Advances in Haploid Production in Higher Plants

Alisher Touraev • Brian P. Forster • S. Mohan Jain
Editors

Advances in Haploid Production in Higher Plants

Editors
Dr. Alisher Touraev
Max F. Perutz Laboratories
University of Vienna
Dr Bohrgasse 9/4
1030 Vienna
Austria

Dr. S. Mohan Jain
University of Helsinki
Department of Applied Biology
FI-00014 Helsinki
Latokartanonkaari 5
Finland

Dr. Brian P. Forster
BioHybrids International Ltd.
Earley, Reading
RG6 5FY United Kingdom

ISBN 978-1-4020-8853-7 e-ISBN 978-1-4020-8854-4

Library of Congress Control Number: 2008831064

© 2009 Springer Science + Business Media B.V.
No part of this work may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording or otherwise, without written permission from the Publisher, with the exception of any material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work.

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

Preface

The importance of haploids is well known to geneticists and plant breeders. The discovery of anther-derived haploid *Datura* plants in 1964 initiated great excitement in the plant breeding and genetics communities as it offered shortcuts in producing highly desirable homozygous plants. Unfortunately, the expected revolution was slow to materialise due to problems in extending methods to other species, including genotypic dependence, recalcitrance, slow development of tissue culture technologies and a lack of knowledge of the underlying processes. Recent years have witnessed great strides in the research and application of haploids in higher plants. After a lull in activities, drivers for the resurgence have been: (1) development of effective tissue culture protocols, (2) identification of genes controlling embryogenesis, and (3) large scale and wide spread commercial up-take in plant breeding and plant biotechnology arenas.

The first major international symposium on "Haploids in Higher Plants" took place in Guelph, Canada in 1974. At that time there was much excitement about the potential benefits, but in his opening address Sir Ralph Riley offered the following words of caution: "*I believe that it is quite likely that haploid research will contribute cultivars to agriculture in several crops in the future. However, the more extreme claims of the enthusiasts for haploid breeding must be treated with proper caution. Plant breeding is subject from time to time to sweeping claims from enthusiastic proponents of new procedures. Mention may be made of induced mutations and induced polyploids. The new techniques usually put an additional weapon in the armoury of the breeder but they rarely provide the total defence initially suggested. Let us study and consider haploids fully but let us be cautious and not take an unrealistic view of the contributions haploids can make to plant breeding real though I expect these to be*." It has taken several years to "study and consider haploids" to a point where these concerns can be addressed and understood, but thanks to advances in recent years we are now at this point.

Today there are published tissue culture protocols in over 200 plant species, ranging from *Aconitum* to *Zingiber*. These have been collated in the book, "Doubled Haploid production in crop plants: a manual" (Maluszynski et al. 2003). This manual is a valuable contribution to the practical application of our topic and we do

not wish to repeat a list of protocols here. Rather we would like to complement the manual by emphasizing the scientific basis of haploid and doubled haploid production in higher plants and to showcase the impact in plant breeding and biotechnology. Similarly we have striven not to duplicate the contents of the recently published book: "Haploids in crop improvement II", Springer (Palmer et al. 2005), which contains information on various aspects of haploidy technology and limitations of haploidy utilisation in breeding programmes. Here we have focused on major advances in understanding, such as the genetic control of embryogenesis. Success in identifying the controlling genes has propelled further investigations in functional genomics in unravelling the mechanisms involved especially in respect to regulation and their manipulation. These studies are now linking in with gene expression, metabolism studies as well as changes in cell ultra-structure. The take up of doubled haploidy by plant breeders has been no less impressive. Doubled haploidy provides the fastest route to the production of pure breeding lines and the coupling of doubled haploidy with genetic markers provides a rapid and commercially attractive means of cultivar production. Doubled haploidy has now become an essential biotechnology in the development of most major crop species and many vegetables, and is rapidly expanding into other species, particularly those of high value, e.g. medicinal, aromatic and ornamental plants. The widespread activity in haploid research has also resulted in unexpected findings and novel applications, which have prompted studies in scientific understanding and patent claims to protect and capitalise on innovative applications.

In February 2006 Vienna hosted a major international symposium on "Haploidy in Higher Plants III", which in itself marked the renaissance in haploidy and doubled haploidy. The symposium attracted top experts from around the world and it is their work plus invited contributions that has formed the basis of this book.

The book is composed of five sections. In the first, 'Technology improvement' reviews are provided that describe advances in the various methods used to produce haploids both generally and specifically for model and major crop species. In the past, many breakthroughs in haploid and doubled haploid production have been achieved through empirical methods. Today the emerging disciplines of functional genomics, proteomics and metabalomics along with increasingly sophisticated methods in cell biology are fuelling the drive for a more scientific basis aimed at understanding the biological processes involved; these approaches are described in section 2 (Biological mechanisms). The third section, 'Breeding and genetics' covers the application of the haploid/doubled haploid technologies to what is traditionally considered to be the primary end users – plant geneticists and plant breeders. In section 4, the expansion of these technologies is aptly demonstrated in 'New species'. Finally, Novel applications are considered.

We, the editors, have striven to provide a current and balanced view of the science and application of haploidy/doubled haploidy in plants, in what is a topic undergoing rapid advancement by scientist and take up by end users. The book, although confined to a relatively restricted topic, has widespread appeal ranging from cell biologists, embryologists, research in plant genetics, biotechnology and genetic engineering, and molecular biologists and with considerable commercial end user relevance.

References

Maluszynski et al. (2003). Doubled haploid production in crop plants: a manual. Kluwer, Dordrecht

Palmer CE et al. (2005). Haploids in crop improvement II", Springer, Berlin

Riley R. (1974). The status of haploid research. In: Kasha K.J. (ed.), Proceeding of the First International Symposium on Haploids in Higher Plants: Advances and Potential. Univ. Guelph, Guelph, Canada, pp 3–9

Contents

Preface v

1 Progress in Doubled Haploid Technology in Higher Plants 1
M. Wędzony, B.P. Forster, I. Żur, E. Golemiec, M. Szechyńska-Hebda, E. Dubas, and G. Gołębiowska

2 Doubled Haploids *via* Gynogenesis 35
B. Bohanec

3 Overview of Barley Doubled Haploid Production 47
P. Devaux and K.J. Kasha

4 Production of Doubled Haploids in *Brassica* 65
J. Gil-Humanes and F. Barro

5 An Overview on Tobacco Doubled Haploids 75
K. Belogradova, I. Lewicka, E. Heberle-Bors, and A. Touraev

6 An Overview of Triticale Doubled Haploids 87
F. Eudes and A. Chugh

7 Patents and Haploid Plants 97
J.M. Dunwell

8 Gene Expression Profiling of Microspore Embryogenesis in *Brassica napus* 115
M.R. Malik and J.E. Krochko

9 Expression Profiles in Barley Microspore Embryogenesis 127
M. Muñoz-Amatriaín, J.T. Svensson, A.M. Castillo, L. Cistué, T.J. Close, and M.P. Vallés

10 Proteomics in Rapeseed Microspore Embryogenesis 135
J. Cordewener, F. van der Wal, R. Joosen, K. Boutilier, and T. America

11 Programmed Cell Death and Microspore Embryogenesis 147
A.L. Varnier, C. Jacquard, and C. Clément

12 Albinism in Microspore Culture 155
A.M. Torp and S.B. Andersen

13 Doubled Haploids in Breeding Winter Oilseed Rape........................ 161
C. Möllers and M.C.M. Iqbal

14 Anther Culture Derived Doubled Haploids in Oat 171
E.M. Kiviharju

15 Barley and Wheat Doubled Haploids in Breeding 179
J. Weyen

16 Rice Doubled Haploids and Breeding.. 189
J. Pauk, M. Jancsó, and I. Simon-Kiss

17 Potato Haploids and Breeding.. 199
V.-M. Rokka

18 Current Status of Doubled Haploids in Medicinal Plants................ 209
A.M.R. Ferrie

19 Microspore Embryogenesis in Selected Medicinal and Ornamental Species of the *Asteraceae* 219
U. Bal and A. Touraev

20 Carrot Doubled Haploids.. 231
K. Górecka, D. Krzyżanowska, W. Kiszczak, U. Kowalska, and R. Górecki

21 Haploids and Doubled Haploids in Fruit Trees................................. 241
M.A. Germanà

22 Haploidy in Tef.. 265
L. Gugsa and J. Kumlehn

23 A Novel and Reversible Male Sterility System Using Targeted Inactivation of Glutamine Synthetase and Doubled Haploidy.......... 285
A. Ribarits, A.N.K. Mamun, S. Li, T. Resch, M. Fiers, E. Heberle-Bors, C.-M. Liu, and A. Touraev

24 Embryogenic Pollen Culture: A Promising Target for Genetic Transformation 295
J. Kumlehn

25 Immature Pollen as a Target for Gene Targeting 307
T. Resch, E. Ankele, R. Badur, B. Reiss, E. Herberle-Bors, and A. Touraev

26 Induction of Semi-Dwarf, Salt Tolerant Rice Mutants from a Tall Salt Tolerant *Indica* Landrace 319
R. Afza, M. Guzmann, F.J. Zapata, E. Tumimbang-Raiz, G. Gregorio, B.P. Forster, and C. Mba

27 Chromosome Doubling in Monocots 329
A.M. Castillo, L. Cistué, M.P. Vallés, and M. Soriano

28 Tracking Gene and Protein Expression During Microspore Embryogenesis by Confocal Laser Scanning Microscopy 339
P.S. Testillano and M.C. Risueño

Chapter 1
Progress in Doubled Haploid Technology in Higher Plants

M. Wędzony, B.P. Forster, I. Żur, E. Golemiec, M. Szechyńska-Hebda, E. Dubas, and G. Gotębiowska

Abstract In the early 1990s, many basic protocols were developed for haploidy and doubled haploidy, but most were inefficient. During the last decade, progress in technology has been achieved mainly by empirical, time and cost consuming testing of protocols; as a consequence success was proportional to the number of laboratories involved. In the most frequently studied crops (barley, wheat, triticale, maize, rice and rapeseed) improved protocols are now used routinely in breeding and although several problems remain the benefits make doubled haploidy well worthwhile. Significant advances have also been achieved in vegetable, fruit, ornamental, woody and medicinal species, though responses in many remain low with legumes being particularly recalcitrant. There has been resurgence in doubled haploids over the last few years with protocols published for almost 200 plant species. The present review aims to show the recent progress in haploid and doubled haploid technology of higher plants.

Keywords Technology, androgenesis, gynogenesis, media composition, wide crossing, regeneration, albinism

Introduction

Doubled haploidy is the fastest route to homozygosity in plants. The core motivation for developing broadly applicable protocols to raise doubled haploids (DHs) are at least three fold: (1) DHs are useful in fixing traits rapidly in desirable combinations

---

M. Wędzony(✉)
Institute of Plant Physiology Polish Academy of Sciences, Niezapominajek 21, 30-239, Kraków, Poland and Pedagogical University of Kraków, Pochorążych 2, 30-084, Kraków, Poland
E-mail: niwedzon@cyf-kr.edu.pl

B.P. Forster
Biohybrids International Ltd, Po Box, 2411, Earley Reading RG6 5FY, UK

I. Żur, E. Golemiec, M. Szechyńska-Hebda, E. Dubas, and G. Gotębiowska
Institute of Plant Physiology Polish Academy of Sciences, Niezapominajek 21, 30-239, Kraków, Poland

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

in a line/variety; (2) DHs facilitate hybrid breeding, (3) DHs applied in marker studies speed up the development of mapping populations and marker/trait associations. Breeders can evaluate DH lines with more speed, accuracy and confidence, especially in respect to quantitatively inherited traits such as yield and quality. Moreover, certain stages of double haploid cultures are useful as targets for inducing mutation and transformation. Androgenic structures induced from microspores in suspension make a perfect model to study plant cell cycle regulation, cell division and early embryogenesis. However, the advantages of DHs for breeding purposes can be fully exploited only when efficient technologies are available in relevant species and genotypes.

Brief History

In 1974 Riley provided an excellent review on 'The status of haploid research'. Here we present a synopsis of that article. The history of doubled haploids (DHs) began with the observation of haploid production in the higher plant, *Datura stramonium* L. (Jimson weed) reported by A.D. Bergner in 1921 (cited by Blakeslee et al. 1922). This was soon followed by similar discoveries in other species e.g. *Nicotiana tabacum* (Clausen and Mann 1924) and *Triticum compactum* (Gains and Aase 1926). The potential of haploids (and doubled haploids) in breeding and genetics was quickly realised and research was initiated to identify methods, limiting factors and ways of improving the frequency of haploid production (reviewed by Kimber and Riley 1963). A wide range of methods was identified including parthenogenesis, pollen irradiation, selecting seed with twin embryos, sparse pollination, alien cytoplasm, wide hybridisation and use of certain genetic stocks (see Kasha and Maluszynski 2003). Chase (1952) is recognised as being among the first to incorporated haploids in a breeding programme, in this case by inducing haploids in maize via parthenogenesis and then doubling their chromosome complement to produce doubled haploids. A major breakthrough came with the discovery that haploid plants could be generated from cultured anthers of *Datura* (Guha and Maheshwari 1964, 1966). The method was tested and became established in many species, but with little knowledge of the underlying mechanisms involved. Today anther culture and its derivative technique, isolated microspore culture are preferred methods, though wide crossing and ovule culture are frequently deployed in producing haploids and doubled haploids. There has been resurgence in both technological innovations and applications of haploidy in higher plants (Forster et al. 2007) with published protocols for close to 200 species being studied (Maluszynski et al. 2003b).

Technologies to Obtain Doubled Haploids

Haploids (Hs) and doubled haploids (DHs) can be obtained from male or female gametic cells. For some monocots and potato both routes are accessible, in most dicotyledonous species the current choice is limited to just one.

In androgenesis sporophytic growth of the male gametic cell, the microspore, is induced. This requires the complete re-programming of the developmental plan, to

embryo rather than pollen grain formation. The microspore is originally programmed to produce pollen consisting of two cells: one vegetative, one generative. Eventually the generative cell gives rise to two male gametes (sperm) during pollen maturation or later, during pollen germination on the stigma. Each pollen cell is highly specialised and performs specific biological function thus, the induction of sporophytic development is only possible at early developmental stages, when the gametic cell appears to be totipotent (Touraev et al. 2001). The microspores are the first generation of male gametic cells and have a haploid chromosome complement, and in certain conditions and in many species, they are amenable to androgenic induction. Microspores are produced in plant anthers in large numbers thus, they are relatively easy to access and manipulate.

In gynogenesis, sporophytic development is induced in the unfertilised egg cell (female gamete). An advantage here is that re-programming is not required; the embryogenic pathway just needs to be switched on without proper fertilisation. To some extent, gynogenesis is similar to parthenogenesis found in nature during apomixes. Gynogenesis is usually not very efficient, partly because plants produce far fewer egg cells than pollen grains, but it is deployed in species where androgenic methods are not effective.

Haploid plants can also be obtained in some genera by “wide crossing”. This involves pollination with a genetically distant male partner or a pollinator of the same genus having special genetic properties, e.g. “haploid inducing genes”. In this technique, fertilisation usually takes place but male genetic material is eliminated from cells of the developing embryo at early stages of its growth. Since the endosperm fails to develop properly, the resulting haploid embryos originating from the female partner in the cross needs to be rescued and cultured *in vitro* to produce haploid plants. Wide crossing is a common technique in cereal and potato breeding, but there is the potential to develop the technique in other species.

Progress in Androgenesis

Androgenesis is the most deeply studied and the most widely effective technology deployed in obtaining DHs. Most methods recommend two vital *in vitro* steps: (1) the induction of the androgenic process, and (2) the regeneration of H/DH plants. The induction phase is often preceded by pre-treatments of plants, inflorescences/flowers or anthers. The regeneration phase is sometimes followed by an *in vitro* rooting phase. Finally, the plantlets obtained are acclimated to the *ex vitro* conditions. In most cases, the chromosome constitution of haploid plants needs to be doubled by treatment with chemicals, in some species however, “spontaneous duplication” of chromosomes occurs during the culture at a sufficiently high rate for practical purposes.

The development of a microspore to a plant can be direct i.e. through formation of an androgenic embryo; often described in the literature as “an embryo-like structure” (ELS). In appropriate culture conditions, ELSs germinate directly into plants. Induced microspores can also form callus tissue that regenerate plants via somatic embryos or shoots. Often all the developmental pathways co-exist in the same culture and their proportion depends on genotype and culture condition used.

Based on the technology used, the androgenic methods are divided into anther culture or isolated microspore culture. For the latter, two alternative methods of microspore isolation are used. Flowers or inflorescences at the optimal developmental stage are cut by hand or blended, microspores are then separated from the debris by a series of washes, sieving and/or centrifugation steps (Zaki and Dickinson 1990; Pechan et al. 1991; Hu and Kasha 1997). Anthers can also be isolated manually from flowers and placed in liquid medium containing mannitol where the microspores are spontaneously released into the medium and then filtered or separated by centrifugation and re-suspended in the induction medium (Touraev et al. 1996a, 1997). In cereals, both isolation methods appear to give satisfactory results according to the authors. In the "shed microspore culture" protocols anthers are initially cultured intact on a liquid or semi-liquid media then the microspores shed into the medium and these are cultured along with the remaining anthers and anther debris. Proven practical methods used on a large scale are described in detail in Maluszynski et al. (2003a).

Androgenesis is routinely applied in breeding programmes to important crops such as cereals and rapeseed. Table 1 lists selected papers describing some important steps in technology improvement in species most frequently studied since 1990. Protocols have also been developed for many other species including trees and ornamentals as reviewed in "Doubled haploid production in crop plants – A manual" edited by Maluszynski et al. (2003a). In the latter book, successful protocols are described in detail for many species and a large reference list is provided.

Tobacco (*Nicotiana tabacum*) is the oldest model species in the study of androgenesis. Its history started in the early 1970s as reviewed by Heberle-Bors et al. (1996). The role of temperature and other environmental factors in androgenic induction (Duckan and Heberle-Bors 1976; Heberle-Bors and Reinert 1981) and the identification of the most appropriate stage of microspore development suitable for androgenic induction (Heberle-Bors and Reinert 1979) were among the first topics to be investigated. Tobacco was also the subject of pioneering work on the genotypic control of androgenesis (Heberle-Bors 1984) and pre-treatment effects such as temperature shock, starvation and/or other stress treatments (Zarsky et al. 1992, 1995; Touraev et al. 1996b).

More recently, the spring variety of rapeseed (*Brassica napus*) cv. 'Topas' became a model genotype for dicotyledonous androgenesis, especially in the area of basic biological studies on cell division and early stages of embryogenesis (Binarova et al. 1993, 1997; Hause et al. 1993, 1994; Custers et al. 1994, 1999, 2001). The results obtained in this species are especially important since data can be compared to the genome and proteome of *Arabidopsis thaliana* (even though no data on successful androgenic process are available for *Arabidopsis*). Promising technology developments were obtained for a number of related *Brassica* species (see Table 1), and *Brassica napus* was the first species to be represented by a DH cultivar; Maris Haplona was released in the UK in the early 1970s (Thompson 1969, 1972).

Androgenesis in monocots has been extensively studied. Success in DH cultivar production occurred early for barley with the release of cv. Mingo in Canada in the mid 1970s (Ho and Jones 1980). Large amounts of exploratory studies were done on the model, and the highly responsive winter cultivar of barley (*Hordeum vulgare* L.) 'Igri' (Foroughi-Wehr et al. 1982; Olsen 1987; Cai et al. 1992; Ziauddin et al. 1990, 1992; Scott and Lyne, 1994; Evans and Batty 1994; Hoekstra et al. 1996, 1997;

Table 1 Androgenesis – selected papers which proved important for protocol improvements. AC – anther culture; MC – microspore culture

Species	Culture	Reference
Avena sp. Oat	AC	Kiviharju et al. (1997, 1998, 2000, 2004); Kiviharju and Pehu (1998); Kiviharju and Tauriainen (1999)
Brassica napus napus Rape seed oil, canola	AC	Wang et al. (1999)
	MC	Pechan et al. (1991); Zaki and Dickinson (1991); Binarova et al. (1993); Boutilier et al. (1994); Custers et al. (1994, 1999); Zhao and Simmonds (1995); Palmer et al. (1996); Ilic-Grubor et al. (1998); Sun et al. (1999); Wang et al. (1999); Zhou et al. (2002); Custers (2003)
Brassica rapa (syn. *B. campestris*)	MC	Ferrie et al. (1995); Ferrie (2003)
Brassica carinata Ethiopian mustard	MC	Barro and Martin (1999); Barro et al. (2001)
Brassica juncea Brown mustard	MC	Thiagarajah and Stringam (1993); Lionetton et al. (2001)
Brassica oleracea Cabbage, broccoli	MC	Duijs et al. (1992); Da Silva Dias (1999, 2001, 2003); Kuginuki et al. (1999); Rudolf et al. (1999); Wang et al. (1999)
Citrus ssp.	AC	Germana (1997)
Hordeum vulgare Barley	AC	Ziauindin et al. (1990, 1992); Cai et al. (1992); Evans and Batty (1994); Hoekstra et al. (1996, 1997); Cistue et al. (2003); Carreda et al. (2000); Castillo et al. (2000); Kasha et.al. (2001); Wojnarowiez et al. (2002); Jaquard et al., (2003); Szarejko (2003)
	MC	Mordhorst and Lörz (1993); Scot and Lyne (1994); Salmenkallio-Martilla et al. (1995); Scott et al. (1995); Kasha et al. (2001, 2003); Li and Devaux (2001); Davies (2003), Monostori et al. (2003); Kumlehn et al. (2005)
Linum usitatissimum Lineseed/flax	AC	Chen et al. (1998, 1999); Pretova and Obert (2000); Nichterlein et al. (1991); Nichterlein (2003); Obert et al. (2004)
	MC	Nichterlein and Friedt (1993); Bergmann and Friedt (1996)
Malus domestica Apple	AC	Höffer and Lespinasse (1996); Höffer, (2003, 2004)
	MC	Höffer (2003, 2004)
Oryza sativa Rice	AC	Harada et al. (1991, 1992); Alemanno and Guirderdoni (1994); Lentini et al. (1995); Xie et al. (1996); Pande and Bhojwani (1999); Bishnoi et al. (2000a, b); Trejo-Tapia et al. (2002); Zatapa and Arias, (2003)
	MC	Xie et al. (1996)
Quercus suber Oak	AC	Bueno et al. (2000, 2003); Raminez et al., (2004)
Populus nigra Poplar	AC	Andersen (2003)
	MC	Deutsch et al. (2004)
Prunus domestica & *P. armeniaca* Plum	AC	Peixe et al. (2000)

(continued)

Table 1 (continued)

Species	Culture	Reference
Solanum ssp.	AC	Jacobsen and Ramanna, (1994); Rokka et al. (1995, 1996); Shen and Veilleux, (1995); Aziz et al. (1999); Rokka (2003), Tai and Xiong, (2003)
Nicotiana tabacum Tobacco	AC	Heberle-Bors and Reinert (1979); Touraev and Heberle-Bors (2003)
	MC	Duckan and Heberle, (1976); Touraev and Heberle-Bors (1999, 2003); Kyo and Harada (1986); Touraev et al. (1996a, b); Garrido et al. (1995)
Triticum aestivum Wheat (hexaploid)	AC	Navarro-Alvarez et al. (1994); Ekiz and Konzak, (1997); Zhou and Konzak (1997); Orshinsky et al. (1990); Barnabaás et al. (2003b); Pauk et al. (2003a)
	MC	Gustafson et al. (1995); Touraev et al. (1996); Hu and Kasha (1997, 1999); Indrianto et al. (1999); Kunz et al. (1999); Reynolds (2000); Zheng et al. (2001, 2002a); Liu et al. (2002a, b); Darko et al. (2004)
Triticum durum Wheat (tetraploid)	AC	Dogramci-Altuntepe et al. (2001); Jauhar (2003)
xTriticosecale Triticale	AC	Karsai and Bedõ (1997); Ryöppy (1997); Immonen and Robinson (2000); Arzani and Darvey (2002); Tuvesson et al. (2003); Wedzony (2003)
	MC	Pauk et al. (2000, 2003a, b); Oleszczuk et al. (2004)
Secale cereale Rye	AC	Flehingous et al. (1991); Deimling and Flehinghous-Roux, (1996); Immonen and Antilla (1996, 1998, 1999, 2000); Rakoczy-Trojanowska et al. (1997); Immonen (1999)
	MC	Pulli and Guo (2003); Ma et al. (2004)
Zea mays Maize	AC	Delalonde and Coumans (1998); Barnabaás et al. (1999); Barnabaás (2003a)
	MC	Barnabaás et al. (1999); Naegeli et al. (1999); Obert et al. (2000); Zheng et al. (2003)

Kasha et al. 2001; Wojnarowiez et al. 2002). ‘Igri’ was also used as a reference in studying recalcitrant barley genotypes (Mordhorst and Lörz 1993; Cistué et al. 1994; Caredda et al. 2000). Recent results covering a wide range of barley genotypes were published, proving the successful practical applications (Castillo et al. 2000; Li and Devaux 2001; Jacquard et al. 2003; Kasha et al. 2001, 2003). Androgenic methods are now also economically feasible in the breeding of hexaploid wheat (*Triticum aestivum* L.) and triticale (x *Triticosecale* Wittm.) (Table 1). Androgenesis (anther and/or microspore culture) of rye (*Secale cereale* L.), durum wheat (*Triticum durum* L.), maize (*Zea mays* L.), oat (*Avena sativa* L.) and rice (*Oryza sativa* L.) can be also induced; however, on average at lower rates.

In many genotypes, DH lines are obtained at an efficiency rate too low for breeding purposes (for references see Table 1). Despite the enormous effort to date, there is no universal protocol effective in each species or every entry of the defined

Table 2 The results of published experiments of androgenesis induction in *Fabaceae*

Species	Authors	Method	Induction	Regeneration
Cicer arietimum	Croser et al. (2004, 2005)	MC	Yes	No
	Huda et al. (2001)	AC	Yes	No
	Mallikarjuna et al. (2005)	AC	Yes	No
Glicine max	Kaltchuk-Santos et al. (1997)	AC	Yes	No
	Cardoso et al. (2004)	AC	Yes	No
	De Moraes et al. (2004)	AC	Yes	Haploid plantlets
	Rodrigues et al. (2004)	AC	Yes	No
Lupinus albus	Ormerod and Caligari (1994)	AC	Yes	No
	Bayliss et al. (2004)	MC	Yes	No
Lupinus angustifolius	Bayliss et al. (2004)	MC	Yes	No
	Skrzypek et al. (2008)	AC	Yes	No
Lupinus polyphyllus	Sator et al. (1983)	AC	Yes	No
	Skrzypek et al. (2008)	AC	Yes	No
Medicago sativa	Zagorska and Dimitrov (1995)	AC	Yes	Shoots in cultivar Boyanista 5

AC- another culture; MC-microscope culture

species – success remains highly genotype dependent. This can be illustrated by the results of attempts to induce androgenesis in the *Fabaceae* family. Although several research groups have made an effort to develop effective procedures, the results are poor (Table 2). The recent intensification in efforts to produce DH methods is worth noting as it corresponds to the economic importance of the species and also to advances in micro-propagation methods.

Technology Improvements in Androgenesis

Several aspects of the androgenic process were studied in detail and helped contribute to the overall progress in developing DH technologies. These are: (1) the role of stress factors in androgenic induction, (2) the source of organic nitrogen in the media, (3) carbohydrate constituents in the culture media and (4) growth regulators. To date more attention has been paid to the induction phase than to the regeneration phase. The occurrence and frequency of chlorophyll deficient plants (albino plants) remains a serious problem in monocot androgenesis. Unfortunately, there appears to be little attention given to safe and efficient methods of chromosome doubling which normally involves the use and disposal of toxic chemicals such as colchicine.

Application of Abiotic Stress

Abiotic stresses play a very important role in androgenic induction as was first established for tobacco (Duckan and Heberle-Bors 1976; Heberle-Bors and Reinert

1981). Low or high temperature shocks are applied as a pre-treatment or at the early stages of induction in most protocols developed for both, mono- and dicotyledonous plants.

At the beginning of the 1990s in cereal androgenesis, treatment of spikes with low temperature of 4–7°C for a period of 3 or 4 weeks was regarded as a prerequisite for androgenic induction in barley (Olsen 1987; Ziauddin et al. 1990; Mordhorst and Lörz 1993; Scott and Lyne 1994; Evans and Batty 1994; Salmenkallio-Marttila et al. 1995). Cold pre-treatment of spikes for over 1 week was also applied to wheat (Gustafson et al. 1995; Ingram et al. 2000; Redha et al. 2000), triticale (Ślusarkiewicz-Jarzina and Ponitka 1997; Marciniak et al. 1998; Gonzalez and Jouve 2000; Immonen and Robinson 2000; Tuvesson et al. 2003; Węgzony et al. 2003) and rye (Immonen and Anttila 2000; Immonen and Tenhola-Roininen 2003). Along with other pre-treatments, the cold pre-treatment favours the synchronization of the developmental process of the microspores (Hu and Kasha 1999).

The role of temperature in induction of androgenesis was clearly shown in the case of *Brassica napus* microspore culture (Telmer et al. 1993; Custers et al. 1994; Binarova et al. 1997; Touraev et al. 1997; Indrianto et al. 1999; Smykal and Pechan 2000). At a temperature of 25°C or lower, microspores in suspension continue their development into pollen, while an 8 hour treatment with at a higher temperature of 32°C was enough to induce the androgenic process.

There is evidence to suggest that prolonged stress treatment raises the proportion of albino plants in monocot cultures. By examination of various treatment lengths, Ohnoutkovă et al. (2000) and Zheng et al. (2001) demonstrated that yield of green plants could be optimised for a range of starting materials including freshly collected anthers. The role of temperature in androgenic induction is now better understood as discussed in Touraev et al. (1997), where it is described as one of many stress factors influencing microspore transition from gametophytic to sporophytic development. Indeed, low temperature stress can be combined or replaced by other stress treatments, sometimes with better success than previously described protocols. Osmotic and starvation stress are nowadays frequently applied to cereals in combination with a relatively short, 3–5 day treatment with low temperature (Touraev et al. 1996a, b; Hoekstra et al. 1997; Hu and Kasha 1997; Caredda et al. 2000; Kasha et al. 2001; Li and Devaux 2001; Wojnarowiez et al. 2002; Jacquard et al. 2003; Cistué et al. 2003; Davies 2003). An improvement in androgenic efficiency in maize was achieved by the application of a cold pre-treatment (7°C) of tassels (Barnabás 2003a) or by pre-treatment at 14°C in a medium containing mannitol (Nageli et al. 1999; Obert et al. 2000; Zheng et al. 2003). Zheng et al. (2002a, 2003) and Liu et al. (2002a, b) used the combination of growth regulators and a short cold pre-treatment of spikes and/or starvation stress in mannitol with satisfactory results.

Novel methods were also published for wheat in which the cold pre-treatment of spikes was replaced with heat shock, 33°C for 48–72 hours (Touraev et al. 1996a, b; Liu et al. 2002a, b). Elevated temperatures were routinely used in androgenic induction of rapeseed microspores. Pechan et al. (1991) demonstrated that a treatment of 32°C for 8 hours provided a synchronous and irreversible switch in the development of embryogenic microspores of cv. Topas. It was confirmed later that,

in certain culture conditions, temperature alone controls the developmental fate of rapeseed microspores and provided a perfect system to study various aspects of the androgenic process (Hause et al. 1993; Binarova et al. 1993; Boutilier et al. 1994; Telmer et al. 1995; Custers et al. 1999, 2001). However, in some microspore suspensions heat shock could be replaced by colchicine treatment (Zhao et al. 1996a, b) and here a high number of the regenerated plants were doubled haploids, an important practical benefit. Androgenic induction was proven to be successful in a number of related *Brassica* species when heat shock protocols were applied (Duijs et al. 1992; Barro and Martin 1999; Sato et al. 2002; Ferrie 2003; Hansen 2003).

Various "stress treatments" have been applied widely. For example, application of low temperature pre-treatment was proven to favour androgenic induction in trees: oak (*Quercus* sp.) (Bueno et al. 2000, 2003; Raminez et al. 2004), apple (*Malus* sp.) (Höfer 2003, 2004) and poplar (*Populus nigra*) (Andersen 2003; Deutsch et al. 2004). It also favours androgenic induction in some *Fabaceae* species (Zagorska and Dimitrov 1995; Kaltchuk-Santos et al. 1997; Croser et al. 2004; De Moraes et al. 2004). Comparative studies by Bayliss et al. (2004) showed that low temperature stress gives better results in comparison to high temperature when applied to *Lupinus* sp.

It can be concluded that in most present protocols to induce androgenesis, stress treatments of various kinds are widely applied while in numerous other studies pre-treatments and/or pre-cultures are omitted. In the latter case, one has to take into consideration that disinfection and isolation procedures are, in themselves, stress treatments thus the way in which material is handled before the start of the induction culture may play an important role; a stress factor(s) not completely controlled and not yet entirely understood.

Compositions of Media

Improvements in the formulation of culture media have also contributed to the progress of androgenic methods. The composition of basic salts and micro-elements is wide and varied. The most often modified components are: (1) the source of organic nitrogen, (2) carbohydrates, and (3) growth regulators.

The first significant step towards better efficiency in barley anther culture was achieved by lowering the ammonium nitrate content and elevating the glutamine level as a source of organic nitrogen (Olsen 1987). The role of various nitrogen sources was later studied in detail by Mordhorst and Lörz (1993). Most recent barley media recommend usage of glutamine at levels above 500 mg/l of medium (Li and Devaux 2001; Ritala et al. 2001; Kasha et al. 2003; Szarejko 2003). Raised level of glutamine or asparagine also became a component of media in DH production of triticale (Pauk et al. 2003b; Wędzony 2003) and rye (Immonen and Tenhola-Roininen 2003; Pulli and Guo, 2003). Glutamine and serine at relatively high amounts are components of media used in *Brassica* ssp. (Custers 2003; Da Silva Dias 2003; Ferrie 2003; Hansen 2003). These data suggest that amino acids serve

as a better nitrogen source for developing ELSs compared to inorganic chemicals containing nitrate. Thus, it is recommended that organic nitrogen levels are raised in the induction medium until the induction rate and quality of ELSs are optimised.

Carbohydrates serve as a source of energy, building material and a component that regulates the osmotic properties of the culture media. The most spectacular success in protocols efficiency was achieved by the replacement of sucrose by maltose in numerous versions of induction media. This led to the patented use of maltose in barley DH production (Hunter 1987). The recommended levels of maltose in barley androgenesis is 62 g/l of the induction medium with half of this amount in the regeneration media (Scott and Lyne 1994; Salmenkallio-Marttila et al. 1995; Caredda et al. 2000; Li and Devaux 2001; Wojnarowiez et al. 2002; Szarejko 2003; Ritala et al. 2001; Kasha et al. 2001, 2003). In wheat, triticale, rye and rice the concentration of maltose ranges from 60 to 90 g/l of induction media (Chu et al. 1990; Orshinsky et al. 1990; Orshinsky and Sadasivaiah 1994; Otani and Shimada 1993; Karsai et al. 1994; Navarro-Alvarez et al. 1994; Bishnoi et al. 2000a, b; Barnabás 2003b; Pauk et al. 2003a; Tuvesson et al. 2003; W&ecedil;dzony 2003). In the examples cited above for cereals, the concentration of sucrose in the regeneration media is in the order of 20–30 g/l, which is a standard amount in many other protocols. This is however, in contrast to rapeseed media, where maltose is not applied but sucrose is present at concentration of 130g/l in the induction medium, and its concentration is subsequently lowered in the regeneration medium (Pechan and Smykal 2001). Sucrose and maltose are the main sugar components of the media throughout the literature with few examples of other carbohydrates tested. An interesting experiment was performed by Ilic-Grubor et al. (1998) who successfully induced androgenesis in rapeseed cv. ‘Topas’ in a medium of low sucrose concentration, but with the addition of polyethylene glycol (PEG) as an osmoticum. Zhou et al. (1991) showed that the osmotic potential of media influences green plant regeneration. This opens up another issue: the role of osmotic pressure in the media, an important physiological parameter rarely reported in papers and not routinely measured in media preparation.

Numerous substances are active as growth regulators *in vitro*, many of them are synthetic analogous of plant hormones. The kind of substance, its dose and the proportions in which several components are composed remain of substantial importance in regulating cell division and morphogenesis. Surprisingly, in many protocols for isolated microspore culture, growth regulators are omitted in the induction medium.

Early attempts to produce doubled haploids in maize by anther culture showed high genotypic dependence of the protocol efficiency (see references in Table 1). Delalononde and Coumans (1998) connected variability in androgenic response with the level of endogenous auxine. In barley androgenic cultures, BAP, IAA, NAA and PAA are added to the induction media alone or in combination at various concentrations (Mordhorst and Lörz 1993; Scott and Lyne, 1994; Davies 2003; Caredda et al. 2000; Jacquard et al. 2003; Szarejko 2003). The improved protocol of Kasha et al. (2001) and Kasha and Maluszynski (2003) contains 1 mg/l of BAP

with 10 mg/l of PAA. On the other hand, in anther culture of wheat, triticale and rye 2,4-D and kinetin are used in the induction media and NAA with kinetin to stimulate regeneration (Otani and Shimada 1993; Simonson et al. 1997; Zhou and Konzak 1997; Redha et al. 1998, 2000; Zheng and Konzak 1999; Pauk et al. 2003a, b). Abscisic acid (ABA) was applied to improve regeneration of induced embryos (Hansen 2000).

