Durham E-Theses

Air investigation of some alternative methods to agrobacterium and protoplast transformation for introducing exogenous genes into plants, based on the use of pollen as a vector

Morris, S.R.

How to cite:

Morris, S.R. (1987) *Air investigation of some alternative methods to agrobacterium and protoplast transformation for introducing exogenous genes into plants, based on the use of pollen as a vector*, Durham theses, Durham University. Available at Durham E-Theses Online: http://etheses.dur.ac.uk/6668/

AN INVESTIGATION OF SOME ALTERNATIVE METHODS

TO AGROBACTERIUM AND PROTOPLAST TRANSFORMATION

FOR INTRODUCING EXOGENOUS GENES INTO PLANTS,

BASED ON THE USE OF POLLEN AS A VECTOR

S.R. MORRIS   B.Sc. Ph.D.

Dissertation submitted in partial fulfilment

of requirements for the degree of

Master of Science, University of Durham.

1987.

2 3 SEP 1992

ABSTRACT

Some of the problems involved in the search for alternative plant transformation vectors have been examined. In particular the use of pregerminated pollen as a direct transformation vector has been investigated. It has been shown that the protocols of Hess et al (1985) and De Wet et al (1985) are not easy to repeat with the levels of success claimed by the authors, and that these methods are unlikely to find general applicability to plant transformation in unmodified form. It has not been shown conclusively that these methods do not work, although the true levels of transformation may be considerably lower than those claimed. Some of the basic information required for application of the methods of de la Peña et al (1987) to <u>Petunia</u> has been elucidated.

CONTENTS

CHAPTER 1   INTRODUCTION

Plants act as primary producers in food chains and as the only renewable energy source available to animals, photosynthetic bacteria being perhaps the only other primary producers. Plants grow autotrophically, requiring only water, carbon dioxide, minerals and sunlight. Their biomass production accounts directly for 90% of human calorie intake and 80% of human protein intake, the rest coming from animal sources which also depend on plants for their nutrition.

About three thousand species have been used by man for food, however, about twenty crop species now account for the vast majority of man's protein and calorie intake. The cereals are the dominant staple crops, with rice and wheat being the major contributors. As the human population continues to expand, pressure is being brought to bear on the capacity of world agricultural resources to support this population. It has been calculated, assuming advanced agricultural practices and highly selected crops, that the currently estimated availability of arable land could comfortably support the extrapolated world population of eight billion in 2001 (Mantell, Matthews and McKee 1986). However, problems of nutritional balance exist with purely vegetarian diets in terms of both essential amino acids and vitamin B12.

The improvements that have been made to both the yield and quality of crop plants in the twenthieth century have partly been due to improved agricultural practices in crop husbandry, use of inorganic fertilisers, pesticides, herbicides and irrigation, and partly due to genetic improvements brought about by the plant breeders. Improvements in the efficiency of crop utilisation in terms of yield, pest

resistance, nutritional quality and environmental range are being continually sought in the endeavour to match agricultural resources to population demand, and it is certain that conventional breeding techniques and chemical contributions to husbandry will play the major role in these improvements in the medium term future. However, the emerging technologies of plant tissue culture and cloning and transfer of genetic material in plants will have an increasing role to play as the techniques are refined and transfer of their application from model plant systems to important crop species accelerates. (Austin et al 1986)

These modern methods which may conveniently be grouped together as 'Plant Biotechnology' have a range of potential applications to crop science. Genetic transformation via a specifically designed gene vector has several potential advantages over conventional plant breeding techniques in the generation of new crop varieties. (Old & Primrose, 1985; Mantell, Matthews and McKee 1986) Introduction of single genes or groups of genes in a well-defined way into major crop plants could short-circuit the conventional method of crossing and back-crossing to obtain desired traits, thereby reducing the development time for new varieties. This would reduce the expense of development and increase both the potential for profit and the range of varieties available. Also the the possibility would exist for insertion of foreign traits not available in the existing 'gene bank' of genetically compatible species, into established varieties.

Along with the possibility of inserting foreign genes, either by facilitation of normally incompatible crosses or by use of an appropriate vector system, goes the possibility of generating completely new genetic variability in either a random fashion, such as with the

phenomenon of somaclonal variation, or in a more planned manner using the ability to alter specific gene sequences by site specific mutagenesis techniques. (Old & Primrose 1985; Mantell, Matthews and McKee 1986; Evans and Sharp 1986; Chaleff 1983) This new variability could yield desired traits suitable for introduction into crop varieties by genetic engineering methods, or in less rigorous situations (eg ornamentals, vegetables) give rise directly to new varieties.

Gene transfer in plants also gives rise to the possibility of growing entirely new 'added value' crops in which the gene for some desirable biological product has been inserted into a crop plant in a way analogous to the current biotechnological production of therapeutic materials derived from animals (eg interferon, growth hormones) in micro-organisms (Old and Primrose 1985). As the necessity for strain stability and the species of plant to be used is not defined other than by the ease with which a suitable product may be obtained, this 'added value' production is probably well within the medium term scope of existing technology.

This project set out to examine some of the potential vector systems available as a alternative to the well-developed Agrobacterium/Ti plasmid vector system. The desire to do this stemmed largely from the perceived limitation of Agrobacterium and associated systems (Old and Primrose 1985; Horsch et al. 1984; Lichtenstein 1986; de la Peña, Lorz and Schell 1987). The Agrobacterium system and associated methods of protplast transformation and development of plant viruses as vectors depend on their potential on the phenomenon of totipotency (Mantell, Matthews and McKee 1986). This is the ability of many (if not all) plant cells to regress to an undifferentiated state, from which under

suitable culture conditions, whole plantlets may be induced to proliferate. This method by-passes the plant's reproductive system altogether.

Considerable success has been obtained in generating whole plants using the Agrobacterium/Ti system expressing foreign genes. The most notable recent success being that of Vaech et al (1987) in producing transgenic tobacco plants protected from insect attack by virtue of the presence of the Dt2 toxin gene from Bacillus thuringiensis. However, the method suffers from having a restricted range of plants susceptible to this kind of manipulation (Old and Primrose 1985; Horsch et al 1984; Lichtenstein 1986; de la Peña, Lörz and Schell 1987). Another disadvantage is the existence of the phenomenon of somaclonal variation (Mantell, Matthews and McKee 1986; Evans and Sharp 1986) with its concomitant problems of retaining strain stability - an essential factor in the development of new staple crops if they are to be commercially competitive. A lesser but perhaps significant problem is the need to incorporate a selectable marker along with the required gene if it is desired to transfer phenotypic tracts which are unidentifiable at the single cell or callus stage. This, of course, involves introducing an additional genetic load into the system which may, for instance, cause significant problems with yield optimisation in the intensely competitive area of cereal cropping.

Transformation of DNA into protoplasts using Ca2+ precipitation along with treatments like PEG or electroporation to destabilise the membrane offers some promise of success in overcoming the species range problems associated with Agrobacterium (Old and Primrose 1985; ??; Hain et al 1985). Success with important monocot crops is to date somewhat

limited, but this appears to be a matter of technical perfection rather than an absolute barrier, as regeneration of whole plants from protoplasts seems to acquire identification of exacting conditions which vary from species to species. Recent advances with rice protoplasts (Fujimara et al 1985; Yamada, Zhi-Qi and Ding-Tai 1986) suggest that these problems can be overcome. However, this method shares with the Agrobacterium system the problems associated with somaclonal variation and the necessity to incorporate selectable markers, as well as being technically much more demanding. The currently ill-developed virus-vectors (Old and Primrose 1985; Grimsley et al 1987) whilst showing some promise again suffer from the problems associated with somaclonal variation and selectable markers. However, it is possible to direct these against whole plant tissue and if they could be directed through the germplasm, the possibility of circumventing the possibility of somaclonal variation exists. The problem of increasing the genetic load may however be exacerbated by the use of virus vectors as it will probably be necessary to insert into the plant genome much more DNA than just the required genes.

Although the systems previously mentioned are probably the front runners in the current drive towards genetic manipulation of crop plants, the limitations involved make the search for alternative vectors based on what may loosely be termed as 'whole plant' reproductive manipulation .an attractive and justifiable quest (de la Peña, Lörz and Schell 1987).

The potential advantages offered by whole plant reproductive manipulation lie in the possibility of allowing the putatively transformed cells to go through a normal developmental cycle within the

whole plant environment rather than exposing them to the apparently genetic destabilising effects of growth in culture conditions where reliance is placed on hormonal induction of meristemoids or somatic embryogenesis to enable production of whole new plants carrying the altered or added genetic material. The rationale is based on similar manipulations in animal systems (Old and Primrose 1985; Gordon and Ruddle 1981; Palmiter et al 1982; Zolakar 1981; Capecchi 1980) which have yielded animals altered in the expression of a single gene trait, but otherwise apparently normal (eg. micro-injection of Drosophila P-elements at the syncytial blastodem stage in developing embryos, micro-injection of mouse germ cells at various stages to produce transgenic mice).

These systems depend upon introducing the desired piece of DNA into animal germ-tissue where they are incorporated in a proportion of cases, by more or less specific recombination events mediated for instance by sequence homology (Palmiter et al 1982) or transposon-like elements (Zolakar 1981). In the case of mice, embryos carrying the introduced DNA are reimplanted into surrogate mothers and allowed to develop normally, expression of the introduced gene is then assayed by genetic complementation of mutant traits or phenotypic expression different from untransformed sibs. This second assay method is extremely useful as no marker is required, only a prediction for the phenotypic effect of the transferred gene.

The fundamental ideas behind these methods of gene manipulation in animals would potentially be of great use in plant genetic engineering, offering the possibility of attaining the highly desirable result of a stable strain with insertion of a single gene or defined group of genes

which will be expressed in the subsequent generations. Obtaining this result would depend on the technological ability to target small amounts of defined DNA to the embryo sac (megagametophyte), and to achieve its incorporation into the zygote in such a way that the stability of the subsequent embryogenesis is not significantly disrupted. Several approaches have been taken to investigate this possibility, which is complicated by the complexity of the process of syngamy in angiosperms, by the inaccessibility of the embryo sac in the intact plant and the near impossibility of making the female generative nucleus amenable to microsurgical and reimplantation techniques similar to those used for in vitro fertilisation and reimplantation in mammals.

Most of the avenues explored to date have involved the special properties of the microgametophyte and the way in which syngamy is achieved by the growth of the pollen tube through the tissues of the female floral parts and eventual release of the sperm nuclei into the embryo sac. These methods may be grouped together as the Pollen Vector Theory.

Of these only ovule culture (Beasely and Jensen 1985) involves resort to artificial culture conditions. This technique involves surgical removal of the ovule for purposes of manipulation (microinjection for instance). Unfortunately, this introduces a large measure of the disruption which it is necessary to avoid, as re-implantation is virtually impossible. The resulting necessity to resort to artificial culture more properly places this technique with the previous methods discussed, as although it does not depend on totipotency, it does involve development which may be aberrant due to the stress of artificial culture.

