 pg (Table 4). All hybrids contained lower amount of DNA than the sum of the DNA values of the parental species (i.e. 50.3+33.2=83.5 pg) (Fig. 4C). These results suggest that the hybrids were aneuploid and were probably derived from fusions with *A. ampeloprasum* 3992 protoplasts containing less DNA than the standard *A. ampeloprasum*. The DNA values of the hybrids are close to the sum of the DNA values of *A. cepa* parent and regenerants from *A. ampeloprasum* 3992 (33.2+36.2=69.4 pg). Hybrid A30-1 showed the lowest DNA content (50.3 pg). This hybrid did also not contain the *Got-2* bands of onion. The non-hybrid regenerants derived from the fusions had a DNA content similar to the regenerants of the parental line *A. ampeloprasum* 3992, except for four non-

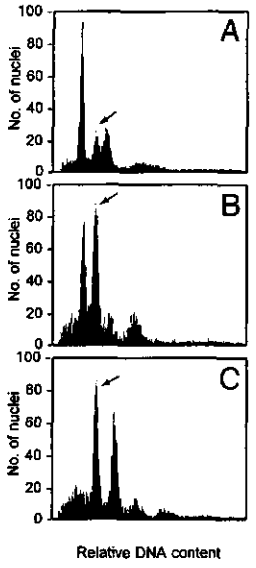

Fig. 4. Flow cytometric analysis of nuclear DNA content, (A) *A. cepa* cv. 'Hyton', (B) protoplast regenerant of parental suspension line *A. ampeloprasum* 3992, (C) Hybrid. Nuclei from leaves of *A. ampeloprasum* cv. 'Porino' were added as an internal standard (arrow).

hybrid regenerants, which had a DNA content up to 4 times higher, probably due to homofusion or polyploidization. These plants were less vigorous and had an abnormal phenotype.

Chromosome analyses

Genomic *in situ* hybridization (GISH) was carried out on six hybrids to characterize their chromosome composition (Table 5). Using total genomic DNA from *A. cepa* as probe together with an excess of unlabeled DNA from *A. ampeloprasum*, it was possible to determine the parental origin of the chromosomes. In control preparations, where metaphases of root tips of *A. cepa* and *A. ampeloprasum* were analyzed, no cross-hybridization could be detected. In the hybrids the chromosome number varied from 41 to 45, which is significantly lower than the additive chromosome numbers of the parental species (32+16=48). In five hybrids the full set of onion chromosomes was present, while the number of leek chromosomes was lower than 32. One hybrid A6-1 showed recombinant chromosomes. This hybrid possessed 30 leek chromosomes, 12 onion chromosomes and 3 recombinant chromosomes. It contained two reciprocal translocations and one interstitial translocation (Fig. 5B).

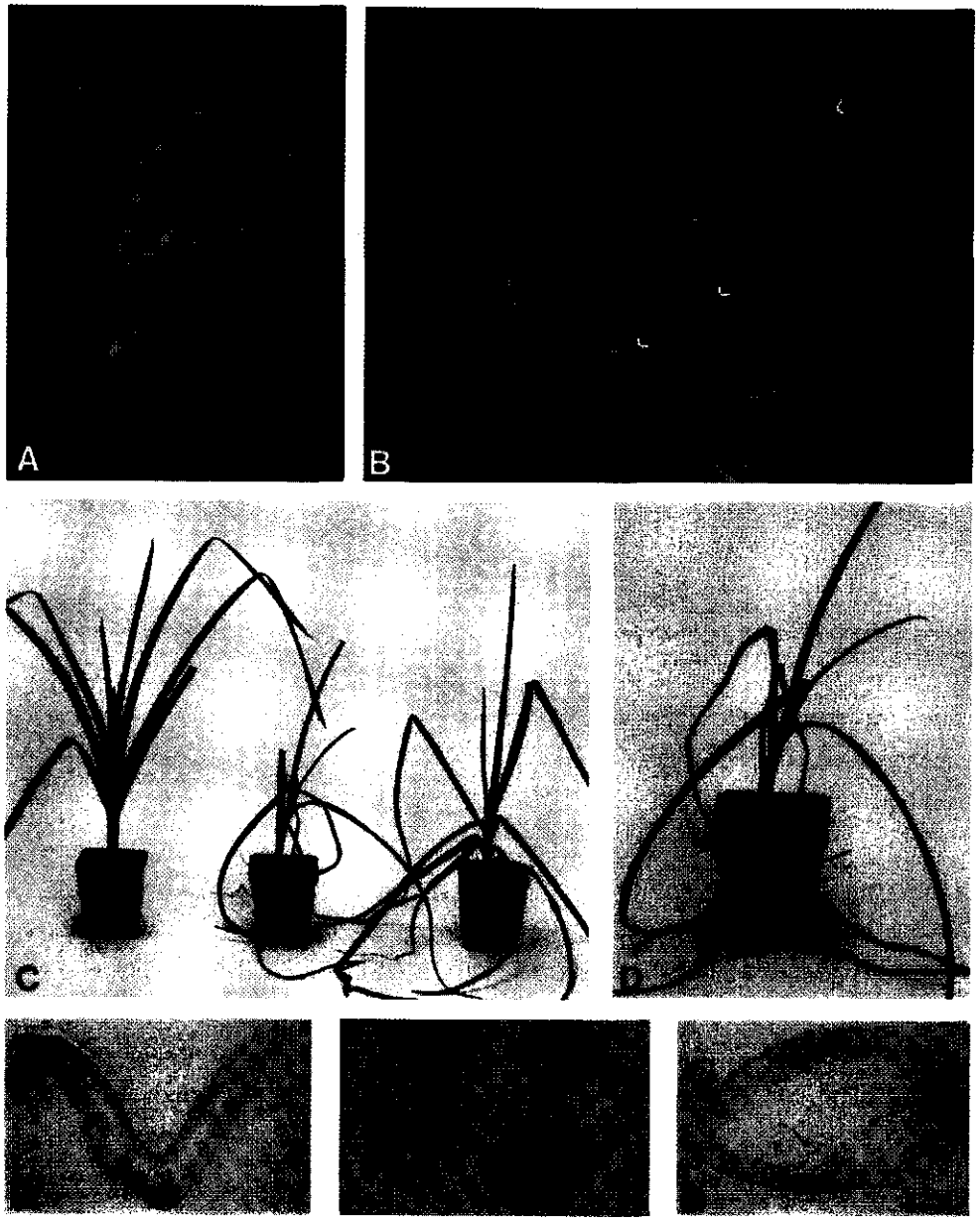

Fig. 5. Detection of leek and onion chromosomes in metaphase cells by genomic *in situ* hybridization (GISH). The onion chromosomes show the yellow fluorescence of FITC. Leek chromosomes fluoresce orange-red with propidium iodide. (A) Metaphase cell of hybrid A17-2 showing 28 leek chromosomes and 16 onion chromosomes. (B) Metaphase cell of hybrid A6-1 with 30 leek chromosomes, 12 onion chromosomes and 3 recombinant chromosomes (arrows). Plant phenotype of hybrid and parents, from left to right: *A. ampeloprasum*, Hybrid and *A. cepa* (C), Hybrid (D) Transverse section of leaves of *A. ampeloprasum* (E), Hybrid (F) and *A. cepa* (G) Bar = 1 mm.

Table 5. Chromosome composition as determined by GISH of somatic hybrids of *A. ampeloprasum* (+) *A. cepa* (cv. 'Hyton' and 'Alamo')[a]

Hybrids	No. of chromosomes	No. of *A. cepa* chromosomes	No. of *A. ampeloprasum* chromosomes	No. of recombinant chromosomes
H19-7	43	16	27	0
H25-5	41	16	25	0
A6-1	45	12	30	3
A9-1	41	16	25	0
A9-8	41	16	25	0
A17-2	44	16	28	0

[a] Chromosome number of the parental species *A. cepa* and *A. ampeloprasum* are 16 and 32 respectively, chromosome number of the parental suspension line *A. ampeloprasum* 3992 is not determined.

Morphological characterization

The somatic hybrids rooted well and were successfully transferred to the greenhouse. They showed good growth and resembled the control leek regenerants in size, although they showed a retarded growth compared to seedling-derived plants of leek and onion (Fig. 5C - D). The leaves of onion are cylindrical, while leek has a keeled leaf blade. The leaf morphology of the hybrid is intermediate between these two parents (Fig. 5E - G). Some hybrids produced a bulb. Unlike onion, these bulbs consisted of several cloves which gave rise to new sprouts.

Discussion

The results obtained in this study show that protoplast fusion makes feasible the production of somatic hybrids between *A. ampeloprasum* and *A. cepa*. To our knowledge, this is the first time that such hybrids have been produced between two sexually incongruent *Allium* species. Selection of the somatic hybrids was possible through the metabolic inactivation of leek protoplasts in combination with the inability of onion protoplasts to divide.

The overall regeneration frequency in the fusion experiments was much higher than in that of the control leek protoplasts. Non-treated leek calli regenerated with a frequency of just 2%. This was unexpected, because the regeneration capacity of the hybrids is presumed to be derived from the leek parent. This could mean that the regeneration capacity is positively affected by the fusion process. To examine this explanation the regeneration frequency should be compared with that of auto-fused leek protoplasts cocultivated with auto-fused onion

protoplasts. In our auto-fusion experiments only very few calli were obtained and no regeneration data are available. Therefore, the regeneration frequency of the non-hybrid calli (4%) was compared with that of the hybrid calli (39%) of experiment 2 (Table 2). This regeneration frequency of the non-hybrid calli is low, but conforms to the regeneration frequency of the control leek calli. Therefore, it is more likely that the higher regeneration frequency in the fusion experiments is indeed due to a higher regeneration capacity of the hybrid calli.

The loss of regeneration capacity of the leek protoplasts can be explained by the fact that these protoplasts were isolated from a suspension culture that consisted of a mixture of aneuploid and normal tetraploid cells (data not shown). Reduction in regeneration capacity in relation to changes in ploidy level (aneuploidy or polyploidy) has been observed in other callus and suspension cultures (Binarova and Dolezel 1988, Coutos-Thevenot *et al* 1990, Creemers-Molenaar *et al* 1992, Moyne *et al* 1993, Pijnacker and Ramulu 1990).