Microspore suspensions are often cultured without the addition of growth regulators although the most successful media are conditioned with ovaries (Hansen and Svinset 1993; Bruins et al. 1996; Puolimatka et al. 1996; Hu and Kasha 1997; Zheng et al. 2002b). Conditioning with an actively growing suspension culture was also successfully applied to induce *in vitro* development of isolated zygotes (Bakos et al. 2005). It can be presumed that the ovaries provide a source of active ingredients, phytohormones or other signalling molecules important for androgenic induction or ELS maturation. However, the data from detailed analysis of conditioned media have not yet been published.

Maize microspore culture was used recently as a model to study androgenic processes (Testillano et al. 2002, 2004; Borderies et al. 2004; Góralski et al. 2005; Mathys-Rochon 2005). Among others, the latter authors showed that arabinogalactan proteins added to the medium improved regeneration in low responsive genotypes. This discovery opens up new possibilities in improving the regeneration process, and may have beneficial effects for other species. It is probable, that other molecules that play regulatory role are secreted into the conditioned media however, to prove this hypothesis more detail studies of media during culture are required.

Regeneration

Regeneration media are usually simpler and differ in sugar and growth regulator compositions compared to induction media. Compositions of regeneration media vary according to the induction medium used. For instance, in most anther culture protocols developed for hexaploid wheat, 2,4-D and kinetin are used in the induction media and in the following regeneration media NAA with kinetin are added (Otani and Shimada 1994; Simonson et al. 1997; Zhou and Konzak 1997; Redha et al. 1998, 2000; Zheng and Konzak 1999; Pauk et al. 2003b).

Less effort has been made into the study of regeneration requirements since it is believed that the quality of androgenic embryo/callus determined during the induction phase is the main factor influencing overall effectiveness of culture. This may be misleading, since in many studies, the regeneration rate is low, large numbers of induced structures are lost, and improvements are desirable and possible. For example, regeneration in rapeseed cultures was substantially increased by the application of cold treatment of 10 days at 2°C at the beginning of the regeneration phase of the culture (Zhou et al. 2002). Treatments with the temperature below 10°C for 1–3 weeks at the start of the regeneration phase are also recommended for other

Brassica species (Da Silva Dias 2001, 2003; Ferrie 2003). On the other hand, physiologic maturation of Brassica embryos can be improved by the addition of ABA to the cultured embryos before their transfer to the regeneration conditions (Custers 2003; Da Silva Dias 2003; Hansen 2003). Additionally, the procedure of embryo desiccation prior to the regeneration phase was recommended by the latter author to aid ‘germination’, thus mimicking seed maturation and germination. In triticale, 7 days at 10°C at the beginning of the regeneration phase is recommended for optimal regeneration and a better green:albino ratio (Wędzony 2003). Regeneration is under the influence of various physical factors of the culture, out of which temperature, light intensity and photoperiod appeared to be important (Ziauddin et al. 1992; Nichterlein and Friedt 1993; Zheng and Konzak 1999; Nowak 2000; Ritala et al. 2001). The cited data raise the issue of the pre-conditioning of the derived androgenic structures during the transition from the induction to the regeneration phase. It may be that more in depth studies are required to substantially raise the overall efficiency of existing protocols.

Albinism

A particular problem of androgenesis in monocots is the high frequency of regenerants devoid of chlorophyll, so called “albino plants” or albinism (see also chapter on’ Albinism in microspore culture’) Experiments with alloplasmic lines of hexaploid wheat, which have identical nuclear, but different plastid genomes, showed significant differences in the response to microspore culture indicating an influence of the given plastid genome (Ekiz and Konzak 1991). However, backcrosses with the male parent plants resulted in a shift in the green: albino ratio back to that of the parent (Zhou and Konzak 1992). In reciprocal crossing experiments between wheat cultivars, which produce a high number, and others, which produce a low number of albino plants, only insignificant reciprocal effects were observed (Tuvesson et al. 1989). These experiments demonstrate that albino production rate is a highly heritable trait and point to the dominant role of the nuclear genome in controlling this phenomenon. Quantitative trait loci (QTLs) for albinism/green plant production were identified on chromosomes 1BL/1RS, an on 2AL, 2BL and 5BL chromosome arms of wheat (Agache et al. 1989; Tuvesson et al. 1991; Torp et al. 2001). Molecular approaches revealed large-scale deletions and rearrangements in the plastid genome of microspore-derived albino wheat (Day and Ellis 1984; Ankele 1998; Hofinger 1999; Hofinger et al. 2000), barley (Day and Ellis 1985; Dunford and Walden 1991; Mouritzen and Bach-Holm 1994) and rice (Harada et al. 1991, 1992).

Although genetically determined, the trait can be tempered to some extent by culture conditions. For instance, the combination of starvation and cold stresses applied simultaneously during 3–4 days elevated microspore survival and lowered the proportion of chlorophyll-deficient (albino) plants versus green plants regenerated from the culture in comparison to 3–4 weeks of cold treatment (Caredda and Clement 1999; Caredda et al. 2000; Kasha et al. 2001). Recent studies have indicated, that the

number of albino plants can be reduced further by an addition of $CuSO_4$ to the pre-treatment “starvation” mannitol medium (Liu et al. 2002a, b; Wojnarowiez et al. 2002; Cistué et al. 2003; Davies 2003). Despite these developments, albinism remains a major problem in monocot androgenesis, while it is rare in dicots. A valuable review on albinism was published by Clement et al. (2005). It points to the genotypic differences among the green-producing and albino-producing cultivars at the level of chloroplast anatomy, visible at the early stage of the uninucleate microspore, the stage when the androgenesis is induced. The hypothesis is put by the authors on the role of the programmed cell death as the process responsible for chloroplast DNA damage during microspore embryogenesis resulting in albino phenotypes (see chapter on ‘Programmed cell death and microspore embryogenesis’).

Gynogenesis

Gynogenesis is usually selected as the alternative method in species where androgenesis fails. It therefore plays a limited role in cereals, although it was induced in certain genotypes of wheat (Zhu et al. 1981; Matzk 1991; Comeau et al. 1992; Matzk et al. 1995), barley (Gaj and Gaj 1996; Gaj 1998) and rice (Zhou and Yang 1981). Gynogenesis resembles apomictic parthenogenesis, thus our understanding of the processes that regulate autonomous (i.e. occurring without fertilisation) embryogenesis can contribute also to improve *in vitro* gynogenesis methods. Genes responsible for the initiation of apomictic embryo development from unfertilised egg cells (parthenogenesis) may also determine/play a role in gynogenesis. This potentially, can open up new perspectives for *in vitro* gynogenesis. At present gynogenesis in breeding is practiced in two cultivated genera: *Alium* sp. and *Beta* sp. though a number of species are responsive, e.g. barley, gerbera, maize, mulberry, squash, sunflower and tef (see chapters ‘Doubled haploids via gynogeneis’ and ‘Haploidy in tef’).

Gynogenesis in onion is achieved *via* the culture of flowers on solid media. An earlier review of methods can be found in Keller and Korzun (1996). The medium developed by the group of Campion and collaborators named BDS (Campion and Alloni 1990; Campion et al. 1992, 1995) is commonly applied. However, Geoffriau et al. (1997) used B5 medium for induction and MS for regeneration. Researchers from Lubliana University (Bohanec and Jakse 1999; Jakse et al. 2003; Jakse and Bohane 2003) and from the Agricultural University of Kraków (Michalik et al. 2000a, b; Nowak 2000; Adamus et al. 2001; Grzebelus and Adamus 2004) contributed vastly to recent progress. Close to one hundred onion genotypes/accessions were subjected to a standard procedure and revealed a high level of genetic variability in gynogenic response (varying from 0 to 10 green plants regenerated per hundred cultured flowers). Most of the regenerated plants appeared to be haploid and artificial chromosome doubling was required to produce the DHs. Colchicine, oryzaline, trifluraline and aminoprophosmethyl (APM) were tested and eventually a medium containing 50 µM APM applied for 24 hours was found to be the most effective (Grzebelus and Adamus 2004). Gynogenic origin of regenerated plants

was confirmed by embryological studies (Musiał et al. 2001). Puddephat et al. (1999) failed to show substantial improvement of the efficiency by plant stress pre-treatment prior to ovary culture. A noval approach was described by Martinez (2003) where polyamines are components of the induction medium and where embryos were subcultured before transfer onto the regeneration medium. Comparison of various protocols is difficult since they have not been tested on a common set of genotypes however, their average efficiency is good enough for application in breeding programmes and they have raised valuable breeding materials (Prof. B. Michalik, personal information).

Early attempts to obtain doubled haploid lines in beets, (*Beta vulgaris*) involved both, androgenesis and gynogenesis (Van Geyt and Jacobs 1986). Gynogenesis induced from isolated ovules appeared to be successful for sugar beet (Bossoutrot and Hosemans 1985; Goska 1985; Doctrinal et al. 1989; Galatowitsch and Smith 1990; Lux et al. 1990; Ragot and Steen 1992; Gürel et al. 2000; Wremerth-Weich and Levall 2003), red beet (Baransky 1996) and fodder beet (Kikindonov 2003). The basic protocol was developed in late 1980s (Bossoutrot and Hosemans 1985; Goska 1985; Doctrinal et al. 1989). Cold treatment of inflorescences at 8°C for 1 week, combined with relatively high temperature (30°C) of the induction phase can be regarded as the latest improvement of the procedure (Wremerth-Weich and Levall 2003). Beet doubled haploids are used in breeding programmes (Zakhariev and Kikindonov 1997), including hybrid breeding (Kikindonov and Kikindonov 2001). A detailed protocol tested in high-throughput DH-production was described recently by Wremerth-Weich and Levall (2003).

Wide Crossing

Wide crossing also exploits haploidy from the female gametic line. In some inter-specific or intergeneric crosses of the *Poaceae* and *Pannicoidae,* fertilisation is followed by paternal chromosome elimination from the hybrid embryo. In these crosses the endosperm is either not formed or poorly developed; therefore, such embryos do not mature in the caryopsis and embryo rescue and *in vitro* culture are necessary. Cereals are therefore in privileged position as here a third way to produce DHs is possible – ‘wide crossing’ (for earlier reviews see: Laurie et al. 1990; Mujeeb-Kazi and Riera-Lizarazu 1997). Intergeneric crosses are applied also in potato however the mechanism does not involve chromatin elimination but parthogenetic induction of the egg cell.

The ‘Bulbosum Method’

The first method in cereals based on ‘wide crossing’ was the ‘*bulbosum* method’ (now widely used in barley breeding). It was discovered over 30 years ago after

pollinating barley (*Hordeum vulgare*) with a wild species, *Hordeum bulbosum*, the cross resulted in the production of haploid *H. vulgare* plants (Kasha and Kao 1970; Lange 1971). The method was further developed and various pre- or post-pollination growth regulator treatments (usually gibberellins and/or IAA) were applied to raise efficiency (Pickering and Devaux 1992; Pickering and Wallace 1994). An endosperm is frequently formed in *H. vulgare* × *H. bulbosum* crosses, but its development is usually disturbed and at 12–14 days the embryos are excised from developing caryopses and cultured *in vitro*. Since the plants obtained are haploid, duplication of the chromosome complement is always necessary. The '*bulbosum* method' plays an important role in the barley breeding since it is relatively cheap, non-genotype dependent and uses techniques breeders are familiar with. About 50 barley varieties registered in various countries were derived *via H. bulbosum* pollination (Prof. Ken Kasha, personal communication). Pollination with *H. bulbosum* also appeared to be effective for DH production in some wheat and triticale genotypes (Inagaki 1985; Sitch and Snape 1986). Crossability barriers between the majority of cultivated wheat and triticale genotypes and *H. bulbosum* limit its wider application to other cereals (Wojciechowska and Pudelska 1993; Inagaki and Tahir 1995). The '*bulbosum* method' is still effectively applied in many barley breeding programmes (Mihăilescu and Giura 1996; Devaux 2003; Hayes et al. 2003, see also chapter on 'Overview on barley doubled haploid production').

Crosses with Maize and Its Relatives

The first reports on embryo formation after pollination of wheat with maize (Zenkteler and Nitzsche 1984) raised the attention of plant breeders. Further studies revealed that maize chromatin is eliminated from the hybrid nuclei during early embryogenesis of the hybrid embryo (Laurie and Bennett 1986, 1989). In crosses between the members of *Pooideae* with maize the endosperm is formed only occasionally and without special treatment *in planta* with growth regulators, embryos abort as early as 5–6 days after fertilization (Laurie and Bennett 1989; Wędzony and van Lammeren 1996; Brazauskas et al. 2004). Various treatments with synthetic auxine analogues to spikes were developed that maintain embryo growth to the stage suitable for embryo isolation and culture for further growth (Riera-Lizarazu et al. 1992; Matzk and Mahn 1994; Mujeeb-Kazi and Riera-Lizarazu 1997; Wędzony et al. 1998a, b; Wędzony 2003).

Since crossability barriers were not found between maize and a number of cereals, green plants were obtained successfully in a wide range of genotypes, including those recalcitrant to androgenesis, e.g. wheat (Almouslem et al. 1998; Inagaki et al. 1998; Saidi et al. 1998; David et al. 1999; Cherkaoui et al. 2000; Knox et al. 2000), barley (Furusho et al. 1991), triticale (Wędzony et al. 1998a, b) and rye (Deimling and Fleihinghaus-Roux 1996). Results obtained for barley and triticale are as good to those of wheat while in rye, embryos are obtained in low number, and they are difficult to rescue. On the contrary, oat could be easily crossed with maize (Rines 2003).

Oat polyhaploids recovered from oat x maize crosses sometime retain maize chromosomes and some of them are partially fertile which is detrimental in the context of breeding, but led to the development of valuable novel aneuploid stocks for genetic and gene introgression studies (Rines 2003). Flowering times of maize and most *Pooide* can be matched in the field conditions in subtropical and tropical climates while in Europe and Canada experiments are run at glasshouse conditions. Unfortunately, there is no effective method to store and maintain the viability of maize pollen.

Some species related to maize could substitute for maize as the pollinators. Ushiyama et al. (1991) used pollen of wild Mexican corn – theosinte (*Zea mays* ssp. *mexicana*) in crosses with wheat. Sorghum (*Sorghum bicolor*) was utilized to pollinate hexaploid and tetraploid wheats (Riera-Lizarazu et al. 1992). Additionally, pearl-millet (*Pennisetum glaucums* synonym *Pennisetum americana*) was successfully applied in several experiments (Inagaki and Mujeeb-Kazi 1995; Ohkawa et al. 1992; Mujeeb-Kazi and Riera-Lizarazu 1997). Use of these species ('haploid inducing pollinators') might be beneficial in hot climates where flowering of mother plants and pollen donor may be easily synchronised. Comparative experiments usually show that sweet corn type hybrid maize are good pollinators.

Maize Doubled Haploids by "Inducer" Line

Very promising results were obtained in maize with a method that utilizes inbred 'inducer' lines (Sarkar et al. 1994; Shatskaya et al. 1994; Chalyk 1999). A new improved inducer line 'RWS' has been recently developed and characterised (Röber et al. 2005). With its use, 10% of progeny appear to be haploids derived from the mother plant. Moreover, RWS contains a colour marker gene that makes it possible to select kernels containing haploid embryos among those containing diploid embryos. QTL analysis showed that the ability to induce haploid embryos is controlled by a large number of loci, but the specific genes involved are unknown (Röber 1999). Microscopy showed that chromatin is eliminated from the nuclei of some embryos after RWS self-pollination. A large number of micro-nuclei in shoot primordia can be observed in 3 week old embryos excised from developing caryopses (Wędzony et al. 2002). Practical protocols for haploid induction and chromosome duplication were developed and interest in the method grows rapidly among breeders (Deimling and Fleihinghaus-Roux 1996; Röber et al. 2005).

Potato Doubled Haploidy by S. tuberosum x S. phureja Crosses

Doubled haploids can be produced from tetraploid genotypes of *Solanum tuberosum* (cultivated potato) by pollination with the diploid potato species *Solanum*

phureja (Mendiburu et al. 1974; De Maine 2003). In about 0.5% of pollinated ovules, both male sperm cells of *S. phureja* take part in formation of functional endosperm, which triggers the parthenogenic development of unfertilised egg cells. The best pollinator lines of *S. phureja* were bred for a dominant purple spot embryo marker, thus seeds containing haploid embryos can be easily distinguished from hybrid *S. tuberosum* x *S. phureja* seeds. Methods of more effective chromosome number duplication were developed more recently and production of potato can now be obtained by androgenic methods with a better efficiency (Jacobsen and Ramanna 1994; Rokka et al. 1996; Tai and Xiong 2003; Rokka 2003). Moreover, androgenesis is applicable to a much wider range of *Solanum* species in comparison to crosses with *S. phureja* (Jacobsen and Ramanna 1994; Rokka et al. 1995; Aziz et al. 1999; Tai and Xiong 2003; Rokka 2003).

Concluding Remarks

Research on DHs accelerated in the last few decades with a growing number of successful applications (Forster et al. 2007). There has been a steady rise in the number of new plant species in which doubled haploidy has been report. Prior to the 1970s there was only a handful of reports on the production of DHs, this grew to about 50 publications in the 1970s, 185 in the 1980s and about 200 in the 1990s (data from Maluszynski et al. 2003b). Currently there is much interest in applying DH technologies to neglected crops, particularly those of high value, e.g. pharmaceutical and aromatic species, where large gains can be achieved is a short space of time (Ferrie 2007, see also chapter on ‘Doubled haploids in medicinal plants’). A major component in the expansion of DH technologies to new species has been the adoption and adaptation of protocols from the model species, tobacco, rape-seed and barley. Published methodologies now cover about 200 species and represent a wide variability of modifications compared to the initial protocols. It is rarely the case that a protocol optimised for one genotype is optimal for another. Adaptation of methods from one species to another often requires substantial changes usually elaborated by empirical comparisons of protocols. As a consequence, success correlates with the number of laboratories engaged in developing new methods. The main difficulty in improving androgenesis lies in the large range of factors that control and influence this process. Even if successful, this will help to understand just the first steps of this complicated process. The same problems apply to gynogenic methods. The wide crossing protocols usually do not need many modifications, but their applicability is limited to a narrow range of species.

Functional genomic approaches that determine genes associated with androgenic induction have revealed several hundreds of up-regulated and down regulated genes, the functions of some have been investigated (see Hosp et al. 2007). The idea of determining one or a few ‘androgenic induction’ gene(s) does not find support in current studies Genomic research on zygotic embryogenesis, apomixes, somatic embryogenesis and plant ‘stem cells’ can potentially also contribute to improvements

in DH technologies. Although for the moment, further improvements can be expected mostly from empirical testing of new variants of known protocols, the new approaches in discovering gene function are expected to have an impact in the future.

Although not perfect, DH systems are broadly applied in breeding (Thomas et al. 2003), genetic mapping (Forster and Thomas 2005) and mutation studies (Barro et al. 2001; Szarejko 2003; Szarejko and Forster 2007). They also provide useful systems for transformation and fixing transformation events (Shim and Kasha 2003), and for fundamental studies on plant embryogenesis and cell differentiation (Custers et al. 2001; Kumlehn et al. 2001; Bakos et al. 2003, 2005). Further progress in methods can be expected with the growing understanding of the processes involved. Joint efforts between scientists from a range of disciplines and breeders are needed to develop the best strategies in deploying DH systems for practical use. Coupled with marker assisted selection DH technology is a powerful tool to speed up breeding and help keep pace with contemporary challenges such as environmental issues (pollution, climate change, etc.) and new social demands for high quality and safer agricultural products.

References

Adamus A, Samek L, Nowak E, Michalik B (2001) Optimization of protocols for gynogenesis induction and haploid plants diploidization in onion (*Allium cepa* L.). Folia Hort, Ann 13:69–74

Agache S, Bachelier B, De Buyser J, Henry Y, Snape JW (1989) Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. Theor Appl Genet 77:7–11

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Reprod 13:432–436

Almouslem AB, Jahuar PP, Peterson TS, Bomminieni VR, Rao MB (1998) Haploid durum wheat production via hybridization with maize. Crop Sci 38:1080–1087

Andersen SB (2003) Doubled haploid production in poplar. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 293–296

Ankele E (1998) Molecular investigations of chloroplast DNA of wheat pollen during different developmental stages *in vivo* and *in vitro*. Diploma thesis, University of Agriculture, Vienna

Arzani A, Darvey NL (2002) Androgenetic response of heterozygous triticale populations using a greenhouse hydroponic system. Euphytica 127:53–60

Aziz AN, Seabrook JEA, Tai GCC, De Jong H (1999) Screening diploid *Solanum* genotypes responsive to different anther culture conditions and ploidy assessment of anther derived roots and plantelets. Amer J Patato Res 76:9–16

Bakos F, Darkó É, Pónya Z, Barnabás B (2003) Regeneration of fertile wheat (*Triticum aestivum* L.) plants from isolated zygotes using wheat microspore culture as nurse cells. Plant Cell Tiss Org Cult 74:243–247

Bakos F, Jäger K, Barnabás B (2005) Regeneration of haploid plants after distant pollination of wheat via zygote rescue. Acta Biol Crac Ser Bot 47:167–171

Baransky R (1996) *In vitro* gynogenesis in red beet. Acta Soc Bot Poloniae 65:57–60

Barnabás B (2003a) Anther culture of maize (*Zea mays* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 103–108

Barnabás B (2003b) Protocol for producing doubled haploid plants from anther culture of wheat (*Triticum aestivum* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 65–70

Barnabás B, Obert B, Kovacs G (1999) Colchicine, an efficient genome-doubling agent for maize (*Zea mays* L.) microspores cultured in anthero. Plant Cell Reprod 18:858–962

Barro F, Martín A (1999) Response of different genotypes of *Brassica carinata* to microspore culture. Plant Breed 118:79–81

Barro F, Fernández-Escobar J, De La Vega M, Martín A (2001) Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. Plant Breed 120:262–264

Bayliss KL, Wroth JM, Cowling WA (2004) Pro-embryos of *Lupinus* spp. produced from isolated microspore culture. Aust J Agr Resour 55:589–593

Bergmann R, Friedt W (1996) Haploidy and related biotechnological methods in linseed (*Linum usitatissimum* L.). In: Jain MS, Sopory SK, Veilleux RE (eds) *In vitro* Haploid Production of Higher Plants. Vol 1. Kluwer, Dordrecht, pp 1–16

Binarova P, Straatman K, Hause B, Hause G, van Lammeren AAM (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. Theor Appl Genet 87:9–16

Binarova P, Hause G, Cenklova V, Cordewener JHG, van Lookeren Campagne MM (1997) A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex Plant Reprod 10:200–208

Bishnoi U, Jain RK, Rohilla JS, Chowdhury VK, Gupta KR Chowdhury JB (2000a) Anther culture of recalcitrant indica – Basmati rice hybrids. Anther culture of indica rice hybrids. Euphytica 114:93–101

Bishnoi US, Jain RK, Gupta KR, Chowdhury VK, Chowdhury JB (2000b) High frequency androgenesis in indica – Basmati rice hybrids using liquid culture media. Plant Cell Tiss Org Cult 61:153–159

Blakeslee AF, Belling J, Farnham ME Bergner AD (1922) A haploid mutant in the Jimson weed, *Datura stramonium*. Science 55:646–647

Bohanec B (2000) COST Action 824 – Biotechnological approaches for utilisation of gametic cells – Final meeting, Bled, Slovenia, July 1–5, 2000. Luxemburg: Office for Official Publications of the European Communities 2001. ISBN 92–894–0225–3. pp 277

Bohanec B, Jakse M (1999) Variations in gynogenic response among long-day onion (*Allium cepa* L.) accessions. Plant Cell Reprod 18:737–742

Borderies G, Bechec M, Rossignol M, Lafitte C, Deunff E, Beckert M, Dumas CH, Matthys-Rochon E (2004) Characterisation of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. Eur J Cell Biol 83:205–212

Bossoutrot D, Hosemans P (1985) Gynogenesis in *Beta vulgaris*. From *in vitro* culture of unpollinated ovules to the production of doubled haploid plat in soil. Plant Cell Reprod 4:300–303

Boutilier KA, Gines MJ, Demoor JM, Huang B, Baszczynski CL, Iyer VN, Miki BL (1994) Expression of the BnmNAP subfamily of napin genes coincides with the induction of *Brassica* microspore embryogenesis. Plant Mol Biol 26:1711–1723

Brazauskas G, Pasakinskiene I, Jahoor A (2004) AFLP analysis indicates no introgression of maize DNA in wheat maize crosses. Plant Breed 123:117–121

Bruins MBM, Rakoczy-Trojanowska M, Snijders CHA (1996) Isolated microspore culture in wheat (*Triticum aestivum* L.): the effect of co-culture of wheat or barley ovaries on embryogenesis. Cereal Res Commun 24:401–408

Bueno MA, Manzanera JA (2003) Oak anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plant – A Manual. Kluwer, Dordrecht/Boston/London, pp 297–302

Bueno MA, Agundez MD, Gomez A, Carrascosa MJ, Manzabera JA (2000) Haploid origin of cork-oak anther embryos detected by enzyme and rapid gene markers. Intl J Plant Sci 161:363–367

Bueno MA, Gomez A, Sepulveda F, Segui JM, Testillano PS, Manzabera JA, Risueno MC (2003) Microspore-derived embryos from *Quercus suber* anthers mimic zygotic embryos and maintain haploids in long-term anther culture. J Plant Physiol 160:953–960

Cai Q, Szarejko I, Polok K, Maluszynski M (1992) The effect of sugars and growth regulators on embryoid formation and plant regeneration from barley anther culture. Plant Breed 109:218–226

Campion B, Alloni C (1990) Induction of haploid plants in onion (*Allium cepa* L.) by *in vitro* culture of unpollinated ovules. Plant Cell Tiss Org Cult 20:1–6

Campion B, Azzimonti MT, Vicini E, Schiavi M, Falavigana A (1992) Advances in haploid plant induction in onion (*Allium cepa* L.) through *in vitro* gynogenesis. Plant Sci 86:97–104

Campion B, Perri E, Azzimonti MT, Vicini E, Schiavi M (1995) Spontaneous and induced chromosome doubling in gynogenic lines of onion (*Allium cepa* L.). Plant Breed 114:243–246

Cardoso MB, Kaltchuk-Santos E, Mundstock EC, Bonadese-Zanettini MH (2004) Initial segmentation patterns of microspores and pollen viability in soybean cultured anthers: indication of chromosome doubling. Braz Arch Biol Technol 47(5):703–712

Caredda S, Clément C (1999) Androgenesis and albinism in *Poaceae*: influence of genotype and carbohydrates. In: Clément C, Pacini E, Audran JC (eds) Anther and Pollen: From Biology to Biotechnology. Springer, Berlin, Heidelberg, Tokyo, pp 211–226

Caredda S, Doncoeur C, Devaux P, Sangwan RS, Clément C (2000) Plastid differentiation during androgenesis in albino and non-albino producing cultivars of barley (*Hordeum vulgare* L.). Sex Plant Reprod 13:95–104

Castillo AM, Valles MP, Cistué L (2000) Comparison of anther and isolated microspore cultures in barley. Effects of culture density and regeneration medium. Euphytica 113:1–8

Chalyk ST (1999) Use of maternal haploids for improving maize inbred lines. MNL 73:54–56

Chase SS (1952) Production of homozygous diploids of maize from monoploids. Agron J 44:263–267

Chen FQ, Hayes PM, Rivin CJ (1991) Wide hybridization of *Hordeum vulgare* x *Zea mays*. Genome 34:603–605

Chen Y, Kenaschuk E, Dribnenki P (1998) High frequency of plant regeneration from anther culture in flax, *Linum usitatissimum* L. Plant Breed 117:463–467

Chen Y, Kenaschuk E, Dribnenki P (1999) Response of flax genotypes to doubled haploid production. Plant Cell Tiss Org Cult 57:195–198

Cherkaoui S, Lamsaouri O, Chlyah A, Chlyah H (2000) Durum wheat x maize crosses for haploid wheat production: Influence of parental genotypes and various experimental factors. Plant Breed 119:31–36

Chu CC, Hill RD, Brule-Babel AL (1990) High frequency of pollen embryoid formation and plant regeneration in *Triticum aestivum* L. on monosacharide containing media. Plant Sci 66:255–262

Cistué L, Ramos A, Castillo AM, Romagosa I (1994) Production of large number of doubled haploid plants from barley anthers pretreated with high concentrations of mannitol. Plant Cell Reprod 13:709–712

Cistué L, Vallés MP, Echavarri B, Sanz J, Castillo A (2003) Barley anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 29–34

Clausen RE, Mann MC (1924) Inheritance in *Nicotiana tabacum*. V. The occurrence of haploid plant in interspecific progenies. PNAS 10:121–124

Clément C, Sangwan RS, Sangwan BS (2005) Microspore embryo induction and development in higher plants: cytological and ultrastructural aspects. In: Nagata T, Lörz H, Idholm JMW (eds) Biotechnology in Agriculture and Forestry, Vol. 56. Springer, Berlin, Heidelberg, Tokyo (ISBN 3–540–22224–3), pp 53–72

Comeau A, Nadeau A, Plourde A, Simard R, Maes S, Kelly S, Harper L, Lettre J, Landry B, St. Pierre CA (1992) Media for the *in ovulo* culture of polyembryos of wheat and wheat-derived interspecific hybrids or haploids. Plant Sci 81:117–125

Croser J, Lulsdorf M, Cheng B, Allen K, Wilson JG, Dament T, Siddique K, Warkentin T, Vandenberg A (2004) Embryogenesis from isolated microspores of chickpea and field pea – Progress towards a doubled haploid protocol as a tool for crop improvement, 4th International Crop Science Congress, Brisbane, Queensland, Australia, The Regional Institute Ltd, 1:pp not available – www.cropscience.org.au

Croser J, Lulsdorf M, Davies P, Wilson J, Sidhu P, Grewal R, Allen K, Dament T, Warkentin T, Vandenberg A, Siddique K (2005) Haploid embryogenesis from chickpea and field pea – progress towards a routine protocol. In: Bennett IJ, Bunn E, Clarke H, McComb JA (eds) ‘Contributing to a Sustainable Future’. Proceedings of the Australian Branch of the IAPTC & B, Perth, Western Australia, September 21–24, 2005. pp 71–79; 81–82

Custers JBM (2003) Microspore culture in rapeseed (*Brassica napus* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 185–194

Custers JBM, Cordewener JHG, Noellen Y, Dons HJM, van Lookeren CampagneMM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Reprod 13:267–271

Custers JBM, Snepvangers SCHJ, Jansen HJ, Zhang L, van Lookeren Campagne MM (1999) The 35S-CaMV promoter is silent during early embryogenesis but activated during non-embryogenic sporophytic development in microspore culture. Protoplasma 208:257–264

Custers JBM, Cordewener JHG, Fiers MA, Massen BTH, van Lookeren Campagne MM Liu CM (2001) Androgenesis in *Brassica*. A model system to study the initiation of plant embryogenesis. In: Bhojwani SS, Soh WY (eds) Current Trends in the Embryology of Angiosperms. Kluwer, Dordrecht, pp 451–470

Da Silva Dias JC (1999) Effect of activated charcoal on *Brassica oleracea* microspore culture embryogenesis. Euphytica 108:65–69

Da Silva Dias JC (2001) Effect of incubation temperature regimes and culture medium on broccoli microspore culture embryogenesis. Euphytica 119:389–394

Da Silva Dias JC (2003) Protocol for broccoli microspore culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 195–204

Darkó É, Ambrus H, Stefanovits-Bányai É, Fodor F, Bakos F, Barnabás B (2004) Aluminium toxicity, Al tolerance and oxidative stress in an Al-sensitive wheat genotype and in Al-tolerant lines developed by *in vitro* microspore selection. Plant Sci 66:583–591

David JL, Dusautoir JC, Raynaud C, Roumet P (1999) Heritable variation in the ability to produce haploid embryos via pollination with maize and embryo rescue in durum wheat. Genome 42:338–342

Davies PA (2003) Barley isolated microspore culture (IMC) method. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 49–52

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: Possible basis for maternal inheritance of chloroplasts. Cell 39:359–368

Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9:671–678

De Maine MJ (2003) Potato haploid technologies. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 241–247

De Moraes AP, Bonadese-Zanettini MH, Callegari-Jaques SM, Kaltchuk-Santos E (2004) Effect of temperature shock on soybean microspore embryogenesis. Braz Arch Biol Technol 47:537–544

Deimling S, Fleihinghaus-Roux T (1996) Haploids in rye. In: Mohan-Jain S, Spory SK, Veilleux RE (ed) In vitro haploid production in higher plants, Kluwer, Dordrecht, pp 181–204

Delalononde M, Coumans MP (1998) Effect of IAA content modulators on peroxidase activity and on endogenous IAA during cold pretreatment of maize anthers prior to androgenesis. Plant Growth Reg 26:123–130

Deutsch F, Kumlehn J, Ziegenhangen B, Fladung M (2004) Stable haploid poplar callus lines from immature pollen culture. Physiol Plant 120:613–622

Devaux P (2003) The *Hordeum bulbosum* (L.) method. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 15–20

Doctrinal M, Sangwan RS, Sangwan-Norreel BS (1989) *In vitro* gynogenesis in *Beta vulgaris* L. Effects of plant growth regulators, temperature, genotypes and season. Plant Cell Tiss Org Cult 17:1–12

Dogramci-Altuntepe M, Peterson TS, Jahuar PP (2001) Anther culture derived regenerants of durum wheat and their cytological characterisation. J Hered 92:56–64

Duckan EJ, Heberle-Bors E (1976) Effect of temperature shock on nuclear phenomena in microspores of *Nicotiana tabacum* and consequently on plantlet production. Protoplasma 90:173–177

Duijs JG, Voorrips RE, Visser DL, Custers JBM (1992) Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica 60:45–55

Dunford R, Walden RM (1991) Plastid genome structure and plastid-related transcript levels in albino barley plants derived from anther culture. Curr Genet 20:339–347

Ekiz H, Konzak CF (1991) Nuclear and cytoplasmic control of anther culture response in wheat: I Analysis of alloplasmic lines. Crop Sci 31:1421–1427

Ekiz H, Konzak CF (1997) Effects of light regimes on anther culture response in bread wheat. Plant Cell Tiss Org Cult 50(1):7–12

Evans JM, Batty NP (1994) Ethylene precursors and antagonists increase embryogenesis of *Hordeum vulgare* L. anther culture. Plant Cell Reprod 13:676–678

Ferrie AMR (2003) Microspore culture of *Brassica* species. In: Maluszynski M, Kasha KJ. Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 205–215

Ferrie AMR (2007) Doubled haploid production in nutraceutical species: A review. Euphytica 130:347–357

Ferrie AMR, Epp DJ, Keller WA (1995) Evaluation of *Brassica rapa* L. genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Reprod 14:580–584

Flehinghaus T, Deimling S, Geiger HH (1991) Methodical improvements in rye anther culture. Plant Cell Reprod 10:397–400

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88

Forster BP, Herberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375

Foroughi-Wehr B, Friedt W, Wenzel G. (1982) On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor Appl Genet 62:233–239

Furusho M, Suenaga K, Nakajima K (1991) Production of haploid barley plants from barley x maize and barley x Italian ryegrass crosses. Jpn J Breed 41:175–179

Gains EF, Aase HC (1926) A haploid wheat plant. Am J Bot 13:373–385

Gaj M (1998) Estimation of parthenogenesis frequency in genotypes of *Hordeum vulgare* (L.) by auxin test. J Appl Genet 39A:98

Gaj M, Gaj MD (1996) Parthenogenetic barley haploids obtained by auxin treatment. Proceedings of the International Conference on Perspectives in Plant Genetics, Warszawa – Ursynów, September 16–17, 1996. J Appl Genet (Genet Pol) 37:187–190

Galatowitsch MW, Smith GA (1990) Regeneration from unfertilized ovule callus of sugar beet (*Beta vulgaris* L.). Can J Plant Sci 70:83–89

Garrido D, Chibi F, Matill A (1995) Polyamines in the induction of *Nicotiana tabacum* pollen embryogenesis by starvation. J Plant Physiol 145:731–735

Geoffriau E, Kahane R, Rancillac M (1997) Variation of gynogenesis ability in onion (*Allium cepa* L.). Euphytica 94:37–44

Germana MA (1997) Haploidy in Citrus. In: Jain MS, Sopory SK, Veilleux RE (eds) In vitro Haploid Production in Higher Plants. Vol. 5. Kluwer, Dordrecht, pp 195–217

Gonzalez JM, Jouve N (2000) Improvement of anther culture media for haploid production in triticale. Cereal Res Com 28:65–72

Goska M (1985) Sugar beet haploids obtained in the *in vitro* culture. Bull Pol Acad Sci Biol Sci 33:31–33

Góralski G, Rozier F, Matthys-Rochon E, Przywara L (2005) Cytological features of various microspore derivatives appearing during culture of isolated maize microspores. Acta Biol Crac0v Series Bot 47/1:75–83

Grzebelus E, Adamus A (2004) Effect of anti-mitotic agents on development and genome doubling of gynogenic onion (*Allium cepa* L.) embryos. Plant Sci 167:569–574

Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of *Datura*. Nature 204:497

Guha S, Maheshwari SC (1966) Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212:97–98

Gürel S, Gürel E, Kaya Z (2000) Doubled haploid production from unpollinated ovules of sugar beet (*Beta vulgaris* L.). Plant Cell Reprod 19:151–159

Gustafson VD, Baeziger SP, Wright MS, Stroup WW, Yen Y (1995) Isolated wheat microspore culture. Plant Cell Tiss Org Cult 42:207–213

Hansen M (2000) ABA treatment and desiccation of microspore-derived embryos of cabbage (*Brassica oleracea* ssp. *capitata* L.) improves plant development. J Plant Physiol 156:164–167

