TOWARDS *IN VITRO* FERTILIZATION, GAMETOSOMATIC CYBRIDIZATION AND DNA TRANSFER IN PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

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TOWARDS *IN VITRO* FERTILIZATION, GAMETOSOMATIC CYBRIDIZATION AND DNA TRANSFER IN PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

Proefschrift

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This thesis describes the results of a research project financially supported by the Programme Committee on Agricultural Biotechnology (PcLB) and performed at the Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen, The Netherlands.

1. Het gebruik van cellen zonder celwand voor genetische transformatie doeleinden bij plantesoorten die ongevoelig zijn voor *Agrobacterium tumefaciens*, is achterhaald door de introductie van de "particle gun" techniek (P Christou, Plant J 2: 275-281 (1992) & dit proefschrift).

2. Bij Engels raaigras kunnen geïsoleerde spermacellen ideale donoren van celkernen zijn voor cybridisatie experimenten (Dit proefschrift).

3. Vitamine C heeft een heilzame werking op de vitaliteit van spermacellen van *Lolium perenne* en *Homo sapiens* (Dit proefschrift & D Bradley, New Scientist 14-3-'92).

4. Specifieke herkenning tussen gameten tijdens de bevruchting bij planten en dieren, vindt plaats via op het oppervlak van de gameten aanwezige complementaire macromoleculen (GP Bolwell, JA Callow, V Evans, J Cell Sc 43: 209-224 (1980) & CJ Stafford, JR Green, JA Callow, J Cell Sc 101: 437-448 (1992) & C Crombach, Trouw 15-4-'92).

5. Duurzame landbouw ligt binnen bereik, wanneer kennis afkomstig van verschillende disciplines die zich bezighouden met deze problematiek gebundeld wordt, en commercieel belang niet op de eerste plaats staat.

6. "Sex creates a conflict of interests" (A Rayner, I Ross, New Scientist 30-3-'91).

7. Voor personeel in tijdelijk dienstverband bij de overheid, geeft zwangerschapsverlof aanleiding tot een rechtsongelijkheid tussen man en vrouw.

8. Het feit dat jonge Nederlandse mannen de meeste kansen hebben op de arbeidsmarkt (W Cornelisse, Intermediair 29: 24 (1993), getuigt van een zekere impotentie bij de werkgever.

9. Dat een portret met de neus gewoon tussen de ogen geplaatst weer mag (Vrij Nederland 23-1-'93), zal de produktie van portretten een nieuwe stimulans geven.

10. Een stelling bedacht om een discussie op gang te brengen, hoeft niet per sé de mening van de bedenker te zijn.

Stellingen behorende bij het proefschrift getiteld: "Towards *in vitro* fertilization, gametosomatic cybridization and DNA transfer in perennial ryegrass (*Lolium perenne* L.)".
Heleen M. van der Maas, 17 januari 1994.

Abstract

In this thesis, research towards in *vitro* fertilization, gametosomatic cybridization and DNA transfer in perennial ryegrass (*Lolium perenne* L.), the most important forage grass of North West Europe is described. Techniques for *in vitro* fertilization and gametosomatic cybridization were aimed at developing a new regeneration technique allowing DNA modification of perennial ryegrass by DNA transfer or by transfer of cytoplasmic traits. For this, isolation and storage techniques for sperm cells and egg cells were developed. The isolated sperm cells proved to be haploid and exhibited a moderate cytological variability. They contain only a small number of DNA containing organelles, which renders these cells suitable as nucleus donors in cybridization experiments. The morphology of isolated egg cells was comparable to the morphology of egg cells *in situ* of other monocotyledons and angiosperms in general. The most likely option to obtain *in vitro* fertilization and gametosomatic cybridization between, respectively, egg cells and sperm cells and sperm cells and cytoplasts, will be one to one fusion under microfusion conditions. Furthermore, to analyze whether promoters of rice genes are adequate for transgene expression in perennial ryegrass a method for direct gene transfer was developed. Stable transformation was obtained using biolistic bombardment of a non embryogenic cell suspension culture. The constitutive promoter of the rice gene *GOS2* proved suitable for expression of a transgene in perennial ryegrass. For production of transgenic plant material in the near future, biolistic bombardment of an embryogenic cell suspension or of other regenerative tissues, will be a more realistic option than via *in vitro* fertilization as regeneration technique in DNA transfer experiments. The performed research has created a broad basis for further research aimed at both cybridization, using sperm cells as nucleus donors, and direct gene transfer in perennial ryegrass, as well as at topics such as gamete interaction, zygote formation and early embryogenesis of higher plants at the cellular and molecular level.

Key words: Perennial ryegrass - *Lolium perenne* L. - *In vitro* fertilization - Gametosomatic cybridization - Gametoplast - Sperm cell isolation - Egg cell isolation - Cytological characterization - Direct gene transfer - Biolistic bombardment - Cell suspension - Transgenic callus lines - *gusA* gene - Rice gene *GOS2*.

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Account

Parts of this thesis have been included into the following publications:

Chapter 2 **Van der Maas HM, Zaal MACM** (1990) Sperm cell isolation of perennial ryegrass (*Lolium perenne* L.). In: Barnabás B, Liszt K (eds) Characterization of male transmission units in higher plants. MTA Copy, Budapest, p 31-35
Van der Maas HM, Zaal MACM, De Jong ER, Van Went JL, Krens FA (1993) Optimization of isolation and storage of sperm cells from pollen of perennial ryegrass (*Lolium perenne* L.). Sex Plant Reprod 6: 64-70

Chapter 3 **Van der Maas HM, De Jong ER, Van Aelst AC, Verhoeven HA, Van Went JL**
Cytological characterization of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.). Protoplasma in press

Chapter 4 **Van der Maas HM, Zaal MACM** (1990) Gametoplasts in perennial ryegrass. Acta Bot Neerl 39: 339
Van der Maas HM, Zaal MACM, De Jong ER, Krens FA, Van Went JL (1993) Isolation of viable egg cells of *Lolium perenne* L. Protoplasma 173: 86-89

Chapter 6 **Hensgens LAM, De Bakker EPHM, Van Os-Ruygrok EP, Rueb S, Van der Mark F, Van der Maas HM, Van der Veen S, Kooman-Gersmannn M, 't Hart L, Schilperoort RA** (1993) Transient and stable expression of *gusA* fusions with rice genes in rice, barley and perennial ryegrass. Plant Mol Biol 22: 1101-1127
Van der Maas HM, De Jong ER, Rueb S, Hensgens LAM, Krens FA Stable transformation and long term expression of the *gusA* reporter gene in callus lines of perennial ryegrass (*Lolium perenne* L.). Plant Mol Biol in press

List of abbreviations

BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
CMS	cytoplasmic male sterility
DAPI	4,6-diamino-2-phenylindole
$DIOC_6(3)$	3,3'-dihexyloxacarbocyanine iodide
DMSO	dimethylsulphoxide
EF	exoplasmic fracture
FCR	fluorochromatic reaction
FCS	fetal calf serum
FU	fluorescence unit
gusA	ß-glucuronidase
h	hour
hpt	hygromycin B phosphotransferase
IMPs	intramembrane particles
MES	2(N-Morpholino)ethane sulphonic acid
MOPS	3(N-Morpholino)propane sulphonic acid monosodium salt
PEG	polyethylene glycol
SSC	sodium sodium citrate
SDS	sodium dodecyl sulphate
SE	standard error of the mean value
T	time
VT50	Estimated storage time after which 50% of the isolated sperm cells retained their viability
PF	protoplasmic fracture

Chapter 1

General introduction

Agriculture is one of the important cornerstones of the world society. Change in approach of agricultural practice, therefore, not only affects the consumer's market of agricultural products, but also has a world wide social-economic and environmental impact. The agricultural approach of the last 40 years has brought cheap products of high quality at a high production rate with low imput of manpower. At the same time, since for this development new technologies were a prerequisite, it was a capital intensive approach of agriculture that only rich countries and companies could afford. The policy behind this approach led in many cases to monopolisation of agricultural production, poisoning of the environment with amongst others herbicides and pesticides and destruction of small scale agricultural practices in less wealthy world communities.

The last 10 years a new strategy of agriculture is in development. It is called plant biotechnology and one of the main promises of this new approach is to solve the environmental problems which were caused by use of toxic agrochemicals. Since a lot of new technology is involved, it is again highly capital intensive.

This thesis describes experimental research towards a new plant biotechnological strategy for breeding of perennial ryegrass (*Lolium perenne* L.). Additionally to the discussion of the results from the performed experimental research, in the general discussion section attention is paid to possible benefits and problems of this new way of agriculture, primarily focused on the environment.

Perspectives for breeding of perennial ryegrass using plant biotechnological techniques

Perennial ryegrass (*Lolium perenne* L.) is the major forage grass in North West Europe. In The Netherlands, pastures occupy the largest part of the total area under cultivation and forage grasses, from which perennial ryegrass has a portion of more than 80%, represent an annual value of over 2,000 million dutch guilders. Perennial ryegrass is an obligate outbreeder and is therefore difficult to manage in breeding and selection schemes, which results in slow progress in breeding success. Today, perennial ryegrass varieties are almost universally synthetic varieties based on several well combining clones, which are sexually propagated together for a few generations before seed reaches the market. This type of varieties thus consists of a mixture of genotypes with a comparable parentage (Den Nijs 1990).

Plant biotechnological techniques might be of interest to the grass breeder in speeding up breeding programmes or in allowing better control. Plant biotechnology involves a combination of two areas of plant science research. The first of these areas is concerned with the isolation and manipulation of single cells and tissues and subsequent growth and regeneration to complete plants. The second field, plant molecular biology, allows detailed manipulation of individual genes in plant genomes.

What agronomically desirable characters for perennial ryegrass are amenable to plant biotechnology? One of these characters may be cytoplasmic male sterility (cms). F1 hybrids between selected lines show improved agronomic traits as a consequence of a better fitness of heterozygotes with respect to homozygotes. A prerequisite for producing 100% pure F1 hybrid seed is male sterility in one of the selected lines to exclude self-fertilization. In perennial ryegrass a stable type of cms is available (Wit 1974). The feature cms is encoded by the dna of the mitochondrion, and causes the production of non-functional pollen. Today, F1 hybrids are not commercially available, because via sexual crossing, it takes about six years of back crossing to obtain a nuclear genetic identical line with the transferred cms trait to the original selected line of agronomical interest. By ways of asymmetric protoplast fusion, the cytoplasm of one line can be combined with the nucleus of a line of agronomic interest. This way, the desired new nucleus/cytoplasm combination can be obtained in one step. Furthermore, by recombination of the mixed mitochondrial dnas of two lines after fusion, new types of cms might arise, a phenomenon that cannot occur through sexual hybridization via crossing since in the majority of higher plants, including perennial ryegrass, cytoplasmic organelles are predominantly maternally inherited (Corriveau and Coleman 1988). Research aimed at transfer of the trait of cms via plant biotechnological techniques has been performed by Creemers-

MOLENAAR et al. (1992). New combinations of cytoplasm and nucleus were obtained at a high frequency which offers promising perspectives for breeding of F1 hybrids.

A second option for plant biotechnology in perennial ryegrass is development of resistance to pests and diseases. The most important pests of temperate grasslands are insects. Leather jackets (larvae of *Tipula paludosa*), cutworms (*Agrotis spp.*) and grass grubs (e.g. *Melolontha melolontha*) cause heavy damage to pastures and a significant amount of general purpose insecticides is used to fight these pests. A genetic based resistance to those insects is unknown in ryegrasses (DEN NIJS 1990). Leather jackets, which belong to one of the major pest in Northern Europe, cause great damage to root systems and seedlings. A possible way to combat these unprofitable herbivores could be to expose them to insecticidal crystal proteins produced by *Bacillus thuringiensis* (*Bt*), since these larvae are among the group of target species for biological control involving these proteins (WAALWIJK 1990). Different strains of *Bacillus thuringiensis* produce more than 25 different, but related insecticidal crystal proteins. These proteins are toxic for larvae of different orders of insects, including disease vectors and many agricultural pest insects. Other insects beneficial to agriculture and mammals are unaffected. Probably the most efficient and economic way of insect exposure to these toxic proteins, is through genetic engineering of the insect's target plants (LAMBERT and PEFEROEN 1992). For tobacco (VAECK et al. 1987), tomato (FISCHHOFF et al. 1987), cotton (PERLAK et al. 1990) and potato (PEFEROEN et al. 1990) insect resistant plants have been obtained by transfer and expression of *Bt* genes coding for insecticidal crystal proteins into the plant genome. The introduction of resistance genes to pests and diseases in perennial ryegrass by ways of genetic engineering, could be economically and environmentally attractive.

Genetic manipulation or modification of a crop by plant biotechnological methods only becomes a alternative to the breeder, if tissue culture and regeneration techniques tailored to the particular crop species are available. Perennial ryegrass belongs to the family of Poaceae, a member of the monocotyledons. This family which incorporates major agricultural crops like rice, wheat, maize, oats and barley is recalcitrant in applying tissue culture techniques, especially when it concerns regeneration from protoplasts (cells without a cell wall isolated from sporophytic tissue) to mature plants. In 1986 when this project was formulated, already some successes on protoplast regeneration had been reported in rice (FUJIMURA et al. 1985; COULIBALY and DEMARLY 1986; YAMADA et al. 1986), but in comparison to successes obtained with species from dicotyledonous families like for instance the Solanaceae, with species such as tobacco, petunia, tomato and potato, the results were still rather poor.

Protoplasts are essential for intra- and interspecific somatic hybridization and cybridization to obtain new combinations of nuclear and cytoplasmic features.

However, because of serious difficulties faced in culture and regeneration of cereal/grass protoplasts, very few attempts had been made to obtain somatic hybrids in the Poaceae (VASIL 1987). Also, it was considered that for gene transfer in gramineous crops, protoplasts were a prerequisite. Cereals and grasses were generally insensitive to the natural vector for gene transfer *Agrobacterium tumefaciens*, suggested to be caused by inability of the bacteria to attach to the cell wall, or by the auxin-cytokinin balance in monocots that reduces the wound response upon infection (SCHÄFER et al. 1987). Therefore, direct gene transfer techniques were needed to obtain stable genetic transformation. Since only methods of direct gene transfer for protoplasts were available at that time, again the low regeneration capacity of protoplasts appeared to be a major obstacle. With direct gene transfer to protoplasts the first gramineous stable transgenic cell lines (no plants) were obtained from wheat (LÖRZ et al. 1985), italian ryegrass (POTRYKUS et al. 1985), maize (FROMM et al. 1986) and rice (UCHIMIYA et al. 1986). With regard to perennial ryegrass, a protoplast isolation and regeneration procedure still had to be developed. Over the last five years, however, a lot of progress has been made tackling these problems, resulting in regeneration techniques from different kinds of explants, cell suspension cultures and protoplasts for perennial ryegrass (reviewed in CREEMERS-MOLENAAR and BEEREPOOT 1992). Also success on gene transfer in a gramineous species using *Agrobacterium tumefaciens*, has been reported (RAINERI et al. 1990).

In 1986 a programme was formulated aimed at developing a new regeneration technique for cells without a cell wall and at the preparation of vectors for direct DNA transfer to gramineous species. The implementation of the programme resulted in a collaboration between the Institute of Molecular Plant Sciences of Leiden State University and the former Foundation for Agricultural Plant Breeding (SVP), now part of the Centre for Plant Breeding and Reproduction Research (CPRO-DLO) at Wageningen. This programme was financially supported by the Programme Committee on Agricultural Biotechnology (PCLB), which was established in 1985 by the Ministry of Agriculture, Nature Conservation and Fisheries. The Institute of Molecular Plant Sciences was concerned with research on conserved regions in regulatory sequences of corresponding genes in rice and perennial ryegrass and designing plant vectors especially for gramineous species. Different promoters of rice had been isolated: a constitutive promoter that produces expression in all tissues of the plant (DE PATER et al. 1992), a shoot specific promoter that induces expression in the leaves of the plant (DE PATER et al. 1990) and a root specific promoter that causes only expression in the roots of the plant (DE PATER and SCHILPEROORT 1992). These promoters have been cloned in a vector in front of the *gusA* reporter gene (JEFFERSON et al. 1987) by HENSGENS et al. (1993) for analysis of the promoter activities after trans-

formation. The cell biological section of the programme that comprised the development of a new regeneration technique, using gametoplasts (cells without a cell wall isolated from gametophytic tissue) and a method for direct gene transfer to test the vectors constructed by HENSGENS et al. (1993), was performed at the Centre for Plant Breeding and Reproduction Research (CPRO-DLO). The ultimate goal was to combine DNA transfer and regeneration in a new to develop technique.

OUTLINE OF THE RESEARCH PROGRAMME

For improvement of the regeneration capacity of cells without a cell wall, gametoplasts can possibly be used as an alternative to protoplasts. Fusion of isolated male and female gametoplasts represents the ultimate stage of *in vitro* fertilization. The hypothesis behind this approach is, that such a fusion product will have a good regeneration capacity because of its biological background. Besides, in the majority of higher plants, including perennial ryegrass, cytoplasmic organelles are predominantly maternally inherited (CORRIVEAU and COLEMAN 1988), which might be circumvented by *in vitro* fertilization, where the contents of both partners are mixed, producing a new type of cytoplasm. On the other hand, mature sperm cells of perennial ryegrass contain a small amount of cytoplasm with only a few mitochondria (this thesis, chapter 3), which renders this cell suitable as a nucleus donor in cybridization experiments with cytoplasts (protoplast without a nucleus) for transfer of traits which are coded for by DNA present in cytoplasmic organelles, e.g. CMS. *In vitro* fertilization combined with direct DNA transfer of chimeric genes regulated by organ specific promoters could be an option to acquire transgenic plants with expression of the transferred gene only in specific parts of the plant.

Gametoplasts isolated from microspore tetrads have already been used in gametosomatic fusion experiments. The regenerated plants from these fusion products were found to be hybrids for both nuclear (PIRRIE and POWER 1986; LEE and POWER 1988) as well as cytoplasmic DNA (PENTAL et al. 1989). It has also been shown possible to induce cell divisions in isolated pollen gametoplasts (ZHOU 1989a,b). With the crop maize, fusion products of egg cells with sperm cells and sperm cells with cytoplasts have been obtained and cultured (KRANZ et al. 1991a; KRANZ et al. 1991b) and regeneration of zygotes obtained through *in vitro* fertilization has been established (Kranz and LÖRZ unpublished results 1993). Thus, gametophytic cells as starting material to obtain cells without a cell wall for further manipulation might be a true alternative to gain a better regeneration capacity compared to protoplasts of sporophytic cells,

which opens up new possibilities for transfer and recombination of nuclear and cytoplasmic genetic information, and direct gene transfer.

Furthermore, when viable male and female gametoplasts are available, topics like membrane interaction during fertilization, zygote formation and early embryogenesis in angiosperms are new targets accessible to research. These topics can produce new insights in the mechanisms that generate successful or unsuccessful fertilization, and could yield a better understanding of the processes involved in embryogenesis. Such knowledge could also benefit the understanding of the regeneration process important for many plant biotechnological techniques.

To attain the forementioned goals of research, a number of techniques needed to be developed for perennial ryegrass. Techniques for isolation, storage and fusion of male and female gametoplasts were required. Also the cytological characteristics of the gametoplasts needed to be investigated. In addition, characterization of the expression of the different chimeric genes, consisting of rice promoters linked to the *gusA* reporter gene (HENSGENS et al. 1993), implied development of a direct gene transfer technique. In the following paragraphs the different parts of this thesis are introduced.

Isolation and storage of male gametoplasts

In the past two decades, several research groups have developed different techniques to isolate viable male gametoplasts from a variety of different microspore and pollen stages of various plant species. Gametoplasts have been obtained from tetrads (BHOJWANI and COCKING 1972; BAJAJ 1974), pollen grains (TANAKA et al. 1987; ZHOU 1988), generative cells (ZHOU 1988; TANAKA 1988) and sperm cells (RUSSELL 1991). Initially the use of microspore tetrads for isolation of male gametoplasts of perennial ryegrass was investigated. However, the period of the tetrad stage during pollen development appeared to be very short, even when plants were grown at low temperatures to slow down the rate of development. Furthermore, it appeared to be very difficult to identify florets with anthers containing tetrads on basis of morphological characteristics (unpublished results). Since florets just before anthesis, which contain mature pollen grains, are simply to recognize and are abundantly present, it was decided to use mature pollen instead of tetrads for the isolation of male gametoplasts.

The pollen of perennial ryegrass is tricellular, like mainly all species from the family of the Poaceae (BREWBAKER 1967), containing a vegetative cell and two sperm cells, which are the male gametes. Isolation of large numbers of sperm cells from tricellular pollen species has already been reported for Plumbago zeylanica (RUSSELL

1986), maize (DUPUIS et al. 1987) and spinach (THEUNIS and VAN WENT 1990). For spinach a squash method was developed, while for Plumbago and maize an osmotic shock procedure was used to release the sperm cells from the pollen grains.

The lifespan of the isolated sperm cells must be considerable to make cytological and fusion studies possible. Therefore, the viability and ways of improvement of the long-term viability of isolated sperm cells were investigated. In chapter 2, an isolation and storage method for sperm cells of perennial ryegrass is described and discussed.

Cytological characterization of male gametoplasts

Ultrastructural analysis of sperm cells of several species from the Poaceae such as barley (CASS 1973; CHARZYNSKA et al. 1988), wheat (ZHU et al. 1980), and maize (MCCONCHIE et al. 1987a; CASS and FABI 1988; WAGNER and DUMAS 1989; MOGENSEN et al. 1990) revealed that the cytoplasm of the sperm cells contains an assortment of cell organelles. For *Plumbago zeylanica* (RUSSELL 1984) and two *Brassica* species (MCCONCHIE et al. 1987b), a dimorphism involving the number of proplastids and mitochondria has been determined, whereas for other species like barley (MOGENSEN and RUSCHE 1985), maize (WAGNER and DUMAS 1989), and spinach (THEUNIS 1992) no indications for a dimorphism have been detected.

For future fusion experiments aimed at cellular modifications and DNA transfer or for studying developmental processes in fertilization and embryogenesis, it is important to obtain detailed knowledge of the isolated sperm cells: i.e. about the ploidy level and condition of the nucleus, the presence of a cell wall, characteristics of the plasma membrane, and DNA containing organelles and their numbers. In chapter 3, a multidisciplinary cytological characterization of isolated sperm cells of perennial ryegrass is described and discussed.

Isolation and characterization of female gametoplasts

The female gametophyte of higher plants is called the embryo sac and is situated in the ovule enclosed by layers of sporophytic tissue. Several types of embryo sacs can be distinguished on basis of their type of megasporogenesis and type of megagametogenesis. The most common programme of development leads to the Polygonum type, which is found most frequently in angiosperm species (WILLEMSE and VAN WENT 1984), including perennial ryegrass (ELGERSMA and SNIEZKO 1988). The embryo sac of the Polygonum type consists of a haploid egg cell and two haploid synergids at the micropylar pole of the ovule, a central cell with two haploid polar nuclei in the centre, and three haploid antipodal cells at the chalazal end of the ovule.

After pollination, pollen tubes carrying two sperm cells emerge from the pollen grains and grow through the stigma and style to the micropyle of the ovule. Before fertilization takes place, a pollen tube penetrates one of the synergids and discharges its content. Via the synergid, the sperm cells are transferred to respectively the egg cell and the central cell. After fusion of the egg cell with one of the sperm cells a zygote is produced, that will develop into an embryo. The other sperm cell fuses with the central cell, which is the start of endosperm development. The antipodals probably have a nutritional function in the female gametophyte (WILLEMSE and VAN WENT 1984; CAMERON and PRAKASH 1990).

Throughout this century research on embryology of angiosperms was performed with techniques of sectioning, clearing and squashing of fixed ovular tissues to study the development of the embryo sac and early embryogenesis (HERR JR. 1971). For *in vitro* fusion experiments, isolated viable female gametophytic cells are essential and their study could also contribute to a better insight in the processes of fertilization and embryogenesis. Different techniques for embryo sac and egg cell isolation like microdissection, squashing and enzymatic degradation of ovular tissue have been developed (reviewed in THEUNIS et al. 1991). For maize that, like perennial ryegrass, possesses an embryo sac of the Polygonum type and a crassinucellate ovule (ovule with a large nucellus), a combination of enzymatic degradation and mechanical manipulation of ovular tissue has been developed to isolate constituents of the embryo sac (KRANZ et al. 1991a)

In chapter 4, three techniques, squashing of ovules, enzymatic degradation of ovary and ovular tissues and a combination of enzymatic degradation and mechanical manipulation of ovules, aimed at isolation of viable embryo sacs and egg cells are presented and discussed. Also for the isolation of female gametoplasts, the mature phase was chosen, as was also decided for isolation of male gametoplasts. The morphology of isolated egg cells is compared to egg cells *in situ* of other gramineous species and angiosperms in general.