The remaining potential methods all depend upon Pollen Vector Theory. Thus, for instance, some success has been claimed for direct microinjection of nucleic acid into the ovary or embryo sac (Hepher et al 1985; Steinbiss et al 1985; Nu et al 1980; Soyfer 1980). The hope is that the exogenous nucleic acid, if present in sufficient quantity, may be taken up during the entry of the pollen tube into the embryo sac. This seems intuitively to be a rather optimistic approach as there are potentially several membrane or tissue barriers to the uptake of the nucleic acid. The success claimed by some workers using injection of RNA (Nu et al 1980), is based on some rather poor assay methods and other workers have been unable to obtain transformation by this method to date (Hepher et al 1985; Steinbiss et al 1985).

A similar method has approached the injection idea by trying to use pollen tubes immediately post fusion with the embryo sac as a potential entry point for DNA (Zhou et al 1983). This certainly seems a plausible method as presumably the breach of the embryo sac and subsequent emptying of the pollen tube does briefly create a direct 'tube' into the embryo sac. Some success with this technique has been reported, again by Chinese workers, using injection of whole purified DNA from a donor strain, and subsequent scoring for plants grown from the resulting seed for transformation to donor phenotype for a number of traits.

It seems that the art of timing and targetting the injection appropriately, along with the observable formation of callose plugs in pollen tubes of many species may make this a technique singularly difficult to reproduce in a general way over a whole range of species. However, further exploration of the technique is probably warranted.

One of the most attractive approaches currently under investigation is the use of active pollen as a vector for the exogenous DNA (Hepher et al 1985; Hess, Dressler and Konle 1985; Pandey 1980; Pandey and Phung 1982; Werner et al 1984; De Wet et al 1985; Snape et al 1983; Powell, Caligari and Haytor 1983). This is similar in principle to the last method, but differs in that the exogenous DNA is incorporated into the pollen tube, or even the germ cells before the tube grows down through the style to fuse with the embryo sac. This approach offers the possibility of leaving the female gametophyte and its supporting tissues completely in situ, thereby causing the minimum disruption of the obviously very fine balance of controls required for the stable development of the new sporophyte which will become the plant whose genome we hope to have effectively engineered.

Varying levels of achievement have been described in this field. A number of demonstrations of the possibility of 'pseudo-fertilisation' by irradiated pollen and subsequent development of a pathenogenetic sporophyte partially transformed by the fragmented DNA of the 'pseudo-fertilising' irradiated pollen have been made (Pandey 1980; Pandey and Phung 1982; Werner et al 1984; Snape et al 1983; Powell, Caligari and Hayter 1983). These provide an interesting insight into what might be possible with pollen, without really providing a mechanism for carrying exogenous genetic material into the progeny. Attempts to introduce exogenous DNA into germinating pollen tubes by microinjection, although offering a possible 'universal method', with a high frequency of transformation being theoretically attainable (cf. animal experiments), have to date been unsuccessful (Hepher et al 1985; Steinbiss et al 1985). These failures may, however, be representative of the

difficulties encountered in establishing effective protocols, rather than any inherent theoretical weakness in the method, therefore continued efforts in this field are to be expected.

Perhaps the most successful pollen vector method to date seems to be that of getting freshly germinated pollen grains to take up exogenous DNA in a way analogous to that used in protoplast transformation (Hess, Dressler and Konle 1985; De Wet et al 1985; Hess et al 1974a; Hess et al 1974b; Hess et al 1975a; Hess et al 1975b). The method is based on areas of naked protoplast available for transforming DNA due either to undeveloped cell wall during early pollen tube emergence (De Wet et al 1985), or to the existence of a pore in the cell wall at the tip of the emergent tube in the very early stages of tube elongation (Hess, Dressler and Konle 1985). Expression of inserted foreign genes and Mendelian transmission to subsequent generations has been reported for various marker genes in Zea mays, Petunia and Nicotiana (Hess, Dressler and Konle 1985). If this technique should prove to be universally applicable it could go a long way to solving the technical problems of genetic engineering in plants.

One remaining idea for transformation of plants via pollen vectors is the less well established, but nevertheless theoretically attractive possibility of finding and appropriately manipulating a plant viral strain capable of lysogeny. The fact that viruses may be transmitted through both seed and pollen is well established (Matthews, R. 1981). If a suitable virus can be found and 'tamed' in a way analogous to 'phage λ (lambda) in E. coli a very efficient cloning/transformation system could be established using pollen vectors and micropropagation techniques.

More recently a method has emerged whereby injection of transforming DNA into the developing flower buds of rye at an appropriate stage of development (14 days before the first miotic metaphase) has given rise to transformed offspring (de la Peña, Lörz and Schell 1987). It is not clear whether it is the male or female germ cells or both which are vulnerable to transformation by this method, although this could perhaps be checked by assaying germinating pollen grains for expression of the marker gene. This method potentially offers a simple and probably general method for transformation of cereal plants, and if it can be repeated consistently must be regarded as a major breakthrough in the field of alternative transformation systems not using Agrobacterium or protoplast regeneration.

The interest of the author was in examining some of these alternative transformation systems, to determine whether reproducible results or effective protocols could be established. Given the range of alternatives it was difficult to assess exactly which direction to take, however, on the basis of technical simplicity and the range of expertise and equipment existing within the department, three alternatives were considered: a) the direct transformation of germinating pollen grains; b) microinjection of germinating pollen tubes; and latterly, c) the injection of transforming DNA into developing flower buds. After some examination of the logistic difficulties attached to transferring pregerminated pollen grains, (which are a prerequisite of microinjection) back onto the stigma of the flower (or some surgically reduced form of the female parts) so that they may carry out a normal fertilisation it was decided that anything more than an investigation into the problems attached to successful microinjection would be beyond

the scope of this short project. Direct transformation of pollen grains, however, is a technique with well defined protocols for maize, Petunia and Nicotiana plants, for which some success with transformation have already been reported (De Wet et al 1985, Hess and Dressler 1984, Hess et al 1985). Also, Petunia and Nicotiana are plants with growth cycles short enough to go from seed to seed within the time span available. It was therefore decided to follow these protocols with a view to establishing whether transformation could be achieved using a good marker gene for which expression would be easy to demonstrate. Thus the methods of de Wet and Hess were followed in detail, and the results are reported here. The gene selected as a marker was the E. coli NPT II gene (Kanamycin resistance) fused to a strong general promoter of plant gene expression derived from gene VI of the cauliflower mosaic virus.

This construct was carried in the PABDI plasmid which also carries an Ampicillin resistance gene for selection in E. coli. As the experiments involved inevitably resulted in some time waiting for crosses made to produce mature seed, this time was occupied learning the technique of embedding and sectioning plant material with a view to examining flower buds of Petunia. The aim of this was to see whether the equivalent stage of development to that used in the rye experiments of Vaech et al could be identified with the ultimate goal of establishing a protocol by which similar experiments could be carried out in Petunia

CHAPTER 2   MATERIALS AND METHODS

1.   Growth of Plant Material

Petunia hybrida cv 'Carousel' plants were grown from a commercial packet of seed supplied by R and G Cuthbert, Upper Dee Mills, Llangollen, Clywd.   Nicotiana elata plants were grown from seed supplied by Dr P Gates.   Plants were sown initially during February in a growth room, and subsequently in two week successions to ensure a supply of experimental material.   At the end of March all plants were transferred to the temperate section of the greenhouse of the Botanical Gardens. Seeds were germinated initially on several thicknesses of Whatman filter paper in a petri dish then pricked out into 4" pots containing a commercial peat-based potting compost supplied by the supervisor at the Botanic Gardens.   The Petunia and Nicotiana plants commenced flowering about ten weeks from the initial sowing dates during the latter part of April.   From this date a continuous supply of flowering material was kept available.   Notes were made concerning various morphological characters of the flowers grown, some of which have an established genetic basis, as it was felt that this information may be of some importance should any transformed offspring be obtained.

2.   Emasculation of Flowers

Receptor (female) flowers for all experiments were emasculated by removal of anthers and petals from the receptor flower buds about 1-2 days prior to anthesis.   Although Hess (1978) suggests that this should be done only 24 hours before the experimental pollination, preliminary experiments done by the author (see results) suggested that this was an

unnecessary precaution, as no difference was detectable in wet pollination efficiencies between flowers emasculated for up to 6 days before pollination occurred.

3. Pollen germination

This was carried out in either Brewbaker's (BB)(Brewbaker and Kwack 1963) medium or Fanrich's (F) (Fanrich 1964) medium according to the experimental protocol. Formulae for these media are given in Appendix 2.

4. Progeny seed propagation

Progeny seeds were surface sterilised by immersion in 20% v/v of a commercial sodium hypochlorite solution ("chloros") for 20 minutes followed by two rinses in sterile distilled water. The seeds were then spread with 0.2-.4ml of sterile H_2O on 0.8% sterile agar containing a slightly modified version of the medium of Wagner and Hess (1974). The formula for this medium is also given in Appendix 2. To the agar medium was added sterile Kanamycin sulphate solution (50mg/ml), to a final concentration varying from 25μg/ml to 200μg/ml according to the experimental protocol.

The Kanamycin sulphate solution was presterilised by pushing it through a sterile 0.2μ bacteriological filter by syringe pressure, into a pre-sterilised McCartney bottle. The solution was stored at -20°c (Lichtenstein and Draper 1985).

Recombinant DNA material

The plasmial used in these experiments, pABDI, was kindly supplied by Dr R. R. Croy. A restriction map of this plasmid is contained in Appendix 1. The salient features of this 5.4kb plasmid are the presence of the E. coli NPT II gene which when expressed conveys resistance to

the amino glycocide antibiotic Kanamycin. This gene is fused at the 5'
end to a strong, general plant promoter from the CaMV gene VI and at the
3' to a sequence containing the poly A signal from the CaMV gene VI.
These features should ensure efficient expression of this construct if
inserted in a plant genome. An ampicillin resistance gene and origin of
replication derived from plasmid pMBI facilitates selection and
amplification of this plasmid in E. coli.

Selected restriction digests were carried out on small samples from
the batches of pABDI supplied to ensure that the patterns obtained were
consistent with the restriction map of the plasmid. This confirmation
was obtained by running the digests on 0.8% agarose gels containing
ethidium bromide, against samples containing a standard size marker
digest (λ Hind III or λ PstI digests).

5. Restriction analysis of pABDI

(a) Restrictions were set up such that 4 μl of restriction buffer
was made up to 20 μl total volume containing 5 units of enzyme and about
1 μg of DNA. The mixtures were incubated for 3 hours at 37°c and
stopped by incubating for 5 minutes at 70°c.

(b) Gel electrophoresis. 1.6g of agarose was dissolved in 180 cm³
of distilled water by boiling. The gel solution was cooled to about
70°c when 20cm³ of stock electrophoresis buffer and 20μl of stock
ethidium bromide solution were added. The gel was cooled further, then
poured into a mould with a twelve-slot sample comb in position about 3cm
from the end of the gel. Equal volumes (10μl) of each restriction
mixture and stop dye were mixed and loaded into individual sample slots
on the gel. The gel was run overnight at 50U and 40mA. The bands were
visualised under U.V. illumination and photographed.

6.   Transformation experiments

These were carried out according to either the method of Hess 1978 or the method of de Wet 1985.   In the case of de Wet's method, certain modifications have been made to adapt the method which was originally designed for *Zea mays* to make it more suitable for *Petunia* and *Nicotiana*.   Hess's method however was originally described for *Petunia* and *Nicotiana* and has been followed as exactly as possible.

