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Combination of *in vitro* angrogenesis and biolistic transformation: an approach for breeding transgenic maize (*Zea mays* L.) lines

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Publications coming out of this work

- Aulinger IE, Peter SO, Schmid JE, Stamp P (2003, in press) Gametic embryos of maize as a target for biolistic transformation: comparison to immature zygotic embryos. Plant Cell Reports.

- Aulinger IE, Peter SO, Schmid JE, Stamp P (2003, in press) Rapid attainment of a double haploid line from transgenic maize (*Zea mays* L.) plants by means of anther culture. In Vitro Cellular and Developmental Biology - Plant. Volume 39, March/April 2003.

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Summary

The combination of *in vitro* androgenesis and genetic transformation has been successfully applied to many species for the rapid development of fully homozygous transgenic lines. Despite repeated efforts to apply this strategy to maize (*Zea mays* L.), success has not yet been achieved.

The original goal of this doctoral work was to develop a protocol for the transformation of haploid or double haploid material in maize, following two basic strategies:

1) the biolistic transformation of isolated microspores and

2) the transformation of gametic embryos by applying a very efficient protocol developed for the biolistic transformation of immature zygotic embryos of maize.

The main aspects that were addressed in the development of these protocols were:

- the optimisation of the androgenic response, e.g. a high yield of induced microspores in the isolated microspore culture and a high number of gametic embryos by means of anther culture,
- the induction of secondary embryogenesis from gametic embryos and the efficient regeneration of the secondary embryos into double haploid plants,
- the analysis of double haploid material with regard to the level of homozygosity achieved,
- the study of the transgene expression in the target material (microspores, gametic embryos and immature zygotic embryos of a transformable genotype as a positive control) and
- the response of the transformed material to the selection method.

Each of the above plays an essential role in the establishment of a transgenic double haploid line.

The experiments performed during this doctoral work showed that the isolated microspore culture does not yield a sufficient number of gametic embryos, despite a significant improvement achieved by adding activated charcoal to the microspore culture. Furthermore, only 30 % of these gametic embryos presented a doubled chromosome set. The regeneration of the gametic embryos into plants was improved by means of an indirect regeneration method, based on the induction of somatic embryogenesis prior to regeneration. Still, the overall efficiency of the isolated microspore method is too low; thus, microspores are an unsuitable target for genetic transformation. Furthermore, transgene expression was not observed when the microspores were bombarded with a visual marker gene construct.

In contrast, the anther culture gave an extremely high yield of gametic embryos. These were responsive to the induction of secondary embryogenesis but were not as efficient as the

positive control zygotic embryos. The secondary embryos of gametic origin showed a satisfactory regeneration capability, though lower than that of the control. On average, 54 % of the regenerated plants were double haploid. A molecular markers study performed on four double haploid lines demonstrated that the material gained by androgenesis has a highly homozygous background. The experiments on the transformation of gametic embryos showed that the transgene was successfully delivered into the gametic embryos. However, the density of transiently transformed cells (by means of the density of cells expressing a visual marker gene) was much higher for the control zygotic than for the gametic embryos. The selection mechanism, based on the resistance to the herbicide Basta conferred by the *pat* transgene, seemed to have a toxic effect on non-transformed and, indirectly, on transformed cells of the gametic embryos.

Compared to the control zygotic embryos, the overall weaker response of gametic embryos to the determinant steps for a successful transformation determined that the establishment of a double haploid line from transformed gametic embryos was not achieved. Nevertheless, the gametic embryos could probably be transformed if some of the key steps were optimised even further.

Since the transformation of haploid or double haploid material was not achieved, another approach was taken to achieve the rapid development of double haploid and transgenic lines. The strategy was based on the extraction of transgenic double haploid lines from the cross between a transgenic (non-androgenic) and an androgenic genotype by means of anther culture. This approach was successful, even though it was very inefficiency. Nevertheless, this was the first time that transgenic and fully homozygous maize lines were achieved by the combination of genetic transformation and *in vitro* androgenesis.

It was concluded from this work that the combination of genetic transformation and *in vitro* androgenesis may be applied for the development of homozygous and transgenic maize lines. The key processes that limit the establishment of a reproducible and efficient protocol have been identified.

Zusammenfassung

Die Kombination von *in vitro* Androgenese und genetischer Transformation wurde erfolgreich für die Erzeugung transgener homozygoter Linien bei verschiedenen Pflanzenarten angewendet. Alle Versuche, diese Strategie bei Mais (*Zea mays* L.) einzusetzen, sind bisher jedoch fehlgeschlagen.
Die Zielsetzung dieser Dissertation war es, ein Protokoll für die Transformation von haploiden oder doppelhaploiden Strukturen bei Mais zu entwickeln. Für diesen Zweck wurden zwei Strategien verfolgt:
1) die biolistische Transformation von Mikrosporen und
2) die Transformation von gametischen Embryonen mittels eines effizienten Protokolls für die Transformation von zygotische Embryonen bei Mais.

In der Entwicklung dieser Protokolle wurden folgende Aspekte betrachtet:

- die Optimierung des androgenetischen Prozesses über Mikrosporen- und Antherenkultur,
- die Induktion der sekundären Embryogenese aus gametischen Embryonen und deren effizienten Regeneration zu doppelhaploiden Pflanzen,
- die Bestimmung des Homozygotiegrades der doppelhaploiden Pflanzen,
- die Analyse der Transgenexpression im Zielmaterial (Mikrosporen, gametische und zygotische Embryonen eines transformierbaren Genotyps als positive Kontrolle) und
- das Verhalten des transformierten Materials unter Selektionsdruck.

All diese Faktoren spielen eine essentielle Rolle in der Etablierung einer transgenen doppelhaploiden Linie.

Die Ergebnisse der in dieser Dissertation durchgeführten Experimente ergaben, dass die Mikrosporenkultur eine zu geringe Zahl von gametischen Embryonen liefert; dies trotz einer gesteigerten Effizienz, welche durch die Zugabe von Aktivkohle in das Kulturmedium erzielt wurde. Nur 30 % dieser gametischen Embryonen hatten einen verdoppelten Chromosomensatz. Die Regenerationsrate von Pflanzen aus gametischen Embryonen wurde durch die Anwendung eines indirekten Regenerationsverfahren verbessert, welches auf die Induktion der somatischen Embryogenese vor der Induktion der Pflanzenregeneration basiert. Dennoch ist die allgemeine Effizienz dieser Methode zu niedrig, um Mikrosporen als Ziel der genetischen Transformation in Betracht zu ziehen. Zudem wurde in einem Transformationsexperiment mit einem visuellen Markergen keine Expression des Transgens in Mikrosporen gefunden.

Im Gegensatz zu der Mikrosporenkultur, gab die Antherenkultur eine sehr hohe Ausbeute von gametischen Embryonen. Diese Embryonen zeigten eine gute Antwort in Bezug auf sekundäre Embryogenese, obwohl diese geringer als die der positiven Kontrolle (zygotische Embryonen) war. Die sekundären Embryonen aus gametischer Herkunft wiesen eine gute Regenerationsfähigkeit auf, die jedoch schlechter als die der Kontrolle war. Durchschnittlich 54 % der Pflanzen aus der Antherenkultur hatten einen verdoppelten Chromosomensatz. Eine Studie mittels molekularer Marker bestätigte, dass die doppelhaploiden Pflanzen einen hohen Homozygotiegrad hatten. Die Ergebnisse der Transformation von gametischen Embryonen zeigten, dass das Transgen vollständig in das Genom integriert wurde. Dennoch war die Menge an transformierten Zellen geringer in gametischen als in zygotischen Embryonen. Der in dieser Arbeit angewendete Selektionsmechanismus basierte auf die Resistenz gegen das Herbizid Basta, welche durch das *pat* Gen verliehen wird. Dieser Mechanismus entwickelte jedoch einen toxischen Effekt auf nicht transformierte und indirekt auf transformierte Zellen.

Die allgemein schlechtere Leistung der gametischen im Vergleich zu zygotischen Embryonen in Bezug auf die analysierten Aspekte bedingte, dass die Erzeugung transgener doppelhaploider Linien aus transformierten gametischen Embryonen nicht erreicht werden konnte.
Dennoch könnten gametische Embryonen potentiell transformiert werden, wenn eine weitere Optimierung bestimmter Schritte erreicht würde.

Nachdem die Transformation von Mikrosporen oder gametische Embryonen nicht erreicht wurde, wurde ein anderer Ansatz unternommen, um innerhalb weniger Generationen homozygote und transgene Pflanzen zu entwickeln. Diese Strategie basierte auf der Erzeugung von doppelhaploiden transgenen Pflanzen aus der Kreuzung zwischen einem transgenen nicht-androgenetischen Genotyp und einem androgenetischen Genotyp mittels Antherenkultur. Dieser Ansatz war erfolgreich, obwohl die Ausbeute mittels dieser Methode sehr gering war. Die Erzeugung transgener und homozygoter Maislinien über die Kombination von der *in vitro* Androgenese und der genetischen Transformation wurde bis *dato* nicht publiziert.

Die vorliegende Arbeit führte zu der Schlussfolgerung, dass die Kombination von *in vitro* Androgenese und genetischer Transformation zur Entwicklung von transgenen und homozygoten Maislinien dienen kann. Die Prozesse, die die Etablierung eines effizienten Protokoll für die Erzeugung von transgenen und doppelhaploiden Linien limitieren, wurden verifiziert.

Resumen

La combinación del fenómeno de androgénesis *in vitro* y de la transformación genética pudo ser aplicada exitosamente en varias especies para la generación de líneas transgénicas y homocigotas en un período de tiempo extremadamente reducido. A pesar de los repetidos esfuerzos para adaptar esta técnica a maíz (*Zea mays* L.), ningún éxito pudo ser alcanzado hasta el momento.

El objetivo de este trabajo fue desarrollar un protocolo para la transformación de material haploide o doble-haploide de maíz, siguiendo básicamente dos estrategias:

1) la transformación de microsporas a través del cañón de genes y

2) la transformación de embriones gaméticos siguiendo el modelo de transformación biolística de embriones cigóticos de maíz.

Los principales aspectos que fueron considerados en el desarrollo de tales protocolos fueron:

- la optimización de la respuesta androgénica, es decir, la obtención de una gran proporción de microsporas inducidas a través del cultivo de microsporas y la generación de un gran número de embriones gaméticos a través del cultivo de anteras,
- la inducción de embriogénesis secundaria a partir de embriones gaméticos y la eficiente regeneración de los embriones secundarios en plantas doble-haploides,
- el análisis del material doble-haploide con respecto al grado de homocigotía alcanzado,
- el estudio de la expresión del transgen en el explanto usado como blanco de la transformación (microsporas, embriones gaméticos y embriones cigóticos como control positivo) y
- la respuesta del material transformado al mecanismo de selección.

Cada uno de estos aspectos tiene una función esencial en el establecimiento de una línea transgénica doble-haploide.

Los experimentos llevados a cabo en esta tesis demostraron que el cultivo de microsporas genera una limitada cantidad de embriones gaméticos, a pesar de un mejoramiento significativo alcanzado a través de la adición de carbón activado a las culturas. Solamente el 30 % de los embriones gaméticos presentó un set de cromosomas duplicado. La regeneración de tales embriones en plantas pudo ser optimizada a través de la aplicación de un sistema de regeneración indirecto, el cual está basado en la inducción de embriogénesis somática previo a la inducción de la regeneración. A pesar de estos progresos, la eficiencia del sistema continúa siendo demasiado baja como para poder considerar a las microsporas un blanco adecuado para la transformación genética. Lo que es más, ninguna expresión del transgen pudo ser detectada cuando las microsporas eran bombardeadas con el gen marcador *gfp*.

El cultivo de anteras, por el contrario, genera una cantidad extremadamente alta de embriones gaméticos. Los mismos muestran una respuesta satisfactoria a la inducción de embriogénesis secundaria, aunque con eficiencias menores a las del control positivo (embriones cigóticos). Los embriones secundarios de origen gamético muestran una buena capacidad de regeneración, aunque inferior a la del control positivo. En promedio, el 54 % de las plantas regeneradas poseen un set de cromosomas duplicado. Un estudio basado en marcadores moleculares confirmó que las líneas doble-haploides presentan un genoma altamente homocigota. Los experimentos de transformación de embriones gaméticos revelaron que el transgen puede ser derivado exitosamente a las células de los embriones gaméticos. Sin embargo, la densidad de células transgénicas resulta ser muy inferior en los embriones gaméticos comparado con el control cigótico. El mecanismo de selección basado en la resistencia al herbicida Basta (conferida a través del gen *pat*) desarrolla aparentemente un efecto tóxico no sólo en las células no transformadas, sino también en las células transgénicas.

En general, las eficiencias de los embriones gaméticos con respecto a todos los aspectos analizados demostraron ser inferiores a las de los embriones cigóticos, por lo que el establecimiento de una línea transgénica doble-haploide a través de la transformación de embriones gaméticos no pudo ser alcanzado. Sin embargo, se demostró que tales embriones pueden potencialmente ser transformados en el caso de que ciertos pasos clave sean optimizados.

A razón de que la transformación de microsporas o embriones gaméticos no resultó ser exitosa, otra estrategia fue ideada para la generación líneas doble-haploides transgénicas; tal estrategia estuvo basada en la extracción de líneas doble-haploides a partir de la cruza entre un genotipo transgénico (sin respuesta andogénica) con un genotipo androgénico, a través del cultivo de anteras. La estrategia resultó ser exitosa, aunque la eficiencia demostró ser extremadamente baja. Sin embargo, ésta fue la primera vez que se logró la generación de líneas homocigotas y transgénicas a través de la combinación de las técnicas de androgénesis *in vitro* y transformación genética.

La conclusión de este trabajo es que los métodos de androgénesis *in vitro* y transformación genética pueden ser combinados con el fin de generar líneas de maíz transgénicas. Los procesos que limitan el establecimiento de un protocolo eficiente fueron claramente identificados.

General Introduction

General introduction

I Modern maize breeding

Modern maize breeding dates back to the beginning of the last century with the work of George H. Shull, who was the first to publish that the crossing of two inbred lines results in high-yielding and very homogeneous progeny (Shull, 1908). Six years later he introduced the term “heterosis”, which means hybrid vigour (Shull, 1914). In the 1930s, the culture of hybrids spread very rapidly replacing open-pollinated maize (Fig. I.1 represents the case in the USA.).

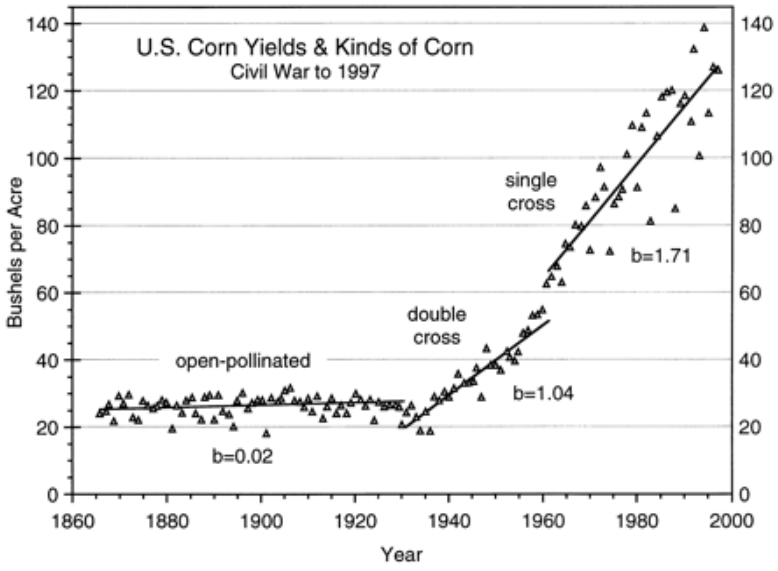

Figure I.1 Maize yield in the USA. The periods dominated by open-pollinated, four-way and two-way crosses are indicated. (1 bushel/acre ≅ 0.06 t/ha) (Crow, 1998).

At first, when the inbred lines gave yields that were too low to be used as seed producers, breeders used very closely related lines to produce higher yielding single crosses, which were later crossed with more distant single crosses to produce the four-way hybrids that were used by the farmers. Three decades later, single crosses slowly replaced double crosses; the selection for high-yielding inbred lines determined that they could be used as seed producers. The single cross hybrids not only gave higher yields than four-way crosses, but were also more uniform.

The basic procedure in maize breeding is based on the development of inbred lines by seven to nine generations of self-pollination, the subsequent evaluation of their general and specific combination ability and, finally, the production of the commercial hybrid seed (Hallauer et al., 1988).

In the last decade, a novel tool for improving quantitative and qualitative traits of crops emerged; transgenic varieties were developed and spread extremely fast. From 1996 to 2001, the global area of GM (genetically modified) crops increased more than 30-fold (Fig. I.2). The total area in 2001 was 52.6 million hectares, grown by 5.5 million farmers. Seven percent of the world's 140 million hectares of hybrid maize were transgenic, corresponding mainly to *Bt*-maize (Monsanto, Syngenta, etc.), a hybrid that produces the protein Cry1Ab from the soil bacterium *Bacillus thuringiensis*, which is toxic to certain insect species.

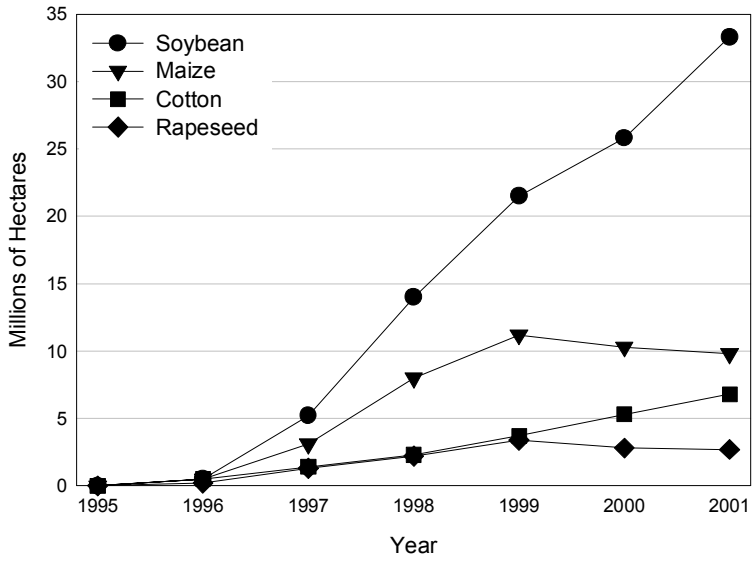

Figure I.2 Global area of four transgenic crops (1995 to 2001) (James, 2001).

Ninety-nine percent of the area covered by GM crops in 2001 was located in the USA, Argentina, Canada and China. In western Europe in particular, the public sector is still concerned with the benefits of GMOs and demands more information about the potential risks to the environment and consumer's health before a transgenic product is released. Spain was the only western European country to grow 30 000 hectares of commercial GMO crops in 2001 (ABE, 2002). The acceptance of genetically modified crops on the one hand, and the demand for more basic information about GMOs on the other hand, resulted in a huge amount of private and public funds being used for research in the field of crop biotechnology (reviewed by James, 2001).

II Biotechnology

Biotechnology aimed at improving crops includes three main areas: *in vitro* culture, genetic transformation and molecular markers analysis:

II.A *In vitro* culture

Plant cells can dedifferentiate and lose the contact-mediated inhibition of growth when isolated from mature tissues, resulting in an uncontrolled reactivation of cell division. These undifferentiated cells are **totipotent**, which refers to their ability to redifferentiate and recapitulate embryogenesis or organogenesis, leading subsequently to the development of fertile plants under certain *in vitro* culture conditions. This process is characterised by the lack of a reproductive phase.

In vitro culture of maize

Initiation of maize *in vitro* culture was first reported by LaRue (1949), but it was not until 1975 that Green and Phillips managed to establish cultures able to regenerate plants. Different explants, i.e. organs, tissues (e.g. the scutella of immature zygotic embryos) or single cells (e.g. protoplasts or microspores), can serve as inoculums for maize *in vitro* culture:

- **Culture of immature zygotic embryos**

Zygotic embryos at an early developmental stage are the most frequently used explants for the initiation of maize tissue culture; when placed in contact with MS (Murashige and Skoog, 1962) culture medium containing 2,4-D (2,4-Dichlorophenoxyacetic acid), proliferation of scutellar cells is induced to form callus (Green and Phillips, 1975). Two types of calli have been characterised according to their morphology, Type I and Type II:

- Type I callus corresponds to compact structures composed of tissues exhibiting shoot meristems and scutellar-like structures (Springer et al., 1979). Plant regeneration occurs via organogenesis by elongation of the meristems (Green and Phillips, 1975; Freeling et al., 1976) or by somatic embryogenesis (Lu et al., 1982): the scutella of immature embryos can proliferate to form somatic embryos (Vasil et al., 1984, 1985), which are similar in structure to zygotic embryos and are capable of regenerating plants (McCain and Hodges 1986). This compact, embryogenic callus is difficult to maintain for more than a few subcultures due to an increasing level of differentiation and the consequent decrease in the callus growth rate (Lu et al., 1982; Vasil and Vasil, 1986).
- Friable, embryogenic tissue cultures (Type II callus) are somatic, highly undifferentiated embryos. The initiation of this type of callus occurs at lower frequencies than Type I callus. Plant regeneration from Type II callus was first described by Armstrong and Green (1985) and Kamo and Hodges (1986).