Hansen M (2003) Protocol for microspore culture in Brassica. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 217–222

Hansen M, Svinset K (1993) Microspore culture of swede (*Brassica napus* ssp. *rapifera*) and the effects of fresh and conditioned media. Plant Cell Reprod 12:496–500

Harada T, Sato T, Asaka T, Matsukawa I (1991) Large-scale deletions of plastid DNA in rice anther culture. Theor Appl Genet 81:157–161

Harada T, Ishikawa R, Niizeki M, Saito K (1992) Pollen-derived rice calli that have large deletions in plastid DNA do not require protein synthesis in plastid for growth. Mol Gen Genet 233:145–150

Hause B, Hause G, Pechan P, van Lammeren AAM (1993) Cytoskeletal changes and induction of embryogenesis in microspores and pollen cultures of *Brassica napus* L. Cell Biol Int 17:153 168

Hause B, van Veenendaal WLH, Hause G, van Lammeren AAM (1994) Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv. Topas. Botanica Acta 107:369–472

Hayes P, Corey A DeNoma J (2003) Doubled haploid production in barley using the *Hordeum bulbosum* (L.) technique. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 5–14

Heberle-Bors E (1984) Genotypic control of pollen embryogenesis in *Nicotiana tabacum* L. Theor Appl Genet 68:475–479

Heberle-Bors E, Reinert J (1979) Androgenesis in isolated pollen cultures of *Nicotiana tabacum*: dependence upon pollen development. Protoplasma 99:237–245

Heberle-Bors E, Reinert J (1981) Environmental control and evidence for predetermination of pollen embryogenesis in *Nicotiana tabbacum* pollen. Protoplasma 109:249–255

Heberle-Bors E, Stöger E, Touraev A, Zarsky V, Vicente O (1996) *In vitro* pollen cultures: progress and perspectives. In: Mohapatra SS, Knox RB (eds) Pollen Biotechnology. Gene Expression and Allergen Characterization. Chapman & Hall, New York pp 85–109

Ho KM, Jones GE (1980) Mingo barley. Can J Plant Sci 60:279–280

Hoekstra S, van Bergen S, van Brouwershaven IR, Schilperoort RA Heidekamp F (1996) The interaction of 2,4-D application and mannitol pretreatment in anther and microspore culture of *Hordeum vulgare* L cv. Igri. J Plant Physiol 148:696–700

Hoekstra S, van Bergen S, van Brouwershaven IR, Schilperoort RA, Wang M (1997) Androgenesis in *Hordeum vulgare* L. Effect of mannitol, calcium and abscisic acid on anther pretreatment. Plant Sci 126:211–218

Höfer M (2003) *In vitro* androgenesis in apple. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 287–292

Höfer M (2004) *In vitro* androgenesis in apple – improvement of the induction phase. Plant Cell Reprod 22:365–370

Höfer M, Laspinasse Y (1996) Haploidy in apple. In: Jain MS, Sopory SK Veilleux RE (eds) In vitro Haploid Production in Higher Plants. Vol. 5. Kluwer, Dordrecht, pp 259–274

Hofinger B (1999) Moleculargenetic analysis of plastid deficiencies in anther culture-derived albino plants of wheat (*Triticum aestivum*). Diploma thesis, University of Vienna, Vienna

Hofinger BJ, Ankele E, Güll y C, Heberle-Bors E, Pfosser MF (2000) The involvement of the plastid genome in albino plant regeneration from microspores in wheat. In: Bohanec B (ed) Biotechnological Approaches for Utilization of Gametic Cells, Brussels. pp 215–228

Hosp J, de Faria Maraschin S, Touraev A, Boutilier K (2007) Functional genomics of microspore embryogenesis. Euphytica 130:275–285

Hu T, Kasha KJ (1997) Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.) through ovary co-culture. Plant Cell Reprod 16:520–525

Hu T, Kasha KJ (1999) A cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum* L.) cv. Chris. Genome 42:432–441

Huda S, Islam R, Bari MA, Asaduzzaman M (2001) Anther culture of Chickpea. Int Chickpea & Pigeonpea Newsl. 8:24–26

Hunter CP (1987) Plant generation method. European patent application, No. 87200773.7

Ilic-Grubor K, Attree SM, Fowke LC (1998) Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. Plant Cell Reprod 17: 329–333

Immonen S (1999) Androgenic green plants from winter rye, *Secale cereale* L., of diverse origin. Plant Breed 118(4):319–322

Immonen S. Anttila H (1996) Success in anther culture of rye. Vortr. Pflanzenzuchtg. 35:237–244

Immonen S, Anttila H (1998) Impact of microspore developmental stage on induction and plant regeneration in rye anther culture. Plant Sci 139:213–222

Immonen S, Anttila H (1999) Cold pretreatment to enhance green plant regeneration from rye anther culture. Plant Cell Tiss Org Cult 57:121–127

Immonen S, Anttila H (2000) Media composition and anther planting for production of androgenic green plants from cultivated rye (*Secale cereale* L.). J Plant Physiol 156:204–210

Immonen S, Robinson J (2000) Stress treatments and ficoll improving green plant regeneration in triticale anther culture. Plant Sci 150:77–84

Immonen S, Tenhola-Roininen T (2003) Protocol for rye anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds.), Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 141–150

Inagaki M (1985) Chromosome doubling of the wheat haploids obtained from crosses with *Hordeum bulbosum* L. Jpn J Breed 35:193–195

Inagaki M, Mujeeb-Kazi A (1995) Comparison of polyhaploid production frequencies in crosses of hexaploid wheat with maize, pearl millet and sorghum. Breed Sci 45:157–161

Inagaki MN, Tahir M (1995) Comparision of crossabilities of tetraploid wheat with *Hordeum bulbosum* and maize. Cer Res Comm 23:339–343

Inagaki MN, Pfeiffer WH, Mergoum M, Mujeeb-Kazi A (1998) Variation of the crossability of durum wheat with maize. Euphytica 104:17–23

Indrianto A, Heberle-Bors E, Touraev A (1999) Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. Plant Sci 143:71–79

Ingram HM, Power JB, Lowe KC, Davey MR (2000) Microspore-derived embryo induction from cultured anthers of wheat. Plant Cell Tiss Org Cult 60:235–238

Jacobsen E. Ramanna MS (1994) Production of monohaploids of *Solanum tuberosum* L. and their use in genetics, molecular biology and breeding. In: Potato Genetics. Bradshaw, JE, Mackay GR (eds) CAB International, Cambridge, pp 155–170

Jacquard C, Wojnarowiez G, Clement C (2003) Anther culture in barley. In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 21–28

Jakse M, Bohanec B (2003) Haploid induction in onion via gynogenesis. In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 281–287

Jakse M, Havey MJ, Bohanec B (2003) Chromosome doubling procedures of onion (*Allium cepa* L.) gynogenic embryos. Plant Cell Reprod 21: 905–910

Jauhar PP (2003) Haploid and doubled haploid production in durum wheat by anther culture. In.: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plant – A Manual. Kluwer, Dordrecht/Boston/London, pp 161–166

Kaltchuk-Santos E, Mariath JE, Mundstock E, Hu Ch, Bodanese-Zanettini MH (1997) Cytological analysis of early microspore division and embryo formation in cultured soybean anthers. Plant Cell Tiss Org Cult 49:107–115

Karsai I, Bedö z (1997) Effect of carbohydrate content on the embryoid and plant production in triticale anther culture. Cereal Res Commun 25(2):109–116

Karsai I, Bedö Z, Hayes PM (1994) Effect of induction medium, pH and maltose concentration on *in vitro* androgenesis of hexaploid winter triticale and wheat. Plant Cell Tiss Org Cult 39:49–53

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225:874–876

Kasha KJ, Maluszynski M (2003) Production of doubled haploids in crop plants. An introduction. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 1–4

Kasha KJ, Simion E, Oro R, Yao QA, Hu TC, Carlson AR (2001) An improved *in vitro* technique for isolated microspore culture of barley. Euphytica 120:379–385

Kasha KJ, Simion E, Oro R, Shim YS (2003) Barley isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 43–48

Keller ER, Korzun L (1996) Haploidy in onion (*Allium cepa* L.) and other Allium species. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 51–75

Kikindonov T (2003) Influence of the genotype on the development of isolated *in vitro* embryos of sugar and fodder beet crosses. Bulg J Agric Sci 9:343–348

Kikindonov G, Kikindonov T (2001) Heterosis effect in hybrid combinations of dihaploid sugar beet lines. Bulgarian J Agric Sci 7:577–581

Kimber G, Riley R (1963) Haploid angiosperms. Botanical Rev 29:480–531

Kiviharju E, Pehu E (1998) The effect of cold and heat pretreatments on anther culture response of *Avena sativa* and *A. sterilis*. Plant Cell Tiss Org Cult 54:97–104

Kiviharju E, Tauriainen A (1999) 2,4-Dichlorophenoxyacetic acid and kinetin in anther culture of cultivated and wild oats and their interspecific crosses: plant regeneration from *Avena sativa* L. Plant Cell Reprod 18:582–588

Kiviharju E, Puolimatka M, Pehu E (1997) Regeneration of anther-derived plants of *Avena sterilis* L. Plant Cell Tiss Org Cult 48:147–152

Kiviharju E, Puolimatka M, Saastamoinen M, Hovinen S, Pehu E (1998) The effect of genotype on anther culture response of cultivated and wild oats. Agric Food Sci, Finland 7 (3):409–422

Kiviharju E, Puolimatka M, Saastamoinen M, Pehu E (2000) Extension of anther culture to several genotypes of cultivated oats. Plant Cell Reprod 19:674–679

Kiviharju E, Laurila J, Lehtonen M, Tanhuanpää P, Manninen O (2004) Anther culture properties of oat x wild red oat progenies and a search for RAPD markers associated with anther culture ability. Agric Food Sci 13:151–162

Knox RE, Clarke JM, De Pauw RM (2000) Dicamba and growth condition effects on doubled haploid production in durum wheat crossed with maize. Plant Breed 119:289–298

Kuginuki Y, Miyajima T, Masuda H, Hida K, Hirai M (1999) Highly regenerative cultivars in microspore culture in *Brassica oleracea* L. var. capitata. Breed Sci 49:251–256

Kumlehn J, Kirik V, Czihal A, Altschmied L, Matzk F, Loerz H, Bäumlein H (2001) Parthenogenetic egg cells of wheat: cellular and molecular studies. Sex Plant Reprod 14:239–243

Kumlehn J, Serazetdinova L, Hensel G, Becker D, Loerz H (2005) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol J.4:251–261

Kunz C, Islam SMS, Berberat J, Peter OS, Büter B, Stamp P, Schmid JE (1999) Assessment and improvement of wheat microspore derived embryo induction and regeneration. J. Plant Physiol 156:190–196

Kyo M, Harada H (1986) Control of the developmental pathway of tobacco pollen *in vitro*. Planta 186:427–432

Lange W (1971) Crosses between *Hordeum vulgare* L. and *H. bulbosum* 1. Production, morphology and meiosis of hybrids, haploids and dihaploids. Euphytica 20:14–29

Laurie DA, Bennett MD (1986) Wheat-maize hybridization. Can J Genet Cytol 28:313–316

Laurie DA, Bennett MD (1989) The timing of chromosome elimination in hexaploid wheat-maize crosses. Genome 32:953–961

Laurie DA Donoughe S Bennett MD 1990. Wheat x maize and other wide sexual hybrids, their potential for genetic manipulation and crop improvement. In: Gustafson JP (ed) Genetic Manipulation in Plant Development II, Plenum Press, New York, pp 95–106

Lentini Z, Reyes P, Martinez CP Roca WM (1995) Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Sci 110:127–138

Li H, Devaux P (2001) Enhancement of microspore culture efficiency of recalcitrant barley genotypes. Plant Cell Reprod 20:475–481

Lionneton E, Beuret W, Delaitre C, Ochatt S, Rancillac M (2001) Improved microspore culture and doubled-haploid plant regeneration in the brown condiment mustard (*Brassica juncea*). Plant Cell Reprod 20:126–130

Liu W, Zheng MY, Konzak CF (2002a) Improving green plant production via isolated microspore culture in bread wheat (*Triticum aestivum* L.). Plant Cell Reprod 20:821–824

Liu W, Zheng MY, Polle EA, Konzak CF (2002b) Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. Crop Sci 42:686–692

Lux H, Herrmann L, Wetzel C (1990) Production of haploid sugar beet (*Beta vulgaris* L.) by culturing unpollinated ovules. Plant Breed 104: 177–183

Ma R, Guo YD, Pulli S (2004) Comparison of anther and microspore culture in the embryogenesis and regeneration of rye (*Secale cereale*). Plant Cell Tiss Org Cult 76(2):147–157

Mallikarjuna N, Jadhav D, Clarke H, Coyne C, Muehlbauer F (2005) Induction of Androgenesis as a Consequence of Wide Crossing in Chickpea. International Chickpea Pigeonpea Newsletter 12:12–15

Maluszynski M, Kasha KJ, Forster BP, Szarejko I (2003a) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London 2003. ISBN 1–4020–1544–5, pp 428

Maluszynski M, Kasha KJ, Szarejko I (2003b) Published protocols for other crop plant species In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 309–336

Marciniak K, Banaszak Z, W&ecedil;dzony M (1998) Effect of genotype, medium and sugar on triticale (×*Triticosecale* Wittm.) anther culture response. Cer Res Comm 26:145–151

Martinez L (2003) *In vitro* gynogenesis induction and doubled haploid production in onion (*Allium cepa* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 275–281

Mathys-Rochon E (2005) Secreted molecules and their role in embryo formation in plants: a minireview. Acta Biol Crac Ser Bot 47:23–29

Matzk F (1991) A novel approach to differentiated embryos in the absence of endosperm. Sex Plant Reprod 4:88–90
Matzk F, Mahn A (1994) Improved techniques for haploid production in wheat using chromosome elimination. Plant Breed 113:125–129
Matzk F, Meyer HM, Bäumlein H, Balzer HJ (1995) A novel approach to the analysis of the initiation of embryo development in Gramineae. Sex Plant Reprod 8:266–272
Mendiburu AO, Peloquin SJ, Mok DWS (1974) Potato breeding with haploids and 2n gametes. In: Kasha KJ (ed) Haploids in Higher Plants. University of Guelph, Guelph, pp 249–258
Michalik B, Adamus A, Nowak E (2000a) Gynogenesis in Polish onion cultivars. J Plant Physiol 156:211–216
Michalik B, Adamus A, Samek L, Nowak E (2000b) Gynogenesis in Polish onion cultivars: effect of temperature during donor plant growth. Biotechnological approaches for utilization of gametic cells. COST 824 final meeting, Bled, Slovenia, July 1–5, 2000, pp 91–94
Mihăilescu A, Giura A (1996) Production of winter barley haploids by Bulbosum system. 2. Influence of barley genotype on *in vitro* haploid regeneration. Romanian Agric Res 5–6:13–20
Monostori T, Lantos C, Mihály R, Pauk J (2003) Induction of embryogenesis without exogenous hormone-supplement in barley microspore culture. Cer Res Commun 31:297–300
Mordhorst AP Lörz H (1993) Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspores as influenced by the amount and composition of nitrogen sources in culture media. J Plant Physiol 142:485–492
Mouritzen P, Bach-Holm P (1994) Chloroplast genome breakdown in microspore cultures of barley (*Hordeum vulgare* L.) occurs primarily during regeneration. J Plant Physiol 144:586–593
Mujeeb-Kazi A, Riera-Lizarazu O (1997) Polyhaploid production in the Triticeae by sexual hybridization. In: Mohan-Jain S, Spory SK Veilleux RE (eds) *In vitro* Haploid Production in Higher Plants. Kluwer, Dordrecht, pp 276–295
Musiał K, Bohanec B, Przywara L (2001) Embryological study on gynogenesis in onion (*Allium cepa* L.). Sex Plant Reprod 13:335–341
Nageli M, Schmid JE, Stamp P, Buter B (1999) Improved formation of regenerable callus in isolated microspore culture of maize: impact of carbohydrates, plating density and time of transfer. Plant Cell Reprod 19:177–184
Navarro-Alvarez W, Baenziger PS, Eskridge KM, Shelton DR, Gustafson VD, Hugo M (1994) Effect of sugars in wheat anther culture media. Plant Breed 112:53–62
Nichterlein K (2003) Anther culture in linseed (*Linum usitatissimum* L.). In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plant – A Manual. Kluwer, Dordrecht/Boston/London, pp 249–254
Nichterlein K, Friedt W (1993) Plant regeneration from isolated microspore culture of linseed (*Linum usitatissimum* L.). Plant Cell Reprod 12:426–430
Nichterlein K, Umbach H, Friedt W (1991) Genotypic and exogenous factors affecting shoot regeneration from anther callus of lineseed (*Linum usitatissimum* L.) Euphytica 58:157–164
Nowak E (2000) Gynogenic onion plants – studies on regeneration and diploidization. Biotechnological approaches for utilization of gametic cells. COST 824 final meeting, Bled, Slovenia 1–5 July 2000, pp 95–99
Obert B, Pret'ová A, Buter B, Schmid JE (2000) Effect of different saccharides on viability of isolated microspores and androgenic induction in *Zea mays*. Biologia Plantarum 43:125–128
Obert B, Dedi ová B, Hricová A, Šamaj J, Pret'ová A (2004) Flax anther culture: effect of genotype, cold treatment and media, Plant Cell Tiss Org Cult 79:233–238
OhkawaY, Suenaga K, Ogawa K (1992) Production of haploid wheat plants through pollination of sorghum pollen. Jpn J Breed 42:891–894
Ohnoutková L, Novotny J, Mullerova E, Vagera J, Kucera L (2000) Is a cold pretreatment really needed for induction of *in vitro* androgenesis in barley and wheat? Proceedings of COST Action 824 –"Biotechnological approaches for utilisation of gametic cells", Bled Slovenia, July 1–5, 2000, pp 33–37

Oleszczuk S, Sowa S, Zimny J (2004) Direct embryogenesis and green plant regeneration from isolated microspores of hexaploid triticale (x *Triticosecale* Wittmack) cv. Bogo. Plant Cell Reprod 2:885–893
Olsen FL (1987) Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carsberg Res Commun 52:393–404
Ormerod AJ, Caligari PDS (1994) Anther and microspore culture of *Lupinus albus* in liquid medium. Plant Cell Tiss Org Cult 36:227–236
Orshinsky BR, McGregor LJ, Johnson GIE, Huel P, Kartha KK (1990) Improved embryoid induction and green shoot regeneration from wheat anthers cultured in medium with maltose. Plant Cell Reprod 9:365–369
Orshinsky BR Sadasivaiah RS (1994) Effects of media on embryoid induction and plant regeneration from cultured anthers of white spring wheats. Plant Sci 102:99–107
Otani M, Shimada T (1993) High frequency of pollen embryo formation in *Triticum aestivum* on maltose containing medium. Cereal Res Commun 21:11–15
Otani M, Shimada T (1994) Pollen embryo formation and plant regeneration from cultured anthers of tetraploid wheat. J Genet Breed 48:103–106
Palmer CE, Keller WA, Arnison PG (1996) Utilization of Brassica haploids. In: Jain SM, Sopory Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants, Vol. 3 Kluwer, Dordrecht, pp 173–192
Pande H. Bhojwani SS (1999) Promotion of Androgenesis in Rice Anther Cultures by Substitution of Sucrose with Maltose and Mannitol. Biologia Plantarum. 42(1):125–128
Pauk J, Puolimatka M, Tók L, Monostori T (2000) In vitro androgenesis of triticale in isolated microspore culture. Plant Cell Tiss Org Cult 61:221–229
Pauk J. Hassan MS, Puolimatka M, Lantos CS, Mihály R, Mesterházy A, Kertész Z, Matuz J (2003a) Microspore- and anther culture improvements for wheat breeding. In: Mujib, A, Cho MJ, Predieri S, Banerjee S (eds) *In Vitro* Application In Crop Improvement: Recent Progress, Science, Enfield, New Hampshire, USA, pp 131–151
Pauk J, Mihaly R, Monostori T, Puolimatka M (2003b) Protocol of triticale (x *Triticosecale* Wittmack) microspore culture. In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 129–134
Pechan PM, Bartels D, Brown DCW, Schell J (1991) Messenger-RNA and protein changes associated with induction of Brassica microspore embryogenesis. Planta 184:161–165
Pechan PM, Smykal P (2001) Androgenesis: Affecting the fate of male gametophyte. Physiol Plant 111:1–8
Peixe A, Campos MD, Cavaleiro C, Barroso J, Pais MS (2000) Gamma-irradiate pollen induces the formation of 2n endosperm and abnormal embryo development in European plum (*Prunus domestica* L., cv. “Rainha Claudia Verde”). Scientia Hort 86:267–278
Pickering RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: Shewry PR (ed) Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. CAB International, Oxford, pp 519–547
Pickering RA, Wallace AR (1994) Gibberellic acid + 2,4-D improves seed quality in *Hordeum vulgare* L. x *Hordeum bulbosum* L. crosses. Plant Breed 113:174–176
Pretová A, Obert B (2000) Progress in flax androgenesis. In: Bohanec B (ed) Biotechnological Approaches for Utilization of Gametic Cells Proceedings of COST 824 Final Meeting, Bled, Slovenia, July 1–5, 2000, pp 165–169
Puddephat IJ, Robinson HT, Smith BM, Lynn J (1999) Influence of stock plant pretreatment on gynogenic embryo induction from flower buds of onion. Plant Cell Tiss Org Cult 57:145–148
Pulli S, Guo JD (2003) Microspore culture of rye. In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 151–154

Puolimatka M, Laine S, PaukJ (1996) Effect of ovary cocultivation and culture medium on embryogenesis of directly isolated microspores of wheat. Cereal Res Commun 24:393–400
Ragot M, Steen P (1992) Genetic and environmental effect on chromosome doubling of sugar beet (*Beta vulgaris* L.) haploids. Euphytica 63:233–237
Rakoczy-Trojanowska M, miech M, Malepszy S (1997) The influence of genotype and medium on rye (*Secale cereale* L.) anther culture. Plant Cell Tiss Org Cult 48:15–21
Raminez C, Testillano PS, Pinto B, Moreno MA, Bueno MA, Risueno MC (2004) Changes in pectins and MAPKs related to cell development during early microspore embryogenesis in *Quercus suber* L. Eur J Cell Biol 83:213–225
Redha A, Attia T, Büter B, Stamp P, Schmidt JE (1998) Single and combined effects of colchicine, L-proline and post-inoculation low temperature on anther culture of wheat, *Triticum aestivum* L. Plant Breed 117:335–340
Redha A, Islam SMS, Büter B, Stamp P, Schmidt JE (2000) The improvement in regenerated doubled haploids from anther culture of wheat by anther transfer. Plant Cell Tiss Org Cult 63:167–172
Reynolds TL (2000) Effects of calcium on embryogenic induction and the accumulation of abscisic acid, and an early cysteine-labeled metallothionein gene in androgenic microspore of *Triticum aestivum*. Plant Sci 150:201–207
Riera-Lizarazu O, Mujeeb-Kazi A, William MDHM (1992) Maize (*Zea mays* L.) mediated polyhaploid production in some Triticeae using a detached tiller method. J Genet Breed 46:335–346
Riley R (1974) The status of haploid research. In: Kasha KJ (ed) Haploids in Higher Plants. Advances and Potential. Proceedings of the First International Symposium on Haploids in Higher Plants. University of Geulph, pp 3–9
Rines HW (2003) Oat haploids from wide hybridization. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 155–160
Ritala A, Mannonen L, Oksman-Caldentey K-M (2001) Factors affecting the regeneration capacity of isolated barley microspores. Plant Cell Reprod 20:403–407
Röber FK (1999) Fortpflanzungsbiologische und geneische Untersuchungen mit RFLP-Markern zur *in-vivo*-Haploideninduktion bei Mais. Dissertation, University of Hohenheim. Grauer Verlag, Stuttgart, pp. 124
Röber FK, Gordillo GA, Geiger HH (2005) *In vivo* haploid induction in maize – Performance of new inducers and significance of doubled haploid line in hybrid breeding. Maydica 50:275–284
Rodrigues LR, Terra TF, Bered F, Bodanese-Zanettini MH (2004) Origin of embryo-like structures in soybean anther culture investigated using SSR marker. Plant Cell Tiss Org Cult 77(3):287–289
Rokka VM (2003) Anther culture through direct embryogenesis in a genetically diverse range of potato (*Solanum*) species and their interspecific and intergeneric hybrids. In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 235–240
Rokka VM, Valkonen JPT, Pehu E (1995) Production and characterization of haploids derived from somatic hybrids between *Solanum brevidens* and *Solanum tuberosum* through anther culture. Plant Sci 112:85–95
Rokka VM, Pietilä L, Pehu E (1996) Enhanced production of dihaploid lines via anther culture of tetraploid potato (*Solanum tuberosum* ssp. *tuberosum*) clones. Am Potato J 73:1–12
Rudolf K, Bohanec B, HansenM (1999) Microspore culture of white cabbage, *Brassica oleracea* var. capitata L.: genetic improvement of non-responsive cultivars and effect of genome doubling agents. Plant Breed 118:237–241
Ryöppy PH (1997) Haploidy in triticale. In: Mohan Jain S, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants, Vol. 4 – Cereals (5):117–131
Saidi N, Chlyah O, Chlyach H (1998) Production of green durum wheat plants by pollination of wheat with maize. Can J Bot 76:652–656

Salmenkallio-Marttila M, Kurten U, Kauppinen D (1995) Culture conditions for efficient induction of green plants from isolated microspores of barley. Plant Cell Tiss Org Cult 43:79–81
Sarkar KR, Pandey A, Gayen P, Mandan JK, Kumar R, Sachan JKS (1994) Stabilisation of high haploid inducer lines. MNL 68:64–65
Sato S, Katoh N, Iwai S, Hagimori M (2002) Effect of low temperature pretreatment of buds or inflorescence on isolated microspore culture in *Brassica rapa* (syn. *B. campestris*). Breed Sci 52:23–26
Sator C, Mix G, Menge U (1983) Investigation on anther culture of *Lupinus polyphyllus*. Plant Res Dev 18:37–46
Scott P, Lyne RL (1994) The effect of different carbohydrate sources upon the initiation of embryogenesis from barley microspores. Plant Cell Tiss Orga Cult 36:129–133
Scott P, Lyne RL, Rees T (1995) Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare* L.). Planta 197:435–441
Shatskaya OA, Zabirova ER, Shcherbak VS Chumak MV (1994) Mass induction of maternal haploids in corn. MNL 68:51
Shen LY, Veilleux RE (1995) Effect of temperature shock and elevated incubation temperature on androgenic embryos yield of diploid potato. Plant Cell Tiss Org Cult 43:29–35
Shim YS, Kasha KJ (2003) The influence of pretreatment on cell stage progression and the time of DNA synthesis in barley (*Hordeum vulgare* L.) uninucleate microspores. Plant Cell Reprod 21:1065–1071
Simonson RL, Baenziger PS Gustafson D (1997) Wheat anther culture as affected by various cultural changes and supplements. J Appl Genet 38:381–392
Sitch LA, Snape JW (1986) Doubled haploid production in winter wheat and triticale genotypes using the *Hordeum bulbosum* system. Euphytica 35:1045–1051
Skrzypek E, Czyczyło-Mysza I, Marcińska I, Wędzony M (2008) Prospects of androgenie induction in Lupinus spp. Plant Cell Tiss Organ Cult 94:131–137
Ślusarkiewicz-Jarzina A, Ponitka A (1997) Effect of genotype and media composition on embryoid induction and plant regeneration from anther culture in triticale. J Appl Genet (Genet. Pol.) 38:253–258
Smykal P, Pechan PM (2000) Stress, as assessed by the appearance of sHsp transcripts, is required but not sufficient to initiate androgenesis. Physiol Plant 110: 135–143
Sun M, Kieft H, Zhou C, van Lammeren A (1999) A co-culture leads to the formation of microcalli derived from microspore protoplasts of *Brassica napus* L. cv. Topas. Protoplasma 208:265–274
Szarejko I (2003) Anther culture for doubled haploid production in barley (*Hordeum vulgare* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 35–42
Szarejko I, Forster BP (2007) Doubled haploidy and induced mutations. Euphytica 130: 359–370
Tai GCC, Xiong XY (2003) Haploid production of potatoes by anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 229–234
Telmer CA, Newcomb W, Simmonds DH (1993) Microspore development in *Brassica napus* and the effect of high temperature on division *in vivo* and in *vitro*. Protoplasma 172:154–165
Telmer CA, Newcomb W, Simmonds DH (1995) Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma 185:106–112
Testillano PS, Raminez C, Domenech J, Coronado MJ, Vergne P, Matthys-Rochon E, Risueno MC (2002) Young microspore-derived maize embryos show two domains with defined features also present in zygotic embryogenesis. Int J Dev Biol 46:1035–1047
Testillano PS, Georgiev S, Mogensen HL, Coronado MJ, Dumas C, Risueno MC, Matthys-Rochon E (2004) Spontaneous chromosome doubling results from nuclear fusion during *in vitro* maize induced microspore embryogenesis. Chromosoma 112:342–349

Thiagarajah MR, Stringam GR (1993) A comparison of genetic segregation in traditional and microspore-derived populations of *Brassica juncea* L. Czern and Coss. Plant Breed 111:330–334

Thomas WTB, Forster BP, Getersson B (2003) Doubled haploids in breedings. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 337–350

Thompson KF (1969) Frequencies of haploids in spring oil-seed rape (*Brassica napus*). Heredity 24:318–319

Thompson, KF (1972) Oil-seed rape. Rep. Pl. Breed. Inst. Cambridge, 1972, 94–96

Torp AM, Hansen AL, Andersen SB (2001) Chromosomal regions associated with green plant regeneration in wheat (*Triticum aestivum* L.) anther culture. Euphytica 119: 377–387

Touraev A, Heberle-Bors E (1999) Microspore embryogenesis and *in vitro* pollen maturation in tobacco. In: Hall BD (ed) Methods in Molecular Biology 111: Plant Cell Culture Protocols, Humana Press, Totowa, NJ, pp 281–291

Touraev A, Heberle-Bors E (2003) Anther and microspore culture in tobacco. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 223–228

Touraev A, Ilham A, Vincente O, Heberle-Bors E (1996a) Stress-induced microspore embryogenesis in tobacco: an optimised system for molecular studies. Plant Cell Reprod 15:561–565

Touraev A, Pfosser M, Vincente O, Heberle-Bors E (1996b) Stress as a major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. Planta 200:144–152

Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. Sex Plant Reprod 9:209–215

Touraev A, Vicente O, Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends Plant Sci 2:297–302

Touraev A, Pfosser M, Heberle-BorsE (2001) The microspore: a haploid multipurpose cell. Adv Bot Res 35:53–109

Trejo-Tapia G, Amaya UM Morales GS, De Js'us S'anchez A, Bonfil BM, Rodriguez-Monroy M, Jim'enez-Aparicio A (2002) The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. Plant Cell Tiss Org Cult 71:41–46

Tuvesson IKD, Pedersen S, Andersen SB (1989) Nuclear genes affecting albinism in wheat (*Triticum aestivum* L.) anther culture. Theor Appl Genet 78:879–883

Tuvesson IKD, Pedersen S, Olesen A, Andersen SB (1991) An effect of the 1BL/1RS chromosome on albino frequency in wheat (*Triticum aestivum* L.) anther culture. J Genet Breed 45:345–348

Tuvesson S, Von Post R, Ljungberg A (2003) Triticale anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 117–121

Ushiyama T, Shimizu T, Kuwabara T (1991) High frequency of haploid production of wheat through intergeneric cross with teosinte. Jpn J Breed 41:353–357

Van Geyt JPS, Jacobs M (1986) Induction of haploids of sugar beet by means of androgenesis and gynogenesis. Bulletin de la Soc Botan De France 133:83–92

Wang M, Farnham MW, Nannes JSP (1999) Ploidy of broccoli regenerated from microspore culture versus anther culture. Plant Breed 118:249–252

Wędzony M (2003) Protocol for anther culture in hexaploid triticale (*Triticosecale Wittm.*). In: Maluszynski M, Kasha KJ, Forster BP Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 123–128

Wędzony M, van Lammeren AAM (1996) Pollen tube growth and early embryogenesis in wheat x maize crosses influenced by 2,4-D. Annals Bot 77:639–649

Wędzony M, Marcińska I, Ponitka A, Ślusarkiewicz-Jarzina A, Woźna J (1998a) Production of doubled haploids in triticale (*xTriticosecale* Wittm.) by means of crosses with maize (*Zea mays* L.) using Picloram and Dicamba. Plant Breed 117:211–215

Wędzony M, Marciñska I, Ponitka A, Ślusarkiewicz-Jarzina A, Woźna J (1998b) Factors influencing triticale doubled haploid production by means of crosses with maize. In: Juskiw P (ed) Proceedings of the Fourth International Triticale Symposium Vol. 1. Lectures. Red Deer, Canada, July 26–30, 1998, pp 45–52

Wędzony M, Röber FK, Geiger HH (2002) Chromosome elimination observed in selfed progenies of maize inducer line RWS. In: XVIIth Int Cong Sexual Plant Repro. Maria Curie-Sklodowska University Press, Lublin 2002, p 173

Wojciechowska B, Pudelska H (1993) Hybrids from reciprocal barley-wheat crosses. Genet Pol 34:1–13

Wojnarowiez G, Jacquard C, Devaux P, Sangwan RS, Clement C (2002) Influence of copper sulfate on anther culture in barley (*Hordeum vulgare* L.). Plant Sci 162:843–847

Wremerth-Weich E, Levall M (2003) Doubled haploid production of sugar beet (*Beta vulgaris* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 255–265

Xie JH, Gao MW, Cai QH, Liang ZQ, Xue QZ (1996) Effects of donor plant growth status preculture temperature on isolated microspore culture in japonica rice (*Oryza sativa* L.). Cer Res Comm 24(2):133–138

Zagorska N, Dimitrov B (1995) Induced androgenesis in alfalfa (*Medicago sativa* L.). Plant Cell Reprod 14:249–252

Zakhariev A, Kikindonov G (1997) Possibilities of haploidy application in the sugar beet breeding. Plant Sci 34:28–30

Zaki MAM, Dickinson HG (1990) Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma Wien 156:149–162

Zaki MAM, Dickinson HG (1991) Microspore-derived embryos in Brassica: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex Plant Reprod 4:48–55

Zarsky V, Garrido D, Rihova L, Tupy J, Vicente O, Heberle-Bors E (1992) Depression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sex Plant Reprod 5:189–194

Zarsky V, Garrido D, Eller N, Tupy J, Vicente O, Schöffl F, Heberle-Bors E (1995) The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ 18:139–147

Zatapa-Arias FJ (2003) Laboratory protocol for anther culture technique in rice. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plant – A Manual. Kluwer/Boston/London, pp 109–116

Zenkteler M, Nitzsche W (1984) Wide hybridization experiments in cereals. Theor Appl Genet 68:311–315

Zhao JP, Simmonds DH (1995) Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. Physiol Plant 95:304–309

Zhao JP, Simmonds DH, Newcomb W (1996a) High frequency production of doubled-haploid plants of *Brassica napus* cv. Topas derived from colchicine-induced microspore embryogenesis without heat shock. Plant Cell Reprod 15:668–671

Zhao JP, Simmonds DH, Newcomb W (1996b) Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. Planta 198:433–439

Zheng MY, Konzak CF (1999) Effect of 2–4-dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture of wheat (*Triticum aestivum* L.). Plant Cell Reprod 19:69–73

Zheng MY, Liu W, Weng Y, Polle E, Konzak CF (2001) Culture of freshly isolated wheat (*Triticum aestivum* L.) microspores treated with inducer chemicals. Plant Cell Reprod 20:685–690

Zheng MY, Liu W, Weng Y, Polle E, Konzak CF (2002a) Production of doubled haploids in wheat (*Triticum aestivum* L.) through microspore embryogenesis triggered by inducer chemicals. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 83–94

Zheng MY, Weng Y, Liu W, Konzak CF (2002b) The effect of ovary-conditioned medium on microspore embryogenesis in common wheat (*Triticum aestivum* L.). Plant Cell Reprod 20:802–807

Zheng MY, Weng Y, Sahibzada R, Konzak CF (2003) Isolated microspore culture in maize (*Zea mays* L.) production of doubled-haploid via induced androgenesis. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants – A Manual. Kluwer, Dordrecht/Boston/London, pp 95–102

Zhou H, Konzak CF (1992) Genetic control of green plant regeneration from anther culture of wheat. Genome 35:957–961

Zhou H, Konzak CF (1997) Influence of genetic and environmental factors on anther culture response of wheat. J Appl Genet 38:393–406

Zhou C, Yang HY (1981) Induction of haploid rice platelets by ovary culture. Plant Sci Lett 20:231–237

Zhou H, Zheng Y, Konzak CF (1991) Osmotic potential of media affecting green plant percentage in wheat anther culture. Plant Cell Reprod 10:63–66

Zhou WJ, Ahgberg P, Tang GX (2002) Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspores in spring *Brassica napus*. Euphytica 128:27–34

Zhu ZC, Wu HS, An QK, Liu ZY (1981) Induction of haploid plantlets from the unpollinated ovaries of *Triticum aestivum* culture *in vitro*. Acta Genet Sin 8:386–389

Ziauddin A, Simion E, Kasha KJ (1990) Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cv. Igri. Plant Cell Reprod 9:69–72

Ziauddin A, Marsolais A, Simion E, Kasha KJ (1992) Improved plant regeneration from wheat anther and barley microspore culture using phenylacetic acid (PAA). Plant Cell Reprod 11:489–498

Chapter 2
Doubled Haploids *via* Gynogenesis

B. Bohanec

Abstract Gynogenic haploid regeneration is an alternative procedure for haploid induction used in several agronomically important species such as sugar-beet and onion. At inoculation, developmental stage of ovules is frequently immature, but in contrast to androgenesis, *in vitro* maturation of embryo sac occurs. The egg cells were found to be the predominant source of haploid embryos while antipodal or synergid origin has been also proposed for some species. For majority of species triggering factors promoting haploid embryogenesis are not apparent, but media constituents such as phytohormons and carbohydrates evidently have some role in reprogramming. The majority of regenerants obtained via gynogenesis are haploid, for optimization of treatments large experimental units are proposed and alternative to the use of antimitotic agents spontaneous duplication via adventitious regeneration is proposed. Major characteristics of successful protocols are listed.