(a)   de Wet's method (modified)

200μl of BB medium was added to about 12 freshly dehisced anthers in a sample bottle.   The mixture was agitated with a pipette tip to loosen the pollen grains, and the bottle placed at an angle to allow settling of the pollen grains in the angle between the base and wall of the bottle.   29μl of the resulting germination medium plus sedimented pollen grains was removed into a sterile Eppendorf tube.   This resulted in a high concentration of pollen grains in the mixture.   The pollen grains were allowed to continue imbibing for two minutes, then 1μl of pABDI at 5mg/μl was added, resulting in a final concentration of pABDI in the mixture of 165μg/ml.   The pollen was allowed to continue germinating for a further twelve minutes before pollinations commenced.   A drop of 3-4 μl of the pollen suspension was then placed on the stigmas of previously emasculated flowers and allowed to dry overnight in the lab.   The plants were then returned to the greenhouse and observed for successful seed set.

In the initial experimental crosses successful germination of the pollen was monitored by excising the stigma and style after about 4 days and examining a squashed preparation stained with aniline blue using epifluorescence U.V. microscopy.   This technique specifically stains the

callose component of pollen tube walls and plugs, thereby facilitating observation of the tubes.

This was later abandoned as it was not an absolutely reliable indicator of seed set, and because of different rates of pollen tube growth in different crosses it was felt that in some cases successful seed set had been preempted by excising the styles too soon.

Control experiments were done by making similar crosses omitting the pABI solution from the pollen germination medium.

(b) Hess' method

For this method anthers were collected 1-2 days before dehiscence. pABDI was added to 200µl Fanrich's pollen germination medium to a final concentration of 165µg/ml. The pollen germination medium also contained 50µg/ml ampicillin to inhibit bacterial growth during the incubation period. This was a slight modification of the original method which utilised ampicillin and cloxacillin in the medium. However, cloxacillin was not available in the department, and examination of the activity spectrum of the antibiotic suggested that omission rather than substitution would not seriously jeopardise the outcome of the experiments.

To this 200µl of incubation medium were added 20-25 of the collected anthers. These were squashed gently to ensure access of the medium to the pollen grains and the mixture was incubated at 27°c in the dark in a gently shaking waterbath.

The experimental crosses were then made by placing one squashed anther plus an additional drop of the remaining pollen suspension on to each stigma. Seed set was recorded as before and seed collected from each successful cross.

7. Experiments on flower bud development

(a) Time Scale of bud development

This was estimated by tagging ten buds from a few different plants at the earliest time when definite commitment to flower formation could be detected by eye. Notes were made on the stage of development through to opening of the bud into a mature flower.

Cellular events during bud developments

Cellular events were followed by harvesting buds, which could be placed in the estimated time scale according to their size, and wax embedding, sectioning, staining and observing using standard Kohler optics light microscopy.

(b) Protocol for wax embedding

The buds were fixed in 1.5% paraformaldehyde 2-5% gultaraldehyde in 0.05M cacodylate buffer, for several days at room temperature, then washed in three changes of cacodylate buffer. Dehydration was achieved through a series of 12.5%, 25%, 50%, 70%, 90%, 100%, dry 100% ethanol solution, giving 2 changes in each solution at 15 minute intervals for the 12.5%, 25% and 50% stages and at 30 minute intervals for the subsequent stages. A further two changes were made in a solution containing 1 part ethanol to 1 part of the commercial wax solvent Histosol. The buds were then left overnight in pure histosol. The solution was changed the following morning and during the course of the day wax shavings were added throughout the day. (The sample bottle was kept hand-hot during this process to aid dissolution of the wax.) Over the next four days the buds were subjected to 7 changes of pure histoplast wax, being maintained at 56°c in an oven dedicated to this purpose during the process. On the morning of the fourth day the last

change of wax was made, and the buds were finally embedded in the afternoon. This last step is achieved by putting fresh histoplast wax into a tray divided into cubical sections (rather like an ice-cube tray from a domestic fridge) and, maintaining this in a just-melted state on a hot plate, quickly removing the infiltrated buds from the oven and setting them at an appropriate orientation in the cubes of new wax. The tray was then removed from the hot plate and left to set hard on an ice-tray.

(c) Sectioning

Sectioning was carried out on a Leitz microtome. The embedded buds with the wax blocks appropriately trimmed were 'glued' to a wooden block using molten wax for fixing to the microtome sample clamp. Sections were cut at 10µ thickness, and the resulting ribbons floated onto degreased, clean glass slides and allowed to dry under gentle heat.

(d) Staining and microscopy

The prepared sections were dewaxed and rehydrated by a procedure adapted form O'Brien and McCully (1981) which involved two 5-minute passages through each of histosol (xylene in O'Brien and McCully), 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, distilled water. The sections were then stained in Aceto-orcein (5 minutes), washed and coverslipped and examined under Kohler illumination for the division stages involved in gametogenesis.

CHAPTER 3   RESULTS

PART 1

TRANSFORMATION EXPERIMENTS

(a)   Pollen pregermination experiments

These were carried out to ensure that pollen of Petunia and Nicotania would germinate satisfactorily in the media to be used and to establish some idea of the time scale involved.

Pollen collected from freshly dehisced anthers were pregerminated in either BB or F medium, either in a 10μl drop of medium on a coverslip, or in a 10μl drop contained in a well of a microtitre plate. Pollen was observed at regular time intervals using Nomorski optical microscopy and the outcome of a typical experiment is illustrated in plate 1.

It was found that pollen of both species of plant germinated quite satisfactorily in both media. The growth rates were not measured critically, however, it was apparent that both the source of pollen and its level of maturity had a substantial effect on the lag time before germination commenced.

For this reason, it was decided to try so far as possible to limit the pollen parents to a few plants to minimise the potential effect of this variability on future experiments. Along with this, great care was taken to collect pollen within a very few hours of complete dehiscence of the anthers, except for the experiments using Hess' methods which required harvesting of anthers just before dehiscence.

(b) Assay for vigorous crosses using pregerminated pollen

A few random crosses using dry pollen had illustrated the fact that not all possible crosses would be viable, presumably due to incompatability mechanisms. Therefore, it was decided to assay the experimental plants for those crosses which work well using pregerminated pollen. A restricted number of pollen parents were crossed to as many of the remaining plants as possible to identify viable crosses. Pollen was pregerminated as described above in microtitre plates and observed for signs of germination. When approximately 10% of the grains showed signs of germination, pollination was undertaken by placing a 3-4µl drop of pollen suspended in the germination medium on the receiving stigma. Successful pollination was assayed by harvesting the stigma and style 3-4 days after pollination, making aniline blue stained squash preparations and visualising the growing pollen tubes by U.V. epifluorescence microscopy.

The results for Petunia and Nicotania are shown in tables 1 and 2 respectively. Table 3 shows the Petunia results analysed in terms of success of pollination relative to the age of the emasculated flower. It can be seen that no significant differences are observable.

Good recipient Petunia plants were identified from these crosses as P2, P3 and P7. Subsequent demonstration of good seed set despite equivocal performance in the above experiment also led to the use of P1, P6, P13, P14 and P16 in experimental crosses. On the basis of lack of seed set despite good pollen germination, P3 was discarded as a potential recipient. These plants were used in the experiments investigating De Wet's method, with P20 and P21 as pollen donors. A similar experiment allowed P5, P8, P9 and P12 to be identified as

potential recipients for the experiments investigating Hess' method using P3 and P8 as pollen parents.

Incompatibility and variable vigour of crosses generally seems to be less of a problem with the Nicotania plants. However, T1 was identified as apparently unable to receive pregerminated pollen from all four donors tried. The other crosses tried all worked well. T1 was therefore eliminated as a experimental plant and the experimental crosses were restricted to the other six plants (see table 2).

In general, the foregoing experiment is fairly speculative with insufficient data for many crosses for firm conclusions to be drawn about viability. However, it must be realised that the objective was not exhaustive investigation of incompatability in Petunia or Nicotania, but to identify a useable number of viable crosses so that experimental material was not wasted on crosses doomed to failure from the outset. (For instance, if one had assumed no such problems and used T1 as an experimental recipient, a large amount of material could have been wasted with no prospect of success.)

(c) Experiment to establish whether pollination could be achieved by applying pregerminated pollen directly to the cut surface of the ovary following excision of the stigma and the style in Petunia flowers.

Following discussion with Dr Gates, this experiment was attempted which, if successful, offered two potential advantages: (a) the possibility of overcoming some incompatability barriers; and (b) the possibility of improving the vigour of crosses done with pregerminated pollen by reducing the amount of tissue penetration which the growing pollen tubes must achieve to give successful transfer of germ nuclei to the embryo sac.

Just prior to application of the pregerminated pollen, the stigma and style were excised from the previously emasculated receptor flower, leaving a cut surface of approximately similar dimensions to the stigma surface. A 3-4μl drop of pollen pregerminated in Brewbaker's medium in a microtitre plate, as previously described, was applied to the cut surface. The flowers were left for about 6 hours for the drop to dry and then the cut surface was sealed with high vacuum silicone grease. Buds were observed for successful seed set, as indicated by swelling of the bud and retention of the green colour of the surface. Non-successful buds shrivel and go brown within about two weeks. The results are presented in Table 4.

It can be seen that only one cross out of nineteen made succeeded. It was concluded therefore that no advantage could be gained by pursuing this method.

(d) Experiment to establish appropriate levels of Kanamycin selection

Although it was known that selection for kanamycin resistance in other experiments carried out in the department was generally carried out at concentrations of around 100μg/ml of kanamycin in the medium, these experiments were usually of the tissue culture type and done with more highly inbred strains than those used in my experiments. Therefore, it seemed wise to take the precaution of establishing an appropriate level for screening my seed by experiment. This was undertaken by setting up plates of agar medium containing 0, 25, 50, 100 and 200 μg/ml concentrations of kanamycin and planting out seed collected from similar crosses to those used experimentally to assay (a) for an appropriate level of kanamycin sufficient to screen out any low-level variation of intrinsic resistance due to variation of genetic

background (important as these experiments were all done as out-crosses); and (b) to establish if any significant background of high-level resistance to kanamycin existed in the seeds used. The results showed that some resistance is apparent to all levels of kanamycin in the sense that the seeds germinate and grow to the hypocotyl stage at all concentrations of kanamycin used. However, it is extremely apparent that all levels of kanamycin inhibit further growth to some extent, and that once the level of 100μg/ml is reached, further inhibition is not detectable in the 200μg/ml plates. The effect is most striking when examined in terms of root growth (see plates 4 and 5) which is stopped completely when the roots reach about 1cm length on the kanamycin containing plates, but which is extensive (many can grow within 14-20 days) on the control plates not containing kanamycin.

As a result of these experiments, it was decided that 100μg/ml kanamycin was an appropriate level of antibiotic for screening of the experimental seeds, as this level seems to be the lowest level which gives complete inhibition of root growth in all non-transformed plants. Thus this level of selection should be sufficient to give the maximum chance of finding a transformed individual, i.e. as low a level as possible to maximise this chance, while being sufficiently high to avoid the inclusion of too many plants within the higher reaches of the normal distribution of natural resistance in the class of putative transformants.

