Biotechnology of Reproductive Processes in Cereals

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Abstract

Sexual reproduction is an essential process in the propagation of flowering plants. Recent advances in plant cell biology and biotechnology have brought new and powerful methodologies to investigate and manipulate the reproductive processes of angiosperms including agronomically important crop plants. Successfull cryopreservation of maize, rye and triticale pollen and young embryos of microspore-and zygote-origine contributes to long term preservation of important plant germ-lines in gene banks. Discovering morphogenetic characteristics of the different developmental pathways taking place in wheat and maize androgenesis in vitro helps to influence the procedure to produce genetically and phenotipically stable homozygous doubled haploid plants for breeding purposes.

Detailed ultrastructural and cell-biological studies on the developmental sequences of male and female gametophyte development in wheat, experimental protocols developed to isolate and micromanipulate egg cell protoplasts, make it possible to use plant gametes and the sexual route itself to produce genetically improved organisms. Plant gametes can become useful tools for crop improvement in the near future. Recent achievements by our laboratory in this field are reviewed in the present paper

Introduction

Plant reproduction research has already been the subject of entire books and a number of recent reviews [17, 23, 25, 26, 27].

This article summarises the latest results that have been achieved by our laboratory in the area and points out how the tools of cell biology and plant biotechnology can be used to address some unanswered questions, and how the integration of various experimental systems ranging from the botanical to the molecular level made it possible that most of our results could be utilised in plant breeding.

Cryopreservation of gametic structures

One special area of reproduction biology research is the examination of pollen viability and factors influencing its maintenance. For a period of time while following anthesis (release of pollen), the mature pollen grain exist as a free organisms (independent from the sporophyte) until it is transported to the stigma of an appropriate pistil. The pollen longevity of different plant species varies between minutes and years depending primarily on the taxonomic status of the plant. For a number of agronomically important taxa, including the short-lived graminaceous pollen, special storage conditions are needed to preserve the viability and fertilising ability of pollen for a long period in order to use stored pollen for hybridization in the case of asynchronous flowering of plant parents or to maintain important germplasms in gene banks[1, 3].

Table 1. Fertilising ability of maize pollen containing various amounts of water

Pollen water content (%)	Seed-set (%)
45-20	81.1
20-10	71.3
10	31.1

Pollen was collected from a single cross hybrid.
Seed set was calculated as an average of 5 self pollinated cobs in each case.

Table 2. Fertilising ability of freshly shed and partly desiccated pollen of rye and triticale

	Rye		Triticale	
Pollen water content (%)	65-45	25-15	65-45	25-15
Seed-set (%)	74.5	60.1	70.0	28.4

Seed set was calculated from 10 previously emasculated and the pollinated ears in each case.

Storage of maize, rye and tiriticale pollen in deep-frozen conditions

Successful long-term storage of Gramineae pollen is generally limited compared to that of other species. This is basicly caused by the high (35-60%) pollen water content at the time of shedding and the rapid dehydration followed by the loss of viability. Maize and rye are cross-pollinating plants and their pollen has a relatively longer (12-24hs) lifespan than the self-pollinating triticale (7hs). Immediately afáer anthesás the moisture content of cereal pollen is 40-60%, depending on the species and on the environmental conditions under which the pollen was formed .In the case of triticale the water content of the pollen was always above 50% while for rye and maize it was often 40-50% [3, 9, 10].

Our earlier studies [7] have shown that the water content of pollen is critical for the success of ultra-low temperature storage. Immediate liquid nitrogen freezing, even if it is rapid enough, would cause irreversible structural damage in the membranes as a consequence of ice formation. Prior to preservation the high water content of the pollen must be reduced to a suitable extent by adequate drying.

As a result of the water loss, the ability of the pollen grains to form pollen tubes and to complete fertilisation weakens to various extents depending on the species (Table 1 and 2)

Maize pollen survived 65-75% loss of the original moisture, rye pollen could also tolerate the extraction of more than 50% of its original water content, while with triticale pollen containing less than 30% water a seed setting of only about 28% was obtained.

With an actual water content of 15-20% which eliminates mechanical damage to the membranes due to the

ice formation during freezing, maize and rye pollen could be stored for more than 10 years in liquid nitrogen at 196°C. Stored pollen regained its viability in 50%, and almost 30% retained their fertilising capacity after thowing it up.

Only a small proportion (4%) of triticale pollen survived cryostorage (Table 3).

Table 3. Seed-setting ability of cryopreserved cereal pollen after various storage periods

Species	Length of cryostorage (days)	Average seed-set (%)
Zea mays	14	38.3
	365	32.4
	730	37.6
	3650	27.1
Secale cereale	14	36.4
	365	30.5
	730	40.0
	3650	37.1
Triticosecale	14	4.1
	365	3.6
	730	3.2
	3650	3.8

In each case five ears were pollinated. Pollen water content: 20%.

Duration of deep-freezing had no influence on the pollen survival. Pollen cryopreservation seems to be the most efficient method for the long-term storage of partly dehydrated pollen grains.

Cryopreservation of young wheat embryos of microspore and zygote origin.