Keywords Ovule stages at inoculation, origin of gynogenic embryos, triggering factors, chromosome doubling procedures

Introduction

Haploid regeneration using un-pollinated female gametophytes is an alternative process for haploid induction. The process is usually described by the term gynogenesis, or haploid parthenogenesis. Often, the term gynogenesis has been reserved for haploid embryogenesis induced by the presence of a male sperm cell, which does succeed in contributing any genetic material to the embryo; it is used as such in the animal kingdom. In plant terms, gynogenic haploid regeneration is widely used for all haploid induction methods in which a female gametophyte is used as the origin of haploid cells, regardless of whether a pseudo-fertilization process is involved or not. In this chapter,

B. Bohanec(✉)
University of Ljubljana Biotechnical Faculty, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.
E-mail: borlut.bohanec@bf.uni-lj-si

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

the term gynogenesis will be used in this sense as a substitute for the longer descriptive explanation – haploid induction from un-pollinated ovules, ovaries or flower buds.

Historically, the first haploid plants originating from female gametophytes were described in *Datura stramonium* (Blakeslee et al. 1922). These haploid plants formed spontaneously were subsequently found in other species at low frequencies. A more recent review of spontaneously formed haploid plants is given by Palmer and Keller (2005) citing the occurrence of haploid plants in tobacco, rice, maize, *Brassica* and barley. The first *in vitro* induced haploid plants of gynogenic origin were achieved by San Noeum (1976) in barley; several earlier attempts to induce haploids by culturing unfertilized ovules failed (Yang and Zhou 1982). Despite other successful examples in a number of plant species, it should be mentioned that culturing unfertilized female ovules has often ended in failure in inducing either un-organized or organized haploid tissues. Some failures and early attempts are reviewed by Laksmi-Sita (1997), Mukhambetzhanov (1997) and, more recently, for fruit crops by Germana (2006) although, in general, failures to induce haploid plants are often not published. The occasionally held belief that female gametophytes, given appropriate stimulation, can be easily induced to form multi-cellular structures and subsequent embryo formation is therefore incorrect. In fact, 86 years after the first discoveries, haploid induction by culturing un-pollinated ovules is still more or less problematic and limited to a relatively small number of plant species. The chapter will concentrate on critical aspects of doubled haploid induction using unfertilized female gametophytes, while emphasizing the numerous and recent literature.

Developmental Stage at Inoculation

The pollen developmental stage is one of most important factors in androgenesis. Late uni-cellular microspore is often the most responsive target for re-programming a pathway from gametophytic into sporophytic. There is a general opinion that, in contrast to *in vitro* androgenesis, it is possible to induce a gynogenic response from ovules over a broad range of developmental stages. Data supporting this opinion are to some extent limited. The stage of ovule development at the time of inoculation has not often been clearly determined. Several authors prefer to describe the inoculation stage according to the developmental stage of the flower bud or stage of pollen development. In several species, male and female gametophytes do not mature simultaneously, a phenomenon known as protandry, the maturation of anthers before carpels (e.g., onion, leek, sunflower, sugar beet, carrot,) and the opposite protogyny (e.g., pearl millet). Direct comparison using the pollen developmental stage is therefore problematic. Microscopic analysis of embryo sacs is tedious and requires a skilful embryologist for interpretation of the results. There are therefore much less data available compared to anther/microspore culture, although the embryo sac developmental stages at the time of inoculation have been determined for a number of species. In sugar beet, flower buds collected 1–3 days before anthesis possessed a mature embryo sac (Ferrant and Bouharmont 1994). A sugar beet embryo sac is capable of being fertilized 5 days before anther dehiscence, but

VanGeyt et al. (1987) reported a degeneration of young spherical ovules after a few days in culture. In sunflower, flowers possess a young but completely developed embryo sac 2–3 days before anthesis (Yang et al. 1986). Uninucleate to four-nucleate embryo sacs, corresponding to late uninucleate or early-binucleate stage of pollen were found in rice (Zhou et al. 1986) and were reported as superior to earlier or mature stages. Similar results were obtained in onion (Musial et al. 2005), in which the smallest flowers predominantly (containing megaspore mother cells) and largest (containing mature embryo sacs) were less responsive than medium-size flowers, containing 2–4 nucleate embryo sacs.

With the exception of mulberry, barley and maize, other species (onion, sugar beet, squash, sunflower, gerbera, *Hyosciamus muticus* and *Melandrium album*) have been reported to be optimally inoculated at earlier flower developmental stages.

In Vitro Maturation of Embryo Sacs

The second characteristic of culturing of un-pollinated ovules is their ability to continue their maturation process after inoculation. This phenomenon is in major contrast with androgenic haploidy induction, in which *in vitro* maturation of microspores into pollen grains can only be induced by specific media and has so far only been demonstrated in a few species. Mature pollen grains are also completely differentiated and unable to re-differentiate into haploid embryos. Data existing for gynogenic embryo development indicate that the maturation process of embryo sacs continues during *in vitro* culture. The developmental pattern of the female gametophyte in excised ovaries *in vitro* has been studied in onion, barley, rice, tobacco, *Cenchus ciliaris* and *Melandrium album* (Musial et al. 2005). In an experiment with onion in which ovaries at different developmental stages were fixed at inoculation and then at 3 day intervals, *in vitro* maturation of the embryo sacs was evident. At day 12 in culture, only mature embryo sacs were present, while the level of degenerated embryo sacs varied according to the initial stage at inoculation (Musial et al. 2005). A maturation process similar to *in situ* development has been found in studied species, the difference from *in situ* maturation being mainly expressed at a higher frequency of degenerating ovules.

Origin of Embryos

Embryo sacs possess haploid egg cells, but also other cells theoretically capable of forming a haploid embryo, such as synergids, antipodal cells or non-fused polar nuclei. The majority of records indicate that it is the egg cell that is the predominant source of haploid embryos: *Beta vulgaris* (Ferrant and Bouharmont 1994), *Allium cepa* (Musial et al. 2001, 2005), *Helianthus annuus* (Gelebart and San 1987), *Hevea brasiliensis* (Guo et al. 1982), *Hordeum vulgare* (Huang et al. 1982),

Melandrium album (Mol 1992) and *Nicotiana tabacum* (Wu and Chen 1982). An antipodal or synergid origin of embryos has been proposed in *Hordeum vulgare* (San Noeum 1979) and *Oryza sativa* (Zhou et al. 1986). A detachment of the egg cell at early stages of development has also been noted at least in onion and sugar beet (Ferrant and Bouharmont 1994; Musial et al. 2001), and suspensor attached embryos have been found in sugar beet and mulberry (Pedersen and Keimer 1996; Bhojwani et al. 2003). These reports, showing different responses in individual species should be seen in the context of the large variation in cell types of mature embryo sac. In angiosperms these are classified as monosporic, bisporic and tetrasporic, with the monosporic *Polygonum* type being the most common (for a review see Yadegari and Drews 2004). In addition to the major morphological differences, several other characteristics, such as early degeneration of antipodal cells or endo-reduplication of the nuclei within sinergids, clearly determine the egg cell as the predominant source of haploid embryos.

Triggering Factors

Stress treatments are the most common factor affecting embryogenesis and androgenesis, with cold/heat shock and/or starvation treatment being common pre-treatments. There are relatively few reports of the same stress treatments playing a decisive role in gynogenic embryo induction. Donor plant growth conditions favouring low temperatures and high illumination has been reported to be beneficial for onion (Puddephat et al. 1999). Cold treatment was found to be beneficial in durum wheat (Sibi et al. 2001) and sugar beet (Lux et al. 1990; Gurel et al. 2000) and heat treatment promoted embryogenesis in cucumbers (Gemes-Juhasz et al. 2002). Despite the cited examples of a positive influence of cold or heat treatment, the opposite has also been reported, for instance in rice (Zhou et al. 1986), in addition no apparent stress treatment has been used in the majority of other protocols.

In vitro culture itself, or common media constituents, can also be seen as a stress factor. The requirement for macro and micro elements in the basal induction medium is interesting, and again in contrast with androgenesis. The macro and micro elements for gynogenesis of seventeen species are listed in Table 1. These are almost exclusively those commonly used for micro-propagation. This suggests that media formulae are designed more for supporting the growth requirements of haploid embryos than for re-programming the gametophytic pathway into a sporophytic development. Other medium constituents vary to a large extent among the listed species, although phytohormones can be regarded as inducing factors. However, as with micro-propagation, the quantity of phytohormones used for gynogenic embryogenesis varies greatly among plant species. The most commonly used are auxins and cytokinins, others are rare or have subsequently been found to be less useful. Novel polyamines have been reported in onion as a possible replacement for auxins and cytokinins (Martinez et al. 2000), and these empirical results were recently supported by physiological evidence (Geoffriau et al. 2006). The

situation with carbohydrates is similar to phytohormones. A high concentration (usually sucrose) is beneficial for gynogenesis, particularly in monocot species (onion, barley, millet, durum wheat and maize), while in some other listed species (e.g., gerbera) the carbohydrate requirement is low.

It remains unclear what the triggering factors are (except pollination inductions). For instance, it is clear that, in general, the addition of appropriate phytohormones stimulates gynogenic development. Omitting 2,4-D from the induction medium in onion genotypes pre-selected for high gynogenic response resulted in only a minor decrease of haploid embryo formation, but with much faster embryo emergence (J. Jakše and B. Bohanec, unpublished). At least in onion, therefore, 2,4-D does to some extent induce development whilst simultaneously delaying the regeneration process. Much more data are needed to draw final conclusions on the triggering factors in non-pollinated ovule culture systems.

Ploidy and Chromosome Doubling Procedures

A characteristic of gynogenic haploid induction is a low percentage of spontaneous chromosome doubling, resulting in the majority of regenerants being haploid. Data (Table 1) giving high proportions of diploid regenerants are often preliminary and based on a low number of haploids, or in some cases not proven to be homozygous. The situation is similar to gynogenic induction induced by pollination treatments also resulting predominantly in haploid regenerants. Approaches to chromosome doubling have been reviewed by Kasha (2005). Only a few other observations should be added. Large numbers of treated individuals are often needed to obtain reproducible results. In the author's experience, diploidization treatments in onion embryos were repeatable when the experimental unit was is high (400–500 embryos per treatment, Jakše et al. 2003). Another observation is that the optimal treatment should be a compromise between the efficiency in chromosome doubling and the mortality of treated tissues. The latter data are often not given. Chemicals used to induce chromosome doubling (spindle inhibitors or anti-microtubule agents) target the whole meristematic domain, resulting in a large proportion of mixoploid plants. An alternative approach to doubling the chromosomes of haploid plants can be based on spontaneous chromosome doubling during adventitious *in vitro* regeneration, instead of chemical treatments. Such treatment of diploid tissues often leads to increased ploidy. For instance in hop, Škof et al. (2007) induced up to 58.6% tetraploids. In this case, doubled regenerants are probably regenerated from a single doubled cell and, as such, often do not possess mixoploid tissues. This approach has already been attempted in haploid onion plants (Alan et al. 2007), in which regeneration from flower buds resulted in 60.7% of spontaneously doubled plants. The low frequency of mixoploidy, low mortality and simultaneous chromosome doubling and clonal multiplication of breeding lines are why an *in vitro* adventitious regeneration approach used for chromosome doubling deserves further attention.

Table 1 List of successful protocols for gynogenic induction using non-pollinated organs: selected key constituents of induction media and the highest frequency of haploid induction and proportion of haploid regenerants obtained

Type of explant	Main constituents of typical induction medium (minor variations not given)	Highest haploid induction frequency obtained*	Proportion of haploid regenerants	References
Onion, Shallot *Allium cepa* L.				
Ovaries, ovules or whole flower buds	B5 (or BDS or MS), 2 mg/l 2,4-D + 2 mg/l BAP, 10% (or 7.5%) sucrose, (often + glutamine 800 mg/l, myo-inositol 500 mg/l)	Varieties up to 22.6%, selected genotypes up to 196.5%	Around 70–90%, with tendency for higher haploid proportions to be detected when large number of regenerants were measured	Muren (1989),; Campion et al. (1992); Cohat (1994); Bohanec et al. (1995); Jakse et al. (1996); Javornik et al. (1998); Bohanec, Jakše (1999); Puddephat et al. (1999); Michalik et al. (2000); Bohanec et al. (2003); Alan et al. (2003); Alan et al. (2004)
Flower buds	BDS, 2 mM putrescine, 10% sucrose,	9.5%		Martinez et al. (2000)
Flower buds	BDS, 2 mg/l 2,4-D, 2 mg/l BAP, CCC 0.1 g/l (CCC sprayed on umbels), 10% sucrose	4.76% regenerants	62.5%	Ponce et al. (2006)
Sugar beet, Red beet *Beta vulgaris* L.				
Ovules	N6 (or MS), 0.5 mg/l IAA, 0.2 mg/l BAP, 60 g/l sucrose	12.7%		Doctrinal et al. (1989); Ferrant and Bouharmont (1994); Barański (1996)
Ovules	MS, 2.0 mg/l BAP, 0.5% activated charcoal, 10% sucrose,	18.3%		Gurel et al. (2000)
Cucumber *Cucumis sativus* L.				
Ovaries	CBM, 0.02 mg/l TDZ + 40 g/l sucrose	18.4%	87.7%	Gemes-Juhász et al. (2002)
Squash *Cucurbita pepo* L.				
Ovaries	MS, 1 or 5 mg/l 2,4-D, 3% sucrose	11.5%	25%	Metwally et al. (1998)

(continued)

Table 1 (continued)

Type of explant	Main constituents of typical induction medium (minor variations not given)	Highest haploid induction frequency obtained*	Proportion of haploid regenerants	References
Tef *Eragostis tef* (Zuccagni) Trotter				
Pistils or panicle segments	MS, 2–4 mg/l 2,4-D, 0.2 mg/l BAP, carbohydrate content not given	12% callus forming pistils, 38.6% of callus formation from panicle segments	2.7%	Gugsa et al. (2006)
Gerbera *Gerbera jamesonii* Bolus ex Hooker				
Ovules	MS, 2 mg/l BAP, 2 mg/l kinetin, 0.5 mg/l IAA, 2% sucrose	Calli with roots		Sitbon (1981)
Ovules	½ MS, 0.3 mg/l benzyl adenine, 0.05 mg/l IAA, 0.1 mg/l GA_3, 2% sucrose	6.2% callus formation	78.4%	Meynet and Sibi (1984)
Ovules	MS, 0.2 mg/l BA, 0.1 mg/l IAA, 1% sucrose	17.5% callus formation	80.4%	Miyoshi and Asakura (1996); Tosca et al. (1999)
Sunflower *Helianthus annuus* L.				
Ovules	N6, 0.5–2.0 mg/l MCPA, 6% sucrose	4.8%		Yang et al. (1986)
Egyptian henbane *Hyoscyamus muticus* L.				
Ovaries	MS, 2.0 mg /l IAA, 0.5 mg/l BAP, carbohydrate not given	35.7% of callus formation (regeneration reported)	25%	Chand and Basu (1998)
Barley *Hordeum vulgare* L.				
Ovaries	N6, 0.5 mg/l IAA, 0.1 mg/l BA, 0.12 mg/l MCPA, 9% sucrose	0.75% regenerated haploid plantlets		Castillo and Cistué (1993)
Easter lily *Lillium longiflorum* L.				
Ovaries	MS, 1 mg/l 2,4-D, 2 mg/l BA, 5% sucrose	45.0%		Ramsay et al. (2003)
Melandrium album (Mill.) Garcke				
Placenta attached ovules	Miller's basal medium, 2.0 mg/l 2,4-D, 6% sucrose	6.6%		Mol (1992)
Mulberry *Morus alba* L.				
Ovaries	MS, 1 mg/l 2,4-D, 0.2 mg/l BAP, 3% sucrose	16%	60%	Thomas et al. (1999)

(continued)

Table 1 (continued)

Type of explant	Main constituents of typical induction medium (minor variations not given)	Highest haploid induction frequency obtained*	Proportion of haploid regenerants	References
Rice *Oryza sativa* L.				
Ovaries	N6 + 2 mg/l NAA, 1.0 mg/l BA, 0.6–0.8% DMSO, 5% sucrose	12.5% callus formation	52%	Rongbai et al. (1998); Rongbai et al. (1999)
Millet *Panicum miliacem* L.				
Ovaries	MS or ½ N6, 0.5 mg/l BA, 0.1 mg/l NAA, 9% sucrose	1.4%		Kashin et al. (2000)
White sails *Spathiphyllum wallisii* Regel				
Ovaries	MS macro, Nitch and Nitch (1969) micro salts, 0.05 mg/l TDZ, 0.4 mg/l PIC, 0 or 4.45 mg/l imazalil, 5.7% sucrose	26.3%	Two diploid homozygous genotypes	Eeckhaut et al. (2001)
Durum wheat *Triticum durum* Desf.				
Ovaries	½ MS, 2 mg/l 2,4-D, 1 mg/l kinetin, 12% sucrose	24.1% of callus formation	100%	Mdarhri-Alaoui et al. (1998)
Ovaries	modified Jähne et al. (2001), 2 mg/l 2,4-D, 0.5 mg/l kinetin, 6% maltose	17.1% buds on calli	Predominantly haploid	Sibi et al. (2001)
Maize *Zea mays* L.				
Ovaries	MS, 3 mg/l 2,4-D, 12% sucrose	8.3%	Predominantly mixoploid	Truong-Andre and Demarly (1984)

*Percentage often reported as embryos per flower bud, which can contain several ovules.

Genetic Influence and Further Prospects

A genotype specific response is a familiar characteristic of almost all tissue culture systems, including somatic adventitious regeneration, androgenesis and gynogenesis. As reviewed by Mukhambetzhanov (1997), genotype has often been reported to be the most important factor for the success of gynogenic induction. The genotype effect has also been confirmed in other more recent publications. In onion, for instance, non-preselected accessions differ in induction frequency among 22 European accessions from 0–17% (Geoffriau et al. 1997), among 39 European, American and Japanese accessions from 0–22.6% (Bohanec and Jakše 1999), among 10 Polish cultivars from 0–10%, but in preselected onion genotypes reached

much higher induction frequencies, such as 196.5% for a doubled haploid line (Javornik et al. 1998) or 82.2% for an inbred line (Bohanec et al. 2003).

Little is known about gene action in gynogenesis. Bohanec et al. (2003), using hybrid low and high yielding onion genotypes, proposed quantitative inheritance of gynogenic regeneration with a dominance towards low production. It should be mentioned that for plant breeding purposes, identification of genotypes with high gynogenic potential is applicable, since such lines can be used as "inducers" for crosses with low responsive breeding material, although the need for backcrossing delays the breeding process.

Even less is known about the molecular genetic mechanisms that trigger gynogenic development. Molecular genetic studies of (to some extent a similar process of embryo development) apomixis at first speculated that apomixis was simply inherited, but it has recently been recognised as being very complex (Matzk et al. 2005). We might speculate that similar to apomixis revealing genetic mechanisms leading to haploid embryo development require similar large scale studies to identify candidate genes. Low and high responsive lines, which exist for species such as onion or sugar beet, might be used as an important genetic base for such studies.

Despite the lack of molecular and basic genetic knowledge, gynogenic haploid systems have already been applied for other purposes, such as genetic studies of inheritance (Kim et al. 2004) or stabilization of characters after interspecific hybridization (Sulistyaningsih et al. 2002; Alan et al. 2003).

Gynogenesis, as a possible approach for haploid induction, has become one of the available options in species with which other methods have failed or have been prone to mutation induction in the process of regeneration. Although mechanisms redirecting female gametophytic cells into sporophytic development have still not been discovered, substantial progress has been made in optimizing induction procedures, at least for economically important species.

References

Alan AR, Mutschler MA, Brants A, Cobb E, Earle ED (2003) Production of gynogenic plants from hybrids of *Allium cepa* L. and *A. roylei* Stearn. Plant Sci 165: 1201–1211

Alan AR, Brants A, Cobb E, Goldschmied PA, Mutschler MA, Earle ED (2004) Fecund gynogenic lines from onion (*Allium cepa* L.) breeding materials. Plant Sci 167: 1055–1066

Alan AR, Lim W, Mutschler MA, Earle ED (2007) Complementary strategies for ploidy manipulations in gynogenic onion (*Allium cepa* L.) Plant Sci 173: 25–31

Bara ski R (1996) *In vitro* gynogenesis in red beet (*Beta vulgaris* L): Effects of ovule culture conditions. Acta Soc Bot Pol 65: 57–60

Bhojwani SS, Chaturvedi R, Raina A (2003) Tissue culture of woody species. In: Bhojwani SS, Soh WY (eds) Agrobiotechnology and plant tissue culture, Science Publishers, Enfield, Plymouth, pp 57–80

Blakeslee AF, Belling J, Farnham ME.; Bergner AD (1922) A haploid mutant in the Jimson weed, '*Datura stramonium*'. Science 55: 646–647

Bohanec B, Jakše M (1999) Variations in gynogenic response among long-day onion (*Allium cepa* L.) accessions. Plant Cell Rep 18: 737–742

Bohanec B., Jakše M, Ihan A, Javornik B (1995) Studies of gynogenesis in onion (*Allium cepa* L.) – induction procedures and genetic analysis of regenerants. Plant Sci 104: 215–224
Bohanec B, Jakše M, Havey MJ (2003) Genetic analyses of gynogenetic haploid production in onion. J Am Soc Hortic Sci 128: 571–574
Campion B, Azzimonti MT, Vicini E, Schiavi M, Falavigna A (1992) Advances in haploid plant induction (*Allium cepa* L.) through *in vitro* gynogenesis. Plant Sci 86: 97–104
Castillo AM, Cistué L (1993) Production of gynogenic haploids of *Hordeum vulgare* L. Plant Cell Rep 12: 139–143
Chand S, Basu P (1998) Embryogenesis and plant regeneration from callus cultures derived from unpollinated ovaries of *Hyoscyamus muticus* L. Plant Cell Rep 17: 302–305
Cohat J (1994) Production of gynogenetic plants by *in vitro* culture of flower buds in shallot (*Allium cepa* L. var aggregatum). Agronomie 14: 299–304
Doctrinal M, Sangwan RS, Sangwan-Norreel BS (1989) *In vitro* gynogenesis in *Beta vulgaris* L. – effects of plant growth regulators, temperature, genotypes and season. Plant Cell Tiss Org Cult 17: 1–12
Eeckhaut T, Werbrouck S, Dendauw J, Van Bockstaele E, Debergh P (2001) Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell Tiss Org Cult 67: 181–189
Ferrant V, Bouharmont J (1994): Origin of gynogenetic embryos of *Beta vulgaris* L. Sex Plant Reprod 7: 12–16
Gelebart P, San LH (1987) Production of haploid plants of sunflower (*Helianthus annuus* L) by *in vitro* culture of non-fertilized ovaries and ovules. Agronomie 7: 81–86
Gemes-Juhasz A, Balogh P, Ferenczy A, Kristof Z (2002) Effect of optimal stage of female gametophyte and heat treatment on *in vitro* gynogenesis induction in cucumber (*Cucumis sativus* L.). Plant Cell Rep 21: 105–111
Germana M (2006) Doubled haploid production in fruit crops. Plant Cell Tiss Org Cult 86: 131–146
Geoffriau E, Kahane R, Martin-Tanguy J (2006) Polyamines are involved in the gynogenesis process in onion. Physiol Plant 127: 119–129
Geoffriau E, Kahane R, Rancillac M (1997) Variation of gynogenesis ability in onion (*Allium cepa* L). Euphytica 94: 37–44
Gugsa, Likyelesh; Sarial, Ashok; Lörz, Horst; Kumlehn, Jochen (2006) Gynogenic plant regeneration from unpollinated flower explants of *Eragrostis tef* (Zuccagni) Trotter. Plant Cell Rep 25: 1287–1293
Guo F, Jia Xj, Chen LX (1982) Induction of plantlets from isolated ovules of *Hevea brasiliensis*. Hereditas 4: 27–28
Gurel S, Gurel E, Kaya Z (2000) Doubled haploid plant production from unpollinated ovules of sugar beet (*Beta vulgaris* L.) Plant Cell Rep 19: 1155–1159
Huang QT, Yang HY, Zhou C (1982) Embryological observations on ovary culture of unpollinated young flowers in *Hordeum vulgare* L. Acta Bot Sin 24: 295–300
Jähne A, Lazzeri PA, Jagergussen M, Lörz H (1991) Plant-regeneration from embryogenic cell-suspensions derived from anther cultures of barley (*Hordeum vulgare* L.) Theor Appl Genet 82: 74–80
Jakše M, Havey MJ, Bohanec B (2003) Chromosome doubling procedures of onion (*Allium cepa* L.) gynogenic embryos. Plant Cell Rep 21: 905–910
Javornik B, Bohanec B, Campion B (1998) Second cycle gynogenesis in onion, *Allium cepa* L, and genetic analysis of the plants. Plant Breed 117: 275–278
Kasha KJ (2005) Chromosome doubling and recovery of doubled haloid plants. In: Palmer CE, Keller WA, Kasha KJ (eds) Biotechnology in agriculture and forestry, Vol. 56. Haploids in crop improvement, Springer, Berlin/Heidelberg, pp 123–152
Kashin AS, Blyudneva EA, Silkin MA (2000) Megagamete activation and regulation of *in vitro* embryogenesis in unpollinated millet ovaries. Russ J Plant Physiol 47: 260–269
Kim S, Binzel ML, Yoo KS, Park S, Pike LM (2004) Pink (P), a new locus responsible for a pink trait in onions (*Allium cepa*) resulting from natural mutations of anthocyanidin synthase. Mol Genet Genomics 272: 18–27

Laksmi-Sita G (1997) Gynogenic haloids *in vitro*. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants. Vol. 5. Kluwer, Dordrecht, pp 175–193
Lux H, Herrmann L, Wetzel C (1990) Production of haploid sugar-beet (*Beta vulgaris* L) by culturing unpollinated ovules. Plant Breed 104: 177–183
Martinez LE, Aguero CB, Lopez ME, Galmarini CR (2000) Improvement of *in vitro* gynogenesis induction in onion (*Allium cepa* L.) using polyamines. Plant Sci 156: 221–226
Matzk F, Prodanovic S, Baumlein H, Schubert I (2005) The inheritance of apomixis in *Poa pratensis* confirms a five locus model with differences in gene expressivity and penetrance. Plant Cell 17: 13–24
Mdarhri-Alaoui M, Saidi N, Chlyah A, Chlyah H (1998) Green haploid plant formation in durum wheat through *in vitro* gynogenesis. CR Acad Sci Serie III – Life 32: 25–30
Metwally EI, Moustafa SA, El-Sawy BI, Haroun SA, Shalaby TA (1998) Production of haploid plants from *in vitro* culture of unpollinated ovules of *Cucurbita pepo*. Plant Cell Tiss Org Cult 52: 117–121
Meynet J, Sibi M (1984) Haploid plants from *in vitro* culture of unfertilized ovules in *Gerbera jamesonii*. Z Pflanzenzucht 93: 78–85
Michalik B, Adamus A, Nowak E (2000) Gynogenesis in Polish onion cultivars. J Plant Physiol 156: 211–216
Miyoshi K, Asakura N (1996) Callus induction, regeneration of haploid plants and chromosome doubling in ovule cultures of pot gerbera (*Gerbera jamesonii*). Plant Cell Rep 16: 1–5
Mol R (1992) *In vitro* gynogenesis in *Melandrium album* – from partenogenetic embryos to mixoploid plants. Plant Sci 8: 261–269
Mukhambetzhanov SK (1997) Culture of nonfertilized female gametophytes *in vitro*. Plant Cell Tiss Org Cult 48: 111–119
Muren RC (1989) Haploid plant production from unpollinated ovaries in onion. Hortscience 24 (5): 833–834
Musial K, Bohanec B, Przywara L (2001) Embryological study on gynogenesis in onion (*Allium cepa* L.). Sex Plant Reprod 13: 335–341
Musial K, Bohanec B, Jakse M, Przywara L (2005) The development of onion (*Allium cepa* L.) embryo sacs *in vitro* and gynogenesis induction in relation to flower size. *In vitro* Cell Dev Biol – Plant 41: 446–452
Nitch JP, Nitch C (1969) Haploid plants from pollen grains. Science 163: 85–87
Palmer CE, Keller WA (2005) Overview of haploidy. In: Nagata T, Lorz H, Widholm JM (eds) Biotechnology in agriculture and forestry – Haploids in crop improvement II. Springer, Berlin/Heidelberg, pp 3–10
Pedersen HC, Keimer B (1996) Haploidy in sugar beet (*Beta vulgaris* L.). Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants. Vol. 3. Kluwer, Dordrecht, pp 17–36
Ponce M, Martinez L, Galmarini C (2006) Influence of CCC, putrescine and gellam gum concentration on gynogenic embryo induction in *Allium cepa*. Biol Plant 50: 425–428
Puddephat IJ, Robinson HT, Smith BM, Lynn J (1999) Influence of stock plant pretreatment on gynogenic embryo induction from flower buds of onion. Plant Cell Tiss Org Cult 57: 145–148
Ramsay JL, Galitz DS, Lee CW (2003) Basal medium and sucrose concentration influence regeneration of easter lily in ovary culture. Hortscience 38: 404–406
Rongbai Li, Pandey MP, Garg GK, Pandey SK, Dwivedi DK, Ashima (1998) Development of a technique for *in vitro* unpollinated ovary culture in rice, *Oryza sativa* L. Euphytica 104: 159–166
Rongbai Li, Pandey MP, Pandey SK, Dwivedi, DK (1999) Agro-morphological characterization of ovary culture-derived plants of rice (*Oryza sativa* L.). Euphytica 106: 197–203
San Noeum LH (1976) Haploides d'hordeum vulgare L. par culture *in vitro* non fecondes. Ann Amelior Plant 26: 751–754
San Noeum LH (1979) *In vitro* induction of gynogenesis in higher plants. Broadening genetic base of crops. Proceeding of the conference, Wageningen, The Netherlands, pp 327–329
Sibi ML, Kobaissi A, Shekafandeh A (2001) Green haploid plants from unpollinated ovary culture in tetraploid wheat (*Triticum durum* Defs.). Euphytica 122: 351–359

Sitbon M (1981) Production of haploid *Gerbera jamesonii* plants by *in vitro* culture of unfertilized ovules. Agronomie 1: 807–812

Sulistyaningsih E, Yamashita K, Tashiro Y (2002) Haploid induction from F-1 hybrids between CMS shallot with *Allium galanthum* cytoplasm and common onion by unpollinated flower culture. Euphytica 125: 139–144

Škof, Bohanec, Kastelec, Luthar Z (2007) Spontaneous induction of tetraploidy in hop using adventitious shoot regeneration method. Plant Breed 126: 416–421

Thomas TD, Bhatnagar AK, Razdan MK, Bhojwani SS (1999) A reproducible protocol for the production of gynogenic haploids of mulberry, *Morus alba* L. Euphytica 110: 169–173

Tosca A, Arcara L, Frangi P (1999) Effect of genotype and season on gynogenesis efficiency in Gerbera. Plant Cell Tiss Org Cult 59: 77–80

Truong-Andre I, Demarly Y (1984) Obtaining plants by *in vitro* culture of unfertilized maize ovaries (*Zea mays* L.) and preliminary studies on the progeny of a gynogenetic plant. Z Pflanzenzucht 92: 309–320

VanGeyt J, Speckmann GJ, Halluin KD, Jacobs M (1987) *In vitro* induction of haploid plants from unpollinated ovules and ovaries of the sugar beet (*Beta vulgaris* L.). Theor Appl Genet 73: 920–925

Wu BJ, Chen KC (1982) Cytological and embryological studies on haploid plant production from cultured unpollinated ovaries of *Nicotiana tabacum* L. Acta Bot Sin 24: 125–129

Yadegari R, Drews GN (2004) Female gametophyte development. Plant Cell 16: S133–S141

Yang HY, Zhou C (1982) *In vitro* induction of haploid plants from unpollinated ovaries and ovules. Theor Appl Genet 63: 97–104

Yang H, Zhou C, Cai D, Yan H, Wu Y, Chen X (1986) *In vitro* culture of unfertilized ovules in *Helianthus annuus* L. In: Hu H, Yang H (eds) Haploids of higher plants *in vitro*. Springer, Berlin, pp 182–191

Zhou C, Yang H, Tian H, Liu Z, Yan H (1986) *In vitro* culture of unpollinated ovaries in *Oryza sativa* L. In: Hu H, Yang H (eds) Haploids of higher plants *in vitro*. Springer, Berlin, pp 167–181

Chapter 3
Overview of Barley Doubled Haploid Production

P. Devaux and K.J. Kasha

Abstract Production of haploids in barley has been widely used for many years for breeding and breeding method research. Initially the Bulbosum method was available and provided a good random sample of female gametes as plants. Anther culture (male gametes) improved more gradually but has been widely used and more recently, isolated microspore culture has been perfected for barley breeding programmes and research. Thus, barley is now considered the cereal model crop species for haploid production and research. Doubled haploid populations have been extensively utilized in molecular marker work in barley providing detailed chromosome maps. Barley microspores are often selected for biochemical and cytological investigations of androgenesis. With the advancement in molecular, biochemical and cytological tools, haploid research has shifted to sequencing of DNA and transcriptome analyses that reveals hundreds of genes are involved in induction and embryogenesis from microspores. These genes can be identified and associated with various biochemical pathways and the proteins and enzymes involved. Transformation in barley is progressing well using haploid systems.

Keywords Bulbosum method, another culture, IMC, breeding, molecular markers, transcriptome analysis, embryogenesis, transformation

P. Devaux
Florimond Desprez Biotechnology Lab, Rue Florimond Desprez,
P.O. Box 41, 594242 Cappelleen Pévèle, France

K.J. Kasha(✉)
Professor Emeritus, Dept. Plant Agriculture University of Guelph,
Guelph, Ontario, N1G2W1, Canada
E.mail: kk.mk@sympatico.ca

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

Introduction

Barley (*Hordeum vulgare* L.) is a diploid species ($2n = 2x = 14$) with a genomic designation of I but the chromosomes are described with the symbol 1H to 7H. This species is self-pollinating under most climatic conditions and therefore the cultivars are usually phenotypically and genetically uniform. This was usually achieved by inbreeding and selection for a few generations but haploid systems can achieve homozygosity in a single generation and therefore, save a few generations of time in the breeding of new cultivars.

Genetic uniformity is a requirement for registration of new cultivars in many countries so that haploidy may be used in the final stages of breeding to achieve that uniformity. Because of this uniformity, populations for molecular mapping studies are often derived as doubled haploids (DH), particularly in barley. Seeds obtained from a doubled haploid can be planted in rows, making it much easier to select various plant types and disease resistance lines (Fig. 1). Haploids or DH lines are also valuable for genetic studies, mutation and selection at the single cell level in cultures and in studying embryo development.

The first demonstration of producing embryos and plants from immature pollen was using anther culture (Guha and Maheshwari 1964, 1966) in Datura and this created much interest in other species. The first haploids produced in barley were by Clapham (1973) while Sunderland (1974) was the early leader in investigating the mechanism of haploid production in barley. Barley was the first crop in which large numbers of haploids could be produced from most genotypes when Kasha and Kao (1970) produced haploids from wide hybridization of *H. vulgare* with *H. bulbosum*. While there was much interest in barley anther and isolated microspore culture (IMC)

Fig. 1 Two rows each from four different doubled haploid plants, showing three different levels of segregation for BYDV (Barley Yellow Dwarf Virus) and the ease of DH selection. The two DHs on the right are highly susceptible, the one on the left is resistant and the other demonstrates an intermediate level

at the First International Symposium on Haploids in Higher Plants (Kasha, 1974a), it was the ‘Bulbosum method’ that enabled researchers to produce doubled haploids and to evaluate the potential of haploidy in breeding programmes (Choo et al. 1985), where it showed great promise. Anther culture improvement proceeded more slowly by ‘trial and error’ due to the many factors involved that were varying with the species. However, it also was used to produce new cultivars and now both anther culture and IMC are being used in breeding programmes. It has been speculated that half of the barley cultivars now grown in central Europe were produced as doubled haploids. Breeders still use the Bulbosum method, particularly when genotypes do not respond to anther culture or IMC.

There have been a number of reviews written about barley haploid production, starting with Sunderland (1974) and Kasha (1974b) and followed by Pickering and Devaux (1992), Jähne and Lörz (1995), Thomas et al. (2003), Maluszynski et al. (2003) Devaux and Pickering (2005), and on barley biotechnology Kasha (2007). Our review will cover the methods of haploid production in barley. Some aspects of genetic, cytological, molecular and biochemical approaches to understanding the control and processes involved during induction and embryogenesis via androgenesis in barley will be summarized.

