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

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Abstract Results are reported on the production and characterization of somatic hybrids between *Allium ampeloprasum* and *A. 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 a lower DNA content than the sum of the DNA amounts of the parental species, suggesting that they were aneuploid. A 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.

Key words Interspecific somatic hybridization · Leek · Onion · *Allium ampeloprasum* · *Allium cepa* · Genomic in situ hybridization

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Introduction

Leek (*Allium 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 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*. While successful crosses between species within the *A. ampeloprasum* complex have been achieved by Kik et al. (1997), 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 respect, somatic hybridization may be a viable alternative that will enable breeders 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 dicotyledon species. This is due to recalcitrance of the protoplasts to regeneration. Until now, successful regeneration of somatic hybrid and cybrid plants has been restricted to members of the *Gramineae*. In rice, several authors, have reported on the production of somatic cybrids and hybrids and the successful transfer of mitochondrial genomes from cytoplasmic male-sterile (CMS) lines (Akagi et al. 1989, 1995; Hayashi et al. 1988; Kyozuka et al. 1989; Terada et al. 1987; Yang et al. 1989). Asymmetric and symmetric hybrid plants were also obtained in *Festuca* and *Lolium* 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 a regeneration procedure for leek was first 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 (IOA)-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 the polymerase chain reaction (PCR) and restriction-site variation of rDNA to detect the nuclear hybrids. In addition, the nuclear composition of the hybrid plants was analysed by flow cytometry and in situ hybridization.

Materials and methods

Plant material and culture conditions

Tetraploid (2n = 4x = 32) leek (*Allium 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* cvs '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 half-strength 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, Tokyo, Japan) and 0.05% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan)). The cell walls were digested for 16 h on a rotary shaker at 40 rpm. To identify the fusion products, we stained the leek protoplasts 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 m*M* IOA) and incubated at 4°C for 15 min. After IOA treatment, leek protoplasts were washed twice by centrifugation and 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 m*M* 2-morpholinoethansulfonic acid (MES) and 3 m*M* 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, 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 *g*). Subsequently, they were resuspended in CPW salts, 15% (w/v) sucrose and 3 m*M* MES (pH 5.7) with an upper layer of 1 ml wash medium (CPW salts, 9% mannitol and 3 m*M* ascorbic acid, pH 5.7) and centrifuged for 10 min at 700 rpm (40 *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) $CaCl_2 \cdot 2H_2O$] was added dropwise without mixing to 2×10^6 protoplasts resuspended in 0.3 ml wash medium. The protoplasts were incubated for 30 min at room temperature. After incubation, 0.8 ml of a 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 in steps of 100 mOsm/kg (by reducing the glucose concentration) to 450 mOsm/kg. After 4–6 weeks, when microcalli had formed, the alginate plates were transferred to solid KM medium containing 3% (w/v) glucose, 1 mg/l 2,4-dichlorophenoxyacetic acid (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

We used the variability in the internal transcribed spacer (ITS) region of rDNA to distinguish between the nuclear genomes of the two fusion parents. 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 m*M* TRIS-HCl, 1 m*M* EDTA (pH 8.0)]. For PCR, 3 μl of a 10 × -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, 1 × Supertaq PCR buffer (HT Biotechnology) and 0.2 U Supertaq (HT Biotechnology). Amplification was carried out under the following conditions: 1 cycle of 94°C, 1 min, 35

cycles of 94°C, for 1 min; 50°C, for 1 min, 72°C, for 2 min, a final single, 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 2 m*M* 8-hydroxyquinoline for 5 h 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, Tokyo, Japan), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) and 0.1% (w/v) Cytohelicase (Sigma) in a 10 m*M* 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 × 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

Two different *A. cepa* cultivars were used in the symmetric protoplast fusions. One fusion experiment was performed with the combination *A. ampeloprasum* (+) *A. cepa* cv 'Hyton' and five experiments with the combination *A. ampeloprasum* (+) *A. cepa* cv 'Alamo' (Table 1). It was possible to identify the heterokaryons on the 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

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 gamma irradiation (Gy)	IOA treatment[a]	Total no. of protoplasts[b]	Number of calli obtained[c]	Number of calli transferred to regeneration	Number of calli regenerated to plants	Total no. of plants
A. amp. (+) *A. cepa* cv Hyton	0	−	1.2×10^7	> 2000	2000	23	127
A. amp. (+) *A. cepa* cv Alamo	0	+	5.9×10^6	1224	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 m*M* 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

was completely inhibited by a treatment of 4 m*M* 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 m*M* IOA and that higher concentrations may be needed to completely inhibit cell division. Therefore, fusions were conducted with 4–7 m*M* 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 three 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 viability, except that the viability of the 300 Gy-treated protoplasts declined to 50% of that of 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 2 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 fragment with *Dde*I, *A. ampeloprasum* and *A. cepa* were distinguishable by their different restriction fragment patterns (Fig. 1). *A. ampeloprasum* showed one band, which was 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.