The identification of somatic hybrid plants by nuclear DNA analysis based on PCR amplification and restriction-site variation showed that most of the regenerants were hybrids. This is remarkable, because in the first experiment no selection with IOA was applied and in the other experiments the treatment was such that many leek protoplasts escaped the IOA inactivation. Therefore, the question arises as to how such a high percentage of hybrids can be obtained without applying any selection. Another interesting observation was that the regeneration capacity of the hybrid calli was much higher in comparison to that of the non-hybrid (leek) calli. It is likely that the loss of regeneration capacity in the leek protoplasts, due to aneuploidy, might be compensated by the onion genome added in the hybrid through fusion. Similar results were described by Jacobsen *et al* (1993), who obtained a high frequency of hybrids (over 70%) between *Solanum tuberosum* and *Solanum brevidens* without using any selection for isolating the hybrid calli. They observed that the somatic hybrids were obtained from relatively fast growing green calli, while the *S. brevidens* parental regenerants originated from late regenerating brown calli. In the present study the hybrid and non-hybrid calli could not be visibly distinguished.

In asymmetric fusion experiments, we have applied gamma irradiation to onion protoplasts to eliminate the chromosomes. This method has been previously used for other monocotyledon species such as rice (Akagi *et al* 1989, Kyozuka *et al* 1989, Yang *et al* 1989) and grasses (Spangenberg *et al* 1994, 1995). Unfortunately, in our research this method was unsuccessful in producing asymmetric hybrids or cybrids. Upon irradiation the frequency of

putative hybrid calli was reduced considerably, suggesting a deleterious effect on viability and cell division of the fusion products. Furthermore, no hybrid regenerants could be obtained from the irradiated series.

The results of the isozyme analysis suggest that in hybrid A30-1 the *Got-2* allele of onion is not expressed or is lost. Van der Valk *et al* (1991) reported that the two *Got* loci appeared to be unlinked. Shigyo *et al* (1994, 1995) determined chromosomal locations of the *Got* genes in shallot (*A. cepa* Aggregatum group), which is very similar to the common onion, by means of isozyme analysis of monosomic addition lines and found that *Got-2* was located on chromosome 6 and *Got-1* on chromosome 2. Thus hybrid A30-1 might have lost chromosome pair 6 or fragments there of. Some hybrids did not show the expected *Got* zymogram (Fig. 3) concerning the ratio of intensity of the bands. If it is assumed that the intensity of the bands from the hybrids bear some relation to the relative contribution made by the alleles from the two fusion partners, these results indicate that the ratio between the leek and onion alleles is shifted. It is possible that these hybrids lack some *Got* alleles of leek or that these alleles are not expressed.

By genomic *in situ* hybridization (GISH) it was possible to identify the onion and leek chromosomes in the somatic hybrids. We were also able to detect some recombinant chromosomes due to reciprocal or interstitial translocations in one hybrid. The occurrence of intergenomic translocations is a common phenomenon in somatic cells of hybrids obtained after fusion. Intergenomic translocations have been previously observed in asymmetric hybrids obtained after irradiation of the donor (Parokonny *et al* 1992, Piastuch and Bates 1990) as well as in symmetric hybrids, obtained without irradiation (White and Rees 1985, Wolters *et al* 1994) and in microprotoplast hybrids (Ramulu *et al* 1996a).

All the hybrid plants obtained in this study were hypohexaploid. The GISH results of six hybrids showed that between 41 and 45 chromosomes were present. The results also showed that one hybrid had a shortage of onion chromosomes, probably due to spontaneous chromosome elimination after fusion. Since aneuploid leek suspension protoplasts were used for fusion, it is possible that the hybrids result from fusions with leek protoplasts with different chromosome numbers. This might explain the shortage of leek chromosomes in the hybrids. The data on FCM reveal a loss of 13 - 40% of nuclear DNA in the hybrids. Though, these hybrids with severely reduced nuclear DNA could be regenerated to plants, it still remains to be seen if they are vital and fertile. However, it is possible that aneuploidy may be tolerated better by these polyploid plants.

In this study we have shown that the use of the ITS marker, which is a useful tool in taxonomic studies (Baldwin 1992), is an effective method to characterize the genomic composition of the somatic hybrids. Combined with PCR amplification, it appeared to be a quick and simple method for identifying the inter genomic hybrids in the calli at a very early stage. The rDNA is tandemly repeated at one or more sites in the genome. For *A. cepa*, the ITS regions are probably located on two to four chromosomes (Cortés and Escalza 1986, Rogers and Bendich 1987, Schubert and Wobus 1985). The use of the ITS regions is a reliable method for detection of symmetric somatic hybrids. In our case, based on results from genomic *in situ* hybridization, we found that one hybrid had lost onion chromosomes. This implies that the percentage of hybrids obtained may be underestimated. However, all plants scored as non-hybrid plants did have the leaf morphology of leek and had a DNA content similar to leek or a duplication thereof.

For successful application of these somatic hybrids for the improvement of leek, backcrosses with the leek parent will have to be performed in order to eliminate undesirable onion traits. A primary requirement for making such backcrosses is hybrid fertility which is yet to be ascertained after flowering. The hybrids will be potentially useful for the production of monosomic or disomic addition plants and recombinant lines after backcrossing, as has been obtained in the case of somatic hybrids (Jacobsen pers. comm.) or microprotoplast hybrids (Ramulu *et al* 1996b).

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Chapter 6

Biased chloroplast and mitochondrial transmission in somatic hybrids of *Allium ampeloprasum* L. and *Allium cepa* L.

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Summary

Somatic hybrid plants, produced by symmetric protoplast fusion between leek (*Allium ampeloprasum*) and cytoplasmic male sterile onion (*Allium cepa*), were analyzed for their organelle composition. Mitochondrial DNA (mtDNA) analyses were performed using PCR amplification of the V7 region of the mitochondrial small ribosomal RNA (srRNA) gene in conjunction with restriction site analysis and Southern hybridization. Of the 55 hybrids analyzed with the PCR method only three hybrids had mtDNA restriction fragment patterns of both parents, while the remaining hybrids displayed a pattern identical to *A. ampeloprasum*. Detailed analysis of 18 of these hybrids with Southern hybridization using 5 mitochondrial probes revealed that 15 hybrids possessed a rearranged mitochondrial genome of both parents, but with a predominance of mtDNA fragments of *A. ampeloprasum*. The other 3 hybrids had the same hybridization pattern as *A. ampeloprasum*, indicating that these hybrids probably contain the unaltered parental *A. ampeloprasum* mitochondrial genome. The same 18 hybrids were analyzed for their cpDNA using the *Petunia* chloroplasts DNA pPCY64 probe. A total of 15 hybrids possessed chloroplasts identical to *A. ampeloprasum* and 3 had *A. cepa* chloroplasts. Therefore, it is suggested that the chloroplasts of the hybrids did not sort out randomly. Also a novel combination of chloroplasts and mtDNA was found in two hybrids.

Introduction

Protoplast fusion provides a way to combine the cytoplasms of two genotypes. Due to the process of segregation this can result in plants with novel combinations of chloroplast, mitochondrial and nuclear genomes. The chloroplasts generally sort out to homogeneity for one of the parental types. Occasionally, hybrid plants containing chloroplasts from both parents are found (Glimelius *et al* 1981). In contrast to chloroplast DNA, the mitochondrial DNA often undergoes extensive rearrangements and recombination after fusion, leading to somatic hybrids with novel, non-parental mtDNA (Kumar and Cocking 1987). In addition, protoplast fusion has proven to be successful for the transfer of cytoplasmically inherited agronomically important traits, such as cytoplasmic male sterility (CMS), from one species to another. A large number of studies on introduction of CMS by protoplast fusion has been published. Introduction of CMS has either been done by transferring an existing sterilising cytoplasm to another line or species, i.e. in *Nicotiana* (Zelcer *et al* 1978, Belliard *et al* 1979), *Petunia* (Boeshore *et al* 1983, Izhar *et al* 1983), *Brassica* (Pelletier *et al* 1983), *Daucus* (Tanno-Suenaga *et al* 1988), *rice* (Kyozuka *et al* 1989) and chicory (Rambaud *et al* 1993). It is also possible to create a new alloplasmic CMS such as in tomato (Melchers *et al* 1992).

Leek (*A. ampeloprasum*, 2n=4x=32) has a rather narrow cytoplasmic genetic basis and there is no CMS currently available for hybrid seed production. Kik *et al* (1997) analyzed mtDNA restriction profiles of several commercial leek cultivars and could observe only three different mitochondrial genotypes among these cultivars. From a breeders point of view it would be interesting to increase the genetic diversity of the cytoplasmic genome of leek, for instance by hybridizing it with species such as onion (*A. cepa*, 2n=2x=16). In onion two different CMS cytoplasms have been found, designated as S- and T-cytoplasm (Jones and Emsweller 1936, Berninger 1965).

Recently, somatic hybrids between leek and onion have been obtained through protoplast fusion (Buiteveld *et al* 1997). The hybrids were isolated from fusion experiments of leek with the onion cultivars 'Alamo' and 'Hyton', containing the S-type and T-type CMS cytoplasm respectively. These hybrid plants, which have been characterized by isozyme analyses and genomic *in situ* hybridization, contain the chromosomes of both parents. In the present article, the chloroplast and mitochondrial DNA of these somatic hybrids was analyzed. Knowledge regarding the fate of the organelle genomes in these plants is of particular interest, in order to determine if protoplast fusion between leek and onion is a feasible tool to extend the cytoplasmic genetic diversity in leek.

Materials and methods

Plant material

The organelle analysis was performed on somatic hybrid plants previously obtained from fusions between suspension protoplasts of *A. ampeloprasum* cv. 'Porino' (3992) and mesophyll protoplasts of *A. cepa* cv. 'Hyton' and 'Alamo' (Buiteveld *et al* 1997). The onion cultivars 'Hyton' and 'Alamo' contained the T-type and S-type CMS cytoplasms respectively, leading to male sterility. In the fusion combination *A. ampeloprasum* (+) *A. cepa* cv. 'Alamo' the leek protoplasts were treated with iodoacetamide (IOA) and the hybrids, designated as 'A'-hybrids were selected on the basis of metabolic complementation. In the fusions with the cultivar 'Hyton' no selection was applied ('H'-hybrids). The hybrid plants used in this study originated from different calli and were identified by PCR and isozyme analyses.