Methods of Producing Barley Haploids

Chromosome Elimination (Bulbosum Method)

This method of haploid production exploits the selective elimination of the entire chromosome set of one parent following wide hybridization crosses. Kasha and Kao (1970) reported the first system to be widely used in barley. When barley is crossed with diploid *Hordeum bulbosum* pollen, fertilization occurs, but the chromosomes of *H. bulbosum* are preferentially eliminated in early embryo development so that haploid embryos are produced. These embryos must be rescued, usually 12–14 days after pollination, and cultured *in vitro* prior to endosperm degeneration. The most commonly used medium is based on B5 (Gamborg et al. 1968) modified by omitting 2,4-D and adding sucrose and agar at 20 g l^{-1} and 7 g l^{-1} respectively (Kasha and Kao 1970). Haploid sporophytes that develop can be treated with a 0.05% (Pickering and Devaux 1992) to 0.1% (Kasha 2005) aqueous colchicine + 2% dimethyl sulphoxide solution for 5 hours to restore the fertility by doubling the chromosome number.

For the first time, a large number of doubled haploid plants (DH) could be produced across most genotypes and without any significant selection of gametophytes developing into plants. As a consequence the method quickly entered into breeding programmes around the world. This was enhanced through extensive research aimed at optimizing the critical factors influencing the method e.g. the *H. bulbosum* genotype (Simpson et al. 1980; Pickering and Rennie 1990), temperature and culture conditions of the plants (Pickering 1985), the nature and concentration of the

growth substances applied as a post-pollination spray to the barley spikes (Devaux and Pickering 2005). For detailed technical information, the recent papers of Hayes et al. (2003) and Devaux (2003) are recommended. As a breeding tool, it has generated more cultivars than any other technique so far (www.scri.sari.ac.uk/assoc/cost851/default.htm). However with the improvement and extended use of *in vitro* androgenesis, both anther and IMC, these procedures are expected to dominate barley cultivar development over the next few years.

To day, the Bulbosum method is still used as an alternative method to obtain barley DH from hybrids that are recalcitrant to androgenesis, which is more frequent in spring than in winter types. The method is also used when an unbiased random sample of gametes is required for a mapping population (Kleinhofs et al. 1993; Devaux et al. 1995). In other cereals, especially wheat, chromosome elimination following the interspecific hybridization with maize has been extensively used to generate wheat haploids with high efficiency (Laurie and Bennett 1988; Mujeeb-Kazi and Riera-Lizarazu 1996; Lefebvre and Devaux 1996).

Anther Culture

The remarkable discovery that culturing anthers of *Datura* could produce haploid embryos and plants was initially reported by Guha and Maheshwari (1964, 1966). Along with isolated microspore culture, anther culture is nowadays the most widely used systems for haploid production in plants. The large numbers of microspores within an anther combined with the multiple anthers per spike provide the potential for the largest number of haploid plants per spike.

Clapham (1973) was the first to report obtaining haploid plants from anther culture in barley. Since then substantial improvements of the technique have been made (reviewed in Pickering and Devaux 1992) and detailed protocols are currently available (for example, see Jacquard et al. 2003; Cistué et al. 2003; Szarejko 2003). Briefly, donor plants are raised in growth rooms or glasshouses at temperatures between 12°C and 15°C with a 16 hour day length regime at an irradiance of 350–450 µE m^{-2} s^{-1}. Spikes are collected when microspores are from mid- to late-uni-nucleate stage that is generally reached when the distance between the flag and the penultimate leaves is 3–6 cm. The switch of cultured microspores from a gametophyte to a sporophytic pathway has been induced by various stresses applied either *in situ* or *in vitro* (reviewed by Zoriniants et al. 2005). In barley, both cold shock at 4°C for 28 days or sugar starvation for 3–4 days in a 0.3–0.7 M solution of mannitol (Roberts-Oehlschlager and Dunwell 1990; Cistué et al. 1994), or alternatively with some macronutrients (Hoekstra et al. 1997) have been extensively used. After the pretreatment, anthers are plated on induction media, either the FHG (Hunter 1987) or BAC3 (Szarejko and Kasha 1991) and incubated at about 26°C in darkness. 3–5 weeks later, microspore-derived embryos >1 mm in size are transferred onto a regeneration medium which is often the same basal medium as the induction one with 2–3% of sucrose instead of maltose and reduced concentrations of growth substances. Regeneration plates are incubated at 22–25°C under low light for 8 hours

day^{-1}. Once green plants reach the 3 to 4 leaf stage they are transferred into soil in a glasshouse.

Although the technique has been extensively used for many purposes, albinism among regenerated plantlets has been a limiting factor. The mechanisms behind albinism have been investigated (reviewed in Devaux and Pickering 2005). Differences in proplastid differentiation, thylakoid development DNA content and ability of cells to divide have been detected between cultivars that regenerate high and low proportions of albino plantlets. The proportion of albino plantlets among regenerants fluctuates according to genotype, pre-treatment and culture conditions. Jacquard et al. (2006) pointed out the annual cycle and the position of the spike as sources of variation in green plant regeneration. This finding is particularly critical for recalcitrant genotypes that regenerate mostly albino plantlets from anther culture.

A key feature of gametophyte-derived plants is that chromosome doubling occurs during the very early stages of embryogenesis leading to plants that are fully fertile. In barley anther culture about 60–80% of the regenerants are spontaneous DH. As 8% are polyploidy and the remaining are haploid, ploidy level determination is required to eliminate the first ones and to perform colchicine treatment on the second ones. Ploidy levels can be determined by flow cytometry with high efficiency and speed. Similar to the Bulbosum method, anther culture has been widely used and many cultivars have been generated (see www.scri.sari.ac.uk/assoc/cost851/default.htm or Forster and Thomas 2005).

Isolated Microspore Culture

In this procedure, microspores are removed mechanically from the anther prior to *in vitro* culture. Because of its potential to produce large numbers of embryos, the use of isolated microspore culture (IMC) is increasing in DH production programmes including barley. In addition, a high frequency of spontaneous chromosome doubling (70–80%) is reached so that colchicine treatment is no longer required (Kasha 2005). Several procedures have been used efficiently for many years (for example see Davies 2003; Kasha et al. 2003) and cultivars have been released in recent years (Forster and Thomas, 2005).

Briefly, IMC differs from anther culture from the time when microspores are isolated from the anthers. For this, individual anthers or 2–3 cm segments of spikes are in most cases blended in either a cold 0.3 M mannitol or a medium solution for a few seconds. Then the slurry is filtered through a 100 µm sieve and the filtrate is centrifuged gently (50–100 g) for a few minutes. After removing the supernatant, the microspore pellet is suspended in cold mannitol solution and subjected to several cycles of washing and centrifugation. Dead cells and small debris are removed by a final centrifugation on a 20% maltose solution. Microspores are cultured in a liquid auxin-containing medium most often the FHG medium reported for anther culture. An antibiotic e.g. cefotaxin may be added to the induction medium to control bacterial contamination.

In comparative studies, Davies and Morton (1998) and Li and Devaux (2005) found that IMC performed better than anther culture although this was not totally

confirmed by Castillo et al. (2000). Today microspore culture is being used extensively in winter barley and to a lesser extent in spring barley as some recalcitrance has been found in European breeding lines of spring barley.

Other Methods of Haploid Production in Barley

Haploids have also been produced in barley by ovule culture (San Noeum 1976) and by the haploid initiator gene (*hap*) (Hagberg and Hagberg 1980). However, difficulties with these systems have not enabled their use in barley. Ovule culture has been improved (Yean-San 1987) but the frequencies still only range from 0.2–1.4% and the procedures are laborious and it does not have the potential to compete with androgenesis systems. The *hap* gene functions by preventing fertilization of the egg cell but the polar nucleus is fertilized and endosperm develops normally. The egg cell is stimulated to grow to produce a haploid embryo so that seed appear normal (Morgensen 1982). Thus, haploid and diploid seeds cannot be distinguished. Haploid seeds can be induced when the gene is both homozygous and heterozygous making it difficult to produce cultivars that do not contain the *hap* gene, as the plants with a haploid embryo will be sterile. Further research is needed on the haploid initiator gene system to make it competitive with the Bulbosum method or androgenesis systems of haploid production in barley.

Comparisons of Haploid Production Methods

The two major systems currently used for DH production i.e. the Bulbosum method and anther/microspore culture differ in several aspects that have to be considered for optimal success. Basically, wide hybridization is less genotype-dependent than *in vitro* androgenesis, especially for spring barley, and generates little or no genetic variation (Snape et al. 1988; Devaux et al. 1993). In addition, distortion of segregation at marker loci is less frequent (Schön et al. 1990; Devaux et al. 1996). These are substantial advantages of the Bulbosum method over the anther/microspore culture.

However spontaneous chromosome doubling is rare in plants generated from the Bulbosum method (Subrahmanyam and Kasha 1975) while it can reach up to 80% in IMC or an intermediate level (60% on average) in anther culture. Therefore ploidy level determination is only required for plants produced by anther culture. Irrespective of their origin, haploid plants have to be treated with colchicine to restore their fertility. Since colchicine is toxic to human, the possibility to regenerate spontaneous DH plantlets is a tremendous advantage as its manipulation can be avoided. In androgenesis, chromosome doubling occurs during the very early stages of embryogenesis and therefore the derived plants are completely doubled and fertile. Compared with colchicine-treated plants, spontaneous DH plants

produce many more grains at first multiplication and this can save one generation for seed increase prior to phenotypic evaluation for quantitative traits.

In most cases, breeders are looking for maximum useful recombination among progeny lines of crosses. In barley, significant increased meiotic recombination has been reported in anther culture-derived (male recombination) *vs H. bulbosum*-derived (female recombination) populations (Devaux et al. 1995). This was recently confirmed in wheat (Guzy-Wróbelska et al. 2007). Although the cost of DH production has remained at the same level between techniques for many years (Pickering and Devaux 1992; Kasha and Maluszynski 2003), it is most likely lower for those derived from IMC due to recent technical improvements (Kasha et al. 2001a; A. Jensen 2000, personal communication; Li and Devaux 2005).

At Florimond Desprez, the barley-breeding programme generates F_1 grains from crosses made in glasshouses and in the fields in late spring/early summer. Spikes can be collected from the F_1 derived plants a few months later from which anther or microspore culture is performed over the whole winter and in spring. Then the Bulbosum method is applied to the F_1 hybrids for which the number of desirable haploids has not been reached. This scheme is appropriate to save maximum time and labour due to dissimilar environmental conditions for donor plant growth and recalcitrance of certain genotypes to androgenesis while ensuring the production of DH from every hybrid.

Genetic Control of Haploid Production

The identification of genes involved in a method of haploid production has been pursued since haploids were first discovered but today molecular technical developments have made it possible to identify hundreds of genes involved in haploid induction via androgenesis. The first haploids found were mostly produced parthenogenetically and genotypes or stocks were found to enhance the frequencies (up to 8%) of haploids (Coe 1959), which were sufficient for producing haploids for breeding purposes in maize (*Zea mays* L.) (Chase 1969). Recently, there has been a revival of parthenogenic systems for maize breeding with many companies using them to procure inbreds for hybrid maize (CFH Longin 2006, personal communications). The *hap* initiator gene in barley (Hagberg and Hagberg 1980) is an example of a dominant mutation for stimulation of egg parthenogenesis accompanied by a normal endosperm that produces a normal appearing seed. Being dominant, this gene is a problem for seed production since the gene must be eliminated for commercial use of seed. The Bulbosum method of barley haploid production following preferential chromosome elimination after wide hybridization was discovered as a genome balance phenomenon (Kasha and Kao 1970). Ho and Kasha (1975) demonstrated by crossing stocks of barley trisomics and telo-trisomics with tetraploid *H. bulbosum* that genetic factors controlling the balance phenomenon were located on both arms of barley chromosome 2H and on one arm of barley chromosome 3H.

The isolation of these factors to determine their function has not been pursued. Pickering (1983) found a gene controlling incompatibility between barley and *H. bulbosum* and located it on barley chromosome 5H. Similar chromosome elimination phenomenon result when wheat and oats are crossed with maize or millet pollen. In all cases, the genotype of the pollen donor plant can have an influence the success of the elimination system.

The sequencing of the *Arabidopsis* genome and most of the rice genome has made it feasible to detect hundreds of genes that are over-expressed or under-expressed during induction of embryogenesis from microspore/anther cultures, and probably could be applied to other systems of haploid production. Initially in androgenesis studies there were strong differences in genotype response and these appeared to arise from many factors such as genes and stresses such as pests, donor plant growth conditions, environmental differences, media components, culture conditions, procedures for induction and microspore viability. Many of these factors were discovered by 'trial and error' but gradually a better understanding of factors began to emerge but they tended to be species specific. In barley, the use of maltose in place of sucrose (Hunter 1987) and the type and proportion of nitrogen sources (Mordhorst and Lörz 1993) were important steps while small changes like auxins (Ziauddin et al. 1992) helped in developing a completely defined media for barley isolated microspore culture (Kasha et al. 2001a). Donor plant growth conditions were critical to good response as were their freedom from pests or stress conditions (Hunter 1987). These and other developments helped to eliminate much of the genotype difference in response, but not all.

The crossing of genotypes with strong differences in responses was one of the early systems used to get some idea of the number of factors involved in induction of microspore embryogenesis or calli formation. When molecular marker maps were first developed for barley (Graner et al. 1991; Kleinhofs et al. 1993), it became feasible to determine gene differences in response as QTLs (Quantitative Trait Loci). Markers located in areas of the chromosomes where genes for differences in response were located tended to show segregation distortion of marker allele differences in the parents. Zivy et al. (1992) and Devaux and Zivy (1994) found four QTL differences associated with response. Two were linked to embryo production and green plant regeneration while the other two were only linked to green plant regeneration. Chen et al. (2007) also identified three such QTLs from a different barley population. Many of the populations developed for marker gene mapping were derived as doubled haploids that enabled researchers to collaborate knowing they were working with identical genotypes. The types of molecular markers available developed rapidly from AFLPs, RAPDs, SSRs, ESTs, and SNPs, with increasingly smaller nucleotide differences so that thousands of markers became available. The SSRs and more recently SNPs are the preferred markers for chromosome mapping, marker assisted selection in breeding, and for genetic diversity studies. Varshney et al. (2007) presented barley chromosome maps with 775 SSR markers with an average distance between markers being 1.38 cM (CentiMorgans). Through mapping it is feasible to isolate regions of DNA and sequencing them to locate the actual gene and its sequence. However, ESTs have become the key tools for gene identification

and isolation. A large collaborative approach has produced a large number of ESTs (over 400,000) in barley that is placed on a web site database (http://www.ucbi.nih.gov/UniGene/). They are thought to include about 40,000 distinct genes or about 85% of the gene complement of barley (Varshney et al. 2005). Such databases provide the opportunity to compare sequences across the cereal and grass genomes.

The biochemical and molecular methods are now making gene isolation and identification much faster and easier than before but it still requires knowledge and facilities that most breeders will not have. However large numbers of ESTs have been used to develop cDNA libraries on microarray plates that can be used to identify genes associated with induction and various developmental stages of microspore induced embryogenesis (Boutilier et al. 2005). One of the first differential display studies in barley was that of Vrinten et al. (1999) who used differential display of isolated RNAs produced from 3 day old embryogenic and non-embryogenic microspore cultures. Three of the most highly up-graded and clear genes were then compared to known gene sequences for identification. They encoded a lipid transfer protein, a glutathione S transferase and an unknown protein that was most likely an Arabinogalactan protein (AGP). The presence of AGP was interesting, as AGPs have been used to prevent plant cell apoptosis. Subsequent studies have shown that commercial AGP sources, AGP isolated from barley and wheat seeds and AGPs found secreted from ovaries used in co-culture with microspores, can be used to improve isolated microspore viability and response (Kasha and Simion 2001; Paire et al. 2003; Letarte et al. 2006; J Letarte and KJ Kasha unpublished). Wang et al. (1999) demonstrated that adding ABA to freshly isolated microspores prevented features of apoptosis from appearing and thus its importance during isolation of microspores. Reynolds (2000) developed a cDNA library from microspore derived embryos (MDE) and then screened the library with expressed sequences from mature pollen and pollen embryoids but identified only one of the many embryoid associated genes, a cysteine-labelled metallothionein whose expression started only 6 hours after embryo induction.

In a collaborative project, Close et al. (2004) developed a micro-array chip (Barley 1 GeneChip) fabricated by Affymetrix which contains about 22,000 unique 25mer 3’ end ESTs screened from over 400,000 ESTs in barley. This chip has been used to screen for large scale transcriptome analysis (Zhang et al. 2004) and for the effect of mannitol on induction of barley microspore embryogenesis (Muñoz-Amatriain et al. 2006) Screening for a four-fold or greater change in gene expression, Muñoz-Amatriain et al. (2006) identified 2,673 genes, of which 887 were up regulated and 1,786 were down-regulated. The up-regulated genes were mostly associated with abiotic and biotic stress responses and changes in developmental programmes. Maraschin et al. (2005a) tested for stress-induced gene expression by cDNA array analysis using a smaller microarray containing 1,421 ESTs covering the early stages of barley microspore embryogenesis. They used two sets of expression profiles, namely the induction phase and pollen development. They identified processes and metabolic pathways associated with induction as well as specific genes that together provide ‘bio-markers’ associated with androgenic response.

Complementary to the isolation and identification of genes involved in various steps of androgenesis, there have been a number of cytological studies following these processes. Indrianto et al. (2001) studied the development of the nucleus prior to the first division of the uni-nucleate microspore. Shariatpanahi et al. (2006) have reviewed the stresses used for reprogramming the plant microspores towards embryogenesis. Clement et al. (2005) reviewed the cytological and ultrastructural aspects of induction and embryo development. González-Melendi et al (2005) presented three-dimensional confocal and electron microscopy imagining of diploidization in the early stages of barley microspore-derived embryogenesis. Maraschin et al. (2005b) reviewed the switch from male gametophyte development to plant embryogenesis.

A problem important in barley anther and microspore culture has been the production of albino plants and the development of the chloroplasts has been studied. Caredda et al. (2004) examined the plastid ultra structure in green producing and albino producing microspore cultures. They observed that the proplastids in albino lines rarely divided and the thylakoids and DNA were scarce. The main probable cause of albinism is thought to be DNA degradation in plastids during induction. The complete barley chloroplast genome has been sequenced and was recently compared to those of other genera more closely related to barley (Saski et al. 2007).

Transformation of Haploid Barley

Interest in the transformation of haploids stems from the problem that in transformation, the newly inserted genes are hemizygous (heterozygous) and another generation of inbreeding and selection is required to obtain plants homozygous for the transgene. In androgenesis systems, fertile doubled haploid plants are produced in high frequencies of 60–80% or sometimes even higher in barley and so investigators have been interested in producing such plants that would be homozygous for the transgene. Theoretically, if one could introduce the gene when the recipient haploid cell is in the G1 stage of the cell cycle, it might become a plant homozygous for the transgene. The method of choice for transformation was initially through particle bombardment of isolated microspores, since the co-cultivation of microspores with *Agrobacterium* required 2 days and the microspore viability was severely reduced during this period. Furthermore, the microspore wall was thought to prevent the *Agrobacterium* from introducing the transgene.

Jähne et al. (1994) were the first to report on the transformation of barley microspores by particle bombardment. Of the five plants analyzed, all were homozygous for the transgene. Later Yao et al. (1997) reported the production of transgenic plants from particle bombardment and all the plants were heterozygous for the transgene, as were those reported by Carlson et al. (2001). All three reported similar numbers of transformed plants and bombardment procedures were similar. However, each used a different induction pretreatment of the microspores that may have led to the microspores being at different stages of the cell cycle. Jähne et al. (1994) used a 28 days cold pretreatment at 4°C and then

bombarded the isolated microspores. Yao et al. (1997) used a 0.3 M mannitol pretreatment at 25°C for 4 days while Carlson et al. (2001) used a 4 days pretreatment in cold + 0.3 M mannitol. Therefore, Shim and Kasha (2003) monitored the stages of the microspore cell cycle using the three different pretreatments from the time of spike collection to the end of the pretreatment and after microspore isolation prior to bombardment. The microspores were uni-nucleate when collected from the donor plants, with most of the microspores at the G1 stage of the cell cycle while some were in the S stage. When the spikes were placed in cold (4°C) + 0.3 M mannitol for 4 days, the microspores appeared to be held at the same stage as when collected. When spikes were pretreated for 4 days at 25°C in 0.3 M mannitol, most microspores had divided by day 2. In cold alone for 21 days, about 75% of microspores were at the uni-nucleate G2 stage (after DNA synthesis) while the remainder were dividing or had divided. A subsequent study by Shim (2006); and KJ Kasha (unpublished) using cold for 21 days or cold plus mannitol for 4 days followed by bombardment revealed that it was possible to obtain homozygous transgenic plants when using cold + mannitol to keep some of the microspores at the G1 stage. This chromosome doubling occurred after the first microspore division and most likely by nuclear fusion (Kasha et al. 2001b). However, bombardment following the cold pretreatment for 21 days also produced homozygous transgenic plants in higher frequencies and more plants were recovered, including a number of plants that were still haploid but transgenic. This would indicate that chromosome doubling probably occurred after the second mitotic division. Relative to producing transgenic plants from microspores, the long cold pretreatment as originally used by Jähne et al. (1994) would appear to be the most efficient bombardment procedure to obtain plants homozygous for the transgene.

However, Hensel and Kumlehn (2004) and Kumlehn et al (2006) revised *Agrobacterium* procedures to transform barley microspore derived cultures at the time when the multi-cellular structures break free from the microspore wall (7–10 days). In a check of 20 transgenic diploid plants, they found that 4 were homozygous for the transgene while the remaining plants were hemizygous for the transgene. They also obtained a number of transgenic haploid plants that could have their chromosome number doubled by colchicine treatment. The frequencies of transgenic plants obtained based upon the numbers of microspores used were similar in the Kumlehn et al. (2006) report and by Shim and Kasha (unpublished). Shim and Kasha (unpublished) obtained a higher proportion of homozygous transgenic plants and most carried a single insertion site. However, *Agrobacterium* is known to have a higher frequency of single transgene copies inserted at a sight than does bombardment (Travella et al. 2005) and therefore produce a higher frequency of stable transgenic plants. Methods to select for plants with a single transgene copy following bombardment are now available (Koprek et al. 2001) and have been used to produce barley plants with desirable traits (von Wettstein, 2004). Meng et al. (2006) observed that the nature of the stress and whether the transgene was single or multi copy influenced the expression or silencing of the transgene. It would appear that *Agrobacterium* transformation in barley microspore cultures might also overcome the problem of poor response across genotypes that was

originally found when using it to transform zygotic embryos as first reported by Tingay et al. (1997). *Agrobacterium* transformation may be favored for transformation microspore cultures at this time but continued efforts on bombardment appear to be warranted.

There are a number or reports of the transfer of important genes into barley, mostly by transformation of zygotic embryos or scutellum by particle bombardment (see Cheng et al. 2004; Kasha, 2007, for reviews). A recent report (Ayliffe et al. 2007) used the Ac/Ds transposable elements from maize (Koprek et al. 2001) to identify dominant mutations by over-expression of endogenous genes in barley. It would be of interest to use barley haploids in such a system, as it should also allow for the expression of recessive mutations.

Conclusions

Production of haploids in barley has been widely used for many years for breeding and breeding method research. Initially the Bulbosum method was available and anther culture improved more gradually in its applications. More recently, isolated microspore culture has been utilized for barley breeding programmes. Barley is now considered as one of the model crop species, particularly in cereals, for research in identifying the genes involved in induction of microspore embryogenesis and for genes involved in the stages of embryo development. Being a diploid species, barley is often selected for biochemical and cytological investigations of microspore androgenesis since the system can easily be viewed as opposed to gynogenesis or zygotic embryos. Doubled haploid populations have been extensively utilized in molecular marker work in barley providing detailed chromosome maps and where genes are located chromosomally. In return, marker mapping has enabled the identification of numbers of critical genes involved in haploid production through androgenesis. With the advancement in molecular, biochemical and cytological tools, the research has shifted to sequencing of DNA and transcriptome analyses that reveals hundreds of genes are involved in induction and embryogenesis from microspores. These genes can be identified and associated with various biochemical pathways and the proteins and enzymes involved. Transformation in barley is progressing well with much interest in using haploid systems because of their availability and the potential for smaller targets from which more uniformly transformed plants can be recovered, many being immediately homozygous for the transgene. Transposable elements can be used to tag genes and to select plants with a single copy of the transgene.

References

Ayliffe MA, Pallotta M, Langridge P, Pryor AJ (2007) A barley activation tagging system. Plant Mol Biol 64:329–347

Boutilier K, Fiers A, Liu C-M, van der Geest AHM (2005) Biochemical and molecular aspects of haploid embryogenesis. In: Palmer D, Keller W, Kasha KJ (eds) Haploids in crop improvement II. Springer, Heidelberg, pp 73–95

Castillo AM, Vallés MP, Cistué L (2000) Comparison of anther and isolated microspore culture in barley. Effects of culture density and regeneration medium. Euphytica 113:1–8

Caredda S, Devaux P, Sangwan RS, Proult I, Clément C (2004) Plastid ultrastructure and DNA related to albinism in androgenetic embryos of various barley (*Hordeum vulgare* L.) cultivars. Plant Cell Tiss Org Cult 76:35–43

Carlson AR, Letarte J, Chen J, Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green fluorescent protein (GFP). Plant Cell Rep 20:331–337

Chase SS (1969) Monoploid and monoploid derivatives of maize (*Zea mays* L.). Bot Rev 35:117–167

Chen X-W, Cistue L, Muñoz-Amatriain M, Sanz M, Romagosa I, Castillo A-M Vallés M-P (2007) Genetic markers for doubled haploid response in barley. Euphytica 158:287–294

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol Plant 40:31–45

Coe EH (1959) A maize line with high haploidy frequency. Amer Nat 93:381–382

Choo TM, Reinbergs E, Kasha KJ (l985) Use of haploids in barley breeding. Plant Breed Rev 3:219–252

Cistué L, Ramos A, Castillo AM, Romagosa I (1994) Production of large numbers of doubled haploid plants from barley anthers pretreated with high concentrations of mannitol. Plant Cell Rep 13:709–712

Cistué L, Vallés MP, Echávarri B, Sanz JM, Castillo A (2003) Barley anther culture. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 29–34

Clapham D (1973) Haploid *Hordeum* plants from anthers *in vitro*. Z Pflanzenzücht 69:142–155

Clement C, Sangwan RS, Sangwan-Norreel B (2005) Microspore embryo induction and development in higher plants: Cytological and ulrastructural aspects. In: Palmer D, Keller W, Kasha KJ (eds) Haploids in crop improvement II. Springer, Heidelberg, pp 53–72

Close TJ, Wanamaker S, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiol 134:960–968

Davies PA (2003) Barley isolated microspore culture (IMC) method In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 49–52

Davies PA, Morton S (1998) A comparison of barley isolated microspore and anther culture and the influence of cell culture density. Plant Cell Rep 17:206–210

Devaux P (2003) The *Hordeum bulbosum* (L.) method. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 15–19

Devaux P, Pickering RA (2005) Haploids in the improvement of Poaceae. In: Palmer D, Keller W, Kasha KJ (eds) Haploids in crop improvement II. Springer, Heidelberg, pp 215–242

Devaux P, Zivy M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701–706Devaux P, Kilian A, Kleinhofs A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241:674–679

Devaux P, Kilian A, Kleinhofs A (1995) Comparative mapping of the barley genome with male and female recombination-derived, doubled haploid populations. Mol Gen Genet 249:600–608

Devaux P, Zivy M, Kilian A, Kleinhofs A (1996) Doubled haploids in barley. In: Scoles G, Rossnagel B (eds) V International Oat Conference & VII International Barley Genet Symp. Univ. Ext. Press, Saskatchewan, Saskatoon, Canada, pp 213–222

Forster BP, Thomas WTB (2005) Doubled haploids in plant breeding. Plant Breed Rev 25:57–88

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

González-Melendi P, Ramirez C, Testillano PS, Kumlehn J, Risueno MC (2005) Three dimensional confocal and electron microscopy imaging define the dynamics and mechanisms of diploidization at early stages of barley microspore-derived embryogenesis. Planta 222:47–57

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G, Herrmann RG (1991) Construction of an RFLP map of barley. Theor Appl Genet 83:250–256

Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of *Datura*. Nature 204:497

Guha S, Maheshwari SC (1966) Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212:97–98

Guzy-Wróbelska J, Labocha-Pawlowska A, Kwasniewski M, Szarejko I (2007) Different recombination frequencies in wheat doubled haploid populations obtained through maize pollination and anther culture. Euphytica 156:173–183

Hagberg A, Hagberg G (1980). High frequency of spontaneous doubled haploids in the progeny of an induced mutation in barley. Hereditas 93:341–343

Hayes P, Corey A, DeNoma J (2003) Doubled haploid production in barley using the *Hordeum bulbosum* (L.) technique. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 5–14

Hensel G, Kumlehn J (2004) Genetic transformation of barley (*Hordeum vulgare* L.) by co-culture of immature embryos with Agrobacterium. In: IS Curtis (ed) Transgenic crops of the world – essential protocols. Kluwer, Dordrecht, pp 35–45

Ho KM, Kasha KJ (1975) Genetic control of chromosome elimination during haploid formation in barley. Genetics 81(2):263–275

Hoekstra S, van Bergen S, van Brouwershaven IR, Schilperoort RA, Wang M (1997) Androgenesis in *Hordeum vulgare* L: effects of mannitol, calcium and abscisic acid on anther pretreatment. Plant Sci 126:211–218

Hunter CP (1987) Plant generation method. European Patent Application No. 87200773.7

Indrianto A, Barinova I, Touraev A, Heberle-Bors E (2001) Tracking individual wheat microspores *in vitro*: identification of embryogenic microspores and body axis formation in the embryo. Planta 212:163–174

Jacquard C, Wojnarowiez G, Clément C (2003) Anther culture in barley. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 21–27

Jacquard C, Asakaviciute R, Hamalian AM, Sangwan RS, Devaux P, Clément C (2006) Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Rep 25:375–381

Jähne A, Lörz H (1995). Cereal microspore culture. Plant Sci 109:1–12

Jähne A, Becker D, Brettschneider R, Lörz H (1994) Regeneration of transgenic, microspore-derived, fertile barley. Theor Appl Genet 89:525–533

Kasha KJ (ed.) (1974a) Haploids in higher plants: advances and potential. Proceedings of the 1st International Symposium, Guelph ON. Univ. Guelph. 421 pp

Kasha KJ (1974b) Haploids from Somatic cells. In: Kasha KJ (ed) Haploids in higher plants: advances and potential. Proceedings of the 1st International Symposium, Guelph ON, Univ. Guelph, pp 67–87

Kasha KJ (2005) Chromosome doubling and recovery of doubled haploid plants. In: Palmer D, Keller W, Kasha K (eds) Haploids in crop improvement II. Springer, Heidelberg, pp 123–152

Kasha KJ (2007) Barley. In: Pua EC, Davey MR (eds) Economic crop biotechnology II. Biotechnology in agriculture and forestry Vol 59, Chapter 6: 129–150

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.) Nature 225:874–876

Kasha KJ, Maluszynski M (2003) Production of doubled haploids in crop plants. An introduction. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 1–4Kasha KJ, Simion E (2001) Embryogenesis and plant regeneration from microspores. International Patent Public. No. W0 01/41557. June 14, 2001

Kasha KJ, Simion E, Oro R, Yao QA, Hu TC, Carlson AR (2001a) An improved *in vitro* technique for isolated microspore culture of barley. Euphytica 120:379–385

Kasha KJ, Hu TC, Oro R, Simion E, Shim YS (2001b) Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. J Exp Bot 52:1227–1238

Kasha KJ, Simion E, Oro R, Shim YS (2003) Barley isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer, Dordrecht, pp 43–47

Kleinhofs A, Killian A, Saghai-Maroof MA, Hayes PM, Chen FQ, Lapitan N, Fenwick A, Blake T, Kanazin V, Ananiewe E, Dahleen L, Kudrna D, Bollinger A, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare* L.) genome. Theor Appl Genet 86:705–712

Koprek T, Rangel S, McElroy D, Louwerse JD, Williams-Carrier E, Lemaux PG (2001) Transposon-mediated single-copy gene delivery leads to increased transgene expression stability in barley. Plant Physiol 125:1354–1362

Kumlehn J, Serazetdinova L, Hensel G, Becker D, Lörz H (2006) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol J 4:251–261

Laurie DA, Bennett MD (1988) The production of haploid wheat plants from wheat x maize crosses. Theor Appl Genet 76:393–397

Lefebvre D, Devaux P (1996) Doubled haploids of wheat from wheat x maize crosses: genotypic influence, fertility and inheritance of the 1BL-1RS chromosome. Theor Appl Genet 93:1267–1273

Letarte J, Simion E, Miner M, Kasha KJ (2006). Arabinogalactans and arabinogalactan-proteins induce embryogenesis in wheat (*Triticum aestivum* L.) microspore culture. Plant Cell Rep 24: 691–698

Li H, Devaux P (2005) Isolated microspore culture over performs anther culture for green plant regeneration in barley (*Hordeum vulgare* L.). Acta Physiol Plant 27:611–619

Maluszynski M, Kasha KJ, Szarejko I (2003) Published protocols for other crop plant species. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: A manual, Kluwer, Dordrecht, pp 309–336

Maraschin SF, Caspers M, Potokina E, Wülfert F, Graner A, Spaink HP, Wang M (2005a) cDNA array analysis of stress-induced gene expression in barley androgenesis. Physiol Plantarum 127:535–550

Maraschin SF, de Priestr WS, Spaink HP, Wang M (2005b). Androgenic switch: An example of plant embryogenesis from the male gametophyte perspective. J Exp Bot 56:1711–1726

Meng L, Ziv M, Lemaux PG (2006) Nature of stress and transgene locus influences transgene expression stability in barley. Plant Mol Biol 62:15–28

Morgensen HL (1982) Double fertilization in barley and the cytological explanation for haploid embryo formation, embryoless caryopses, and ovule abortion. Carlsberg Res Comm 47:313–354

Mordhorst AP, Lörz H (1993) Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspores are influenced by the amount and composition of nitrogen sources in culture media. J Plant Physiol 142:485–492

Mujeeb-Kazi A, Riera-Lizarazu O (1996) Polyhaploid production in the Triticeae by sexual hybridization. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants, Vol 1. Kluwer, Dordrecht, pp 275–296

Muñoz-Amatriain M, Svensson JT, Castillo A-M, Cistue L, Close TJ (2006) Transcriptome analysis of barley anthers: effect of mannitol treatment on microspore embryogenesis Physio Plantarum 127:551–560

Paire A, Devaux P, Lafitte C, Dumas C, Matthys-Rochon E (2003) Proteins produced by barley microspores and their derived androgenic structures promote in vitro zygotic maize embryo formation. Plant Cell Tiss Org Cult 73:167–176

Pickering RA (1983) The location of a gene for incompatibility between *Hordeum vulgare* L. and *Hordeum bulbosum* L. Heredity 51:455–459

Pickering RA (1985) Partial control of chromosome elimination by temperature in immature embryos of *Hordeum vulgare* L. x *H. bulbosum* L. Euphytica 34:869–874

Pickering RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: Shewry PR (ed) Barley: genetics, molecular biology and biotechnology. CAB International, Wallingford, pp 511–539

Pickering RA, Rennie WF (1990) The evaluation of superior *Hordeum bulbosum* L. genotypes for use in a doubled haploid barley breeding programme. Euphytica 45:251–255

Reynolds TL (2000) Effects of calcium on embryogenic induction and the accumulation of abscisic acid, and an early cysteine-labeled methallothionein gene in androgenic microspores of *Triticum aestivum*. Plant Sci 150:201–207

Roberts-Oehlschlager SL, Dunwell JM (1990) Barley anther culture: pre-treatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tiss Org Cult 20:235–240

San Noeum LH (1976) Haploïdes d'*Hordeum vulgare* L par culture *in vitro* d'ovaires non fecondés. Ann Amélior Plant 26:751–783

Saski C, Lee S-B, Fjelljeim S, Guda C, Jansen RK, Luo H, Tomkins J, Rognli OA, Daniell H, Clarke JL (2007). Complete chloroplast genome sequences of *Hordeum vulgare, Sorghum bicolour* and *Agrostis stolonifera,* and comparative analyses with other grass genomes. Theor Appl Genet 115:571–590

Schön C, Sanchez M, Blake T, Hayes PM (1990) Segregation of Mendelian markers in doubled haploid and F_2 progeny of a barley cross. Hereditas 113:69–72

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006). Stresses applied for the reprogramming of plant microspores towards *in vitro* embryogenesis. Physiol Plantarum 127:519–534

Shim YS, Kasha KJ (2003) The influence of pretreatment on cell stage progression and the time of DNA synthesis in barley (*Hordeum vulgare* L.) uninucleate microspores. Plant Cell Rep 21:1065–1071

Shim YS (2006) Improvement of Microspore Transformation in Barley (*Hordeum vulgare* L). Ph D Thesis University of Guelph, pp170

Snape JW, Sitch LA, Simpson E, Parker BB (1988) Tests for the presence of gametoclonal variation in barley and wheat doubled haploids produced using the *Hordeum bulbosum* system. Theor Appl Genet 75:509–513

Simpson E, Snape JW, Finch RA (1980) Variation between *Hordeum bulbosum* genotypes in their ability to produce haploids of barley, *Hordeum vulgare*. Z Pflanzenzüchtg 85:205–211

Subrahmanyam NC, Kasha KJ (1975) Chromosome doubling of barley haploids by nitrous oxide and colchicine treatments. Can J Genet Cytol 17:573–583

Sunderland N (1974) Anther culture as a means of haploid induction. In: Kasha KJ (ed) Haploids in higher plants – advances and potential, Univ of Guelph, pp 91–122

Szarejko I (2003) Anther culture for double haploid production in barley (*Hordeum vulgare* L) In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: A manual. Kluwer, Dordrecht, pp 35–42

Szarejko I, Kasha KJ (1991) Induction of anther culture derived doubled haploids in barley. Cereal Res Commun 19:219–237

Thomas WTB, Forster BP, Gertsson B (2003) Doubled haploids in breeding. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: A manual. Kluwer, Dordrecht, pp 337–350

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J 11:1369–1376

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. Plant Cell Rep 23:780–789

Varshney RK, Sigmund R, Borner A, Korzun V, Stein N, Sorrells ME, Langridge P, Graner A (2005) Interspecific transferability and comparative mapping of barley EST-SSR markers in wheat, rye and rice. Plant Sci 168:195–202

Varshney RK, Marcel TC, Ramsay L, Russell J, Roder MS, Stein N, Waugh R, Langridge P, Niks RE, Graner A (2007) A high density barley microsatellite consensus map with 775 SSR loci. Theor Appl Genet 114:1091–1103

von Wettstein D (2004) Transgenic barley. In: Spunar J, Janikova J (eds) Proceedings of the 9th International Barley Genetics Symposium, Brno CZ, pp 144–150

Vrinten PL, Nakamura T, Kasha KJ (1999) Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (*Hordeum vulgare* L.). Plant Mol Biol 41(4):455–463

Wang M, Hoekstra S, van Bergen S, Lamers GEM, Oppedijk BJ, van der Heijden MW, de Priester W, Schilperoort RA (1999) Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. Plant Mol Biol 39:489–501

Yao QA, Simion E, William M, Krochko J, Kasha KJ (1997) Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.) Genome 40:570–581

Yean-San LH (1987) Gynogenèse *in vitro*. Variabilité des haploides doublés issus d'androgenèse *in vitro* et de croisement interspécifique chez *Hordeum vulgare* L. Thèse Doctorat d'Etat, Université de Paris-sud, France

Zhang H, Sreenivasulu N, Weschke W, Stein N, Rudd S, Radchuk V, Potkina E, Scholz U, Schweizer P, Zierold U, Langridge P, Varshney RK, Wobus U, Graner A (2004) Large-scale analysis of the barley transcriptome based on expressed sequence tags. Plant J 40: 276–290

Ziauddin A, Marsolais A, Simion E, Kasha KJ (1992) Improved plant regeneration from wheat anther and barley microspore culture using phenylaceticacid (PAA). Plant Cell Rep 11:489–498

Zivy M, Devaux P, Blaisonneau J, Jean R, Thiellement H (1992) Segregation distortion and linkage studies in microspore derived doubled haploid lines of *Hordeum vulgare* L. Theor Appl Genet 83:919–924

Zoriniants S, Tashpulatov AS, Heberle-Bors E, Touraev A (2005) The role of stress in the induction of haploid microspore embryogenesis. In: Palmer D, Keller W, Kasha K (eds) Haploids in crop improvement II. Springer, Heidelberg, pp 35–52

Chapter 4
Production of Doubled Haploids in *Brassica*

J. Gil-Humanes and F. Barro

Abstract The production of doubled haploid (DH) plants is one of the most important developments in biotechnology. Haploids and DH have been produced in *Brassica ssp*. using anther culture or isolated microspores. The microspore culture is a simple and affordable technique for production of DH plants, and therefore is the method of choice for plant genetic research and breeding programmes in oilseed *Brassicas*. Microspore culture is a useful tool in producing doubled homozygous lines for breeding since lines exhibiting desired agronomic traits can be rapidly obtained. Microspore culture is also useful in gene transfer, biochemical and physiological studies and in the production of desired traits such as herbicide resistance and fatty acid modification through mutagenesis and selection.