Despite the fact that Type I and Type II calli may originate from the same initial cells within the scutella (Green 1982; Green et al., 1983), the differences appear to be due to effects of embryo genotype (Carvalho et al., 1997), the stage of embryo development, environmental variation affecting the donor plants (Tomes, 1985a, b) and the culture media; Armstrong and Green (1985) demonstrated that the addition of L-proline to the N6 medium (Chu et al., 1975) induces the formation of embryogenic Type II callus. A high heritability of callus initiation and plant regeneration was determined by Beckert and Qing (1984). Indeed, callus initiation can be

improved by crossing recalcitrant genotypes with highly responsive genotypes (Tomes, 1985a, b).

- **Protoplast culture**

Another milestone in the development of *in vitro* cultures of maize was the establishment of cultures from single cells devoid of their cell wall (protoplasts). Potrykus et al. (1977) reported for the first time callus formation from protoplasts isolated from stem sections. It took until the late 1980s to regenerate fertile plants from maize protoplast cultures (Prioli and Söndahl 1989; Shillito et al., 1989). The technique was improved to such extent, that even maize transgenic plants derived from the delivery of DNA into protoplasts was achieved (Golovkin et al., 1993; Omirulleh et al., 1993).

A challenging approach was the initiation of protoplast cultures from maize microspores (Mitchell and Petolino, 1991; Sukhapinda et al., 1993). Plants were regenerated but they remained at a haploid stage and were, therefore, infertile. A more efficient way to regenerate maize plants from intact microspores, either isolated or *in anthero*, was developed in the late 1970s: the technique is referred to as *in vitro* androgenesis.

- ***In vitro* androgenesis**

Androgenesis arises as a deviation from the normal process of **microgametogenesis** (the formation of pollen, Fig. II.1). The process of microgametogenesis takes place in the anthers and is initiated when a sporophytic cell divides, giving give rise to a tapetal initial cell and a pollen mother cell. The latter undergoes meiosis, leading to a tetrad of haploid cells, which are released as free **microspores**. The microspores undergo two mitotic divisions: the first division is asymmetric and yields a large vegetative and a small generative cell. A second division of the generative cell yields two sperm cells. After the first mitotic division, the vegetative cell receives most of the cytoplasm. Most of the examined specific pollen genes are expressed only in the vegetative cell nucleus.

The vegetative cell plays a role in the delivery of the sperm cells to the ovule through the pollen tube. In the process of double fertilisation, one of the sperm cells fertilises the egg cell to form the diploid zygote, whereas the other sperm cell fuses with the polar nuclei to form the triploid endosperm.

A pollen grain has a two-layered cell wall composed of an inner intine and an outer exine wall (Buchanan et al., 2000, Fig. II.1).

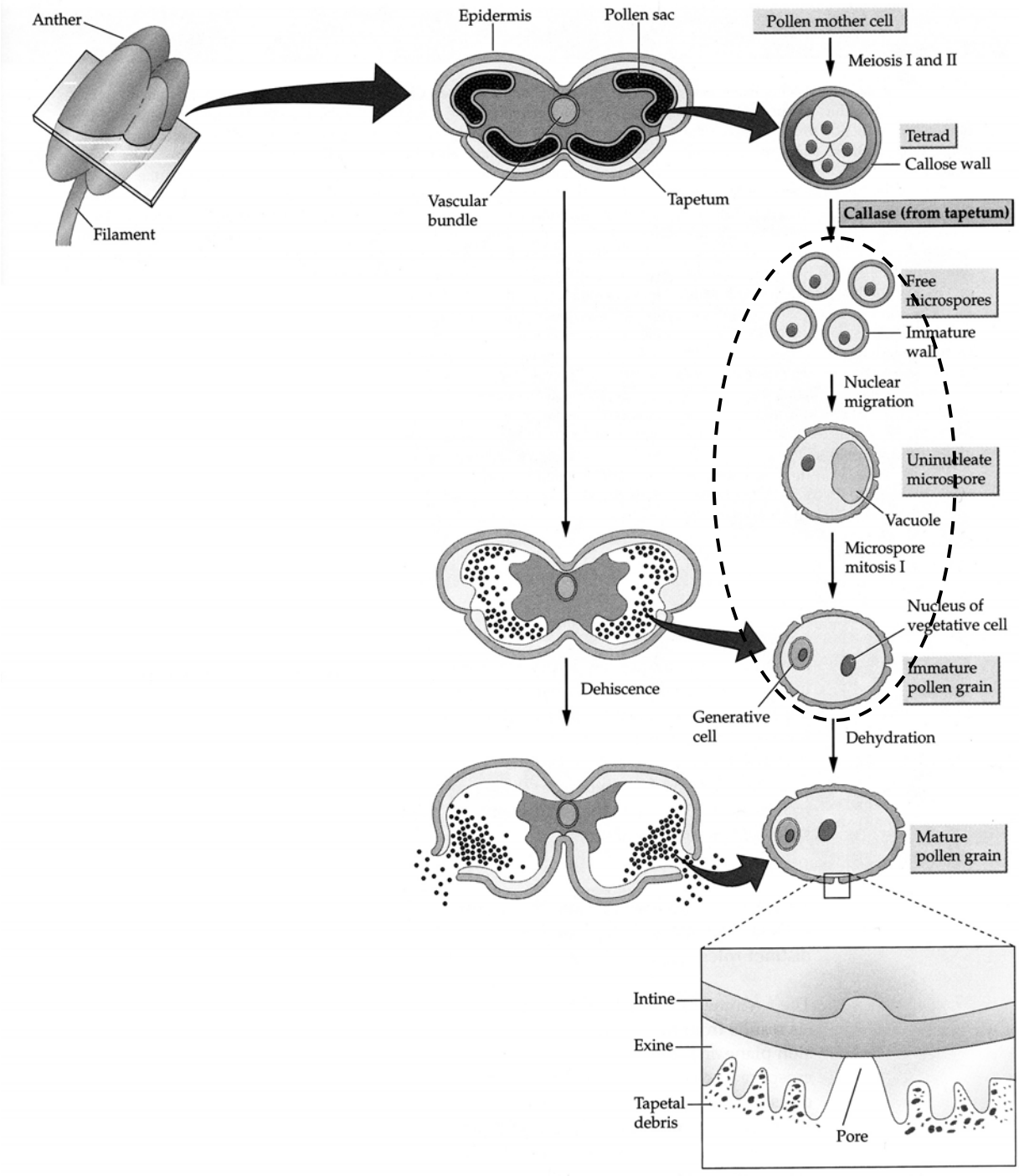

Figure II.1 Pollen development. Meiosis occurs in the anthers and yields four haploid microspores. The first mitotic division of the microspores yields a large vegetative and a small generative cell. At dehiscence, pollen is released from the anther (Buchanan et al., 2000). The broken oval indicates the stage at which microspore development is interrupted for the induction of the androgenic process.

Under specific conditions, it is possible to artificially interrupt normal pollen development (Fig. II.1) and induce microspores to form haploid or double haploid multi-cellular and organised structures called "gametic embryos", which are able to regenerate into whole plants. This process is referred to as ***in vitro*** **androgenesis** or the **DH (double haploids) technique** when chromosome doubling is artificially induced during the process of androgenesis (Fig. II.2).

After the first pollen mitosis, the vegetative nucleus usually remains in the G1 phase and DNA synthesis occurs only in the generative cell. In contrast, it is possible to induce DNA synthesis in the vegetative cell when a stress is applied. This can be achieved by means of cold, heat, drought, colchicine, gamma and UV-irradiation or sugar starvation treatments. The stress leads to the release of the cell from the G1 arrest and enables the reactivation of the cell cycle and the commencement of the androgenic process. The synthesis of the cell wall is initiated right after the first stress-related cell division takes place in the late uni- to early bi-nucleated microspore (Fig. II.2 A). Induction of embryogenesis is characterised by a symmetrical cellular division after the migration of the nucleus towards the cell centre in the late uni-cellular microspore (Fig. II.2 B) or by altered division planes in the early bi-cellular pollen grain. In the latter case, the vegetative cell divides symmetrically, thus contributing to the androgenic process. Once the cell has undergone more than two divisions, androgenesis generally proceeds to the globular stage (Fig. II.2 C). After a few divisions, the developing embryo ruptures the exine (Fig. II.2 D) and expands further (reviewed by Pechan and Smykal, 2001). The resulting gametic embryos (Fig. II.2 E) can regenerate directly into whole plants or, by callus induction and subsequent somatic embryogenesis, give rise to multiple embryos (**secondary embryogenesis**, Fig. II.2 F), all of which carry the potential to regenerate into a plant (Fig. II.2 G and H) (Cao et al., 1981; Gu et al., 1983).

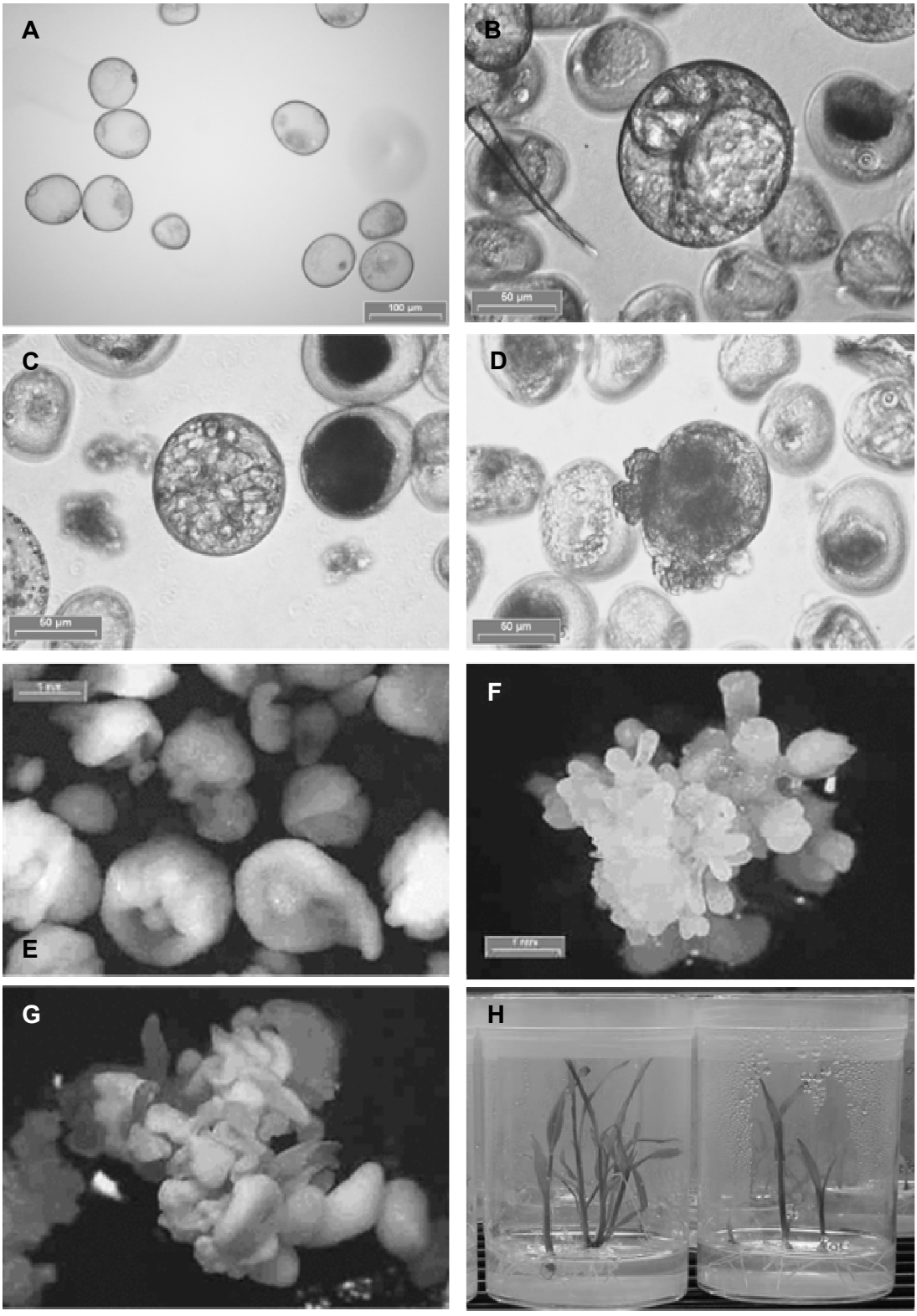

Figure II.2 *In vitro* androgenesis in maize and regeneration of haploid or double haploid plants. (A) Microspores at a uni- to bi-nucleated stage, immediately after isolation. **(B, C)** Dividing microspore as observed 5 and 10 d after isolation. **(D)** Embryo rupturing the exine 15 d after isolation. **(E)** Gametic embryos 25 d after isolation. **(F)** Somatic embryogenesis from gametic embryos (Type I callus). **(G)** Regeneration of shoot and root organs. **(H)** Establishment of a haploid or double haploid plant.

Applications of androgenesis

There are numerous applications for the haploid or double haploid technique, including basic research and breeding programs (reviewed by Niemirowicz-Szezytt, 1997). Examples of its implementation in basic research include mapping and genetic analysis, induction of mutations, transformation, protoplast culture and somatic hybridisation, biochemical and physiological studies, artificial seed production and germplasm storage. The advantages for plant breeding include:

- a shorter period of development of inbred lines by circumventing several generations of self-pollination and
- high levels of homozygosity in the double haploids.

Microspore and anther culture in maize

Androgenesis by means of isolated microspore and anther culture in maize was first reported in the late 1970s by Nitsch (1977) and the 401 Research Group in China (Kuo et al., 1978), respectively. Since then, the technique has been improved considerably. The main factors determining the utility and efficiency of the double haploids method in maize are:

- **Genotype dependency**: an extremely limited number of genotypes were found which respond to anther and/or microspore culture (Petolino and Jones, 1986b). The androgenic response has been shown to be a polygenic heritable trait. Cowen et al. (1992), Murigneux et al. (1994), Beaumont et al. (1995) and Marhic et al. (1998) presented evidence of the existence of diverse chromosomal segments associated with responsiveness to *in vitro* androgenesis. The androgenic trait can, therefore, be transmitted to recalcitrant genotypes by inter-crossing with an androgenic genotype, as demonstrated by Jumpatong et al. (1996) and Saisingtong et al. (1996a, b).
- **Androgenic responsiveness**: since the origins of maize microspore and anther culture, the yield of gametic embryos has risen to a very great extent. Stress treatments at a determined developmental stage of the microspores were identified as elicitors of the

androgenic process (Gaillard et al., 1991; Pechan and Smykal, 2001). The most commonly applied stress is a cold treatment (Genovesi, 1990), but heat treatments (Coumans et al., 1989; Genovesi 1990) or the addition of L-Proline to the culture medium (Büter et al., 1991) have also been applied. The importance of the carbohydrate source (Nägeli et al., 1999; Obert et al., 2000), amino acids (Büter et al., 1991), growth regulators (Duncan and Widholm, 1988; Wassom et al., 2001), osmotic pressure, activated charcoal (Büter et al., 1993) and the sterilisation method (Büter et al., 1993) of the culture media were studied in detail.

- **Chromosome doubling**: chromosome doubling of maize haploids can happen spontaneously at a rate of 5 to 10 % (Kuo et al., 1978; Nitsch et al.1982) to 22 % (Dieu and Beckert, 1986) or can be induced artificially by means of doubling agents. The most commonly used doubling agent is colchicine (Martin and Widholm, 1996; Saisingtong et al., 1996b; Barnabás et al., 1999). This alkaloid acts as a spindle inhibitor and prevents tubulin's polymerization into microtubules, arresting the chromosomes at metaphase and preventing anaphase. This results in an unreduced number of chromosomes before cell division takes place.
- **Plant regeneration efficiency**: it is a common feature of double haploid plants that they present several morphological and physiological abnormalities, which hamper the self-pollination of the DH regenerants and, therefore, the establishment of a DH line. The most common abnormalities are: *i)* the lack of the male or female inflorescence, *ii)* the development of tassel-seeds, *iii)* the unsynchronised flowering of the tassel and the ear, *iv)* the lack of pollen or *v)* the lack of anthesis.
- **Homozygosity**: during *in vitro* androgenesis, additional *in vitro* culture and chromosome doubling, mutations may arise which can lead to a certain level of heterozygosity in the DH material. Experiments performed at molecular and morphological levels (Murigneux et al., 1993a, b) confirmed the effective attainment of homozygosity by means of the double haploids technique when a direct regeneration pathway (without an intervening callus phase) was applied.

II.B Genetic transformation

Gene transfer techniques enable the addition of new or modified genetic traits to existing varieties, circumventing the sexual process and, thus, enabling an acceleration of the whole breeding process. Several methods of transformation have been developed to transfer novel genes to the plant genome:

- ***Agrobacterium***-mediated transformation is commonly used to transform dicotyledonous species; the technique is based on the capability of the bacterium's Ti (tumour-inducing) plasmid to transfer and insert a particular segment of its DNA (T-DNA, for transfer DNA) into the plant genome. Host recognition, transfer and integration of the T-DNA are controlled by a series of plasmid "*vir*" genes. The T-DNA can be manipulated so that it contains genes of interest which will, by infection, be integrated in the target plant species. A large advantage of this technique over other transformation methods is that, in most of the cases, only a single copy of the T-DNA is found to be integrated in the plant genome. The fact that monocotyledonous species are not natural hosts of *Agrobacterium* and, therefore, recalcitrant to the transformation method, determined that this technique is limited to the most relevant crops, i.e. rice, wheat and maize. Nevertheless, recent papers report that rates of transformation have increased, mainly due to *i)* the use of super binary vectors containing extra copies of the *vir* genes that enhance host recognition (Negrotto et al., 2000), *ii)* the addition of antioxidants during the transformation process, which are expected to prevent host cell death by inhibiting the hypersensitive response to *Agrobacterium* (Frame et al., 2002), *iii)* the enhancement of the signal-induced expression of the gene transfer elements of *Agrobacterium* by the addition of host-recognition factors (e.g. xenognosin and/or acetosyringone) during the transformation process (Zhang et al., 2000) and *iv)* the use of mutant plasmids, which synthesise high levels of the T-DNA strand in an inducer-independent manner (Ke et al., 2000).

- **Microinjection** is a technique used to deliver DNA to specific intracellular compartments of living cells. Micro-capillaries and microscopic devices are used to inject DNA into individual cells.
- The **PEG** (polyethylene glycol) treatment has been used widely to transform protoplasts from monocotyledonous plants. PEG increases the permeability of the cell membrane by hydrating its surface and causing agglutination of the DNA with the membrane.
- **Electroporation** induces reversible semi-permeable areas on the cell membrane, resulting from an electric pulse that enables molecules such as DNA to enter or leave the cell. The technique has been used widely to transform protoplasts of both dicotyledonous and monocotyledonous species.
- **Silicon-carbide whiskers** are micro fibres, 10 to 80 µm long and 0.6 µm in diameter. When whiskers are mixed with plant cells and plasmid DNA, cell penetration occurs. Whiskers probably function as needles, facilitating the entry of DNA into cells during the mixing process.
- The "***biolistic***" (for "*bio*logical bal*listics*") transformation method is the most widespread technique for the transformation of monocotyledonous species. This transformation method is based on the direct delivery of DNA to plant cells on the surface of accelerated micro-projectiles (Klein et al., 1987).

  A variant of a particle bombardment device is the so-called PIG (Particle Inflow Gun, Fig. II.3). The PIG is based on the acceleration of DNA-coated tungsten or gold particles, usually 1 to 3 µm in diameter, using pressurised helium in combination with a partial vacuum. The particles with the DNA are accelerated in an aqueous environment directly into the target. The construction of the device was described in detail by Finer et al. (1992).

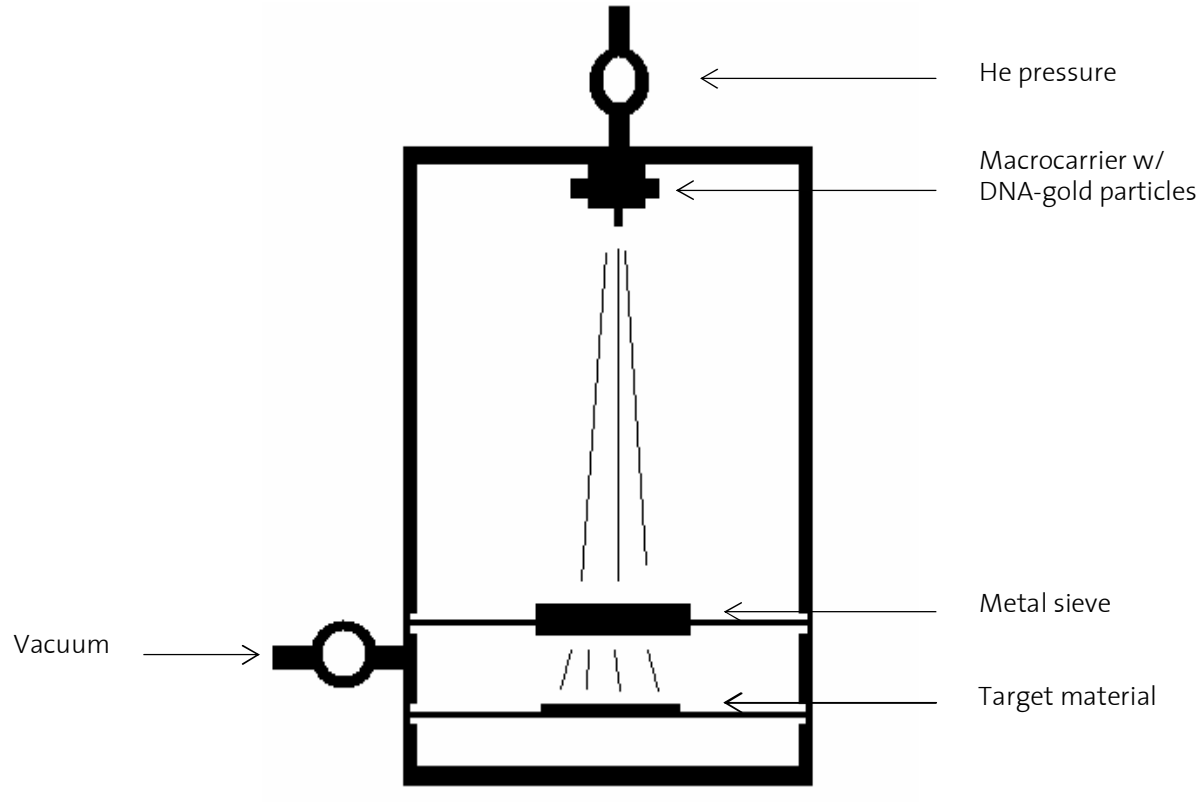

Figure II.3: Scheme of the Particle Inflow Gun. The helium source is connected to the metal sieve plate containing the gold-DNA particles. Upon release of the He pressure, the particles are accelerated in the direction of the target cells. During bombardment, the chamber is partially evacuated.