PCR amplification and restriction site analysis

PCR amplification of a portion of the mitochondrial genome in conjunction with restriction endonuclease digestion was used to detect a polymorphism between the mitochondrial genomes of the fusion parents. Total DNA was isolated from leaves of the cultivars 'Porino', 'Alamo', 'Hyton' and from calli of line 3992 by the method of Cheung *et al* (1993). The precipitated DNA was resuspended in 50 µl TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). A 3 µl sample of a 10x-diluted DNA solution was used for PCR. Two primers '$mtV7_{p1}$' (5'-TATGAACAACAAAACCTGTCTTTAACGGGATGG-3') and '$mtV7_{p2}$' (5'-GCGGACTTG-ACGTCATCCCCCACCTTCCTCCAG-3') were used for amplification of the variable V7 region of the mitochondrial small ribosomal RNA (srRNA) gene (Neefs *et al* 1990, Russell 1994). The PCR reaction mixture contained 3 µl of template DNA, 100 ng of each primer, 0.4 mM dNTPs, 1x-Supertaq PCR buffer (HT Biotechnology) and 0.2 units Supertaq (HT Biotechnology) in a final volume of 50 µl. The conditions for the PCR reaction were 1 cycle of 94 °C, 1 min, 35 cycles of 94 °C, 1 min, 57 °C, 1 min, 72 °C, 2 min followed by a final extension step of 72 °C for 5 min. In order to reveal the polymorphism approximately 0.25 ng of the amplified DNA product was digested with *RsaI* under the conditions recommended by the manufacturer (GIBCO BRL). The DNA restriction fragments were separated on a 2% agarose gel and were photographed under UV illumination. Subsequently, the hybrids were analyzed in the same way.

Southern analysis

Mitochondrial (mtDNA) and chloroplast (cpDNA) analysis was performed by Southern hybridization on total DNA of 18 somatic hybrids. Leaves were used for DNA isolation of the hybrids and the parental onion cultivars 'Alamo' and 'Hyton'. DNA from the leek parent was isolated from leaves of the cultivar 'Porino' and from calli of suspension line 3992. Total DNA was isolated by a procedure of Koes *et al* (1986). After DNA isolation an extra purification step was carried out. The DNA solution was mixed with NaCl to a final concentration of 1 M and kept at -20 °C for 15 min. Subsequently, the solution was centrifuged at 14000 rpm in an Eppendorf centrifuge at 4 °C for 5 min. The pellet was dissolved in TE buffer, precipitated with sodium acetate and ethanol, and dissolved again in TE buffer.

For characterization of the mitochondrial DNA the following plant mitochondrial gene-specific probes were used: the 1.4 kb *Bam*HI fragment of the ATPase α-subunit gene (*Atpα*)

from *Oenothera* (Schuster and Brennicke 1986), the 2.7 kb *Hin*dIII fragment of maize containing the ATPase subunit 6 gene (*Atp6*) (Dewey *et al* 1985a), the 2.2 kb *Xba*I fragment of the maize ATPase subunit 9 gene (*Atp9*) (Dewey *et al* 1985b), the 2.4 kb *Eco*RI fragment of *Cox*II from maize (Fox and Leaver 1981) and the 1.1 kb *Eco*RI-*Pst*I fragment of *Cox*III from *Oenothera* (Hiesel *et al* 1987).The chloroplast DNA was characterized using the 2.4 kb *Bam*HI fragment of *Petunia* probe pPCY64 (de Haas *et al* 1986).

Probes were labeled with α^{32}P-dATP by random priming (Feinberg and Vogelstein 1983). A sample of 5 µg of total DNA of the hybrids and parents was digested with restriction endonucleases *Eco*RI and *Hin*dIII. The digested DNA was separated on a 0.8% (w/v) agarose gel and blotted to nylon membranes (Hybond N^+) according to the protocol as described by the supplier (Amersham). Hybridizations were performed according to the method of Church and Gilbert (1984). After hybridization the blots were washed to a stringency of 2x SSC, 0.1% SDS at 65 °C. Hybridizations were visualized by autoradiography.

Results

Analysis of mitochondrial DNA

First, a pre-screening of the mtDNA composition of 55 somatic hybrids was performed, based on PCR amplification of a part of the mitochondrial genome in conjunction with restriction endonuclease digestion. Amplification of the V7 region of the mitochondrial srRNA gene resulted in a single product of more or less the same size for both parental species (approx. 0.5 kb) when analyzed on a 2% agarose gel. After digestion with *Rsa*I it was possible to

Table 1. Analysis of the mitochondrial DNA, based on restriction-site variation in the V7 region of the mitochondrial srRNA gene of 55 independent hybrid plants obtained from 2 fusion experiments between *A. ampeloprasum* and *A. cepa*.

Fusion combination	IOA treatment[a]	No. of hybrids analyzed	MtDNA[b]		
			A	C	A+C
A. ampeloprasum (+) *A. cepa* cv. 'Hyton'	–	16	15	0	1
A. ampeloprasum (+) *A. cepa* cv. 'Alamo'	+	39	37	0	2
Total		55	52	0	3[c]

[a] Iodoacetamide treatment (4 - 7 mM) of *A. ampeloprasum* protoplasts.
[b] MtDNA restriction pattern of *A. ampeloprasum* (A), *A. cepa* (C) and of both parents (A+C).
[c] Hybrid A30-1, A28-1 and H4-3.

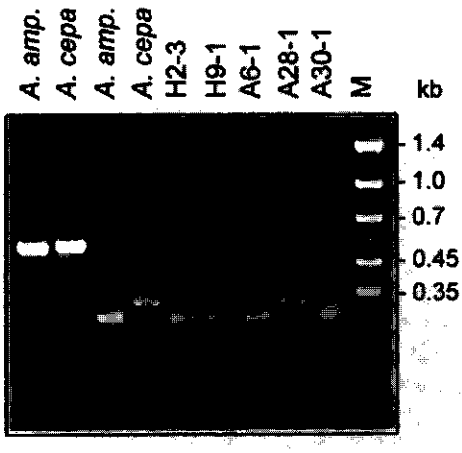

Fig. 1. Mitochondrial DNA analysis by PCR amplification of the V7 region of the srRNA gene and restriction site variation of somatic hybrids. Amplification products of *A. ampeloprasum* 3992 (lane 1) and *A. cepa* (lane 2), restriction fragment pattern of amplification product after digestion with *Rsa*I of *A. ampeloprasum* 3992 (lane 3), *A. cepa* (lane 4) and some hybrids (lane 5 - 9). M: *Taq*I-digested pEMBL18 as molecular size standard (lane 10).

distinguish between the mitochondrial genotypes of *A. ampeloprasum* and *A. cepa* based on their different restriction fragment patterns (Fig. 1). *A. ampeloprasum* showed a band of approximately 0.3 kb, which was slightly smaller than the *A. cepa* band (approx. 0.32 kb). Both species showed a second small band of approximately 0.2 kb. In this way the mtDNA of 55 somatic hybrid plants was amplified followed by *Rsa*I digestion. These results revealed that 3 hybrids (H4-3, A28-1 and A30-1) showed the restriction fragment patterns of both parents. The remaining 52 hybrids had a restriction fragment pattern identical to *A. ampeloprasum* (Table 1). In Figure 1 examples of restriction fragment patterns of the different types of hybrids detected are shown.

Subsequently, the mt genomes of 18 somatic hybrids, including the three hybrids with the additive patterns, were analyzed by Southern hybridization. For this purpose five mtDNA-specific probes (*Atpα*, *Atp6*, *Atp9*, *Cox*II and *Cox*III) and two restriction enzymes (*Eco*RI and *Hin*dIII) were used. All probe/enzyme combinations, except one (*Atp9*/*Hin*dIII), revealed a polymorphism between the mtDNAs of the parents. No differences in hybridization patterns were detected between the leek genotypes 'Porino' and 3992 and between the two onion cultivars 'Alamo' and 'Hyton'.

Table 2. Analysis of chloroplast type and mitochondrial DNA composition of 18 independent somatic hybrid plants obtained from symmetric fusions between *A. ampeloprasum* and *A. cepa*. 'H'-hybrids were derived from the fusion combination *A. ampeloprasum* (+) *A. cepa* cv. 'Hyton', 'A'-hybrids from *A. ampeloprasum* (+) *A. cepa* cv. 'Alamo'.

Plant	Chloroplast type[a]	MtDNA fragments[b]			
		A	C	R	Total
Parents					
A. ampeloprasum 3992	A	14	–	–	14
A. cepa cv. 'Hyton'	C	–	10	–	10
A. cepa cv. 'Alamo'	C	–	10	–	10
Hybrids					
H2-3	A	13	6	7	26
H4-3	A	11	8	11	30
H9-1	A	14	8	16	38
H18-5	A	14	6	1	21
H21-2	A	14	5	10	29
H22-3	A	14	4	4	22
H25-5	A	14	4	1	19
H34-3	A	11	6	7	24
H36-3	A	12	6	7	25
A3-1	A	14	0	0	14
A6-1	A	14	0	0	14
A8-1	A	14	4	5	23
A9-1	C	13	10	9	32
A15-5	C	14	2	1	17
A16-2	A	14	0	0	14
A17-2	A	14	4	4	22
A28-1	C	14	1	2	17
A30-1	A	14	10	10	34

[a] Chloroplast genome of *A. ampeloprasum* (A) or *A. cepa* (C).
[b] Number of mitochondrial DNA hybridization fragments, counted for 9 hybridization probe/restriction enzyme combinations, A, *A. ampeloprasum*-specific; C, *A. cepa*-specific; R, new hybridizing fragment.