(e) Transformation experiments

Having established (see above) that successful pollinations were achievable using pregerminated pollen and which crosses were mostly

likely to succeed, the next step was to attempt to reproduce Hess' and De Wet's methods in the experimental material.

Experimental crosses were therefore set up utilising these methods and using pABDI as the potential transforming agent. Initially successful pollination was monitored using the aniline blue stain to monitor pollen tube growth by excising the stigma and style and doing squash preparation. The results of some of these are shown in plates 2 and 3. However it was noticed after the first two experiments that (a) observation of successful tube growth was not a guarantee of seed set (nor to a lesser extent did failure to observe growth guarantee failed seed set), and (b) I felt that on at least some occasions, due to the variable nature of the ratio of pollen tube development, I may have actually pre-empted successful seed set by excising the stigma and style too early. This method was therefore abandoned in favour of waiting to see if seed set occurred.

De Wet's method was attempted first, as its greater technical simplicity makes it the more attractive candidate for a general method. The tables 5-7 show the crosses made and the levels of success obtained for both de Wet's and Hess' method as applied to Petunia. Tables 8-10 show similar results for Nicotania. In all cases control crosses were made using pregerminated pollen treated in an identical manner but without pABDI in the mixture. It was not felt necessary to include alternative DNA in the mixture as in both De Wet's and Hess' experiments as the sought-after effect should be clear cut (i.e. Kanamycin resistance) and presence of the plasmid DNA in putative transformants would be confirmable by probing DNA prepared from the transformants using nick translated pABDI as a probe, in Southern blotting

experiments. Thus any resistance observed should be easily identifiable and unlikely to be an effect brought about by a non-specific effect of the DNA treatment.

Seed from the successful crosses was collected fastidiously and screened for Kanamycin resistance at 100μg/ml concentration of Kanamycin in the medium. Control crosses were used to set up control plates with and without kanamycin, which were used as a standard against which to judge the experimental seed. The comparison of these control plates illustrating to clear-cut nature of the selection is shown in plate 5.

The results of these screening experiments, showing the numbers of seed screened, the percentage germination and the numbers of transformants obtained are shown in tables 7 and 10.

Unfortunately, some problems were encountered with contamination during the seed screening and a substantial proportion of the seeds were lost particularly in the experiment investigating Hess' method in Petunia. In strict accordance with Parkinson's Law, this happened to be the experiment for which least experimental seed was obtained in the first place, and therefore the resulting number successfully screened in this experiment is too low for useful conclusions to be drawn. In other circumstances, it would have been simple enough to repeat this experiment, but in this short project the waiting time would have been unacceptable.

PART TWO: BUD DEVELOPMENT EXPERIMENT

The observations made of bud development may be summarised in terms of counting back from the day of anthesis and the results are shown in Table 11.

These observations have been correlated with a series of photomicrographs of wax embedded sections of the developing flower buds and anthers.   It was found that the meiosis which gives rise to the pollen tetrads occurs when the bud has recognisable petals approximately 1cm in length.   The pollen tetrads are produced in a continuous way from the microsporocytes over a short period of time when the bud is this size.   The cells at different stages of meiosis occur over a period of about four days at about 15 days before anthesis.   Plate 4 shows these cells being produced from the pollen mother cells (microsporocytes), these are the layer of cells showing intense division activity.   Various types of meiotic division are apparent.   Plate 5 shows the final appearance of the tetrads before they separate into the haploid microspores.

<u>Table 1</u>

Crosses made and proportion of successful pollen germination in <u>Petunia</u> plants.

Pollen Parent

Female Recipient	P20		P21	
	No. of flowers	Successful germination	No. of flowers	Successful germination
1	–	–	1	0
2	3	3	4	4
3	3	3.	1	1
4	–	–	1	0
5	–	–	1	1
6	2	1	2	1
7	4	4	4	2
8	–	–	1	0
10	4	0	2	0
11	1	0	1	1
12	1	1	–	–
13	2	0	1	0
14	–	–	1	0
15	–	–	1	1
16	1	0	–	–
18	2	2	–	–
Totals	23	14	21	11

Table 2

Crosses made and proportion of successful pollen germination in _Nicotania_ plants.

Pollen Parent

Female Recipient	T1		T2		T4		T6		T7	
	N	S	N	S	N	S	N	S	N	S
1	2	0	1	0	2	0	3	0	–	–
3	2	1	2	2	2	2	5	5	–	–
4	2	2	2	1	–	–	–	–	4	2
5	2	2	3	3	2	2	–	–	–	–
6	1	1	2	2	1	1	–	–	–	–
7	2	2	2	2	3	3	2	1	3	1

Table 3

Success of Petunia crosses related to age of female flower on date of pollination.

Days emasculated prior to pollination	Pollen Parent	Number of crosses	Number of successes	% success
3-5	P20	15	8	
	P21	10	7	70
0-2	P20	16	9	
	P21	10	4	40

<u>Table 4</u>

Crosses made and proportion of successful seed set in experiments with excising the stigma and style prior to pollination

Pollen Parent

Female recipient	P20		P21	
	No. of flowers	Successful seed set	No. of flowers	Successful seed set
1	-	-	1	0
3	3	0	1	0
4	1	0	1	0
6	-	-	1	1
7	2	0	1	0
11	-	-	1	0
12	1	0	-	-
16	1	0	2	0
17	1	0	-	-
19	1	0	1	0
Totals	10	0	9	0

Table 5

Experimental and Control Crosses made to investigate De Wet's Method in
Petunia

Pollen Parent

| | P20 | | P21 | |
Female recipient	No. of flowers	Successful seed set	No. of flowers	Successful seed set
1	5	5	-	-
2	6	3	-	-
6	-	-	5	2
7	1	1	3	2
13	7	2	-	-
14	5	4	-	-
16	5	3	-	-
Totals	29	18	8	4

Controls

2	2	2	-	-
6	-	-	2	1
7	1	0	1	1
13	2	1	-	-
16	1	1	-	-
Totals	6	4	3	2

Table 6

Experimental and Control Crosses made to investigate Hess' Method in Petunia

Pollen Parent

	P3		P8	
Female recipient	N flowers	Successful seed set	No. of flowers	Successful seed set
3	5	0	-	-
5	5	0	4	0
8	5	0	5	3
9	5	0	4	3
12	2	0	2	2
Totals	22	0	15	8

Table 7

Number of Seeds screened for Kanamycin Resistance in Petunia Experiments

Method	Number plated	Number germinated (%)	Number of resistant plants
De Wet	2386	2267 (95%)	0
Hess	447	427 (96%)	0
TOTAL	2833	2684	0

Table 8

Experimental Crosses made to Investigate De Wet's Method in Nicotania

Pollen Parent

Female recipient	T2		T4		T6	
	N	S	N	S	N	S
3	5	3	5	1	12	3
4	–	–	–	–	7	0
5	2	0	2	0	10	1
7	4	0	3	3	2	0
Totals	11	3	10	4	31	4

Controls

3	–	–	–	–	4	1
4	–	–	–	–	4	3
7	–	–	–	–	4	2

Table 9

Experimental Crosses made to Investigate Hess' Method in Nicotania

Pollen Parent

Female recipient	T2		T4		T6	
	N	S	N	S	N	S
3	5	2	5	2	5	3
5	2	0	2	1	2	2
7	3	0	4	1	4	3
Totals	10	2	11	4	11	8

Table 10

Number of Seeds Screened for Kanamycin Resistance in _Nicotiana_ Experiments

Method	Number plated	Number germinated	Number of Resistant plants
De Wet	1702	1668 (98%)	0
Hess	2978	2889 (97%)	·0
TOTAL	4680	4557	

Table 11

Flower Opening Dates and Flower Development (days prior to opening)

Flower number	Anthers dehisced (date)	Closed petal whorl ½" length	Recognisable Flower bud
14.1	15/5	-14 days	-21 days
11.1	23/5	-12 days	-19 days
11.2	25/5	-12 days	-21 days
11.3	23/5	-12 days	-19 days
15.1	28/5	-9 days	-17 days
15.2	28/5	-10 days	-17 days
17.1	30/5	-8 days	-19 days
17.2	22/5	-11 days	-18 days
12.2	1/6	-10 days	-18 days
15.3	15/5	-12 days	-16 days
Means with standard deviations		11.0+/-1.7	18.5+/-1.6

CHAPTER 4   DISCUSSION

The potential advantages of direct transformation of the zygote within the context of the normal plant reproductive cycle have been discussed by many authors, and have been ably summarised by Zhou (1985) who adumbrated the following major benefits:

(1)   A much shorter time is required for generation of transformed plants compared to genetic manipulation starting with a plant cell culture stage.

(2)   Existing economic cultivars may be manipulated directly, and the economic importance of the transformed plant judged directly, eliminating the tedious cell selection procedure, and avoiding the uncertainty about the genetic value of the final product due to passage through artificial culture conditions.

Zhou concluded that these direct techniques, having the outlined potential advantages and also the benefit of being technically less demanding, should be more practical than current methods in the realisation of plant genetic engineering.   In addition, these methods allow the possibility of addressing the problem of transforming monocots, especially the economically important cereals, which have largely resisted manipulation either by Agrobacterium mediated transformation, to which they do not seem to be susceptible, or by direct transformation of protoplasts due to the difficulty in regenerating complete plants from cereal protoplasts (Lichtenstein 1987).   This latter problem has recently shown signs of being a surmountable problem, with some success being gained with the

regeneration of rice protoplasts (Fujimura et al 1985, Yamada et al 1986).

As Old and Primrose (1985) point out in their text on genetic manipulation, in the course of a discussion of the merits of pursuing the development of the caulimoviruses as plant vectors, research on alternative vectors for plants transformation would probably be totally justified in economic terms if they contributed only one successful transformed cultivor of one of the major crop plants, such is the scale of the agricultural industry. Of the methods considered during this project, microinjection of exogenous DNA directly into pollen tubes or embryo sacs has yet to show any success (Hepher et al 1985, Steinbiss et al 1985). Introduction of exogenous DNA via direct transformation of the pregerminating pollen has been reported on several occasions (De Wet et al 1985, Hess 1978, Hess 1979, Hess and Dressler 1984, Hess, Dressler and Konle 1985). Also direct injection of transforming DNA into the proximity of apparently naturally competent cells at different stages of the development cycle has shown some success (Zhou et al 1983, Zhou 1985, de la Peña, Lörz and Schell 1987).