Transgenic maize

Several methods have been developed to obtain transgenic maize plants. These include the techniques of electroporation (D´Halluin et al., 1992), biolistics (Finer et al., 1992), micro-injection (Kranz et al., 1991), silicon-carbide whiskers (Petolino et al., 2000), PEG (Wang et al., 2000) and *Agrobacterium* (Negrotto et al., 2000). The main targets of these transformation procedures are *i)* protoplasts and *ii)* immature zygotic embryos or callus derived from them.

Brettschneider et al. (1997) developed a very efficient method of maize transformation by the bombardment of scutellar tissue of immature zygotic embryos. Their protocol is based on *i)* the culture of freshly isolated immature zygotic embryos for one to six days on Modified N6 medium (D'Halluin et al., 1992) supplemented with 1 mg/l 2,4-D, *ii)* the transfer of the embryos to hyper-osmotic MS medium (Murashige and Skoog, 1962) four hours prior to the

bombardment in order to reduce the turgor pressure of the cells and, thus, minimise the damage caused by the bombardment, *iii)* the bombardment of the embryos with the gold-DNA particles and *iv)* the induction of secondary embryogenesis and further regeneration in combination with a selection mechanism. In their publication, Brettschneider et al. (1997) also demonstrated clearly that it is unnecessary to establish Type II callus as a target for transformation. This protocol, which has become a standard procedure for maize transformation, has the disadvantage that it is most efficient when applied to hybrid target material, which performs better *in vitro* than inbred lines, and shows better developed regenerants (R. Brettschneider, personal communication). This derives in the fact that the transgene is integrated in a highly heterozygous background, making it very difficult to determine the phenotype of the novel gene. To develop homozygous material, up to seven generations of self-pollination are needed.

Transformation of microspores or microspore-derived material

As a target for transformation, isolated microspores have the advantage that they are haploid and uni-cellular. Thus, the transformation of these microspores and the subsequent chromosome doubling can lead to the recovery of diploid transgenic plants in only one generation. These plants are homozygous, not only for the transgene, but also for the entire genome. Also the transformation of double haploid microspore-derived structures (gametic embryos) would lead to the same results, although a further generation of self-pollination would be required to convert the transgene from a hemi- to a homozygous stage.

The transformation of microspores or microspore-derived material and the establishment of transgenic DH lines was successful in tobacco, oil seed rape, barley, rice and wheat. Whereas the transformation of rice was achieved by the PEG treatment of microspore-derived protoplasts (Chaïr et al., 1996), the transformation of tobacco (Stöger et al., 1995), oil seed rape (Fukoka et al., 1998), barley (Jähne et al., 1994; Yao et al., 1997; Carlson et al., 2001) and wheat (Mentewab et al., 1999; Folling and Olesen, 2001) was achieved by bombarding late uni- to early bi-nucleated microspores or callus derived from them. Folling and Olesen (2001) stressed the

importance of subjecting the wheat microspores to a pre-culture phase of four days prior to bombardment. During the pre-culture, the microspores expand in volume, the cytoplasm undergoes structural reorganisation and the structure of the cell wall and organisation of the plasma membrane change, factors which may affect the penetration of the gold particles. The most important aspect of the pre-culture is probably that the microspores started to divide at the time of the bombardment. The nuclear membrane may constitute an important barrier to the import and integration of plasmid DNA. Being completely or partially devoid of the nuclear membrane, M-phase cells may be more competent since they have a more accessible genome. The volume of the vacuoles also diminishes, thereby increasing the probability that particles are delivered outside the vacuole. Folling and Olesen (2001) experimented simultaneously with the transformation of isolated microspores and the biolistic transformation of wheat microspore-derived callus. This latter approach was superior to the first, yielding nine transgenic wheat lines from 1 000 bombarded explants.

All attempts at transforming maize microspores have failed thus far; the transient expression of the *gus* (β-glucuronidase) gene was observed on bombarded (Jardinaud et al., 1995a) as well as on electroporated microspores (Jardinaud et al., 1995b). Also the *cat* (chloramphenicol acetyl transferase) gene, delivered by means of PEG and electroporation to microspores, showed transient transgene expression (Fennel and Hauptmann, 1992). Shukhapinda et al. (1993) obtained stable expression in electroporated protoplasts isolated from microspore-derived cell suspension cultures and were even able to regenerate a few transgenic plants. All the regenerants were, nevertheless, haploid and therefore sterile, hindering the establishment of a transgenic DH line. Transgenic maize lines were established in this doctoral work through the combination of androgenesis and transformation methods.

II.C Molecular markers

The use of these markers is determined by the enormous amount of naturally occurring polymorphism at a molecular (either protein or DNA) level. In many cases, there is a correlation between the molecular markers and phenotypic traits; this is the basis of "marker assisted breeding", which enables breeders to predict the phenotype of an organism by performing simple molecular analysis of a small amount of plant material. There are two major categories of DNA-based markers: RFLPs (Restriction Fragment Length Polymorphism), which reflect the polymorphism generated by the restriction of genomic DNA at specific locations by means of restriction enzymes, and markers based on PCR (Polymerase Chain Reaction) amplification. The latter are based on the length polymorphism of regions flanked by conserved motives. These regions can be amplified by PCR using specific oligonucleotide primers matching the flanking motives. Polymorphism is detected by differential fragment migration by gel electrophoresis (according to molecular weight).

Simple Sequence Repeats (SSR)

Compared to other DNA-based molecular marker systems, the SSRs (or micro-satellites) have several advantages: they are abundant in plant genomes, highly polymorphic within species, can be assayed relatively rapidly and inexpensively and can be used to consistently identify specific chromosome regions across populations (Chin et al., 1996; Taramino and Tingey, 1996; Chen et al., 1997; Smith et al., 1997). Simple sequence repeats occur in many plant genomes including those of maize (Shattuck-Eidens et al., 1990; Senior and Heun, 1993). Polymorphism is based on tandem repeated mono- to hexa-nucleotide motifs which are present in different numbers from one genotype to another. SSR loci are individually amplified by PCR using pairs of primers specific to conserved DNA sequences flanking the SSR sequence. Gel electrophoresis enables the detection of differences in the number of repeats targeted and amplified.

Application of molecular markers to the double haploids technique

Molecular markers (mostly RFPLs) have been used to identify chromosomal regions involved in androgenic responsiveness (Cowen et al., 1992; Wan et al., 1992; Murigneux et al., 1994; Beaumont et al., 1995; Marhic et al., 1998).

Another interesting use of molecular markers is to classify double haploid lines with respect to their level of homozygosity. Murigneux et al. (1993a, b) tested 189 DH lines at more than 100 loci by means of the RFLP method. The results confirmed the homozygous nature of the DH lines.

Dufour et al. (2001) analysed the segregation of 145 AFLP (Amplified Fragment Length Polymorphism, a PCR-based molecular marker system) markers during *in vitro* androgenesis in maize. They found a link between distortions at marker loci and genes controlling microspores embryogenesis, attributed to a wide range of selective factors resulting from the partial or total elimination of gametes or zygotes.

III The INCO-DC project

The studies described in this thesis were performed within the framework of an INCO-DC (International Co-operation with Developing Countries) project entitled "Breeding early maturing maize by conventional methods and biotechnology" (Contract number: ERBIC18CT980304), financed by the European Commission. The project consisted of two major modules dealing with the development and analysis of earliness in maize. The development of earliness was approached by means of *i)* conventional breeding and biotechnological methods, through the extraction of recombinant inbred lines from promising populations by self-pollination and by andro- and gynogenesis, respectively, and *ii)* by genetic transformation. The analysis of early maturing maize was based on *i)* the identification of QTLs (quantitative trait loci) and the isolation of genes related to earliness in maize and *ii)* the physiological and agronomical evaluation of early maturing maize. The role of the Group of Agronomy and Plant Breeding of the ETH Zurich in this project was to establish a protocol for the generation of

transgenic and homozygous maize lines by combining *in vitro* androgenesis and genetic transformation techniques, and the contingent application of the novel protocol for the introduction of candidate genes responsible for earliness into maize.

IV Goals of this doctoral work

The objective of this doctoral work was to develop a method for the **rapid** gain of **transgenic** and fully **homozygous** maize lines by combining the *in vitro* androgenesis and the biolistic transformation techniques.

Full homozygosity is relevant not only for maize breeding, where pure lines are a pre-requisite for hybrid production, but also for basic research; to study the expression of transgenes controlling quantitative traits, the transgene must be integrated in a homogeneous background in order to avoid the masking of the transgene expression by the phenotypic variation originated by a heterozygous background. To illustrate this, a candidate gene responsible for early flowering in dicotyledonous species (*fpf1*, for flowering promoting factor) (Melzer et al., 1999) was transformed into maize zygotic embryos of the cross A188 x H99 at the beginning of the INCO-DC project mentioned above. The phenotypic analysis of the transgenic offspring with regard to earliness gave no clear results due to the segregation of several traits, which hampered the discrimination of variation due to the heterozygous background and variation due to the novel gene.

In conventional transgenic maize breeding, a limited number of maize genotypes (mostly hybrids) showing good transformation capability are used as targets for DNA delivery. Further time-consuming inbreeding or back-crossing to the line of interest and the selection of the transgenic trait in each generation are required in order to integrate the transgene into a homozygous background. The process of *in vitro* androgenesis permits a fast generation of double haploid and, therefore, homozygous plants in one generation. If novel genes are transferred to microspores or gametic embryos, the regeneration and self-pollination of these materials could lead to the establishment of transgenic and homozygous lines. The

transformation of isolated microspores in maize has been tackled using several methods, without success. There are no publications on the transformation of maize gametic embryos.

The establishment of a method to achieve transgenic and double haploid maize lines was approached in this doctoral work by the direct transformation of haploid or double haploid material, using the biolistic method. Both microspores and gametic embryos were considered as target material for the genetic transformation.

The first goal which was addressed in the development of the transformation protocol, was to determine which one of these explants is more suitable for genetic transformation, based on its *in vitro* characteristics. If microspores were to be transformed, then high induction rates of the androgenic process would be required. If gametic embryos were to be transformed, then a large amount of this target material would have to be produced, either by the isolated microspore or the anther culture methods. A further aspect of this work that applies to both strategies of microspore and gametic embryo transformation was the efficient induction of secondary embryogenesis from gametic embryos. Secondary embryogenesis is essential because it enables the multiplication of the material and a subsequent higher output of regenerated plants. If this process is combined with selection pressure after transformation of the target material with a selectable marker gene, then only transgenic cells will undergo the formation of transgenic secondary structures capable of regenerating into a fully transgenic plant. Thus, a high regeneration capability of the secondary embryos was also tackled in this work.

In addition to these aspects, the efficiency of artificial chromosome doubling was analysed. Since mutations may arise during androgenesis and further *in vitro* culture, a molecular marker study was performed in order to determine the quality of the double haploid lines with regard to the level of homozygosity achieved.

A further goal in the development of the protocol was to analyse the transient and stable transgene expression in the target material, either microspores or gametic embryos, after the delivery of the transgene construct by means of the biolistic method. For this respect, the visual marker gene *gfp* and the selective marker gene *pat* were used.

In summary, the following essentials for the establishment of an efficient protocol for the transformation of microspores or gametic embryos were analysed in detail:

- the androgenic response
- the induction rate of secondary embryo formation
- the ability of secondary embryos to undergo the regeneration of double haploid and fully homozygous plants
- the transient and stable transgene expression on the target material
- the selection mechanism.

Induction of gametic embryos formation and their characteristics *in vitro*

1. Induction of gametic embryos formation and their characteristics *in vitro*

1.1 Abstract

An important step in establishing a protocol for the transformation of microspores and gametic embryos in maize is the achievement of adequate *in vitro* performance of these target materials. The response to isolated microspore culture of the three androgenic genotypes ETH-M24, ETH-M72 and ETH-M82 was studied and improved by the addition of activated charcoal to the cultures; the rate of plant regeneration was also enhanced by the application of an indirect regeneration pathway through secondary embryogenesis. At the same time, the response of the three genotypes to anther culture and the ability of their gametic embryos to undergo somatic embryogenesis and further regeneration of double haploid plants were studied. The results indicate that the microspore culture approach results in a very poor androgenic response, despite the improvement achieved by adding activated charcoal (a max. of 27 embryos per 80 000 microspores). The anther culture protocol, on the other hand, results in a high number of gametic embryos (a max. of 790 embryos per 100 anthers) that undergo somatic embryogenesis and regenerate into diploid plants at a high rate. For these reasons, preferentially gametic embryos from anther culture, rather than microspores, should be considered as targets for genetic transformation.

1.2 Introduction

The first goal in the establishment of a transformation protocol is an adequate *in vitro* performance of the target material, which should ensure a rapid proliferation of the transgenic cells and the efficient regeneration of fertile plants. The genetic transformation of androgenic material can be achieved either by gene transfer to isolated microspores (Carlson et al., 2001; Folling and Olesen, 2001) or gametic embryos (Folling and Olesen, 2001, page 22 of the General Introduction).

As mentioned on page 21 of the General Introduction, a very efficient protocol for the transformation of maize zygotic embryos was developed by Brettschneider et al. (1997). A key step in this transformation protocol is the induction of secondary embryos of the callus Type I (Armstrong and Green, 1985) after the delivery of the DNA-construct. This is essential, since it enables the isolated transgenic cells to divide and give rise to an organ capable of regenerating into a plant. It is assumed that the same protocol of zygotic embryo transformation can be applied to transform gametic embryos, too. In addition to a good androgenic response (whether by microspore or anther culture) and a satisfactory rate of diploidisation, high rates of somatic embryo formation should be given for this respect.

For the successful transformation of isolated microspores, the basic requirements are extremely high rates of androgenic induction and plant regeneration; in the case that the microspore is transgenic, these factors increase the probability that the microspore undergoes androgenesis, giving rise to a transgenic plant.

The aims of the experiments described in this chapter were to improve the existing protocols for the production of the explants used as targets for transformation (induced microspores and gametic embryos) and to determine which type of explant is more suitable as a target for transformation, based on their *in vitro* performance.

The optimisation of the isolated microspore culture was based on a protocol developed at the ETH, by which a stress is applied to the microspores by a pulse of high concentrations of sucrose (Nägeli et al., 1999). An overall improvement of the androgenic induction of microspores was recently achieved by the Group of Agronomy and Plant Breeding of the ETH by adding activated charcoal (AC) to the microspore cultures. An attempt was made to improve the yield of gametic embryos by applying AC to Nägeli's protocol. The increase in the number of regenerants per gametic embryo was tackled by applying secondary embryogenesis prior to the induction of plant regeneration.

For the production of gametic embryos as targets for transformation, the very efficient anther culture protocol developed at the ETH by Saisingtong et al. (1996b) was used. In order to

augment the output of regenerants and as a pre-requisite for the success of transformation, secondary embryogenesis was applied to the gametic embryos.

The protocol of Brettschneider et al. (1997) was applied previously to transform immature zygotic embryos of the genotype ETH-M72 (one of the androgenic genotypes used in this study); ETH-M72 zygotic embryos were, therefore, used as positive control for the response of gametic embryos to secondary embryogenesis and plant regeneration.

1.3 Materials & Methods

1.3.1 Plant donor material

The genotypes used in this study were selected for androgenic responsiveness at the Experimental Station of the ETH Zurich. These genotypes are ETH-M24 (Saisingtong et al., 1996b), ETH-M82 and ETH-M72. The public line H99, which is not responsive to androgenesis, was used as negative control. The donor plants were grown in the greenhouse at 25 °C (day) and 18 °C (night), a photoperiod of 16 h and a light intensity of 250 µmol m^{-2} s^{-1}.

1.3.2 *In vitro* androgenesis

Tassel harvest and cold stress treatment

Tassels with microspores at a uni- to early bi-nucleated developmental stage (approximately 2.5 months after sowing) were removed from the plants wrapped in their leaf whorl and wrapped in moist paper towels and aluminium foil. The material was kept at 8 to 9 °C for 10 to 14 d for a cold stress treatment.

Sterilisation of the spikelets

The spikelets were removed from the main branch of the tassel and put into a 50 ml tube. Approximately 30 ml of a 2.5 % (w/v) sodium hypochlorite solution, supplemented with 0.1 % (v/v) detergent (Mucasol, Merz & Co, Frankfurt, Germany), were added. The tube was rocked gently for 15 min; the spikelets were rinsed three times in sterile, double distilled water. This material was used either for anther or microspore culture.

Microspore culture (sucrose pulse method)

The following procedure was originally developed by Nägeli et al. (1999) and then modified for simplification:

Sterile spikelets were pre-cultured in a 50 ml tube containing about 30 ml of cold Pre-culture Medium (page 91 of the Appendix) for 3 d at 15 °C and in the dark. Thereafter, the spikelets in the pre-culture medium were transferred to the container of a Waring blender (125 ml), which was sealed with aluminium foil. Two pulses of 10 s each at speed 3 (10 900 rpm) were applied to release the microspores. The slurry was passed through a 100 µm sieve, and the filtrate was collected in a 50 ml tube. After centrifugation at 160 x g for 3 min, the supernatant was discarded and 30 ml of cold Induction Medium Sucrose IMS (page 91 of the Appendix) were added; this sucrose pulse was applied for 10 min. Thereafter, three washes with Induction Medium Maltose IMM (page 91 of the Appendix) were performed. The final density was adjusted to about 40 000 microspores per ml IMM. Two ml of the suspension was put into each Petri-dish, 2 cm diameter.

The protocol was varied by adding six small cubes (0.5 cm x 0.5 cm x 0.5 cm) of IMM (lacking TIBA) supplemented with 5 g/l activated charcoal and solidified with 5 g/l phytagel to each petri-dish.

The cultures were kept at 27 °C in the dark for approximately one month until the developing gametic embryos reached a diameter of 1.0 to 1.5 mm.

Seven repetitions of five petri-dishes each (ca. 80 000 microspores per petri-dish) were performed for each genotype and protocol (with and without the addition of AC to the cultures).

Anther culture

The three anthers in the larger floret of the spikelet were isolated using forceps under sterile conditions. Twenty-five anthers were placed in petri-dishes (6 cm in diameter) containing 8 ml of Induction Medium Liquid IML supplemented with 250 mg/l colchicine (page 90 of the Appendix) and cultivated for 7 d at 15 °C in the dark. Thereafter, the anthers were transferred to petri-dishes containing Induction Medium Semi Solid IMSS (page 90 of the Appendix) and cultivated for approximately one month in the dark at 27 °C until the emergence of the gametic embryos (according to the protocol of Saisingtong et al., 1996b).

1.3.3 Culture of immature zygotic embryos

Ears of self-fertilised ETH-M72 plants were harvested 11 to 13 d after pollination and kept at 4 °C for 2 to 4 d. The kernels were separated from the cob and sterilised in a 2.5 % (w/v) sodium hypochlorite solution supplemented with 0.1 % (v/v) detergent (Mucasol, Merz & Co, Frankfurt, Germany) for 15 min. The kernels were rinsed three times in sterile double distilled water. The embryos (1.5 to 2.0 mm in length) were isolated with a scalpel under sterile conditions and transferred to medium for secondary embryogenesis.

1.3.4 Secondary embryogenesis

Secondary embryos were induced from gametic and immature zygotic embryos on Modified N6 medium supplemented with 1 mg/l 2,4-D (D' Halluin et al., 1992, page 92 of the Appendix). The zygotic embryos were placed on the medium with the scutellar side up. The cultures were kept in the dark at 27 °C and sub-cultured onto fresh medium at two week intervals.

1.3.5 Plant regeneration

Secondary embryos of zygotic and gametic origin (in the case of an indirect regeneration system) or embryos directly explanted from microspore or anther culture (in the case of a direct regeneration system) were placed in perti-dishes, 10 cm in diameter, containing autoclaved Sigma MS medium (Murashige and Skoog, 1962) supplemented with 3 % (w/v) sucrose and solidified with 3 mg/l phytagel; the pH was adjusted to 5.8. The cultures were maintained at 27 °C in constant light (100 µmol m^{-2} s^{-1}). The cultures were transferred to fresh medium every second week, and the regenerating shoots were sub-cultured to Magenta boxes containing the same medium. Plantlets of approximately 8 cm were transferred to soil and covered with a translucent plastic beaker for one week to enable the leaves to adapt to dryer conditions. When possible, plants of gametic embryo origin were self-pollinated in order to establish a DH line.