Factors influencing DH production as well as current applications of *Brassica* doubled haploidy are discussed in this work.

Keywords *Brassica*, doubled haploid, heat shock, microspore culture

Introduction

Rapeseed (*Brassica napus* L.) is the third most important source of vegetable oil in the world with 27.4 million hectares cultivated in 2005 (FAOSTAT 2007, http://faostat.fao.org). The oilseed *Brassicas* are found within *B. napus, B. juncea, B. rapa* and *B. carinata*. Modification of the fatty acid composition is an important objective of breeding of these crops (Friedt and Luhs 1998) and there is considerable commercial interest in the development of high erucic acid and/or low glucosinolates

J. Gil-Humanes
Instituto de Agricultura Sostenible, CSIC. 14080-Córdoba, Spain

F. Barro(✉)
Instituto de Agricultura Sostenible, CSIC. 14080-Córdoba, Spain
E-mail: ge1balof@uco.es

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

lines targeted towards industrial end-use and in the development of low (or zero) erucic acid, low linoleic and high oleic lines for food industries. Currently, biodiesel production using oilseed *Brassicas* is being widely investigated since the trans-esterification of vegetable oils constitutes an efficient method to provide a fuel with chemical properties similar to those of diesel fuel. For this purpose, the fatty acid composition of the oils plays an important role in the production of biodiesel. The presence of erucic acid increases the free fatty acid content and prevents the conversion of *Brassica* oil in its methyl ester (Dorado et al. 2004).

The production of haploid (H) and doubled haploid (DH) plants is one of the most important developments in biotechnology. Haploids and DHs have been produced in *Brassica ssp*. using anther culture or isolated microspores. The first successes using *Brassica* anthers cultures were reported by Keller et al. (1975) and Thomas and Wenzel (1975). Alternatively, in 1982, Lichter developed a culture system for isolated *B. napus* microspores. Microspore culture has been demonstrated to be more efficient than anther culture for embryo production (Siebel and Pauls 1989) and most genotypes respond better to isolated microspore culture (Cao et al. 1994). In addition, Wang et al. (1999) reported that DHs obtained from microspore culture showed a lower frequency of ploidy variation within clonal groups derived from individual embryos than those obtained from anther culture.

The microspore culture is a simple and affordable technique for production of DH plants, and therefore is the method of choice for plant genetic research and breeding programmes (Palmer et al. 1996). Microspore culture consists of culturing male gametophytic cells and their induction into haploid embryos instead the formation of mature pollen cells.

Important advances have been obtained in isolated microspore culture technology in all major *Brassica* species, and nowadays the techniques have been applied for H and DH production in *B. napus* (Weber et al. 2005; Zhou et al. 2002), *B. oleracea* (Phippen 1990), *B. carinata* (Barro et al. 2001; Barro et al. 2003), *B. juncea* (Lionneton et al. 2001), *B. rapa* (Ferrie and Keller 1995a; Gu et al. 2003).

Microspore culture is a useful tool in producing doubled homozygous lines for breeding since lines exhibiting desired agronomic traits can be rapidly obtained (Maluszynski et al. 1995; Morrison and Evans 1988). Microspore culture is also useful in gene transfer, biochemical and physiological studies and in the production of desired traits such as herbicide resistance and fatty acid modification through mutagenesis and selection.

Aspects Influencing Doubled Haploid Production

Figure 1 summarizes the different stages of DH production from isolated microspores. The success is influenced at every stage of the process i.e. microspore isolation, microspore culture, embryo selection, plant regeneration and chromosome duplication.

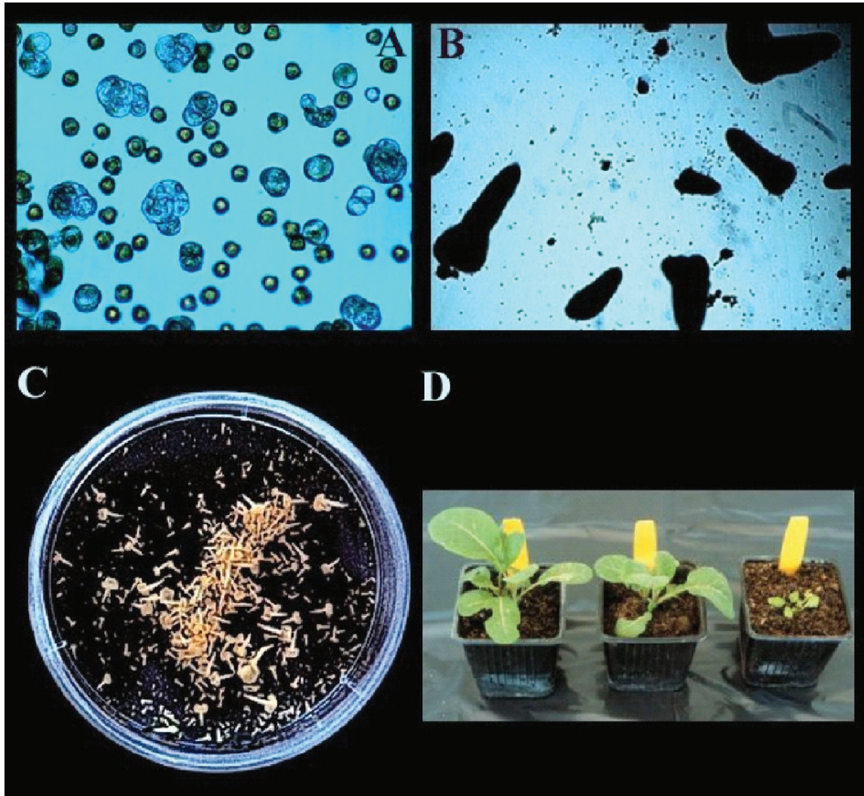


Fig. 1 Microspore culture of *B. carinata* line BC71. **A**. Cell division and cell clusters after 5 days in culture. **B.** Torpedo-shaped and cotyledoneous embryos after 3 weeks of culturing. **C**. Well developed embryos after 5 weeks in culture. **D**. *B. carinata* haploid plants in three developmental stages

Microspore Culture

Microspore culture is a critical determining stage in the production of H and DH lines. Microspore embryogenesis can be influenced by genotype and growth conditions of the donor plant, microspore developmental stage, microspore pretreatments and culture conditions.

Genotype and Growth Conditions of the Donor Plant

Brassica microspore culture is highly genotype-dependent, as reported in most *Brassiceae* species (Ferrie et al. 1995b; Lichter 1989; Phippen 1990). This variability is outlined in Fig. 2, in which 10 out of 16 lines of *B. carinata* tested (63%) showed cell division and embryo formation (Barro and Martin 1999).

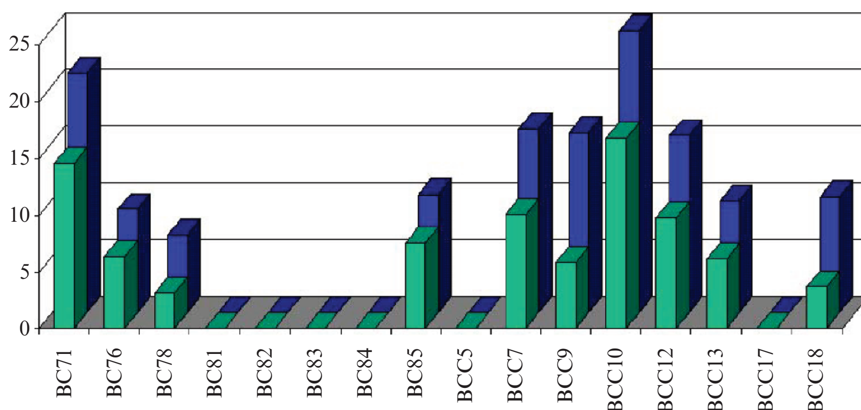


Fig. 2 Frequency of cell division (green) and embryos formation (blue) from microspores isolated from different lines of *B. carinata*

Donor plants have to be grown in an environmentally controlled growth chamber which minimizes stress. Optimal growth conditions will produce healthy plants and enhance embryogenic responses. Factors such as temperature, light, water and nutrients are important in order to obtain healthy plants. It is known that the most responsive microspores are obtained from plants grown at low temperatures (Dunwell 1985). For example, *B. carinata* plants are grown at 25°C/15°C day night temperature cycle for 1 month, and then at 15°C/10°C for 2 months. Plants can be used as donors for a period of up to 2 weeks after the first flower has opened. During this time it is important to remove all flowers and avoid pod formation.

Bud Length

Morphological and physiological studies have shown that only microspores at the late uni-nucleate or early bi-nucleate stages will develop into embryos (Fan et al. 1988; Kott et al. 1988a; Pechan and Keller 1988). Kott et al. (1988a) studied the cytological aspects of isolated microspore culture in two genotypes of *B. napus* and found that the highest frequency of microspore embryogenesis was observed with microspore at late uni-nucleate stage. In addition, Kott et al. (1988b) observed a reduction of the conversion of microspores to embryos in microspore cultures of *B. napus*, caused by a toxin generated by the cultured microspores themselves. The negative effects of the toxin were correlated with the presence of bi-nucleate microspores in the culture. Therefore, the choice of buds containing microspores at the right stage is critical for increasing the frequency of cell division and embryo formation. Bud length is an easy parameter in order to select buds containing the highest proportion of microspores at right nucleus stage. For *B. carinata*, the highest values of cell division were obtained using buds ranging from 2.5 to 3.5 mm

Table 1 Frequency of *B. carinata* microspores at different developmental nucleus stages and frequency of cell division as influenced by bud length. Nucleus stages: A, spores in tetrad; B, early uni-nucleate; C, mid uni-nucleate; D, late uni-nucleate; E, bi-nucleate and F, tri-nucleate

	Nucleus stage (%)						
	Uni-nucleate		Bi-nucleate			Tri-nucleate	
Bud length (mm)	A	B	C	D	E	F	Cell division (%)
2.0	14	16	38	32	0	0	3.1 ± 0.4
2.5	5	6	32	58	0	0	10.8 ± 1.3
3.0	0	0	31	66	2	1	11.5 ± 1.5
3.5	0	0	12	52	25	11	8.6 ± 0.9
4.0	0	0	2	32	40	21	2.7 ± 0.3

in length, which contained the highest proportion of microspores at the late uni-nucleate stage (Table 1).

Heat Shock

Stress is an essential component during embryogenesis induction in microspore culture. The type of stress can vary from heat to gamma irradiation and colchicine (Pechan and Keller 1988; Zaki 1995; Zhao et al. 1996). Nevertheless, heat treatment is mostly used in *Brassica* species in order to induce embryogenesis in microspore culture. Pechan (2001) reported that the 32°C temperature treatment is an absolute temperature requirement for inducing androgenesis in *B. napus*. In *B. carinata*, embryogenic response occurred within 1–4 days of pre-treatment at 32°C for all buds tested. However, for buds between 2.5 and 3.5 mm the highest values of both cell division and embryo yield were obtained with 3 days of incubation at 32°C. Further incubation did not improve cell division, but embryo yield was drastically reduced (Barro and Martin 1999).

Embryo Selection and Plant Regeneration

Generally, 21 days after microspore isolation and culture haploid embryos can be transferred to solid regeneration medium. In terms of plant development from microspore-derived embryos, the stage at which embryos are transferred to solidified medium is critical for the plantlet formation (Niu et al. 1999). Burnett (1992) reported that embryos transferred at the cotyledonary stage resulted in the highest frequency of plant regeneration from isolated microspores. So, it is important that embryos have reached morphological maturity and have an elongated root/shoot axis with two cotyledons surrounding the shoot apex before transfer to solid medium.

Medium composition determines the success of plant regeneration on solid medium. The B5 medium (Gamborg et al. 1968) supplemented with 0.8% agar (w/v) and 0.1 mg/l of gibberellic acid (GA_3) gives excellent results. However, some authors have reported that ½ MS (Murashige and Skoog 1962) or ½ B5 media have better effect on the plantlet formation than full MS or B5 media (Gland-Zwerger 1995). After 3 weeks on a solid medium, plantlets are well developed and can be transferred to soil and grown on to maturity.

Chromosome Doubling

DH plants are obtained by colchicine treatment of H plants. Spontaneous doubling of chromosomes is frequent. Barro et al. (2003) found a spontaneous chromosome doubling rate of 50% in *Brassica carinata* plants regenerated from microspore culture. However, it is reported that 70–90% of regenerated plants from rapeseed microspore culture are haploid (Charne 1988; Chen and Beversdorf 1992).

Chromosome doubling consist of immersing the roots (Gland 1981) or the whole plant in a colchicine solution (Fletcher et al. 1998), or culturing plantlets in colchicine-containing medium in the glasshouse (Mathias 1991). Other methods inject a 0.2% solution of colchicine into secondary buds (Lichter 1981) or apply a cotton swab soaked in colchicine to auxiliary buds (Gland 1981). Such methods often result in the formation of chimeric plants with relatively small sectors of diploid tissue which will produce few diploid seeds. Consequently, an extra cycle of seed multiplication becomes necessary in the glasshouse before the first field planting.

Concentrations of 50–500 mg/l of colchicine and treatment duration of 18–24 hours have been used to optimise doubling rates. Mollers (1994) reported a chromosome doubling rate of 80–90% after a 24 hour treatment of *Brassica napus* microspores with 50 mg/l colchicine, while Zhou et al. (2002) obtained a doubling efficiency of 83–91% from 500 mg/l colchicine treatment for 15 hours.

Current Applications of *Brassica* Doubled Haploidy

Microspore culture and anther culture provide the opportunity of producing haploid embryos at high frequencies in many *Brassica* species and their commercial cultivars and, combined with other biotechnologies such as marker assisted selection and induced mutations, can speed up breeding programmes (Maluszynski et al. 1995; Morrison and Evans 1988).

Some possible uses of DH technology combined with mutation are the production of *Brassica* lines with desirable traits such as disease resistance, herbicide resistance and altered fatty acid content. There are two main mutation methods for isolated microspores: chemical mutation using mutagenic agents such as ethyl methane sulphonate (EMS) or sodium azide (NaN_3) and physical mutation using

ultraviolet (UV) or gamma radiation. Many mutated varieties have been obtained using these mutation methods. Mutants exhibiting herbicide resistance were obtained in *B. napus* and Chinese cabbage (*B. campestris* subsp. *pekinensis*) by Swanson et al. (1988, 1989). Other studies obtained *B. napus* mutant lines showing resistance to black spot disease (*Alternaria brassicicola*) (Ahmad et al. 1991) and white rot (*Sclerotinia sclerotiorum*) (Liu et al. 1997), and Chinese cabbage (*B. campestris* subsp. *pekinensis*) with soft-rot resistance (*Erwinia carotovora*) (Zhang and Takahata 1999).

Altered fatty acid lines have been developed by radiation mutation and chemical mutation. Using UV radiation, Barro et al. (2003) enhanced the content of erucic acid from 42.8% to 49.5% in *B. carinata* lines. Ferrie (1999) also used radiation to obtain mutant variation in the content of fatty acids increasing oleic acid from 47.1% to 50%, decreasing linoleic acid from the normal 11.4% to less than 8%, and decreasing the saturated fatty acids from 5.7% to 5%.

On the other hand, using chemical mutation, oleic acid content was enhanced from 60% to 85% and linoleic acid content were decreased from 10% to 3% by Kott (1996) in *B. napus* mutant lines, while Barro et al. (2001) obtained *B. carinata* mutant lines with low erucic content (showing contents below 25%) and high erucic content (showing up to 52%) using EMS treatment as chemical mutant agent.

References

Ahmad I, Day JP, Macdonald MV, Ingram DS (1991) Haploid culture and UV mutagenesis in rapid-cycling *Brassica napus* for the generation of resistance to chlorsulfuron and *Alternaria brassicicola*. Ann Bot 67:521–525

Barro F, Martin A (1999) Response of different genotypes of *Brassica carinata* to microspore culture. Plant Breed 118:79–81

Barro F, Fernandez-Escobar J, De La Vega M, Martin A (2001) Doubled haploid lines of *Brassica carinata* with modified erucic acid content through mutagenesis by EMS treatment of isolated microspores. Plant Breed 120:262–264

Barro F, Fernandez-Escobar J, De la Vega M, Martin A (2003) Modification of glucosinolate and erucic acid contents in doubled haploid lines of *Brassica carinata* by UV treatment of isolated microspores. Euphytica 129:1–6

Burnett L (1992) Embryogenesis and plant regeneration from isolated microspores of *Brassica rapa* L ssp *oleifera*. Plant Cell Rep 11:215–218

Cao MQ, Li Y, Liu F, Dore C (1994) Embryogenesis and plant regeneration of pakchoi (*Brassica rapa* L *ssp chinensis*) via *in vitro* isolated microspore culture. Plant Cell Rep 13:447–450

Charne DG (1988) Embryogenesis following cryopreservation in isolated microspores of rapeseed (*Brassica napus* L). Plant Cell Rep 7:407–409

Chen JL, Beversdorf WD (1992) Cryopreservation of isolated micropores of spring rapeseed (*Brassica napus* L) for *in vitro* embryo production. Plant Cell Tiss Org 31:141–149

Dorado MP, Ballesteros E, Lopez FJ, Mittelbach M (2004) Optimization of alkali-catalyzed transesterification of *Brassica carinata* oil for biodiesel production. Energ Fuels 18:77–83

Dunwell JM (1985) Influence of genotype plant growth temperature and anther incubation temperature on microspore embryo production in *Brassica napus* ssp *oleifera*. J Exp Bot 36:679–689

Fan Z, Armstrong KC Keller WA (1988) Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma 147:191–199

Ferrie AMR (1999) Combining microspores and mutagenesis. In: PBI Bulletin National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada, pp 17–19

Ferrie AMR, Keller WA (1995a) Development of methodology and applications of doubled haploids in *Brassica rapa*. In: GCIRC Proceedings of the Ninth International Rapeseed Congress. GCIRC, Cambridge, UK, pp 805–809

Ferrie AMR, Epp DJ, Keller WA (1995b) Evaluation of *Brassica rapa* L genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Rep 14:580–584

Fletcher R, Coventry J, Kott LS (1998) Doubled haploid technology for spring and winter *Brassica napus* (revised ed.). In: Publication O (ed) Technical Bulletin. University of Guelph, Ontario, Canada, p 42

Friedt W, Luhs W (1998) Recent developments and perspectives of industrial rapeseed breeding. Fett-Lipid 100:219–226

Gamborg OL, Miller RA, Ojima K (1968) Nutrients requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gland AA (1981) Doubling chromosomes in interspecific hybrids by colchicine treatment. Eucarpia Cruciferae Newsl 6:20–22

Gland-Zwerger A (1995) Culture conditions affecting induction and regeneration in isolated microspore cultures of different *Brassica* species. In: GCIRC Proceedings of the Ninth International Rapeseed Congress. GCIRC, Cambridge, UK, pp 799–801

Gu HH, Zhou WJ, Hagberg P (2003) High frequency spontaneous production of doubled haploid plants in microspore cultures of *Brassica rapa* ssp *chinensis*. Euphytica 134:239–245

Keller WA, Rajhathy T, Lacapra J (1975) *In vitro* production of plants from pollen in *Brassica campestris*. Can J Genet Cytol 17:655–665

Kott LS, Polsoni L, Berversdorf WD (1988a) Cytological aspects of isolated microspore culture of *Brassica napus*. Can J Bot 66:1658–1664

Kott LS, Polsoni L, Ellis B, Beversdorf WD (1988b) Autotoxicity in isolated microspore cultures of *Brassica napus*. Can J Bot 66:1665–1670

Kott LS (1996) Production of mutants using the rapeseed doubled haploid system. In: Induced Mutation and Molecular Techniques for Crop improvement. IAEA/FAO Proceedings of an International Symposium on the Use of Induced Mutations and Molecular Techniques for Crop Improvement. Vienne, Austria, pp 505–515

Lichter R (1981) Anther culture of *Brassica napus* in a liquid culture medium. Z Pflanzenphysiol 103: 229–237

Lichter R (1982) Induction of haploid plants from isolated polen of *Brassica napus*. Z Pflanzenphysiol 105: 427–434

Lichter R (1989) Efficient yield of embryoids by culture of isolated microspores of different *Brassicaceae* species. Plant Breed 103:119–123

Lionneton E, Beuret W, Delaitre CO, Chatt S, Rancillac M (2001) Improved microspore culture and doubled-haploid plant regeneration in the brown condiment mustard (*Brassica juncea*). Plant Cell Rep 20:126–130

Liu Y, Liu HY, Zheng ZY (1997) The application of microspore culture screening disease resistance to *Sclerotinia sclerotiorum* in oilseed rape Southwest China. J Agr Sci 10(s):108–112

Maluszynski M, Ahloowalia BS, Sigurbjornsson B (1995) Application of *in vivo* and *in vitro* mutation techniques for crop improvement. Euphytica 85:303–315

Mathias RR (1991) Effective diploidization of microspore-derived haploids of rape (*Brassica napus* L.) by *in vitro* colchicine treatment. Plant Breed 106:82–84

Mollers CC (1994) Efficient production of doubled haploid *Brassica napus* plants by colchicine treatment of microspores. Euphytica 75:95–104

Morrison RA, Evans DA (1988) Haploid plants from tissue culture: new plant varieties in a shortened time frame. Bio/technology 6:684–690

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Niu YZ, Liu YZ, Wang LZ, Yuang YX, Li SC, Fang QJ (1999) A preliminary study on isolated microspore culture and plant regeneration of resynthesized *Brassica napus*. Journal of Sichuan Agricultural University 17:167–171

Palmer CE, Keller WA, Arnison PG (1996) Experimental haploidy in *Brassica* species. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* Haploid Production in Higher Plants. Kluwer, Dordrecht, The Netherlands, pp 143–172

Pechan PM (2001) Androgenesis: Affecting the fate of the male gametophyte. Physiol Plant 111:1–8

Pechan PM, Keller WA (1988) Identification of potentially embryogenic microspores in *Brassica napus*. Physiol Plant 74:377–384

Phippen C (1990) Genotype plant bud size and media factors affecting anther culture of cauliflowers (*Brassica oleracea* var *botrytis*). Theor Appl Genet 79:33–38

Siebel J, Pauls KP (1989) A comparison of anther and microspore culture as a breeding tool in *Brassica napus*. Theor Appl Genet 78:473–479

Swanson EB, Coumans MP, Brown GL, Patel JD, Beversdorf WD (1988) The characterization of herbicide tolerant plants in *Brassica napus* L. after *in vitro* selection of microspores and protoplasts. Plant Cell Rep 7:83–87

Swanson EB, Herrgesell MJ, Arnoldo M, Sippell DW, Wong RSC (1989) Microspore mutagenesis and selection Canola plants with field tolerance to the imidazolinones. Theor Appl Genet 78:525–530

Thomas E, Wenzel G (1975) Embryogenesis from microspores of *Brassica napus*. Z Pflanzenzuecht 77–81

Wang M, Farnham MW, Nannes JSP (1999) Ploidy of broccoli regenerated from microspore culture versus anther culture. Plant Breed 118:249–252

Weber S, Unker F, Friedt W (2005) Improved doubled haploid production protocol for *Brassica napus* using microspore colchicine treatment *in vitro* and ploidy determination by flow cytometry. Plant Breed 124:511–513

Zaki M (1995) Modification of cell development *in vitro*: the effect of colchicine on anther and isolated microspore culture in *Brassica napus*. Plant Cell Tiss Org 40:255–270

Zhang FL, Takahata Y (1999) Microspore mutagenesis and *in vitro* selection for resistance to soft-rot disease in Chinese cabbage (*Brassica campestris* L subsp *pekinensis*). Breed Sci 49:161–166

Zhao JP, Simmonds DH, Newcomb W (1996) Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L cv Topas. Planta 198:433–439

Zhou WWJ, Tang GGX, Hagberg PP (2002) Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regul 37:185–192

Chapter 5
An Overview on Tobacco Doubled Haploids

K. Belogradova, I. Lewicka, E. Heberle-Bors, and A. Touraev

Abstract Androgenesis is a process of redirection of normal pollen development towards the formation of haploid embryos and ultimately doubled-haploid plants. This review gives a historical overview of different procedures used to induce androgenesis in tobacco. The various factors that influence this developmental switch including growth of donor plants, pre-treatments, culture media, as well as cytological aspects of cell and tissue changes that occur during the induction and development of the microspore-derived embryos are described. Finally, this review will cover recent molecular data on mechanisms of embryogenic induction in cultured tobacco microspores.

Keywords Tobacco, anther and microspore culture, gene expression, double haploids

Introduction

Nicotiana tabacum (tobacco) is a member of the agriculturally important *Solanaceae* family. Tobacco has been cultivated for more than 500 years and is the most valuable non-food crop in the world. *Nicotiana tabacum* is a natural allotetraploid ($2n = 4x = 48$) likely resulted from a cross between *N. sylvestris* (2n = 24) and *N. tomentosiformis* (2n = 24) (Murad et al. 2002). Nowadays tobacco is one of the model plants in biology with established procedures of cell culture, transformation

K. Belogradova, I. Lewicka, and E. Heberle-Bors
Max F Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Plant Molecular Biology, Dr. Bohrgasse 9/4, 1030 Vienna, Austria

A. Touraev(✉)
Max F Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Plant Molecular Biology, Dr. Bohrgasse 9/4, 1030 Vienna, Austria
E-mail: alisher.touraev@univie.ac.at

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

and doubled haploid production, and is extensively used in studies on gene expression. Apart from being employed in breeding programs tobacco microspore/pollen embryogenesis technology combined with tobacco genome sequencing data can become a powerful tool to study plant cell totipotency.

Tobacco Microspore Embryogenesis

Though tobacco haploids had been known of from early twenties of the last century (Clausen and Mann 1924) significant progress in tobacco haploid production has been made only almost 50 years later.

History Behind Success

Pollen grains represent the male gametophytic generation in higher plants, are produced in large numbers and are easily accessible. Each pollen grain has a unique genome due to pairing, recombination, and segregation of chromosomes at meiosis. All those explain the great interest of plant geneticists and breeders in microspore-derived plants.

Microspores, immature and mature pollen grains have been cultured in vitro since the works of Tulecke with *Ginkgo biloba* (Tulecke 1953), *Taxus* (Tulecke 1959), *Torreya nucifera* (Tulecke and Sehgal 1963) and of Konar with *Ephedra foliate* (Konar 1963). The results of these works were only the formation of undifferentiated callus structures from these mature gymnosperm pollens. Guha and Maheshwari (1964) studying normal male gametophyte development in *Datura innoxia* observed in anthers cultured in vitro nothing less than embryo-like structures which, as they showed later (Guha and Maheshwari 1966), were haploid.

Several reports on the successful recovery of haploid plants in tobacco anther cultures appeared throughout the late 1960s–early 1970s (Bourgin and Nitsch 1967; Nakata and Tanaka 1968; Nitsch and Nitsch 1969; Sunderland and Wicks 1969; Sunderland and Wicks 1971). Since then extensive studies have been made to find the factors that facilitate the development of microspores into haploid plants. Numerous experiments in *Nicotiana* and *Datura* led to the conclusion that for these species the culture medium can be simple: sugar and mineral salts, but availability of iron is crucial. When the iron level was not enough, development of embryos stopped around the globular stage of embryogenesis (Nitsch 1972). But the key factor in success and rapid development of anther culture was the recognition of the precise stage of pollen development when pollen within cultured anthers could be switched from its normal program and continue divisions to form haploid plants. Thus, in tobacco the critical stage occurs around the time of the first pollen mitosis: just before, during or just after. Maximal response in terms of percentage of anthers induced, percentage of anthers giving plantlets and number of plantlets per anther

was obtained when microspores within the excised anthers were at the late unicellular stage or underwent mitosis (Sunderland and Wicks 1971; Nitsch 1972; Sunderland and Dunwell 1977). Several additional factors, including growth conditions of the donor plants, pre-culture treatments and culture conditions, have been shown to increase the embryogenic potential of pollen. Thus, in *Nicotiana tabacum* a difference in photoperiod (8 hours compared with 16 hours at certain light intensity) produces a five-fold difference in the yield of pollen plants (Dunwell 1976). Another factor, which strongly affected the response of anthers, was the age of the donor plants. The decline throughout the flowering period in *Nicotiana tabacum* was shown to correlate with a drop of one-half in the number of anthers producing plants and a drop of two-thirds in the number of plants produced (Sunderland and Dunwell 1977). Pollen viability is an important factor for pollen plant formation and the treatments, which maintain pollen viability, can be beneficial. Pretreatments such as chilling treatment of buds, anaerobic treatment of anthers, pre-treatment of excised anthers in a water-saturated atmosphere were shown to be effective (Nitsch and Norreel 1973; Duncan and Heberle 1976; Sunderland 1978; Imamura and Harada 1981; Dunwell 1981). *Nicotiana tabacum* and several other species of *Nicotiana* are hormone-independent species and only a simple basal medium is required for both inducing and sustaining growth of the pollen to the plantlets. Auxins, cytokinins, gibberellins, purine and pyrimidine bases do not positively affect the number of anthers induced, and several of these compounds are inhibitory at higher concentrations (Sunderland 1971). The inclusion of activated charcoal to agar media increases in *Nicotiana tabacum* and other species the proportion of anthers producing plantlets presumably because it absorbs inhibitory substances from the culture medium or the agar, or toxic compounds that emanate from the senescing cells of the wall (Anagnostakis 1974; Kohlenbach and Wernicke 1978; Horner et al. 1977).

In tobacco anther culture the number of embryos produced varies considerably from one anther to another, both within the same bud and between different buds and range from less than 400 to 10,000 per anther (Sunderland and Wicks 1971). Plants produced range from 1–135 per anther (Sunderland and Wicks 1971; Nitsch 1972; Horner et al. 1977). In general the proportion of pollen grains competent for embryogenesis in anther culture varies between less than 0.5% to about 5% of the grains, what corresponded to the percentage of a type of anomalous pollen grain found in nearly mature tobacco flowers *in situ* (Horner and Street 1978). The anomalous pollen grains were characterized by their smaller size and stained relatively lightly with general stains like acetocarmine. These pollen grains could undergo additional divisions in vivo and their subsequent gametophytic development was arrested: they were not able to germinate and fertilize. This phenomenon has been called pollen dimorphism and was studied in *Paeonia*, where it is prevalent (Sunderland and Dunwell 1977), and in tobacco (Horner and Street 1978; Heberle-Bors and Reinert 1979, 1980), and was also observed in *Hordeum vulgare* (Sunderland and Dunwell 1977) and *Secale cereale* (Wenzel and Thomas 1974). The cytological features between anomalous pollen in vivo and the pollen grains undergoing sporophytic development in cultured anthers in vitro were similar. Thus, it was suggested that pollen embryogenesis in tobacco could be predetermined

(Horner and Street 1978). The proof that anomalous pollen grains formed in situ were competent for embryogenesis was given by Heberle-Bors and Reinert (1980). The anomalous pollen grains were separated by Percoll gradient centrifugation from normal tobacco pollen grains and when cultured in liquid medium in vitro, they gave rise to normal embryos, abortive embryos and multinucleate structures. These direct pollen cultures were introduced as a new culture procedure – isolated pollen cultures. It was shown that the induction of P-pollen (pollen competent for embryogenesis) occurs early in flower development – during the meiotic prophase (Heberle-Bors 1982). The thorough study of this phenomenon in tobacco revealed that the frequency of P-pollen formation was strictly determined by the growth conditions of the donor plants and genotype under standard growth conditions (Heberle-Bors and Reinert 1981). In tobacco induction of P-pollen could be triggered by chemical treatments of plants with agents known to feminize flowers, namely auxins, antigibberellins, or by ionic effects such as nitrogen starvation (Heberle-Bors 1983).

While working with isolated tobacco pollen cultures Imamura et al. (1982) found out that the pollen embryos and plantlets could be obtained through isolated pollen cultures without prior treatment of donor plants or prior culture of anthers or buds. Pollen isolated at early binucleate stage of the development was first cultured in media without sucrose (water or medium contained macronutrients of H medium (Nitsch 1969)) for various periods and then transferred to H medium containing 2% sucrose and 5 mM glutamine. It was shown that the optimum medium for the initial culture (in terms of embryo formation) was water and an initial culture period of 5–7 days was required for the formation of plantlets. Based on these findings Kyo and Harada (1985, 1986) developed a new method for culture of isolated pollen of *Nicotiana rustica* and then *Nicotiana tabacum*. Two factors were intriguing: culture conditions and pollen developmental stage. Authors reported that sugar and nitrogen starvation applied to highly homogeneous population of immature pollen grains at the mid-binucleate stage allowed directing pollen development on sporophytic pathway in vitro in both species. Two processes were reported to be involved in the switch to sporophytic pathway: the dedifferentiation of the pollen during the period of starvation and its redifferentiation after the resupply of necessary nutrients (Imamura et al. 1982; Kyo and Harada 1986; Harada et al. 1988). Authors also proposed that in anther culture the same stressful conditions within anthers could switch a certain population of microspore to sporophytic pathway and that the embryogenic pathway through P-grain development might not be the only one (Kyo and Harada 1986). But although the frequency of induction of pollen cell divisions using this method in tobacco was high (40–70%), the frequency of embryo or callus formation was lower than that in *N. rustica*. Benito-Moreno et al. (1988) proved that iron is required for normal embryo development and plantlets formation in tobacco isolated pollen cultures and thus confirmed findings made earlier in tobacco anther culture (Nitsch 1972).