The work of Dieter Hess in particular, has laid on extensive groundwork of evidence that pregerminated pollen tubes are competent to take up exogenous DNA in both Petunia and Nicotiana. The recent success of De Wet in achieving transformation of maize uses methods based on the same principle. This apparent confirmation of Hess's ideas suggests that these relatively simple methods are worth examination, for if these methods could be applied more generally, a considerable revolution in plant genetic engineering would take place. This work set out to examine the methods of Hess and also the potentially simpler method of

De Wet, to see if they could be repeated with _Petunia_ and _Nicotiana_. Success in this venture would lend credence to the claim of Zhou (1985) that his method and that of Hess promise to raise plant genetic enginering to a level of practical benefit to crop improvement. The results have shown that despite screening a substantial number of successfully germinated plants (3935 for De Wet's method and 3316 for Hess's method) no transformants were found. This contrasts with the findings of the original authors who found relatively high frequencies of transformation for the characters which they were examining. Thus De Wet et al (1985) found 72 out of 6303 seedlings of a true breeding white cob variety of maize transformed to red cob phenotype by their transforming DNA, obtained from a red cob variety (a staggering 1.1% transformation rate), they also found 2 out of 6303 seedlings transformed to rust resistance (a 0.03% transformation rate). Hess et al (1985) express many reservations, but reading through the group's results over the many years in which they have been researching the use of pollen vectors, they appear to suggest a transformation rate in _Petunia_ of the order of 0.2% using their direct transformation method. In all cases (except perhaps the red cob character), the authors find some difficulty in differentiating completely satisfactorily the transformed progeny from background levels of expression of similar phenotypes in untransformed progeny.

The plasmid pABDI used for these experiments was specifically selected because it would be a relatively simple matter to unambiguously demonstrate its presence in, and transmissibility to progeny of, transformed individuals. Paszkowski et al (1984) constructed pABDI and used it in protoplast transformation experiments with _Nicotiana tabacum_.

Presence of the plasmid was selected and demonstrated by resistance to 200μg/ml kanamycin, and confirmed both by assay of APH(3')II enzyme activity and by Southern blot hybridisation of nick-translated probes for plasmid DNA sequences. Thus, should genuinely transformed plants be evidenced by the screening process, confirmation of the presence of the plasmid can be unambiguously demonstrated, a level of confirmation never quite achieved either by De Wet or Hess. Paszkowski et al were also able to demonstrate that the acquired resistance was genetically transmissible, segregating with a 1:1 ratio in one transformant strain, indicating incorporation of only one functional locus for kanamycin resistance which is acting as a Mendelian dominant factor. This evidence suggests that incorporation of a single functional copy of the resistance gene is sufficient to confer a resistant phenotype.

Why then was no success observed in this case? The probability of observing at least one transformant for a given sample size and transformation frequency is calculable using the binomial theorem. The values of these probabilities for the highest to the lowest observed transformation frequencies, relative to the sample sizes achieved in the experiments are shown in table twelve. It can be seen that if the highest levels of transformation were obtained, similar to those quoted for transformation of maize to red cob colour by De Wet et al (1985), the probability of observing at least one transformant in the sample would be very high. As the transformation frequency reduces, so do the probabilities of observing a transformant for each sample size. Thus it can be seen that for the lowest transformation rate of 0.03% reported by De Wet et al (1985) the sample size would have to rise to 10,000 for a reasonable certainty of observing a transformant to be obtained.

However, even at this transformation rate a better than 50% probability exists of seeing a transformant in all but the experiments having the lowest sample sizes. For the lower transformation rate of 0.01% (which is still higher than the rates claimed by Paszkowski et al 1984 for protoplast transformation with this plasmid, which are at least one order of magnitude lower), it can be seen that the probability of observing at least one transformant drops away quickly, with the probability being very small indeed for a sample of 427 and only rising to 0.39 for a sample of 5000. Thus even if it were legitimate (which it surely is not) to pool the results of all the experiments the probability of having seen a transformant at this transformation rate would still only be around 0.50. It is however reasonable to pool the results over a method and thus it can be seen that for all the putative transformation rates observed by other authors using their methods, the lowest probability of finding a tranformed plant in these pooled experiments was .63, quite a high probability of success. For transformation frequencies of the order of 0.1% such as those observed by Dieter Hess's group, a high probability exists of finding a transformant in all the experiments taken individually except the sample for Hess's method using Petunia which shows a probability of only 0.35 of observing a transformant. For the highest transformation frequency observed by De Wet (1.0%) even the smallest sample gives an extremely high probability of having observed a transformed plant.

The conclusion from this must be that if the methods of either Hess or De Wet are looked at in terms of their general applicability, then with any of the transformation rates which these authors obtained, even at the lowest rate of transformation, the experiments done here had a

reasonable chance of finding a transformed plant, and at the higher rates a transformed plant should almost certainly have been observed. If each experiment is examined as an individual case, it is still true for Hess's transformation rates there was high probability of having identified a transformant (the lowest is 0.81). The exception to this is for the experiment using Hess's method with _Petunia_ where at least 900 seeds were lost to a major incident of contamination, and no more seeds were available to screen, even this experiment gives p = 0.35, which is about a 1 in 3 chance of observing a transformant.

It seems then that the experiments have been unable to confirm the general applicability of Hess's and De Wet's methods. Certainly the experiments have not confirmed the high rates of transformation obtained by these workers. For the specific case of Hess's method as applied to _Petunia_, which in terms of the literature evidence, unfortunately, was the experiment most likely to work, the sample size obtained is really insufficient to do anything other than deny rates of transformation in excess of 1%. For the other cases the evidence suggests that transformation rates of 0.1% are unlikely and that even if transformation rate are as low as 0.03% it is probable that a transformant would have been found if the methods have general applicability. It would have been desirable to achieve even higher sample sizes in the screening experiments, and except for the _Petunia_ crosses using Hess's method, ample seed remains unscreened due to lack of time. However if the true transformation rate is of the same order of magnitude as that obtained by protoplast transformation (and the methods are in principle very similar), sample sizes would need to be very high indeed (around $5 \times 10^5 - 2 \times 10^6$) in order to achieve a high

probability of observing a transformant. With pollen transformation these population sizes are easily attainable in the target population of pollen, but as only a relatively small proportion of these will successfully enter an embryo sac and complete fertilisation, the seed sampling problem is immense. An alternative approach which takes advantage of this ability to attain high pollen target population would exist if it was possible to demonstrate expression of the transformed phenotype in the gametophyte itself (i.e. the pollen tube). This would allow either direct screening of the pollen for transformation using techniques such as anther culture, or enrichment of the pregerminated pollen for transformed phenotype by exposure to kanamycin containing medium during the wet pollination procedure.

This latter suggestion could be particularly appropriate for utilisation in association with the methods of de la Peña et al (1987), where the transforming DNA is incorporated during the process of gametogenesis. The probability of DNA introduced during this phase of development being integrated and functional during the metabolically active stage of the gametophyte must be quite high, and therefore the possibility of enriching for transformants exists.

The method of de la Peña et al (1987) is an attractive alternative to those of Hess, De Wet and Zhou. It is similarly dependent on the introduction of transforming DNA at a stage of development where nturally competent germ-line cells exist. Considerable groundwork of cytological work specific to rye was laid down before these transformation experiments were attempted (de la Peña, Puertas and Merino 1981, Puertas et al 1984), and therefore it would have been inappropriate to attempt such experiments with other species before a

time scale for the identification of the same competent stage in development had been established. The results outlined in part two of the last chapter have gone some way towards identifying the analogous stages in _Petunia hybrida_. The timescale in which buds differentiate and move to the meiotic stage during which the microspore tetrads are formed has been established. However, as the overall timescale of flower development is considerably shorter in _Petunia_ than _Secale cereale_ it seems unlikely that the treatment time of 10-14 days prior to this is appropriate for _Petunia_, as it is clear that buds are not committed to flower formation this early before meiosis. It had been hoped that a protocol would be established for effective injection of transforming DNA into developing _Petunia_ buds so that experiments could be done to establish the appropriate time for the injections using methods similar to de la Peña, Puertas and Merino (1981) and Puertas et al (1984), time did not allow this.

Table 12

Probability values of finding at least one transformed plant for given

sample sizes and transformation frequencies

Transformation frequency

Sample Size	1%	0.1%	0.03j%	.01%
427	.98	.35	.12	.04
1668	.99	.81	.39	.15
2267	>.99	.87	.49	.20
2889	"	.94	.59	.25
3316	"	.98	.63	.28
3933	"	.98	.69	.32
5000	"	.99	.77	.39
10000	"	>.99	.95	.63

Plate 1   Freshly harvested pollen of _Petunia hybrida_ after about 30 minutes pregermination in Brewbaker and Kwack's medium.
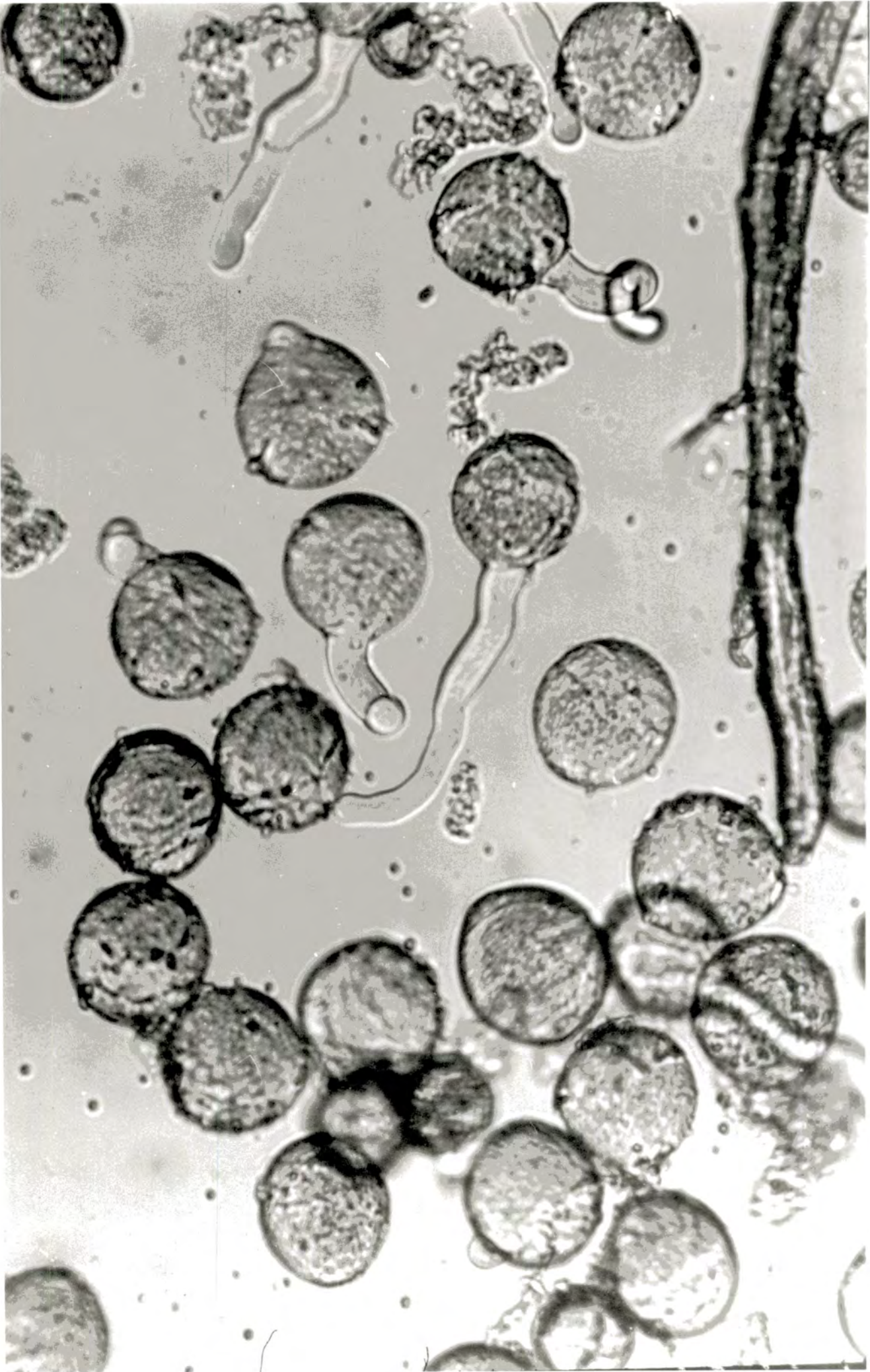
Plates 2 and 3   The effect of Kanamycin selection on germinating
                   seeds of <u>Petunia hybrida</u>. The plates compare the
                   luxurious growth and long roots of seeds growing on
                   a control plate with the much poorer foliage and
                   stunted roots caused by selection on plates
                   containing 100μg/ml kanamycin.