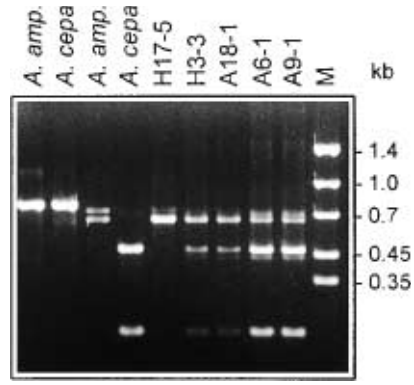


Fig. 1 Nuclear DNA analysis by PCR amplification and restriction site variation of putative hybrids. PCR product of ITS region of nuclear ribosomal DNA of parental line *A. ampeloprasum* 3992 (*lane 1*) and *A. cepa* cv 'Hyton' (*lane 2*). Restriction fragment pattern of PCR product after digestion with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

To determine the frequency of hybrids that was obtained, we analysed calli and plants from two experiments 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

Table 2 Nuclear DNA composition determined by PCR analysis of calli and regenerants from two different fusion experiments between *A. ampeloprasum* and *A. cepa* (*n.d.* not determined)

Experiment no.	Fusion combination	IOA treatment	Number of calli transferred to regeneration medium[a]	Number of hybrid calli (%)	Number of calli regenerated to plants		Total no. of calli regenerated to plants (%)	Percentage of hybrid plants
					Hybrid	Leek		
1	*A. amp.* (+) *A. cepa* cv Hyton	−	2000	n.d.	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

were hybrids. Thus, in both experiments the percentage of plants with a hybrid nature was high, 70% and 93%, respectively. 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 the GOT and SKD isozymes in 28 plants regenerated from independent calli (data from two experiments)

Exp. no.	Fusion combination	Number of plants analysed	*Got-1*	*Got-2*	*Skd*
1	*A. amp.* (+) *A. cepa* cv Hyton	13	H[a]	H	H
		2	L	L	L
2	*A. amp.* (+) *A. cepa* cv Alamo	11	H	H	H
		1	H	L	H
		1	L	L	L

[a] H, Hybrid pattern; L, leek pattern

Isozyme analysis

Further evidence on the hybridity of the regenerated plants was obtained by isozyme analysis. In Fig. 2 GOT and SKD isozyme patterns of the parents and some of the hybrids are presented. Of the 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, 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 Fig. 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