Analysis of conditions for fusion

For *in vitro* fertilization and gametosomatic cybridization experiments, it is of great interest to acquire information about how fusion between gametoplasts, and gametoplasts and cytoplasts can be achieved. Since isolation protocols for large numbers of sperm cells (This thesis, chapter 2) and cytoplasts (VAN ARK et al. 1992) were available, these cells were used in a preliminary study, discussed in chapter 5.

Direct gene transfer

In order to analyze the promoter activity of isolated constitutive and organ specific rice promoters (De Pater et al. 1990; De Pater and Schilperoort 1992; De Pater et al. 1992) in perennial ryegrass a method for direct gene transfer was required. DNA uptake by protoplasts can be realized by inducing permeability of the cell membranes of the protoplasts with polyethylene glycol (PEG) combined with a high salt concentration (Krens et al. 1982), or with electroporation, using electrical pulses (Shillito et al. 1985). During the course of the programme, next to direct DNA transfer methods with protoplasts, new methods of direct gene transfer were developed that could accomplish DNA uptake through the cell wall of the plant cell. Uptake of DNA and transient expression in dry and viable embryos of wheat has been realized by imbibition in a DNA solution (Töpfer et al. 1989). Stable integration of the transferred DNA might then be achieved by induction of transgenic callus on the imbibed embryos under selection conditions, which might eventually lead to regeneration of transgenic plants from the callus material. Using high-velocity microprojectiles, nucleic acids can be delivered into plant cells (Klein et al. 1987). By this biolistic method, transgenic plants have been obtained from soybean (McCabe et al. 1988), maize (Gordon-Kamm et al. 1990), papaya (Fitch et al. 1990), cotton (Finer et al. 1990), rice (Christou et al. 1991), sugarcane (Bower and Birch 1992) and wheat (Vasil et al. 1992).

In chapter 6, experiments with three methods of direct gene transfer, PEG transformation, embryo imbibition and biolistic bombardment, are presented. As a start, transient expression of the chimeric gene construct consisting of the *gusA* reporter gene regulated by the promoter of the constitutive rice gene *GOS2*, constructed by Hensgens et al. (1993) is evaluated in different tissues of seedlings and in cell suspension material. Stable expression of the constitutive regulated *gusA* gene was estimated at different times after DNA transfer, to monitor long-term stability of transgene expression.

CHAPTER 2

ISOLATION AND STORAGE OF SPERM CELLS FROM POLLEN OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

HELEEN M. VAN DER MAAS, MONIQUE A.C.M. ZAAL, ELIZA R. DE JONG, JAC L. VAN WENT and FRANS A. KRENS

Summary. A sperm cell isolation procedure for perennial ryegrass (*Lolium perenne* L.) was developed. Sperm cells were released from pollen grains by osmotic shock. The percentage of sperm cells released from the pollen grains was determined by both pH and osmolality. An optimum yield of sperm cells was achieved with only a specific combination of pH and osmolality. Aimed at improvement of yield and long-term viability, various combinations of vitamins E, C and fetal calf serum were tested. Their possible mode of action as important components for improvement of yield and long-term viability is discussed. Under optimized conditions, a yield of 12% was established and the storage time after which 50% of the sperm cells were still viable was improved to 60 hours. Cytological observations demonstrated that the isolated sperm cells are true protoplasts, which may allow future fusion experiments.

INTRODUCTION

The pollen grains of perennial ryegrass (*Lolium perenne* L.) are tricellular. Like in mainly all species from the family of the Poaceae (BREWBAKER 1967), the pollen grain contains a vegetative cell and two sperm cells, which are the male gametes. Isolation of large numbers of sperm cells from tricellular pollen grains has already been reported for *Plumbago zeylanica* (RUSSELL 1986), maize (DUPUIS et al. 1987) and spinach

(THEUNIS and VAN WENT 1990). For spinach a squash method was developed, while for *Plumbago* and maize an osmotic shock procedure was used to release the sperm cells from the pollen grains.

Pollen from the family Poaceae undergoes rapid and severe desiccation in the atmosphere after release from the anther. Due to this desiccation, the pollen grains rapidly loose their viability (HESLOP-HARRISON 1979). During desiccation of pollen in general, an increase of organic free radicals is observed (PRIESTLEY et al. 1985). In maize, pollen desiccation results in a decrease of the ratio of membraneous unsaturated and saturated fatty acids, and an increase of the amount of free fatty acids, which are degradation products of lipids (HOEKSTRA et al. 1989). Furthermore, it was reported that dehydration of maize pollen causes oxidative damage to the vegetative cell plasma membrane of the pollen grain (KERHOAS et al. 1987). Such degradive processes of the plasma membrane could also be affecting the viability of sperm cells during isolation and storage.

Indications for a positive effect of an antioxidative system such as vitamins E and C, and fetal calf serum on the yield and longevity of isolated sperm cells of perennial ryegrass were obtained earlier (VAN DER MAAS and ZAAL 1990). Vitamins E and C form a synergistic antioxidative system which occurs naturally in plant tissues (FINCKH and KUNERT 1985). Fetal calf serum has been demonstrated to have a beneficial effect on pollen protoplast culture (ZHOU a,b 1989) and protoplast culture of gramineous crops, like rice and perennial ryegrass (YAMADA et al. 1986; CREEMERS-MOLENAAR et al. 1989).

In this chapter an isolation procedure for sperm cells from perennial ryegrass is presented. Aimed at improvement of yield and long-term viability, various combinations of vitamins E,C and fetal calf serum were tested.

MATERIAL AND METHODS

Plant material

Three varieties of *Lolium perenne* L., Peramo, Talbot and Tranie, which differ in period of anthesis, being respectively early, intermediate and late, were used. Plants were grown in a greenhouse under a light/dark cycle of 14/10 h at controlled temperature conditions of 17°/12°C. Spikes were collected at the beginning of flowering. For aseptic conditions spikelets were sterilized in 1% (w/v) sodium hypochlorite and 1 droplet Tween 20 during 10 minutes and were subsequently rinsed with sterilized water during 1 hour with four changes of water. From the spikelets only the anthers

which were in a stage just before anthesis, were collected and used for sperm cell isolation. The quality of the pollen was tested, using the fluorochromatic reaction (FCR) assay as described by HESLOP-HARRISON et al. (1984). When ≥90% of the pollen was FCR positive, the material was used for experiments.

Isolation procedure

A large number of parameters was tested to improve yield and long-term viability of isolated sperm cells. As a result of these experiments the following procedure was developed. To release the sperm cells from the pollen grains, 100 mg of anthers was squashed in 2 ml isolation medium at 30°C in a glass Petri dish. The optimal isolation medium consisted of a modified BKS medium (BREWBAKER and KWACK 1963), pH 6.0, containing 10% (w/v) sucrose (360 mosmol/ kg H_2O), 5 mM MOPS (3(N-Morpholino)propane sulphonic acid monosodium salt), 0.1 mM Vitamin E (DL-α-tocopherol phosphoric acid ester disodium salt) and 10 mM vitamin C (L-ascorbic acid). After filtering the suspension through a 20 µm gauze, the filtrate was kept at 0°C (filtrate 1). The residue was resuspended and squashed again in the same volume of isolation medium, after which a storage medium was added to the suspension at a 1 to 1 basis. The optimal storage medium consisted of a modified RY medium (YAMADA et al. 1986), pH 6.0, containing 17.5% (w/v) sucrose (780 mosmol/kg H_2O), 5 mM MOPS, 0.1 mM vitamin E, 10 mM vitamin C and 6.5% (v/v) fetal calf serum. The suspension was filtered (20 µm) and the filtrate was mixed with filtrate 1. Subsequently, storage medium was added to a final 1:2 ratio (isolation:storage). For purification, the filtrate mixture was layered onto a discontinuous gradient of 35% and 10% (v/v) Percoll in storage medium at 0°C. After centrifugation at 10,000 g for 15 minutes at 4°C, the sperm cells were pipetted from the interphase between the 35% and 10% Percoll layers with a Pasteur pipet. The Percoll was washed away from the purified sperm cell fraction by dilution with storage medium and centrifugation at 10,000 g for 5 minutes at 4°C. The pellet, containing the isolated sperm cells, was suspended in storage medium at 0°C.

Cytological characterization

The isolated sperm cells were analyzed by phase contrast microscopy (MATTHYS-ROCHON et al. 1987) and fluorescence microscopy after staining with 4,6-diamino-2-phenylindole (DAPI) (COLEMAN and GOFF 1985). The FCR assay as described by HESLOP-HARRISON et al. (1984), was used to assess the viability of the sperm cells.

The presence of a cell wall was tested for by calcofluor white and analine blue staining (TANAKA 1988).

Sperm cell number determination

The weight of one anther is 0.4 mg ± 0.03 (SE) and the mean number of pollen grains/anther was determined at 5853 ± 214 (SE) ≈ 6000. The number of sperm cells present in the starting material was determined by multiplying the number of mg anthers by 3×10^4 ($2.5 \times 6000 \times 2$ sperm cells/pollen grain). After isolation 95 - 100% of the sperm cells, observed with phase contrast microscopy, were FCR positive. Therefore, the number of sperm cells counted in a haemocytometer using phase contrast microscopy, was considered as indicative for the number of viable sperm cells. The number of isolated sperm cells from the pollen grains, was estimated by dividing the number of counted sperm cells by the calculated number of sperm cells in the starting material times 100%.

Effects of pH and osmolality on sperm cell isolation

The values of pH and osmolality were varied to assess the optimal osmotic shock conditions for sperm cell release from the pollen grain. The pH of the isolation medium was varied in a range of 5.0 to 8.0 at 780 mosmol/kg H_2O. The buffer at pH 5.0 and 5.5 was 5 mM MES (2(N-Morpholino)ethane sulphonic acid). For higher pH values the buffer was 5 mM MOPS. For sperm cell isolation at different pH values, the concentration of fetal calf serum in the storage medium was 0.8% (v/v) instead of the optimal concentration of 6.5% (v/v) as described in the isolation procedure. The osmolality of the isolation medium was varied with sucrose 10 - 20% (w/v) from 360 mosmol to 780 mosmol/kg H_2O at pH 6.0. The effects of pH and osmolality on sperm cell isolation, were measured by estimating the number of disrupted pollen grains in relation to the number of intact viable pollen grains × 100%, after osmotic shock, and by determining the yield of viable sperm cells.

Effects of vitamins E, C and fetal calf serum on yield and long-term viability

Eight different combinations of vitamin E, vitamin C and fetal calf serum were tested in two series of three experiments under optimal osmotic shock conditions (*table* 1). Vitamins E and C were added to both the isolation medium and the storage medium, whereas fetal calf serum was added only to the storage medium. In the first series of experiments, effects of vitamins E and C in the presence of fetal calf serum were

investigated. In the second series of experiments, the effects of vitamins E and C without fetal calf serum were investigated. The combinations with all (1) and without any of the components (5) were used as standards in both experiments. After isolation (isolation phase) the sperm cells were stored at 0°C in storage medium (storage phase). To analyze the yield and long-term viability of the isolated sperm cell population, the number of viable sperm cells was determined at 8 points of time in a time interval from T=0 hours (h), directly after isolation, up to T=137.5 h. The estimated storage time after which 50% of the isolated sperm cell population retained their viability was defined as the VT50 (Viability Time). The standards (1) and (5) (*table* 1) were used to determine at what phase, isolation and/or storage, the components had their main effect. After isolation in the two standards and determination of the yield, the sperm cells were equally divided in two and stored in their original or the other standard medium. Subsequently, the long-term viability of the sperm cell population was determined as forementioned. This experiment was repeated two times and statistically analyzed with the Wilcoxon matched-pairs signed-ranks test.

Statistical analysis

If not stated otherwise, the data were $\arcsin\sqrt{(x+0.5)}$ transformed and statistically analyzed with a one-way analysis of variance, using SPSS (SPSS INC 1986). The differences between the data within the experiments, were analyzed with the Student-NEWMAN-KEULS procedure.

Table 1. Combinations of vitamins E and C and fetal calf serum in isolation and storage medium.

	Isolation phase					Storage phase		
	Isolation medium		Storage medium			Storage medium		
	0,1 mM	10 mM	6,5%	0,1 mM	10 mM	6,5%	0,1 mM	10 mM
1	E	C	FCS	E	C	FCS	E	C
2	–	C	FCS	–	C	FCS	–	C
3	E	–	FCS	E	–	FCS	E	–
4	–	–	FCS	–	–	FCS	–	–
5	–	–	–	–	–	–	–	–
6	E	C	–	E	C	–	E	C
7	–	C	–	–	C	–	–	C
8	E	–	–	E	–	–	E	–

Fetal calf serum (FCS)

Results

To isolate sperm cells, pollen were released from the anthers by mechanical forces generated by squashing. Simultaneously, sperm cells were released from these pollen grains by disruption of the vegetative cell wall at the germ pore through osmotic shock induced by the isolation medium. Within 5 minutes the isolated sperm cells transformed from a spindle shape, which is the natural form within the pollen grain, into a spherical shape in the medium (*fig.* 1a-c). Directly after isolation, 95 - 100% of the isolated sperm cells were FCR positive and showed a dense cytoplasm (*fig.* 1d-e). After staining isolated sperm cells with calcofluor white for the presence of cellulose and analine blue for the presence of callose, no positive reaction was observed.

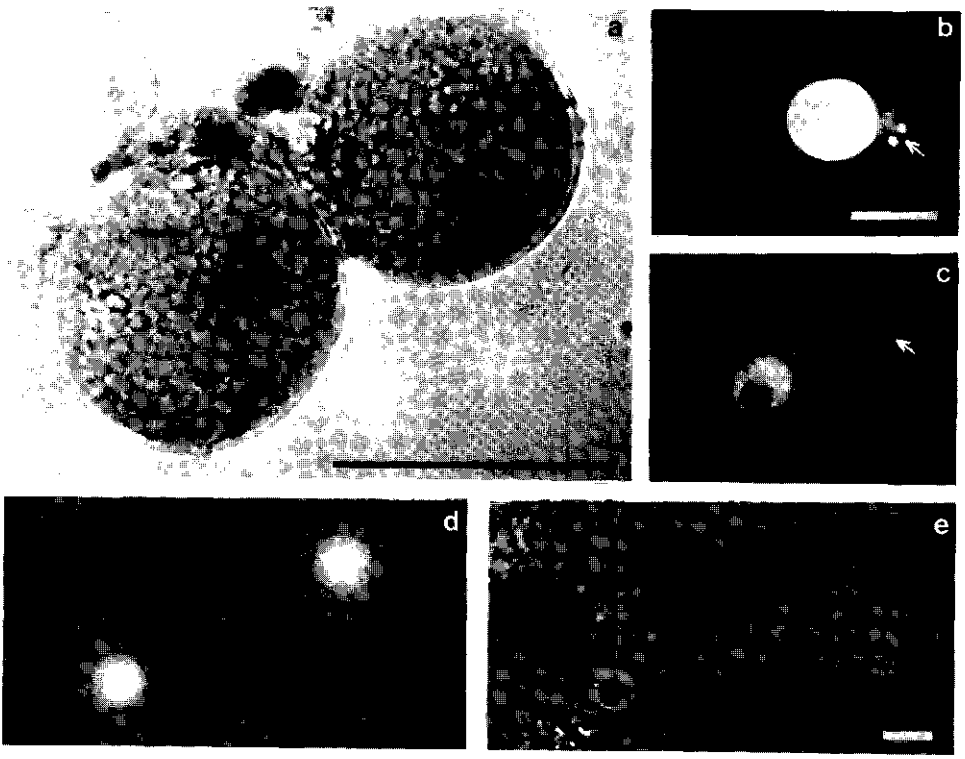

Fig. 1. Sperm cells of perennial ryegrass (*Lolium perenne* L.) before and after isolation. **a.** Tricellular pollen grains after staining with acetocarmine (spc: sperm cell; vn: vegetative nucleus). Bar=30 µm. **b.** Sperm cells outside the pollen grain after osmotic shock and staining with DAPI. Bar=30 µm **c.** FCR positive sperm cells outside the pollen grain after osmotic shock. **d.** Isolated FCR positive sperm cells. **e.** Isolated sperm cells visualized by phase contrast microscopy. Bar=10 µm.

For the release of sperm cells from the pollen grains, a specific combination of pH and osmolality of the isolation medium appeared to be crucial. When analyzing the effects of these parameters on sperm cell isolation, the optimal isolation conditions were not always used, because development of the procedure was still in progress. Therefore, optimal yields of sperm cells were not obtained. At the tested pH values of the isolation medium, differences for both the percentage of disrupted pollen grains and the yield of isolated sperm cells were found, with an optimum at pH 6.0 (*fig.* 2a-b). Varying the osmolality in a range from 360 to 780 mosmol/kg H_2O at pH 5.5, the percentage of disrupted pollen grains remained unchanged, whereas at pH 6.0 the osmolality influenced both the percentage of disrupted pollen grains as well as the yield of isolated sperm cells, with an optimum at 360 mosmol/kg H_2O (*fig.* 2c).

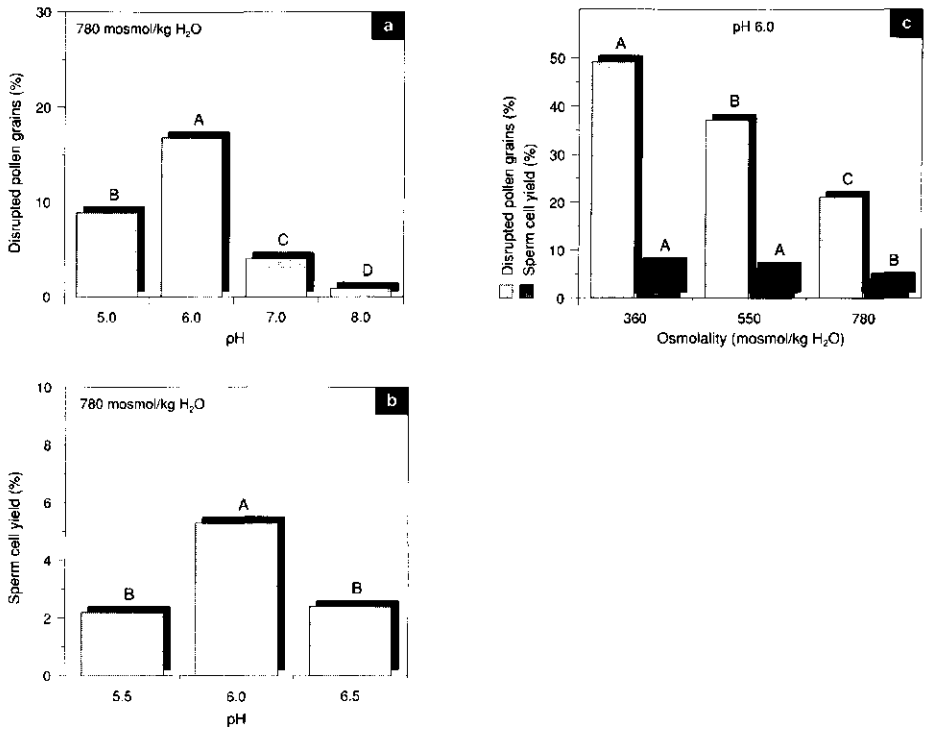

Fig. 2. Effect of pH and osmolality of the isolation medium on the disruption of pollen grains and the yield of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.). Different letters on top of bars indicate significant differences ($P<0.05$). **a.** Percentage of disrupted pollen grains at different pH values and 780 mosmol/kg H_2O ($F_{(3,76)}=56.3$; $P<0.001$). **b.** Yield of sperm cells at different pH values and 780 mosmol/kg H_2O ($F_{(2,24)}=36.3$; $P<0.001$). The storage medium in this experiment contained 0.8% (v/v) fetal calf serum. **c.** Percentage of disrupted pollen grains ($F_{(2,111)}=96.6$; $P<0.001$) and yield ($F_{(2,68)}=16.4$; $P<0.001$) at different osmolalities and pH 6.0.

Comparing the results, the significant increase of the percentage of disrupted pollen grains between 360 and 550 mosmol/kg H_2O, did not result in a significant improvement of the sperm cell yield. Using optimal osmotic shock conditions with pH 6.0 and 360 mosmol/kg H_2O in the isolation medium, no variety dependent response to the isolation conditions was found with the three varieties that were tested.

To improve the yield and long-term viability of the isolated sperm cells, various combinations of vitamins E, C and fetal calf serum in the isolation and storage medium (*fig.* 3) were analyzed. Generally after isolation, an increasing number of sperm cells shrank, lost their spherical shape and became FCR negative until no viable sperm cells could be observed. In *fig.* 3a, the effects of fetal calf serum in combination with vitamins E and C are shown. Comparing the results of the tested combinations, fetal calf serum separately and combined with vitamin C improved the yield and VT50 (*fig.* 3d), whereas vitamin E had no effect. In *fig.* 3b, the effects of vitamins E and C without fetal calf serum are shown. Comparing the results of the tested combinations, vitamin C alone improved the yield and in combination with vitamin E the VT50 (*fig.* 3d) was increased. In order to determine at what step in the procedure the presence of vitamin E, C and fetal calf serum has its main effect, the components were omitted at the separate steps of isolation and storage (*fig.* 3c). The sperm cell population that was isolated in standard (1) and stored without the components (1/5), showed a decline in the VT50 from 60 to 22 hours (*fig.* 3d), compared to the sperm cell population that was isolated and stored in the presence of the components (1). The sperm cell population, isolated without the components and stored with the vitamins and fetal calf serum (5/1), showed an improvement of the VT50 from 10 to 22 hours, compared to the sperm cell population that was isolated and stored without the components (5).

In conclusion it can be stated that optimal yield and long-term viability were obtained in the presence of vitamin C and fetal calf serum during isolation and storage. The yield was increased from 5 to 12% and the storage time during which 50% of the sperm cell population retained their viability was improved from 10 to 60 hours. Note that fetal calf serum was only present in the storage medium, because addition of fetal calf serum to the isolation medium did not improve the yield.

Discussion

Pollen grains from small grain cereals and grasses rapidly loose their viability after desiccation (Heslop-Harrison 1979). Perennial ryegrass pollen has the same trait. Therefore, instead of mature pollen after anthesis, pollen just before anthesis was used for sperm cell isolation.

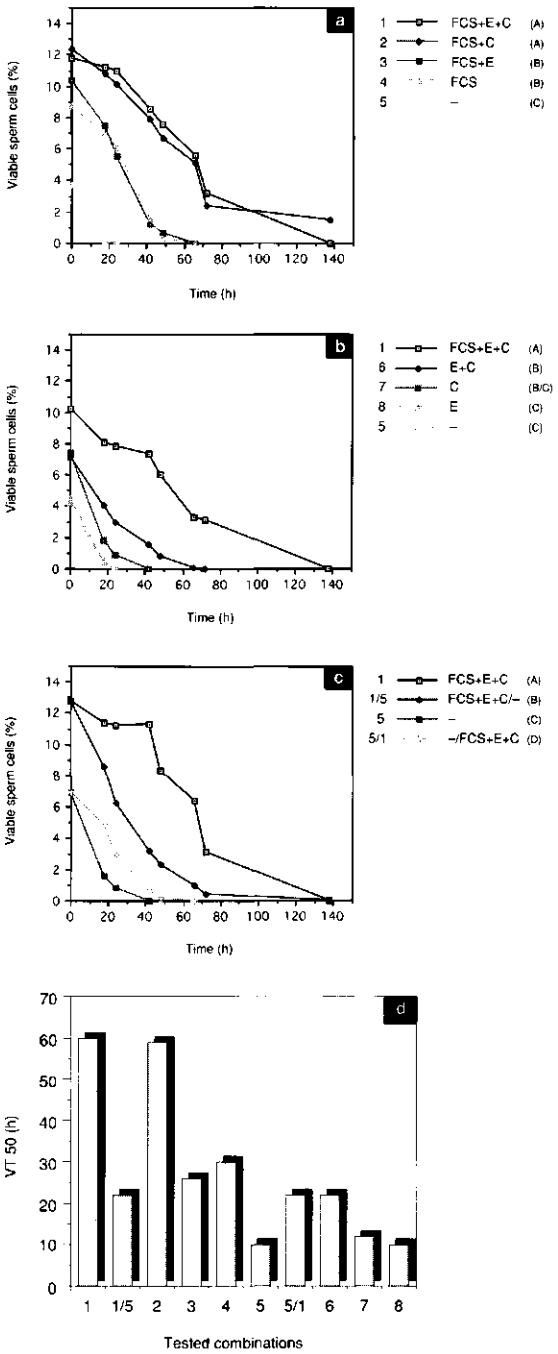

Fig. 3. Effects of vitamins E, C and fetal calf serum (FCS) on the yield and long-term viability of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.). Different letters behind the legends indicate significant differences ($P<0.05$) between the long-term viabilities of the tested combinations. Statistical analysis on the yields (at T=0 h) are presented by placing the comparisons in a descending order of their mean value. Groups linked by a common line were not significantly different ($P>0.05$). a. Effects of vitamins E, C in combination with fetal calf serum on the yield ($F_{(4,53)}=21.8$; $P<0.001$, 2 1 3 4 5) and long-term viability ($F_{(4,371)}=77.1$; $P<0.001$). b. Effects of vitamins E and C on the yield ($F_{(4,55)}=15.9$; $P<0.001$, 1 7 6 5 8) and long-term viability ($F_{(4,452)}=67.8$; $P<0.001$). c. Yield and long-term viability of isolated sperm cells of perennial ryegrass in dependence of isolation and storage conditions with or without vitamins E, C and fetal calf serum. Analysis of difference between 1 and 1/5, Wilcoxon: T=0 N=44; $P<0.001$. Analysis of difference between 5 and 5/1, Wilcoxon: T=13.50 N=28; $P<0.001$. d. VT50 of the tested combinations, estimated from *fig.* 3a-c. VT50: storage time after which 50% of the isolated sperm cells are still viable. The VT50 of standard 1 and 5 are the average results from *fig.* 3a-c.