Either parental, additive or novel hybridization patterns could be detected. In 15 out of the 18 hybrids new mtDNA hybridization patterns were found. A Southern blot with *Eco*RI-digested DNA probed with the *Atp6* gene is shown in Figure 2A. The probe hybridized to a 5 kb and an 8.3 kb fragment of *A. ampeloprasum* and to only one 3.1 kb fragment of *A. cepa*. The DNA of 12 hybrids displayed an additive pattern, with both the *A. ampeloprasum* and *A. cepa* fragments. Only the hybrid A9-1 showed a new unique 5.1 kb fragment. Figure 2B shows the hybridization patterns obtained with the *Eco*RI-digests and the *Cox*III probe. With this probe/enzyme combination 10 out of 18 hybrids had an *A. ampeloprasum* parental mtDNA pattern. With all probe/enzyme combinations, except with *Atpα/Eco*RI, new hybrid-specific fragments could be detected. The *Atpα* and *CoxII* regions showed the highest number

of new hybrid-specific fragments, while the least number of new fragments occurred in the *Atp9* region. Hybrids with common new fragments were also frequently found. For example, when the *Atpα* probe was hybridized to *Hin*dIII-digested DNA, 14 out of 18 hybrids displayed a common novel fragment of 4.1 kb (Fig. 2C). With the *Cox*II probe hybridized to *Eco*RI-digested DNA a 3.1 kb fragment was observed in 11 hybrids, while a common 10 kb fragment appeared in 9 hybrids when *Hin*dIII-digested DNA was hybridized with the *Atp6* probe (not shown). For each hybrid the total number of *A. ampeloprasum*-specific, *A. cepa*-specific and new hybridizing fragments for all probe/enzyme combinations is summarized in Table 2. It can be seen that the 15 hybrids with rearranged mtDNA possessed mitochondrial DNA fragments of both parents as well as new non-parental fragments. All hybrids retained the 14 mtDNA fragments specific for *A. ampeloprasum*, except 5 hybrids that missed 1 to 3 fragments. On the other hand most hybrids had lost many *A. cepa*-specific fragments, except for H4-3, H9-1, A9-1 and A30-1. These hybrids contained all or almost all fragments from both parents as well as a high number of new hybridizing fragments. In these hybrids the total number of fragments exceeded that of the sum of the parents. Three hybrids A3-1, A6-1 and A16-2 had exclusively mtDNA fragments specific for *A. ampeloprasum*. These hybrids had lost all *A. cepa*-specific fragments, while no new fragments were present. These results suggest that these hybrids only have the mitochondrial genome of *A. ampeloprasum*. In Table 3 an overview of the different mitochondrial DNA hybridization patterns observed in the

Table 3. Summary of the different mitochondrial DNA hybridization patterns observed in 18 *A. ampeloprasum* (+) *A. cepa* somatic hybrids specified for each hybridization probe/restriction enzyme combination.

Probe/enzyme	Mitochondrial hybridization pattern[a]							
combination	A	A+C	A+C+R	A+R	A+C*+R	A*+C+R	A*+R	A*+C*+R
*Atpα/Eco*RI	4	14	–	–	–	–	–	–
*Atpα/Hin*dIII	4	–	14	–	–	–	–	–
*Atp6/Eco*RI	5	12	1	–	–	–	–	–
*Atp6/Hin*dIII	5	2	6	2	–	1	2	–
*Atp9/Eco*RI	13	1	4	–	–	–	–	–
*Cox*II/*Eco*RI	6	–	2	5	2	–	1	2
*Cox*II/*Hin*dIII	7	–	7	4	–	–	–	–
*Cox*III/*Eco*RI	10	–	2	6	–	–	–	–
*Cox*III/*Hin*dIII	10	4	4	–	–	–	–	–
Total	64	33	40	17	2	1	3	2

[a] A and C, hybridization patterns of *A. ampeloprasum* and *A. cepa* respectively; A* and C*, incomplete hybridization patterns of *A. ampeloprasum* and *A. cepa* respectively; R, hybridization pattern with non-parental bands.

hybrids is given, indicating that the *A. ampeloprasum* hybridization pattern predominates in the hybrids. Also an additive pattern (including the complete hybridization patterns of both parents) and a novel pattern consisting of the complete patterns of both parents as well as non-parental bands were frequently observed in the hybrids. Novel patterns with some parental

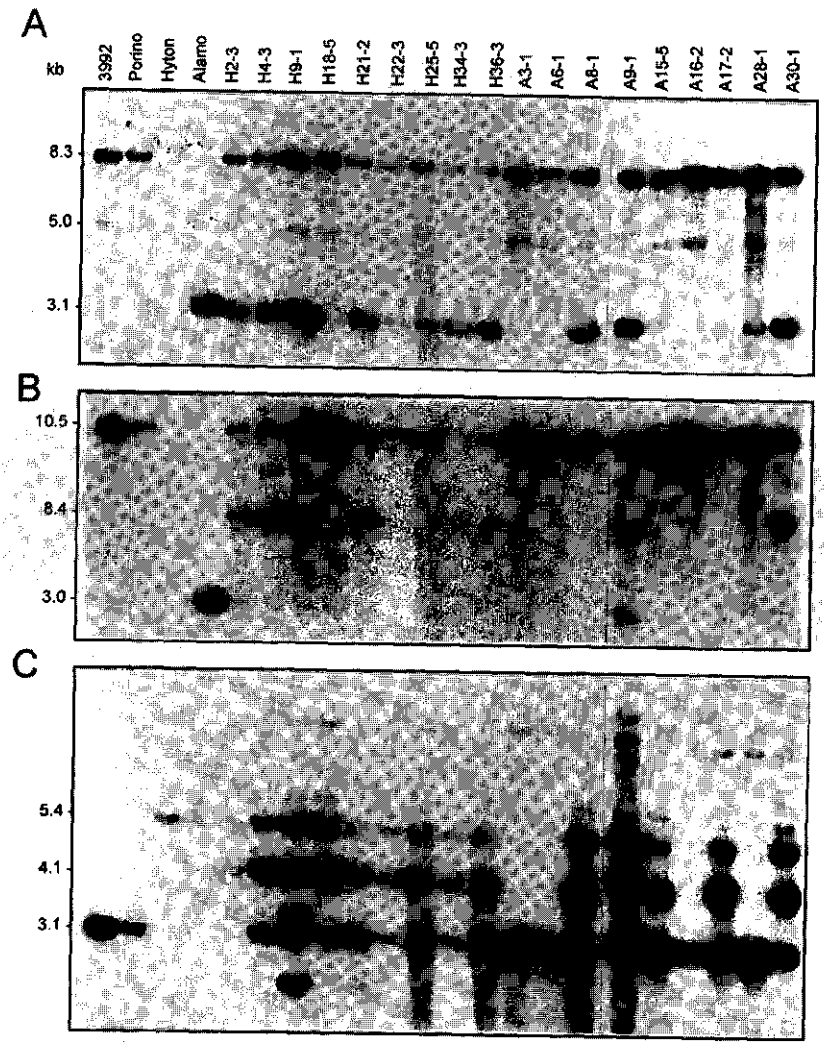

Fig. 2. Analysis of mitochondrial DNA. Autoradiographs of a Southern blot with *Eco*RI-digested total DNA, hybridized with the mtDNA probe *Atp6* (A), the mtDNA probe *Cox*III (B) and of a Southern blot with *Hin*dIII-digested total DNA hybridized with the mtDNA probe *Atpα* (C). Lane 1 - 2, *A. ampeloprasum* 3992 and 'Porino'; lanes 3 - 4, *A. cepa* cvs 'Hyton' and 'Alamo'; lane 5 - 22, somatic hybrids.

Fig. 3. Analysis of chloroplast DNA. Autoradiograph of a Southern blot with *Eco*RI-digested total DNA, hybridized with the cpDNA probe pPCY64. Lane 1 - 2, *A. ampeloprasum* 3992 and 'Porino'; lanes 3 - 4, *A. cepa* cvs 'Hyton' and 'Alamo'; lane 5 - 22, somatic hybrids.

fragments and some new fragments were less frequently found, while none of the hybrids showed an *A. cepa*-like hybridization pattern.

'A'-Hybrids were derived from fusion experiments where the *A. ampeloprasum* protoplasts were treated with IOA, while the 'H'-hybrids were obtained without selection. No differences were found between these two groups of hybrids concerning their mtDNA constitution, which indicates that the IOA treatment did not influence the mtDNA segregation.

Analysis of chloroplast DNA

For the chloroplast DNA analysis of the 18 somatic hybrid plants a Southern blot of total DNA restricted with *Eco*RI was hybridized with the pPCY64 probe. This probe hybridized to a 5.5 kb fragment of *A. ampeloprasum* and to a 4.7 kb fragment of *A. cepa* (Fig. 3). In total 15 of the 18 hybrids had the cpDNA of *A. ampeloprasum*, while the remaining 3 hybrids (A9-1, A15-5, A28-1) possessed the chloroplasts of *A. cepa* (Table 2, Fig. 3). These results indicate that sorting out of the chloroplasts was not random ($\chi_1^2 = 8$, $P < 0.05$). No mixtures of parental chloroplasts were found in the hybrids.

When the cpDNA and mtDNA compositions of the hybrids are compared, we see that in two hybrids novel combinations of organelles were found. Hybrids A28-1 and A15-5 both had *A. cepa* chloroplasts and predominantly the mitochondrial hybridization fragments of *A. ampeloprasum*.

Discussion

This research has shown that a biased segregation towards the *A. ampeloprasum* chloroplasts occurred in the somatic hybrids between *A. ampeloprasum* and *A. cepa*. Analysis of the mitochondrial DNA with Southern hybridization showed that most of the hybrids had a rearranged mitochondrial genome; only 3 of the 18 hybrids showed indications of an unaltered genome of the *A. ampeloprasum* parent with the enzymes and probes used. None of the hybrids contained an unaltered mt genome of *A. cepa*. The hybrids with the rearranged mtDNA patterns predominantly contained the mtDNA fragments of *A. ampeloprasum*. On the other hand, the high number of additive patterns found in the hybrids might indicate that not for all regions segregation was complete. To investigate whether or not the mitochondrial genomes of the hybrids have stabilized yet, the analyses should be repeated in a later stage, e.g. after seed set. Furthermore, two plants (A15-5 and A28-1) with a novel organelle combination were obtained, indicating that chloroplasts and mitochondria sorted out independently. These plants, containing the *A. cepa* chloroplasts and a prevailing mt genome of *A. ampeloprasum*, could not have been produced by sexual crossing. Both hybrids showed a normal growth. The existence of this novel organelle combination indicates that *A. cepa* chloroplasts and *A. ampeloprasum* mitochondria are compatible enough to give rise to viable plants.