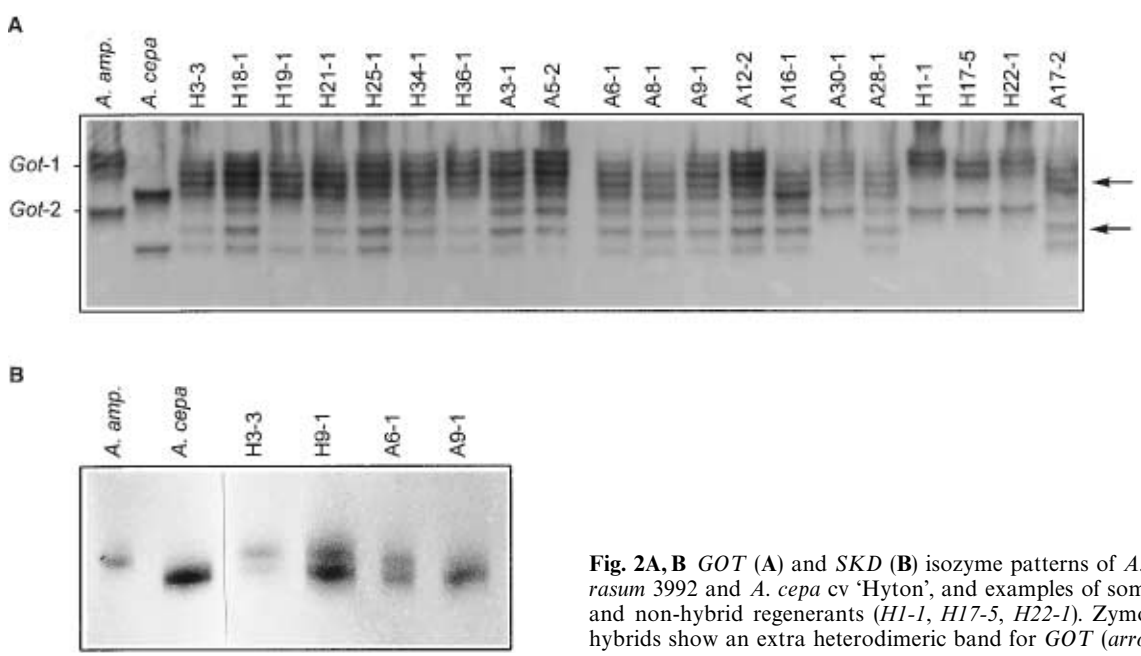


Fig. 2A, B *GOT* (**A**) and *SKD* (**B**) isozyme patterns of *A. ampeloprasum* 3992 and *A. cepa* cv 'Hyton', and examples of some hybrids and non-hybrid regenerants (*H1-1*, *H17-5*, *H22-1*). Zymograms of hybrids show an extra heterodimeric band for *GOT* (*arrows*)

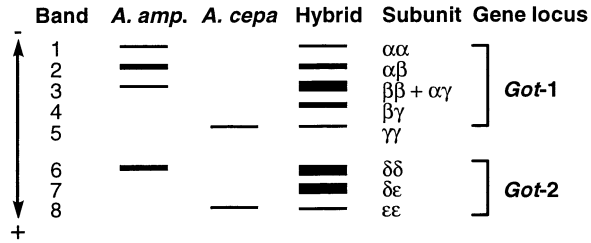


Fig. 3 Schematic illustration of glutamate oxaloacetate transaminase zymograms of fusion parents and expected hybrid. From *left* to *right*, *lane 1 A. ampeloprasum* 3992, PPpp (*Got-1*) and PPPP (*Got-2*), *lane 2 A. cepa*, CC, *lane 3* expected hybrid, PPppCC (*Got-1*) and PPPPCC (*Got-2*)

Fig. 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 $\alpha\gamma$ migrates at the same position as the homodimeric protein $\beta\beta$.

From Fig. 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 (FCM) 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 (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 of the hybrids contained a 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 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

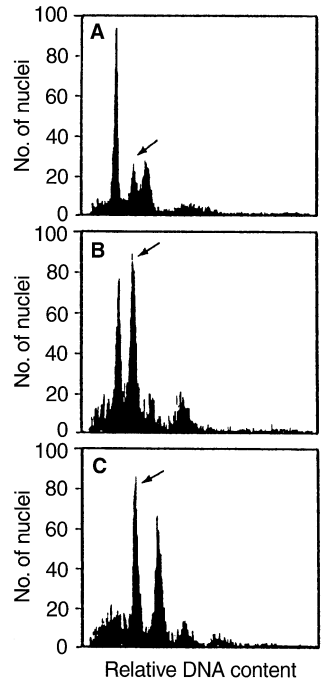


Fig. 4A–C Flow cytometric analysis of nuclear DNA content. **A** *A. cepa* cv 'Hyton', **B** protoplast regenerant of parental suspension line *A. ampeloprasum* 3992, **C** hybrid. Nuclei from leaves of *A. ampeloprasum* cv 'Porino' were added as an internal standard (*arrow*)

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	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

sum of the DNA values of the *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 also did not contain the *Got-2* bands of onion. The non-hybrid regenerants derived from the fusions had a DNA content similar to that of the regenerants of the parental line *A. ampeloprasum* 3992, except for 4 non-hybrid regenerants, which had a DNA content up to four 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 6 hybrids to characterize their chromosome composition (Table 5). Using total genomic DNA from *A. cepa* as a probe together with an excess of unlabelled DNA from *A. ampeloprasum*, we were able to determine the parental origin of the chromosomes. In control preparations, where metaphases of root tips of *A. cepa* and *A. ampeloprasum* were analysed, 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 5 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).

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.