Isolation of large numbers of sperm cells from tricellular pollen grain species has already been reported for *Plumbago* (RUSSELL 1986), maize (DUPUIS et al. 1987) and spinach (THEUNIS and VAN WENT 1990). For spinach a squash method was developed, while for *Plumbago* and maize an osmotic shock procedure was used. The crucial effect of the pH on the osmotic shock, is possibly established by modifications of the vegetative plasma membrane as reported by HESLOP-HARRISON and HESLOP-HARRISON (1980). The importance of the pH for sperm cell isolation has been reported earlier by ROECKEL et al. (1990) and VAN DER MAAS and ZAAL (1990). The result, that at optimal pH for osmotic shock conditions, the number for disrupted pollen grains was optimal at 360 mosmol/kg H_2O, whereas the sperm cell yield did not differ between 360 and 550 mosmol/kg H_2O, is possibly caused by disruption of a percentage of sperm cells at the lower osmolality. Therefore, the osmolality of the storage medium was set at 780 mosmol/kg H_2O, being the optimal osmolality used for pollen germination of perennial ryegrass (AHLOOWALIA 1973). At this osmolality, disruption of pollen grains during germination was minimal.

For sperm cell isolation from *Plumbago*, maize and spinach, a BKS medium was used for both isolation and storage. However, in the case of perennial ryegrass, the BKS medium proved to be inadequate in retaining the viability of the sperm cells after release from the pollen grains. Therefore a modified RY medium, which is a rich protoplast culture medium for rice (YAMADA et al. 1986) and perennial ryegrass (CREEMERS-MOLENAAR et al. 1989) was used as a storage medium. To improve the yield and the long-term viability, the addition of various combinations of vitamins E, C and fetal calf serum was tested. The fact that vitamin E alone did not improve the yield and long-term viability, is in agreement with negative results obtained with vitamin E to extend the longevity of pollen grains during dry storage (HOEKSTRA and BARTEN 1986). Vitamin C alone, an important biological antioxidant (LARSON 1988) did show a positive effect on the yield, whereas in combination with vitamin E also the long-term viability was improved. Interaction between vitamins E and C in plants as an antioxidative system was reported by FINCKH and KUNERT (1985) and consists of regenerating oxidized vitamin E by vitamin C. They also observed that when a low vitamin C to vitamin E ratio was found in the plant material, no protection to lipid peroxidation could be established. Their hypothesis that in that case the antioxidative potential of vitamin C is too low for efficient regeneration of vitamin E, might also apply to our negative results obtained with vitamin E alone.

The positive effect of fetal calf serum can be explained in several ways. Bovine serum albumin (BSA), which is a major component of fetal calf serum, could act as a substrate for proteases, which may be released during isolation of the sperm cells from the pollen grains, although the yield did not improve when fetal calf serum was

also present in the isolation medium. This function of BSA has been suggested as an explanation for the beneficial effect on yield and plating efficiency of potato mesophyll protoplasts (TAYLOR et al. 1989). Additionally, the yield and viability of isolated sperm cells of maize was found to be improved with BSA (ZHANG et al. 1992). Its possible function was thought to be a binder of free fatty acids released by cell rupture, that prevents membrane damage (LATIES and TREFFRY 1969; SOUTHWORTH and KNOZ 1988). When the effects of fetal calf serum and BSA on sperm cell yield were compared, no difference between BSA and fetal calf serum was observed (unpublished results). This indicates that at least part of the positive effect of fetal calf serum can be explained by a function of its major component BSA as a substrate for proteases or as a free fatty acid binder. Next to BSA, also the antioxidative enzyme superoxide dismutase was reported to be present in fetal calf serum (JOENJE 1989). Whether superoxide dismutase is one of the components of fetal calf serum that induces a positive effect on the viability of the isolated sperm cell population needs further investigation.

Both during isolation as well as during storage, vitamins E, C and fetal calf serum were found to be important in maintaining the viability of the sperm cells. Although the function of fetal calf serum is not completely clear, the positive effects of vitamins E and C without fetal calf serum as a synergistic antioxidative system and vitamin C separately, suggest that peroxidation occurs during isolation and storage of sperm cells and that sperm cells of perennial ryegrass are sensitive to lipid peroxidation. The suggested sensitivity of sperm cells of perennial ryegrass to oxidative damage induced after release from the pollen grain, is in agreement with the reported involvement of similar processes in the generally low desiccation tolerance of gramineous pollen (HOEKSTRA et al. 1989). Because vitamin E had no effect on the yield and the long-term viability in the presence of fetal calf serum, the optimal combination of vitamin C and fetal calf serum will be used in further research on isolated sperm cells.

From the absence of cellulose and callose, it can be concluded that sperm cells of perennial ryegrass are gametoplasts as was found for isolated sperm cells from other species (RUSSELL 1991).

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Chapter 3

Cytological characterization of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.)

Heleen M. van der Maas, Eliza R. de Jong, Adriaan C. van Aelst, Harry A. Verhoeven, Frans A. Krens and Jac L. van Went

Summary. The cytoplasmic content and the distribution of intramembrane particles (IMPs) of the plasma membrane of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.) have been characterized using flow cytometry, transmission electron microscopy, confocal scanning laser microscopy and freeze-fracture study. The isolated haploid sperm cells contain a variety of cell organelles with the exception of microtubules. Proplastids and plastids with starch were observed, although only rarely. Vacuoles containing remnants of organelles and stacked lamellae of endoplasmatic reticulum with cytoplasmic inclusions were observed frequently, indicating that autophagy takes place. The number of mitochondria varies from 11 to 26 with an average of 17. Generally, the nucleus has a lobed shape and displays various interphasic stages of chromatin condensation. The analysis of the number of mitochondria and the nuclear morphology did not show evidence of sperm cell dimorphism. The cytological variability observed, could be explained by differences in developmental stages already present *in situ* at the moment of isolation. No correlation between the number of mitochondria and the nuclear median-section area and/or the condensation state of the chromatin could be found. The density of intramembrane particles of the plasma membrane on the exoplasmic fracture face is more than twice that on the protoplasmic fracture face. That is the opposite to what was found for sporophytic cells of perennial ryegrass.

Introduction

In angiosperms the mature pollen grain is either bicellular, consisting of a vegetative cell and a generative cell, or tricellular containing a vegetative cell and two sperm cells. The pollen of perennial ryegrass (*Lolium perenne* L.) is tricellular, like most species of the Poaceae (Brewbaker 1967). Ultrastructural analyses of sperm cells of several species of the Poaceae such as barley (Cass 1973; Charzynska et al. 1988), wheat (Zhu et al. 1980), and maize (McConchie et al. 1987a; Cass and Fabi 1988; Wagner and Dumas 1989; Mogensen et al. 1990) have shown that the cytoplasm contains an assortment of cell organelles. However, microtubules and plastids have never been observed. For *Plumbago zeylanica* (Russell 1984) and two *Brassica* species (McConchie et al. 1987b), a dimorphism between the two sperm cells of one pollen grain has been observed with respect to the number of proplastids and mitochondria, whereas for other species such as barley (Mogensen and Rusche 1985), maize (Wagner and Dumas 1989) and spinach (Theunis 1992) no indications of such a dimorphism could be detected.

The plasma membrane is considered to play an important role in the functioning of sperm cells. As such cells are predestined to fuse with either the egg cell or the central cell of the female gametophyte during fertilization, the plasma membrane of the sperm cell may have specific features related to this fusion process. In this regard, *in vitro* fusion of isolated male and female gametes of maize required only mild electrofusion conditions to obtain a high fusion frequency (Kranz et al. 1991a), in comparison to fusion of isolated protoplasts. Furthermore, freeze fracture studies of isolated sperm cells of spinach have revealed that the exoplasmic fracture (EF) face contains more intramembrane particles (IMPs) than the protoplasmic fracture (PF) face (Van Aelst et al. 1990). In contrast, the plasma membrane of other kinds of plant cells such as sporophytic cells of potato (Wilkinson and Northcote 1980) and vegetative cells of pollen of several species (Platt-Aloia et al. 1986, Kerhoas et al. 1987), contain more particles on the PF side.

For perennial ryegrass procedures for isolation of sperm cells (this thesis, chapter 2) and egg cells (this thesis chapter 4) have already been developed. For future fusion experiments aimed at cellular modifications and DNA transfer or for studying developmental processes during fertilization and embryogenesis, it is important first to obtain insight into the ploidy level of the nucleus, the presence or absence of a cell wall and the presence and numbers of DNA-containing organelles. Consequently, a multidisciplinary cytological characterization of isolated sperm cells of perennial ryegrass has been performed.

Methods

Plant material

Two diploid (2n) varieties of perennial ryegrass (*Lolium perenne* L.), Peramo and Tranie were used, which differ in their period of anthesis, being respectively early and late. Plants were grown in a greenhouse under a light/dark cycle of 14/10 h with controlled temperature conditions of 17°/12°C. Sperm cell isolation was performed as described in chapter 2, this thesis. Isolated sperm cells were suspended in sperm cell storage medium (this thesis, chapter 2) and kept at 4°C until use. The FCR assay as described by Heslop-Harrison et al. (1984), was used to assess the viability of the isolated sperm cells.

Determination of the ploidy level using flow cytometry

For confirmation of the haploid ploidy level of the sperm cells, the DNA content of the sperm cell fraction was determined. Young leaf material of the same varieties from which the sperm cells were isolated were used as a reference to determine the nuclear DNA content of diploid cells. Leaf material was chopped in a nucleus extraction buffer according to Verhoeven (1989) and then sieved over a 15 µm gauze. The suspension of isolated sperm cells was diluted 1:8 with the nucleus extraction buffer. Samples were analyzed using a Partec Pas-II flow cytometer according to Verhoeven (1989). DNA contents were expressed in arbitrary C values, using diploid plants as a reference for the 2C level.

Characterization of cytoplasmic organelles using transmission electron microscopy

Isolated sperm cells were fixed in 3% (v/v) glutaraldehyde according Theunis (1990) dissolved in sperm cell storage medium (this thesis, chapter 2). Subsequently, the material was rinsed 3 times with 66 mM cacodylate buffer supplemented with 20% (w/v) sucrose, pH 7.2 at 4°C. The material was post fixed in 1% (w/v) OsO_4 dissolved in cacodylate buffer, pH 7.2, for 1 hour at 21°C. Following three rinses of 10 minutes with distilled water, the material was dehydrated through an acetone series and embedded in Spurr's resin. Ultrathin sections were cut using an LKB ultramicrotome and mounted on Formvar-coated mesh grids. The sections were stained with uranyl acetate and lead citrate and examined using a JEOL 1200 transmission electron microscope.

Determination of cell diameter, number of mitochondria, nuclear median-section area and nuclear condensation state using confocal scanning laser microscopy

Isolated sperm cells were embedded in a thin layer of sperm cell storage medium (this thesis, chapter 2) solidified with 0.8% (w/v) low melting agarose on a cover slip coated with a solution of 96% (v/v) ethanol and 1.6% (w/v) low melting agarose (1:1) according to VERHOEVEN et al. (1990). Mitochondria were visualized by staining with 10 µg/ml 3,3'-dihexyloxacarbocyanine iodide (DIOC6(3)) according to MATZKE and MATZKE (1986). Cell organelle movement was blocked using 10 µg/ml cytochalasin B according to VERHOEVEN and BLAAS (1992). All analyses were performed with a MCR 500 confocal scanning laser microscope mounted on a ZEISS ICM405 inverted microscope, equipped with a computer-controlled stage (VERHOEVEN et al. 1990). Excitation of DIOC6(3) was achieved using 2.5 mW at 488 nm and analyzed using the A2 ficteroic mirror/filter combination. Ethidium bromide was excited using 2.5 mW, 514 nm light, with a 9HS filter. Embedded sperm cells were observed with phase contrast microscopy according to MATTHYS-ROCHON et al. (1987) and the coordinates of their positions were stored using a computer-controlled cell finder system according to VERHOEVEN et al. (1990).

DIOC6(3) fluorescence. The positions of the sperm cells were relocated and the DIOC6(3) fluorescence of the sperm cells was determined with 4 scans at equidistant planes of 1.5 µm through the sperm cell. The scans were digitally stored for later analysis of the number of mitochondria (n=20) and diameter of the sperm cell (n=40). The diameter of each analysed sperm cell was determined from the scan with the largest diameter.

Ethidium bromide fluorescence. For examination of the nuclear median-section area and condensation state, 100 µl nucleus extraction buffer containing 5 µg/ml ethidium bromide (VERHOEVEN 1989) was pipetted onto the thin agarose layer containing the sperm cells and was incubated for 30 minutes at room temperature. The positions of the sperm cells were again relocated and the ethidium bromide fluorescence was determined for the median-section scan of the nucleus (n=40). The nuclear condensation state was determined by quantification of the fluorescence intensity of the ethidium bromide staining and was expressed in arbitrary fluorescence units (FU). Additionally, the median-section area of the fluorescence was measured. For analysis of sperm cell nuclei within the pollen grain, pollen from florets just before anthesis were released by gentle disruption of the anthers in a modified sperm cell isolation medium (this thesis, chapter 2) at 4°C, pH 5.5. The pollen suspension was mixed with an equal volume of nucleus extraction buffer containing 5 µg/ml

ethidiumbromide. The fluorescence intensity and mean cross-section area of the nucleus was determined by 4 scans at equidistant planes of 2 μm through the sperm cells (n=18). The data of the 4 scans per sperm cell were then averaged. The fluorescence intensity of the sperm cell nuclei could only be used to compare the nuclear condensation state of two sperm cells inside the same pollen grain, because of auto fluorescence of the pollen grain wall and the varying positions of sperm cells inside different pollen grains.

The digitally stored scans were analyzed using the image analyzing computer programme of the MRC 500 confocal scanning laser microscope. Correlation coefficients between the measured properties were calculated. The variation between the mean nuclear cross-section areas of two sperm cells within a pollen grain was compared to the variation in nuclear mean-section areas of the sperm cells from different pollen grains using a one-way analysis of variance.

Determination of the distribution of intramembrane particles (IMPS) in the plasma membrane using freeze-fracture

The distribution of IMPS in the plasma membranes of isolated sperm cells and sporophytic cells was analyzed. The sporophytic cells originated from a non embryogenic cell suspension culture which was initiated from mature embryos of perennial ryegrass (CREEMERS-MOLENAAR et al. 1989). Isolated sperm cells and suspension cells were freeze-fractured in, respectively, storage medium (this thesis, chapter 2) which contained 18% (w/v) sucrose, and cell suspension culture medium containing 3% sucrose (CREEMERS-MOLENAAR et al. 1989). Freeze-fracturing was performed according to VAN AELST et al. (1990) using a BAF 400 (Balzers). The replicas were rinsed with an increasing concentration (≤ 10% (w/v)) of sodium hypochlorite and subsequently with distilled water. The replicas were observed using a JEOL 1200 transmission electron microscope. For each replica the average number of IMPS/μm^2 plasma membrane was calculated by averaging 5 random counts. Seven sperm cells and 3 cell suspension cells were analyzed. The freeze-fracture nomenclature follows Branton et al. (1975).

RESULTS

Determination of ploidy level

Using flow cytometry, the DNA content of isolated sperm cells was compared to the DNA content of sporophytic cells originated from young diploid leaves (*fig.* 1). The

histogram of the DNA content of leaf nuclei shows two peaks corresponding to the 2C and 4C DNA level (*fig.* 1a). The 2C peak consists of diploid nuclei at the G1 stage of the cell cycle, whereas the 4C peak consists of diploid nuclei in the G2 phase. The histogram of the isolated sperm cells shows a single peak (*fig.* 1b), indicating that the sperm cell isolate was pure and not contaminated with sporophytic cells. In *fig.* 1C, the histogram of a mixture of sperm cells and diploid leaf cells is shown. The position of the peak for sperm cell nuclei corresponds to a DNA content equivalent to 1C value, as determined by its position relative to that of the 2C peak of the diploid cells.

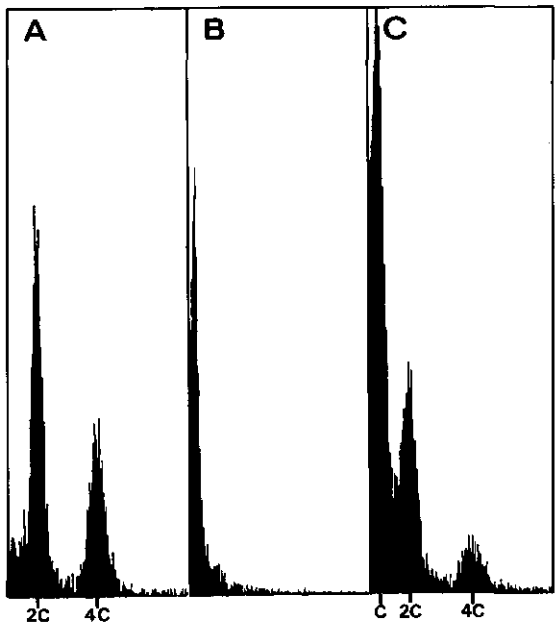
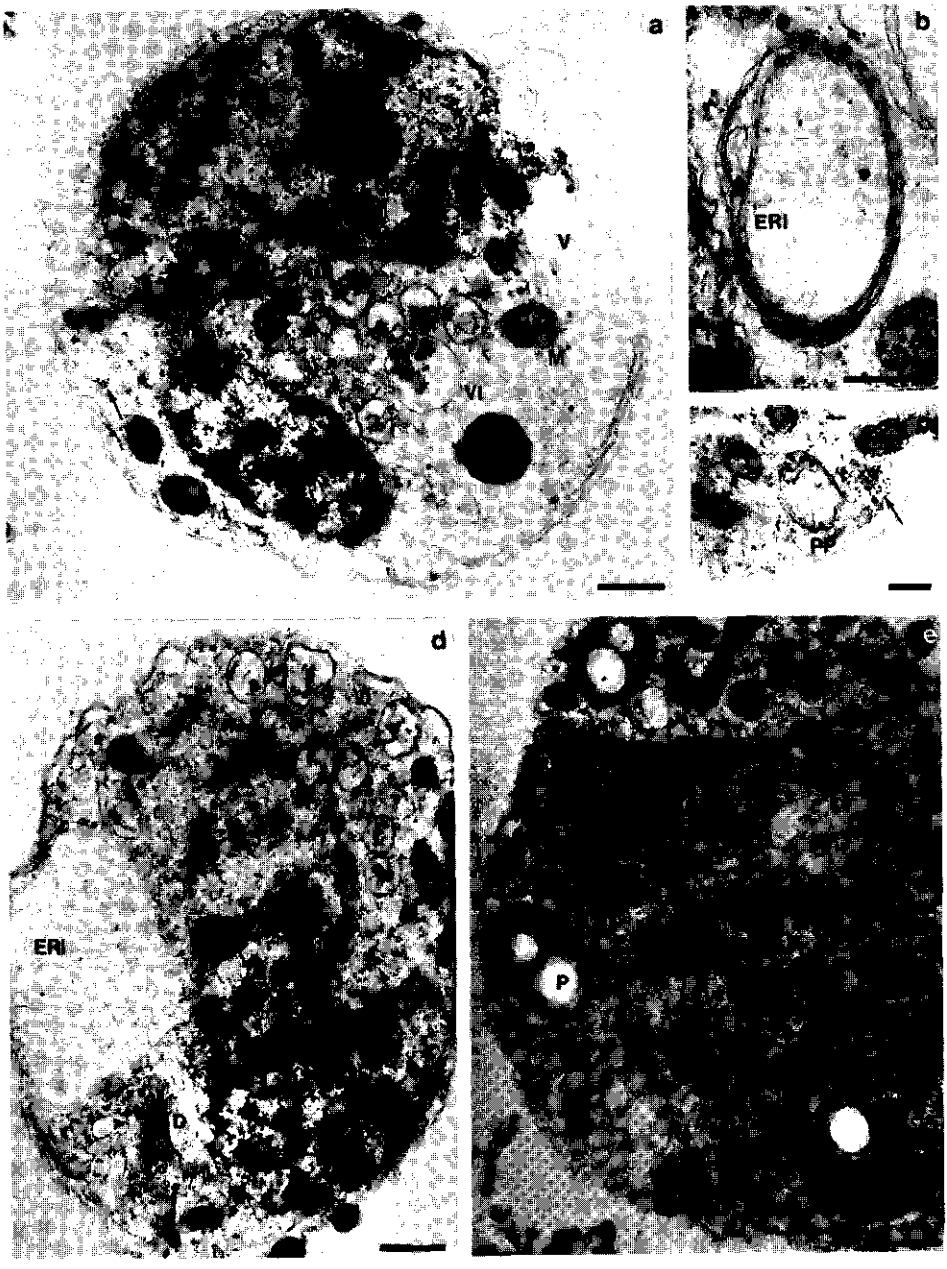


Fig. 1. Histograms of the DNA contents of isolated sperm cells and diploid young leaf tissue of perennial ryegrass (*Lolium perenne* L.). **a.** Diploid tissue of young leaves. **b.** Suspension of isolated sperm cells. **c.** A mixture of isolated sperm cells and diploid leaf tissue.

Fig. 2. Cell constituents of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.) observed by transmission electron microscopy. **a.** A section of a spherical isolated sperm cell indicating a nucleus (N) with a dense spherical nucleolus (NO), mitochondria (M), vacuoles with inclusions of degenerated organelles (VI), a large vacuole (V) and ribosomes (arrow). Bar=500 nm. **b.** Detail of a small cytoplasmic inclusion of stacked lamellae of endoplasmatic reticulum (ERI). Bar=200 nm. **c.** Detail of a proplastid (PP) and ribosomes (arrow). Bar=200 nm. **d.** Section of an isolated sperm cell indicating dictyosomes (D), vacuoles with inclusions of degenerated organelles (VI) and a large cytoplasmic inclusion of stacked lamellae of endoplasmatic reticulum (ERI). Bar=500 nm. **e.** Section of an isolated sperm cell indicating plastids with starch grains (P) and spherical and oblong mitochondria (M). Bar=500 nm.


a
V
M
VL
b
ERI
PP
d
ERI
D
e
P

Characterization of cell organelles

After isolation from the pollen grains, the sperm cell shape changes from a spindle-like, which is the natural form within the pollen grain, to a spherical shape in liquid medium (*fig.* 2a). Using transmission electron microscopy, 50 sections of isolated sperm cells were examined. A cell wall was not observed. The nucleus is distinctly lobed and contains a spherical, condensed nucleolus (*fig.* 2a). The nuclear DNA displays interphasic stages of chromatin condensation (*fig.* 2a,d,e). Large vacuoles were only occasionally observed (*fig.* 2a). The mitochondria are spherical to oblong in shape (*fig.* 2a,e) and were observed in every section. Abundant ribosomes are scattered singly through the cytoplasm or are concentrated as polysomes (*fig.* 2a,c). The mainly smooth endoplasmatic reticulum is situated at the periphery of the sperm cell (*fig.* 2a) or is present as circular stacked lamellae, containing small or large cytoplasmic inclusions (*fig.* 2b,d). Dictyosomes, with or without vesicles, were infrequently observed (*fig.* 2d), whereas vacuoles containing inclusions of organellar remnants were detected in many sections (*fig.* 2a,d). Proplastids (*fig.* 2c) and plastids containing starch (*fig.* 2e) were detected, although only rarely, in respectively 3 and 1 sections. The presence of microtubules could not be demonstrated.