                   Plate 1 shows the plates viewed from the foliage
                   side, Plate 2 the same plates viewed from the
                   underside showing the dramatic difference in root
                   development.
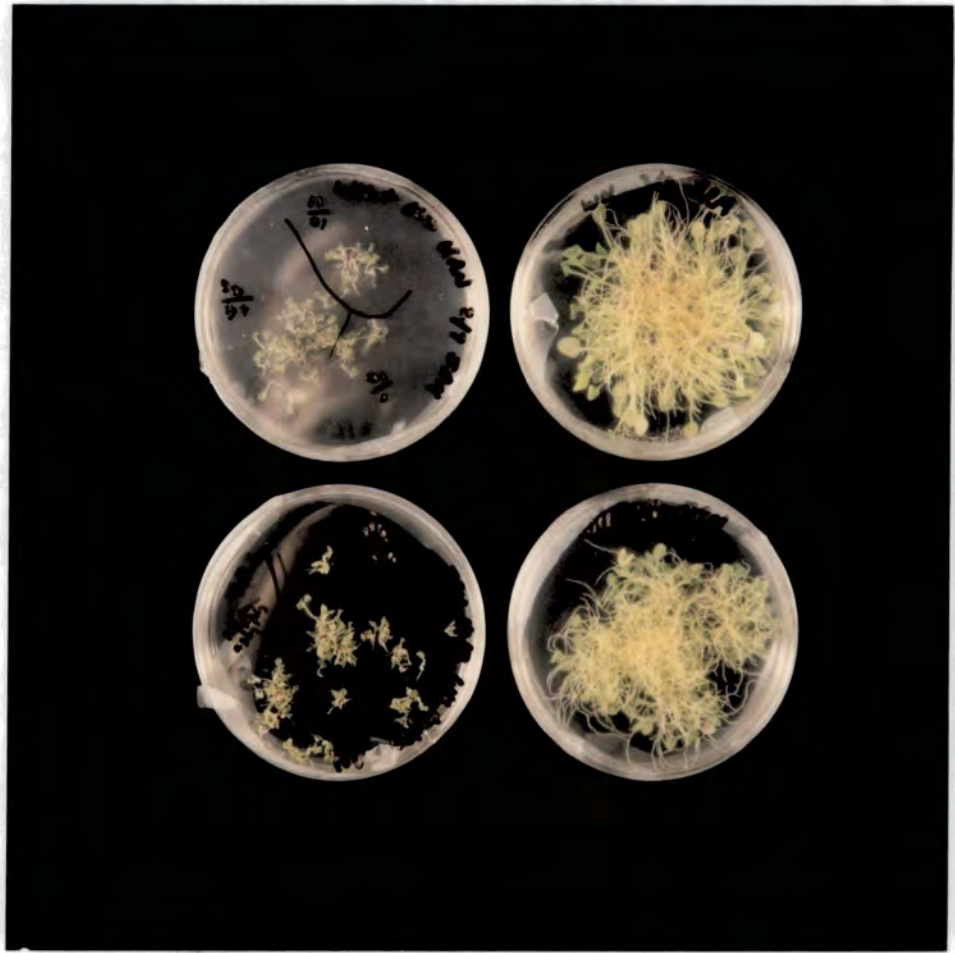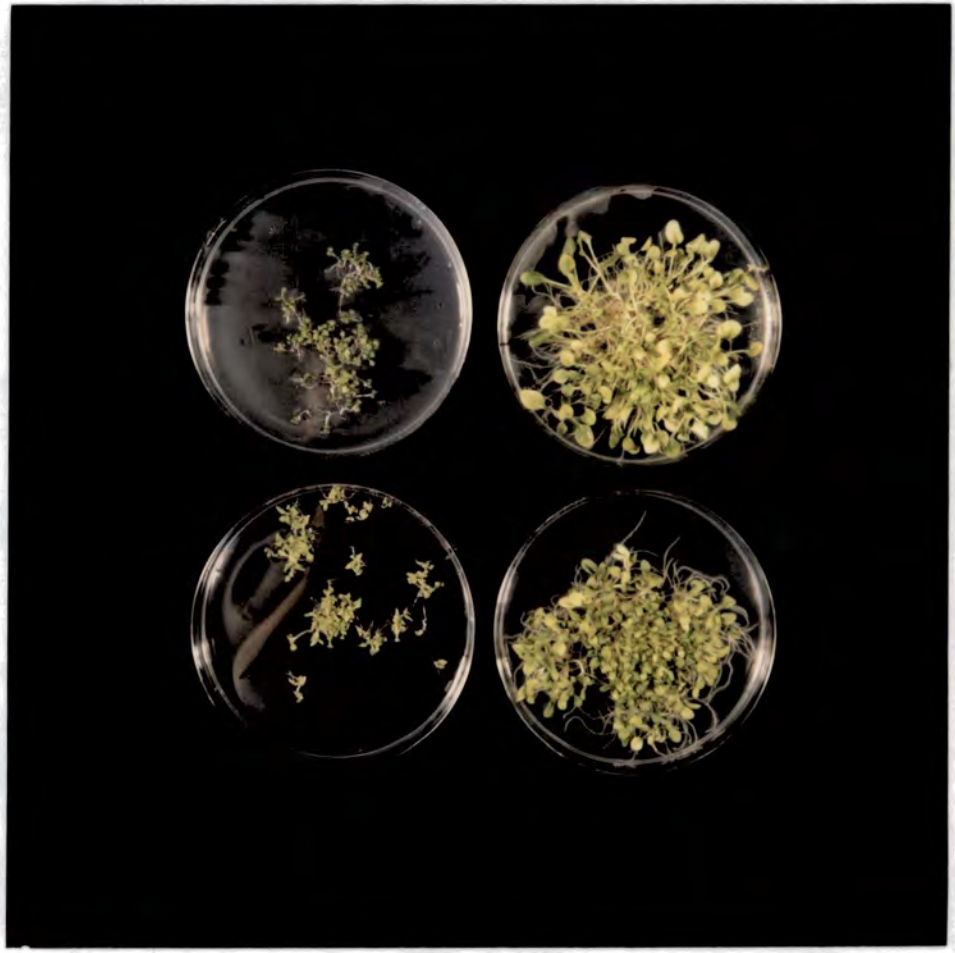
Plate 4   Pregerminated pollen of _Petunia hybrida_ showing continued development in situ on a receptive stigma. The pollen tubes are stained with decolourised Aniline blue stain and the squash preparation (x400) photographed under UV illumination.

Plate 5   Pollen tubes of _Petunia hybrida_ showing massive growth down the style after pollination with pollen pregerminated in Brewbaker's medium containing pABDI. Preparation and staining as above.
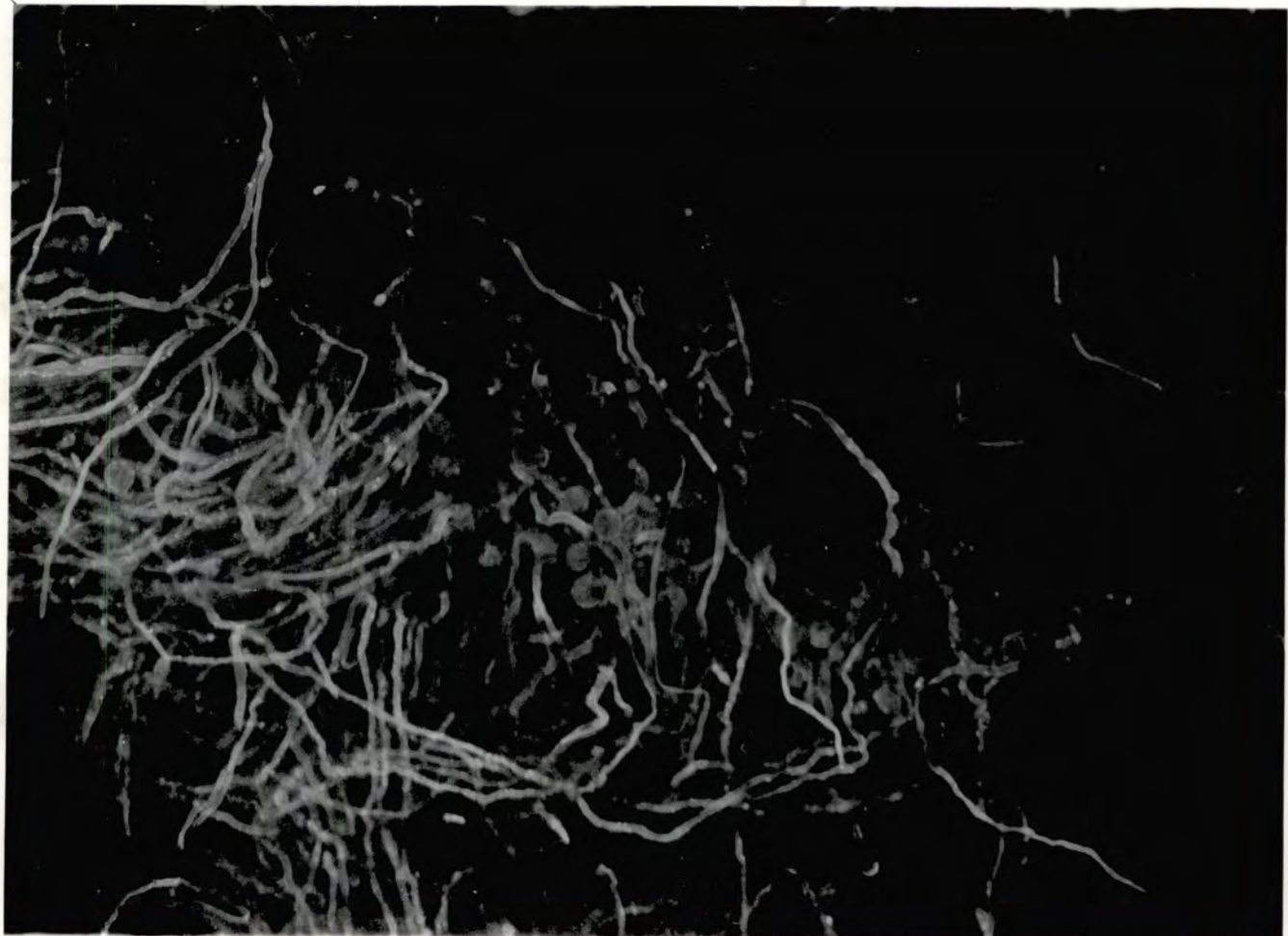
Plate 6   Microsporogerous tissue showing cells in various stages of