Since the effect of particular factors on the organelle segregation was not studied, one can only speculate which factor is responsible for the biased segregation of *A. ampeloprasum* chloroplasts and the predominance of their mt regions in our hybrids. Cell type is a factor that has extensively been studied by others for its effect on organelle segregation. In a comparison of mesophyll and hypocotyl protoplasts in reciprocal fusions between different *Brassica* species, it was found that organelle segregation in the somatic hybrids was not influenced by the parental cell type (Landgren *et al* 1994, Sundberg *et al* 1991, Walters and Earle 1993). In fusions between mesophyll and suspension-derived protoplasts, the hybrids had predominantly the chloroplasts of the mesophyll parent (Rose *et al* 1990, Li and Sink 1992). This is in contrast with our observations that the organelles of the suspension-derived protoplasts of *A. ampeloprasum* are preferentially transmitted and not the ones of the mesophyll protoplasts of *A. cepa*. Therefore, it seems unlikely that the cell type played a major role in the organelle transmission in our hybrids.

A biased organelle segregation might also be due to differences in ploidy level between the

fusion parents. However, there is some discrepancy in the literature about the effect of parental ploidy level. Sundberg *et al* (1991) found a biased chloroplast segregation in *Brassica* species towards the type of the tetraploid species and not the diploid species. Since the number of plastids is related to the nuclear DNA content (Butterfass 1989), they suggested that the biased segregation might be due to an unequal input of organelles. Wolters *et al* (1995) found no correlation between the nuclear genome constitution, i.e. one or two potato genomes, and the segregation of cpDNA and mtDNA of somatic hybrids between *Solanum tuberosum* and *Lycopersicon esculentum*. Similarly, Hung *et al* (1993) found a random segregation, irrespective of the genome constitution and, therefore, they suggested that the initial chloroplast input does not affect chloroplast segregation. However, the results of Sundberg *et al* (1991) are in agreement with our observed preferential segregation of the organelles of the tetraploid *A. ampeloprasum* parent. In some way they have a selective advantage compared to the organelles of the diploid *A. cepa*. Our hybrids are hexaploid, because the nuclear genomes of both parents are present. This may indicate that the organelles of tetraploid *A. ampeloprasum* can cooperate better with the hexaploid nuclear genome of the hybrid. However, the fact that the organelles were derived from the same species that predominates in the nucleus the occurrence of nucleo-cytoplasmic incompatibility between *A. ampeloprasum* and *A. cepa* could also be a plausible explanation.

The possibility of using a PCR-method based on amplification of a part of the mitochondrial rDNA followed by restriction site analysis for a rapid pre-screening of the cytoplasms of the somatic hybrids was investigated. With this mitochondrial marker it was easy to discriminate between the cytoplasms of the parental species. However, the occurrence of recombination and segregation of the mitochondrial genomes after fusion complicates the analysis of the hybrids using this mitochondrial marker, because it covers only a small portion of the rDNA in the mitochondrial genome. With this marker only 3 of the 15 hybrids with an altered mt genome could be detected and thus provided insufficient information. Therefore, this method is only reliable when used together with other mitochondrial PCR markers covering the whole genome, or when used to complement the Southern blot analyses.

The hybrids analyzed in this study were derived from two different fusion combinations. In the fusion combination with *A. cepa* cv. 'Alamo' the suspension protoplasts of *A. ampeloprasum* were treated with iodoacetamide prior to fusion to inhibit cell division and select the heterokaryons ('A'-hybrids). The 'H'-hybrids were derived from the fusion with the *A. cepa* cv. 'Hyton' and were not selected by iodoacetamide. Pre-treatment with IOA is

widely used for selection of fusion products. Occasionally, an effect of IOA on elimination of the mitochondria is reported. For instance, Walters and Earle (1993) found that treating hypocotyl protoplasts of *B. oleracea* with iodoacetate reduced their mtDNA contribution to the fusion products and also Liu *et al* (1996) obtained results that suggest that the introduction of mitochondria into a new background was more efficient when treating the nuclear donor with IOA. Our results do not support these observations because there were no differences between the mtDNA composition of the 'A'- and 'H'-hybrids.

Hybrids with common non-parental fragments were found for all probes. This phenomenon, which suggests that mt recombination involves site-specific sequences, has been described for several other species combinations, i.e. within *Solanum* (Kemble *et al* 1986), *Brassica* (Temple *et al* 1992, Walters and Earle 1993, Landgren and Glimelius 1994), *Lolium* (Creemers-Molenaar *et al* 1992) and *Lycopersicon* (Derks *et al* 1991).

These results show that most of the hybrids (83%) had a rearranged mitochondrial genome. Most of the CMS hybrids or cybrids obtained by protoplast fusion contain a reorganized mitochondrial genome (Akagi *et al* 1989, Medgyesy 1990). This high percentage of hybrids with mtDNA rearrangements agrees well with other studies that have demonstrated high frequencies of recombinants (Walters and Earle 1993, Landgren and Glimelius 1994). These hybrids with recombined mitochondrial genomes between leek and onion might bring the necessary cytoplasmic diversity in leek and, therefore, represent interesting material to be incorporated in breeding programmes.

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Chapter 7

General discussion

Introduction

In this thesis the possibility of transferring cytoplasmic male sterility (CMS) to leek via protoplast fusion has been investigated. Transfer of CMS via protoplast fusion relies on two processes: a method for regeneration of protoplasts and a method for protoplast fusion, followed by regeneration of plants from fusion products.

As shown in this thesis the development of a technology for protoplast regeneration of leek was the critical aspect in recovering somatic hybrids. In this chapter some key factors that are important for the development of a regeneration system will be evaluated. Also problems encountered by establishing such a regeneration procedure and some possibilities to optimize the system will be discussed. Furthermore, the potential of protoplast fusion as a way to introduce a suitable and economical attractive hybrid seed system in leek will be evaluated and will be compared with alternative approaches to introduce male sterility in leek, such as mutation breeding, interspecific hybridization via sexual crosses and genetic transformation.

Friable embryogenic callus: a major key factor in obtaining totipotent leek protoplasts

In leek, two types of embryogenic callus could be distinguished: compact callus and friable callus (Chapter 2 and 3). The first type can be indicated as a white, compact and nodular callus with pro-embryogenic structures. The second type of callus is characterized by a soft and white friable appearance and contains numerous globular embryoids, mostly embedded in a mucilaginous substance. This friable type of callus, for the first time reported in maize in 1982 (Green), has since then been observed in many other species such as oat (Bregitzer *et al* 1989), cassava (Taylor *et al* 1996), hevea (Montoro *et al* 1993) and sorghum (Elkonin *et al* 1995). Friable embryogenic callus has two valuable attributes: it is excellent for the initiation of suspension cultures, because of its loose texture and it has a high regeneration ability for a long period.

As shown in this thesis, the use of a friable embryogenic type of callus for the establishment of fast growing, embryogenic suspension cultures was a major key factor in

obtaining regenerative protoplasts. However, in contrast to compact callus this type of callus is much more difficult to initiate. In leek, compact callus could be formed with frequencies up to 90%, whereas friable callus was only produced up to 33% (Chapter 3).

Parameters influencing friable embryogenic callus initiation

In leek immature embryos are a suitable explant source for the initiation of friable embryogenic callus. The suitability of this type of explant for the induction of friable embryogenic callus has been demonstrated in several other species such as maize (Green 1982), oat (Bregitzer *et al* 1989) and *Sorghum bicolor* (Elkonin *et al* 1995). The developmental stage of the embryo and the genotype of the donor plant were the two most crucial factors for friable embryogenic callus induction in leek (Chapter 3). Embryos of 0.5 - 2.5 mm gave the highest frequency of friable callus production. Embryos larger (older) than 2.5 mm significantly lose their potential to initiate friable callus and a switch to compact callus production was observed. This decline in friable embryogenic callus response is likely due to physiological changes within the explants during the course of embryo development. In maize, several external factors, e.g. basal medium, L-proline, sucrose concentration and $AgNO_3$ are decisive for the formation of either compact or friable callus (Vasil *et al* 1984, Armstrong and Green 1985, Songstad *et al* 1991). Probably, embryo cells change in their sensitivity for these factors during embryo development. Our results showed that compact and friable callus can develop simultaneously, which might suggest that they have different sites of origin. In maize, it is demonstrated that compact callus arises from a broad meristematic zone that appears in the scutellum, while friable callus originates from a small zone of proliferating epidermal and subepidermal cells of the scutellum (Vasil *et al* 1985, Fransz and Schel 1987, 1991).

The genotype-dependency, also observed in leek, is a general characteristic of friable embryogenic callus production. In some plant species, such genetic factors could be compensated by changing external factors. For instance, the performance of the recalcitrant B73 maize immature embryos and its derivates could be importantly improved by adding $AgNO_3$ to the culture medium (Songstad *et al* 1991).

Limitations of friable embryogenic callus induction

In general, the efficiency to produce friable embryogenic callus in leek was low. Highly responding donor plant genotypes did occur, but the performance of these genotypes varied considerably over the years, probably due to environmental or physiological factors. The use of immature embryos has also some major limitations, especially for practical approaches. Dissectioning of the embryos and screening for the right type of callus is a laborious task. Besides this, it remains difficult to determine the optimum stage of the immature embryo as there are differences in developmental stages between embryos within one flower and between flowers of one inflorescence. Leek cultivars are heterozygous and, because they are open-pollinated populations, extremely heterogeneous. Each embryo represents an individual with unknown genotype. This also means that high-responding genotypes are lost in the next generation. Another disadvantage of immature embryos is that they are not always available, since year round seed production in leek is not possible. For these reasons, other possibilities for optimization of the efficiency of friable embryogenic callus production in leek should be exploited.