Determination of cell diameter, number of mitochondria, nuclear median-section area and nuclear condensation state

By storing position coordinates of embedded sperm cells using a computer controlled cell finder system, it was possible to combine the results of both a vital ($DIOC_6(3)$) staining, to visualize the mitochondria and plasma membrane, and a non vital (ethidium bromide) procedure to visualize subsequently the nucleus of the same sperm cell (*fig.* 3a). In this way, the number of mitochondria and the cell diameter could be related to the nuclear median-section area and the condensation state of the chromatin. Movement of mitochondria was blocked using cytochalasin B, which made determination of the number of mitochondria possible (*fig* 3b,c). The lobed shape of the nucleus, as observed using transmission electron microscopic analysis, was also detected after ethidium bromide staining using confocal scanning laser microscopy (*fig.* 3d,e). There is little variation in cell diameter among the measured sperm cells, whereas the number of mitochondria and the median-section area and condensation state of the nucleus show variability (*table* 1), which had a gradual character. A significant correlation coefficient between the measured properties ($P<0.001$) was only observed between the nuclear median-section area and the condensation state of the chromatin.

The nuclei of the two sperm cells within a single pollen grain, have a comparable condensation state (data not shown). Variation between mean nuclear cross-section areas of sperm cells between different pollen grains is considerably larger than that between the two nuclei within a single pollen grain ($P<0.001$).

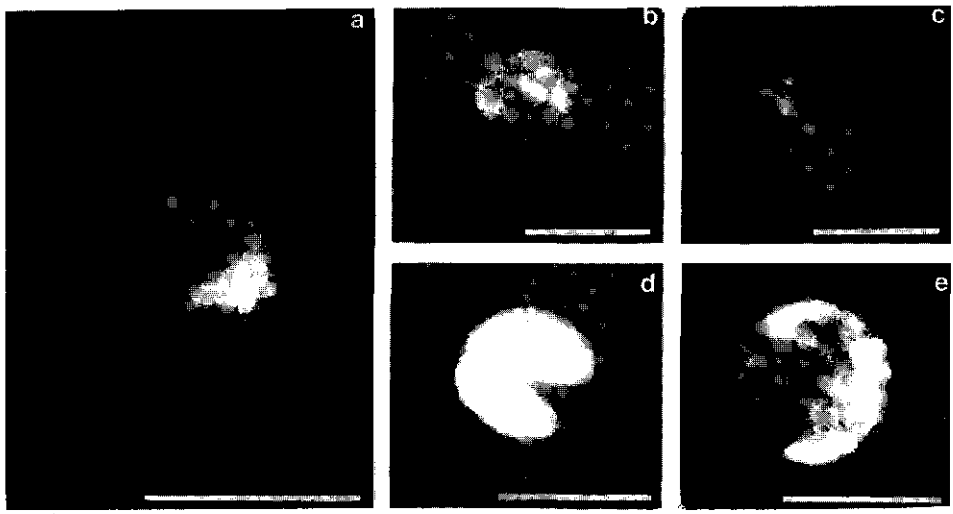


Fig. 3. Confocal scanning laser microscopy of isolated sperm cells of perennial ryegrass (*Lolium perenne* L.). Isolated sperm cells were subsequently stained with DIOC6(3) which visualized the mitochondria and plasma membrane, and with ethidium bromide which visualized the nucleus. a. Compilation of DIOC6(3) fluorescence (yellow) and ethidium bromide fluorescence (red) of the same sperm cell. b.,c. Scan of DIOC6(3) fluorescence for analysis of the number of mitochondria per sperm cell. d.,e. Scan of ethidium bromide fluorescence to determine the nuclear median-section area and condensation state. Bars=6 µm.

Table 1. Confocal scanning laser microscopic analysis of sperm cells of perennial ryegrass (*Lolium perenne* L.).

		x ± SE	n	min - max
isolated sperm cells	diameter (µm)	6.1 ± 0.1	40	5.4 - 6.8
	number of mitochondria	17 ± 1	20	11 - 26
	nuclear median-section area (µm²)	13.6 ± 0.6	36	8.5 - 25.1
	nuclear condensation state (FU)[a]	148 ± 6	36	82 - 206
non isolated sperm cells	mean nuclear cross-section area (µm²)	6.8 ± 0.5	18	3.2 - 9.9

[a] FU = fluorescence units, an arbitrary value to express the nuclear condensation state, which was measured by the stain intensity of the nucleus.

Determination of the distribution of IMPs in the plasma membrane

The distribution of IMPs in the plasma membranes of the isolated sperm cells and sporophytic suspension cells was compared. The IMPs are regularly dispersed both on the exoplasmic fracture (EF) and protoplasmic fracture (PF) faces of both sperm and suspension cells (*fig.* 4). However, the density on the EF face of sperm cells is more than two times higher than that on the PF face. In contrast, for the sporophytic cells the opposite was found (*fig.* 5).

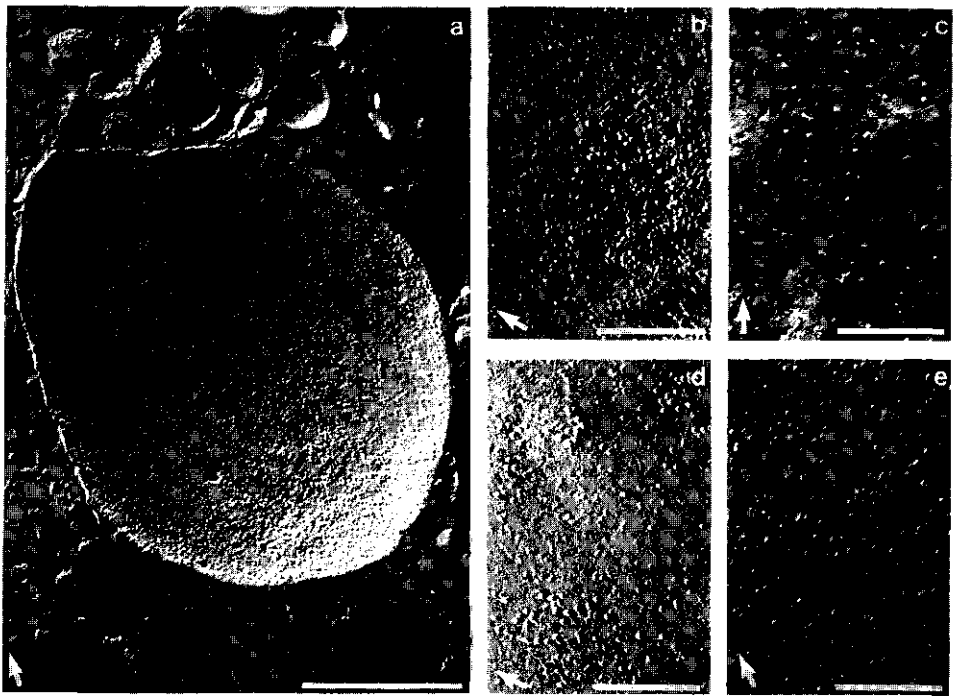


Fig. 4. Freeze-fracture study of the distribution of IMPs in the PF and EF face of the plasma membranes of isolated sperm cells and sporophytic cells of perennial ryegrass (*Lolium perenne* L.). Arrows in lower left-hand corner indicate the direction of shadowing. **a.** EF face of an isolated sperm cell. Bar=500 nm. **b.** Detail of EF face of an isolated sperm cell. Bar=200 nm. **c.** Detail of EF face of a sporophytic cell. Bar=200 nm. **d.** Detail of PF face of an isolated sperm cell. Bar=200 nm. **e.** Detail of PF face of a sporophytic cell. Bar=200 nm.

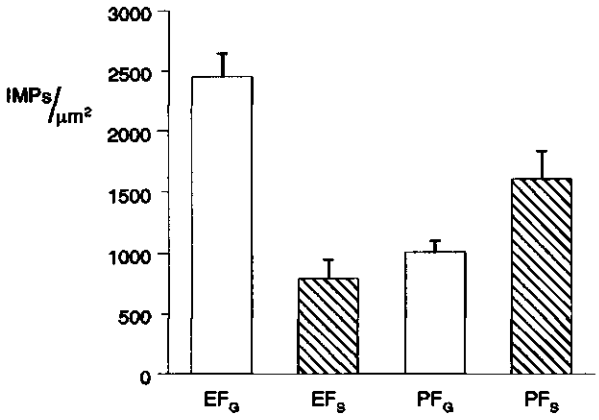


Fig. 5. Number of IMPs per µm² (IMPs/µm²) plasma membrane of the EF and PF face of sperm cells (gametophytic, (G)) and sporophytic (S) cells of perennial ryegrass (*Lolium perenne* L.). The histogram shows average values with their standard errors.

Discussion

Using flow cytometry we have been able to confirm that our previously developed procedure for the isolation of sperm cells of perennial ryegrass (this thesis, chapter 2) yields a pure population of true haploid cells. While isolation includes preliminary squashing of dissected anthers, which could easily release sporophytic cells, it is clear that isolates are free from any contamination by diploid cells and thus requires no further purification before use.

The sperm cells contain a variety of cell constituents although microtubules could not be detected. Proplastids and plastids containing starch were observed, but only rarely. Proplastids have also been observed in sperm cells of several cereals (Hagemann and Schröder 1984), but so far, plastids containing starch grains have never been reported in sperm cells of any angiosperm species. Since the plastids were only observed in one section, it can be concluded that in perennial ryegrass their presence is extremely rare. The absence of microtubules is possibly the primary cause of the shape transformation of the sperm cells from being spindle-shaped inside the pollen grain to being spherical after isolation. Isolated lily generative cells lacking microtubules also become spherical after isolation, despite the presence in this case of a cell wall (Tanaka et al. 1989). The absence of a cell wall around sperm cells of perennial ryegrass, which has been determined by histochemical analysis of viable, isolated sperm cells (this thesis, chapter 2), might be due to loss of wall components during isolation and manipulation, since the sperm cell wall is supposed to be sensitive to treatments such as fixation or isolation (Van Aelst and Van Went 1992).

It is known that in a number of species, during maturation of the sperm cells, a reduction in organelle number and cytoplasmic content occurs, additionally to condensation of the nucleus (CLAUHS and GRUN 1977; WILMS and LEFERINK-TEN KLOOSTER 1983; MOGENSEN and RUSCHE 1985). For sperm cells of barley it has been observed that both cytoplasm and organelle reduction is achieved by vesiculation and pinching off from the plasma membrane (MOGENSEN and RUSCHE 1985). In perennial ryegrass, organelle and cytoplasm reduction appears to be achieved by autophagic activity as is suggested by the presence of vacuoles and stacked lamellae of endoplasmatic reticulum containing respectively, organellar remnants and cytoplasm. Such autophagy has also been observed in isolated sperm cells of maize (WAGNER and DUMAS 1989) and during pollen maturation in other species (CLAUHS and GRUN 1977; VAUGHN et al. 1980). Autophagy is a common feature of plant cells, undergoing extensive differentiation (MATILE 1975).

Isolated sperm cells of perennial ryegrass contain a less variable number of mitochondria (11-26), than isolated sperm cells of spinach (THEUNIS 1992) or maize (MOGENSEN et al. 1990). These species contain mitochondria numbers varying from 5 to 25 and from 7 to 74 respectively. Mainly interphasic stages of chromatin condensation were detected for the nuclei of isolated sperm cells of perennial ryegrass, although isolated sperm cells of maize (WAGNER and DUMAS 1989) and spinach (THEUNIS 1990) were found to contain either heterochromatic or euchromatic nuclei. Furthermore, sperm cell nuclei of different pollen grains show a noteworthy variation in the mean nuclear cross-section area, whereas sperm cells of the same pollen grain display no difference in mean nuclear cross-section area. For spinach it has been demonstrated that isolated sperm cells within the same pollen grain contain comparable numbers of mitochondria (THEUNIS 1992). Therefore, since organelle reduction and nuclear condensation take place during pollen maturation, the gradual variation observed in mitochondria number and nuclear condensation state are unlikely to be the result of sperm cell dimorphism, but rather may be the result of different developmental stages of the isolated sperm cells already existing in the plant material. This has also been suggested for isolated sperm cells of maize (WAGNER and DUMAS 1989; MOGENSEN et al. 1990)) and spinach (THEUNIS 1990; 1992). A significant correlation between the number of mitochondria and the nuclear condensation state was not observed. This suggests that organelle reduction and change in the nuclear condensation state, which occurs during maturation of the sperm cells, are two asynchronous processes in these cells. For cellular manipulation, e.g. fusion using sperm cells, it is still unknown whether the mitochondria and plastids present in the isolated sperm cells will be able to play a role in determining the cytoplasmic constitution of the fusion product. The organelles of the sperm cells, that were not

eleminated during pollen maturation, might have lost their DNA as has been reported for wheat; although sperm cells of wheat still contain structures like plastids and mitochondria (ZHU et al. 1980; HAGEMANN and SCHRÖDER 1984), mitochondrial and plastid nucleoids are no longer present after the second pollen mitosis (MIYAMURA et al. 1987).

The characterization of the IMP distribution in the sperm cell plasma membrane, revealed that the EF face contains more IMPS than the PF face, which is in agreement with what has been determined for isolated sperm cells of spinach (VAN AELST et al. 1990). This is in contrast to the arrangement of IMPS in the plasma membrane of other types of plant cells such as sporophytic cells of perennial ryegrass, sporophytic cells of potato (WILKINSON and NORTHCOTE 1980) and the vegetative cell of pollen of several species (PLATT-ALOIA et al. 1986; KERHOAS et al. 1987). In these cases more IMPS have been observed on the PF face. The resemblence of the plasma membranes of isolated sperm cells from a dicot (VAN AELST et al. 1990) and a monocot species, is an indication that a higher density of IMPS on the EF face may be a general feature of angiosperm sperm cells. This might suggest that IMPS on the EF face of the plasma membrane of sperm cells play a role in cell recognition or membrane fusion during fertilization.

From our results it can be concluded that the isolated sperm cell population has a high degree of purity and exhibits a moderate cytological variablity.

ACKNOWLEDGEMENTS

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CHAPTER 4

ISOLATION OF VIABLE EGG CELLS OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

HELEEN M. VAN DER MAAS, MONIQUE A.C.M. ZAAL, ELIZA R. DE JONG, FRANS A. KRENS and JAC L. VAN WENT

Summary. For isolation of viable egg cells of perennial ryegrass (*Lolium perenne* L.), three different techniques, squashing, enzymatic maceration and a combination of enzymatic maceration and mechanical manipulation, were tested. Only with the third method viable egg cells could be isolated. After enzyme incubation, ovules disintegrated into loose cells upon mechanical manipulation. The egg cells could be identified between the bulk of sporophytic cells derived from the macerated ovules. The morphology of the isolated egg cell corresponds to the morphology of the egg cell *in situ* and is comparable to the morphology of egg cells *in situ* of other monocotyledons and angiosperms. Two hours after isolation the egg cells were still viable. The developed protocol proved reproducible and the yield was determined at 10%.

INTRODUCTION

For development of an isolation procedure for egg cells of perennial ryegrass (*Lolium perenne* L.), it is important to obtain information about the morphology of the embryo sac, the female gametophyte. The mature embryo sac of perennial ryegrass belongs to the Polygonum type, consisting of an egg cell and two small synergids located at the micropylar end, a central cell and three giant antipodals at the chalazal

end of the ovule (ELGERSMA and SNIEZKO 1988), enclosed within a crassinucellate ovule.

Various techniques to isolate embryo sacs have been developed (reviewed in THEUNIS et al. 1991). Squashing of ovules to release the embryo sacs, has been mainly applied to fixed plant material for studying embryo sacs in Poaceae (D'CRUZ and REDDY 1967) or in combination with enzymatic maceration of unfixed material (WEBB and GUNNING 1990). ZHOU and YANG (1985) and HU et al. (1985) succeeded in developing an enzymatic maceration technique to isolate viable embryo sacs of several angiosperms. For each species, a precise combination of enzymes, pH, osmotic pressure, incubation temperature and time is required to isolate the embryo sac in a living state (ZHOU and YANG 1985). With this knowledge, enzymatic methods to isolate viable embryo sacs have been developed for a spectrum of species (reviewed in THEUNIS et al. 1991). Isolated egg cells are obtained by a further enzymatic maceration of the isolated embryo sacs (MOL 1986; HUANG and RUSSELL 1989; WAGNER et al. 1989; VAN WENT and KWEE 1990) or manual dissection (KRANZ et al. 1991a).

In this chapter the development of an egg cell isolation procedure is described. Squashing, enzymatic maceration and a combination of enzymatic maceration and mechanical manipulation of ovaries and ovules were employed. The morphology of the isolated viable egg cells is compared to the morphology of egg cells *in situ* of other monocotyledons and angiosperms in general.

MATERIAL AND METHODS

Plant material

Plants of an early and a late flowering variety of perennial ryegrass (*Lolium perenne* L.), respectively Peramo and Tranie, were grown in a greenhouse under a light/dark cycle of 14/10 h at controlled temperature conditions of 17°/12°C. Spikes were collected at the beginning of flowering and stored at 4°C until use. Florets just before flowering were used to dissect the ovaries. For development of an isolation method of viable egg cells, the use of incised ovaries and intact and incised ovules was compared.

Cytological characterization of the embryo sac

For analysis of cell wall material of the embryo sac, ovules were fixed in FPA (formalin - propionic acid - 50% ethanol, 1:1:18 mixed by volume) according to HUANG and RUSSELL (1989). After rehydration, the ovules were stained with Calcofluor White

and Analine Blue dissolved in distilled water (TANAKA 1988) and Auramine O (HESLOP-HARRISON 1977). After gently squashing of the ovule to release the embryo sac, the cell wall of the embryo sac was observed with UV microscopy.

In situ embryo sac morphology

For *in situ* observation of unfixed embryo sacs, ovules were carefully mounted in a drop of medium on a slide, with the placental attachment side of the ovule upwards and gently covered with a cover slip. Observations were made with bright field and Nomarski interference contrast microscopy. The medium consisted of CPW salts according to FREARSON et al. (1973), and supplemented with 0.7 M mannitol (780 mosmol/kg H_2O), 5 mM MES (2(N-morpholino)ethane sulphonic acid), 0.5% (w/v) BSA (bovine serum albumin) at pH 6.0.

Egg cell isolation

Three different methods for egg cell isolation, squashing, enzymatic maceration and a combination of enzymatic maceration and mechanical manipulation, were tested. For the first 2 methods the viability of isolated embryo sac constituents was assessed according to HESLOP-HARRISON et al (1984) in relation to a number of parameters, such as osmolality, pH, salt mixture of the medium and incubation time and temperature. The osmolality of the medium was varied from 99 to 1000 mosmol/kg H_2O with sucrose or mannitol. Different concentrations of $CaCl_2$ and potassium dextran sulphate and the salt mixtures of BKS (BREWBAKER and KWACK 1963), RY (YAMADA et al. 1986) and CPW medium (FREARSON et al. 1973) were analyzed, and the pH was varied in a range from 4.0 to 6.0. The temperature and length of the incubation period were varied respectively from 4 to 30°C and from 10 minutes to 16-18 hours.

Squashing. Ovules, intact or incised, and incised ovaries were mounted in 1 ml of medium followed by gently squashing.

Enzymatic maceration. Ovules, intact or incised, and incised ovaries were incubated in different combinations of hemicellulase (SIGMA), cellulase R10, cellulase "ONOZUKA" RS (YAKULT), macerozyme R10, driselase (SIGMA) pectinase (SERVA), pectolyase Y23 (SEISHIN) and cytohelicase (SIGMA) in concentrations varying from 0.01 - 2% (w/v).

Combination of enzymatic maceration and mechanical manipulation. This isolation procedure was derived from the egg cell isolation technique described for maize by KRANZ et al. (1991a). 20 ovules were incubated for 10 - 15 minutes at 24°C and 40 minutes at 4°C in 1.5 ml of the enzyme mixture used for maize, with 0.5% (w/v) pec-

tinase (SERVA), 0.2% (w/v) pectolyase Y23 (SEISHIN), 0.3% (w/v) hemicellulase (SIGMA) and 0.3% (w/v) cellulase "ONOZUKA" RS (YAKULT) supplemented with 0.7 M mannitol (780 mosmol/kgH_2O), CPW salts (FREARSON et al. 1973), 5 mM MES and 0.3% (w/v) BSA, pH 5.5. The conditions for osmolality, pH and salt mixture were derived from results obtained with the first 2 methods (*table 1*). After enzyme incubation, the maceration medium was replaced by the medium that was also used for *in situ* observation. Subsequently, the macerated ovules were pierced with a steel needle and gently shaken to release the ovule content into the medium.

After isolation, the suspension of plant material, consisting mainly of tissue, loose cells and protoplasts originating from ovaries and/or ovules was analyzed for the presence of egg cells, synergids, antipodals and central cells, using bright field microscopy at a magnification of 160×. The yield was determined by dividing the number of isolated egg cells or other embryo sac constituents by the number of macerated ovules times 100%.

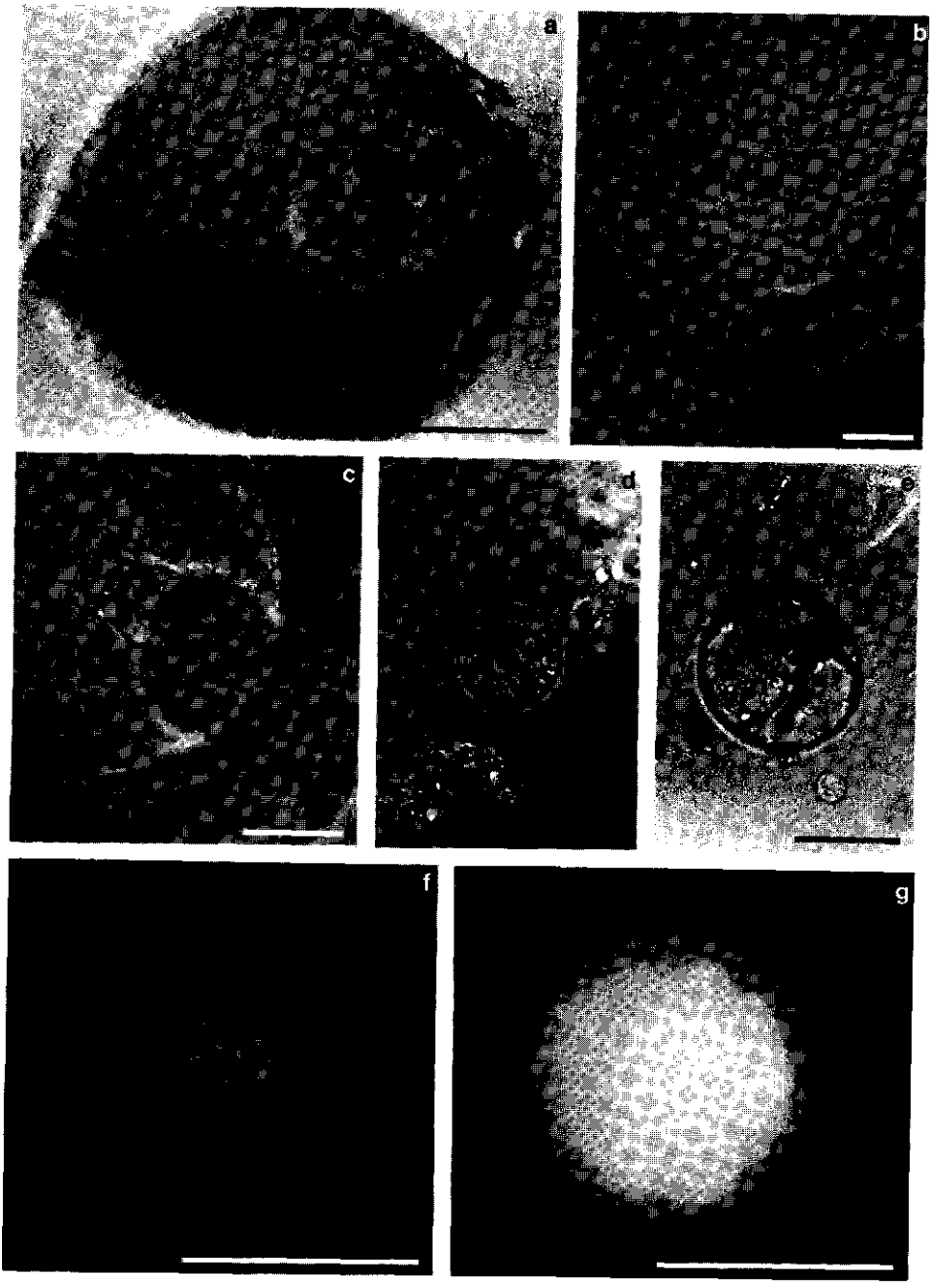
Cytological characterization of the isolated, non fixed embryo sac constituents

The maceration medium was replaced by a staining solution prepared in the medium that was also used for *in situ* observation, with the pH adjusted to 6.0. The fluorochromatic reaction (FCR) based on fluorescein diacetate was used to determine the viability of the isolated gametophytic cells (HESLOP-HARRISON et al. 1984). The egg cell nucleus was visualized by staining for DNA using a 20 minutes incubation with 40 µg/ml HOECHST 33342. Observations were made with UV-microscopy.