1.3.6 Determination of the ploidy level

The Ploidy Analyser I (Partec GmbH, Germany) was used to determine the ploidy level of regenerated shoots. Using a sharp razorblade, a leaf segment of approximately 1 cm^2 was cut into small pieces in 2 ml of cold (8 °C) DAPI staining solution (5 µg/ml, Partec GmbH, Germany) and passed through a nylon gauze (50 µm mesh size). The filtrate was used for flow cytometric analysis; at a par gain FL1 of 412 to 420 (relative fluorescence), a peak set at 100 and 200 FL (corresponding to the G1- and G2- or M- phases, respectively) was interpreted as corresponding to diploid or double haploid material (Fig. 1.1 A). A peak set at 50 and 100 FL was interpreted as corresponding to haploid material (Fig. 1.1 B).

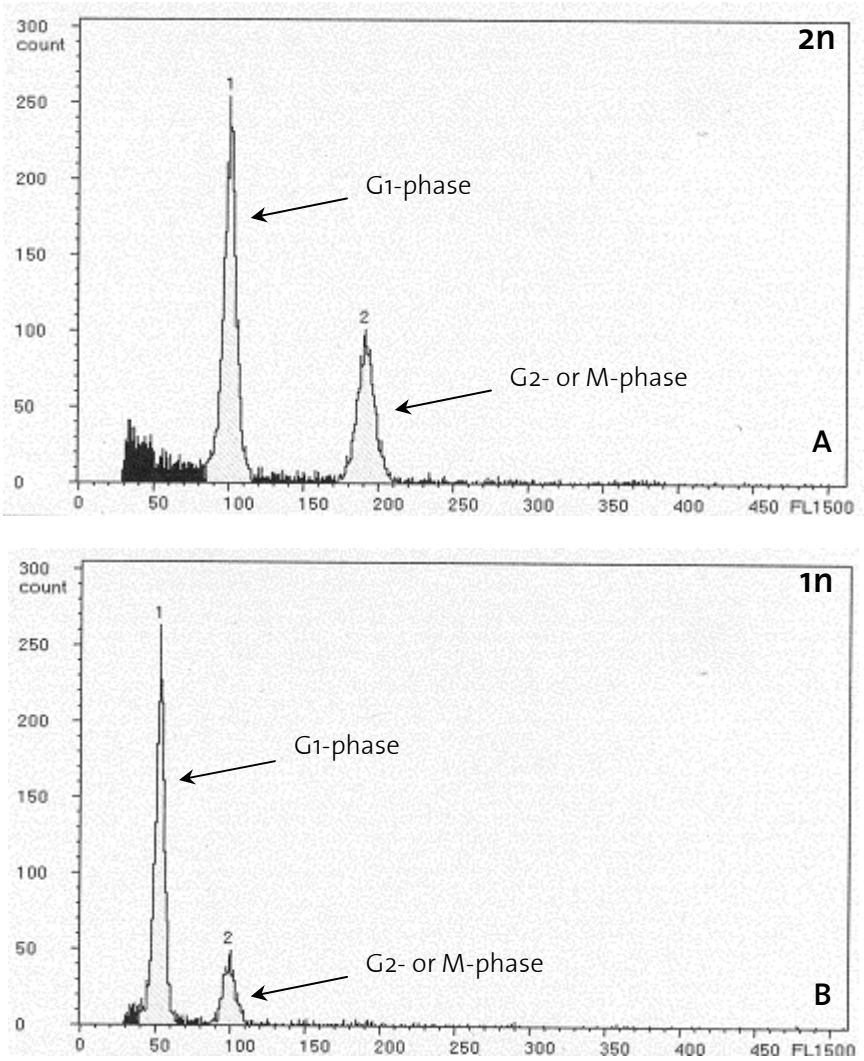

Figure 1.1 Flow cytometric analysis of the ploidy level. The x-axis of the histogram represents the intensity of DNA fluorescence in relative units; the y-axis represents the number of nuclei counted per histogram channel. **(A)** A representative peak set for diploid or double haploid material is shown. **(B)** Peaks corresponding to a typical haploid individual are represented.

1.4 Results

1.4.1 Microspore culture

The androgenic genotypes ETH-M24, ETH-M72 and ETH-M82 and the non-androgenic negative control line H99 were tested for their response to isolated microspore culture. A simplified version of a protocol developed by Nägeli et al. (1999) and a variation of it, which involved the addition of AC to the induction medium, were used.

When cultured on medium without AC, the androgenic genotypes ETH-M24 and ETH-M72 and the control genotype H99 did not respond to the microspore culture (Table 1.1). Only the genotype ETH-M82 yielded an average of 6 and a maximum of 27 gametic embryos per 80 000 isolated microspores.

The addition of AC to the cultures resulted in an average of 15.5 (and a maximum of 58), 4.3 and 0.4 gametic embryos per petri-dish for the genotypes ETH-M82, ETH-M24 and ETH-M72, respectively (Table 1.1). The control line H99 did not show any androgenic response. The response to the androgenic induction differed significantly ($p = 0.05$) with and without the addition of AC to the culture medium, as depicted for the genotype ETH-M82 in Fig. 1.2.

Moreover, a clear morphological difference was determined between the gametic embryos developed in the presence or absence of AC. Whereas the embryos developed in AC were compact and had a defined morphology, those grown on medium without AC consisted mainly of a soft and brownish clump of undifferentiated cells (Fig. 1.2).

Due to the significantly stronger response of the genotype ETH-M82 to the microspore culture, it was selected for further experiments on plant regeneration.

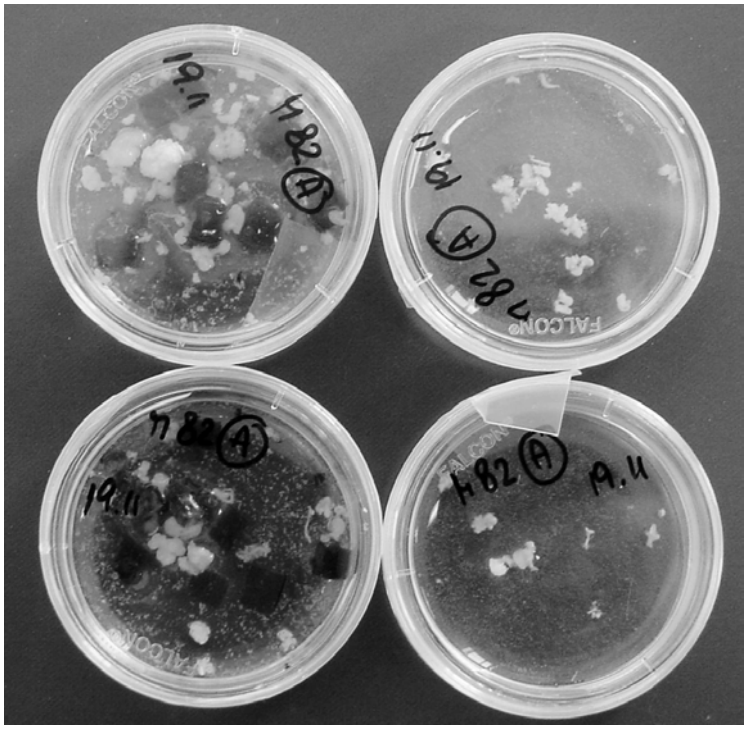

Figure 1.2 Microspore cultures of the genotype ETH-M82 in response to the addition of AC to the medium. Small cubes, consisting of IMM (without TIBA), AC and phytagel, were added to the microspore cultures (dishes on the left). Microspores on medium with AC had a higher induction rate and the resulting embryos showed a higher level of differentiation than the embryos developed without AC (dishes on the right). The latter consisted mainly of a cluster of undifferentiated and brownish cells.

Table 1.1 Response to microspore culture of four genotypes and effect of AC. The number of embryos per 80 000 microspores grown in medium without and with AC (IMM - AC and IMM + AC, respectively) is shown. Data followed by the same letter are not significantly different from each other at p = 0.05.

	Genotypes selected for androgenic response			Control genotype
Medium	ETH-M82	ETH-M24	ETH-M72	H99
IMM - AC	5.8 ± 7.4	0 ± 0 **a**	0 ± 0 **a**	0 ± 0 **a**
IMM + AC	15.5 ± 17.4	4.3 ± 4.4	0.4 ± 0.6	0 ± 0 **a**

ETH-M82 gametic embryos generated in medium with and without AC were subjected to regeneration via a direct and an indirect regeneration pathway: embryos were either transferred immediately after isolation to MS medium or cultivated for four weeks on Modified

N6 medium supplemented with 2,4-D for the induction of secondary embryogenesis, prior to regeneration on MS. Five repetitions were performed per treatment (20 embryos per repetition).

Whereas the direct regeneration method yielded 0.06 vs. 0.01 regenerants per embryo developed on medium without and with AC, respectively, the indirect regeneration method yielded 0.45 and 0.30 regenerants (Table 1.2). Despite the clear differences in the regeneration ability of the gametic embryos developed with and without AC, the yields did not differ significantly at $p = 0.05$. Significant differences were determined between the direct and indirect regeneration pathways.

The regenerated plants were analysed for their spontaneous diplodisation rate. From almost 100 tested individuals, 30.10 % showed a doubled chromosome number.

Table 1.2 Regeneration ability of microspore culture derived embryos. Embryos developed in IMM with and without AC were subjected to a direct and an indirect regeneration pathway. Data followed by the same letter are not significantly different from each other at $p = 0.05$.

	Direct regeneration	Indirect regeneration
Regenerants per embryo developed without AC	0.06 ± 0.07 **a**	0.45 ± 0.28 **b**
Regenerants per embryo developed with AC	0.01 ± 0.02 **a**	0.30 ± 0.26 **b**

1.4.2 Anther culture

Approximately 5 200 anthers of ETH-M24, 5 800 of ETH-M72, 1 400 of ETH-M82 and 1 400 of H99 were isolated and subjected to conditions that induce gametic embryogenesis. Although a very high standard deviation was generally observed in these experiments, androgenic responsiveness differed significantly among all the tested genotypes. With an average of 297

and a maximum yield of 790 embryos per 100 isolated anthers, ETH-M82 was superior to the other genotypes, while the control line H99 did not respond to androgenesis (Table 1.3).

Table 1.3 Androgenic response and diploidisation rate. Three ETH genotypes (selected for androgenic response) and the control genotype H99 were compared with regard to androgenic response and rate of diploidisation. Data followed by the same letter are not significantly different from each other at p = 0.05.

	Genotypes selected for androgenic response			Control genotype
	ETH-M24	ETH-M72	ETH-M82	H99
Embryos/ 100 anthers	85.6 ± 75.6	39.2 ± 51.2	297.3 ± 263.5	0.0 ± 0.0
Percentage DHs	60.7	44.6	56.0	-

The ability of the gametic embryos of ETH-M24, ETH-M72 and ETH-M82 to undergo secondary embryogenesis was assessed for eight weeks and compared to the induction of secondary embryogenesis of zygotic embryos of the genotype ETH-M72. Approximately four weeks after the induction on Modified N6 medium containing 2,4-D, the callus obtained from zygotic embryos showed structures corresponding to the Type I callus (Fig. 1.3 A). The gametic embryos yielded secondary structures resembling those developed from zygotic embryos (Fig. 1.3 B).

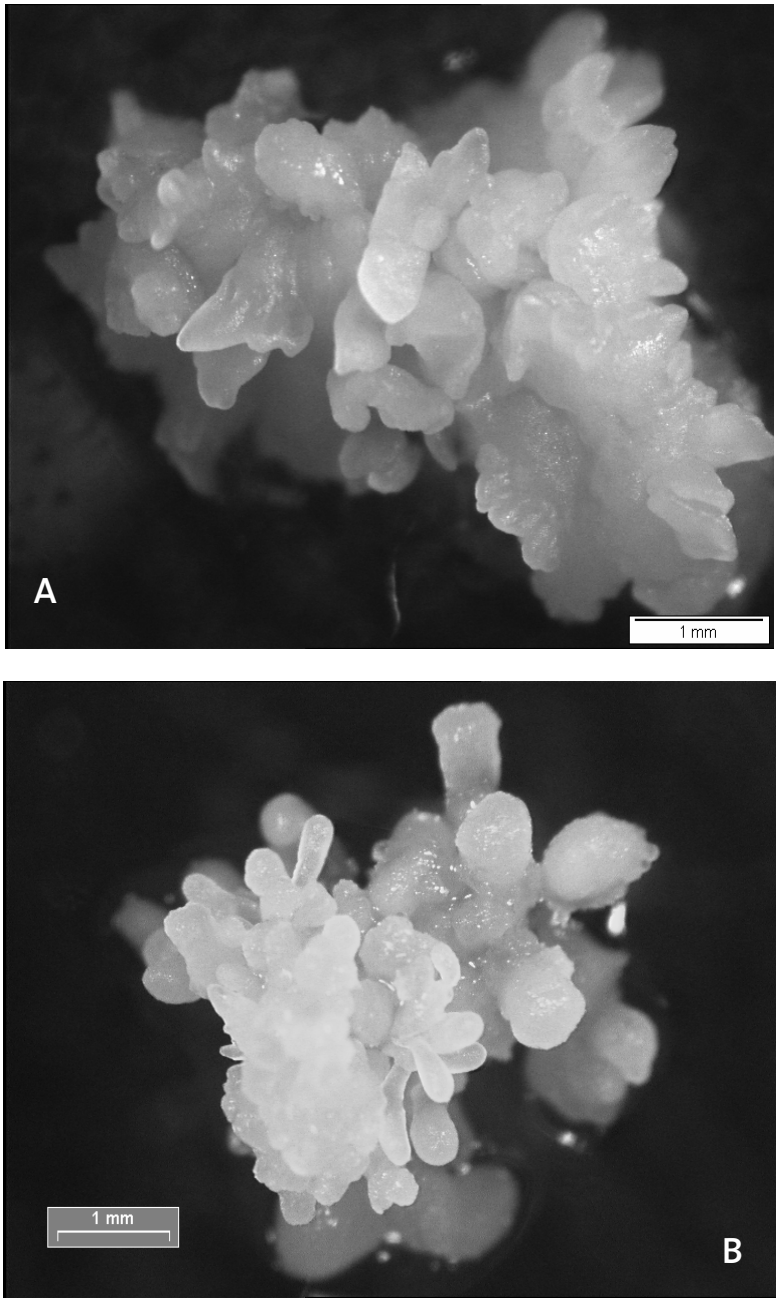

Figure 1.3 Secondary embryogenesis. Somatic embryogenesis from zygotic (genotype ETH-M72) **(A)** and gametic (genotype ETH-M24) **(B)** embryos was induced by cultivating the embryos on Modified N6 medium supplemented with 2,4-D. The callus structures correspond to the callus Type I described by Armstrong et al. (1995).

During the eight-week period, an exponential proliferation of secondary embryos was observed in all cases. A significantly stronger response was found for the zygotic than for the average gametic embryos (Fig. 1.4 A). Within the gametic embryos, no significant differences were

found. After four weeks of culture on Modified N6 medium, the zygotic embryos yielded an average of 12 secondary embryos per explanted embryo, whereas the mean of the gametic embryos was five secondary embryos per explanted embryo. After eight weeks, the zygotic embryos yielded 67 and the gametic embryos 14 secondary embryos per explanted embryo, indicating a five-fold stronger response of the zygotic embryos.

Furthermore, the ability of the secondary embryos (which emerged from the gametic and zygotic embryos) to regenerate into a plant, depending on the duration of their exposure to N6 medium containing 2,4-D, was studied (Fig. 1.4 B). Secondary embryos of zygotic and gametic origin were transferred to regeneration medium after 2, 4, 6 and 8 weeks of cultivation on N6 medium and the total number of regenerating shoots was determined.

Compared to the secondary embryos of gametic origin, those of zygotic origin showed a superior response and yielded approximately 0.5 regenerants per secondary embryo, when they were two to four weeks old. With increasing age, there was a sudden decrease in the number of regenerants per secondary embryo. Secondary embryos of gametic origin yielded approximately 0.11 regenerants per secondary embryo when they were two to six weeks old and an average of 0.06 regenerants per secondary embryo when they were eight weeks old. No significant differences were found for the secondary embryos derived from gametic embryos. In all cases, the potential of secondary embryos to regenerate clearly decreased with increasing cultivation time on N6 medium (Fig. 1.4 B).

The final yield of regenerated shoots per explanted embryo increased linearly with increasing cultivation time on N6 medium for all genotypes (Fig. 1.4 C). The yield of regenerants per zygotic embryo was always significantly higher than the yield per gametic embryo.

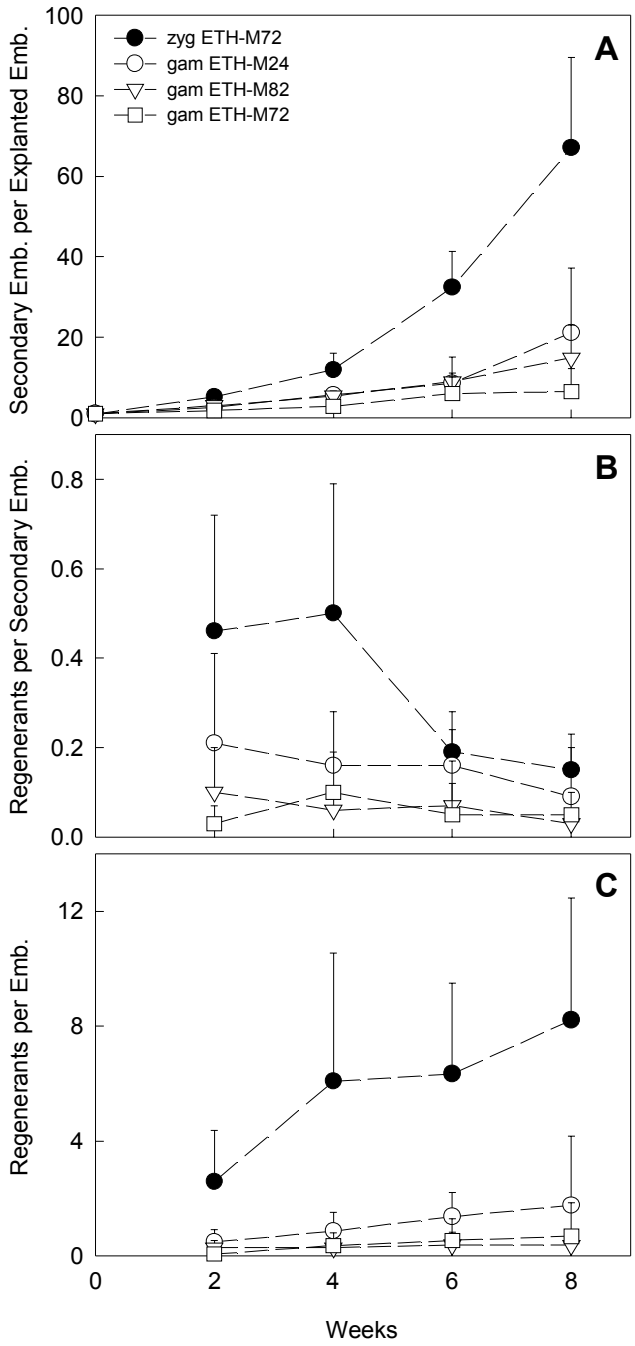

Figure 1.4 Secondary embryogenesis and regeneration potential of zygotic vs. gametic embryos. (A) The yield of secondary embryos per explanted embryo is shown for zygotic material of the genotype ETH-M72 and gametic material of the genotypes ETH-M72, ETH-M82 and ETH-M24. The yield was determined 2, 4, 6 and 8 weeks after transfer to N6 medium containing 1 mg/l 2,4-D. **(B)** On each of these dates, secondary embryos of zygotic and gametic origin were transferred to MS medium, exposed to light, and the number of regenerants was assessed in the following weeks. **(C)** On each date, the number of regenerated shoots per explanted embryo was calculated on the basis of the data presented in (A) and (B).

The ploidy level of approximately 200 regenerants of the gametic embryos of each genotype, ETH-M24, ETH-M72 and ETH-M82, was determined (Table 1.2). On average, 54 % of the individuals were double haploids. The genotype ETH-M24 tended to have a higher rate of diploidisation (61 %) than ETH-M82 (56 %) and ETH-M72 (45 %).

1.5 Discussion

1.5.1 Microspore culture

The microspore culture protocol originally developed by Nägeli et al. (1999) and then simplified, was adopted to test three androgenic ETH genotypes and the non-androgenic control line H99. As expected, the negative control line did not respond. Surprisingly, the androgenic response of the genotypes ETH-M82, ETH-M24 and ETH-M72 was very low compared to the yields achieved by Nägeli et al. (1999). This might be due to the use of a simplified version of their protocol, which lacks a gradient separation of the microspores that enables the selection of qualitatively better microspores but, most probably, due to the use of other genotypes.