Touraev et al. (1996a) established a highly efficient in vitro system of embryogenesis from isolated tobacco microspores, where the combination of two stresses was applied: sugar and nitrogen starvation and heat shock treatment at 33°C, known

to induce pollen embryogenesis in *Brassica napus* (Pechan and Keller 1988). The microspore population used for culture initiation was a mixture of microspores in the mid to late unicellular stage and included a low percentage, 5–10%, of mitotic and early bicellular pollen, the same stages, that were reported to be responsive to embryogenesis in anther culture. Under optimal induction conditions all viable microspores in the culture became embryogenic and developed subsequently into pollen embryos by culture at 25°C in a sugar-containing medium, with induction frequencies of more than 70% with respect to the initial microspore population. The remaining ca. 30% of the initial microspore population did not survive the isolation procedure or the inductive stress treatment, and no mature starch-containing pollen grains could be observed in these cultures (Touraev et al. 1996a). The inductive treatment obviously had a strong synchronizing effect on the cultured microspores as from heterogeneous population of freshly isolated microspores highly homogeneous population of embryogenic microspores was obtained. Thus, this method allowed both to avoid time-consuming steps of purification of the starting microspore population or of the embryogenic microspore population and to obtain highly homogeneous samples of embryogenic pollen and/or pollen embryos in the amounts required for biochemical and molecular work. Touraev et al. (1996b) investigated in tobacco the possible reversibility of the process of embryogenic induction and concluded that the stress treatments (starvation alone or combined with a heat shock) applied to isolated microspores irreversibly blocked normal gametophytic development. Transfer of stressed tobacco microspores to a maturation medium which contained a higher sucrose concentration than the embryogenesis medium, as well as amino acids and nucleosides, did not lead to a continuation of gametophytic pollen development but to a few cell divisions followed by degeneration (Touraev et al. 1996b). Thus, the stress treatment did not simply arrest pollen development but actually reprogrammed them towards a sporophytic type of development.

Thousands of haploid plants can be obtained via isolated microspore/pollen cultures. Approximately 10–12% of plants are spontaneous diploids. The remaining haploid plants need to be diploidized (usually by means of colchicines treatment). The detailed protocols on tobacco doubled haploid production are published elsewhere (Touraev and Heberle-Bors 2003).

Mechanisms of Pollen Embryogenesis Induction in Tobacco

The ability of pollen grain to regenerate into plant intrigued researches for several decades and raised a lot of questions concerning the biological mechanisms associated with induction of pollen embryogenesis. Is pollen embryogenesis an atavism or an expression of totipotency? Why and how does stress stimulate microspore embryogenesis? Are there common or specific biochemical processes involved? How general is the concept of inducing microspore embryogenesis by stress in other species? Does it also apply to somatic cells?

Ultrastructure of embryogenic pollen, cellular/subcellular changes, cell cycle events and cytoskeletal changes associated with its formation as well as the division pathways in its further development in vitro in *Nicotiana tabacum* were studied in detail in anther culture (Sunderland and Wicks 1971; Dunwell and Sunderland 1974a, b, 1975), isolated microspore culture (Garrido et al. 1991; Touraev et al. 1996b) and during the formation of P-grains in vivo (Rashid et al. 1981) and were analysed thoroughly in reviews by Touraev et al. (2001) and Aionesei et al. (2005). In tobacco it is the vegetative cell that divides to give rise to an embryo. The first division in ca. 50% of microspores within an anther or in isolated microspores cultured under stress conditions is asymmetrical (the normal first pollen mitosis), but a low fraction of pollen grains contained two vegetative-like nuclei, products of symmetrical division. Stress applied to microspores induces the cell cycle arrest. Microspores in the G1 phase of the cell cycle pass through S phase during the stress treatment and arrest in the G2 phase. Microspores in the G2 phase divide during the stress treatment and become arrested in G1 phase (Touraev et al. 1996b). In mid-bicellular pollen the vegetative cells, which in vivo are arrested in G1, undergo DNA replication during the stress treatment and remain arrested in the G2 phase (Zarsky et al. 1992). Six to ten days after release from stress or after the first mitosis (symmetrical or asymmetrical) in anther culture the vegetative cells start to divide and multicellular structures are formed. The generative cell can also divide, but if it divides, it does so only once or twice. In the initial stages of growth, cell division proceeds faster than cell enlargement, and the daughter cells accordingly decrease in size with each successive division. Rupture of the pollen wall occurs about 20 day of culture. The place where the exine bursts determines the polarity of the future pollen embryos. The liberated mass of cells grows rapidly into a globular structure, root and shoot ends become differentiated, and embryos develop into plantlets through “heart”, “torpedo” and cotyledonary stages, closely resembling a zygotic embryo development.

Establishment of a highly efficient in vitro system of microspore embryogenesis from isolated microspore culture in tobacco, rapeseed, barley and wheat opened up possibilities to study the molecular mechanisms underlying the developmental switch from gametophytic toward sporophytic pathway in pollen development.

Comprehensive reviews on biochemical and molecular aspects of haploid embryogenesis in different species and in tobacco in particular were given by Touraev et al. (2001), Boutilier et al. (2005), Maraschin et al. (2005), Hosp et al. (2007a).

The three-step mechanism of induction of embryogenesis in tobacco microspores/immature pollen grains was proposed (Touraev et al. 1996b). First, it requires the stress-induced, irreversible block of normal gametophytic development, which is necessary but not sufficient condition for subsequent embryo development. Second, changes at the molecular level take place, leading to the formation of embryogenic cells in which the pathway of sporophytic development, i.e. embryo formation, is activated. And third, the underlying sporophytic programme is released by the initiation of cell divisions after transfer of the induced microspores or pollen grains to a simple, sucrose-containing medium. The first two steps taking place *in vitro* can be compared with the formation of anomalous pollen

grains, P-pollen, *in vivo* if the growth conditions leading to increased population of P-pollen among normal pollen grains (Heberle-Bors and Reinert 1981) are considered as a stress applied to the whole plant.

Though the mechanisms underlying the stress-induced switch from gametophytic to sporophytic pathway are still largely unknown, a number of common cellular processes taking place during transition to the embryogenic state have been revealed. First, stressed microspores undergo a transition towards a more dedifferentiated state. This transition is exhibited in changes in their overall morphology: cellular enlargement, typical "star-like" vacuolization and cytoskeletal rearrangements resulted in the migration of the nucleus towards a more central position (Raina and Irfan 1998; Zaki and Dickinson 1990; Garrido et al. 1995; Touraev et al. 1997). Second, the degradation of cellular components and certain proteins takes place and is considered a prerequisite for developmental re-programming in many organisms (Maraschin et al. 2005). Third, in tobacco and rapeseed the stress-treated microspores undergo a cell cycle arrest, which is relieved when microspores are transferred to non-stress conditions (Zarsky et al. 1992; Touraev et al. 1996b; Binarova et al. 1993).

Molecular studies aimed to reveal the changes that occur during the induction of microspore embryogenesis have largely focused on the identification of genes that are differentially expressed during this developmental transition or at early stages of pollen embryo development. Garrido et al. (1993) reported that in tobacco *de novo* synthesis of specific mRNAs takes place during culture of mid-bicellular pollen in the starvation medium and provided evidence that transcription during the induction phase is required for subsequent embryo formation in the sugar-containing medium. Zarsky et al. (1995) demonstrated that the expression of at least one low-molecular-weight heat shock protein (LMW *hsp*) gene, *Nthsp 18P*, is developmentally regulated during induction and the early stages (up to the heart-shaped stage) of pollen embryogenesis in tobacco. *Nthsp 18P*, encoding a small heat shock protein (18 kDa), was the first characterized gene transcriptionally activated in parallel with the acquisition of embryogenic competence in immature tobacco pollen.

Recently, different functional genomics approaches have been used to investigate the alterations in transcriptional and translational profiles in stress-treated microspores (Hosp et al. 2007b). To identify genes involved in the switch between the two pathways and to analyse gene expression profiles differential screening techniques such as differential display, subtraction hybridisation and dedicated cDNA arrays have been applied in tobacco, rapeseed, wheat and barley (Hosp et al. 2007b and references therein). In tobacco, suppression subtractive hybridization (SSH) followed by differential reverse Northern hybridizations revealed in stressed embryogenic microspores 90 distinct up-regulated sequences. Based on sequence analyses by homology searches half of them were classified into several functional clusters such as metabolism, chromosome remodelling, signalling, transcription and translation, while functions of another half cDNAs remain unknown and those sequences probably can be regarded as uncommon or rare genes (Hosp et al. 2007a). Among the 90 cDNAs 10 sequences were identified in earlier studies on differential gene expression in embryogenic tobacco bi-cellular pollen (Kyo et al. 2002, 2003; Hosp et al. 2007a).

Recent results on metabolic profiling of reprogrammed tobacco microspores revealed, in addition, dramatic changes in various metabolites (Hosp et al. 2007a). The level of fermentable sugars dropped to almost zero, while isomaltose, the product of amylopectin catabolism, was found in high abundance. The amounts of the triamine, spermidine and some amino acids including arginine and ornithine were significantly increased in stressed microspores. Several matches between transcription and metabolic profiles were observed: lack of proline in stressed microspores and highly increased expression of the proline oxidase gene (Hosp et al. 2007a); abundance in the organic acids citrate and isocitrate (early components of the citric acid cycle that takes place within mitochondria) and the upregulation of several genes encoding mitochondrial ATP-synthase subunits (Hosp et al. 2007a) that, in turn, correlated with the morphology of mitochondria of embryogenic microspores (Dunwell and Sunderland 1976) and illustrated reprogrammed microspores as actively respiring cells; specific protein phosphorylation patterns and an increase in protein kinase activities (Harada et al. 1988; Garrido et al. 1993; Kyo et al. 2002; Hosp et al. 2007a) and others (Hosp et al. 2007a).

Tobacco haploids are now applied in a number of research areas including genome mapping, identification of quantitative trait loci (QTL), genetic transformation, marker-assisted selection in breeding for new cultivars (Wernsman 1993; Tai 2005; Milla et al. 2005). Lately tobacco doubled haploid production was combined with reversible male sterility to develop an environment-friendly F1 hybrid system (Ribarits et al. 2007).

The sequencing of plant genomes performed for *Arabidopsis thaliana*, *Oryza sativa,* poplar and grapevine is currently underway for numerous other crop plants of commercial value such as maize, wheat, tomato and tobacco. A number of important research goals will be greatly enhanced with data generated within tobacco genome sequencing projects. A complete gene catalogue will provide basic information to investigate physiological and genetic processes in the tobacco plant, a widely used model in plant biotechnology. Tobacco genomics may lead to the elucidation of genetic factors that impact constituents associated with tobacco consumption. Understanding these processes may potentially contribute to achieving the goal of reducing the harm associated with cigarette smoking. In addition, important agronomic traits such as disease and pest resistance genes would be identified, and thus be available for use in traditional and molecular breeding projects aimed at enhancing the performance of tobacco as a crop in different environments and for various purposes, including production of pharmaceuticals in gene pharming. Finally, *Nicotiana tabacum*, is a member of the agriculturally important *Solanaceae* family, which also includes tomato, potato, eggplant and pepper crop plants. All of these plants may benefit from gene discovery in tobacco.

References

Aionesei T, Touraev A, Heberle-Bors E (2005) Pathways to microspore embryogenesis. In: Palmer CE, Keller WA, Kasha KJ (eds) Haploids in Crop Improvement II. Springer, Berlin/Heidelberg/New York, pp 11–34

Anagnostakis SL (1974) Haploid plants from tobacco – Enhancement with charcoal. Planta 115: 281–283

Benito-Moreno RM, Macke F, Hauser M-T, Alwen A, Heberle-Bors E (1988) Sporophytes and male gametophytes from in vitro cultured immature tobacco pollen. In: Cresti M, Jori P, Pacini E (eds) Sexual Reproduction in Higher Plants. Springer, Berlin/Heidelberg/New York, pp 137–142

Binarova P, Straatman K, Hause B, Hause G, Van Lammeren AAM (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus L*. Theor Appl Genet 87: 9–16

Bourgin JP, Nitsch JP (1967) Obtention de *Nicotiana* haploids à partir détamines cultivées in vitro. Ann Physiol 9: 377–382

Boutilier K, Fiers M, Liu C-M, Geest AHM (2005) Biochemical and molecular aspects of haploid embryogenesis, In: Palmer D, Keller W, Kasha K (eds) Haploids in Crop Improvement II. Springer, Heidelberg, pp 73–96

Clausen RE, Mann MC (1924) Inheritance in *Nicotiana tabacum* V. The occurrence of haploid plants in interspecific progenies. Proc Natl Acad Sci USA 10: 121–124

Duncan EJ, Heberle E (1976) Effect of temperature shock on nuclear phenomena in microspores of *Nicotiana tabacum* and consequently on plantlet production. Protoplasma 90: 173–177

Dunwell JM (1976) A comparative study of environmental and developmental factors which influence embryo induction and growth in cultured anthers of *Nicotiana tabacum*. Environ Exp Bot 16: 109–118

Dunwell JM (1981) Stimulation of pollen embryo induction in tobacco by pretreatment of excised anthers in a water-saturated atmosphere. Plant Sci Lett 21: 9–13

Dunwell JM, Sunderland N (1974a) Pollen ultrastructure in anther cultures of *Nicotiana tabacum* I. Early stages of culture. J Exp Bot 25: 352–361

Dunwell JM, Sunderland N (1974b) Pollen ultrastructure in anther cultures of *Nicotiana tabacum* II. Changes associated with embryogenesis. J Exp Bot 25: 363–373

Dunwell JM, Sunderland N (1975) Pollen ultrastructure in anther cultures of *Nicotiana tabacum* III. The first sporophytic division. J Exp Bot 26: 240–252

Dunwell JM, Sunderland N (1976) Pollen ultrastructure in anther cultures of *Datura innoxia*. J Cell Sci 22: 469–501

Garrido D, Charvat B, Benito-Moreno RM, Alwen A, Vicente O, Heberle-Bors E (1991) Pollen culture for haploid plant production in tobacco. In: Negrutiu I, Gharti-Chhetri G (eds) A Laboratory Guide for Cellular Molecular Plant Biology. Birkhäuser, Basel, pp 59–69

Garrido D, Eller N, Heberle-Bors E, Vicente O (1993) De novo transcription of specific messenger RNAs during the induction of tobacco pollen embryogenesis. Sex Plant Reprod 6: 40–45

Garrido D, Vicente O, Heberle-Bors E, Rodriguez-García MI (1995) Cellular changes during the acquisition of embryogenic potential in isolated pollen grains of *Nicotiana tabacum*. Protoplasma 186: 220–230

Guha S, Maheshwari SC (1964) In vitro production of embryos from anthers of *Datura*. Nature 204: 497

Guha S, Maheshwari SC (1966) Cell division differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212: 97–98

Harada H, Kyo M, Imamura J (1988) The induction of embryogenesis in *Nicotiana* immature pollen in culture. In: Applications of plant cell tissue culture. Ciba Foundation Symposium, Wiley, Chichester, pp 59–74

Heberle-Bors E (1982) On the time of embryogenic pollen grain induction during sexual development of *Nicotiana tabacum L* plants. Planta 156: 402–406

Heberle-Bors E (1983) Induction of embryogenic pollen grains and sebsequent embryogenesis in *Nicotiana tabacum L* by treatments of the pollen donor plants with feminizing agents Physiol Plant 59: 67–72
Heberle-Bors E, Reinert J (1979) Androgenesis in isolated pollen cultures of *Nicotiana tabacum*: dependence upon pollen development. Protoplasma 99: 339–347
Heberle-Bors E, Reinert J (1980) Isolated pollen cultures and pollen dimorphism. Naturwiss 67: 311
Heberle-Bors E, Reinert J (1981) Environmental control and evidence for predetermination of pollen embryogenesis in *Nicotiana tabacum* pollen. Protoplasma 109: 249–255
Horner M, Street E (1978) Pollen dimorphism – origin and significance in pollen plant formation by anther culture. Ann Bot 42: 763–777
Horner M, McComb JA, McComb AJ, Street HE (1977) Ethylene production and plantlet formation by Nicotiana anthers cultured in the presence and absence of charcoal. J Exp Bot 28: 1365–1372
Hosp J, Tashpulatov A, Roessner U, Barsova E, Katholnigg H, Steinborn R, Melikant B, Lukyanov S, Heberle-Bors E, Touraev A (2007a) Transcriptional and metabolic profiles of stress-induced, embryogenic tobacco microspores. Plant Mol Biol 63: 137–149
Hosp J, Maraschin SF, Touraev A, Boutilier K (2007b) Functional genomics of microspore embryogenesis. Euphytica 158: 275–285
Imamura J, Harada H (1981) Stimulation of tobacco pollen embryogenesis by anaerobic treatments. Z Planzenphysiol 103: 259–263
Imamura J, Okabe E, Kyo M, Harada H (1982) Embryogenesis and plantlet formation through direct culture of isolated pollen of *Nicotiana tabacum* cv Samsum and *Nicotiana rustica* cv Rustica. Plant Cell Physiol 23: 713–716
Kohlenbach HW, Wernicke W (1978) Investigations on the inhibitory effect of agar and the function of active carbon in anther culture. Z Planzenphysiol 86: 463–472
Konar RN (1963) A haploid tissue from the pollen of *Ephedra foliate*. Boiss Phytomorphology 13: 170–174
Kyo M, Harada H (1985) Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica*. Plant Physiol 79: 90–94
Kyo M, Harada H (1986) Control of the developmental pathway of tobacco pollen *in vitro*. Planta 168: 427–432
Kyo M, Yamaji N, Yuasa Y, Maeda T, Fukui H (2002) Isolation of cDNA coding for NtEPb1-b3, marker proteins for pollen dedifferentiation in a tobacco pollen culture system. Plant Sci 163: 1055–1062
Kyo M, Hattori S, Yamaji N, Pechan P, Fukui H (2003) Cloning and characterization of cDNAs associated with the embryogenic dedifferentiation of tobacco immature pollen grains. Plant Sci 163: 1055–1062
Maraschin SF Priester W de Spaink HP Wang M (2005) Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective J Exp Bot 56: 1711–1726
Milla SR, Levin JS, Lewis RS, Rufty RC (2005) RAPD and SCAR markers linked to an introgressed Gene Conditioning Resistance to *Peronospora tabacina* D.B Adam in tobacco. Crop Sci 45: 2346–2354
Murad L, Yoong Lim K, Christopodulou V, Matyasek R, Lichtenstein CP, Kovarik A, Leitch AR (2002) The origin of tobacco's T genome is traced to a particular lineage within *Nicotiana tomentosiformis* (Solanaceae). Am J Bot 89: 921–928
Nakata K, Tanaka M (1968) Differentiation of embryoids from developing germ cells in anther culture of tobacco. Jpn J Genet 43: 65–71
Nitsch JP (1969) Experimental androgenesis in *Nicotiana*. Phytomorphology 19: 389–404
Nitsch JP (1972) Haploid plants from pollen. Z Pflanzenzüchtg 67: 3–18
Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163: 85–87
Nitsch C, Norreel B (1973) Effet dun choc thermique sur le pouvoir embryogene du pollen de Datura innoxia cultive dans lanthere ou isole de lanthere CR Acad Sci Ser D 276: 303–306
Pechan PM, Keller WA (1988) Identification of potentially embryogenic microspores in *Brassica napus*. Physiol Plant 74: 377–384

Raina SK, Irfan ST (1998) High frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. Plant Cell Rep 17: 957–962

Rashid A, Siddiqui AW, Reinert J (1981) Ultrastructure of embryogenic pollen of Nicotiana tabacum var Badischer Burley. Protoplasma 107: 375–385

Ribarits A, Mamun AN, Li S, Resch T, Fiers M, Heberle-Bors E, Liu CM, Touraev A (2007) Combination of reversible male sterility and doubled haploid production by targeted inactivation of cytoplasmic glutamine synthetase in developing anthers and pollen. Plant Biotechnol J 5: 483–494

Sunderland N (1971) Anther culture: a progress report. Sci Prog Oxf 59: 527–549

Sunderland N (1978) Strategies on the improvement of yields in anther culture. In: Proceedings of Symposium on Plant Tissue Culture. Science Press, Peking, pp 65–86

Sunderland N, Dunwell JM (1977) Anther and pollen culture. In: Street HE (ed) Plant Tissue and Cell Culture. Oxford, Blackwell, pp 223–265

Sunderland N, Wicks FM (1969) Cultivation of haploid plants from tobacco pollen. Nature 224: 1227

Sunderland N, Wicks FM (1971) Embryoid formation in pollen grains of *Nicotiana tabacum*. J Exp Bot 22: 213–226

Tai GCC (2005) Haploids in the improvement of Solanaceous species. In: Palmer CE, Keller WA, Kasha KJ (eds) Haploids in Crop Improvement II. Springer, Berlin/Heidelberg, pp 173–190

Touraev A, Heberle-Bors E (2003) Anther and microspore culture in tobacco. In: Maluszynski M et al. (eds) Doubled Haploid Production in Crop Plants. Dordrecht, Netherlands: Kluwer Academic Publishers pp 223–228

Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996a) Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep 15: 561–565

Touraev A, Pfosser M, Vicente O, Heberle-Bors E (1996b) Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. Planta 200: 144–152

Touraev A, Vicente O, Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends Plant Sci 2: 285–303

Touraev A, Pfosser M, Heberle-Bors E (2001) The microspore: a haploid multipurpose cell. Adv Bot Res 35: 53–109

Tulecke WR (1953) A tissue derived from the pollen of *Ginkgo biloba*. Science 117: 599–600

Tulecke WR (1959) The pollen cultures of C.D. LaRue: a tissue from the pollen of *Taxus*. Bull Torrey Bot Club 86: 283–289

Tulecke WR, Sehgal N (1963) Cell proliferation from the pollen of *Torreya nucifera*. Contrib Boyce Thompson Inst 22: 153–163

Wenzel G, Thomas E (1974) Observations on the growth in culture of anthers of *Secale cereale*. Z Pflanzenzüchtg 72: 89–94

Wernsman EA (1993) Varied roles for the haploid sporophyte in plant improvement. In: Stalker HT, Murphy JP (eds) Plant Breeding in the 1990s. CAB International, Wallingford, pp 461–484

Zaki MAM, Dickinson HG (1990) Structural changes during the first divisions of embryos resulting from anther and microspore culture in *Brassica napus*. Protoplasma 156: 149–162

Zarsky V, Garrido D, Rihova L, Tupy J, Vicente O, Heberle-Bors E (1992) Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sex Plant Reprod 5: 189–194

Zarsky V, Garrido D, Eller N, Tupy J, Vicente O, Schöffl F, Heberle-Bors E (1995) The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ 18: 139–147

Chapter 6
An Overview of Triticale Doubled Haploids

F. Eudes and A. Chugh

Abstract This review report on the progress made to develop doubled haploid methods in triticale. Wide hybridization, anther culture and isolated microspores methods have been well established in triticale, and adopted in breeding and germplasm development programs for diverse genotypes. The development of these methods and culture media has co-evolved with those for its wheat counterpart. Essentially the critical factors for success remain the same: genotype, growing condition of mother plants, stress-like pretreatment and the culture medium composition. Today, C17, N6 and NPB99 induction medium for microspore culture are preferred. They carry maltose, sucrose or a combination of both, amino acids such as glutamine or proline, a low concentration of auxin and/or cytokinin. Spontaneous doubling has been reported at a low frequency in microspore derived plants making chromosome doubling by colchicine treatment an absolute need, as for wide hybridization derived plants. Isolated microspore culture is a method particularly attractive that will open new opportunities for gene transfer in triticale.

Keywords Anther culture, isolated microspore culture, wide hybridization, review

Introduction

Triticale (X *Triticosecale* Wittmack) is the fruit of scientific work and a dream of combining wheat quality attributes with the robustness of rye in one new species. Modern triticale has many desirable agronomic features, technical merit for feed

---

A. Chugh
Lethbridge Research Centre, Agriculture and Agri-Food Canada, P.O. Box 3000, 5403-1st Avenue South, Lethbridge, Alberta, Canada T1J 4B1

F. Eudes(✉)
Lethbridge Research Centre, Agriculture and Agri-Food Canada, P.O. Box 3000, 5403-1st Avenue South, Lethbridge, Alberta, Canada T1J 4B1
E-mail: eudesf@agr.gc.ca

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Her Majesty the Queen in Right of Canada, as represented by the Minister of Agriculture and Agri-Food Canada

and industrial usages, and has potential for human nutrition (Baier and Gustafson 1996). It is bred like its wheat parent, although continuous development of primary triticale or crosses with either parent, especially wheat, is needed to increase its genetic diversity. These interspecific crosses make triticale breeding laborious and fastidious. The production of haploid or doubled haploid plants provides a solution for accelerating the development of new germplasm and cultivars. It is being used as an alternative to the numerous cycles of inbreeding or backcrossing usually needed to obtain pure lines in conventional plant breeding.

The first observations of cell division of uni-nucleate triticale pollen grain and development of haploid plantlets were reported in the early 1970s (Wang et al. 1973). Experiences established in wheat were transferred to triticale anther culture. Calluses and embryoids were produced at frequencies as high as 30%, and green and fertile plants regenerated (Bernard et al. 1976). Merits of anther culture served the major purpose of rapidly fixing recombinants of this new species and accelerate cytogenetic studies, which were very common at the time. Similar to wheat, genetic differences were observed in the response of triticale lines and species (6x, hexaploid and 8x, octoploid) to induction culture media. Genetic instability of these early hexaploid and octoploids may have contributed to the challenge of regenerating triticale plants. The chromosome re-arrangements observed in the plants regenerated probably pre-existed in microspores of the F_1 hybrids (asyndetic hybrid), especially those involving R-genome chromosomes (Charmet et al. 1986). Cytoplasm and/or its interaction with the nucleus was reported to significantly influence induction and regeneration of triticale plants, with timopheevii cytoplasm exhibiting a positive effect on anther culture (Arzani and Darvey 1996; Gordei and Sorokin 1991).

A range of critical factors that are well known today for exerting influences on anther culture were rapidly reported: the uni-nucleate microspore stage and growing conditions of mother plants, e.g. longer photoperiod (16–19 hours) and higher light intensity (May–June natural light), cool temperature (12–15°C), were critical to the response of triticale microspores in anther culture (Bernard 1977). Reproducibility of growing conditions of mother plants has occasionally been a challenge and high quality growing facilities are a prerequisite for success. Glasshouse hydroponic systems or dedicated growth cabinets provide good examples of uniform, controlled and optimal growth environment (Arzani and Darvey 2002; Eudes and Amundsen 2005). Incubation of the anther culture in the dark at 25–28°C was routine. First reports of heat shock treatment at 31–33°C for the first days of anther culture indicated an increase of green plant frequency (Luk' yanyuk et al. 1988). Anthers kept at 4°C during the early stages of culture also gave rise to significantly higher numbers of green plants (Schumann 1988). Similarly, cold pretreatment (2–4°C) of tillers for a few days increased the final percentage of green plants and reduced that of chlorophyll-deficient plants (Luk' yanyuk et al. 1988). Still, albino plantlets were recovered at a very high frequency and chromosome doubling of the green haploid plants remained a challenge (Bernard 1980; Eudes and Amundsen 2005; Sozinov et al. 1980).

By the end of the 1980s the efficiency of octoploid triticale anther culture was practically at the same level as that of common wheat, that is, 2.5 green pollen

plants per 100 anthers. Promising triticale octoploid fixed lines were identified that constituted new 8x germplasm with agronomic performance similar to their parents, and used to introgress the D and R genomes in the 6x triticale (Tong and Bao 1989). Doubled haploidy was extensively employed to support cytogenetic strategies to broaden the germplasm and produce new triticale lines, including new forms with variability produced by somaclonal or androgenic variation through *in vitro* culture (Jouve and Soler 1996). Double haploids (DHs) can be derived in cereals either through interspecific crosses, e.g., using *Hordeum bulbosum* or *Zea mays*, or androgenesis. Considerable progress has been achieved in establishing successful processes for the production of triticale DH lines, especially through tissue culture of anther and microspores. Isolated microspore culture is further opening up new applications in triticale.

Evolution of Media Composition

An extensive effort has been deployed to improve the induction and regeneration media for triticale haploid culture. Most of the progress made in triticale has followed the development in wheat procedures. In addition, although most studies report concurrent results for the interaction between the treatments studied and genotypes, some differences exist and may reflect the genotypic diversity of triticale used among research groups.

Basal Media

Induction media based on N6, B5, MS, P2, P4, W5, C17, CHB3, MN6 and NPB99 media were tested in anther culture and isolated microspore culture. Significant differences were observed for the production of multi-cellular structures and plantlets: N6 and B5 media were found better than MS medium (Sun et al. 1980); C17 medium respectively better than B5, N6, and MS medium (Park et al. 1995); Potato II medium better than B5 medium, N6, MN6 and C17 (Wang and Hu 1984). Today, some of the defined basal media (C17, N6 and NPB99) are preferred and, are in common use (Eudes and Amundsen 2005; Gonzalez and Jouve 2005; Gut et al. 2006; Ponitka and Slusarkiewicz Jarzina 2007). These authors have reported regeneration on specialised media, 190–2, R9, or hormone free MN6, GEM (Eudes and Amundsen 2005; Karsai and Bedo 1997). Culture on liquid induction medium was consistently more successful than culture on solid medium (Ponitka and Slusarkiewicz Jarzina 2007; Sun et al. 1980). Green plant production was considerably improved by culture on a semi-liquid medium containing ficoll (Eudes and Amundsen 2005; Immonen and Robinson 2000). However, the most significant progress was achieved by supplementing these basal media with optimal carbon source, amino-acids and growth regulators.

Carbon Source

The influence of carbon source (e.g. sucrose, maltose, melezitose) in the induction medium and pretreatment, has been investigated for microspore response. At first, high concentrations of sucrose (6–12%) were associated with enhanced microspore response (Ponitka et al. 1999; Sun et al. 1980). However, later for most of the donor genotypes tested, maltose tested superior over sucrose for the number of green plants produced (Karsai and Bedo 1997; Marciniak et al. 1998). In most cases, the application of 0.21 or 0.26 M maltose resulted in higher embryo induction. High concentration of maltose (100 g/l) or equal amount of maltose and sucrose (50 g/l) were also reported to support the growth of a large number of embryoids and regenerated green plants (Gonzalez and Jouve 2000).

Amino Acids

Induction medium supplemented with glutamine, proline, oxyproline, and hydroxyproline promoted embryoid formation and increased percentage of plantlets (Ignatova and Luk' yanyuk 1982; Sozinov et al. 1981). Glutamine at 400–1,000 mg/l is now routinely supplemented in the induction medium (Eudes and Amundsen 2005; Gonzalez and Jouve 2005; Oleszczuk et al. 2004).

Plant Growth Regulators

Among various auxins and cytokinins used either individually or in combination, the induction medium supplemented with 2,4-D at 2 mg/l produced the highest average frequency of tissues and green plantlets (Hassawi et al. 1990). Consequently, 2,4-D at 2 mg/l has been preferred and extensively used in the induction medium (Gonzalez and Jouve 2005; Ponitka and Slusarkiewicz Jarzina 2007). Low concentration of cytokinins (benzyladenine (BA), 0.5 mg/l; kinetin, 0.2 mg/l) was also occasionally supplemented in the induction medium (Eudes and Amundsen 2005; Oleszczuk et al. 2004). As auxin lead to the production of calli, growth regulator-free medium has been considered for its positive effect on quality of triticale embryo production and plant regeneration (Lantos et al. 2005).

Haploids Using the Wide Hybridization Method

Wide hybridization with *Hordeum bulbosum* or *Zea mays* has been used with variable success to produce triticale haploid plants. After the formation of the hybrid embryo, paternal chromatin is eliminated at the early stages of embryogenesis. This wide

hybridization method includes auxin treatment of the florets to maintain growth of the haploid embryos and to induce ovary enlargement similar to kernel development until a stage where embryos can be isolated and rescued *in vitro* (about 12 days post pollination). Since rescued embryos are haploid, they require colchicine treatment to double their chromosome constitution leading to homozygous fertile plants and seed set.

The *Hordeum bulbosum* system has been applied to various hybrid genome constitutions as a substitute to genotype dependant anther culture. The method however, resulted in low seed set (Lehmann and Krolow 1991). As the seeds stopped their development at an early stage, it was not possible to rescue the embryos by embryo culture. The recessive *kr* alleles for cross compatibility with rye had a favourable effect on the frequency of zygotic haploids from selective chromosome elimination and embryo culture of triticale-wheat hybrid derived genomes. Up to 13.5 haploid plants/1,000 pollinated florets were produced (Sorokin and Gordei 1992).

Triticale DH lines were also obtained by crossing with maize. This method has been extensively adopted in Poland, as an alternative to androgenic methods since it is less influenced by genotype and also benefits from the experience gained in wheat (Wedzony et al. 2000). As many as 20% pollinated florets could produce haploid embryos, and up to 1.5% of pollinated florets were reported to produce doubled haploid triticale lines at a lower cost than from anther culture (Pratap et al. 2006).

Anther Culture

Growing Donor Plants

The quality of donor plants is critical for the success of androgenesis, and is often said to explain 50% of the success. For instance, an increase in day/night temperature from 18°C/14°C to 28°C/25°C resulted in decrease in the proportion of regenerated tissue, especially in winter triticale (Luk' yanyuk et al. 1983). Small grain cereals vary in their temperature and daylength requirements. Phytotron offers an alternative to mimic such unique growing conditions of donor plants all year long: pest free triticale donor plants, temperatures as low as 15°C/12°C, up to 19 hours photoperiod, high intensity lighting (up to 300 μmol m^{-2} s^{-1}). Though all these ideals conditions are rarely met collectively and reported in publications.

Pretreatments

Induction of sporophytic re-programming of cereal microspores is stress induced. Stress reported in triticale include: (1) tillers, through storage in the cold for 2–3 weeks; (2) spikes or anthers, by exposing them to 0.35 M mannitol at 2°C, heat

1shock at 30–33°C for up to 3 days, or low temperature for no more than 48 hours, or aluminium supplemented in the induction medium. Combination of cold pre-treatment complemented with heat shock improved androgenetic response of tested cultivars (Immonen and Robinson 2000).

Genotype Effect

The genotype effect has been associated with the microspore response since the onset of androgenesis. This was particularly true for protocols relying on callus induction (Sharma et al. 1982). There was an optimum time to transfer calluses to regeneration, e.g. 40–60 days. Older calli tended to produce only albino plants, while younger ones would not regenerate (Park et al. 1995). However, protocols relying on direct embryo formation have witnessed a relative attenuation of genotypic effects (Tuvesson et al. 2000).

Callus and embryoid induction proved to be controlled by additive and especially dominant genes, with over-dominance effects predominating, also indicating the absence of any significant epistatic effects (Balatero et al. 1995; Gonzalez et al. 1997; Matveenko et al. 1994). Loci associated with embryo frequency have been located on chromosomes 3RL, 5R (reduces frequency) and on 1AL, and loci associated with green-plant regeneration frequency are located on 4R, 1A, 1R and 3R (alleles on the last two reduce frequency; (Martinez et al. 1994). The triticale lines with the maximum number of dominant alleles controlling the character have been used for raising the efficiency of embryoid formation from hybrid triticale in breeding programmes (Kaminskaya et al. 1996). It has been suggested that the ability to create embryoids being passed onto the next generation would be better with genotypes originating from two way crosses and the heritability of the ability to create green plants would be similar between the two and three way types of hybrids (Gut et al. 2006). All ploidy levels have been observed to be amenable to anther culture and regenerate fertile green plants, this includes tetraploid triticale (Lehmann and Krolow 1991).

Ploidy Level

To date triticale produces less spontaneous doubling than its common wheat parent and requires colchicine treatment. At best, up to 57% green regenerated plants were reported to be fully fertile. Colchicine solution applied in the regeneration medium, in tubes, at low temperature (8°C) for 10 days and 16 hours photoperiod, reduced deleterious effect and enabled recovery of 66–90% fertile plants, in a genotype dependent manner (Slusarkiewicz Jarzina and Ponitka 2003). However, morphological abnormalities, possibly caused by aneuploidy and associated with loss of fertility, did occur and were observed in up to 23% of the plants (Immonen and Robinson 2000). Prolonged cold pretreatment generally increased the proportion of green plants as well as the proportion of spontaneous doubling (Immonen and Robinson 2000).

Best Practices

Once best determining factors (as discussed above) are combined with the established expertise for triticale anther/microspore culture ('the Arts meeting the Science'), high microspore response and doubled haploid plant regeneration yields were achieved. Across a large number of genotypes and F_1, F_2 hybrids, best practices enabled the production of 90–320 embryos per 100 anthers on either media, C17 containing maltose, 190.2 containing sucrose, or PII with sucrose, and supplemented with auxin (1.25–2 mg/l) and cytokinin (0.5 mg/l kinetin). An average of 6 green plants per 100 anthers, and up to 24 green plants, could be produced (Ponitka et al. 1999; Tuvesson et al. 2000; Wedzony et al. 2000).

Isolated Microspore Culture

Suspension culture of triticale microspores differs from anther culture only in the extraction and purification steps of microspores from spikelets or anthers collected, using a blender (Keller 1991). It is not substantially different from wheat microspore extraction and purification, although from our experience the amount of tissues, amount of debris created by blending, and the yield of microspores recovered is higher. All the processes, from blending to purification in a centrifuge, have to be conducted in a very timely controlled fashion, at 4°C, to reduce the rate of microspore mortality that is generally observed in the first few days (Keller 1991). However, genotypes and culture conditions remain major determinants in microspore mortality during the early phase of the culture (Gonzalez and Jouve 2005). By increasing induction medium viscosity and helping microspore to float, Ficoll emerged as a major determinant for the increased numbers of embryos formed and green plants regenerated (Eudes and Amundsen 2005).

Microspore density has been reported as low as 3×10^4 cells per ml to as much as 2×10^5 cells per ml of culture (Eudes and Amundsen 2005; Oleszczuk et al. 2004). A higher density has been associated with a higher induction rate of embryo-like structures. Number of green plants reported per spike varied from 0 to 15 in spring triticale cultivars whereas an average of 55 green plants in winter triticale cv. Bogo has been reported (Eudes and Amundsen 2005; Oleszczuk et al. 2004). Despite these successes, the frequency of albinism varied greatly, ranging from 9% to 50%, and the frequency of haploid green plants ranged from 30% to 90%, though more often on the higher side (Eudes and Amundsen 2005; Oleszczuk et al. 2004; Pauk et al. 2000).