For instance, friable callus can sometimes arise as spontaneously occurring sectors after prolonged subculture of compact callus (Vasil *et al* 1985, Bregitzer *et al* 1989). Moreover, a switch from compact to friable callus can be achieved by adjusting the medium composition. Friable callus has been generated from compact maize callus by reducing the sucrose concentration (Vasil *et al* 1984). In cassava, organized embryogenic structures can be converted to a prolific, friable embryogenic callus by using the basal medium of Gresshoff and Doy (1974) in the presence of picloram as an auxin (Taylor *et al* 1996). This specific basal salt/auxin type interaction probably promotes the formation of friable embryogenic callus, although how it works is not clear. Montoro *et al* (1993) could in *Hevea brasiliensis* enhance the callus friability by using 12 mM Ca^{2+} in the medium instead of usually 3 mM. An advantage of these procedures is that the friable callus production is not dependent on the primary explant type.

Another possibility to enhance the efficiency of friable embryogenic callus production is by addition of some substances to the medium which promotes this type of callus. Addition of asparagine and L-proline induces friable embryogenic callus in Sorghum *bicolor* (Elkonin *et al* 1995). The stimulatory effect of L-proline was earlier demonstrated in maize (Armstrong

and Green 1985), where it also promotes the induction of compact callus (Pareddy and Petolino 1990). $AgNO_3$ is another compound that has been shown to have a stimulatory effect on the initiation of friable embryogenic callus in maize (Vain *et al* 1989a, 1989b, Songstad *et al* 1991, 1992). The Ag^+ changes the effect of ethylene production, through competing with ethylene for its binding site (Beyer 1976). In maize tissue cultures the control of ethylene production is apparently important, but this does not necessarily holds for other species, for example in *Sorghum bicolor* the addition of $AgNO_3$ increases the compactness of the callus (Elkonin *et al* 1995).

Further, to circumvent the problem with retaining the genotype when using immature embryos, also alternative explants such as young inflorescences can be considered. For instance in maize, friable embryogenic callus formation of immature tassels (Songstad *et al* 1992) was obtained, however the frequency was lower than that of immature embryos.

The induction of friable callus cultures in leek can not yet be considered a simple method and needs further improvements. The above mentioned possibilities to optimize the production of this type of callus should therefore also be tested for leek. Out of these the effect of L-proline has been investigated for leek with no beneficial effect on the friable callus production. (Chapter 3). Also some preliminary experiments have been performed with young flower buds as an alternative explant. Results of these experiments showed that young flower buds of leek can produce friable callus, although at a lower frequency than immature embryos and, therefore, this method deserves further investigation and optimization. In addition, preliminary results showed that differences in friable callus response were obtained between seedling-derived donor plants and *in vitro*-derived donor plants and that the response was influenced by the environmental conditions under which the donor plants were grown. A better understanding of these physiological and environmental factors may also contribute to increase the efficiency and the reproducibility of the system.

Cell suspensions and protoplast regeneration

In common with other monocotyledonous species, totipotent leek protoplasts have to be isolated from highly embryogenic cell suspensions. As stated earlier, such suspension cultures

could be established only from friable embryogenic callus. These suspension cultures are difficult to establish and to maintain for long term use. The suspension cultures retain their high capacity for regeneration for about 8 months. In order to have the availability of a continuous source of regenerative protoplasts, new friable callus cultures and new suspension cultures have to be repeatedly established. Limitations of the suitability of leek suspension cultures are the loss of embryogenic capacity after prolonged subculture and the aneuploidization of suspension cells and subsequent protoplasts. In order to avoid problems associated with such suspension cultures, it would be of interest if alternative sources of totipotent protoplasts could be identified for leek.

In garlic, plant regeneration was established from protoplasts isolated from tissue-cultured shoot primordia (Ayabe *et al* 1995). Protoplast isolation directly from callus cultures, without the suspension phase, is another possibility. Plant regeneration of protoplasts from primary, immature embryo-derived callus cultures has been successful in rice (Kyozuka *et al* 1987, Wu and Zapata 1992) and barley (Kihara and Funatsuki 1995). Some preliminary experiments were performed on isolating leek protoplasts directly from friable, embryogenic callus. Although the plating efficiency of these protoplasts is still rather low, high yields of protoplasts could be obtained and, therefore, this method might be a promising alternative for using suspension cultures. Recently, regeneration of protoplasts isolated from scutellar tissue of immature zygotic embryos of rice has been reported (Biswas *et al* 1994). It is generally believed that mesophyll protoplasts of graminaceous plants are not capable of dedifferentiation and re-entry into the cell cycle. Gupta and Pattanayak (1993) proved that this view is too pessimistic and showed that mesophyll protoplasts of rice are totipotent. An important factor for their success was the use of a feeder layer of embryogenic suspension cells.

The culture and regeneration of leek protoplasts did not have stringent requirements concerning medium composition. Leek protoplasts appear to require a rather high plating density, while immobilizing the protoplasts in an alginate matrix had beneficial effects on the division rate.

In general, the regeneration capacity of the leek protoplasts reflected that of the donor material. This is in agreement with other studies on protoplast regeneration of monocotyledonous species, in which suspension cultures are used for protoplast isolation.

Protoplast fusion: fate of the extra-chromosomal DNA

In chapter 5 the production of somatic hybrids between leek and CMS onion is described using a PEG-based fusion method. This technique resulted in high numbers of somatic hybrid plants. These plants represent interesting material to be incorporated in leek breeding programmes. Provided that these hybrids show at least some degree of female fertility, they can be used as female parents in backcrosses with leek. When the hybrids obtained express the male sterility trait, it should be considered that the identification of CMS plants is complicated by other factors to which sterility also can be ascribed. Since cybridization was not successful the hybrids obtained, contain the fused nuclear background of both parental species and, therefore, these hybrids might be hindered by some nucleus-nucleus incongruity. It is generally known that interspecific somatic hybrids of symmetric combinations may show a reduced fertility (Harms 1983). Furthermore, the hybrids were aneuploid, due to the use of aneuploid suspension-derived leek protoplasts for fusion. Aneuploidy may also cause meiotic irregularities, which can lead to sterility of the hybrid.

Analysis of the somatic hybrids

From the analysis of the organelle composition of the hybrids, it was concluded that most of these hybrids possess the chloroplasts of the leek parent and have an rearranged mitochondrial genome (Chapter 6). The unidirectional segregation to one parental type of the chloroplasts and the occurrence of recombination and/or rearrangements in the mitochondrial genome in these hybrids are in agreement with other publications with respect to the fate of organelles after fusion (reviewed in Wolters *et al* 1994). In order to establish a well-functioning somatic hybrid, a strictly concerted interaction between the nuclear, chloroplast and mitochondrial genome is required. Imbalance between these three genomes may result in all kinds of problems, such as poor growth, chlorophyll deficiency and low fertility. When the nuclear DNA and the organellar composition are predominantly derived from the same parent less nuclear-cytoplasmic incompatibility is expected. Thus, ideally, hybrids with leek chloroplasts should contain the mitochondrial DNA of leek with only a minor part of the onion mitochondrial DNA containing the CMS-specific sequences. In our case, the hybrids indeed

contained predominantly the mtDNA of leek and only a small part of the onion mitochondrial genome.

Comparison of somatic hybrids with sexually obtained hybrids

Recently, interspecific hybrids between *Allium cepa* and *Allium ampeloprasum* have been generated via sexual hybridization (Peterka *et al* 1997). These hybrids contain the unaltered S-cytoplasm of the female onion parent. Although the hybrids are sterile, which is probably due to irregular meiotic chromosome pairing of the parental genomes in the triploid hybrid and thus backcrossing with leek is not easy, these results show that it is theoretically possible to transfer CMS to leek via sexual hybridization. In alloplasmic CMS plants, produced by interspecific crosses followed by recurrent backcrosses, the original cytoplasm is replaced by the cytoplasm of another species. Occasionally, the male sterility in these plants is accompanied by major deleterious phenotypic and physiological effects, such as low female fertility, delayed flowering, reduced plant vigour, reduced seed set, premature seed sprouting, parthenogenetic development, haploidy and aneuploidy. For instance in tobacco and wheat, some of these features have caused a setback in hybrid seed production (Kaul 1988, Kihara 1982). These negative side-effects are obviously related to the fact that the entire cytoplasm is transferred instead of a small part of the organelle genome. In this sense the production of CMS plants via somatic hybridization offers an advantage over sexual crossing. Via this method less of such side-effects will be expected as the largest part of the cytoplasmic genome of leek, including the whole chloroplast genome, remains unaltered.

Transfer of only a small part of the donor mitochondrial genome, instead of the whole genome has an advantage, but on the other hand the chance that the CMS trait, in which we are interested, is transferred is thereby substantially reduced. This implies that high numbers of hybrids have to be produced and screened. Yielding large numbers of hybrids necessitates a high efficiency of the method. In leek, problems concerning efficiency are more related to the regeneration part of the method, which is discussed before, than to the fusion part. Efforts to optimize the number of fusion products should therefore be directed towards the regeneration of the protoplasts. A stringent screening of high numbers of hybrids also demands an efficient selection method. In this case, the use of a diagnostic probe capable of detecting the CMS-specific sequences would facilitate the identification of plants carrying the CMS trait. Because

the CMS onion carrying the S-cytoplasm probably results from an interspecific cross (Holford *et al* 1991), it is likely that any differences between mtDNA of fertile onion and CMS lines will segregate with the CMS phenotype and this might be exploited to generate a CMS-specific probe. For instance, a possible relation between the *cob* gene and CMS in onion has been suggested. N-cytoplasm and S-cytoplasm onions appear to show differences in the transcriptional pattern of this gene, whereas the dominant nuclear restorer (*Rf*) genes were found to alter the transcriptional pattern of *cob* (Satoh and Mikami 1994). Such a gene could be used to generate a probe capable of specifically identifying the CMS-associated sequences in the hybrid plant. If such a CMS-specific probe is also used together with mtDNA-derived hybridization probes capable of detecting the absence of other onion-specific mtDNA sequences, this may importantly accelerate the identification of interesting CMS hybrids at a young non-flowering stage.