RESULTS

Ovules could be easily dissected from the ovaries. For histochemical analysis of the wall material of the embryo sac, fixation of the ovules was necessary to allow penetration of the stain through the ovular tissue. By gentle squashing of the fixed ovule a

Fig. 1. Embryo sacs *in situ* and isolated egg cells of perennial ryegrass (*Lolium perenne* L.). a. Embryo sac *in situ*. Bar=400 µm. b. Egg apparatus and central cell *in situ*. Bar=50 µm. c. Egg apparatus and polar nuclei *in situ*. Bar=50 µm. d. Isolated egg cell. Bar=50 µm. e. Vacuolate sporophytic cell from the ovule. Bar=50 µm. f. Nucleus of isolated egg cell stained with HOECHST 33342. Bar=50 µm. g. Isolated FCR positive egg cell. Bar=50 µm. Figure a. and b. were obtained with Nomarski interference contrast microscopy, c., d and e. with bright field optics, f. and g. with UV-microscopy.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complete embryo sac or parts of it could be released from the ovular nucellus tissue. In the peripheral wall of the embryo sac the presence of cellulose and hemicellulose was demonstrated. Cutine was observed in between the two integumental layers, between the inner integumental layer and the nucellus tissue and along the micropyle of the ovule.

After mounting unfixed dissected ovules with the placental attachment side upwards on a slide, in approximately 50% of the 100 ovules studied, the embryo sac with its constituents was clearly visible (*fig. 1a*). The three prominent antipodals are situated at the chalazal end of the embryo sac. The central cell is highly vacuolate and has two partly fused polar nuclei, containing large vacuolate nucleoli, situated at the chalazal end of the egg apparatus (*fig.* 1b-c). At the micropylar side of the embryo sac the egg apparatus is situated (*fig.* 1b-c). The pear shaped egg cell contains granular cytoplasm (*fig* 1b) and the nucleus is located in the centre of the cell, showing a large nucleolus in which small vacuoles can be observed (*fig.* 1c). The contours of the synergids were faint and they could not be recognized as individual cells. Frequently, bursting of the synergids was observed.

For isolation of viable egg cells, the methods of squashing and enzymatic maceration were not successful. Squashing caused damage to the fragile embryo sac. Under optimized conditions only the envelope of the embryo sac could be easily isolated, although it was still attached to the micropyle of the ovule. The content of the embryo sac was no longer clearly distinguishable (*table* 1). Using the method of enzymatic maceration, tissue, loose cells and protoplasts derived from the macerated ovary and ovule tissue were dispersed throughout the medium, due to shaking during the enzyme incubation (*table* 1). To stop the maceration, the enzymes needed to be removed by washing. This could only be done by centrifugation. When the pellet was resuspended, a lot of tissue remained attached to the centrifuge tube wall and was subsequently lost. Under optimized conditions, clusters of antipodals with remnants of the central cell were isolated (*table* 1). For both methods sucrose and mannitol gave comparable results. From these experiments the required osmolality, pH and salt mixture were deduced for the method consisting of the combination of enzyme incubation and mechanical manipulation (*table* 1). Following enzyme incubation, the ovules disintegrated into loose cells upon mechanical manipulation. After 10 minutes incubation at 24°C and 40 minutes incubation at 4°C followed by mechanical dissection, 20% of the ovules yielded large vacuolate central cells or clusters of central cells with antipodals. The central cell disintegrated or shrunk rapidly after isolation. With BSA in the isolation medium, this deterioration could be delayed till 5 to 10 minutes after isolation. When 15 minutes of incubation at 24°C and 40 minutes of incubation at 4°C was applied, subsequent mechanical dissection resulted in the

Table 1. Conditions of analyzed parameters for isolation of egg cells of perennial ryegrass (*Lolium perenne* L.) with three tested methods

analyzed parameters	squashing	enzymatic maceration	enzymatic + mechanical
plant tissue	ovary without style, incised at micropylar end of ovule (variable numbers/ml)	ovary without style, incised at chalazal end of ovule (60/ml)	ovule (20/ml)
pH	5.5 - 6.0	5.0	5.5 - 6.0
osmolality (mosmol/kg$H_2$0)	370	780	780
mineral salts	CPW	CPW	CPW
other additives	none	none	0.5% BSA
incubation	none	0.1% cytohelicase, 0.2% pectinase, 0.06% hemicellulase, 0.06% cellulase R10, 0.06% pectolyase Y23, 25°C, 120 rpm, 16 hours	0.5% pectinase, 0.3% hemicellulase, 0.3% cellulase RS, 0.2% pectolyase Y23, 10'(A) - 15'(B) 24°C, 40' 4°C
treatment after incubation	none	enzyme mixture replaced by medium without enzymes via centrifugation and resuspension	enzyme mixture replaced by medium without enzymes by pipetting
yield	embryo sac envelope without structurally intact contents (50%)	clusters of antipodes (0-5%)	A10': central cell with antipodals(20%) B15': egg cells (10%)
viability	-	-	A:-/+-, B:+

isolation of single egg cells, whereas clusters of central cells and antipodals were no longer detectable. The egg cells (*fig.* 1d) could be clearly distinguished from the bulk of sporophytic cells. Generally, the sporophytic cells are vacuolate cells of which the nucleus contains two small nucleoli without vacuoles (*fig.* 1e). Egg cells were always detected as single spherical cells. The size (Ø 50-60 µm) and the granular appearance of the cytoplasm of the isolated egg cell correspond to the characteristics of the egg cell *in situ* as well as the centred nucleus containing one large nucleolus with small vacuoles. After DNA staining of the isolated egg cells, a large nucleus with a faint fluorescence was observed (*fig.* 1f). Two hours after isolation the egg cells were still viable as was demonstrated by positive FCR staining (*fig.* 1g). The yield was determined at 2 egg cells out of 20 ovules (10%) and was shown to be reproducible in three independent experiments, using 100 ovules.

Discussion

By observation of the embryo sac *in situ*, the morphology of the egg cell of perennial ryegrass could be studied to allow recognition of the egg cell after isolation. The egg cell *in situ* was recognized by its pear shape, and the presence of a centred nucleus carrying a large vacuolate nucleolus. This morphology has also been reported for other monocotyledons as maize (Diboll and Larson 1966), barley (Cass and Jensen 1970) and wheat (You and Jensen 1985).

For isolation of viable egg cells of perennial ryegrass squashing and enzymatic maceration were not successful, probably because of the harsh treatment or the many manipulations respectively. The observation that after fixation complete embryo sacs of perennial ryegrass or constituents could be isolated by squashing, as has been found for other species of the Poaceae (D'Cruz and Reddy 1967), suggests that a fresh embryo sac is much more fragile than a fixed embryo sac. Still, egg cells could be isolated with a combination of enzymatic maceration and mechanical manipulation of the dissected ovules, comparable to the technique which has been developed for maize (Kranz et al. 1991a), which is a much more gentle technique than the other two methods tested. The osmolality, pH and salt mixture of the medium combined with the length and temperature of the enzymatic incubation appeared to be crucial for obtaining viable egg cells of perennial ryegrass with the successful gentle procedure.

The result that after 10 minutes of incubation at 24°C clusters of a central cell and antipodals were isolated and after 15 minutes only egg cells were detected, might be explained by a higher concentration of cell wall material deposited at the micropylar

end of the egg cell, which is generally observed for egg cells of angiosperms (WILLEMSE and VAN WENT 1984). After 10 minutes of incubation the egg cell might still be attached to the micropylar end of the ovule, whereas contact between the central cell and the egg cell is broken. Additionally, after 15 minutes, clusters of central cells with antipodals are disintegrated and the egg cell is released from the micropylar end of the ovule.

BSA, an additive to the medium in the method consisting of enzymatic maceration and mechanical manipulation, was used to retain the structure of the isolated large vacuolate central cell and might also have positively effected the viability of the isolated egg cells. This needs further investigation, since no experiments of egg cell isolation with the third method were performed without BSA. Likewise, the two methods that were not successful in isolating viable embryo sac constituents (*table* 1), could possibly have scored better results if BSA would have been added. BSA has a beneficial effect on the yield and plating efficiency of potato mesophyll protoplasts (TAYLOR et al. 1989) and on the yield and viability of isolated sperm cells of maize (ZHANG et al. 1992) and perennial ryegrass (this thesis, chapter 2). It is suggested that BSA acts as a substrate for proteases that might be released during the isolation procedure, and that contaminate commercial enzyme preparations used for enzymatic maceration (TAYLOR et al. 1989). Also, it is thought that BSA prevents membrane damage by binding free fatty acids released by cell rupture (LATIES and TREFFRY 1969; SOUTHWORTH and KNOX 1988).

The isolated viable egg cell has similar features as the egg cell *in situ*. Although the egg cells were no longer attached to the embryo sac, and in spite of the fact that they became spherical shaped, they could be identified among the sporophytic cells originated from the macerated ovules, because of the specific cellular organization. The granular structure of the cytoplasm is probably due to starch grains as has also been reported for egg cells of barley (CASS and JENSEN 1970), maize (CHEBOTARU 1981; FAURE et al. 1993) and wheat (YOU and JENSEN 1985). The faint fluorescence of the nucleus after DNA staining indicates the presence of a large nucleus with decondensed DNA, which is a characteristic feature of the nucleus of egg cells in angiosperms in general (WILLEMSE and VAN WENT 1984).

Exclusion of the possibility that the isolated cells were synergids instead of egg cells, is argued by the observed disruption of synergids, detected during *in situ* embryo sac observation. Also, in embryo sacs of perennial ryegrass which were fixed before pollination, only remnants of synergids were observed (ELGERSMA and SNIEZKO 1988, unpublished results). Lack of intact synergids before pollination was also reported for wheat, and it has been suggested that the synergids are sensitive to the employed experimental conditions (YOU and JENSEN 1985). Furthermore, the

presence of vacuolate nucleoli is only observed in the nuclei of the egg and central cell and not in the synergids of the embryo sac in maize (DIBOLL and LARSON 1966), barley (CASS and JENSEN 1970), wheat (YOU and JENSEN 1985) and angiosperms in general (WILLEMSE and VAN WENT 1984).

Since the isolated viable cells demonstrate the same morphological features as the egg cell *in situ*, and since they have characteristics specific for the egg cells of other monocotyledons and angiosperms in general, it was concluded that viable egg cells of perennial ryegrass were isolated.

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Chapter 5

Gametosomatic cybridization experiments using sperm cells and cytoplasts of perennial ryegrass (*Lolium perenne* L.)

Heleen M. van der Maas and Eliza R. de Jong

Summary. Mass PEG fusion between sperm cells and cytoplasts of perennial ryegrass (*Lolium perenne* L.) was tried in order to develop a method for obtaining new nucleus/cytoplasm combinations. Major obstacles were encountered in concentrating the cytoplasts due to their low specific gravity and in purification of the sperm cell fraction, removing cell remnants. Agglutination and fusion between cytoplasts were observed. However, probably due to the adherence of sperm cells to cell remnants present in the sperm cell fraction, fusion of sperm cells with cytoplasts or among sperm cells could never be examined. The most likely option for fusion of sperm cells and cytoplasts of perennial ryegrass will be microfusion (1 to 1).

Introduction

To obtain new nucleus/cytoplasm combinations in perennial ryegrass (*Lolium perenne* L.) varieties for e.g. transfer of the trait of cytoplasmic male sterility, somatic cybridization with isolated protoplasts can be employed, as was successfully performed by Creemers-Molenaar et al. (1992). An alternative could be the use of isolated sperm cells and cytoplasts. Mature sperm cells contain a small amount of cytoplasm with a low number of cell organelles compared to plant cells in general. This is

due to unequal distribution of cytoplasm during the first pollen mitosis in combination with disappearance of cell organelles from the gametes during maturation after the first and/or second pollen mitosis (WILMS and LEFERINK-TEN KLOOSTER 1983; MOGENSEN and RUSCHE 1985; MIYAMURA et al. 1987; CHARZYNSKA et al. 1988). Also in isolated sperm cells of perennial ryegrass a relative low number of mitochondria was found (this thesis, *chapter* 3), probably without DNA (MIYAMURA et al. 1987). This feature might render isolated sperm cells particularly suited as nucleus donors in cybridization experiments with cytoplasts.

Isolated male gametoplasts from different stages of pollen development have been used in fusion studies. Gametoplasts of tetrad microspores have been fused with mesophyll protoplasts (PIRRIE and POWER 1986; LEE and POWER 1988; PENTAL et al. 1989), gametoplasts of generative cells and vegetative cells were fused with each other (UEDA et al. 1990), and gametoplasts of sperm cells have been fused to cytoplasts from mesophyll protoplasts and to egg cells (KRANZ et al. 1991b; KRANZ et al. 1991a). So far, cell divisions in fusion products and regeneration into plants have been obtained with the sperm cell - egg cell fusion products (KRANZ et al. unpublished results 1993) and from the tetrad microspore - protoplast combinations. The regenerated plants from the tetrad microspore - protoplast fusions were found to be hybrids for both nuclear (PIRRIE and POWER 1986; LEE and POWER 1988) as well as cytoplasmic DNA (PENTAL et al. 1989).

Two techniques, resp. based on a chemical and electric treatment, have been developed for mass fusion of plant protoplasts. KAO et al. (1974) have reported the occurrence of high frequency of intergeneric fusion of plant protoplasts by a procedure with polyethylene glycol (PEG) to agglutinate the protoplasts followed by dilution of the PEG with a high Ca^{2+}/high pH solution, which presumably disturbs membrane charges and promotes the fusion of membranes in close contact (FISH et al. 1988). A protocol for fusion by electrical pulses has been developed by ZIMMERMANN and SCHEURICH (1981). Protoplasts become bipolar under high frequency alternating electric current, after which they migrate to regions of higher electric field intensity and attract each other, establishing close contact between plasma membrane sites. These protoplasts can be subjected to short direct current pulses inducing transient formation of pores that lead to fusion of contacting membranes (Fish et al. 1988). Since with protoplasts of perennial ryegrass the chemical method yields a higher frequency of viable fusion products (CREEMERS-MOLENAAR et al. 1992) than using electrofusion, this method was used.

Material and methods

Sperm cell isolation was performed according to the method described in this thesis, chapter 2. For cytoplast isolation the suspension culture LP9A of perennial ryegrass (*Lolium perenne* L.), initiated and maintained as described by Creemers-Molenaar et al. (1989), was used. The suspension culture was more than 3 years old and had lost its capacity to regenerate green shoots. Cytoplast isolation was performed according to Van Ark et al. (1992). The isolated sperm cells and cytoplasts were suspended in medium consisting of CPW salts according to Frearson et al. (1973) supplemented with 13% (w/v) mannitol (Creemers-Molenaar et al. 1989), in densities of respectively 1 - 1.4 x 10^6 and 3 - 5 x 10^5/0.1 ml. Fusion between isolated sperm cells and cytoplasts was performed according to Creemers-Molenaar et al. (1992). Beside the PEG solution according to Creemers-Molenaar et al. (1992) also PEG solutions with CPW-salts supplemented with sucrose concentrations in a range of 4 - 20% (w/v) were used. Fusion was performed in small drops of medium on a slide, in Eppendorf tubes or in 10 ml polystyrene tubes (Greiner). During the fusion procedure samples were taken for microscopic analysis. The FCR assay as described by Heslop-Harrison et al. (1984), was used to assess the viability of the sperm cells and cytoplasts.

Results and discussion

Because sperm cells and cytoplasts with a diameter of 4 - 7 µm, have a smaller volume than isolated protoplasts of perennial ryegrass, higher densities were needed than used for fusion of protoplasts of perennial ryegrass. Attempts to enhance the number of cytoplasts per volume by pelleting using centrifugation were not successful, probably because of the low specific gravity of the cytoplast due to the fact that they do not contain a nucleus. Also when KCl with a much lower molecular weight than mannitol was used as an osmoticum, no pellet could be recovered after centrifugation. The isolated sperm cell fraction, although purified using a discontinuous Percoll gradient, still contained cell organelles, probably mostly originating from the vegetative cell of the pollen grain, which is disrupted during sperm cell isolation (this thesis *chapter* 2). When the sperm cells were concentrated in a smaller volume, also the density of the cell remnants increased. In the presence of PEG, the sperm cells and the cell remnants strongly adhered together. Further purification of the sperm cell fraction with various Percoll gradients or filtration steps was impossible (unpublished results).

Cytoplasts survived the fusion treatment, whereas sperm cells did not survive the PEG treatment, unless CPW salts were added to the PEG solution. Salts in the medium were also essential for the viability of isolated gametoplasts and fusion products of generative cells and vegetative cells derived gametoplasts from lily, just as the use of sucrose instead of mannitol (UEDA et al. 1990). The viability of the sperm cells of perennial ryegrass was not improved using different sucrose concentration in the PEG solution during fusion. Still, medium containing sucrose instead of mannitol during the fusion procedure, might prove beneficial to sperm cell viability, since in earlier experiments comparing mannitol, maltose, sucrose and glucose in relation to sperm cell viability, the use of sucrose appeared to result in the best viability (unpublished results).

Agglutination and fusion among cytoplasts was observed. However, fusion of sperm cells with cytoplasts or among sperm cells was never found, probably due to the adherence of sperm cells and cell remnants generated by the sperm cell isolation procedure. The result that the sperm cells adhered to cell debris whereas the cytoplasts could fuse with each other, might be caused by a different plasma membrane constitution of sperm cells and cytoplasts. Freeze-fracture studies of isolated sperm cells and sporophytic suspension cells, from which the cytoplasts were isolated, revealed that isolated sperm cells contain substantial more intramembrane particles at the exoplasmic fracture face than sporophytic suspension cells (this thesis, chapter 3).

The inability to concentrate the cytoplasts and to purify the sperm cell fraction may also represent major obstacles for electrofusion. Another aspect that may interfere with electrofusion is the fact that cytoplasts remain floating and sperm cells do sink in the electrofusion medium due to difference in specific cell gravity (unpublished results). This could inhibit establishing close contact between plasma membranes of sperm cells and cytoplasts, which is a prerequisite for mass electrofusion between the two fusion partners. Therefore, the most likely option for fusion of sperm cell gametoplasts and sporophytic cytoplasts in perennial ryegrass, will be one to one fusion under microfusion conditions as also has been described in maize for gametosomatic fusion (KRANZ et al. 1991b).

If fusion between sperm cells and cytoplasts could be accomplished via microfusion, what will be the probability for cell division and eventually plant regeneration of the fusion product? In gametosomatic fusions between sperm cells and cytoplasts of maize no cell divisions occurred (KRANZ et al. 1991b). However, the protoplasts from which the cytoplasts were isolated, originated from mesophyll tissue and it is known that mesophyll protoplasts of gramineous crops are generally not able to divide under tissue culture conditions (VASIL 1987). This feature makes mesophyll protoplasts unsuitable as a source for cytoplasts in gametosomatic fusions aimed at

plant regeneration. Therefore, when cytoplasts that are isolated from an embryogenic cell suspension of perennial ryegrass are used, the probability to obtain plants from fusion products between sperm cells and cytoplasts will be increased. Additionally, it has been shown by UEDA et al. (1990), that in fusion products between gametoplasts of generative cells and vegetative cells, the morphology and further development of the generative cell nucleus becomes similar to that of a vegetative nucleus, probably induced by the incorporated vegetative cytoplasm. In the same way the physiological state of the sperm cell nucleus could be influenced by the cytoplasm of the cytoplast, by which cell division and regeneration might be induced.

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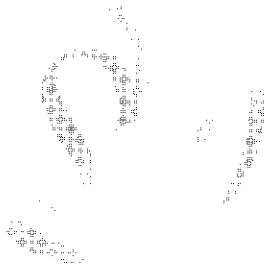
CHAPTER 6



DIRECT GENE TRANSFER AND EVALUATION OF EXPRESSION OF THE *GUSA* REPORTER GENE IN PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.)

HELEEN M. VAN DER MAAS, ELIZA R. DE JONG, MONIQUE A.C.M. ZAAL, SASKIA RUEB, LAMBERT A.M. HENSGENS and FRANS A. KRENS

Summary. Three different techniques of direct gene transfer, using the *hpt* selection and the *gusA* reporter gene, were tested for transformation potential of perennial ryegrass (*Lolium perenne* L.). The techniques were gene transfer to protoplasts using polyethylene glycol (PEG), embryo imbibition, and biolistic bombardment of seedlings and cell suspension cultures. PEG transformation yielded only false positives. Using embryo imbibition, seedlings from embryos treated with or without plasmid DNA exhibited endogenous GUS-like activity, which made analysis of DNA uptake by transient GUS expression impossible. However, stable and transient transformation was achieved using biolistic bombardment of seedlings and of a non embryogenic cell suspension culture. For stable transformation, bombarded suspension cells were transferred to selection medium with hygromycin 24 hours post bombardment. Hygromycin resistant callus lines were isolated at the first and second subculture respectively 4 and 8 weeks post bombardment. Using various hygromycin concentrations, the transformation frequency varied from 1 to 9 transgenic callus lines per bombardment (1.4×10^6 cells). Stable integration of the *hpt* and *gusA* genes into the plant genome was demonstrated by Southern analysis of DNA, isolated from hygromycin resistant callus lines. The *gusA* reporter gene, which was regulated by the constitutive promoter of the rice gene *GOS2*, was expressed in both transient and stable transformation assays, indicating that this promoter is suitable for expression of a transferred chimeric gene in perennial ryegrass. Callus lines with multiple copy integrations of the *gusA* gene did not demonstrate GUS expression. Long term GUS expression was observed in 50% of the analyzed transgenic callus lines.

Introduction

Over the last 10 years, several techniques for direct gene transfer aimed at producing transgenic plants from members of the Poaceae, have been developed. Using modified protocols for transformation of plant protoplasts by polyethylene glycol (Krens et al. 1982), Italian ryegrass (Potrykus et al. 1985), Guinea grass (Vasil et al. 1988), orchardgrass (Horn et al. 1988) and recently tall fescue (Wang et al. 1992) have been stably transformed. Only with orchardgrass and tall fescue transgenic plants were produced, but lack of vigour prevented the survival of transgenic orchardgrass plants (Conger 1991). Because of poor regeneration capacity of the protoplasts, this method did not lead to many successes in obtaining transgenic plants in grass species up till now.

More recently developed techniques are capable of inducing uptake of plasmid DNA through the cell wall of the plant cell, circumventing problems with low protoplast regeneration frequencies. By imbibition of dehydrated, viable embryos in a DNA solution, uptake of DNA and transient expression could be realized in wheat (Töpfer et al. 1989). Stable integration of the transferred DNA might then be achieved by transgenic callus induction on the imbibed embryos under selection conditions, eventually leading to regeneration of transgenic plants from the selected callus material.

Using high-velocity microprojectiles, nucleic acids can be delivered into plant cells (Klein et al. 1987). By this biolistic method, transgenic plants have been obtained from soybean (McCabe et al. 1988), maize (Gordon-Kamm et al. 1990), papaya (Fitch et al. 1990), cotton (Finer et al. 1990), rice (Christou et al. 1991), wheat (Vasil et al. 1992) and sugarcane (Bower and Birch 1992).

Aimed at transformation of perennial ryegrass (*Lolium perenne* L.), PEG transformation, embryo imbibition and biolistic bombardment were tested. Using biolistic bombardment, transient and stable expression of the *gusA* gene, regulated by the promoter of the constitutive rice gene *GOS2*, was analyzed in seedlings and cell suspension material. The organization of the integrated plasmid DNA and long term GUS expression in selected transgenic callus lines was studied and discussed.