The addition of activated charcoal to the cultures led to an almost 6-fold increase in yield in the case of the genotype ETH-M82. A notable difference was found in the morphology of the embryos developed in the presence or absence of activated charcoal. Whereas embryos developed with AC showed differentiated structures, those devoid of AC had a highly undifferentiated morphology and were brown in colour, attributable to the synthesis of phenolic compounds (Pan and van Staden, 1998). The higher levels of differentiation observed for the former embryos may be due to the absorption of the phenolic compounds and other substances inhibiting both growth and differentiation by the activated charcoal. For example, the Group of Agronomy and Plant Breeding of the ETH demonstrated that AC plays a role in the gradual absorbance of the exogenous hormone TIBA. It is interesting that this substance, which inhibits auxin transport (Choi et al., 1997), is essential for the induction of androgenesis but stops it shortly thereafter when not removed from the medium.

The different morphology of both types of embryo had an effect on their ability to undergo somatic embryogenesis: when transferred to N6 medium containing 2,4-D, the brown coloration of the embryos disappeared shortly thereafter and cell division was reactivated. A high ability to undergo callus formation and subsequent secondary embryogenesis was observed, a phenomenon attributable to the higher proportion of undifferentiated cells; this factor probably simplified the reactivation of cell differentiation. An extremely high regeneration rate of 0.45 regenerants per embryo was achieved. On the other hand, embryos developed on medium with activated charcoal did not multiply at such a high rate, leading to a lower output of regenerants (0.30 regenerants per embryo). These differences between both types of embryo were, however, insignificant due to a very high variability between repetitions, which led to extremely high standard deviations.

A highly significant difference was determined between the yields obtained by the direct and the indirect regeneration pathways. The indirect pathway resulted in a 10 times stronger response with regard to regenerants per embryo, yielding a mean of 0.38 regenerants per embryo. This yield is significantly higher than that reported by Gaillard et al. (1991), Nägeli et al. (1999) and Szarka et al. (2001).

A spontaneous doubling efficiency of 30 % was observed, which is higher than the doubling efficiency reported by Nägeli et al. (1999).

Despite the improved response of the ETH-M82 genotype to the microspore culture and an excellent rate of plant regeneration by means of the indirect regeneration pathway, the procedure is not sufficiently efficient for the satisfactory production of gametic embryos and double haploid plants. The results clearly show that microspores are hardly able to develop without the tapetal cells of the anthers. Even extremely responsive genotypes like ETH-M82 do not produce the number of gametic embryos required for further applications.

This drastically diminishes the chance of achieving regenerants from genetically transformed microspores.

1.5.2 Anther culture

The response to anther culture, i.e. the ability of microspores to form gametic embryos *in anthero*, was studied for the three highly androgenic genotypes (ETH-M24, ETH-M72 and ETH-M82): whereas the control line H99 showed no response, ETH-M82 exhibited the highest yields ever observed (Büter et al., 1993; Saisingtong et al., 1996b; Barnabás et al., 1999; Szarka et al., 2001; Wassom et al., 2001). ETH-M24 and ETH-M72 also provided a constant supply of gametic embryos. The high standard deviation in androgenic responsiveness is explained by *i)* the extreme sensitivity of the system to variations in environmental conditions, *ii)* the developmental stage of the donor material and *iii*) the segregation of traits that influence androgenic responsiveness, which was shown to be a heritable polygenic trait (Murigneux et al., 1994). A mean diploidisation rate of 54 % was achieved as a result of the colchicine treatment, in agreement with the results of Saisingtong et al. (1996b).

Armstrong and Green (1985) described two types of callus (I and II) which can originate from the cultivation of immature zygotic embryos on medium containing 2,4-D. In general, Type I callus is white and compact and seems to be more differentiated than the Type II callus. The latter is soft, can retain totipotency after longer periods in culture than the Type I callus, but its establishment is difficult and highly genotype-dependent.

The culture of ETH-M72 zygotic embryos on the Modified N6 medium induced Type I callus only, which was found to be suitable for transformation (D'Halluin et al., 1992; Brettschneider et al., 1997). When ETH-M24, ETH-M72 and ETH-M82 gametic embryos were cultured on the same medium, structures resembling the Type I callus were obtained, indicating that this explant might be as suitable for transformation experiments as the zygotic embryos.

The number of secondary embryos of zygotic and gametic origin multiplied exponentially but rapidly lost their ability to regenerate after four weeks. This is typical of the Type I callus and was proposed by Jiménez and Banghert (2001) to correlate with endogenous IAA levels.

The ability of zygotic embryos to undergo secondary embryogenesis after four weeks of culture on Modified N6 medium was five-fold lower than for zygotic embryos. The cells of gametic

embryos do not undergo somatic embryogenesis to the same extent as the scutellar cells of the zygotic embryos (McCain and Hodges, 1986).

The combination of an exponential multiplication of secondary embryos and a reduction in their ability to regenerate into a plant resulted in an increase in the number of regenerants per original zygotic and gametic embryo during the eight weeks of the experiment. Rates of 8.2 and 1.0 regenerants per original embryo were achieved for eight-week-old zygotic and gametic embryos, respectively. Büter et al. (1993) and Saisingtong et al. (1996b), who applied a direct regeneration procedure, reported a maximum of 0.2 regenerants per gametic embryo. It is expected that, after a prolonged culture on N6 medium, regenerants would no longer form because of the age-correlated decrease in the regeneration ability of secondary embryos, which overcompensates the proliferation achieved by secondary embryogenesis.

In conclusion, the gametic embryos were responsive to the induction of secondary embryogenesis and plant regeneration from secondary embryos. Despite the fact that the zygotic control was much more efficient than the gametic embryos with regard to all the studied factors, the results indicate that the gametic embryos have the characteristics required for their use as a target for genetic transformation.

SSR marker-based analysis of the homozygous nature of DH lines

2. SSR marker-based analysis of the homozygous nature of DH lines

2.1 Abstract

An indirect regeneration method of gametic embryos, based on the induction of somatic embryogenesis in a medium containing 2,4-D prior to the induction of plant regeneration, was used for the studies presented here. This procedure, together with the androgenic pathway, may cause mutations in the genome and, thus, alter the homozygous nature of the DH lines. In order to determinate whether these variations occurred, four DH lines were analysed for polymorphism at 19 different loci by means of SSR markers. Twenty-two individuals per DH line were tested; whereas different band patterns were detected between the lines, polymorphism was not found within the lines. The results confirm the high degree of homozygosity of the DH material; the androgenic process and the prolonged cultivation on an auxin containing medium did not bring out detectable DNA rearrangements; therefore, the applied procedure is suitable for the regeneration of homozygous plants.

2.2 Introduction

The double haploid technique has a great potential for the rapid attainment of fully homozygous lines. Nevertheless, during androgenesis and further *in vitro* culture, variations at a genetic level may occur as a result of gene and chromosome mutations, DNA amplification or activation of transposable elements, which can alter the homozygous nature of the DH lines. These variations are classified as follows:

- gametoclonal variation, which is generated by a deviation from normal segregation and occurs during gamete formation when gametophytic cells or tissues are subjected to *in vitro* culture processes (Rode et al., 1987; Dogramaci-Altuntepe et al., 2001),
- variation induced during artificial chromosome doubling (e.g. by means of colchicine) (Niemirovicz-Szczytt, 1997) and

- somaclonal variation, which is triggered by prolonged periods of tissue culture (Phillips, 1989; Linacero et al., 2000; Dogramaci-Altuntepe et al., 2001; Rahman and Rajora, 2001).

Whereas gametoclonal variation does not affect the homozygous nature of the regenerants from microspore cultures, because the mutation took place before chromosome doubling, genomic alterations generated by the chromosome doubling treatment and somaclonal variation do lead to heterozygosity of the regenerated material.

Murigneux et al. (1993a) performed a study to determine the homozygous nature of maize lines obtained by anther culture and further *in vitro* culture. They analysed 189 double haploid lines at more than 100 loci by means of the RFLP method. They found traces of heterozygosity which they attributed to somaclonal variation; polymorphism was generated after the diploidisation process and probably during the *in vitro* culture. In general, the DH lines presented levels of homozygosity of 99 %, similar to the homozygosity of lines after six to seven generations of inbreeding. Murigneux et al. (1993a) concluded that their system of direct regeneration (without an intervening callus phase) may have determined the low frequency of somaclonal variations.

For the studies presented here, an indirect regeneration procedure, based on secondary embryogenesis, was applied to augment the output of regenerants and as a prerequisite for the genetic transformation of embryos, as discussed on page 21 of the General Introduction. Prolonged callus phases and additional *in vitro* culture may lead to an accumulation of mutations and, thus, to additional genetic variation among DH plants, in contrast to the direct regeneration pathway. In order to evaluate the extent to which this procedure altered the homozygous nature of the lines, four double haploid lines were analysed at a molecular level for the presence of polymorphism. PCR-based SSR markers showing a high level of allelic polymorphism (Smith et al., 1997; Pejic et al., 1998; Senior et al., 1998) were applied.

2.3 Materials & Methods

2.3.1 Plant material

Four double haploid lines were used in this study. The lines were developed by anther culture (as described on pages 32 to 35 of the Chapter 1) from heterozygous material corresponding to the following double cross: (ETH-M80 x ETH-M72, androgenic) x (A188 x H99, non-androgenic). Prior to regeneration on MS medium (Murashige and Skoog, 1962), the gametic embryos were subjected to cultivation on Modified N6 medium (page 92 of the Appendix) containing 1 mg/l 2,4-D for four weeks for the induction of secondary embryogenesis.

2.3.2 SSR analysis

Genomic DNA was extracted from the four double haploid lines using the GenElute™ Plant Genomic DNA Miniprep Kit (G2N, Sigma) and used as template for the SSR analysis.

A set of 96 standard SSR primer sequences (available from the Maize Database, USDA) was chosen arbitrarily (list of primers on page 94 of the Appendix). The primers were synthesised by MWG-Biotech AG in Munich, Germany.

The protocol for maize SSRs described by the CIMMYT Applied Molecular Genetics Laboratory (Hoisington et al., 1999) was used with some adjustments:

Each PCR reaction contained 50 ng of genomic DNA per individual, 150 µM of each dNTP, 1 U *Taq* enzyme, 0.25 µM of each primer, 2.5 mM of $MgCl_2$, 1x *Taq* buffer and sterile ddH_2O. The final volume of the reaction was 20 µl.

The thermocycler model OmniGene (Thermo Hybaid, U.K.) was used for DNA amplification and the following PCR program was applied:

I one initial cycle of denaturation (94 °C, 2 min),

II thirty cycles of denaturation (94 °C, 30 s), primer annealing (54 °C, 1 min) and elongation (72 °C, 2 min) and

III a final elongation (72 °C, 5 min).

The amplification product was run for 1 h 15 min at 160 V on a 4 % (w/v) agarose gel containing 2 % (w/v) Resophor agarose (Eurobio, France) and 2 % (w/v) Agarose STG (Eurobio, France).
In a first step, the parental lines ETH-M72, ETH-M80, A188 and H99 were tested for polymorphic band patterns with all 96 primer sets. Thereafter, 22 individuals per double haploid line were analysed with a selection of polymorphic primers. In order to reduce the number of assays, the genomic DNA of two individuals was pooled (in one PCR reaction, 50 ng DNA of individual A plus 50 ng DNA of individual B were used as template for the amplification).

2.4 Results

From the 96 primer sets tested on all four parental genotypes, 55 clearly revealed at least two different band patterns for the four parental genotypes (Fig. 2.1). Nineteen primer sets (two per chromosome except for chromosome 7) were selected for further analysis of the DH lines, according to the quality and polymorphic level of the bands (list of selected primers on page 95 of the Appendix).

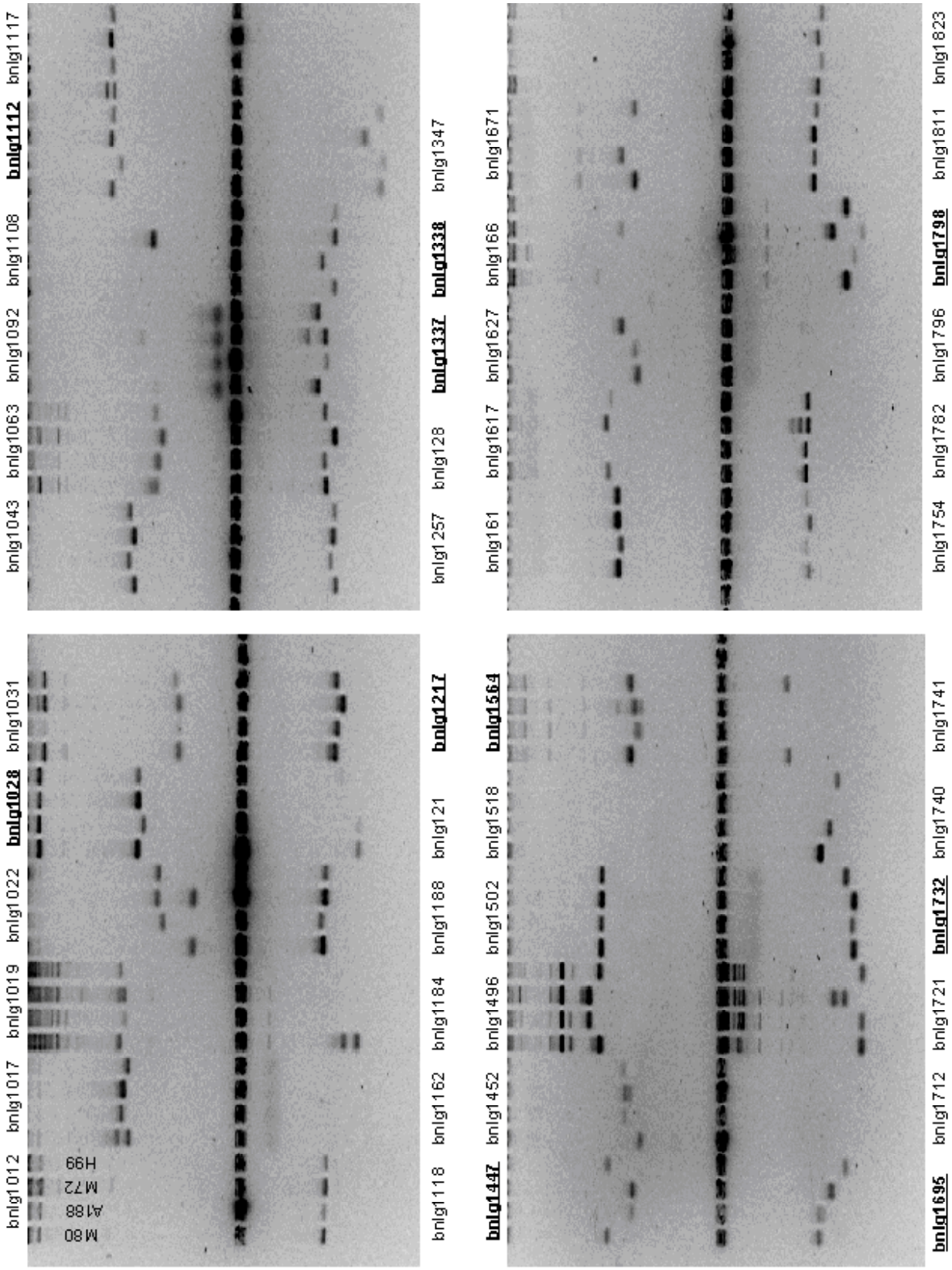

Figure 2.1 SSR primers test on the parental genotypes. The parental genotypes ETH-M80, ETH-M72, A188 and H99 were tested for polymorphism at 96 different loci. The underlined primers correspond to those selected for the analysis of the DH lines.

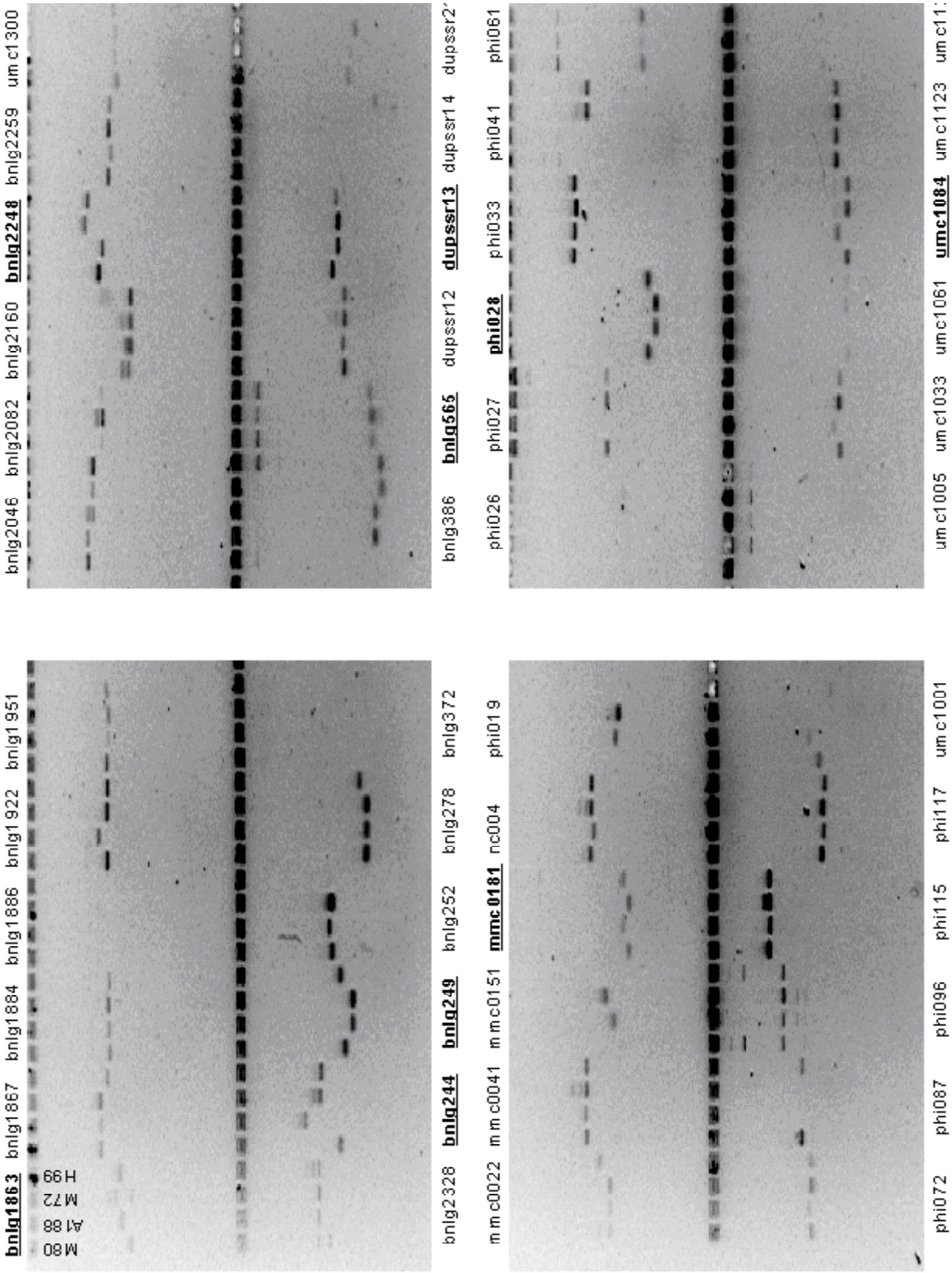

Figure 2.1 SSR primers test on the parental genotypes (cont.)

Twenty-two individuals per DH line were tested with the selected primers. Nine of the 19 primers revealed a different band pattern between at least two lines. Alleles of all four parental genotypes were present in the DH lines in equal proportions. No variation in the band pattern was detected within the lines (Fig. 2.2).

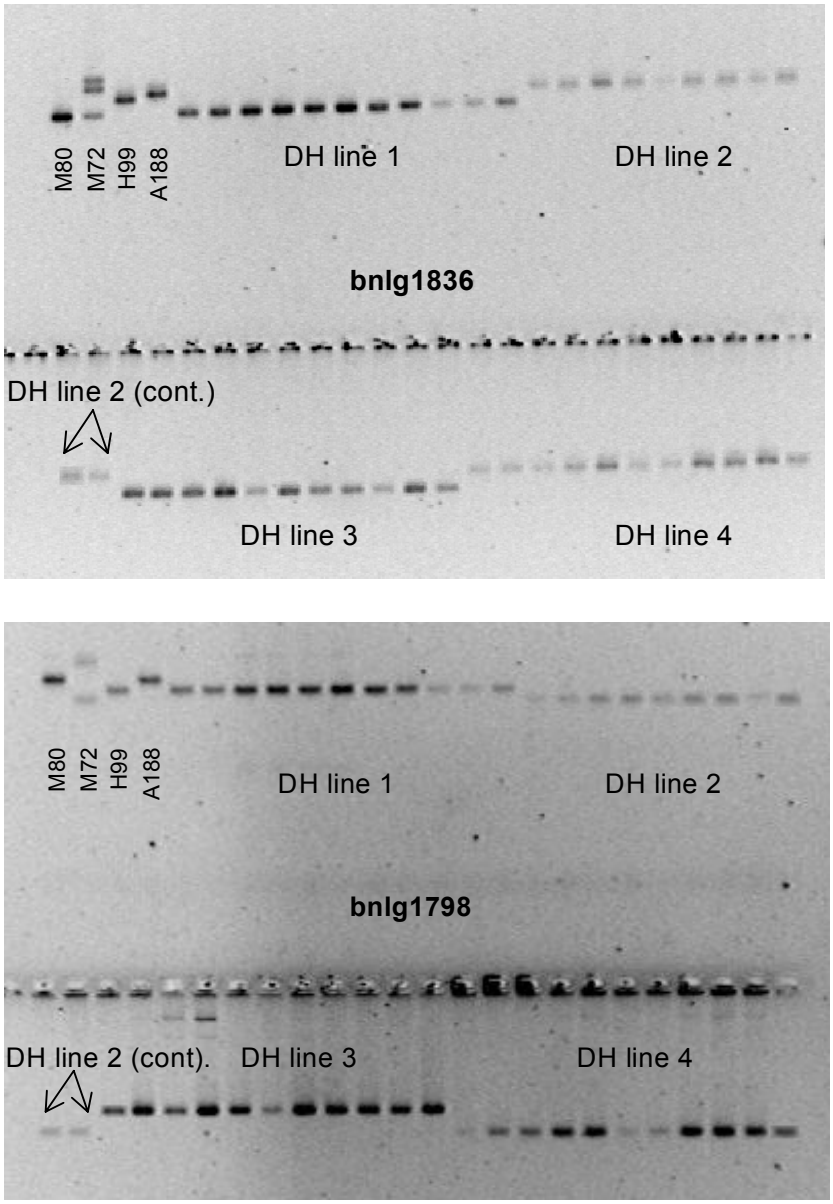

Figure 2.2 SSR analysis of DH lines. Four DH lines (DH line 1 to 4) were analysed at different loci by means of SSR markers. Twenty-two individuals (pooled in two individuals per PCR reaction) per line were tested. The primers showed polymorphism between the parental genotypes (ETH-M80, ETH-M72, A188 and H99). Whereas polymorphism was detected between the four lines, no variation was detected within the lines. The amplification patterns of the primers bnlg1836 and bnld1798 are shown.