the meiotic division which results in the formation of
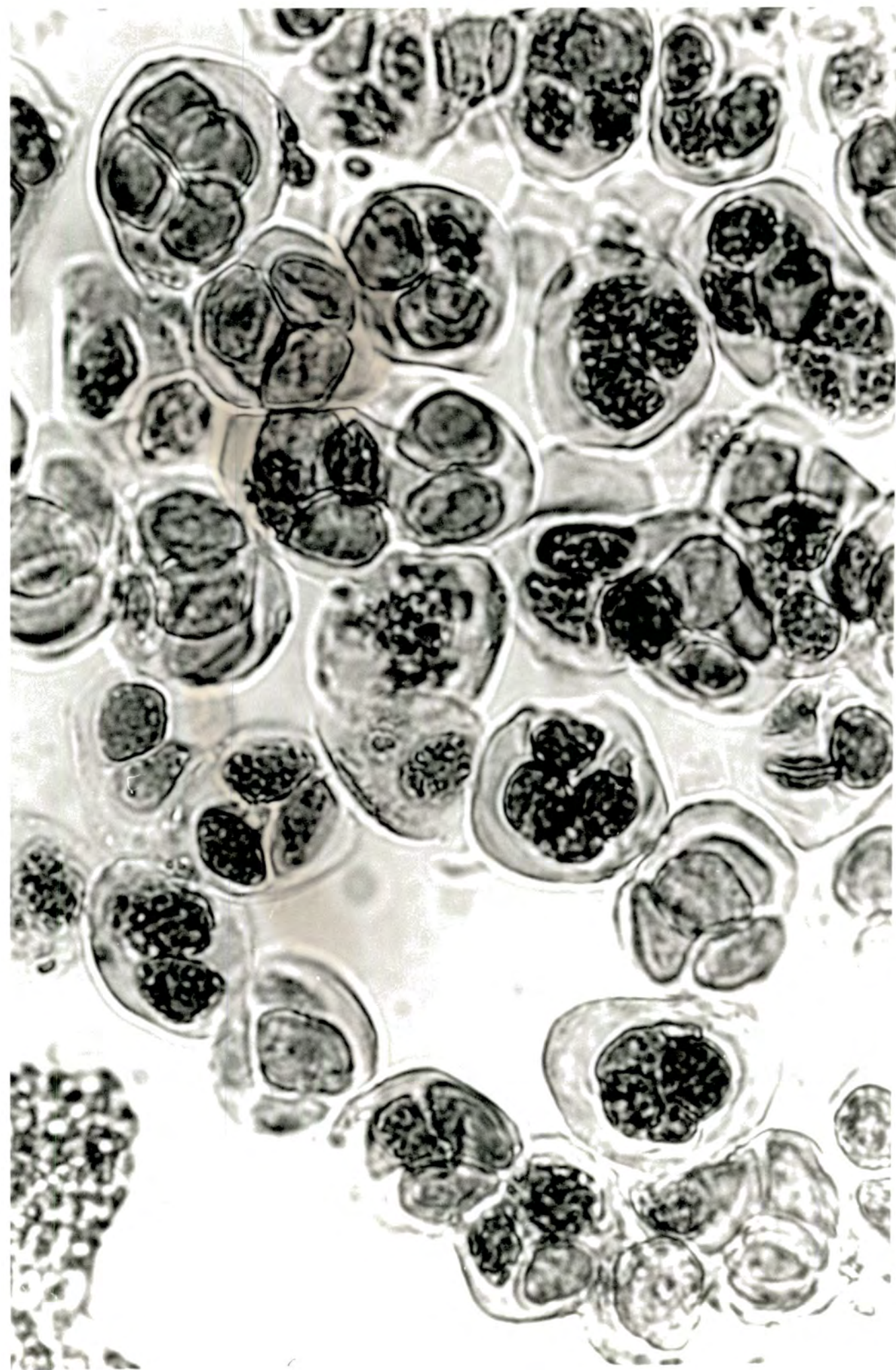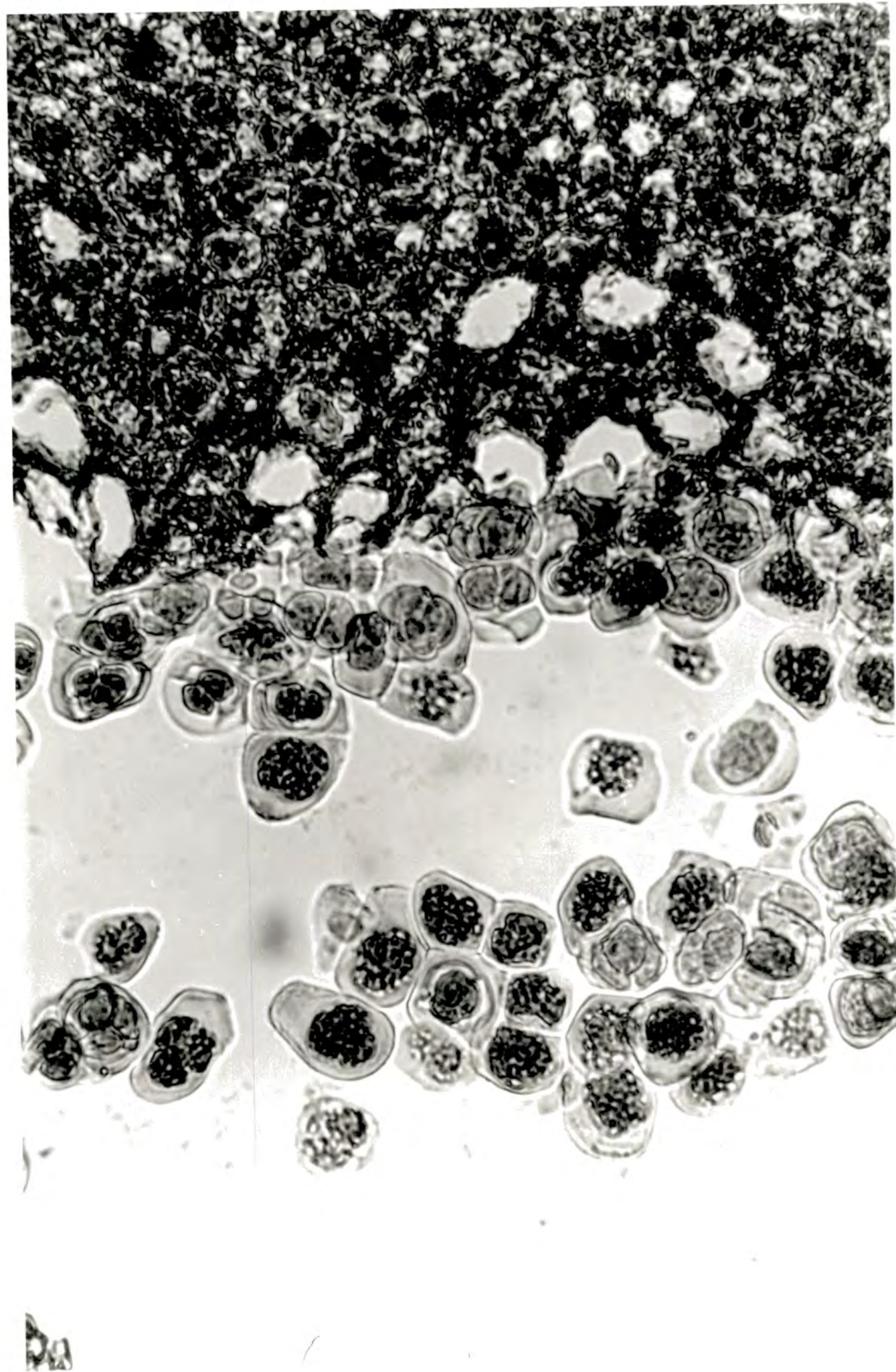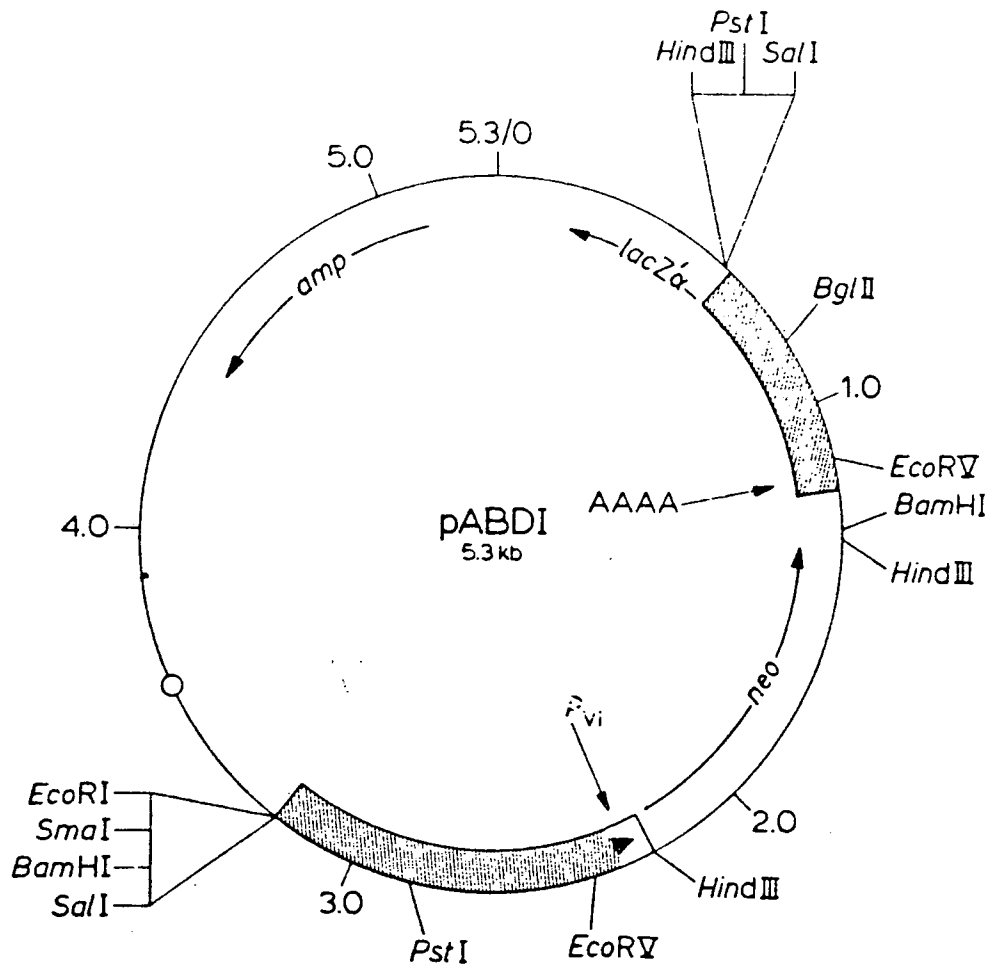
microspore tetrads.

Plate 7   The final appearance of the microspore tetrads before they

separate   into   haploid   microspores,   at   about   14-15   days

before anthesis.