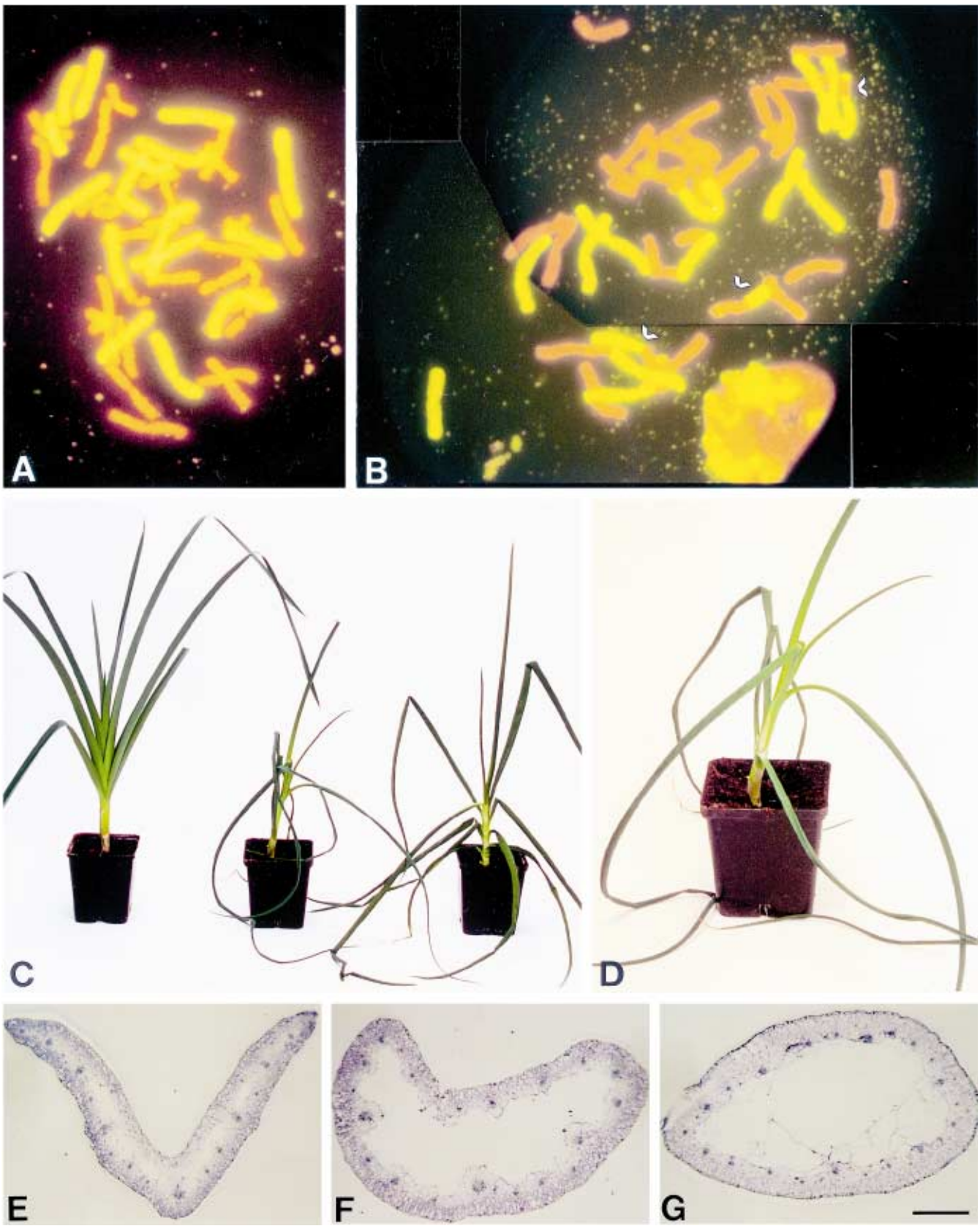
The overall regeneration frequency in the fusion experiments was much higher than in that of the control leek protoplasts. Non-treated leek calli regenerated with a frequency of just 2%. This was unexpected, because the regeneration capacity of the hybrids is presumed to be derived from the leek parent. This could mean that the regeneration capacity is affected by the fusion process. To examine this explanation we should have compared the regeneration frequency with that of auto-fused leek protoplasts cocultivated with auto-fused onion protoplasts. In our auto-fusion experiments, however, only very few calli were obtained and no regeneration data is 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

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

Hybrids	Number of chromosomes	Number of *A. cepa* chromosomes	Number of *A. ampeloprasum* chromosomes	Number 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; the chromosome number of the parental suspension line *A. ampeloprasum* 3992 was not determined


A
B
C
D
E
F
G

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 *S. 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 the asymmetric fusion experiments we 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.

Fig. 5A–G 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*). **C** Plant phenotype of hybrid and parents, from *left* to *right*: *A. ampeloprasum*, hybrid and *A. cepa*, **D** hybrid. **E–G** Transverse section of leaves of *A. ampeloprasum* (**E**), hybrid (**F**), and *A. cepa* (**G**). Bar = 1 mm

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 1 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 6 hybrids showed that between 41 and 45 chromosomes were present. The results also showed that 1 hybrid had a shortage of onion chromosomes, probably due to spontaneous chromosome elimination after fusion. Since aneuploid leek suspension protoplasts are 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% 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 intergenomic 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 2–4 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 1 hybrid had lost onion chromosomes. This implies that the percentage of hybrids obtained may have been underestimated. However, all plants scored as non-hybrid plants did have the leaf morphology of leek and had a DNA content similar to that of 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 (E. Jacobsen personal communication) or microprotoplast hybrids (Ramulu et al. 1996b).

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