2.5 Discussion

A SSR marker-based spot test was performed on four double haploid lines at 19 loci spread throughout all chromosomes. The parentals of the DH lines correspond to two androgenic genotypes (ETH-M80 and ETH-M72) and two non-androgenic genotypes (H99 and A188).

For several SSR primers, polymorphic band patterns were found between the parental genotypes and, as expected, the four analysed DH lines varied at nine loci.

Dufour et al. (2001) and Murigneux et al. (1993a) demonstrated the occurrence of segregation distortions during *in vitro* androgenesis and attributed them to a range of selective factors resulting from the partial or total elimination of gametes, which lead to a higher representation in the DH lines of alleles belonging to the androgenic genotype. In the case of the DH lines analysed in the present study, a greater number of alleles of both genotypes ETH-M72 and ETH-M80 was not detected, suggesting that no biased segregation occurred in favour of the androgenic lines. Nevertheless, for a statistical analysis of segregation distortions in the present material, more loci should be analysed.

Within each DH line, the band patterns were always homogeneous. The plants regenerated from four-week old callus of gametic origin did not present chromosomal aberrations due to incorrect chromosome doubling or somaclonal variations at any of the 19 studied loci. Indeed, Müller et al. (1990) demonstrated a correlation between the duration of the callus culture phase and a high degree of DNA rearrangements. On the other hand, Brown et al. (1991) suggested that differentiation and organogenesis act as a selecting phase against novel DNA rearrangements. According to them, callus formation from somatic tissue of the inbred line A188 resulted in significant deviations from the DNA pattern compared to the seed-grown control. Nevertheless, DNA variation decreased during organogenesis; most of the regenerated plants showed high genetic similarities to the control.

Based on the data obtained from the spot test on four DH lines at 19 loci, it is concluded that the lines obtained by the anther culture and plant regeneration protocols available for this study did not show aberrations or rearrangements at a chromosome level. Even though no

conclusions can be drawn about the presence of base pair or single gene mutations or the occurrence of epigenetic variation, the results indicate that the additional *in vitro* process of secondary embryogenesis did not have a negative impact on the homozygosity of the DH regenerants; therefore, the technique is suitable for the production of diploid and highly homozygous maize lines.

Transformation of microspores and gametic embryos

3. Transformation of microspores and gametic embryos

3.1 Abstract

The *in vitro* performance of microspores and gametic embryos as targets for the biolistic transformation was described in Chapter 1. A second determinant in the establishment of a transformation protocol is the level of transgene expression on the target material and its response to the selection mechanism. In this respect, microspores, gametic embryos and immature zygotic embryos (positive control) were bombarded with a visual gene (*gfp* gene) and a selectable marker gene (*pat* gene). The transformation of isolated microspores showed no transient *gfp* expression, suggesting that these explants are unsuitable for transformation under the experimental conditions. The gametic embryos showed transient and stable expression of the *gfp* transgene, although with less efficiency than the control. The selection method based on the resistance to the herbicide Basta, conferred by the *pat* gene, seemed to have a toxic effect on transformed cells, probably due to an insufficient expression level of the *pat* gene. A positive selection mechanism should be applied in further experiments on transformation of gametic embryos.

3.2 Introduction

The transformation of androgenic material can be achieved by gene transfer to microspores (Carlson et al., 2001; Folling and Olesen, 2001) or gametic embryos (Folling and Olesen, 2001).

Chapters 1 and 2 described the potential of using maize gametic embryos and isolated microspores as targets for genetic transformation, based on their *in vitro* performances and the level of homozygosity reached. The gametic embryos of the three genotypes ETH-M24, ETH-M72 and ETH-M82 have the *in vitro* characteristics required for a successful transformation, such as a satisfactory induction of secondary embryogenesis and a satisfactory plant regeneration capability, even though with significantly less efficiency than the control, transformable zygotic embryos of self-pollinated ETH-M72 plants. A high rate of diploidisation was achieved, and the homozygous nature of the double haploid regenerants was confirmed

by SSR analysis. On the contrary, microspores had a very low rate of androgenic induction (an average of one embryo per 5 300 microspores for the genotype ETH-M82). This fact makes the establishment of transgenic DH lines from these explants unlikely, unless the weak androgenic response can be overcompensated by an extremely high rate of microspore transformation.

The aim of the experiments presented in this chapter was to study the transient and stable expression of a transgene after its delivery to microspores and gametic embryos by means of particle bombardment. The visual marker gene *gfp* (green fluorescent protein, reviewed by Steward Jr, 2001) and the selectable marker gene *pat* (phosphinothricin acetyl transferase, De Block et al., 1987), conferring resistance to the herbicide Basta, were used for this purpose.

The transient transgene expression (screened by the *gfp* expression shortly after bombardment) is important, because it indicates the number of nuclei reached by the plasmid. Nevertheless, transient expression does not indicate the integration of the transgene in the plant genome, since the transgene can be transcribed from the non-integrated plasmid and be translated after its mRNA is transported through the nuclear membrane into the cytoplasm, giving a positive fluorescence signal. This transient expression can be observed for a few days until the DNAses degrade the plasmid if it did not integrate. Therefore, only the growth of tissue expressing the *gfp* marker gene is indicative of stable expression. This confirms that the transgene was integrated into the genome and is, therefore, inherited through mitosis.

The expression level of the transgene is essential at the moment a selective agent, such as the herbicide Basta, is applied. Even though the *pat* transgene is integrated and expressed, the level of the PAT activity may be insufficient to ensure the deactivation of the selective agent. In that case, selection will not occur.

In this study, microspores were bombarded with the visual marker gene *gfp* and its transient and stable expression was determined one day after bombardment, according to a study of Carlson et al. (2001) of the transformation of barley microspores. As positive control, microspores of a transgenic line expressing *gfp* were used.

For the transformation of gametic embryos, the *gfp* and the selective marker gene *pat* were used in a co-transformation approach. The transient and stable integration of the transgene

were determined by the time course of *gfp* expression during callus growth. In order to study the expression level of the transgene, *pat* and *gfp* co-transformed gametic embryos were subjected to Basta selection. Since the *pat* expression could not be determined *in vivo*, the *gfp* gene expression was used as an indicator of transient transgene expression in general. As positive control system for the transformation of gametic embryos, immature zygotic embryos of the transformable genotype ETH-M72 were used.

3.3 Materials & Methods

3.3.1 Genetic transformation

Target material

Gametic embryos of the genotypes ETH-M24, ETH-M72 and ETH-M82, generated by anther culture (pages 32 to 34 of Chapter 1), were used as target material for the transformation immediately after their isolation, when they were about 1.5 to 2.0 mm in diameter. Freshly isolated immature zygotic embryos of self-pollinated ETH-M72 plants were used as a positive control (page 34 of Chapter 1).

The transformation of isolated microspores of the genotype ETH-M82 was also attempted. Microspores were isolated at a late uni- to early bi-nucleated stage, as described on page 33 of Chapter 1. Cultures showing a good androgenic response were used as targets of the bombardment one week after their isolation.

Gene constructs

A construct containing the *gfp* gene and the strong maize ubiquitin promoter was used as visual marker (construct provided by C. Sautter, ETH Zurich). As selective marker gene, the *pat* gene encoding for the Basta herbicide detoxifying enzyme PAT was applied. This construct, which contains the *pat* sequence and the CaMV 35S- promoter, was provided by R. Brettschneider, University of Hamburg, Germany.

DNA delivery by particle bombardment

A Particle Inflow Gun (Finer et al., 1992, page 20 of the General Introduction) was used to deliver the marker gene constructs ubi::*gfp* and 35S::*pat* into the target material in a co-transformation approach. Approximately 625 µg of 1.5-3.0 µm gold particles (Sigma Aldrich 32.658-5) were coated with plasmid DNA; 12.5 µl of a gold suspension (50 mg/ml in glycerol 50 %) were mixed with 1.5 µg of each plasmid. The DNA was precipitated onto the particles by adding 12.5 µl $CaCl_2$ (2.5 M) and 5 µl of Spermidine-HCl (0.1 M). After vortexing for 1 min, 150 µl of ice-cold 96 % ethanol were added and the suspension was incubated for 30 min at -20 °C. After centrifugation, the supernatant was discarded and 12.5 µl of sterile ddH_2O were added. All the suspension was used for one shot.

Freshly isolated gametic and zygotic embryos (the latter with the scutellar side facing up) were placed onto Hyper-Osmotic Modified N6 medium (page 93 of the Appendix) five hours before the bombardment.

According to Jardinaud et al. (1995a), one week after their isolation, an optimum of approximately 100 000 microspores of androgenically responding cultures were placed in the centre (in a diameter of ca 1.5 cm) of petri-dishes, 6 cm in diameter, containing IMM medium (page 91 of the Appendix) solidified with 3 g/l phytagel and covered with a sector of autoclaved thin filter paper, 5.5 cm in diameter.

The target material, gametic and zygotic embryos or the microspores, was bombarded with the DNA-coated gold particles at a He pressure of 6 bar and with a chamber vacuum strength of -0.9 bar. The shooting distance was set at 9 cm.

Twenty-four hours after bombardment, the embryos were transferred to Modified N6 medium, as described for the induction of secondary embryogenesis on page 34 of Chapter 1. Secondary embryogenesis was induced during the next two weeks.

The microspores were suspended immediately after the bombardment in IMM; the density of the cultures was adjusted to 40 000 microspores per ml and distributed in petri-dishes, 2 cm in diameter (2 ml per dish). Six cubes of AC (page 33 of Chapter 1) were added to each petri-dish.

3.3.2 Selection

Visual screening of *gfp* expression

One, 8, 15 and 22 d after DNA transfer, gametic and zygotic embryos were screened for the transient expression of the *gfp* gene under a fluorescence microscope (Olympus AX-70 Provis, Olympus U-RFL-T UV-light burner equipped with an Olympus U-MSWB filter set). The expression was quantified as the number of green spots per embryo.

The microspores were observed one day after the bombardment for transient *gfp* expression under the fluorescence microscope. Microspores of a transgenic ETH-M72 line (developed by the group of Agronomy and Plant Breeding at the ETH Zurich) expressing the green fluorescent protein were the positive control for *gfp* expression. Microspores of a wild-type ETH-M72 plant were used as negative control.

Selection of Basta-resistant material

To select the transgenic material, secondary embryos were routinely transferred to Selective Modified N6 medium (supplemented with Basta, page 93 of the Appendix) two weeks after bombardment. After cultivation for four weeks in the dark, the embryos were transferred to Selective MS medium (Sigma MS supplemented with 3 % (w/v) sucrose, 0.3 % (w/v) phytagel

and 5 mg/l Basta of Bayer CropScience) and exposed to light for the induction of plant regeneration. The plantlets were transferred to soil and sprayed twice with a solution containing 0.13 % (v/v) Basta and 0.5 % (v/v) Tween 20, once at the third and once at the fourth leaf stage (4 d later).

3.4 Results

3.4.1 Transformation of microspores

Seven series of transformation experiments, each with five shots, were performed. Approximately 100 000 microspores were bombarded per shot. Whereas fluorescing microspores were found in the positive transgenic control (Fig. 3.1 A), none were found in the negative wild-type control (Fig. 3.1 B). The bombarded microspores did not show transient expression of the *gfp* (Fig. 3.1 C) one day after the bombardment.

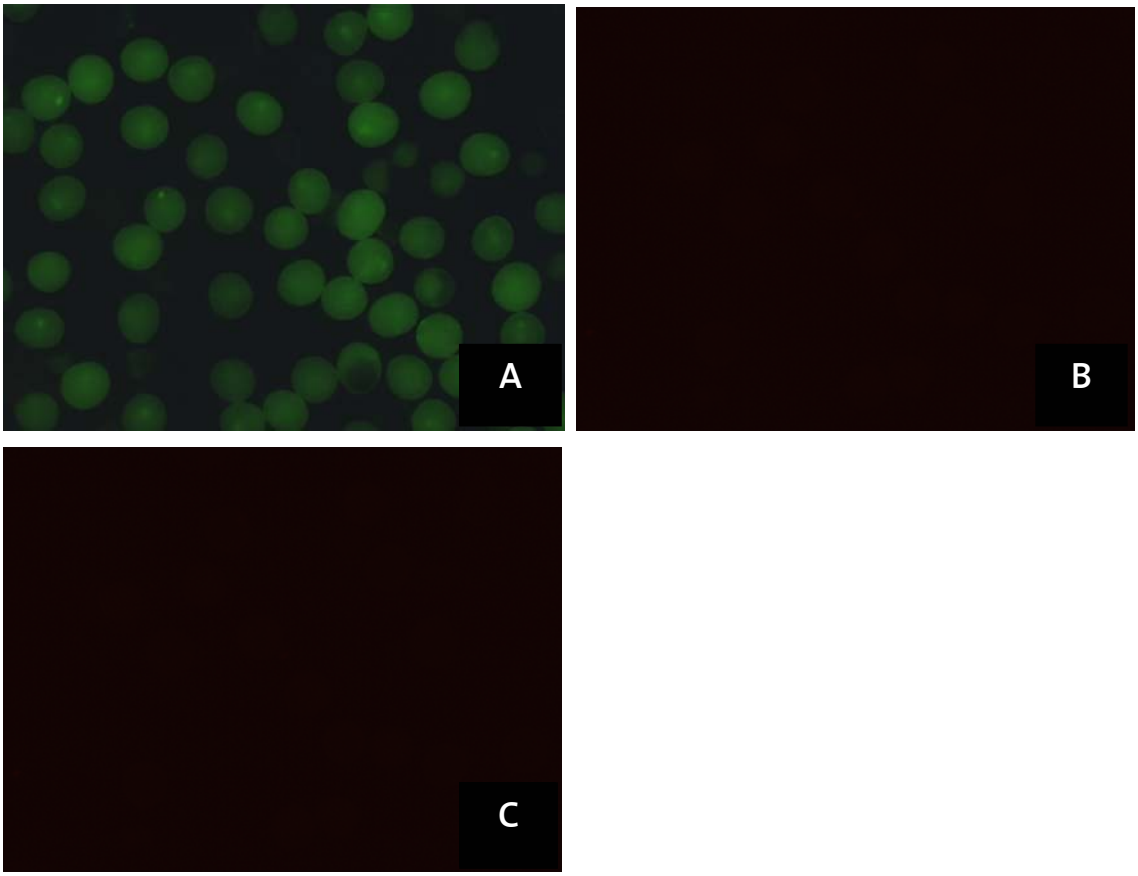

Figure 3.1 *gfp* expression in microspores. (A) Microspores of the stable transformed genotype ETH-M72 encoding for the *gfp* were the positive control. **(B)** Microspores of a wild-type genotype (non-transformed ETH-M72) were the negative control. **(C)** Microspores of the genotype ETH-M82 were bombarded with the *gfp* construct, and transient transgene expression was observed by means of a fluorescence microscope.

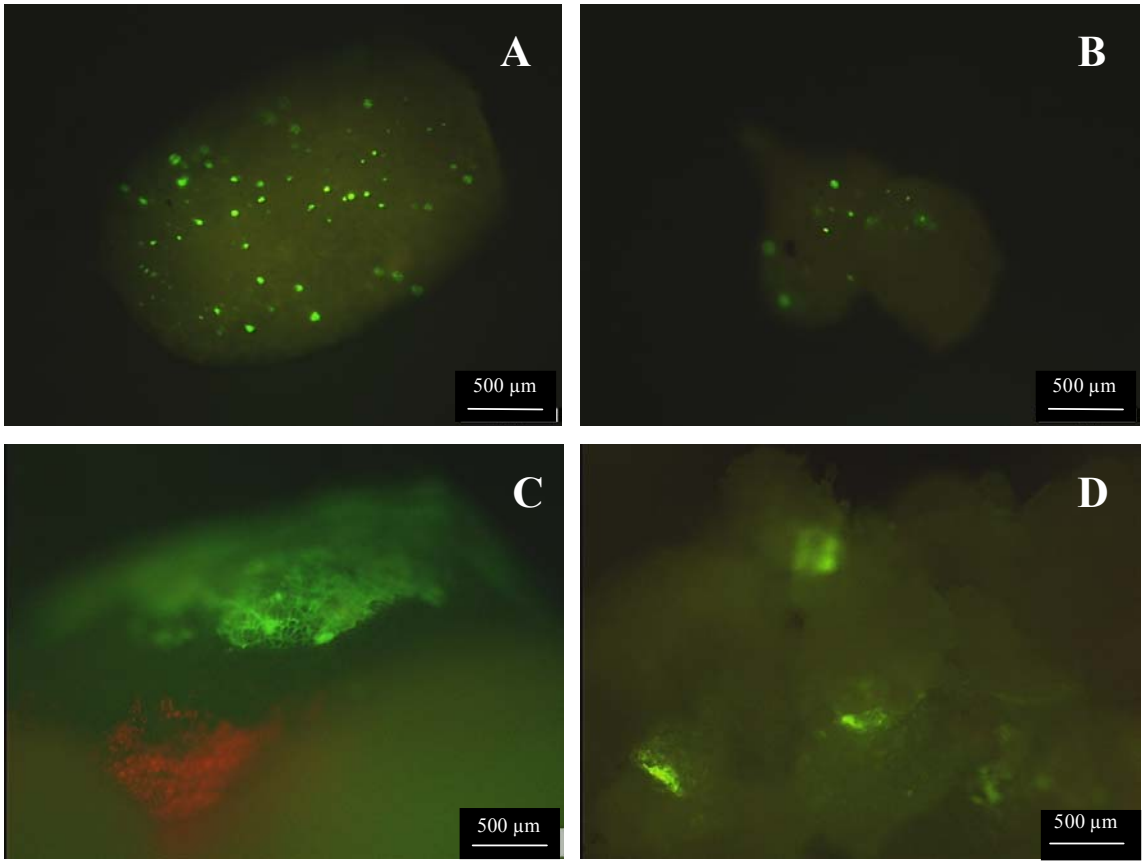

Figure 3.3 Transient and stable *gfp* expression. (A) Zygotic and **(B)** gametic embryos were bombarded to deliver the *gfp* gene. The transient expression was quantified one day after bombardment as the number of green spots per embryo. **(C, D)** Stable transformation of gametic embryos was observed two weeks after bombardment when no selection pressure was applied. Transformed cells expressing the *gfp* gene underwent division and formation of transgenic tissue.

3.4.2 Transformation of gametic embryos

Approximately 450 zygotic embryos of ETH-M72 and 420, 590 and 440 gametic embryos of ETH-M72, ETH-M82 and ETH-M24, respectively, were co-transformed by particle bombardment with the ubi::*gfp* and 35S::*pat* constructs. One day after the bombardment, the transient *gfp* expression was quantified by counting the number of green spots per embryo (Fig. 3.3 A, B). A significantly higher density of *gfp* expressing cells was found in zygotic (19 spots per embryo) than in gametic material (three spots per embryo) (Table 3.1). Within the gametic embryos, a significantly higher level of expression was found for the genotype ETH-M24. Selection pressure was applied routinely 15 d after bombardment by adding Basta to the regeneration medium. A transformation rate (expressed in percentage of stable transgenic lines per bombarded embryo) of 0.6 % was achieved for the zygotic embryos of the genotype ETH-M72. This result was confirmed after spraying the regenerants with Basta and conducting a Southern Blot analysis using a *pat*-specific gene probe. The bombardment of gametic embryos did not result in transgenic regenerants (Table 3.1).

Table 3.1 Transient and stable transformation. Zygotic embryos of the genotype ETH-M72 and gametic embryos of the three ETH genotypes were scored for transient *gfp* expression one day after bombardment. Data followed by the same letter are not significantly different from each other at p = 0.05. Stable transformation rates (percentage of transformation events per bombarded embryo) are shown.