Promising preliminary results have been achieved in the field of embryo cryopreservation. Young androgenic and zygotic embryos (in 1 mm size) of wheat could be stored in liquid nitrogen after pretreatment with 1% DMSO (dimethyl sulfoxide) dissolved in Ms [19] medium for 1 hour. After 3-month storage the small embryos were capable of developing further into a normal plant, or of callusing and produce somatic embryos on the surface of the calli. The plant differentiation frequency was ranged between 7.1-89% depending on the genotype used.

Production of genetically stable homozygous doubled haploid (DH) plants

Application of anther culture in cereal breeding is strongly dependent on the production of large numbers of microspore-derived plants and on high-frequency induction of chromosome doubling. Wheat anther culture can provide a number of haploid plants [8, 13, 14] and a simple and efficient genome redoubling technique is now available for wheat breeders to produce homozygous doubled haploid offspring with high and stable plant fertility [6]. Chromosome doubling induced by colchicine in the time of the first microspore mitosis in culture seemed to be significantly more efficient than the conventionally used techniques, such as treatment to the young plant. To ensure homozygosity, chromosome doubling should preferably occur in the induced microspores.

The potential of doubled haploids in maize breeding has long been recognized [11]. However, the successful application of anther culture techniques in breeding is largely dependent on the androgenic response of the genotypes and on the frequency of induced or spontaneous genome doubling in the regenerants [5, 12, 30].

The construction of maize genotypes with high haploid induction capacity made it possible to study the ef-

fect of colchicine on maize androgenesis in vitro. The highly androgenic DH lines had been produced from exotic plant material through anther culture. They were good sources for the introduction of haploid induction ability into non-responsive elite lines via crossing. Anther cultures of these hybrids were treated with low concentration of colchicine (0.02% and 0.03%) for 3 days at the begining of microspore induction. Colchicine added to the induction medium did not reduce the androgenic responses. Cytological examinations revealed that colchicine treatment before the first microspore division efficiently arrested mitosis. The drug had no influence later on the division symmetry of the microspore nucleus, and unequal divisions remained dominant as it was shown previously by ultrastructural studies [2]. Plant differentiation occured more frequently from calli than from microspore embryos. The plant regeneration ability of microspore derived structures was not influenced by the colchicine treatments. A 3-day exposure of cold-pretreated anthers (at 7°C for 10 days) to a low concentration (0.03%) of colchicine at the oneset of culturing resulted in a significantly higher number of fertile DH plants [4] than in the case of untreated cultures [5].

Gamete micromanipulations in wheat

Fertilisation events of flowering plants occur in the female gametophyte enclosed within the ovules. The understanding of reproductive processes of agronomically important crop plants such as wheat is partly derived from comprehensive information obtained from classical flower biology, light and electron microscopic studies [20, 28, 29, 32]. Another new source of information is based on the isolation, culture and in vitro manipulation of cereal gametes in order to carry out test-tube fertilisation [31, 18].

In vitro fertilisation with isolated, single gametes presents an ideal opportunity to analyse the sequences of embryonic development in plants and for the long run, it will provide a new route for direct transformation of the egg cell using external DNA sources. If manipulations of gametophytic cells are to be successful they must be carried out in the context of comprehensive knowledge of structure and function and at the correct phase of plant ontogeny. Our work on wheat represents the comencement of a series of investigations on the functionality of the female gametophyte and on its suitability for mi-

cromanipulation. Wheat is protogynous, so the oc-
tonucleate embryo sac is already differentiated when de-
velopment of the male gametophyte has reached only first
microspore mitosis [28]. The life span and receptivity
period of the female gametophyte in wheat are fairly long:
10-15% seed-set can still be achieved by hand pollinating
emasculated spikes of winter wheat cultivars even on the
16th day after heading [24].

Examinations of cell structure of wheat egg cell pro-
toplasts isolated from young (3 days prior to anthesis)
and overaged (12 days after anthesis) cryopses confirmed
the experiences of breaders, that wheat caryopses have a
long life span. During maturation, the egg cell increased
in volume (Table 4), and appreared as a metabolically ac-
tive structure.

Table 4. Changes in the volume of wheat egg cell protoplasts dur-
ing ageing

Time of isolation	Cell colume (m3)
3 dba	9.483
anthesis	10.755
3 daa	11.820
6 daa	15.174
9 daa	16.766
12 daa	13.600

dba: days before anthesis
daa: days after anthesis

The overaged egg (12 days after anthesis) showed
some features of apoptosis [21].

An expeditious and highly efficient technique of mi-
croinjection has been elaborated with the aim of in-
troducing exogenous DNA into egg cells of wheat. Egg
protoplasts were dissected mechanically and then were ex-
posed to high frequency AC /Alternating Current/-field
for immobilisation. In this way approx. 15 egg cells
could be microinjected per an hour. Upon injection with
reporter genes: GUS and GFP (Green Fluorescent Protein),
the egg cells gave a positive response in transgene ex-
pression assays. The transformation rate appeared to be a
factor depending upon the time of egg cell isolation. Im-
mature egg protoplasts isolated 1 day before anthesis
showed the highest frequency (73.9%) of transient gene

expression.

Micromanipulation techniques have been elaborated
in our laboratory [15, 21, 22] can offer at the applied lev-
el of research a means of delivering genes of interest into
flowering plants by exploiting the sexual route. Perhaps
the greatest potential of these in vitro systems might be
that these can countribute to dissecting the very early
events of zygotic embryogenesis.

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