MATERIAL AND METHODS

PEG transformation

Plant material

Protoplasts were isolated from logphase suspension cells of the fast growing suspension culture LP9A, which was initiated from mature embryos of perennial ryegrass (*Lolium perenne* L.) (CREEMERS-MOLENAAR et al. 1989). The cell suspension was over 2 years old and had lost its potential to regenerate green shoots. The cell suspension was weekly subcultured and grown in the dark at 25°C in culture medium, that is MS medium (MURASHIGE and SKOOG 1962) supplemented with 3% sucrose (w/v), 5 mg/l 2,4-D and 1 mg/l thiamine-HCl according to CREEMERS-MOLENAAR et al. (1989).

Direct gene transfer

The plasmid PLAMHH271 (HENSGENS et al. 1992), carrying the *hpt* (hygromycin B phospotransferase) gene (GRITZ and DAVIES 1983), which confers hygromycin resistance as a selection gene and the *gusA* (ß-glucurodinase) gene (JEFFERSON et al. 1987) as a reporter gene, was used for transformation. The *hpt* gene is under control of the 35SCAMV promoter and the nopaline synthase polyadenylation signal. The expression of the *gus*A gene is regulated by the 2' promoter of the octopine type Agrobacterium tumefaciens TR-DNA, and the terminator from the TR mannopine synthase 0' gene. The chimeric gene constructs have been subcloned in PIC plasmids (MARSH et al. 1984). Plasmid isolation was performed according the Birnboim method (SAMBROOK et al. 1989) followed by two to three phenol extractions until sufficiently pure (A260/A280 ratio).

The procedure is based on incubation of protoplasts with plasmid DNA in the presence of polyethylene glycol (PEG) and post incubation in a high salt solution, initially developed by KRENS et al. (1982). 7 x 10^5/ml protoplasts were shortly incubated with 10 µg plasmid DNA, followed by addition of 0.5 - 1 ml PEG (40% (w/v)) solution and further incubated for 20 - 30 minutes, at 24°C. Subsequently, the PEG was diluted with F-medium (KRENS et al. 1982), by pipetting 4 times 2 ml during 20 minutes. The treated protoplasts were centrifuged for 5 minutes at 700 rpm and washed in culture medium. Subsequently, the protoplasts were cultured according to CREEMERS-MOLENAAR et al. (1989). Using the protocol of Krens et al. (1982), different parameters, such as PEG concentration, PH value, plasmid concentration, carrier DNA (sheared and phenoled herring sperm DNA) and cations like Ca^{2+} and Mg^{2+} in the salt mixture, were evaluated. Furthermore, protocols developed by MEIJER et al. (1991)

and NEGRUTIU et al. (1988) were tested. The effects of all these parameters were analyzed in relation to the plating efficiency (determined according to CREEMERS-MOLENAAR et al. 1989), transient GUS expression, and stable transformation after employing selection conditions.

Selection of transgenic callus lines
Several selection schemes were investigated, varying from 20 - 40 mg/l hygromycin starting from 1 to 6 weeks after transformation treatment. After six weeks of culture, the selection medium consisted of suspension culture medium, solidified with 0.2% (w/v) gelrite and supplemented with hygromycin. At the second subculture the concentration of hygromycin was varied from 50 to 100 mg/l. From the third subculture, the selected callus material was maintained on selection medium with 100 mg/l hygromycin.

GUS assay
After 24 and 48 hours of culture, the protoplasts were separated from the culture medium by centrifugation. The pellets, containing the protoplasts, were stored at -80°C until use for assay of transient GUS expression. Presence of transient GUS expression in the protoplasts was assayed with a fluorometric assay according to Jefferson (1987) using a Fluoroskan II version 3.1.

Embryo imbibition

Plant material
Seeds from the variety Aurora of perennial ryegrass (*Lolium perenne* L.), a kind gift from BARENBRUG HOLLAND BV (Oosterhout, The Netherlands), were sterilized according to CREEMERS-MOLENAAR et al. (1989). Subsequently, embryos were dissected from the seeds and dried in a laminar flow cabinet overnight.

Direct gene transfer
The same plasmid as for PEG transformation was used. 80 - 100 embryos were incubated in 500 µl 0.1 SSC (sodium sodium citrate), 20% (v/v) DMSO (dimethylsulphoxide) and 100 µg plasmid DNA for 2 hours on a gyratory shaker 40 rpm, 28°C in the dark, according to TÖPFER et al. (1989). As a control the same number of embryos were incubated without DNA. Subsequently, the imbibed embryos were washed 3 times in 0.1 SSC and cultured at 24°C in the light (2000 lux with a 16 h/8 h light/dark period) on germination medium, consisting of MS medium (MURASHIGE and SKOOG

1962), solidified with 0.2% (w/v) gelrite and supplemented with 3% (w/v) sucrose, pH 5.8.

GUS *assay*

After 10 days, the developing seedlings were histochemically analyzed for GUS expression. Various protocols were tested to suppress endogenous GUS-like activity in the germinated seedlings. Best results were obtained using the X-gluc buffer according RUEB and HENSGENS (1989), pH 7.5 with 4 ml X-gluc buffer in 10 ml Falcon tubes for 48 hours at 25°C, placed on a rotating cell mixer (VAN DER MARK pers. comm.). Subsequently, the seedlings were bleached in a alcohol-series of increasing volume-percentages (JEFFERSON 1987) and the presence of blue staining was monitored with stereo microscopy and light microscopic observations of cut sections of bleached seedlings.

Biolistic bombardment

Plant material

Seeds and a fast growing cell suspension culture from the variety Aurora of perennial ryegrass (*Lolium perenne* L.), a kind gift from Barenbrug Holland BV (Oosterhout, The Netherlands), were used. Seeds were sterilized according to CREEMERS-MOLENAAR et al. (1989) and germinated on germination medium (see section on embryo imbibition). The cell suspension culture was initiated and cultured according to CREEMERS-MOLENAAR et al. (1989); it was over two years old and had lost its potential to regenerate green shoots (for detailed information on culture see section on PEG transformation). Three days after subculture, 0.25 g (fresh weight) logphase cells (1.4×10^6 cells) were evenly dispersed onto the surface of a Ø 42 mm filter disc (SCHLEICHER & SCHUELL #604). The medium was removed by vacuum filtration. Subsequently, 0.5 ml of fresh culture medium was added and the filters were placed onto culture medium solidified with 0.2% (w/v) gelrite and left overnight at 25°C in the dark. The next day the filters were used for bombardment.

For transient transformation experiments using seedlings, on the day of transformation, filters with suspension cells were transferred to germination medium and half of the filter surface was covered with 16 seedlings of 10 days old. Transient and stable transformation experiments, using cell suspension material, were performed with filters carrying only cell suspension material.

Direct gene transfer

The plasmid pORCEHyg constructed by HENSGENS et al. (1993), was used. This plasmid contains the *hpt* selection gene and the *gusA* reporter gene as in plasmid pLAMHH271, which was used in the other two applied direct gene transfer techniques. pORCEHyg has a different promoter region in front of the *gusA* gene, which originates from the constitutively expressed rice gene GOS2 (DE PATER et al. 1992) The terminator derived from the TR mannopine synthase 0' gene is the same as in pLAMHH271. The chimeric *hpt* gene construct is the same as in plasmid pLAMHH271 (see section on embryo imbibition). The gene constructs have been subcloned in pBluescript.

The biolistic bombardment protocol developed for rice by HENSGENS et al. (1993) was applied. Two independent experiments (A and B) were performed with different batches of the same cell suspension line and the same batch of seed. Plasmid DNA was adsorbed to tungsten particles according to HENSGENS et al. (1993) and for each bombardment 2 µl particle mix with 0.6 µg plasmid DNA was used. As controls, filters were bombarded using particles without DNA. After bombardment, the filters with suspension cells were transferred to solidified culture medium and the seedlings were kept on germination medium. The material was maintained in the dark at 25°C.

Selection of transgenic callus lines

One day post bombardment, the filters carrying suspension cells were transferred to selection medium. The filters of experiment A were placed onto a selection medium consisting of culture medium with 80 mg/l hygromycin solidified with 0.2% (w/v) gelrite and cultured for 1 week. Subsequently, the plant material was removed from the filters and carefully spread out onto selection medium with 150 mg/l hygromycin. The material of experiment B was directly spread out onto selection medium with 150 mg/l, one day post bombardment. The callus lines were subcultured every 4 weeks on selection medium with 150 mg/l hygromycin. Proliferating callus tissue was selected 4 and 8 weeks post bombardment and maintained separately.

GUS assay

Transient and stable expression of the *gusA* gene was histochemically determined according to RUEB and HENSGENS (1989). Transient expression in the bombarded seedlings was monitored 48 hours post bombardment (see section on embryo imbibition). For assaying transient expression in suspension cells, 24 hours after bombardment, 200 µl X-gluc buffer was pipetted onto the filter discs which were incubated for 2 hours at 37°C and 48 hours at 25°C. Subsequently, the tissue was scored for blue cell clusters, which indicated transient GUS expression. Expression of the *gusA* gene in selected hygromycin resistant callus lines was determined, using the same X-

gluc buffer and an incubation of 2 hours at 37°C and 48 hours at 25°C, on small pieces of callus tissue (Ø 5 mm) in a time period up to 1 year post bombardment.

Southern blot analysis

Total DNA was isolated from hygromycin resistant and control callus lines according to METTLER (1987). The control callus originated from bombarded control tissue unable to grow on selection medium and for DNA isolation subsequently maintained on culture medium. DNA was digested with a fivefold excess of *Eco*RI under conditions recommended by the supplier (AMERSHAM). 15 µg DNA was size-separated by electrophoresis on a 0.8% (w/v) agarose gel and transferred (SOUTHERN 1975) to Gene Screen Plus membranes, according to conditions recommended by the supplier (DUPONT). Prehybridization was performed for 1 hour at 65°C in 1% (w/v) SDS (sodium dodecyl sulphate), 1 M NaCl, 10% (w/v) dextransulphate and 100 µg/ml sheared herring sperm DNA. The membranes were hybridized in a hybridization mixture identical to the prehybridization mixture supplemented with a random-prime labelled (FEINBERG and VOGELSTEIN 1983) 32p-probe at 65°C for 24-48 hours. The membranes were washed in 2, 0.5 and 0.2 x SSC, 1% (w/v) SDS for respectively 5, 15 and 15 minutes at 65°C. Hybridization patterns were visualized by autoradiography using X-Omat AR film (Kodak). For rehybridization, the membranes were stripped with 0.1 x SSC, 1% (w/v) SDS for 30 minutes at 100°C. As probes the 1.0 kb *Bam*HI fragment of plasmid pLP90 carrying the *hpt* gene (GRITZ and DAVIES 1983) and the 1.8 kb *Bam*HI fragment containing the *gusA* gene from the plasmid pcaliGa, a kind gift from the Walbot Laboratory (Stanford University), were used.

RESULTS

PEG transformation

Varying the PEG concentration, it was found that 13.6% (w/v) PEG lowered protoplast survival to 50%, but increased the plating efficiency in comparison to untreated protoplasts. In further experiments this PEG concentration was applied. Also Ca^{2+} compared to Mg^{2+}, pH values of 6.5 and 7.0 compared to 5.6 and 6.0 and carrier DNA positively affected the plating efficiency. The method of KRENS et al. (1982) generally yielded higher plating efficiencies than the methods of NEGRUTIU et al. (1988) and MEIJER et al. (1991). Under conditions according to KRENS et al. (1982) the plating efficiency reached 0.5%.

Presence of transient GUS expression could not be demonstrated, 24 and 48 hours after transformation treatment. In 4 out of a total of 70 experiments, several callus lines could be isolated under selection conditions. These callus lines originated from experiments using the method of KRENS et al. (1982) at pH 6.0, 6.5 and 7.0 and using carrier DNA under selection conditions starting 6 weeks after transformation treatment with 30 mg/l hygromycin. Applying PCR (poly chain reaction, MULLIS et al. 1986) to isolated total DNA from the selected callus lines, the presence of the *hpt* and the *gusA* genes could not be demonstrated.

Embryo imbibition

Preliminary experiments with small numbers of imbibed embryos yielded a high frequency of germinated seedlings with blue staining (42%), after histochemically GUS assay. However, also in the controls a low percentage with blue staining was found (16.7%) (I, *table* 1). When experiments were performed with higher numbers for as well the DNA treated as the control embryos, the percentages of germinated seedlings with blue staining were determined at 66.6% for DNA treated embryos and 62.4% for embryos treated without DNA (II, *table* 1). Because the high percentage of blue staining in the controls is unacceptable for further experimentation, various protocols were investigated aimed at suppression of the blue staining in control tissue. With the protocol described in material and methods this was achieved, although not completely (III, *table* 1). The experiments always yielded higher percentages of blue staining in DNA treated embryos than in the controls. The blue staining was located in remnants of scutellar tissue and in vascular bundles of roots from germinated embryos.

Table 1. Results of blue staining in germinated embryos of perennial ryegrass (*Lolium perenne* L.), 10 days after embryo imbibition.

	frequency of blue staining in imbibed embryos (%)			
	dna treated	n[a]	controls	n
I[b]	42.4	33	16.7	18
II	66.6	240	62.4	233
III	12.2	99	4.0	90

[a] Number of embryos.

[b] I: sum of the results of 2 small experiments, II: sum of the results of 3 experiments, III: result of experiment using the GUS assay with optimal suppression of intrinsic GUS-like activity (see section of material and methods).

Biolistic bombardment

Transient and stable expression of the gusa reporter gene

Two experiments, A and B, which differ in the employed selection conditions for stable transformation, were performed independently. Both experiments showed transient GUS expression in individual cells of leaf, root and coleoptile tissue of bombarded seedlings and cell suspension material respectively 48 and 24 hours post bombardment (*fig.* 1, *table* 2).

Under selection conditions, slow growth of control tissue was still detected after one week of culture on selection medium containing 80 mg/l hygromycin in experiment A. Therefore, these cells were removed from the filters and spread onto selection medium with 150 mg/l hygromycin. The material of experiment B was directly spread onto selection medium with 150 mg/l hygromycin 24 hours post bombardment. At this hygromycin concentration, growth of control tissue was blocked. However, when retransferred to culture medium after maintenance on selection medium for 4 weeks, control tissues started to proliferate again, indicating that the selective agent did not cause complete cell death at the concentration used.

Three weeks post bombardment, the first actively growing calli could be distinguished. At the first subculture, four weeks after bombardment, these calli were isolated and maintained separately as hygromycin resistant callus lines on selection medium. The rest of the material was transferred to fresh selection medium. From this subculture, new hygromycin resistant callus lines were isolated 8 weeks post bombardment at the second subculture. At the first subculture, 27 hygromycin resistant callus lines in experiment A and 4 callus lines in experiment B were isolated. At the second subculture the number of resistant callus lines reached a total of 54 for experiment A and 6 for experiment B (*table* 2).

Table 2. Results of experiment A and B on transient and stable transformation of perennial ryegrass (*Lolium perenne* L.), using biolistic bombardment.

	seedlings		cell suspension							
	transient GUS expression (number of blue cells)		transient GUS expression (number of blue cell clusters)		hygromycin resistant callus lines			GUS phenotype[a] of hygromycin resistant callus lines		
								++	+	-
	x ± SE	n[b]	x ± SE	n	Σx	x ± SE	n			
A	8 ± 5	4	33 ± 11	2	54	9 ± 1	6	9	22	23
B	3 ± 2	4	38 ± 36	2	6	1 ± 1	5	1	4	1

[a] GUS expression was histochemically analyzed with the substrate X-gluc. GUS phenotypes were classified in completely blue (++), partially blue (+) and no blue staining (-).

[b] Σx, x ± SE represent, respectively, total number, average ± standard error of mean value per bombarded filter (1.4 x 10^6 cells). Number of bombarded filters = n.

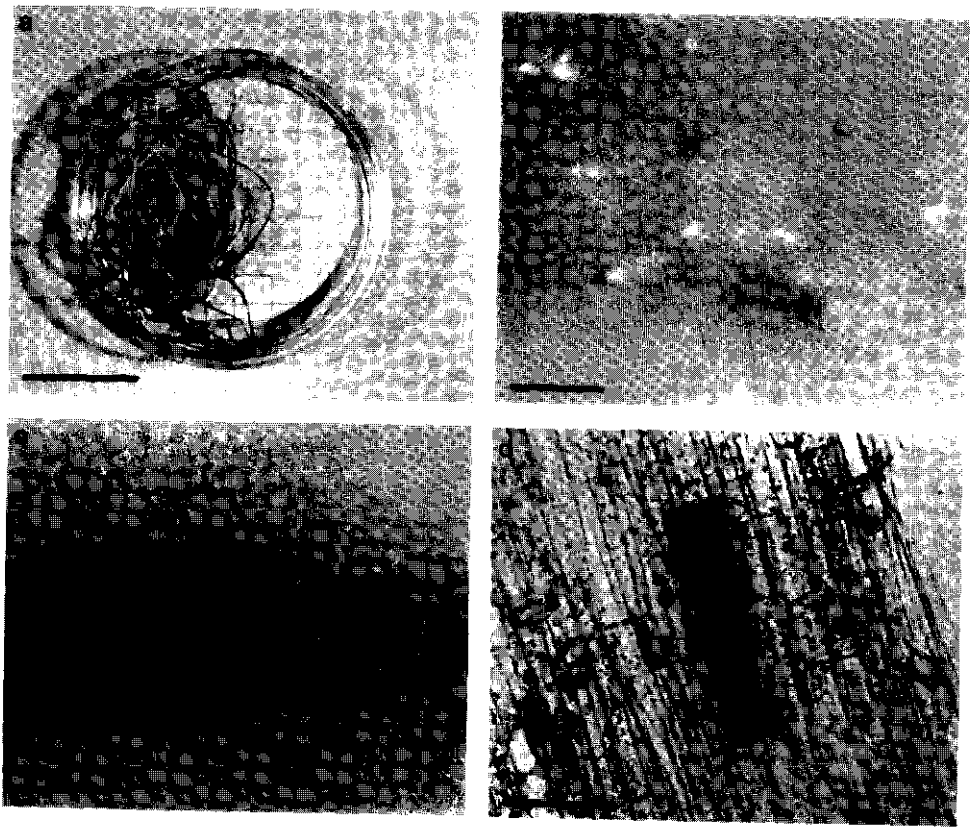


Fig. 1. Transient GUS expression in cell suspension and seedling material of perennial ryegrass (*Lolium perenne* L.), respectively 24 and 48 hours post bombardment. a. Set up of the bombardment of seedlings. Cell suspension was used as a control to determine whether the bombardment was effective. Bar=2 cm. b. Blue cell clusters in cell suspension material 24 hours post bombardment. Bar=500 µm. c. Cell with GUS expression in coleoptile of seedling. Bar=500 µm. d. Cell with GUS expression in leaf of seedling. Bar=75 µm.

Analysis of GUS expression in selected callus lines

The GUS expression in the selected callus lines was initially determined approximately 2.5 months post bombardment. Different patterns of blue staining were observed ranging from callus in which all the cells turned blue to callus with no blue

cells. A classification of the GUS phenotypes was made in completely blue (++), partially blue (+) and no blue (-) stained callus parts (*table* 2).

To analyze the stability of GUS expression upon further culture, the GUS assay was repeated in time. Five of the 10 callus lines which had a completely blue GUS phenotype at the first GUS assay showed a partially blue GUS phenotype 6 months post bombardment. The other 5 calli retained a complete blue GUS phenotype. One year post bombardment a group of 12 callus lines, consisting of 4 callus lines of each classified GUS phenotype determined at the first GUS assay, was assayed again (*table* 3). From the 8 callus lines which exhibited blue staining at the first assay, 5 callus lines showed decrease of blue staining at the second assay. The 4 callus lines with a negative GUS phenotype at the first X-gluc assay remained negative.

Table 3. GUS expression in transgenic callus lines of perennial ryegrass (*Lolium perenne* L.), 2.5 months and 1 year post bombardment.

	gus phenotype[a]	
callus lines	2.5 months	1 year
1	-	-
2	-	-
3	-	-
4	-	-
5	+	-
6	+	+
7	+	-
8	+	-
9	++	+
10	++	+
11	++	++
12	++	++

[a] GUS expression was histochemically analyzed with the substrate X-gluc. GUS phenotypes were classified in completely blue (++), partially blue (+) and no blue staining (-).

DNA analysis of selected callus lines

One year post bombardment, stable integration of the transferred *hpt* and *gusA* genes was confirmed by Southern analysis of the 12 callus lines, which were also tested for long term GUS expression. All the 12 callus lines that were analyzed, displayed individual integration patterns, when the band patterns visualized with the HPT and GUS probe, are combined, indicating independent transformation events. *Eco*RI digestion yielded the expected bands of 1.0 and 1.8 kb, hybridizing with the HPT probe, suggesting the presence of intact *hpt* copies (*fig.* 2). Using the GUS probe, always a 5.6 kb band was detected (*fig.* 2). *Fig.* 3a and c show integration patterns of the *hpt* and *gusA* gene of 2 representatives of each class of GUS phenotype. Callus lines 2 and 3 both show multiple copy integrations of the *hpt* gene as well as the *gusA* gene. Number 6, 7 and 12 only display the 1.0 and 1.8 kb bands of the *hpt* gene. The integration patterns of the *gusA* gene of these callus lines show the

5.6 kb fragment and larger molecular weight fragments. Callus line 11 shows the 1.0 and 1.8 kb fragments and a larger molecular weight band with the HPT probe, whereas with the GUS probe, next to the 5.6 kb fragment lower molecular weight bands are displayed. Undigested DNAs of the 12 analyzed callus lines always showed high molecular weight bands. In *fig* 3b and c, undigested DNA of 2 representatives of each GUS phenotype (*table* 3), hybridized with the two probes, are displayed. For an estimation of the copy number of the *hpt* and *gusA* genes present in the transgenic callus lines, the band pattern of the EcoRI digested DNA was used together with 1 and 5 copy standards per diploid genome (see legend *fig.* 2). In the 4 callus lines with a negative GUS phenotype at the first and second GUS assay, integration patterns of multiple *gusA* and *hpt* copies were detected, whereas the 8 remaining callus lines, contained 1 to approximately 5 *gus* copies, 1 year post bombardment.

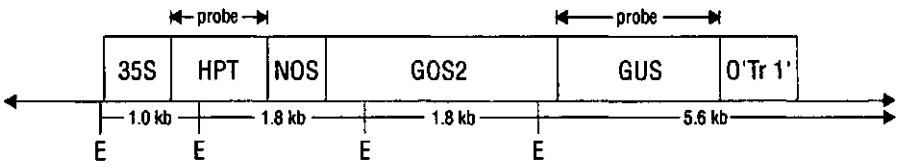


Fig. 2. EcoRI digestion of PORCEHyg plasmid DNA yields the fragments as indicated in the physical map. The sequences corresponding to the *hpt* and *gusA* genes that were used as probes in Southern analyses are indicated above the map.

Discussion

Comparing the three tested methods of direct gene transfer, it is clear that only with biolistic bombardment convincing evidence was obtained for expression and integration of transferred plasmid DNA.