Embryo type	Zygotic	Gametic		
Genotype	ETH-M72	ETH-M72	ETH-M82	ETH- M24
Transient *gfp* expression [no. spots per embryo]	19.05 ± 18.05	2.24 ± 6.85 **a**	2.06 ± 4.95 **a**	5.97 ± 12.73
Stable transformation [%]	0.58	0	0	0

To determine whether the expression level of the *pat* gene in the transformed cells of gametic embryos was sufficient to enable the effective selection by Basta, selection pressure was applied one or 15 d after the bombardment of the gametic embryos of ETH-M24 with the *pat* and *gfp* constructs (Fig. 3.2). The *gfp* marker gene was used as an indicator of transgene expression. Without selection pressure, the percentage of cells expressing *gfp* decreased by almost 50 % during the first week after bombardment and stabilised thereafter, indicating the division of transformed cells (Fig. 3.3 C, D). When Basta was applied on the 15th d after bombardment, transgene expression decreased rapidly and disappeared after another week. When Basta was applied one day after bombardment, the percentage of cells expressing *gfp* decreased drastically by 80 % during the first week and disappeared during the following week.

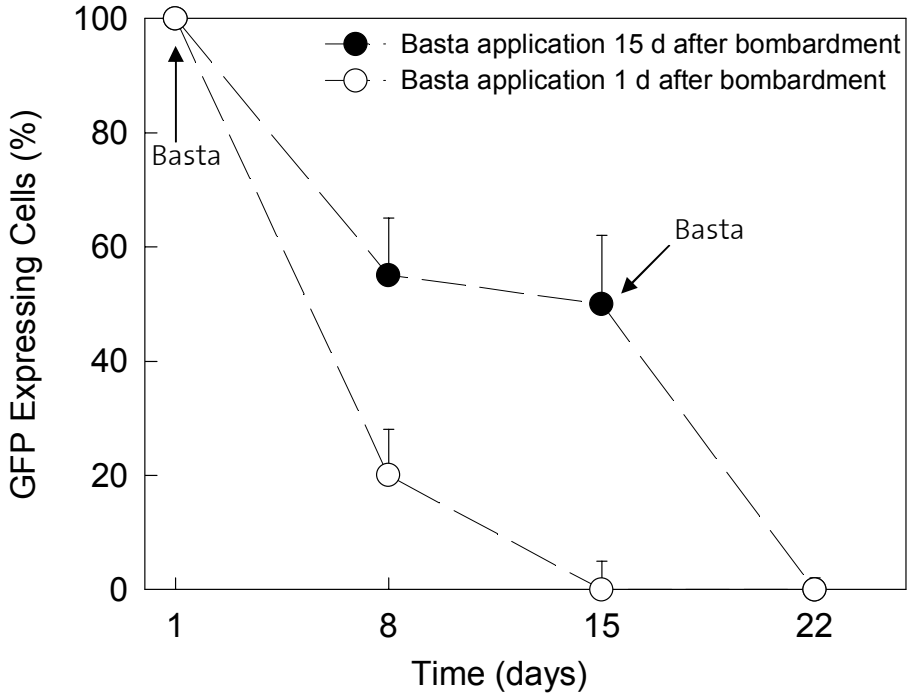

Figure 3.2 Time course of *gfp* expression. Gametic embryos of the genotype ETH-M24 were bombarded with the 35S::*pat* and ubi::*gfp* constructs on day zero. *gfp* expression (spots per embryo) was observed on day 1 and standardised as 100 %. Immediately after observation, half the embryos were transferred to the medium inducing secondary embryogenesis supplemented with Basta. The other half of the embryos was transferred to the same medium without Basta; on day 15, these embryos were also transferred to medium containing Basta. Eight, 15 and 22 d after bombardment, *gfp* expression was observed and expressed as percentage of green spots relative to the expression found on day 1.

3.5 Discussion

The aim of this study was to evaluate the transient and stable expression of the *gfp* gene on the target material (gametic embryos and microspores) and to determine the expression level of the *pat* transgene on gametic embryos as a determinant of the efficiency of selection. Since *pat* activity could not be observed *in vivo*, co-transformation with the visual marker gene *gfp* was performed. In that sense, the *gfp* expression served as a measure of transgene activity.

When dividing microspores were bombarded with the *gfp* construct using the biolistic method, no transient expression was observed, contrary to the results published by Jardinaud et al.

(1995a), who reached a recovery of 0.07 % *gus*-expressing maize microspores. The reason for these poor results might be the extremely low proportion of viable microspores in relation to the non-viable microspores at the moment of bombardment. Therefore, it is quite likely that most of the gold-DNA particles reached non-viable cells which can no longer give any signal. In addition to the results presented in Chapter 1, these results give a strong indication that the transformation of microspores is, at present, not a feasible approach.

After transformation, gametic embryos had on average a five-fold lower density of cells transiently expressing the *gfp* marker gene than zygotic embryos had. The more compact structure of the gametic embryos may result in a less effective delivery of gold particles into their cells than into the cells of the zygotic embryos. Of the gametic embryos, those of the genotype ETH-M24 showed a significantly higher density of cells expressing the transgene than the embryos of ETH-M72 and ETH-M82, indicating that the activation of transgene expression is a genotype-dependent feature.

The selection mechanism based on the herbicide Basta (Block et al., 1987) seemed to have a toxic effect on wild-type as well as on transiently transformed cells of gametic embryos. The *gfp* expression ceased right after the application of Basta. This may have occurred because *i*) the *pat* gene was poorly or not expressed and, therefore, could not detoxify Basta (PPT) to a sufficient extent, *ii*) gametic cells were more sensitive to large amounts of ammonium, which were released into the medium by metabolic active cells as a consequence of Basta intoxication, or *iii*) the density of the transformed cells was too low and, therefore, multiplication of such cells did not take place under selection pressure. When selection pressure was not applied, the transformed cells spread, suggesting that the transgene was stably integrated (Fig. 3 C, D). Nevertheless, no regeneration of chimeras from these tissues was obtained.

In conclusion, the gametic embryos showed a positive response to the transgene delivery by means of the biolistic transformation, as measured by the density of cells transiently expressing the marker gene *gfp*. Even though with less efficiency than the control zygotic embryos, transgene expression indicates that the transformation of gametic embryos is

feasible. A major limiting factor of the proliferation and regeneration of transgenic cells was the selection procedure; in future experiments, the selection of transgenic material based on a **positive** selection mechanism, such as the *pmi* (phosphomannose isomerase) gene (Wang et al., 2000; Wright et al., 2001), should be applied. In contrast to the Basta selection method, positive selection methods have less toxic side effects on the plant material. A visual selection mechanism, based merely on the physical separation of tissues expressing *gfp* (Kaeppler et al., 2001; Steward CN Jr, 2001), might also be considered.

Attainment of a DH line from transgenic maize plants by anther culture

4. Attainment of a DH line from transgenic maize plants by anther culture

4.1 Abstract

A strategy for establishing a transgenic double haploid maize line from heterozygous transgenic material by means of anther culture is presented. Since the efficiency of the procedure is highly genotype-dependent, transgenic, non-androgenic plants carrying a herbicide resistance marker gene (*pat*) were crossed with a highly androgenic genotype. The transgenic progenies were used as donor plants for the anther culture. One transgenic and three non-transgenic double haploid lines were established in approximately one year. Southern blot analysis revealed that the transgenic donor plants and their double haploid progeny exhibited the same integration pattern of the *pat* gene. Segregation of the herbicide resistance trait was not observed in the progeny of the transgenic double haploid line.

4.2 Introduction

The original objective of this doctoral work was to establish a protocol for the direct transformation of maize androgenic material. The results presented in Chapters 1 and 3 clearly show that the transformation of isolated microspores or gametic embryos is complex and requires a great deal of work before an efficient protocol is achieved. An alternative approach for the rapid fixation of homozygous transgenic plants, originally suggested by Kunz et al. (2000) for wheat, is presented here; it is based on the two-step fixation of transgenic DH lines from anther cultures initiated from previously transformed heterozygous material.

Since androgenic responsiveness can be transmitted to recalcitrant genotypes by inter-crossing (Jumpatong et al., 1996; Saisingtong et al., 1996b), donor material for anther culture was generated by crossing heterozygous transgenic material, lacking androgenic responsiveness, with a highly androgenic genotype in order to establish a pure transgenic line from the DH

regenerants. Using *in vitro* haploid techniques, this can be achieved in approximately one year, while conventional inbreeding takes place over seven to eight generations.

4.3 Materials & Methods

4.3.1 Donor material for anther culture

The transgenic heterozygous genotypes 109.2 and 116.1, which express the *pat* (phosphinothricin acetyl transferase) gene, which confers resistance to the herbicide Basta, were obtained from R. Brettschneider (University of Hamburg, Germany). They were generated by particle bombardment of zygotic embryos, isolated from the line H99 (yellow kernels) after pollination with A188 (white kernels). The transgenic regenerants were back-crossed to A188. This material segregated for the markers kernel colour and herbicide resistance. The highly androgenic genotype ETH-M82 (derived from a cross between ETH-M80 and ETH-M72) was pollinated with Basta-resistant individuals of 109.2 and 116.1. Transgenic anther donor plants (G1 generation, Fig. 4.1) were selected by spraying them with Basta.

4.3.2 DH line establishment by anther culture

The protocols used for anther culture, secondary embryogenesis and regeneration were as described in Chapter 1 (pages 32 to 35). Plants of the genotype ETH-M82 were used as the negative control and plants of 116.1 and 109.2 were used as the positive control for the androgenic response.

The regenerated plantlets (G2 generation, Fig. 4.1) were explanted to soil when their shoots were approximately 8 cm long. To establish a DH line (G3 generation, Fig. 4.1), regenerants were self-pollinated whenever possible. In parallel, ETH-M82 was pollinated by the G2 plants (G3' generation, Fig. 4.1) to ensure the availability of material for segregation analysis of the transgenic trait.

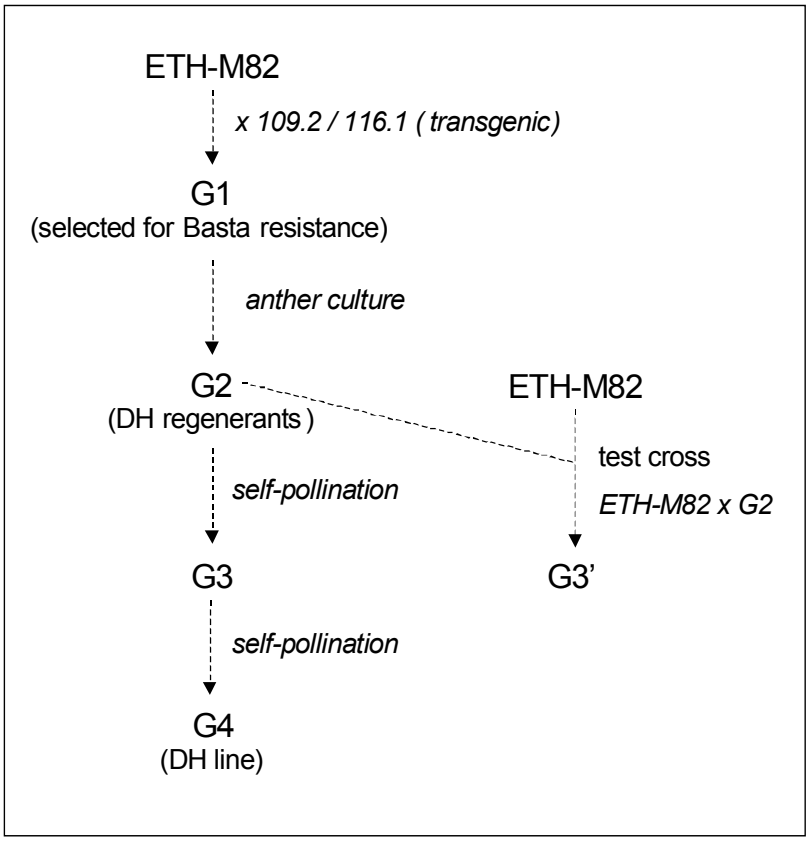

Figure 4.1 Strategy for the establishment of a pure transgenic maize line by anther culture. Transgenic anther donor plants (G1) were selected from a cross between androgenic responsive material (ETH-M82) and transgenic genotypes (109.2 and 116.1). Double haploid (DH) regenerants (G2) were self-pollinated to establish DH lines (G3, G4); a test-cross (G3') enabled an assessment of the homozygosity of G2.

4.3.3 Selection of Basta-resistant plants

Plants were sprayed at the three to four leaf stage with a 0.13 % (v/v) solution of Basta (Bayer CropScience) supplemented with 0.5 % (v/v) Tween 20. The final concentration of the active compound was 250 mg/l bialaphos. The treatment was repeated after three days, and the herbicide resistance of the plants was assessed three days later. The same procedure was performed for transgenic 116.1 (positive control) and non-transgenic plants (negative control).

4.3.4 Southern hybridisation

Plants were analysed on the molecular level for the presence of the *pat* transgene by Southern blot hybridisation.

Plant genomic DNA was extracted from lyophilised leafs according to the protocol of Hoisington et al. (1999). Twenty µg of DNA were digested with the enzymes *EcoRI* and *HindIII*; this combination of enzymes cuts out the entire marker construct, which includes the 35S promoter and the *pat* gene. The resulting DNA fragments were separated on a 0.7 % agarose gel, transferred to a nylon membrane (Schleicher & Schuell, Germany) by alkaline transfer (Sambrook et al., 1989) and hybridised with a digoxigenin-labelled PCR fragment of the *pat* gene. The hybridisation was performed with the Roche™ DIG detection kit according to the instructions of the supplier. For signal detection, the membrane was exposed to X-ray film (Kodak BioMax MR-1) for 45 to 90 min at ambient temperature.

The hybridisation probe was synthesised and labelled with the Roche™ PCR DIG Probe Synthesis Kit using plasmid DNA as template and the following *pat*-specific oligonucleotide primers, located at the positions 579 to 598 and 1092 to 1112 of the synthetic gene, respectively:

*pat*1: 5'-GAG ACC AGT TGA GAT TAG GCC-3'

*pat*2: 5'-ATC TGG GTA ACT GGC CTA ACT-3'

The PCR conditions were as follows:

I one denaturation step (94 °C, 2 min),

II 30 cycles of primer annealing (59 °C, 30 s), elongation (72 °C, 30 s) and denaturation (94 °C, 30 s) and

III a final elongation step (72 °C, 10 min).

4.4 Results

4.4.1 Androgenic response, plant regeneration efficiency and establishment of DH lines

Of 82 individuals derived from the crosses ETH-M82 x 109.2 and ETH-M82 x 116.1, 38 Basta-resistant plants were selected and used as donor plants for anther culture.

A total of 6 025 isolated anthers yielded 837 gametic embryos, which regenerated to 162 plantlets (G2 generation). The crosses ETH-M82 x 116.1/109.2 gave an average yield of 17/25 embryos per 100 anthers with a very high variability within the repetitions (Table 4.1); from 100 of these embryos, an average of 19/27 plants were regenerated, resulting in a yield of 3/7 regenerants per 100 isolated anthers (of each of the two crosses). Whereas the androgenic response was negative for 109.2 and 116.1, the genotype ETH-M82 gave an extremely high yield of gametic embryos and showed an outstanding plant regeneration efficiency.

Table 4.1 Androgenic response of G1 and controls. Number of embryos per 100 anthers, regenerants per 100 embryos and regenerants per 100 anthers of the genotypes ETH-M82 x 109.2, ETH-M82 x 116.1, the positive control genotype ETH-M82 and the negative control genotypes 109.2 and 116.1 (mean and standard deviation of ca. 3 000 anthers per genotype).

	Embryos per 100 anthers	Regenerants per 100 embryos	Regenerants per 100 anthers
ETH-M82 x 109.2	25.2 ± 46.0	27.5 ± 27.5	7.4 ± 15.8
ETH-M82 x 116.1	16.6 ± 23.2	19.0 ± 30.3	2.7 ± 3.9
ETH-M82	279.3 ± 263.5	50.2 ± 45.4	140.61 ± 98.33
109.2	0.0 ± 0.0	-	-
116.1	0.0 ± 0.0	-	-

Four DH descendants of 109.2 but no descendants of 116.1 set seeds after self-pollination and gave rise to four DH lines (G3 generation). This overall poor rate of DH line production was due

to *i*) the low survival rate of the regenerants when transferred to soil, *ii*) developmental disorders such as the lack of the male or female inflorescence, *iii*) male sterility or *iv*) unsynchronised flowering.

While the parental genotypes 109.2 and 116.1 segregated for kernel colour, the four DH lines had either all white or all yellow kernels.

4.4.2 Inheritance of the *pat* gene

Of 82 herbicide treated individuals of the G1 generation, 38 (46.3 %) exhibiting resistance to Basta were selected as donors for anther culture.

Southern analysis of 55 regenerants (G2 generation) revealed a positive hybridisation signal for 14 (25.5 %) plants.

The small number of seeds obtained by self-pollination of the G2 individuals did not enable a segregation analysis in the G3 generation. Therefore, wild-type plants of the genotype ETH-M82 were pollinated with the transgenic and the three non-transgenic G2 individuals. This strategy enabled to test 40 individuals of each cross (G3' generation) for Basta-resistance. All the progenies of the non-transgenic G2 plants proved to be Basta-sensitive, while all the progenies of the transgenic G2 plant were resistant (Fig. 4.2). Sixteen of the resistant G3' plants were also analysed at the molecular level and gave a positive hybridisation signal when probed for the *pat* gene.

Figure 4.2 Effect of Basta on transgenic and non-transgenic individuals: whereas the non-transgenic plants succumb to the treatment with the herbicide Basta (left), the plants expressing the *pat* gene remain unaffected by the treatment with the selective agent. The transgenic material belongs to the G3´ generation descending from the transgenic DH line. The susceptible material descends from a non-transgenic DH line.

The integration pattern of the foreign gene in all the tested transgenic individuals of the generations G2, G3 and G3' was identical to that of the transgenic parental line (109.2). A representative blot with two individuals of the G3 generation and the parental genotypes ETH-M82 and 109.2 is shown in Fig. 4.3.

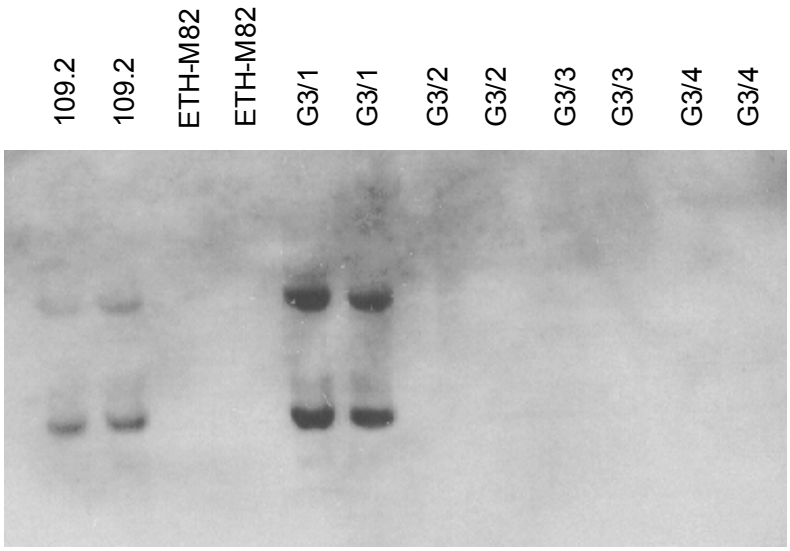

Figure 4.3 Southern hybridisation with a *pat* probe of *HindIII-EcoRI* digested genomic DNA of G3 plants and the parental lines ETH-M82 and 109.2. Two individuals of the parental genotypes and two different G3 progenies of each of the four G2 plants were tested; one of the four double haploid plants inherited the transgene.

The inheritance and expression of the transgenic trait in the established DH line was studied for a further generation. One transgenic G3 plant could be self-pollinated; of the resulting G4 generation, 43 individuals were sprayed with Basta and all proved to be resistant. As expected, the negative controls died, while the positive controls were not affected by the Basta treatment.

4.5 Discussion

A strategy for establishing pure transgenic maize lines from heterozygous transgenic donor material by means of anther culture is presented. To demonstrate the inheritance and expression of a foreign gene in plants derived from the androgenic pathway, a herbicide resistance marker was used.

The androgenic response was shown to be a polygenic heritable trait. Cowen et al. (1992), Murigneux et al. (1994), Beaumont et al. (1995) and Marhic et al. (1998) presented evidence of the existence of diverse chromosomal segments associated with responsiveness to *in vitro*

androgenesis, regeneration efficiency and the establishment of DH lines. While ETH-M82 is highly androgenic and the genotypes 116.1 and 109.2 do not show androgenic response, their crosses exhibit an intermediate responsiveness (Table 4.1). The high degree of variation in the embryo yield and regeneration frequency within repetitions (Table 4.1) is due to the segregation of traits influencing the androgenic response in the G1 generation. Despite the considerable loss in efficiency compared to ETH-M82, the frequency of embryo induction, plant regeneration and establishment of DH lines were sufficient to obtain four independent lines. Further optimisation of the plant regeneration procedure would certainly lead to a higher yield of fertile DH lines.