Future prospects

The quest for CMS leek plants will continue. Protoplast fusion has proven its usefulness for transferring CMS from one species to another, as shown within the genera *Brassica* and *Oryza*. Therefore, of all possible ways to introduce the CMS trait in leek, protoplast fusion remains an important option. With the appearance of somatic hybrids between leek and onion, the first phase in the development of CMS leek via protoplast fusion has attained and it is probably only a matter of time and effort, especially concerning regeneration aspects, whether or not this method will be successful in producing CMS leek plants. Protoplast fusion offers at least two possible ways of introducing CMS: transfer of an existing sterile cytoplasm and creation of a new alloplasmic type of CMS. In our research, the former has been used, namely the transfer of CMS-S and CMS-T cytoplasm. The approach of creating a new type of CMS has been demonstrated by Melchers *et al* (1992), they induced CMS as a result of fusion between the distantly related fertile species tomato and potato. This approach is also an interesting option for the distantly related species *A. ampeloprasum* and *A. cepa*, when a fertile cytoplasm of *A. cepa* is used as a donor.

Other approaches to obtain CMS in leek such as searching for spontaneous cases of male

sterility within leek cultivars or members of the *A. amp*eloprasum group (Kik *et al* 1997) and interspecific crossing (Peterka *et al* 1997) has not been thoroughly explored and deserve further attention. The approach to induce CMS in leek by mutation breeding using chemicals or somaclonal variation so far turned out to be unsuccessful. Treatment of leek seeds with the chemical N-nitroso-N-methyl urea resulted in a higher percentage of male sterile plants in the M_1 generation, but the male sterility in these plants could be explained by nuclear genes. Major difficulties in obtaining a CMS mutant are the small chance for fixation of a mutation in a mitochondrial genome population and the recovery of the cytoplasmic mutation at the plant level (Silvertand 1996). As an alternative, Silvertand (1996) suggested to combine *in vitro* culture with mutagenesis. Induction of CMS by *in vitro* culture has been reported in *Nicotiana sylvestris* (Li *et al* 1988). However, strategies for efficient *in vitro* selection of such mutants are limited.

At present, in leek nuclear male sterility (NMS) is the only possible way to produce hybrid seed. However, the fact that NMS does not permit the production of a uniformly male sterile population remains a serious drawback. For the maintenance of the male sterile plants to be used as females in the hybrid seed production of different crops several solutions have been proposed: vegetative propagation; marker assisted selection; cytogenetic methods; temporary restoration of fertility and use of functional male sterility (Lasa and Bosemark 1993). For leek, vegetative propagation of male sterile plants seems to be economically feasible and hybrid cultivars are commercially produced based on this system (Nunhems Zaden). However, it is questionable if this system will still be competitive after the earlier mentioned approaches to induce CMS or genetically engineered systems of NMS become available for leek.

Genetically engineered male sterility

A transgenic approach to induce nuclear-encoded male sterility has been investigated for leek (Wang 1996). In this system, developed by Mariani *et al* (1990), male sterility is induced by a chimeric gene composed of an anther-specific promoter (p*TA29*) and a ribonuclease gene (*barnase*). This gene encodes a cytotoxic protein that will disrupt the normal development of the tapetal cells and will prevent pollen formation. Male fertility can be restored by the chimeric ribonuclease-inhibitor gene *TA29-barstar* (Mariani *et al* 1992). This method has

been successfully used to engineer male sterility in maize and oilseed rape (Williams 1995). At present, the method has not yet been successful in recovering male sterile leek plants. Transformation of leek with the particle bombardment technique is possible. However, only co-transformation of calli with the *barnase* gene and the *barstar* gene resulted in transgenic plants. When transforming the calli with the chimeric *barnase* gene alone no plants could be obtained. It appeared that the *barnase* gene was already often expressed in leek callus tissue and detrimentally influenced the callus growth. Even if the problems of non-specific expression of the *barnase* gene can be solved it remains to be seen that stable gene integration and expression of the male sterility trait is possible in leek. Problems related to direct gene transfer and gene silencing, e.g. low integration frequencies, rearrangements, multi copy insertions may also occur in leek. Additionally, this system requires treatment with selective herbicides to eliminate the 50% male fertile/herbicide-sensitive plants.

Commercial use of such a system demands high efficiencies concerning transformation and regeneration. An important factor for transformation by particle bombardment is to target the appropriate cells that are competent for both transformation and regeneration. Penetration of the particles seems to be restricted to the first few cell layers. In this respect the use of friable embryogenic callus as a target for transformation might be interesting. In other species, like cassava, it has been demonstrated that the newly formed embryos are located at the surface of the embryogenic units, which make them better accessible for transformation than the ones in compact callus (Raemakers *et al* 1997). Besides this, friable callus is a highly regenerable target tissue.

In the immediate future, an attractive approach to produce hybrid leek cultivars might be transformation of mitochondria in order to introduce MS or CMS-associated genes. Genes which can be considered to be used for transformation are the tapetal-specific expressed RNase gene (*TA29-barnase*) (Mariani *et al* 1990) or the mitochondrial T-*urf13* gene from CMS-T maize (Dewey *et al* 1987). Recently, the expression of T-*urf13* in other plant species has been investigated. Introduction of the T-*urf13* gene in the nuclear genome of tobacco showed that URF13 expression confers toxin and methomyl sensitivity to transgenic tobacco. However, a correlation with male sterility has not yet been proven (Von Allmen *et al* 1991, Chaumont *et al* 1995).

Transformation of yeast and algal mitochondria (Butow and Fox 1990, Randolph-

Anderson *et al* 1993) has been described, although transformation of the higher plant mitochondrial genome is not yet feasible. In this respect chloroplast transformation is an alternative possibility, which already has become an important tool in higher plant research. There are several significant advantages of transforming the chloroplast genome instead of the nuclear genome. Of course, organelle transformation ensures the maternal inheritance of the introduced gene. This also implies that transfer of the transgene to other species by cross-pollination is prevented. The high copy number, up to 50000 (Bendich 1987), of the plastid genome per cell bears the potential of achieving high levels of transgene expression leading to high concentrations of gene products (Bride *et al* 1995). Further, in case of plastid transformation genes are precisely targeted to specific sites, since integration of foreign sequences occurs via homologous recombination. Targeted integration via homologous recombination potentially avoids uncontrolled 'position effects' and thus limits the variation in transgene expression (Maliga 1993).

Chloroplast transformation is so far mainly described for the genus *Nicotiana*, but will probably soon also be exploited more frequently for other plant species. Two methods to obtain stable plastid transformants of tobacco are available, particle bombardment (Staub and Maliga 1992, Svab and Maliga 1993) and PEG treatment (Golds *et al* 1993, Koop *et al* 1996). For leek, both methods may provide interesting strategies for transforming plastids. In case of particle bombardment, friable embryogenic callus is a good target, whereas in case of the latter method protoplasts can be used. Efficient regeneration of both target tissues has been described in this thesis.

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Summary

The vast majority of the present day leek cultivars is of poor quality. The genetic constitution of leek makes it a difficult crop to breed and consequently mass, or family selection methods, both of which have a low efficiency, are mainly used. F_1 hybrid breeding seems the appropriate strategy for improvement of leek. With such a breeding system a higher uniformity of the crop, better fixation of desirable traits, such as pest and disease resistance and a better exploitation of heterosis effects can be achieved. Large-scale seed production of hybrid cultivars requires a hybridization system based on cytoplasmic male sterility (CMS), because of the considerable advantage of this system in maintaining the male sterile parent line. Since no source of CMS has yet been found in leek or related forms of *A. ampeloprasum*, several researchers are focused on introducing cytoplasmic male sterility into leek. In the present thesis the possibility of introducing CMS into leek via somatic hybridization with *A. cepa* as CMS donor was investigated. Successful application of such a technique requires an efficient system for regeneration of plants from protoplasts. At the beginning of the research project in 1990 such a protoplast-to-plant system for leek was not available. Therefore, substantial effort was directed towards the development of efficient plant regeneration methods from embryogenic callus cultures, suspension cultures and protoplasts.

Initially, much attention was focused on development of regenerative callus cultures from different explants and cultivars. The highest compact callus response was obtained when mature, zygotic embryos were cultured on MS medium, containing 30 g/l sucrose and 1 mg/l 2,4-D. Significant differences were found between the cultivars and accessions for shoot formation frequency. In addition, a genotype-dependent response of leek embryo explants for the formation of shoots was evident.

In contrast to plant regeneration from compact embryogenic callus of leek, which is efficient and easy to achieve, the establishment of suspension cultures from this type of callus was less successful. Although compact callus cultures in liquid medium retain their ability to form somatic embryos and shoots for a long period, they do not become a finely-dispersed suspension culture. For this purpose, a new friable embryogenic type of callus was induced on immature embryos instead of mature embryos. This friable callus comprised numerous

globular embryoids, embedded in mucilage and is highly regenerative when plated on a cytokinin containing medium. It was found that the developmental stage of the immature embryo and the genotype of the donor plant significantly influenced the callus response. Characterization of the two callus types by a histological examination revealed striking differences and supported the suggestion that friable callus is more suitable for initiating suspension cultures than compact callus. A stringent selection within these friable callus cultures was necessary to obtain highly-embryogenic suspension cultures.

A procedure was described for the isolation, culture and regeneration of plants from protoplasts derived from these embryogenic suspension cells. Imbedding in alginate was an important factor in increasing the plating efficiency. The regeneration frequency of the protoplast-derived calli was primarily affected by the type of callus that developed. The protoplast-to-plant system described was reproducible for at least three genotypes. Plants were regenerated within 6 months after protoplast isolation.

Utilising this regeneration procedure for protoplasts, a method for symmetric hybridization between leek and onion as a CMS donor was set up. The fusion experiments yielded large numbers of hybrid calli and plants. The aneuploid status of the hybrid plants could be explained by the use of leek protoplasts, derived from an aneuploid suspension culture. This also implied that using cell suspensions as a protoplast source for fusion remains a restrictive factor in the establishment of a successful hybridization system for leek. The leaf morphology of the hybrids was intermediate between the two parents. It appeared that most of the hybrids possessed leek chloroplasts and a rearranged mitochondrial genome of both parents, but with a predominance of mtDNA fragments from leek.

With the methods described in this thesis the first steps to transfer CMS to leek have been realized. The results described here show that a cytoplasm of onion can be successfully transferred to leek via somatic hybridization. Future research should focus on further improvement of the system. For this, optimization of some aspects of the regeneration process and developing an efficient selection system for the desired hybrids, containing the onion specific CMS mtDNA sequences should be accomplished. The research has shown that somatic hybridization has a high potential to obtain CMS leek plants.