PEG transformation

Using the PEG transformation method of protoplasts, the low plating efficiency of 0.5% combined with the average transformation frequency of 10^{-5}, generally found for PEG transformation, should not have been an obstacle to obtain transgenic mate-

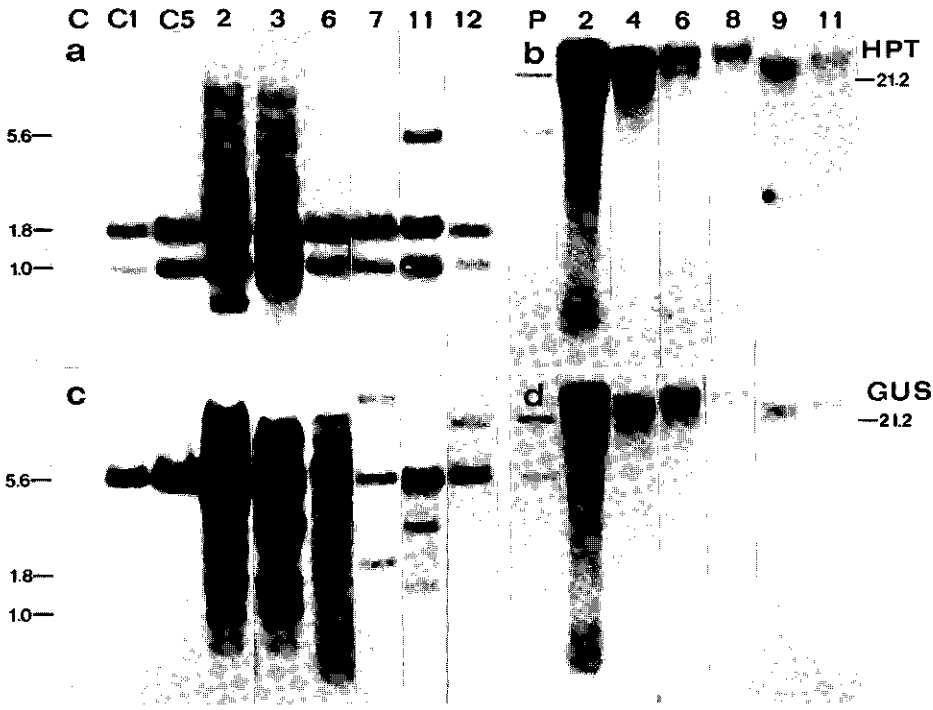


Fig. 3. Southern blot analysis of *EcoRI* digested (a.,c.) and undigested DNA (b.,d.) from hygromycin resistant callus lines of perennial ryegrass (*Lolium perenne* L.), one year post bombardment. Lanes of a. and b., hybridized with the HPT probe, are the same lanes as in c. and d., here, hybridized with the GUS probe. C, C1 and C5 represent control DNA and control DNA mixed with 1 (35 pg) and 5 (175 pg) copy standards of *EcoRI* digested plasmid PORCEHyg per diploid genome (15 µg). The figures above the lanes correspond to the callus lines in *table 3*. The lane indicated with "P" represents intact PORCEHyg plasmid.

rial. For tall fescue, with a plating efficiency of 0.1% and a transformation frequency of 10^{-5}, transgenic plants have been obtained (HA ET al. 1992), although in this report electroporation of protoplasts has been used as DNA transfer technique. However, the cell suspension culture genotype, LP9A, which was used in these experiments, yielded the lowest number of cell clusters exhibiting transient GUS expression, using biolistic bombardment in a later experiment including more genotypes (unpublished results). Although stable transgene expression after plasmid integration into the plant genome and transient expression are two different processes, which might not be related to each other, for both processes uptake of plasmid DNA into the nucleus is a prerequisite. This might be genotypically dependent.

Embryo imbibition

Attempts to obtain transient GUS expression in germinated embryos by imbibition with plasmid DNA of dried embryos, which would indicate expression of the introduced plasmid DNA were unsuccessful. Seedlings emerged from DNA treated embryos and controls always exhibited the same type of blue staining, which was not restricted to particular cells and was often observed in vascular bundles of the roots, although the frequency of DNA treated embryos exhibiting blue staining was consistently higher. The presence of a GUS-like endogenous activity has been reported in various tissues of several seed plants and especially in dehydrated mature embryos and germinated seedlings (HU et al. 1990). This suggests that the observations in perennial ryegrass are also caused by endogenous GUS-like activity. Furthermore, the fact that after one preliminary report on DNA uptake and transient expression using embryo imbibition by TÖPFER et al. (1989), no further scientific evidence has been presented on stable transformation via embryo imbibition, does indicate that this technique has not lived up to its expectations yet.

Biolistic bombardment

Using biolistic bombardment, transient and stable expression of the *gusA* gene in respectively seedlings and cell suspension material has been obtained. In the bombarded seedlings, endogenous GUS-like activity was found at a low frequency, but it was distinguishable from the GUS expression observed in individual cells.

Integration of transferred plasmid DNA in the genomes of the selected callus lines was confirmed by Southern analysis. The fact that next to higher and lower molecular weight bands always the 1.0 and 1.8 kb fragments using the HPT probe, and the 5.6 kb fragment using the GUS probe, were observed, suggests that integration might have occurred preferably through recombination between the rice *GOS2* sequences, located in front of the *gusA* gene (*fig.* 2), and homologous sequences in the *Lolium* genome. Whether this could affect the regulation of the GUS expression in the transgenic callus lines is unknown. Alternatively, several independent integration events of the *hpt* and *gusA* gene, derived from different plasmid DNA molecules, might have taken place. The determination of the precise mode of integration is subject to further investigation. Since a high homology was observed between rice *GOS2* sequences and isolated homologous genomic clones of perennial ryegrass, as determined by DNA sequencing (Hensgens, unpublished results), a potential way to direct the site of integration can be envisaged.

The difference in number of resistant callus lines per bombardment between experiment A and B, is probably not the result of variation in conditions of DNA delivery, since the number of cell clusters exhibiting transient GUS expression was comparable. Furthermore, comparable transient expression is also an indication for the same physiological conditions of the two batches of the suspension culture (RUEB, pers. comm.) In experiment A the cells were manipulated after one week of culture, but it is unlikely that the higher number of transgenic callus lines resulted from disintegration of transgenic cell clusters, since one week post bombardment the transgenic cell clusters derived from one transformed cell are too small to fall apart. Additionally, the individual integration patterns displayed by Southern analysis of transgenic callus lines confirmed separate, independent transformation events. Therefore, the difference in selection conditions between experiment A and B the first week post bombarded, might have caused the variation in the number of transgenic callus lines per bombardment. In contrast to experiment B, the bombarded tissue of experiment A demonstrated slow growth of control tissue the first week after DNA transfer. During this period integration and initiation of expression of the *hpt* gene could still have been established, which could explain the higher transformation rate of experiment A. Although this conclusion needs more experimental evidence, it could argue for application of milder selection conditions shortly after DNA transfer, preventing immediate growth termination, to obtain higher transformation frequencies. However, by using selection conditions that are too mild, false positives might develop, as was observed for the PEG transformation experiments.

The variation in blue staining patterns of stable GUS expression found in the transgenic callus lines of perennial ryegrass is in agreement with results obtained for maize (KLEIN et al. 1989) and, using the same plasmid under comparable conditions as used here, for rice (HENSGENS et al. 1993).

Levels of GUS expression found in maize and rice, assayed by quantitative analysis, generally corresponded to the degree of staining from the histochemical GUS assay (KLEIN et al. 1989, HENSGENS et al. 1993). Therefore, the reduction in blue staining in 50% of the tested callus lines of perennial ryegrass during further culture could be interpreted as a decrease of GUS expression. This decline is not the result of disappearance of the *gusA* gene from the callus DNA, as was proven by Southern analysis one year post bombardment. A similar decrease in GUS expression was found in transgenic rice callus lines (HENSGENS et al. 1993) and cell suspensions (MEIJER et al. 1991). Furthermore, for the *nptII* selection gene a cessation of expression was demonstrated in 50% of transgenic rice plants after regeneration without selection pressure of NPTII resistant callus material (DAVEY et al. 1991). These results show that expression of a delivered gene can decrease or be suppressed in time.

Inactivation of gene expression after multiple copy integration, as was found in transgenic callus lines of perennial ryegrass, has also been reported for, among others, transgenic plants of Petunia (LINN et al. 1990), and tobacco after additional integration of a second gene construct (MATZKE et al. 1989; FUJIWARA et al. 1993). The suppression of expression appeared to be caused by methylation of promoter sequences according to LINN et al. (1990) and MATZKE et al. (1989), or remained unexplained (FUJIWARA et al. 1993). The fact that the HPT resistance phenotype is not inactivated despite multiple copy integration is explained by the employed selection pressure, necessitating the presence of at least one active copy.

This is the first report on transformation of perennial ryegrass. Earlier, POTRYKUS et al. (1985) have been successful in transformation by polyethylene glycol treatment of protoplasts of italian ryegrass (*Lolium multiflorum*), a species of the same *genus.* With perennial ryegrass stable integration and expression of the *hpt* selection gene and the *gusA* reporter gene were obtained. An average of 9 transgenic callus lines per bombardment of 1.4×10^6 cells was established under favourable selection conditions, which is lower than has been reported for maize with 56 transgenic callus lines per 2×10^5 cells (KLEIN et al. 1989), but comparable to rice with 1 - 10 transgenic callus lines per 10^6 cells (HENSGENS et al. 1993). These results provide perspectives for obtaining transgenic plants with a regenerable suspension culture of perennial ryegrass initiated according to CREEMERS-MOLENAAR et al. (1989), using biolistic bombardment as the method of direct gene transfer. The promoter from the constitutive rice gene GOS2 (DE PATER et al. 1992), which was active in transient and stable expression of the *gusA* gene in seedlings and in non embryogenic cell suspension and callus material, appears to be suitable for expression of an introduced gene in perennial ryegrass. In 50% of the analyzed callus lines long term expression of the *gusA* reporter gene was demonstrated. Whether the decrease of GUS expression upon further culture is the result of e.g. methylation of promoter sequences or of other cellular processes at transcriptional or translational level needs further investigation.

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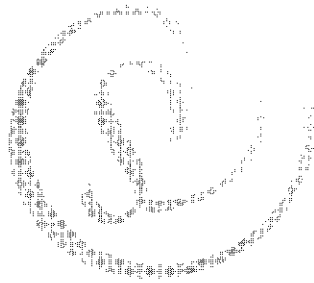
CHAPTER 7



GENERAL DISCUSSION

TOWARDS *IN VITRO* FERTILIZATION, GAMETOSOMATIC CYBRIDIZATION AND DNA TRANSFER

Isolation of viable sperm cells and egg cells has been accomplished. For the sperm cells it has been determined that these cells are true gametoplasts, which is important for *in vitro* fusion. For the egg cells this still needs to be investigated. However, since the egg cells are treated with cell wall degrading enzymes, it is likely that the isolated egg cells are gametoplasts, as was observed for isolated egg cells of maize (KRANZ et al. 1991a; FAURE et al. 1992). At the start of the research programme, it was thought that mass fusion, which is generally applied in somatic hybridization and cybridization, could also be feasible for *in vitro* fertilization and gametosomatic cybridization. However, since egg cell isolation was successful only by using a labour intensive method of combined enzymatic maceration and mechanical manipulation, the number of egg cells that can be isolated in a defined period of time is a restrictive factor that will not allow mass fusion. This leaves one to one fusion of sperm and egg cells, as has also been described for maize (KRANZ et al. 1991a) the most likely option. Also for gametosomatic cybridization it was found that one to one fusion presents the most probable way to obtain fusion products (this thesis, *chapter* 5).

The possibility of inducing new types of CMS through *in vitro* fertilization is probably not attainable, since the isolated sperm cell contains a small amount of cytoplasm with only a few mitochondria (this thesis, *chapter* 3) compared to the isolated egg cell with cytoplasm containing probably abundant mitochondria like has been

reported for the egg cell of maize (DIBOLL and LARSON 1966) and wheat (YOU and JENSEN 1985). This diminishes the chance of recombination between mitochondria of male and female origins. Furthermore, it has been described for wheat that although organelle structures were still present in mature sperm cells (ZHU et al. 1980; HAGEMANN and SCHRÖDER 1984), these cytoplasmic organelles have lost their DNA content during pollen maturation (MIYAMURA et al. 1987). Still, if the few mitochondria and plastids observed in sperm cells of perennial ryegrass have lost their genomes, it renders the isolated sperm cells very suitable as nucleus donors in gametosomatic cybridization experiments with cytoplasts as cytoplasm donors (this thesis, *chapter* 5). In this way CMS can be transferred to an inbred line of agronomic interest without interference by recombination of mitochondria, which usually are still present in the other type of nucleus donors, as used in somatic cybridization in perennial ryegrass when the mitochondria of the nucleus donor are chemically inactivated (CREEMERS-MOLENAAR et al. 1992).

Whether *in vitro* fertilization will be used in combination with DNA transfer via i.e. microinjection in the egg cell with DNA followed by fusion with a sperm cell to allow regeneration into a transgenic plant, will depend on the frequency of DNA integration and regeneration. The egg cell might be a perfect cell for DNA transfer because of its decondensed nucleus. On the other hand, the introduced DNA might easily integrate in regions of the genome which become heterochromatic during further development, hereby blocking expression of the transgene. Still, via microinjection and *in vitro* fertilization, ultimate control of the process of DNA transfer and regeneration will be possible. This way questions concerned with regeneration and DNA transfer might be answered in a more specific way than with trial and error, which is often the case dealing with millions of cells and a low frequency of success. However, for production of transgenic plant material in the near future, biolistic bombardment of an embryogenic cell suspension culture or of other regenerative tissues will be a more realistic option, since for *in vitro* fertilization as a regeneration technique in DNA transfer experiments a long period of research aimed at developing the necessary techniques, i.e. a microfusion technique, a single cell culture system and the right conditions for regeneration, will be a prerequisite. DNA transfer will only be interesting to apply, when regeneration of the artificial zygote can be accomplished.

The observation that the constitutive promoter of the rice gene GOS2 (DE PATER et al. 1992) is active in perennial ryegrass (this thesis, *chapter* 6), could indicate that the isolated organ specific rice promoters (DE PATER et al. 1990; DE PATER and SCHILPEROORT 1992) will also be active in perennial ryegrass, allowing regulated expression. Further research on this topic is needed since these organ specific promo-

ters might be important for sustainable integrated pest resistance management (see this thesis, *chapter 7*, next section). Furthermore, a high degree of homology was observed between isolated rice genes and their homologous isolated genomic clones from perennial ryegrass, determined by DNA sequencing (HENSGENS unpublished results). Via homologous recombination between the rice and perennial ryegrass sequences a potential way to direct the site of integration can be hypothesized. A possible indication for gene targeting by this phenomenon has already been observed in the integration patterns of the chimeric gene construct of the *gus*A reporter gene, regulated by the promoter of the rice gene GOS2 (this thesis, *chapter 6*). This could be an interesting subject to further investigations.

With regard to fundamental research, the viable isolated male and female gametes provide a challenging means to use perennial ryegrass as a model system in experiments aimed at studying cell recognition, zygote formation and early zygotic embryogenesis of angiosperms at the cellular and molecular level. However, before *in vitro* fertilization can become a model for studying *in situ* fertilization, the differences between the two should be analyzed. Because one of the main differences will be the manipulation of gametes before the *in vitro* fusion process, differences between the two fertilization processes might originate from this fact. Maturation stage, plasma membrane constitution and physiological state of the isolated gametoplasts could be different from the gametes *in situ* and might be important factors in determining the success of *in vitro* fertilization and further development of the artificial zygote. Still, gametes are highly differentiated cells which probably not easily "forget" their biological function under artificial conditions.

An interesting starting point for research at the molecular level arises from the observation that both the isolated sperm cell fraction is pure, when the cell type is concerned, and that intramembrane particles in the exoplasmic face of the plasma membrane of the sperm cells might play a role in cell recognition during fertilization (this thesis, *chapter 3*). This information could allow isolation of genes coding for enzymes which are crucial for cell recognition between the female and male gamete and subsequent cell fusion.

Environmental effects of modern intensive agriculture versus plant biotechnology

This thesis has shown, that especially in the field of DNA transfer, for a spectrum of species the experimental phase of the biotechnology has come to an end and can be followed up by large scale application for commercial purposes. Therefore, a compa-

rison between the possible effects of the introduction of products from plant biotechnology in agriculture and the general effects of the agricultural practice over the last 40 years on the environment is at its place. Because The Netherlands are the gene pole of many grass species, special attention is addressed to the possible impact of genetic engineered varieties of perennial ryegrass on the natural environment.

The two major threats of agriculture today are pests and weeds. Modern intensive agriculture has often triggered the occurrence of pest outbreaks by confronting pest organisms with monocultures of genetically identical plants grown in close proximity under high water and fertilizer conditions. In the past years classical breeding was mostly concerned with plant features to improve production and profit like e.g. dwarfism, absence of seed dormancy and non-shattering of fruit and seed. Frequently, breeding for such features resulted in loss of resistance and tolerance to many kinds of pests from the genetic basis of the agricultural crop. Furthermore, crop-weed hybridization led in some cases to the evolution of aggressive weeds, difficult to control because they share so many traits with the crop (BARRETT 1983). For instance, hybridization and introgression between cultivated *Sorghum* species and Johnsongrass (*Sorghum halapense*) are held responsible for the creation of weedy hybrids of Johnsongrass (DE WET 1966; BAKER 1972). Cultivated radish has become a weed due in part to introgression with a weedy relative (BAKER 1972). The need to protect this agricultural production system from major losses to pests and weeds has created a billion-dollar industry of agrochemical companies in just a few decades. The outcome of this chemical approach is that more than 450 insect species have developed resistance to one or more chemical insecticides (GEORGHIOU 1986). In the USA, despite a tenfold increase in the use of insecticides from 1945 to 1988, annual crop losses to insects rose from 7 to 13%. World wide that loss is about 15% (HINDMARSH 1991). Additionally, at least 80 herbicide resistant weed species have been identified (GREEN et al. 1990). Today, weeds reduce agricultural productivity by at least 12%, which is an increase of 50% since the 1940's (KEELER 1989). Although new chemicals offer a short-term solution, this approach to pest control alone will rarely provide a viable long-term strategy.

Two of the main promises of plant biotechnology for the problems in modern agriculture are: 1. the capacity to yield pest resistant crops to reduce pesticide usage, to counteract growing resistance of insects to conventional pest control methods and to offer more precision than broad spectrum insecticides, and 2. the capacity to yield herbicide tolerant/resistant crops to replace hazardous herbicides with "environmentally mild ones". To develop crops that are pest resistant in the long-term, a lot can be learned from the past experience with adaptation to pesticides by pests. The tools of

genetic engineering could be used to produce crop cultivars that could delay or prevent adaptation of pests to resistance genes in previously inconceivable ways (GOULD 1988). For instance, insect resistant crops, which are generated by introducing the genes coding for the insecticidal crystal proteins from *Bacillus thuringiensis*, will not yield a solution in the long-term when the resistance genes are continuously expressed in the whole plant. This way, resistance of the insect to *Bt* insecticidal crystal proteins could evolve and this would reduce the efficacy of new resistant crops, and might eventually change the role that the insecticidal protein plays in the natural ecosystem. By production of crops that express the resistance genes only at times and in places in the crop where they are required, spatial and temporal refugia are created for the pest. Through this strategy, it might be possible to protect a crop while only a small fraction of the pest population is killed. This will minimize the selection pressure for development of resistance of pests (GOULD 1988). To manipulate the expression of a gene, the promoter sequences that regulate gene expression, play a crucial role. Promoters that are organ specific (DE PATER et al 1990; DE PATER and SCHILPEROORT 1992), wounding (SANCHEZ-SERRANO et al. 1987) and/or chemically (WILLIAMS et al. 1992) regulatable, linked to a pest resistance gene, might play an important role in replacing environmentally hazardous pesticides and in future, integrated pest resistance management. If more resistance genes are available against the same pest, these genes linked to the forementioned promoters could be alternated each growing season. To cope with the weed problem, more than 79 corporate/state research programmes are developing over 23 herbicide tolerant crop lines, including cotton, maize, potato, rice, soybean, wheat, tomato, alfalfa and sugarcane (HINDMARSH 1991). In perennial ryegrass herbicide tolerant lines have already been produced by conventional recurrent selection programmes (DEN NIJS 1990). By introducing resistance to herbicides that are less hazardous to the environment, a more environmental friendly way of weed control could evolve. But like it has been concluded from weed control of the last 40 years, the chemical way can not be a solution on the long-term, because of evolution of resistance of the weeds to the new generation of herbicides. One way to postpone the development of resistance of weeds to the new herbicides might be to introduce several genes of herbicide resistance into one crop and very carefully manage the use of the herbicides, to minimize the selection pressure.

Pest and herbicide resistance genes introduced by plant biotechnological techniques (transgenes), could be transferred to related plant species in the natural ecosystem. In the past, one was not concerned with gene flow from cultivated crops to the natural environment; primarily because there was less concern for the environment, and secondly the traits selected for in breeding programmes would mostly be maladaptive in the natural milieu. But some traits, that are introduced by plant bio-

technological techniques, like e.g. pest and disease resistance, might confer a fitness advantage to a wild relative in the natural vegetation (ELLSTRAND and HOFFMAN 1990). If an engineered crop is to be grown in the same region as a compatible wild relative, the engineered trait will be introduced in the natural environment. The hybrid originating from sexual hybridization between the crop and the wild relative may allow the engineered trait to enter the wild population of related species through the process of introgression. Economical and environmental damage from the transfer of a transgene to the natural environment, will depend on the function of the trait and whether it will improve the fitness of the wild relative. In The Netherlands, which is the gene-pool of many grass species, next to perennial ryegrass also related *Lolium* species such as *L. multiflorum*, *L. rigidum*, *L. temulentum* and *L. remotum* occur in the natural environment. Moreover, the genus *Lolium* is very closely related to a part of the genus *Festuca*, also a domestic genus. The perennial ryegrass varieties that are used in cultivated meadows and pastures descend from domestic sources of perennial ryegrass. Crossings and intermediate plants between the various *Lolium* and *Festuca* species are quite generally observed, indicating that sexual hybridization and introgression between cultivated perennial ryegrass and related species are very likely to occur (DEN NIJS and WARDENAAR 1989). The gene flow from pastures to the wild natural environment is established by pollen and seed. Perennial ryegrass is a wind-pollinator and up to 5% seed found 1 kilometre from the tested plot contains marker genes present in the plant material from the tested plot, which implies that even at larger distances pollination can take place (HABEKOTT 1986). Additionally, at the ripening of seeds, the seeds are shed and be scattered by agricultural cultivation, the wind and herbivores. From the pollen and seed dispersal it can be concluded that genes from cultivated pastures and meadows will always escape into the wild environment. Whenever the bred varieties have many features superior to the related grass species in the natural vegetation, genetic narrowing of the wild population will occur or possibly already has occurred (DEN NIJS and WARDENAAR 1989). The same story holds for genetic engineered perennial ryegrass varieties. Exchange of genes between the cultivated pastures and the wild environment via sexual hybridization can never be completely prevented, even when nuclear male sterility or triploidy is introduced to prevent pollen spread. Therefore, the solution to this problem will not be to reduce the chances for sexual hybridization with wild relatives, but to evaluate the impact of the introduced transgene on the fitness of the wild relative.

For perennial ryegrass the insect resistance gene from *Bt*, might be interesting for a future breeding programme to reduce the damage of leather jackets, larvae of the crane fly (*Tipula paludosa*) (this thesis, *chapter* 1). In an integrated pest management programme to prevent development of resistance of the leather jackets to the insecti-

cidal crystal protein produced by the *Bt* gene, expression will have to be induced only at a certain time in a certain organ, creating spatial and temporal refugia. Furthermore, due to the genetic variability of the natural vegetation, leather jackets will not become a plague in the natural vegetation as frequently occurs in the cultivated pastures and meadows with their narrow genetic basis. Therefore, it is probably unlikely that the insect resistance gene will increase the fitness of the wild relative. Herbicide resistance genes will not provide a fitness advantage to grass populations in the wild environment, because the natural vegetation is not sprayed with herbicides. But grasses possess certain weedy characteristics and are even considered as weeds in some cases (CONGER 1991). When grasses with the herbicide resistance gene from the wild population or from a cultivated pasture could invade parcels with other crops, modified with the same herbicide resistance, these grasses will diminish the effect of the introduced herbicide resistance in the other crop, necessitating spraying with more toxic herbicides. Both assumptions on effects of introduction of insect and herbicide resistance genes to wild relatives of perennial ryegrass in the natural environment need of course be substantiated by scientific research.

Public perception

In the past, introduction of new agricultural techniques, agrochemicals or new crop varieties was not questioned by public and authority, simply because there was no concern for the environment, and cheap high quality products had priority. Fortunately, today concern with the environment has a high priority in politics and public opinion. It is generally accepted that agrochemicals are hazardous to public health and environment. Introduction of exotic plants and animals in the natural environment is not self-evident any more, and future weed-crop hybridization must be prevented. The success of genetic engineering for crop improvement depends on public confidence that there is sufficient oversight to minimize the possibility that a transgenic plant might cause adverse environmental effects. Since, on one side the genetic engineering industry persistently claims that the risks of releasing genetically engineered organisms are negligible and on the other hand critics fear the possibility of pandemics, it is rather confusing for the public to choose between the contradicting opinions. Recently, both in the USA and in the EC a public opinion poll has been carried out on modern biotechnology. In the USA 66% of the population thinks genetic engineering will bring changes that will improve their quality of life. A majority of 55% would approve an application that would significantly increase agricultural productivity even when the risk of losing some local species were as high as 1 to 1000. 65% of the population says it would not approve an application if the risks were

unknown (OFFICE OF TECHNOLOGY ASSESSMENT 1988). In the EC 54% of the population thinks that modern biotechnology will improve their quality of life. However, the more people know about modern biotechnology (especially in the northern member states) the more objections arise. The sources of information that are trusted by the public are for 50% consumers associations and environmental protection organizations. One third of the public has confidens in schools, universities and organizations involved in the welfare of animals. 20% considers the public authorities are reliable and only 5% trusts information of industry, political parties and trade unions (SCHENKELAARS 1991). From these data it can be stated that public acceptance of biotechnological products, including genetically engineered crop plants is an important issue to take into account. This debate is likely to continue several years and is badly in need of facts and a rational approach from both supporters and opponents of modern biotechnology.