Replicon	: pMB1^2 (*E. coli*)
Size	: 5.3 kb
Selectable phenotype for plant cells	: Neo^R
Selectable phenotype for *E. coli*	: Amp^R
Cloning sites	: *Eco*RI, *Sma*I

Plasmid pABDI can be used for transfer of DNA sequences to plant cells. The plasmid contains the neomycin-resistance (*neo*) gene, the expression of which is controlled by the promoter (*p*vi), polyadenylation signal and transcription terminator of cauliflower mosaic virus (CaMV) gene VI. The plasmid does not contain T-DNA sequences, but cloned genes can be transferred to the plant genome by direct transfer (ref. 38) with selection of transformed cells by means of their Neo^R phenotype. The plasmid pABDI carries the ampicillin-resistance (*amp*) gene and an origin of replication for selection and propagation in *E. coli*.

Plasmid pABDI comprises a 2.6 kb *Sal*I fragment containing the CaMV sequences and the *neo* gene, inserted into the *Sal*I site of pUC8 (I-A-iv-20). The orientation of the *Sal*I fragment in pABDI is not given; one of the two possibilities has been chosen for the drawing.

APPENDIX II FORMULAE OF MEDIA USED

1. Pollen germination media.

(a) Brewbaker's Medium (Brewbaker and Kwack 1961)

Solution A: H_3BO_3 500mg/dm^3, $Ca(NO_3)_2$ 1,500mg/dm^3,

   KNO_3 500mg/dm^3, Mg SO_4 $7H_2O$ 1,000mg/dm^3

Solution B: 50% w/v sucrose.

Final medium 1 part solution A : 1 part solution B : 3 parts distilled water.

(b) Fähnrich's Medium (Fähnrich 1964)

   Final solution: H_3BO_3 0.001μM, Sucrose 0.7M.

2. Seed germination medium (Wagner & Hess 1973)

   mg/dm^3: $Ca(NO_3)_2$. $2H_2O$, 719.5; KNO_3, 125;

      KH_2PO_4, 125; $MgSO_4$. $7H_2O$, 125; $MnSO_4$. $4H_2O$, 3;

      Casein hydrolysate, 500; Myo-inositol, 500;

      Glycine, 7.5; Niacin, 1.25; $ZnSO_4$. $7H_2O$, 0.5;

      H_3BO_3, 0.5; $CuSO_4$. $5H_2O$, 0.025;

      Na_2MoO_4, 0.025; Pyridoxin. HCl, 0.25;

      Thiamin HCl, 0.25; Riboflavin, 0.25.

   g/dm^3: sucrose, 50; Difco Bacto-Agar, 10.

To this medium was added 5ml of EDTA Fe Na, 7.35g/dm^3.

REFERENCES

Austin RB, Flavell RB, Henson IE, Lower HJB (1986) *Molecular biology
and crop improvement; a case study of wheat, oilseed rape and faba
bean.* Cambridge University Press.

Beasely PA, Jensen WA (1985) 'Cotton ovule culture'. In: Chapman GP,
Mantell SH, Daniels (eds). *Experimental Manipulation of ovule
tissues.* Longman. pp. 221-39.

Brewbaker JL, Kwack BH (1963) 'The essential role of calcium ion in
pollen germination and pollen tube growth'. *American Journal
of Botany* 50:859-65.

Capecchi M (1980) 'High efficiency transformation by direct micro-
injection of DNA into cultured mammalian cells' *Cell* 22:479-88.

Chaleff RS (1983) 'Isolation of agronomically useful mutants from plant
cell culture'. *Science* 219:676-82.

Chapman GP (1985) 'Nuclear events and the angiosperm megagametophyte:
A perspective'. In: Chapman GP, Mantell SH, Daniels (eds).
Experimental Manipulation of Ovule Tissues. Longman. pp. 1-26.

Cornu A, Maizonnier D (1982) 'The Genetics of Petunia'. In:

de la Peña A, Puertas MJ, Merino F (1981) 'Bimeiosis induced by
caffeine'. *Chromosoma* 83:241-48.

de la Peña, Lörz H, Schell J (1987) 'Transgenic rye plants obtained by
injecting DNA into young floral tillers'. *Nature* 325:274-76.

De Wet JMJ, Bergquist RR, Harlan JR, Brink DE, Cohen CE, Newell CA,
De Wet AE (1985) 'Exogenous gene transfer in maize (*Zea mays*)

using DNA treated pollen'. In: Chapman GP, Mantell SH, Daniel

(eds). Longman. pp.179-209.

De Wet JMJ, Newell CA, Brink DE (1984) 'Counterfeit hybrids between

Tripsacum and *Zea* (Graminae)'. *American Journal of Botany* 71:

245-51.

Evans DA, Sharp WR (1986) 'Applications of somoclonal variation'.

Biotechnology 4:528-32.

Fahnrich P, (1964) 'Untersuchungen über den Einfluss des Bors bei der

Pollenkeimung und beim Pollenschlauchwachstum'. In Linskens HF

(ed) *Pollen Physiology and Fertilisation* 102-27. North Holland,

Amsterdam.

Gordon JW, Ruddle FH (1981) 'Integration and stable germ line

transmission of genes injected into maise pronuclei'. *Science*

214:1244-46.

Grimsley N, Hohn T, Davies JW, Hohn B (1987) 'Agrobacterium mediated

delivery of infectious maize streak virus into maize plants'.

Nature 325:177-79.

Hain R, Stabel P, Czernilofsky AP, Steinbiss HH, Herrera-Estrella L,

Schell J (1985) 'Uptake, integration, expression and genetic

transmission of a selectable chimeric gene by plant cell culture'.

Molecular and General Genetics 199:161-68.

Hess D, Gresshoff PM, Fielitz U, Gleiss D (1974) 'Uptake of protein and

bacteriophage into swelling and germinating pollen of *Petunia*

hybrida'. *Zeitschrift für Pflanzenphysiologie* 74:371-76.

Hess D, Lörz H, Weissert E-M (1974) 'Uptake of bacterial DNA into

swelling and germinating pollen grains of *Petunia hybrida* and

Nicotiana glauca'. *Zeitschrift für Pflanzenphysiologie* 74:52-63.

Hess, D, Schneider G, Lörz H, Blaich G (19796) 'Investigations on the tumour induction in *Nicotiana glauca* by pollen transfer of DNA isolated from *Nicotiana langsdorffii*'. *Zeitschrift für Pflanzenphysiologie* 77:247-54.

Hess D (1978) 'Genetic effects in *Petunia hybrida* induced by pollination with pollen treated with lac transducing phages'. *Zeitschrift für Pflanzenphysiologie* 90:119-32.

Hess D (1979) 'Genetic effects in *Petunia hybrida* induced by pollination with pollen treated with Gal transducing phages'. *Zeitschrift für Pflanzenphysiologie* 93:429-36.

Hess D (1981) 'Attempts to transfer kanamycin resistance of bacterial plasmil origin in *Petunia hybrida* using pollen as vectors'. *Biochemistrie Physiologie Flanzen* 176:322-38.

Hess D, Dressler K (1984) 'Bacterial transferasc activity expressed in *Petunia* progenies'. *Journal of Plant Physiology* 116:261-72.

Hess D, Dressler K, Konle S (1985) 'Gene transfer in higher plants using pollen as vectors: Bacterial transferasc activity expressed in *Petunia* progenies'. In: Chapman GP, Mantell SH, Daniels (eds) *Experimental Manipulation of Ovule Tissues.* pp.224-39.

Horsch RB, Fraley RT, Rogers SG, Sanders PR, LLoyd A, Hoffman N (1984) 'Inheritance of functional genes in plants'. *Science* 223:496-98.

Lichtenstein CP, Draper J (1985) 'Genetic engineering of plants'. In: Glover (ed) *DNA Cloning: a practical approach* Volume II IRL pp.67-119.

Lichtenstein CP (1986) 'A bizarre vegetal bestiality'. *Nature* 312:678.

Lichtenstein CP (1987) 'Transgenic cereals by direct injection of DNA into plants'. *Nature* 325:192.

Matthews REF (1981) *Principles of Plant Virology.* 2nd edition.

    Academic Press, New York.

Mantell SH, Matthews JA, McKee RA (1985) *Plant Biotechnology.*

    Blackwell Ltd.

Nu, et al, 'Genetic manipulation in higher organisms III. Detection of

    soya protein in seeds from soy on RNA treated rice'. *Scientia*

    Sinica XXIII:119-23.

Old, Primrose (1985) *Principles of gene manipulation.* Blackwell Ltd.

Palmiter RD, Bruister RL, Hammer RE, Trumbauer ME, Rosenfeld MG,

    Birnberg NC, Evans RM (1982) 'Dramatic growth of mice that develop

    from eggs micro-injected with metallothionein-growth hormone

    fusion gene'. *Nature* 300:611-15.

Pandey KK (1980) 'Further evidence for egg transformation in *Nicotiana.*'

    Heredity 45:15-30

Pandey KK, Phung (1982) '"Hertwig effect" in plants: Induced

    parthenogenesis through use of irradiated pollen'. *Theoretical and*

    Applied Genetics 62:295-300

Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I

    (1984) 'Direct gene transfer to plants'. *The EMBO Journal*

    3:2717-22.

Powell W, Caligari PDS, Hayter AM (1983) 'The use of irradiated pollen

    in barley breeding'. *Theoretical and Applied Genetics* 65:73-76.

Puertas MJ, de la Peña A, Estades B, Merino F (1984) 'Early sensitivity

    to colchicine in developing anthers of rye'. *Chromosoma* 89:121-

    26.

Snape, JW, Parker BB, Simpson E, Ainsworth CC, Payne PI, Law CN (1983)

    'The use of irradiated pollen for differential gene transfer in

wheat (*Triticum aestivum*). *Theoretical and Applied Genetics* 65:103-11.

Soyfer VN (1980) 'Hereditary variability of plants under the action of exogenous DNA'. *Theoretical and Applied Genetics* 58:225-35.

Steinbiss H-H, Stabel P, Töpfer R, Hirtz RD, Schell J (1985) 'Transformation of plant cells by micro-injection of DNA'. In: Chapman GP, Mantell SH, Daniels (eds), *Experimental Manipulation of Ovule Tissues*. Longman. pp.64-75.

Tisserat B (1986) 'Embryogenesis, organogenesis and plant regeneration'. In: Dixon RA (ed), *Plant Cell Culture: a practical approach*. IRL.

Vaeck M, Reynaerts A, Höfte H, Jansens S, De Beuckeleer, Dean C, Zabeau M, Van Montagu M, Leemans J (1987) 'Transgenic plants protected from insect attack'. *Nature* 328:33-37.

Wagner, G, Hess D (1973) 'Invitro-Befruchtungen bei Petunia hybrida'. *Zeitschrift für Pflanzenphysiologie* 69:262-69.

Zhou G-Y, Weng J, Zeng Y, Huang J, Qian S, Liu G (1983) 'Introduction of exogenous DNA into cotton embryos'. *Methods in Enzymology* 101:433-81.

Zhou G-Y (1985) 'Genetic manipulation of the ovule after pollination'. In: Chapman GP, Mantell SH, Daniels (eds), *Experimental Manipulation of Ovule Tissues*. Longman. pp.240-50.