Samenvatting

Prei is een belangrijk vollegrondsgroentegewas in Nederland en de ons omringende landen. De kwaliteit van de thans beschikbare preirassen kan nog veel verbeterd worden. Het zijn rassen die niet erg uniform zijn en waar nog nauwelijks resistenties zijn ingebracht. Een combinatie van eigenschappen van prei: het is een tetraploid, kruisbevruchtend en tweejarig gewas, maakt dat de huidige veredelingsmethoden van massa- en/of familieselectie weinig efficiënt zijn. Een geringe mate van uniformiteit is nadelig voor zowel de veredelaar als de teler. Een geringe uniformiteit kan voor de veredelaar problemen veroorzaken bij de rassenregistratie. Tijdens de teelt betekent een geringe uniformiteit extra arbeid, aangezien het mechanisatie bemoeilijkt. Hybriderassen zijn dan ook zeer gewenst. Met een hybride veredelingssysteem kan het probleem van een geringe uniformiteit opgelost worden, is een betere benutting van het heterotisch potentieel mogelijk en kunnen belangrijke eigenschappen, zoals ziekteresistenties sneller worden ingekruist. Voor prei heeft een hybridisatiesysteem op basis van cytoplasmatische mannelijke steriliteit (CMS) de voorkeur, gezien de aanzienlijke voordelen van dit systeem bij de instandhouding van de mannelijk steriele moederlijnen. In prei zijn echter geen CMS bronnen bekend en introductie van een stabiele CMS bron heeft dan ook de aandacht van onderzoekers.

In dit proefschrift wordt een onderzoek beschreven naar de mogelijkheden om via interspecifieke somatische hybridisatie CMS preiplanten te verkrijgen. Een essentiële voorwaarde voor succesvolle toepassing van protoplastenfusie is de beschikbaarheid van een reproduceerbaar systeem voor regeneratie van planten uit protoplasten. Bij de start van het onderzoek in 1990 was een dergelijk protoplast-tot-plant systeem niet voorhanden bij prei. Het onderzoek heeft zich dan ook voor een belangrijk deel geconcentreerd op het ontwikkelen van methoden voor regeneratie vanuit calluscultures, suspensiecultures en protoplasten.

In eerste instantie werd bepaald welke explantaattypen en rassen het meest geschikt zijn voor het initiëren van embryogene calluscultures. Rijpe, zygotische embryo's bleken een betere callusinductierespons te geven dan bladexplantaten. De hoogste frequentie embryogeen callus (tot 90%) werd gevonden wanneer de rijpe embryo's werden uitgelegd op een 1 mg/l 2,4-D en 30 g/l sucrose bevattend MS medium. Het callus dat gevormd werd kan het beste

omschreven worden als een compact, nodulair type met pro-embryogene structuren. Gebruikmakend van rijpe embryo's als explantaat werden in totaal 12 rassen en accessies vergeleken voor callusinductie en regeneratie. Er waren duidelijke verschillen voor wat betreft het percentage embryo's dat compact callus produceert en het percentage regeneratief callus. De cultivars en accessies konden op grond van regeneratiecapaciteit in 3 groepen worden ingedeeld. In de groep met de hoogste regeneratiefrequentie werden gemiddeld 6,7 scheuten per callus gevormd. De accessies waren het minst regeneratief. Tevens was de variatie in scheutregeneratie groot binnen de rassen. Het genotype speelt dus een belangrijke rol bij het initiëren van regeneratieve calluscultures bij prei.

In tegenstelling tot de hoge mate van efficiëntie waarmee uit compact callus planten geregenereerd kunnen worden, was de initiatie van suspensiecultures uit dit callus minder succesvol. Het compacte callus is in een vloeibare cultuur in staat tot de vorming van somatische embryo's en scheuten gedurende een lange periode, het valt echter niet gemakkelijk in kleine celaggregaten uiteen. Om deze reden werd getracht op onrijpe embryo's een 'friable' type callus te induceren, dat wel tot een fijn-verdeelde celsuspensie leidt. Dit 'friable' callus bestaat uit meerdere globulaire embryoiden, ingebed in een mucose substantie. Het ontwikkelingsstadium van de onrijpe embryo's en het genotype van de donorplant bleken belangrijke factoren voor inductie van dit callus. Onrijpe embryo's van 0,5 - 2,5 mm gaven de hoogste frequentie van dit callus. De productie van 'friable' callus werd niet beïnvloed door het basale medium. De regeneratiecapaciteit van dit callus was zeer hoog. Afhankelijk van het genotype konden tot 1600 scheuten per gram callusmateriaal worden geregenereerd. Het feit dat 'friable' callus geschikt voor het initiëren van een suspensiecultuur blijkt te zijn werd ondersteund door microscopische waarnemingen. Het 'friable' callus is opgebouwd uit aggregaten van embryogene cellen en somatische pro-embryo's, waarbij het 'friable' karakter wordt ontleend aan de intercellulaire holtes. In dit callus wordt geen vaatweefsel waargenomen. Compact callus is verder georganiseerd: het bestaat uit een meristematische zone en bezit vaatelementen. Intercellulaire holtes ontbreken in dit callus. Tien onafhankelijk geïnduceerde calluscultures werden getest op hun geschiktheid voor het initiëren van suspensiecultures. Na strenge selectie op snelle groei en vorming van kleine groepjes cytoplasmarijke cellen konden twee goede suspensiecultures verkregen worden.

Gebruikmakend van deze embryogene suspensiecultures, afkomstig van 'friable' callus is

een procedure ontwikkeld voor isolatie, cultuur en regeneratie van protoplasten. Inbedding van de protoplasten in alginaat bleek een belangrijke factor te zijn om de 'plating efficiency' te verhogen. Verder lieten de resultaten zien dat de geteste regeneratiemedia en lichtintensiteiten gedurende de regeneratiefase geen invloed hebben op de regeneratiefrequentie van de protoplastencalli, maar dat deze primair afhangt van het type callus dat gevormd wordt. Regeneratie van calli verliep in principe via somatische embryogenese. Wanneer niet-embryogene calli gevormd werden, verliep dit proces via scheutvorming. De hele procedure van protoplast tot plant duurde ongeveer 6 maanden. Alle regeneranten waren normaal tetraploid.

Nadat een regeneratieprocedure ontwikkeld was, werden fusies uitgevoerd tussen suspensieprotoplasten van prei en bladprotoplasten van CMS-lijnen van ui. Naast symmetrische werden ook asymmetrische fusies uitgevoerd, waarbij getracht werd door bestraling het kerngenoom van ui te elimineren. Bestraling van protoplasten van ui had een drastisch negatief effect op de ontwikkeling van calli uit gefuseerde protoplasten, zodat de asymmetrische fusies geen hybriden opleverden. Uit de symmetrische fusies werden grote aantallen calli en regeneranten verkregen. Het hybride karakter van de regeneranten werd vastgesteld door middel van PCR- en isozymanalyse. Hieruit bleek dat een zeer hoog percentage van de regeneranten hybriden waren. Het DNA gehalte van de hybriden bleek lager te zijn dan de som van de DNA gehaltes van de oudersoorten. De oorzaak van het aneuploid zijn van de hybriden kan door het gebruik van preiprotoplasten, die geïsoleerd zijn uit een aneuploide suspensiecultuur het meest eenvoudig verklaard worden. Aan de hand van mitotische chromosoompreparaten, die differentieel door *in situ* hybridisatie gelabeld werden, kon tussen de prei en ui chromosomen eenvoudig een onderscheid worden gemaakt. In de meeste gevallen waren alle 16 ui chromosomen aanwezig, maar was er een tekort van 2 tot 7 prei chromosomen in de hybriden. Qua bladmorfologie verschilden de planten duidelijk van de ouderplanten. Het blad van de meeste hybriden heeft een vorm die intermediair is tussen dat van ui (cylindrisch) en prei (kielvormig). Van een aantal hybriden werden zowel de chloroplasten als de mitochondriën geanalyseerd. In de meeste gevallen waren in de hybriden de preichloroplasten aanwezig, hetgeen op een niet-random sortering van de chloroplasten duidt. De hybriden bleken uitsluitend een prei mitochondriëel genoom of een gecombineerd mt-genoom van beide ouders te bevatten.

Met het beschikbaar komen van procedures voor regeneratie en fusie zijn de eerste stappen, die essentieel zijn voor de overdracht van CMS via somatische hybridisatie, gerealiseerd. 'Friable' callus bleek een belangrijke sleutelfactor voor het verwezenlijken van dit systeem te zijn. Dit callus type biedt eveneens goede perspectieven om via andere biotechnologische strategieën tot CMS preiplanten te komen. Op dit moment worden somatische hybriden, die voldoende vrouwelijke fertiliteit en die de gewenste CMS-ui sequenties bezitten, geselecteerd en in veredelingsprogramma's opgenomen.

Curriculum vitae

Joukje Buiteveld werd geboren op 6 augustus 1964 te Drachten. In 1983 behaalde zij haar diploma Atheneum-B aan de Rijksscholengemeenschap 'Ooststellingwerf' te Oosterwolde. In datzelfde jaar begon zij een studie plantenveredeling aan de Landbouwuniversiteit te Wageningen. Haar afstudeervakken waren plantenveredeling, fytopathologie, weefselkweek en somatische celgenetica. Tijdens de studie werd stage gelopen bij het Department of Scientific and Industrial Research (DSIR) te Auckland, Nieuw Zeeland. In juni 1989 behaalde zij haar ingenieursdiploma.

Van februari 1990 tot april 1996 was zij werkzaam bij het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO) te Wageningen. Gedurende deze periode werkte zij aan achtereenvolgens de projecten 'Ontwikkeling van regeneratiemethoden bij prei' en 'Ontwikkeling van CMSpreiplanten via protoplastenfusie tussen prei en ui'. De resultaten hiervan staan beschreven in dit proefschrift.

Momenteel is zij werkzaam bij het DLO-Instituut voor Bos- en Natuuronderzoek (IBN-DLO) te Wageningen.