Concluding remarks

It is evident that the problems caused by agricultural practice in the past such as pollution by hazardous agrochemicals, development of resistance of pests and weeds to agrochemicals and development of more persistent weeds, necessitates a new approach. Plant biotechnology next to classical breeding and ecological ways of agriculture might contribute to an economically and environmentally friendly way of sustainable agriculture. A better understanding between the above mentioned approaches could lead to a fruitful interaction which could cope with the agricultural problems we are facing today. Companies and public authority should corporate and both invest money in risk assessment of genetic engineered crop plants. More knowledge about the behaviour of transgenes in the natural vegetation could loosen up safety measurements concerned with field tests or could overcome a waste of investment of developmental costs when the product does not apply to stringent safety standards.

Whether plant biotechnology will take over a major part in the agricultural production, depends on success of its products in the future, the power of large multinational companies that have invested and still invest billions of dollars in this new branch of agroproduction, and the political point of view on monopolisation of agricultural production by multinationals. The agricultural approach with the highest price does not necessarily need to be the best option for sustainable agriculture.

Sustainable agriculture in the future will only be accomplished when there is equity between peoples communities all over the world (ZADOKS 1993).

Summary

Aim of research

In this thesis research towards *in vitro* fertilization, gametosomatic cybridization and DNA transfer in perennial ryegrass (*Lolium perenne* L.), the most important forage grass of North West Europe is described. Techniques for *in vitro* fertilization and gametosomatic cybridization were aimed at developing a new regeneration technique allowing DNA modification of perennial ryegrass by DNA transfer or by transfer of cytoplasmic male sterility. To analyze whether promoters of rice genes are adequate for transgene expression in perennial ryegrass, a method for direct gene transfer was developed. Furthermore, when viable and female gametoplasts are available, also topics like membrane interaction during fertilization, zygote formation and early zygotic embryogenesis of angiosperms are amenable to research.

Isolation and storage of male and female gametoplasts

Initially, techniques for isolation and storage of male and female gametoplasts were required. A sperm cell isolation procedure was developed. Sperm cells were released from pollen grains by osmotic shock. A specific combination of pH and osmolality appeared to be important to achieve optimal osmotic shock conditions. Aimed at improvement of yield and long-term viability, various combinations of vitamins E, C and fetal calf serum were tested. Their possible mode of action as important components for improvement of yield and long-term viability is discussed. Under optimized conditions, a yield of 12% was established and the storage time after which 50% of the sperm cells were still viable was extended to 60 hours. Cytological observations demonstrated that sperm cells of perennial ryegrass are true protoplasts.

For isolation of viable egg cells, three different techniques, squashing, enzymatic maceration and a combination of enzymatic maceration and mechanical manipulation, were tested. Only with the third method viable egg cells could be isolated. After enzyme incubation, ovules disintegrated into loose cells upon subsequent mechanical manipulation. The egg cells could be identified between the bulk of sporophytic cells derived from the macerated ovules. Two hours after isolation the egg cells were still viable. The developed protocol proved reproducible and the yield was determined at 10%. Since the ovules are treated with cell wall macerating enzymes, it is likely that the isolated egg cells are gametoplasts, as was also observed for isolated egg cells of maize, which are isolated with the same technique (Kranz et al. 1991a; Faure et al. 1992).

Cytological characterization of isolated male and female gametoplasts

For future fusion experiments it is important to obtain insight into the cytological constitution of the isolated gametoplasts. Consequently, a multidisciplinary cytological characterization of the isolated sperm cells, comprising the ploidy level and condition of the nucleus, the presence or absence of a cell wall, the presence and numbers of DNA-containing organelles and freeze-fracture studies of the plasma membrane of the sperm cell, has been performed. The isolated sperm cells are haploid and contain a variety of cell organelles with the exception of microtubules. Proplastids and plastids with starch were observed, although only rarely. Vacuoles containing remnants of organelles and stacked lamellae of endoplasmatic reticulum with cytoplasmic inclusions were observed frequently, suggesting that autophagy takes place in the isolated sperm cells. The number of mitochondria varies from 11 to 26 with an average of 17. Generally, the nucleus has a lobed shape and can display various interphasic stages of chromatin condensation. The analysis of the number of mitochondria and the nuclear state did not show evidence of sperm cell dimorphism. The cytological variability observed, could be explained by differences in developmental stages already present *in situ* at the moment of isolation. No correlation between the number of mitochondria and the nuclear median-section area and/or the condensation state of the chromatin could be found. The number of intramembrane particles on the exoplasmic fracture face of the plasma membrane is more than twice that on the protoplasmic fracture face. That is the opposite to what was found for other types of plant cells, such as sporophytic cells of perennial ryegrass, sporophytic cells of potato (WILKINSON and NORTHCOTE 1980) and the vegetative cel of pollen of several species (PLATT-ALOIA et al. 1986; KERHOAS et al. 1987).

For the isolated egg cells the morphology was compared to egg cells of other monocotyledons and angiosperms in general. The egg cell has a centred euchromatic nucleus with a large nucleolus, containing small vacuoles. The granular structure of the cytoplasm is probably due to starch grains as has also been reported for egg cells of barley (CASS and JENSEN 1970), maize (CHEBOTARU 1981; FAURE et al. 1993) and wheat (YOU and JENSEN 1985).

Fusion conditions for gametosomatic cybridization and *in vitro* fertilization

Since isolated sperm cells of perennial ryegrass contain only a small number of mitochondria, these cells might be suitable as nucleus donors in gametosomatic cybridization experiments with cytoplasts as cytoplasm donors. To explore this assumption, mass PEG fusion between sperm cells and cytoplasts of perennial ryegrass was tried.

Major obstacles were encountered in concentrating the cytoplasts and in purification of the sperm cell fraction in order to remove cell remnants. Agglutination and fusion between cytoplasts was observed. However, probably due to the adherence of sperm cells to cell remnants present in the sperm cell fraction, fusion of sperm cells with cytoplasts or among sperm cells were never found. The most likely option for fusion of sperm cells and cytoplasts to obtain gametosomatic cybridization will be microfusion.

Especially, the number of egg cells that can be isolated in a defined period of time will also be a restrictive factor for mass fusion. Therefore, for *in vitro* fertilization one to one fusion of isolated sperm cells and egg cells under microfusion conditions will also be the most promising option.

Direct gene transfer and evaluation of expression of the *gusA* reporter gene

Three different techniques of direct gene transfer, PEG transformation, embryo imbibition and biolistic bombardment, using the *hpt* selection gene and the *gusA* reporter gene, were tested. PEG transformation yielded only false positives. Germinated seedlings from imbibed embryos with or without plasmid DNA exhibited endogenous GUS-like activity, which made analysis of DNA uptake by monitoring transient GUS expression impossible. Transient and stable transformation, however, was achieved using biolistic bombardment of seedlings and of a non embryogenic cell suspension culture. For stable transformation, bombarded cells were transferred to selection medium with hygromycin 24 hours post bombardment. Hygromycin resistant callus lines were isolated at the first and second subculture respectively 4 and 8 weeks post bombardment. Using various hygromycin concentrations, the transformation frequency varied from 1 to 9 transgenic callus lines per bombardment (1.4×10^6 cells). Stable integration of the *hpt* and *gusA* genes into the plant genome was proven by Southern analysis of DNA, isolated from hygromycin resistant callus lines. The *gusA* reporter gene, which was regulated by the constitutive promoter of the rice gene GOS2 (DE PATER et al. 1992; HENSGENS et al. 1993), was transiently and stably expressed, indicating that this promoter is suitable for expression of a transferred chimeric gene in perennial ryegrass. Callus lines with multiple copy integrations of the *gusA* gene did not demonstrate GUS expression. GUS expression proved stable in 50% of the analyzed transgenic callus lines upon culturing for 1 year.

Concluding remarks

The availability of viable isolated sperm cells and egg cells provide a challenging means to use perennial ryegrass for studying gamete recognition, zygote formation and early zygotic embryogenesis of angiosperms at the cellular and molecular level. Both for realization of *in vitro* fertilization and of gametosomatic cybridization, a long period of research aimed at developing the necessary techniques, i.e. a microfusion technique, a single cell culture system and the right conditions for regeneration, will be a prerequisite. DNA transfer will only be interesting to apply, when regeneration of the "artificial zygote" can be accomplished. Therefore, allowing production of transgenic plant material of perennial ryegrass in the near future, biolistic bombardment of an embryogenic cell suspension culture or of other regenerative tissues will be a more realistic option.

Samenvatting

Doel van het onderzoek

In dit proefschrift wordt onderzoek beschreven gericht op *in vitro* bevruchting, gametosomatische cybridisatie en gen overdracht in Engels raaigras (*Lolium perenne* L.), het belangrijkste voedergras in Noord West Europa. Technieken voor *in vitro* bevruchting en gametosomatische cybridisatie waren bedoeld voor de ontwikkeling van respectievelijk een nieuwe regeneratie methode ten behoeve van DNA overdracht en overdracht van cytoplasmatische mannelijke steriliteit (CMS). Om te kunnen analyseren of promoters van rijstgenen geschikt zijn voor expressie van transgenen in Engels raaigras werd een methode voor directe gen overdracht ontwikkeld. Verder, wanneer geïsoleerde vitale mannelijke en vrouwelijke gameten beschikbaar zijn, kunnen ook nieuwe onderzoeksthema's zoals membraan interactie tijdens de bevruchting, zygote vorming en vroege embryogenese bij angiospermen worden aangepakt.

Isolatie van mannelijke en vrouwelijke gametoplasten.

In eerste instantie waren technieken voor isolatie van mannelijke en vrouwelijke gametoplasten (cellen zonder celwand geïsoleerd uit gametofytisch weefsel) noodzakelijk. Spermacellen werden geïsoleerd uit de pollenkorrels via een osmotische shock. De juiste combinatie van pH en osmotische druk bleek daarbij van belang te zijn. Voor verhoging van de opbrengst en verlenging van de levensduur werden verschillende combinaties van vitamine E, C en foetaal kalf serum getest. Hoe deze komponenten de opbrengst en de levensduur beïnvloedden wordt bediscussieerd. Onder optimale omstandigheden werd een opbrengst van 12% gehaald en werd de bewaringstijd tot wanneer 50% van de geïsoleerde spermacellen nog vitaal waren, verlengd tot 60 uur na isolatie. Cytologische observatie toonde aan dat de spermacellen geen celwand hebben en dus gametoplasten zijn.

Voor isolatie van vitale eicellen, werden 3 verschillende technieken, squashen, enzymatische behandeling en een combinatie van enzymen en mechanische manipulatie, getest. Alleen met de laatst genoemde methode bleek het mogelijk vitale eicellen te isoleren. Na de enzymincubatie disintegreerden de zaadknoppen tot losse cellen door toedoen van mechanische manipulatie. Het was mogelijk de eicellen te herkennen tussen de overmaat van sporofytische cellen, afkomstig van de verteerde zaad-

knoppen. Twee uur na isolatie waren de eicellen nog steeds vitaal. De ontwikkelde methode bleek reproduceerbaar en de opbrengst was 10%. Vanwege het feit dat de eicellen werden behandeld met celwand verterende enzymen, wordt verondersteld dat, net zoals voor geïsoleerde eicellen van maïs (KRANZ et al. 1991a; FAURE et al. 1992), de geïsoleerde eicellen van Engels raaigras geen celwand hebben en dus kunnen worden beschouwd als gametoplasten.

Cytologische karakterisering van geïsoleerde mannelijke en vrouwelijke gameten

Voor toekomstige fusieëxperimenten is het belangrijk informatie te hebben over de cytologische eigenschappen van de geïsoleerde gametoplasten. De geïsoleerde spermacellen werden onderworpen aan een multidisciplinair cytologisch onderzoek, dat bestond uit analyse van het ploïdie niveau en conditie van de kern, de aanwezigheid van de aantallen DNA-bevattende organellen en een vriesbreekstudie van de plasma membraan van de spermacel. De spermacellen bleken haploid en bevatten de verschillende cel organellen met uitzondering van microtubuli. Proplastiden en zetmeelhoudende plastiden werden een enkele keer gesignaleerd. Vacuoles met resten van celorganellen en op elkaar gestapelde lamellae van endoplasmatisch reticulum met afsnoeringen van cytoplasma werden regelmatig gezien, wat aangeeft dat er waarschijnlijk autofagie plaatsvindt in de geïsoleerde spermacellen. Het aantal mitochondriën varieert van 11 tot en met 26 met een gemiddelde van 17 per cel. In het algemeen heeft de celkern een gelobde vorm met verschillende tussenfases van chromatine condensatie. Het aantal mitochondriën en de mate van chromatine condensatie van de celkern gaven geen aanwijzingen voor de aanwezigheid van een spermaceldimorfisme. De cytologische variabiliteit die werd gevonden zou kunnen worden verklaard uit de verschillen in rijping van de spermacellen aanwezig *in situ* op het moment van isolatie. Er werd geen correlatie gevonden tussen het aantal mitochondriën en de oppervlakte van de mediane kerndoorsnede en/of de staat van condensatie van het chromatine in de celkern. De dichtheid van intramembraan partikels aanwezig op het exoplasmatische breekvlak van de plasmamembraan is meer dan 2 keer zo hoog dan op het protoplasmatisch breekvlak. Dit is het tegenovergestelde voor wat werd gevonden bij andere type cellen, zoals sporofytische cellen van Engels raaigras, van aardappel (WILKINSON and NORTHCOTE 1980) en de vegetatieve cel van pollen afkomstig van verschillende soorten (PLATT-ALOIA et al. 1986; KERHOAS et al. 1987).

De morfologie van de geïsoleerde eicellen werd vergeleken met de morfologie van *in situ* eicellen van andere monocotylen en van angiospermen in het algemeen. De

eicel heeft een centraal gelegen euchromatische celkern met een grote cirkelvormige nucleolus, die kleine vacuoles bevat. De granulaire structuur van het cytoplasma wordt waarschijnlijk veroorzaakt door de aanwezigheid van zetmeelkorrels, zoals ook is gevonden in eicellen van gerst (CASS en JENSEN 1970), maïs (CHEBOTARU 1981; FAURE et al. 1993) en tarwe (YOU en JENSEN 1985).

Fusie-omstandigheden voor gametosomatische cybridisatie en *in vitro* bevruchting

Vanwege het feit dat geïsoleerde spermacellen van Engels raaigras slechts een klein aantal mitochondriën bevatten, en daarom waarschijnlijk geschikt zijn als kerndonor in gametosomatische cybridisatie experimenten met cytoplasten als cytoplasmadonor, werd PEG fusie met grote aantallen spermacellen en cytoplasten uitgeprobeerd. Problemen met het verhogen van de concentratie van de cytoplasten en met het zuiveren van de spermacelfractie van celresten, bleken onoverkomelijk. Agglutinatie en fusie tussen cytoplasten onderling werd gesignaleerd. Maar, waarschijnlijk vanwege klontering van spermacellen met celresten, aanwezig in de spermacelsuspensie, bleek fusie tussen cytoplasten en spermacellen en spermacellen onderling niet mogelijk. Daarom lijkt microfusie de beste optie voor het verkrijgen van gametosomatische cybridisatie bij gebruik van geïsoleerde spermacellen en cytoplasten.

Het aantal eicellen dat in een bepaalde tijd geïsoleerd kan worden is ook een beperkende factor voor fusie van grote aantallen. Daarom lijkt voor *in vitro* bevruchting 1 op 1 fusie tussen spermacellen en eicellen onder microfusie omstandigheden ook de beste optie.

Directe gen overdracht en evaluatie van expressie van het *gusA* reportergen

Drie verschillende technieken voor directe gen overdracht, PEG transformatie, embryo imbibitie en "biolistic bombardment", werden uitgetest, waarbij het *hpt* selectiegen en het *gusA* reportergen gebruikt werden als modelgenen. PEGtransformatie leverde alleen vals-positieven op. Zaailingen afkomstig van embryo's geïmbibeerd met en zonder DNA vertoonden endogene GUS-achtige activiteit, wat analyse van DNA opname via bepaling van de transiënte GUS expressie onmogelijk maakte. Met "biolistic bombardment" van zaailingen en niet-embryogeen celsuspensie materiaal, werd wel transiënte en stabiele transformatie verkregen. Voor stabiele transformatie werden gebombardeerde cellen 24 uur na beschieting op selectiemedium gezet. Hygromycine resistente calluslijnen werden de eerste en tweede subcultuur, respectievelijk 4 en 8 weken na beschieting, geïsoleerd. Afhankelijk van de hygromycine

concentratie tijdens de selectie, varieerde de transformatie frequentie vam 1 tot 10 transgene calluslijnen per beschieting (1.4×10^6 cellen). Stabiele integratie van het *hpt* en *gusA* gen in het *Loliumgenoom* werd aangetoond met Southern analyse van DNA, geïsoleerd uit hygromycine resistente calluslijnen. Het *gusA* gen dat gereguleerd werd door de constitutieve promoter van het rice gen GOS2 (DE PATER et al. 1992; HENSGENS et al. 1993), kwam transiënt en stabiel tot expressie in verschillende weefsels, wat aangeeft dat deze promoter geschikt is voor expressie van een transgen in Engels raaigras. Calluslijnen met een hoog aantal geïntegreerde *gusA* kopieën vertoonden geen GUS expressie. Op de lange termijn was de GUS expressie stabiel in 50% van de geanalyseerde transgene calluslijnen.

Conclusies

De beschikbaarheid van vitale geïsoleerde mannelijke en vrouwelijke gameten maakt het interessant om Engels raaigras te gebruiken voor onderzoek naar gameet-interacties tijdens de bevruchting, zygote vorming, en vroege embryogenese bij angiospermen op cellulair en moleculair niveau. Voor zowel de realisering van *in vitro* bevruchting als voor gametosomatische cybridisatie is nog een lange weg te gaan van techniekontwikkeling, zoals een microfusie techniek, een cultuursysteem voor individuele cellen en natuurlijk de juiste condities voor regeneratie. Genoverdracht wordt pas interessant als het mogelijk wordt vanuit de "kunstmatige zygote" een plant te verkrijgen. Voor de productie van transgeen materiaal van Engels raaigras op korte termijn lijkt "biolistic bombardment" van embryogeen celsuspensie materiaal of van andere regeneratieve weefsels, meer geschikt.

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Nawoord

Als eerste wil ik Henk Huizing noemen, wiens idee het was om in plaats van somatische cellen, gametophytische cellen te gebruiken voor plantbiotechnologische doeleinden bij Engels raaigras. Dit idee vind ik nog steeds heel inspirerend.

Om als onervaren onderzoekertje te beginnen aan zo'n onderzoeksproject is in het begin toch even slikken geweest. Maar gelukkig was daar Monique Zaal. Met haar ervaring en creatieve geest zijn we samen het diepe ingedoken om daar stap(je) voor stap(je) de sluiers op te lichten en de doelstelling van het onderzoek te naderen. Ook in de allerlaatste periode van het onderzoek. Daarnaast was het ook echt een hele leuke tijd.

Het bleek voor ons zogenaamde "entkast vlooien" al een heel probleem om het gras het hele jaar door te laten bloeien. Maar gelukkig met de ervaring van Gert Winkelhorst, Ab de Vos en aanwijzingen van de firma Barenbrug kregen we het voor elkaar. We hebben wat afgezaaid, verspeend, gevernaliseerd en met bakken gesleept van de ene naar de andere kas. Gerrit Stunnenberg wil ik hartelijk bedanken voor het verzorgen van de planten en het tijdig ingrijpen als zich weer een meeldauw- of luizenplaag aandiende. Onderzoek valt of staat bij de kwaliteit van het uitgangsmateriaal.

Na 2 jaar kwam Eliza de Jong de plaats overnemen van Monique Zaal. Even zoveel gesleep met bakken gras en even zoveel sluiers om op te lichten. Tijdens mijn zwangerschapverlof heeft zij in de rommelige tijd van verhuizen en reorganisatie binnen het CPRO, toch het onderzoek weten voor te zetten. En dat ging zeker niet altijd even makkelijk. Daar heb ik veel respekt voor. Gelukkig konden we uiteindelijk de resultaten oogsten waar we al zolang op zaten te wachten.

In de tijd van reorganisatie hier en herstructurering daar, heb ik een aantal begeleiders zien komen en gaan. Maar ondanks dat was er Frans Krens die altijd belangstelling had en tijd maakte als er gepraat moest worden, ook in de tijd dat hij geen begeleider was. Dieptepunten werden dan ook snel weggepraat en hoogtepunten met veel enthousiasme ontvangen. Co-promotor wordt je niet zo maar, daar moet je wel wat voor doen.

Op het gebied van gametenisolatie en -karakterisering was al veel kennis aanwezig bij de Vakgroep Planten Cytologie en Morfologie van de LU, bij de start van dit onderzoek. Ondanks dat er geen officieel samenwerkingsverband was kon ik daar toch terecht met mijn vragen. De gesprekken in eerste instantie met Cees Theunis en later met Adriaan van Aelst en Jac van Went waren voor mij erg leerzaam. Ook heb ik veel gehad aan de adviezen van Folkert Hoekstra van de Vakgroep Planten Fysiologie van de LU, met betrekking tot het verbeteren van de vitaliteit van pollen en de daaruit te isoleren spermacellen. Vooral de sfeer van openheid, in deze tijd van concurrerende wetenschappelijke instellingen, heeft mij zeer aangesproken. Deze gesprekken hebben een grote bijdrage geleverd aan de totstand koming van dit proefschrift. Daarnaast toonde Jac van Went zijn vertrouwen in het onderzoek en ging zich langzaam aan meer met het onderzoek bemoeien wat heeft geleid tot zijn promotorschap. Dit vertrouwen heeft mij een grote stimulans gegeven, om het ook daadwerkelijk tot een promotie te laten komen.

De samenwerking met de MOLBASgroep van de RUL, binnen het kader van het project, heeft geleid tot het verkrijgen van transgeen Engels raaigras. Lambert Hensgens, Saskia Rueb en niet te vergeten Frits van der Mark van TNO, te Leiden, hebben hieraan een grote bijdrage geleverd.

Alle collega's van wiens expertise ik gebruik heb mogen maken, als het ging om o.a. statistische analyse, moleculair biologische, microscopische en celbiologische technieken enz., en het uiteindelijk kritisch lezen van manuscripten voor publikatie, ik dank jullie zeer. Ook was de gezelligheid en collegialiteit, zowel onder het regime van de SVP als het CPRO, onovertroffen en ik zou zeggen:" ga zo door."

Het liefdevol verzorgen van andermans kinderen, is een vak waar je talent voor nodig hebt. Johanna Brummelman deed haar werk zo goed, dat ik me ook weer op mijn werk kon concentreren, zonder mij ook maar één keer zorgen te maken. En nog steeds.

Wim Eussen heeft getekend voor de vormgeving en lay-out van dit boekje. Het heeft mij veel "computerfrustraties" en hoofdbrekens bespaard wat voor jou erg prettig moet zijn geweest. Het is toch leuk dat ondanks dat onze werkterreinen zo uitéén liggen, we dit toch samen hebben kunnen doen.

Zonder vrienden, familie, Wim en Hajo was het niet gelukt. Uit de gezellige avonden, het vertrouwen, de liefde, poepluiers en kinderhumor, heb ik de energie kunnen putten om dit onderzoeksproject tot een goed einde te brengen.

Curriculum vitae

Helène Marianne van der Maas werd op 25-8-1963 geboren in Leeuwarden. Zij behaalde het VWO-B diploma in 1981 aan het Chr. Lyceum te Alphen aan den Rijn. Na 5 maanden au pair in Canada en 1 semester studie aan een "college" in de Verenigde Staten, begon zij in 1982 met de studie Biologie aan de Rijksuniversiteit Leiden. In augustus 1987 werd het Doctoraal examen behaald met als specialisaties celbiologie (10 maanden), moleculaire biologie (5 maanden) en de eerste graads onderwijsbevoegdheid. Van maart 1988 tot juni 1992 was zij werkzaam binnen de Stichting voor Plantenveredeling (SVP) te Wageningen, die in 1991 is opgegaan in het Centrum voor Plantenveredelings- en Reproduktie onderzoek (CPRO-DLO). In deze periode heeft zij gewerkt aan het PCLB thema cel- en moleculair biologisch onderzoek in grassen gericht op versnelling van veredelingsprocedures, waarvan de resultaten zijn weergegeven in dit proefschrift.