As detected by Southern hybridisation, the parental genotype 109.2 contained at least two copies of the *pat* gene. The progenies in the G1 generation showed a 1:1 segregation rate of herbicide-resistant and herbicide-sensitive individuals, as was expected for a single heterozygous dominant trait, indicating a close link between these copies. The original hybridisation pattern of 109.2 was preserved in all the transgenic individuals throughout the tested generations. The integration of the transgene was strictly associated with the Basta-resistant phenotype. On the other hand, non-transgenic plants, which exhibited herbicide resistance ("escapes"), were not observed. This clearly demonstrates that the passage along the androgenic pathway did not cause any detectable rearrangements of the transgene at the DNA level; the expression of the transgenic phenotype was unaffected.

Since exclusively heterozygous herbicide-resistant plants were chosen as anther donors, a 1:1 segregation between resistant and sensitive individuals of the G2 generation was expected. However, only 25 % of the 55 tested regenerants inherited the *pat* gene, although G1 plants segregated in a Mendelian manner. This phenomenon can be explained by the fact that gametes containing a higher proportion of genes belonging to the androgenic, rather than to the non-androgenic genotype carrying the *pat* gene, will preferentially undergo the androgenic process, resulting in a biased formation of DH regenerants containing a low proportion of the genes (including the *pat* gene) of the transgenic line.

Because of the generally weak performance of *in vitro* regenerated plants and the resulting low kernel yield of self-pollinated G2 plants, it was impossible to asses the uniformity of the G3 generation with regard to herbicide resistance. In order to prove the homozygosity of the G2 generation, the G3' generation was generated by pollination of wild-type material by the G2 plants. The lack of segregation in the G3' generation with respect to herbicide resistance confirmed the occurrence of correct chromosome doubling.

Four lines with uniform kernel colour were established by self-pollination of G3 individuals (G4 generation), but only one of the lines was herbicide-resistant. The purity of the transgenic DH line was further confirmed by spraying it with Basta.

Despite the presence of multiple transgene copies and the fully homozygous nature of the transgenic DH line, there was no indication of the occurrence of "repeat-induced" (Matzke and Matzke, 1998; Jakowitsch et al., 1999) or "homology-dependent" (Vaucheret et al., 1998; Fagard and Vaucheret, 2000) transgene silencing.

The application of the DH technique to establish a homozygous transgenic line did not result in adverse effects on the DNA structure or transgene expression and may, thus, be considered to be an alternative to inbreeding via the sexual pathway.

Although the yield of DH lines is too low for the routine application of anther culture, for example, for the generation of large numbers of recombinant inbred lines, this technique may be advantageous in cases when the rapid fixation of a transgene is required. Androgenic material could be introgressed into the target material before the transformation is carried out. In order to save time, transgenic material could be crossed out to androgenic lines right after regeneration.

Conclusions & Outlook

Conclusions

The aim of this doctoral work was to develop a method for the rapid attainment of transgenic and homozygous maize lines, a goal which meets the requirements of plant breeders as well as of basic researchers. An alternative to the conventional method of transformation of heterozygous material and further inbreeding or back-crossing to a line of interest was considered, i.e. to transfer the transgene to microspores or gametic embryos. The method of microspore transformation was successfully applied to several species such as wheat, rice, barley, oil seed rape and tobacco. Folling and Olesen (2001) reported much higher rates of transformation when the transgene was delivered into wheat gametic embryos rather than to microspores. Despite continuous efforts to establish a protocol for the transformation of maize microspores for more than a decade (Fennel and Hauptmann, 1992), no success was reported thus far. The literature does not provide information about whether the development of a protocol for the transformation of maize gametic embryos has ever been attempted.

The studies performed during the coarse of this doctoral work dealt with: *i)* the improvement of the existing protocol for androgenesis via isolated microspore and the performance of gametic embryos with regard to the rate of secondary embryogenesis and the efficiency of plant regeneration (Chapter 1), *ii*) the analysis of the homozygous nature of DH lines (Chapter 2), *iii)* the analysis of the transient and stable expression of the transgene in the target materials (microspores and gametic embryos, as well as zygotic embryos of a transformable genotype as a positive control), as well as their response to the selection method based on the resistance to the herbicide Basta conferred by the *pat* transgene (Chapter 3) and *iv)* the rapid fixation of a transgene contained in a heterozygous, non-androgenic material, in a homozygous background. This was achieved by means of anther culture performed from the transgenic offspring of the cross between the heterozygous transgenic genotype and an androgenic genotype (Chapter 4).

The results of the experiments described in Chapters 1 and 3 show that, whereas the microspores of the genotypes ETH-M24, ETH-M72 and ETH-M82 are unsuitable for

transformation because of their very low androgenic response and the lack of transient transgene expression, the gametic embryos of these genotypes could be considered as targets for transformation. They were responsive to the induction of secondary embryogenesis, plant regeneration from secondary embryos and the transient expression of the transgene after bombardment. These results indicate that the generation of transgenic and homozygous plants by means of the biolistic transformation of gametic embryos can potentially be achieved. However, the zygotic control was much more efficient with regard to all the studied factors.

The basis of further experiments on the transformation of gametic embryos should be the establishment of Type II rather than Type I callus. The Type II callus proliferates at a higher rate, is softer and can retain the ability to regenerate into a plant for much longer periods than Type I. These factors would enable the bombardment after the induction of secondary embryogenesis, when the callus is softer, which may lead to a higher efficiency of transgene delivery and, therefore, to a higher density of transformed cells. Furthermore, the higher rate of cell proliferation could contribute to the faster formation of transgenic secondary structures capable of competing out non-transformed tissue, ideally in combination with a positive selection mechanism that does not lead to toxicity in the environment of the transgenic cells. Furthermore, more transgenic plants could be regenerated from an initial target explant.

This strategy could, on the other hand, lead to the accumulation of somaclonal variations. As discussed in Chapter 2, the application of 2,4-D to the gametic embryos for four weeks did not lead to a notable accumulation of mutations at a chromosomal level. Nevertheless, a longer period of cultivation may lead to variations and, therefore, alter the homozygosity of the DH lines. In addition to the variability triggered by the *in vitro* culture, Bregitzer et al. (1998) reported a second source of somaclonal variation in transgenic barley lines, resulting from the particle bombardment. The typical steps of the biolistic transformation, such as the vacuum, the bombardment itself and the selection mechanism, the latter of which leads to a change in pH and the release of secondary metabolites by the dying cells, can trigger mutations.

The plant material used for further experiments on the transformation of gametic embryos should combine both a high androgenic response with the ability to generate Type II callus. Since both features are extremely genotype-dependent (Petolino and Jones, 1986 and Carvalho et al., 1997, respectively), achieving a material which combines the desired characteristics would probably demand a long-term breeding program, which exceeds the scope of this doctoral work.

The method of establishing a transgenic DH line described in Chapter 4 showed that the transgene was diploidised correctly and that alterations with regard to its expression due to transgene silencing did not occur. Nevertheless, the efficiency of the system was extremely low and there is the necessity of crossing the transgenic line with an androgenic line in order to achieve a sufficient number of double haploid regenerants. It is also to be expected that the recombinant lines extracted from the transgenic anther culture donor plants will exhibit segregation distortions in favour of the androgenic parent, since androgenesis and regeneration are traits controlled by a large number of genes dispersed along the whole genome (Marhic et al., 1998). This technique would be useful for studies on transgene expression but not for breeding purposes; the former option would be valid only in the event that the technique is optimised by paying special attention to the process of the plantlet transfer to the soil, which was shown to be the principal cause of material loss. Indeed, material derived from the DH technique has been registered for rice, barley, wheat, tobacco and oilseed rape (reviewed by Niemirowicz-Szezytt, 1997) but not for maize.

In summary, the key processes that hampered the establishment of an efficient protocol for the generation of transgenic double haploid maize lines have been clearly identified. The steps that must be taken to reach the goal are *i)* bombarding Type II callus derived from gametic embryos, *ii)* using a positive selection mechanism and *iii)* improving the survival rate of DH plantlets when transferred to soil. However, from all the work published so far and the own experience, it is concluded that the suggested strategy of combining genetic transformation and *in vitro* androgenesis is unsuitable for breeding programs or basic research, mainly due to the very low

efficiency of the system, the extremely high genotype dependency of the androgenic process and the presence of segregation distortions in the progeny of lines derived from microspores.

Given the technologies of marker assisted breeding, it is much more efficient to follow the conventional, well-established strategy of directly transforming the standard material (e.g. H99 x A188) and performing a marker assisted back-crossing. In that way, time can be saved and the transgene can be introgressed into any material of the desired agronomical, nutritional or industrial characteristics.

The transformation by particle bombardment of immature zygotic embryos of some inbred lines is also feasible, although the transformation efficiency is, in general, much lower for a homozygous than for a hybrid target material.

Outlook

Another alternative for the rapid attainment of homozygous and transgenic lines, in line with the original idea of this doctoral work, is the combination of the *in vivo* haploid induction system and genetic transformation:

In vivo haploid inducer lines were first reported at the end of the 1940s by Chase (1949). These lines, if used as pollen donors, induce haploids from the male gamete, the unfertilised egg or other cells of the embryo sac. Depending on the origin of the haploid embryo, these lines can be classified as *in vivo* maternal or *in vivo* paternal inducer lines. The most commonly used *in vivo* maternal inducer lines belong to the strain "Stock 6" (Coe, 1959). A new maternal haploid inducer line (MHI, for Moldovian Haploid Inducer) has been characterised recently. This line shows efficiencies of up to 8 % for the generation of haploid individuals (Eder and Chalyk, 2002). A paternal haploid inducer line containing the *ig* gene (for indeterminate genotype) was described by Kermicle in 1969. Generally, the induction can be applied successfully to dent, flint or flint x dent maternal material. In general, higher levels of induction are obtained through *in vivo* maternal than *in vivo* paternal haploid inducer lines.

It is clear that the *in vivo* haploid induction has important advantages over the *in vitro* haploid induction, since:

i) the system is genotype-independent, so that DH lines can be extracted from any genetic pool of special interest,

ii) an *in vitro* culture phase, which may induce somaclonal variations and alter, therefore, the homozygosity of the lines, is unnecessary and

iii) the output of fertile lines is very high and therefore applicable to extensive breeding programs and genomic mapping (T. Presterl, University Hohenheim, Germany, personal communication).

The existence of these lines opens the possibility of approaching the generation of homozygous and transgenic lines in a more realistic way; it is assumed that the haploid or double haploid zygotic embryos, which were generated after pollination of individuals belonging to a population of interest with an *in vivo* maternal haploid inducer line, could be used as target for biolistic transformation according to the efficient protocol of Brettschneider et al. (1997). In that way, transgenic double haploid lines, segregating from the original population, could be established.

Alternatively, material corresponding to an interesting genetic pool could be transformed in a first step to establish a transgenic population. The transgenic lines could be extracted from the population by means of the *in vivo* haploid induction system. This system would have advantages over the first suggested system in that the somaclonal variation, which may be triggered by the transformation process itself (Bregitzer et al., 1998), would be eliminated by the subsequent haploidisation process. Time and labour would probably be reduced, too, because only a small number of transformation events on the maternal heterozygous material would be necessary.

Appendix

Appendix

Culture media

- Anther culture

Components (mg/l)	IML	IMSS
KNO_3	2 500	2 500
NH_4NO_3	165	165
$CaCl_2$ x 2 H_2O	176	176
KH_2PO_4	510	510
$MgSO_4$ x 7 H_2O	370	370
$MnSO_4$ x 1 H_2O	4.40	4.40
$ZnSO_4$ x 7 H_2O	1.50	1.50
H_3BO_3	1.60	1.60
KJ	0.80	0.80
Na_2EDTA x 2 H_2O	41.00	41.00
$FeSO_4$ x 7 H_2O	27.80	27.80
Thiamine HCl	0.25	0.25
Nicotinic acid	1.30	1.30
L-proline	125	125
L-glutamine	150	150
L-asparagine	15	15
TIBA (Triiodobenzoic acid)	0.10	0.10
Activated charcoal	5 000	5 000
Sucrose	90 000	90 000
Colchicine	250	-
Phytagel	-	1 500

The pH of the IML was adjusted at 5.8. Twenty-four hours after autoclaving, the activated charcoal was filtered out and the medium was sterile-filtered (Millipore Sterivex GS filter, 0.22 µm). In the case of the IMSS (pH 5.8), the activated charcoal remained in the medium after autoclaving; the medium was dispensed in petri-dishes of a 6 cm diameter.

- Microspore culture

Components (mg/l)	**Pre-culture medium**	**IMM (maltose)** **IMS (sucrose)**
Sucrose or maltose	-	60 000
D-mannitol	109 300	-
KNO_3	-	2 500
$(NH_4)NO_3$	-	165
$CaCl_2*2H_2O$	-	176
KH_2PO_4	-	510
$MgSO_4*7H_2O$	-	370
$MnSO_4*4H_2O$	-	4.40
$ZnSO_4*7H_2O$	-	1.50
H_3BO_3	-	1.60
KJ	-	0.80
Na_2EDTA	-	41
$FeSO_4*2H_2O$	-	27.80
Thiamine HCl	-	0.25
Nicotinic acid	-	1.30
L-ascorbic acid	50	-
TIBA	-	0.10
L-proline	125	125
L-asparagine	-	15
L-glutamine	-	125

The pH of both media was adjusted to 5.8; the media were sterile-filtered (Millipore Sterivex GS filter, 0.22 µm).

- Secondary embryogenesis

Modified N6 medium

Components	**mg/l**
$(NH_4)_2SO_4$	463
KNO_3	2 500
KH_2PO_4	510
$MgSO_4$ x 7 H_2O	370
$CaCl_2$ x 2 H_2O	176
$MnSO_4$ x 1 H_2O	3.87
$ZnSO_4$ x 7 H_2O	1.50
H_3BO_3	1.60
KJ	0.80
Na_2EDTA x 2 H_2O	37.3
$FeSO_4$ x 7 H_2O	27.8
Glycine	2.00
Thiamine HCl	1.00
Pyridoxine-HCl	0.50
Nicotinic acid	0.50
Caseinhydrolysate	100
Inositol	100
L-proline	2 900
$AgNO_3$	1.7
Sucrose	20 000
2,4-D	1
Phytagel	6 000

The pH of the medium was adjusted to 5.8. After autoclaving, the medium was dispensed in petri-dishes of a 10 cm diameter.

Hyper-Osmotic Modified N6 medium

Same as Modified N6 medium but supplemented with 0.7 M sucrose.

Selective Modified N6 medium

Same as Modified N6 medium but supplemented with 3 mg/l of Basta (containing 250 mg/ml of the active compound bialaphos, Bayer CropScience, Germany).

SSR primers (from The Maize Database, USDA)

Primers tested for polymorphism on the parental lines

Primer	bin	Primer	bin	Primer	bin
bnlg1012	9.05-06	bnlg 1017	2.02	bnlg 1019	4.09-10
bnlg1022	3.05	bnlg1028	10.05-06	bnlg1031	8.06
bnlg1043	6.00	bnlg1063	5.03	bnlg1092	2.01
bnlg1108	3.08	bnlg1112	1.01	bnlg1117	3.05
bnlg1118	5.07	bnlg1162	4.03	bnlg1184	2.06
bnlg1188	6.01	bnlg121	2.06	bnlg1217	4.05
bnlg1257	3.09	bnlg128	9.07	bnlg1337	4.11
bnlg1338	2.01	bnlg1347	1.10	bnlg1372	9.02
bnlg1447	3.04	bnlg1452	3.04	bnlg1496	3.09
bnlg1502	1.09	bnlg1518	10.04	bnlg1564	1.07
bnlg161	6.00	bnlg1617	6.05	bnlg1627	1.02
bnlg166	2.04	bnlg1671	1.10	bnlg1677	10.10; 10.06-07
bnlg1695	5.07	bnlg1712	10.03	bnlg1721	2.08
bnlg1732	6.05	bnlg1740	6.07	bnlg1741	4.06
bnlg1754	3.09	bnlg1782	8.05-06	bnlg1796	3.06
bnlg1798	3.06	bnlg1811	1.04	bnlg1823	8.07
bnlg1863	8.04	bnlg1867	6.01	bnlg1884	9.05
bnlg1886	1.05	bnlg1922	6.05	bnlg1951	3.07
bnlg2046	8.05	bnlg2082	8.03	bnlg2160	7.01
bnlg2248	2.03	bnlg2259	7.04	bnlg2328	7.05
bnlg244	9.02	bnlg249	6.01	bnlg252	4.06
bnlg278	5.04-05	bnlg372	4.00	bnlg386	5.09
bnlg565	5.02	dupssr12	1.08	dupssr13	7.04
dupssr14	8.09	dupssr21	2.05	mmc0022	3.00; 3.04-05
mmc0041	1.08	mmc0151	5.00	mmc0181	8.06-07
nc004	4.03	phi019	4.11	phi026	4.05
phi027	9.03	phi028	9.01	phi033	9.01
phi041	10.00	phi061	9.03	phi072	4.00-01
phi087	5.06	phi096	4.04	phi115	8.03
phi117	10.00	umc1001	7.03-04	umc1005	8.08-09
umc1033	9.02	umc1061	10.06	umc1084	10.07
umc1123	1.06	umc1128	1.07	umc1300	3.04-05

Primer sets selected for the analysis of the DH lines

Ch. 1	**Ch. 2**	**Ch. 3**	**Ch. 4**	**Ch. 5**
bnlg1112	bnlg1338	bnlg1447	bnlg1217	bnlg565
bnlg1564	bnlg2248	bnlg1798	bnlg1337	bnlg1695
Ch. 6	**Ch. 7**	**Ch. 8**	**Ch. 9**	**Ch. 10**
bnlg249	dupssr13	mmc0181	phi028	bnlg1028
bnlg1732	-	bnlg1863	bnlg1863	umc1084

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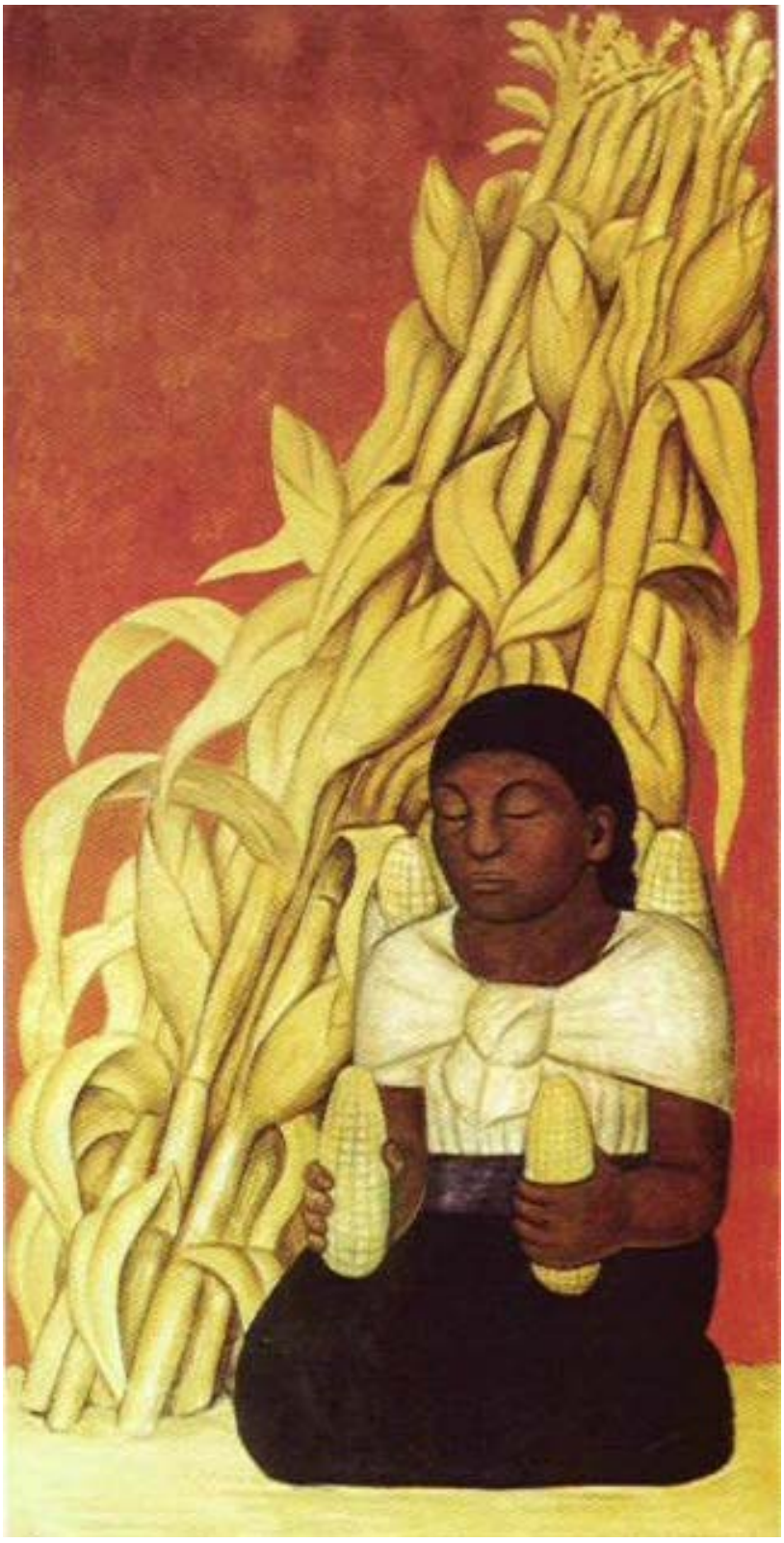

Diego Rivera

Mujer indígena con tallos de maíz