Gene Transfer

Gene transfer can be achieved in different ways, mainly through conventional and interspecific crosses, germplasm development and breeding, as well as direct transgene delivery in plant cell, referred as genetic engineering. Doubled haploids are

being produced from secondary triticale in support of well funded breeding programmes. Haploid lines and haploidy on intergeneric hybrids have been proven useful to bridge the D and R genomes in the 6x triticale and for creating alien addition lines of distant hybrid F_1s (Sbeva et al. 1987; Wang and Hu 1993). Direct gene transfer method to triticale haploid embryo-like structures using the Dupont PDS He/1000 apparatus, for biolistic transformation, has been established (Rubio et al. 2004). We have also tested successfully the efficacy of various promoters and genes in triticale microspore–derived embryos via biolistics (A Chugh and F Eudes 2007, unpublished results). To conclude, the microspore presents unique attributes that make it an attractive target for future development of genetic engineering methods. It is a single cell, haploid, with an incredible capacity to regenerate a full green and fertile plant; no other plant cell cumulates these characteristics. We envision DNA introductions into triticale uni-nucleated microspore as a targeted site, which can then be developed into doubled haploid cells and transmitted to fertile offspring.

References

Arzani A, Darvey NL (1996) Positive effect of timopheevii cytoplasm on anther culture response of triticale. Triticale: today and tomorrow, Dordrecht, Netherlands: Kluwer Academic Publishers pp 391–393

Arzani A, Darvey NL (2002) Androgenetic response of heterozygous triticale populations using a greenhouse hydroponic system. Euphytica 127:53–60

Baier AC, Gustafson JP (1996) Breeding strategies for triticale. Triticale: today and tomorrow, Dordrecht, Netherlands: Kluwer Academic Publishers pp 563–569

Balatero CH, Darvey NL, Luckett DJ (1995) Genetic analysis of anther-culture response in 6x triticale. Theoretical and Applied Genetics February 90:279–284

Bernard S (1977) Study of some factors contributing to the success of androgenesis by in vitro anther culture in hexaploid triticale. Annales de l' Amelioration des Plantes 27:639–656

Bernard S (1980) In vitro androgenesis in hexaploid triticale: determination of physical conditions increasing embryoid and green plant production. Zeitschrift fur Pflanzenzuchtung 85: 308–321

Bernard S, Picard E, Buyser Jd (1976) Obtaining haploid plants from Triticale hexaploides (X Tritosecale wittmack) by in vitro anther culture. C R Hebd Seances Acad Sci, Ser D Sci Nat, July 19 283:235–238

Charmet G, Bernard S, Bernard M (1986) Origin of Aneuploid Plants Obtained by Anther Culture in Triticale. Canadian Journal of Genetics and Cytology 28:444–452

Eudes F, Amundsen E (2005) Isolated microspore culture of Canadian 6x triticale cultivars. Plant Cell Tissue and Organ Culture 82:233–241

Gonzalez JM, Jouve N (2000) Improvement of anther culture media for haploid production in triticale. Cereal Research Communications 28:65–72

Gonzalez JM, Jouve N (2005) Microspore development during in vitro androgenesis in triticale. Biologia Plantarum 49:23–28

Gonzalez M, Hernandez I, Jouve N (1997) Analysis of anther culture response in hexaploid triticale. Plant Breed 116:302–304

Gordei IA, Sorokin AP (1991) Alloplasmic effect in the anther culture of hexaploid triticales. Genetika Moskva 27:565–567

Gut M, Bichonski A, Mikulski W, Bielerzewska H (2006) Anther culture response in heterozygous triticale (x Triticosecale Wittmack) populations. Cereal Research Communications 34:1029–1036

Hassawi DS, Qi J, Liang GH (1990) Effects of growth regulator and genotype on production of wheat and triticale polyhaploids from anther culture. Plant Breed 104:40–45

Ignatova SA, Luk' yanyuk SF (1982) Amino acids in the anthers of triticale following in vitro culture. Nauchno tekhnicheskii Byulleten' Vsesoyuznogo Selektsionno geneticheskogo Instituta 3:26–31

Immonen S, Robinson J (2000) Stress treatments and ficoll for improving green plant regeneration in triticale anther culture. Plant Science 150:77–84

Jouve N, Soler C (1996) Triticale genomic and chromosomes' history. Triticale: today and tomorrow, Dordrecht, Netherlands: Kluwer Academic Publishers pp 91–118

Kaminskaya LN, Khotyljova LV, Matveenko SN (1996) Genetic control of anther culture induction in triticale. Triticale: today and tomorrow, Dordrecht, Netherlands: Kluwer Academic Publishers pp 395–399

Karsai I, Bedo Z (1997) Effect of carbohydrate content on the embryoid and plant production in triticale anther culture. Cereal Research Communications 25:109–116

Keller J (1991) Influence of media conditioning on in vitro development in pollen suspensions of triticale. Biologisches Zentralblatt 110:207–214

Lantos C, Jancso M, Pauk J (2005) Microspore culture of small grain cereals. Acta Physiologiae Plantarum 27:631–639

Lehmann C, Krolow KD (1991) Experiments on haploid production from tetraploid triticales by the Hordeum bulbosum system and anther culture. Cereal Research Communications 19:283–290

Luk' yanyuk SF, Ignatova SA, Sozinov AA (1983) Use of in vitro techniques for producing haploids in barley and triticale. Tagungsbericht, Akademie der Landwirtschaftswissenschaften der Deutschen Demokratischen Republik 207:41–48

Luk' yanyuk SF, Makhnovskaya ML, Ignatova SA, Sherer NV (1988) Significance of growing conditions in obtaining haploids of wheat and triticale. Ispol' zovanie iskusstvennogo klimata 89–92

Marciniak K, Banaszak Z, Wedzony M (1998) Effect of genotype, medium and sugar on triticale (xTriticosecale Wittm.) anther culture response. Cereal Research Communications 26:145–151

Martinez I, Bernard M, Nicolas P, Bernard S (1994) Study of androgenetic performance and molecular characterization of a set of wheat-rye addition lines. Theoretical and Applied Genetics December 89:982–990

Matveenko SN, Khotyleva LV, Kaminskaya LN (1994) Genetic analysis of efficiency of triticale anther culture in vitro. Genetika 30:1238–1242

Oleszczuk S, Sowa S, Zimny J (2004) Direct embryogenesis and green plant regeneration from isolated microspores of hexaploid triticale (x Triticosecale Wittmack) cv. Bogo. Plant Cell Reports 22:885–893

Park KG, Ha YW, Yoon KM (1995) Anther culture response of triticale varieties and plant regeneration as callus induction media and period. RDA Journal of Agricultural Science Biotechnology 37:213–220

Pauk J, Puolimatka M, Toth KL, Monostori T (2000) In vitro androgenesis of triticale in isolated microspore culture. Plant Cell, Tissue Organ Culture 61:221–229, Dordrecht: Kluwer

Ponitka A, Slusarkiewicz Jarzina A (2007) The effect of liquid and solid medium on production of winter triticale (x Triticosecale Wittm.) anther-derived embryos and plants. Cereal Research Communications 35:15–22

Ponitka A, Slusarkiewicz Jarzina A, Wedzony M, Marcinska I, Wozna J (1999) The influence of various in vitro culture conditions on androgenetic embryo induction and plant regeneration from hexaploid triticale (X Triticosecale Wittm.). Journal of Applied Genetics 40:165–174

Pratap A, Sethi GS, Chaudhary HK (2006) Relative efficiency of anther culture and chromosome elimination techniques for haploid induction in triticale x wheat and triticale x triticale hybrids. Euphytica 150:339–345

Rubio S, Jouve N, Gonzalez JM (2004) Biolistic transfer of the gene uidA and its expression in haploid embryo-like structures of triticale (xTriticosecale Wittmack). Plant Cell Tissue and Organ Culture 77:203–209

Sabeva Z, Dorossiev L, Stoinova Y, Staneva M (1987) Using haploid androgenetic regenerates obtained from triticale (2n = 56) in hybridization with triticale (2n = 42). Genetika i Selektsiya 20:17–21

Schumann G (1988) Investigations into albinism in anther cultures of triticale. Archiv fur Zuchtungsforschung 18:115–122

Sharma GC, Wang WC, Sapra VT (1982) Effect of genotype, media and temperature pretreatment on callus initiation in triticale, wheat, and rye anther cultures. Cereal Research Communications 10:143–150

Slusarkiewicz Jarzina A, Ponitka A (2003) Efficient production of spontaneous and induced doubled haploid triticale plants derived from anther culture. Cereal Research Communications 31:289–296

Sorokin AP, Gordei IA (1992) Genotype dependence and the effect of nucleocytoplasmic interactions in the process of allohaploid induction in triticale and wheat. Genetika Moskva 28:93–103

Sozinov A, Lukjanjuk S, Ignatova S (1981) Anther cultivation and induction of haploid plants in triticale. Zeitschrift fur Pflanzenzuchtung 86:272–285

Sozinov AA, Luk' yanyuk SF, Ignatova SA (1980) Induction of haploid plants of triticale by means of anther culture. Genet, selekts i agrotekhn tritikale 7–20

Sun JS, Zhu ZQ, Wang JJ, Tigerstedt PMA (1980) Studies on anther culture of Triticale. Acta Botanica Sinica 22:22–31

Tong Q, Bao W (1989) Man made triticeae genomes by chromosome recombination Ii. Screening for haploid triticale clones with high anther culture efficiency. Acta Agronomica Sinica 15:74–79

Tuvesson S, Ljungberg A, Johansson N, Karlsson KE, Suijs LW, Josset JP (2000) Large-scale production of wheat and triticale double haploids through the use of a single-anther culture method. Plant Breed 119:455–459

Wang X, Hu H (1984) The effect of potato II medium for triticale anther culture. Plant Science Letters 36:237–240

Wang YB, Hu H (1993) Gamete composition and chromosome variation in pollen-derived plants from octoploid triticale x common wheat hybrids. Theoretical and Applied Genetics 85:681–687

Wang YY, Sun CS, Wang CC, Chien NF (1973) The induction of the pollen plantlets of Triticale and Cap sicum annuum from anther culture. Scientia Sinica 16:147–151

Wedzony M, Marcinska I, Golemiec E, Zur I (2000) Major factors influencing triticale anther culture. Biotechnological approaches for utilisation of gametic cells COST 824:1–5

Chapter 7
Patents and Haploid Plants

J.M. Dunwell

Abstract One of the important themes in any discussion concerning the application of haploids in agricultural biotechnology or elsewhere is the role of Intellectual Property Rights (IPR). This term covers both the content of patents and the confidential expertise, usually related to methodology and referred to as "Trade Secrets". This review will explain the concepts behind patent protection, and will use the international patent databases to analyse the content of these patents and trends over the last 20 years. This analysis from regions including North America, Europe, and Asia reveals a total of more than 30 granted patents and a larger number of applications. The first of these patents dates from 1986, and although the peak of activity was in the late 1990s, there has been continuous interest to the present day. The subject matter of these patents and applications covers methods for anther and pollen culture, ovule culture, the use of specific haploid-inducing genes, the use of haploids as transformation targets, and the exploitation of genes that regulate embryo development. The species mentioned include cereals, vegetables, flowers, spices and trees.

Keywords Intellectual property, plant breeding, genetics, homozygous, pollen, anther, ovule

Introduction

Although the present and future status of haploid methods of plant breeding has been the subject of several recent reviews (Palmer et al. 2005; Forster and Thomas 2005; Forster et al. 2007; Maluszynski et al. 2003), there has been no assessment

J.M. Dunwell(✉)
School of Biological Sciences, University of Reading, Whiteknights, Reading RG6 6AS, UK
E-mail: j.dunwell@reading.ac.uk

© Springer Science+Business Media B.V. 2009

of the role of intellectual property in any of these publications. The present review is designed to provide this missing perspective and will extend to a discussion of intellectual property from the perspective of the research scientist (Shear and Kelley 2003) and of those interested in international developments (Koo et al. 2004), globalization (Parayil 2003) and the more general ethical aspects of the public- and private-sector relationships (Hails 2004).

Historical Perspective

More than a century ago in July 1899 an international conference organised by the Royal Horticultural Society (RHS) was held in London. The subject of the conference was hybridisation and in one of the many speeches given at the banquet the leading British judge, Lord Justice Lindley made the following prediction: "I have heard something about hybridisation of which I know little. I have heard something which leads me to suppose that the development of that art may react with the profession to which I have the honour to belong. Without being a prophet, I seem to see before me a vista of patent hybrids! What a treat for the patent lawyers! And what an accession of work for her Majesty's Judges!" (Anon 1900).

By 1906, the emphasis on patents had already been demonstrated in the chemical sector where it was reported (Anon 1906) that "the German company Bayer had achieved a monopoly position in novel chemicals, with 1000 patents at home and 1200 overseas". However, the first real discussion of patents in relation to plant breeding is probably that from the subsequent Third International Conference on Genetics, organised by the RHS in 1906 and most famous for the coining of the term "genetics" by William Bateson (Dunwell 2007). During this meeting, there was a session entitled: "Copyright" for Raisers of Novelties (Anon 1907). It is reported that Mr. George Paul whilst discussing the absence of several well known plant breeders from the conference, commented "The fact is, these gentlemen do not like to tell us, or to show, what they have done in their experiments, because once their knowledge become public, they have not the slightest chance of receiving any pecuniary reward for their labours. If they were properly protected from being deprived of the due reward of their labours, they would no doubt be much more willing to come forward and help us and place their experience at our disposal".

Following these early prescient comments and debates, it was to be several years before any legal protection for plants was enacted, and then only for clonal material in the USA. The first US plant patent (PP00001), issued for a climbing rose in 1931 (Cook 1931a), was soon followed by further examples (Cook 1931b, 1933). It is notable that even at that time the topic remained the subject of controversy from both scientific and legal experts (Allyn 1933; Barrons 1936; Cook 1936; Fay 1937). Much of the debate today, almost 100 years after the first discussion, follows the same themes.

What are Patents?

The history of patent law dates back several centuries, but in summary, "A patent gives an inventor a period of exclusive exploitation (up to 20 years in the UK) in return for a disclosure of the invention" (Huskisson 1996). According to the UNCTAD site (http://www.iprsonline.org/guide/index.htm) a patent application must satisfy the patent examiner that the invention is:

- Useful (i.e., has industrial application): ideas, theories, and scientific formulae are not sufficiently useful or industrially applicable to be patentable.
- Novel: the invention should be recent and original, but perhaps most importantly it should not already be known (in the public domain). In most countries (except the USA) the patent is awarded to the first person to apply, regardless of whether this person was the first to invent.
- Non-obvious or must involve an inventive step: not obvious to a person skilled in the technology and more inventive than mere discovery of what already exists in nature (such as a gene with no known function). The invention must be disclosed to the patent examiners in a detailed way that would enable a skilled technician to make and use it.

This disclosure of an invention takes the form of a publication from the relevant patent office. In the case of most authorities, the patent application is published 18 months after the date of filing and it is then available for inspection. Exceptionally, until 15 March 2001, the US maintained secrecy until the time the patent was granted, a period which can range from an average of 2–3 years to more than 20 years. As an example of the length of time sometimes involved, it took approximately 20 years for resolution of a dispute concerning key patents which cover elements of *Agrobacterium*-based transformation. Under the agreement, announced on 4 February 2005, Max Planck Society, Bayer CropScience, Garching Innovation, and Monsanto will cross license their respective technologies worldwide.

An important difference between the US and other patent systems is that the 17 year duration of a US patent filed prior to 2001 only starts from the time at which it was granted, whereas in Europe (and now in the US) the 20 year period of exclusivity starts from the time of filing the application. Some of the consequences of this change will be discussed in more detail below.

Sources of Patent and other Relevant Information

During the preparation of this review extensive use has been made of the freely available patent databases in the US (http://www.uspto.gov/patft/index.html), World International Patent Organisation (http://pctgazette.wipo.int/), Europe (http://ep.espacenet.com/), and other international sites (eg http://www.google.com/patents; http://www.surfip.gov.sg/; http://www.pat2pdf.org/; http://www.freepatentsonline.com/) and most especially the

Patent Lens section (http://www.bios.net/daisy/bios/patentlens.html) of BiOS, Biological Innovation for Open Society, an initiative of CAMBIA (Center for the Application of Molecular Biology to International Agriculture). Another relevant site with a summary of granted US ag-biotech patents from 1976–2000 is provided by the Economic Research Service (ERS) of the US Department of Agriculture (USDA) (http://www.ers.usda.gov/Data/AgBiotechIP/). It should be noted that the most detailed forms of patent analysis require commercial subscription from companies such as Derwent (http://www.derwent.com), MicroPatent (http://www.micropat.com/static/index.htm), or patentmaps.com (http://patentmaps.com/shop/v2/shophome.htm).

Patents and Plant Biotechnology

Apart from the natural form of genetic protection provided by F_1 hybrids, there are a range of legal processes that can be used to protect novel types of plants produced by one company from being exploited by commercial competitors, with these methods varying from one country to another (Cahoon 2000; Locke 2007). An introduction to the various approaches, namely patents (Chan 2006) and plant breeders' rights (known collectively as Intellectual Property Rights – IPR), is available from several authors (Brown 2003), and from BiOS (http://www.bios.net/daisy/bios/patentlens/tutorials.html).

Information relating to the systems in individual countries is available at the respective patent offices. For example, the latest note on patenting of plants in the UK "Examination Guidelines for Patent Applications relating to Biotechnological Inventions in the UK Intellectual Property Office" was published by the Intellectual Property Office in November 2006 (http://www.ipo.gov.uk/biotech.pdf). Similar information is available in the US (Merrill et al. 2004), Europe (Fleck and Baldock 2003; Schrell et al. 2007), New Zealand (Ministry of Economic Development 2002) and China.

There have been several, extensive reviews of the implications, and consequences of applying patent (and other IPR) protection to plants (Farnley et al. 2004; Adcock 2007) and the reader is referred to these publications, most of which are freely available on the web. In one of the most comprehensive of these reviews (Binenbaum et al. 2003), the important conclusion is reached that as patenting becomes ever more prevalent in biotechnology (Wright 2006; Wright and Pardey 2006) and elsewhere (Straus 2007), the diversity of innovations utilized in developing modern cultivars means a corresponding increase in the number of separate rights needed to produce a new innovation (Tokgoz 2003). Where ownership of the relevant rights is sufficiently dispersed, the problem of multilateral bargaining can become very difficult to resolve. For example, many of those who develop new technology by building on existing technologies are often unaware of either the extent to which the latter have been claimed as IP (Kryder et al. 2000) or the strength of any such claims. Both the conduct of research and development and subsequent commercialization therefore

entail navigating through a potential minefield of patent applications that have been filed but remain invisible pending publication by the patent office. Fortunately, the uncertainty arising from such "submarine" patent applications is becoming less important as the US has harmonized with the rest of the world by publishing (from November 2000) patent applications within 18 months of filing.

Three specific areas in which IPR issues have had significant impact are those relating to international trade and development (Binenbaum et al. 2003; Gaisford et al. 2007; Garrison 2006; Giannakas 2001; Helfer 2004; Léger 2007), the issue of public and private research (Cantley 2004; Donnenwirth et al. 2004; Graff et al. 2003; Heisey et al. 2005), and the consolidation of companies within the agricultural biotechnology sector (Brennan et al. 2005; Chan 2006; Schimmelpfennig and King 2006).

Despite this complexity of IPR in the biotechnology industry (Eisenberg 2006; Kukier 2006), and the difficulties of making accurate predictions over extended time scales (Yerokhin and Moschini 2007) it should be noted that a similar situation exists in the electronics and other similar industries where products are assembled from numerous components, sourced from many countries and covered by a multiplicity of patents.

Patents Related to Haploid Plants

The strong commercial interest in methods for the production and exploitation of haploid plants is exemplified by the extensive number of granted patents and patent applications in this subject area. The most easily accessible information is available from the USA (http://www.uspto.gov/patft/index.html) (see above) and granted patents and patent applications from this jurisdiction are summarised in Tables 1 and 2, respectively. Information from the Patent Cooperation Treaty (PCT) system of the World Intellectual Property Organisation (WIPO) is provided in Table 3, together with patents/applications from other countries and regions. These include Europe, Canada, China, Russia and Japan. Selected examples from these lists are discussed, according the category of invention, in further detail below.

Some of the patents and applications are very general in their coverage, while others are limited to very specific areas of technology. An example of the first type is the Japanese patent JP11318249 which claims both male and female-derived haploids. Another interesting example concerns the case of "Reverse breeding" in plants (US 20060179498). This process involves a method for efficiently producing homozygous plants from a heterozygous individual. It consists of selecting a heterozygous individual, allowing it to produce haploid cells (the products of meiosis), creating homozygous doubled haploids, and selecting the individuals with the desired genome. Most importantly, during production of the haploid cells no recombination occurs, in order to obtain a limited number of genetically different haploid cells. In one example, the prevention or suppression of recombination is achieved by interfering with one or more target genes involved in recombination.

Table 1 Granted U.S. patents

Number	Date issued	Species	Inventor(s)
4835339	February 1986	Tomato	Evans et al.
4840906	June 1989	Barley	Hunter
5049503	September 1991	Brassica	Swanson et al.
5066594	November 1991	Maize	DeBonte et al.
5066830	November 1991	Capsicum	Morrison et al.
5272072	December 1993	Barley	Kaneko and Ito
5306864	April 1994	Maize	Petolino
5322789	June 1994	Maize	Genovesi et al.
5445961	August 1995	Maize	Genovesi et al.
5492827	February 1996	Cucumber	Dirks
5547866	April 1996	Taxus	Durzan et al.
5602310	February 1997	Maize	Petolino
5629183	May 1997	Tobacco	Saunders et al.
5639951	June 1997	Maize	Bosemark et al.
5749169	May 1998	Maize	Briggs
5770788	June 1998	Maize	Jia
5840557	November 1998	Tobacco	Heberle-Bors et al.
5900375	May 1999	Brassica	Simmonds et al.
5929300	July 1999	Tobacco	Burke et al.
6200808	March 2001	Brassica	Simmonds et al.
6316694	November 2001	Brassica	Dormann et al.
6362393	March 2002	Wheat	Konzak et al.
6407314	January 2002	Tobacco	Oldenhof
6764854	July 2004	Rice	Konzak et al.
6812028	November 2004	Wheat	Kasha et al.
6861576	March 2005	Brassica	Drouaud et al.
7135615	November 2006	Maize	Kato
7148402	December 2006	Arabidopsis	Niu and Chua
7151170	December 2006	Brassica	Boutilier et al.
7297838	November 2007	Flax	Chen and Dribnecki

Table 2 U.S. patent applications

Number	Date Published	Species	Inventor(s)
20020151057	October 2002	Maize	Zheng et al.
20040210959	October 2004	Flax	Armstrong et al.
20040226059	November 2004	Wheat	Kasha et al.
20050071898	March 2005	Arabidopsis	Zuo et al.
20050198711	September 2005	Maize	Mathews
20050289663	December 2005	Orchid	Ichihashi
20050289673	December 2005	Maize	Armstrong al.
20060179498	August 2006	Brassica et al.	Dirks et al
20060185033	August 2006	Maize	Zhao et al
20060260005	November 2006	Brassica	Chen and Celio
20070107077	May 2007	Maize	Chen and Tulsieram
20070204366	August 2007	Maize	Deppermann et al.
20070292951	December 2007	Brassica	Ilic-Grubor et al.

Table 3 Non-USA patent documents

Number	Date	Species	Inventor(s)
(a) World Patent System			
WO 86/00495	January 1986	Wheat	Picard
WO 92/14828	September 1992	Barley	Tallberg et al.
WO 94/01999	February 1994	Barley	Holm et al.
WO 02/01940	January 2002	Wheat	Jensen et al.
WO 2004/032607	April 2004	Orchid	Miyoshi
WO 2004/042066	May 2004	Tobacco	Touraev et al.
WO 2005/004586	January 2005	Maize	Bordes et al.
WO 2005/084420	September 2005	Wheat	Shen
WO 2006/116876	November 2006	Saponaria	Ferrie et al.
WO 2006/125310	November 2006	Apiaceae	Ferrie et al.
WO 2006/128707	December 2006	Tomato	Dirks et al.
WO 2007/038075	April 2007	Maize	Barton et al.
(b) European patents			
0 127 313	July 1989	Wheat	Sherba et al.
0 171 310	April 1990	Wheat	Picard
(c) Chinese patents			
CN1080114	January 1994	Seaweed	Chaoyuan et al.
CN1212828	April 1999	Wheat	Weng
CN1331914	January 2002	Tobacco	He et al.
CN1418537	May 2003	Capsicum	Li et al.
CN1418950	May 2003	Capsicum	Gu et al.
CN1473462	February 2004	Camelina	Zhang et al.
CN1484945	March 2004	Radish	Mei
CN1555678	December 2004	Rice	Xu et al.
CN1653885	August 2005	Pumpkin	Wang et al.
CN1723767	January 2006	NA	Mei
CN101011028	August 2007	Chrysanth.	Wang
(d) Japanese patents			
JP59205922	November 1984	NA	Samueru et al.
JP1067130	March 1989	Rice	Negishi et al.
JP3280817	December 1991	Cucumber	Fujishita et al.
JP4112730	April 1992	Brassica	Hamaoka et al.
JP5219849	August 1993	Melon	Kato et al.
JP6237657	August 1994	Melon	Kato et al.
JP8126444	May 1996	Carnation	Sato et al.
JP9172893	July 1997	Cyclamen	Kitaura et al.
JP11318249	November 1999	NA	Kato et al.
(e) Canadian patents			
CA 1236700	May 1988	Wheat	Sherba et al.
CA 2019989	December 1990	Maize	Mitchell et al.
CA 2444797	October 2002	Maize	Bidney et al.
(f) Russian patents			
SU1497212	July 1989	Cedar	Ruguzov et al.
SU1520096	November 1989	Barley	Rodin et al.
SU921138	October 1996	Maize	Tyrnov et al.
RU2150823	June 2000	Flax	Poljakov
(g) Bulgarian patent			
BG51816	September 1993	Tobacco	Kintja et al.
(h) Spanish patent			
ES2180385	February 2003	Cork oak	de la Vega et al.

The target genes can be involved in double strand breaks, chromosome pairing, crossing-over and separation of sister chromatids. In this way an individual heterozygous plant can be converted into a heterozygous (F_1 hybrid) variety without the necessity of vegetative propagation but as the result of the cross of two homozygous lines derived from the original selected plant.

Anther and Microspore Culture

Probably the first patent relating to induction of haploids *in vitro* was that granted to Campbell Soup Co (US 4835339) in 1986. This described a method for anther culture of tomato and chromosome doubling of the resultant haploids. In detail the method involved incubating an inflorescence at 3–5°C for 2–6 days; excising anthers containing microspores at the late uni-nucleate stage; culturing the anthers at 34–38°C for 7–9 days in darkness, on a medium comprising 7–12% sucrose, 5×10^{-8} to 5×10^{-9} M of an auxin, and 5×10^{-8} to 5×10^{-9} M of a cytokinin; transferring the cultured anthers to a temperature of 22–30°C with a 12–18 hours light period for 3–5 days; reculturing them on a medium comprising 3–6% sucrose, and 5×10^{-7} to 5×10^{-8} M of a cytokinin for 6–10 weeks; visually inspecting the anthers to identify embryos; and transferring the embryos to a hormone-free medium to form a haploid plant.

With a few exceptions, most patents in this area in the succeeding years covered the major field crops. One of the first, US patent 4840906, was issued to Shell Oil Co for a novel method of regenerating barley plants from microspores cultured on a medium containing at least one sugar selected from sucrose; glucose; and oligosaccharides and polysaccharides containing at least two glucose residues; wherein the concentration of sucrose and glucose in the medium is not greater than 0.03 mol/l. This invention principally concerned the use of maltose in anther/microspore culture and lead to great interest in the use of this sugar in culture media. A later patent application (WO 94/01999) described the use of barley microspores in nurse culture of cells or protoplasts.

Several patents and applications cover the development of improved techniques for maize (corn). Two of these (US 5306864, 5602310) entitled “Increasing the anther culturability of maize” were granted to United Agriseeds and describe a process for producing germplasm with an enhanced response to anther culture. The anther culture procedure itself is used as a selection criterion for genes favoring *in vitro* androgenesis. After subjecting anthers to standard anther culture regeneration procedures, the regenerated plants are intercrossed and self-pollinated to generate valuable genetic variability for improved culture response. The transfer of increased anther culturability to other selected germplasm is also possible.

Related US Patents 5322789 and 5445961 (DeKalb) describes the regeneration of fertile maize plants from anther or microspore cultures. The method involves the synergistic effects of pretreatment with a combination of stresses, for example, a carbon source such as mannitol in the preculture medium that is capable of inducing

embryogenesis at cold temperatures (e.g. 10°C). A solid, porous support system was developed to transfer isolated microspores through a series of subcultures containing different media. Chromosome doubling methods for haploid anther and isolated microspore cultures are also described.

A similar application (US 20020151057) also describes methods for generating doubled haploid and/or haploid maize plants from microspores. This includes the steps of: (a) selecting plants with microspores at a developmental stage amenable to androgenic induction; (b) incubating the microspores in incubation medium at a temperature and osmolarity effective to induce androgenesis to obtain temperature-treated microspores; (c) isolating such microspores; (d) cultivating them in medium of osmolarity between 300 and 500 mOsm and with at least one cytokinin and at least one auxin, and with either at least one live ovary and/or ovary-conditioned medium to produce regenerative tissue; and (e) regenerating plants from this tissue. In addition, the Canadian application CA 2019989 (DowElanco) covers the subject of generating suspension culture from maize microspores.

There are also several patents based on methods for wheat. For example, US Patent 6362393 (North West Plant Breeders) provides protocols for generating doubled haploid and/or haploid plants from microspores. In the preferred method inflorescences are selected at a stage when the microspores are at a developmental stage amenable to androgenic induction. The treated microspores are isolated by density centrifugation utilizing a solution of 0.3 M mannitol layered over a higher density solution of a sugar, preferably maltose. The isolated, treated microspores are then cultured in a liquid nutrient suspension medium supplemented with at least one plant ovary or with an aliquot of plant ovary conditioned medium, until the microspores develop into embryoids. The embryoids are transferred to a regeneration medium and incubated until they develop into plants. A similar patent (US 6812028) describes methods for embryogenesis and regeneration from wheat and barley microspores. Inflorescences from donor plants are harvested and pre-treated to maintain microspore at a uninucleate cell cycle G1 phase. Pre-treatment conditions comprise cold water or an aqueous solution of about 0.2–1.0 mol/l sugar alcohol, for example mannitol. Microspores are then isolated and embryogenesis is induced in an induction medium, prior to regeneration of green plants. Arabinogalactan protein, auxin and ovary co-culture may be added to the induction medium to enhance embryogenesis. The use of ovary-conditioned medium is also the subject of applications US 20020104128 and WO 02/01940. In a related Chinese application (CN1212828) a method for increasing the differentiation of green plantlets from wheat microspores involves the use of potato extract and an increased concentration of iron in the induction medium.

Patents describing methods for rice include US Patent 6764854 (Northwest Plant Breeding Company) which involves temperature and stress pretreatment, and culture as described above for wheat in patent US 6362393. In the system described in Japanese application JP1067130 anthers or microspores are cultured in a liquid medium containing potato extract, and the microspore-derived calluses are subsequently transferred to solid culture medium for differentiation. A Chinese application CN1555678 also describes a haploid based breeding scheme for paddy rice.

Apart from the methods developed for cereals there are also several patents concerning culture techniques for Brassica. For example, US Patent 5049503 “Method for affecting fertility in plant variants” (Pioneer) suggests that embryogenesis from cultured, immature gametic cells can be exploited to overcome the problem of phenotypic sterility. The subsequent patents US 5900375 and US 6200808 describe induction of embryogenesis using cytoskeleton inhibitors or protein synthesis inhibitors. Embryogenesis from Brassica microspores is routinely induced with a 16–24 hours treatment at 32.5°C, whereas continuous culture at 25°C results in pollen development. However, microspore treatment with anti-cytoskeletal agents, or protein synthesis inhibitors, at the non-inductive temperature of 25°C, can induce embryogenesis, thus demonstrating that heat shock is not required for embryogenic induction. Furthermore, when anti-microtubule agents (e.g. colchicine or trifluralin) are used, embryo induction and chromosome doubling occur simultaneously, thus generating doubled haploids, whereas heat induction generates haploids. More recently, the US application 20070292951 describes a method for culturing immature somatic or microspore-derived embryos to maturity in the presence of a metabolizable carbon source (about 2%) and a specific water potential, to promote the development of somatic or microspore derived embryos similar in size and morphology to zygotic embryos. The Japanese application JP4112730 describes a method for anther culture of a vegetable crucifer such as Chinese cabbage in which the inflorescences after bolting are cut and kept at 1–10°C for 1–3 days prior to culture of the anthers in an appropriate medium, whereas Chinese application CN1473462 relates to anther culture of a hybrid between the two oil-producing species *Camelina sativa* and *C. macrocarpa* f. *longistipata*. Another Chinese application (CN1484945) relates to a method of anther culture of radish.

Other patents that cover vegetable crops include US Patent 5066830 entitled “Pepper gametoclonal variation” (DNA Plant Technology Corp.). This relates to a method of recovering variants of species of the genus *Capsicum* by *in vitro* culture which comprises: (a) culturing excised anthers on a first medium which promotes the initiation of embryos, (b) subculturing these anthers in a second medium which promotes embryo development, the second medium comprising a solid layer containing an effective amount of activated charcoal and an aqueous layer containing no charcoal; and (c) recovering variant plantlets from embryos so produced. The Chinese application CN1418537 also describes an anther culture technique for hot-pepper in which hybrid species are used as a source of anthers, whereas the Chinese application CN1418950 claims a generally improved method for anther culture in which the anthers are floated on liquid medium, thus allowing any microspore-derived embryos to float free from the anther. A Japanese application (JP3280817) describes a method for anther culture of *Cucumis melo* or *Cucumis sativa* on medium containing selected amino acids.

Non-vegetable crops are also the subject of patent coverage. For example, application WO 2006/125310 describes methods for species of the Apiaceae including but not limited to fennel, carrot, dill, anise, lovage, parsnip and laceflower. Related application WO 2006/116876 covers *Saponaria*. Methods for flax are described in applications CA 2505499, US 20030093829 and US 20040210959. A method of breeding a tobacco

variety resistant to the black leg pathogen is described in application CN1331914; the anthers are treated with γ-rays, and disease resistant plants are selected in culture medium containing black leg toxin. A Bulgarian application (BG51816) also claims an improved medium for anther culture of tobacco. Other applications describe anther culture methods for cedar (SU1497212), cork oak (ES2180385), cyclamen (JP9172893) and chrysanthemum (CN101011028).

Ovule Culture

Patents and applications describe the use of this technique for carnation (JP8126444), cucumber (US 5492827) (Nunhems Zaden BV), pumpkin (CN1653885) and melon (JP5219849). This latter example with *Cucumis melo* var. conomon also involves the use of X-irradiated pollen (100K roentgen) prior to isolation and culture of the ovule. A related application (JP6237657) describes the additional use of an aqueous solution of amiprophos-methyl to double the chromosome number of the regenerated haploid plant.

Parthenogenesis and Apomixis

In wheat, interspecific pollination with hexaploid Triticale is claimed as a method to produce homozygous diploid seed (WO 2005/084420), and similar development of apomictic haploid embryos is induced in wheat by the use of gametocidal chemicals (WO 86/00495; EP 0 171 310; EP 0 127 313; JP59205922; CA 451846). The oxo or thio pyridazines and the phenyl-substituted 4-oxo(thio)nicotinates are the preferred chemicals used in this treatment. A related Russian patent application (RU2150823) relates to a method of producing flax apomictic haploids that involves treatment of plant at the time of first flowering with 0.1–0.2 ml of dimethylsulfoxide solution (1%). It is claimed that this treatment ensures the production of flax apomictic haploids in large numbers (5–10-fold more than any known method).

Two patent applications concern methods for orchid. The first (WO 2004/032607) describes the use the use of inactivated pollen to induce parthenogenesis, whereas the second (US 20050289663) (Sapporo Breweries Ltd) describes a method of isolating orchid haploids by treating unfertilized flowers with auxin and then selecting among the parthenogenetic seed produced.

Haploid Inducing Genes/Genotypes

There are several specific genes that are known to control normal reproductive development in plants and mutations in some of these lead to increases in the frequency of either male or female derived haploids after normal pollination. For example, US

Patent 5639951 (Sandoz) claims use of the indeterminate gametophyte (*ig*) gene. A subsequent US Patent (5749169) (Pioneer) describes a methodology for ascertaining gene function entailing selection of mutations in androgenetic haploids produced by fertilizing a maize plant carrying the *ig* gene with pollen obtained from a mutagenized plant. Genes that control quantitative characters can be identified, for example, by fertilizing a first inbred carrying the *ig* gene with pollen from a second inbred that has been mutagenized. Changes in the phenotype of the hybrid progeny are then identified and characterized. More recently the *ig* gene has been sequenced and its use for a range of purposes has been claimed (US 20050198711). Another related application (US 20060185033) (Pioneer), also for maize, describes the use of various haploid inducer lines, also carrying a scorable colour marker such as an anthocyanin gene, *R-nj*, GFP, and lec1 promoter driving CRC. The advantage of such a combination is that it allows the non-destructive identification of seed with haploid embryos. Other patent applications that claim the use of specific haploid inducing lines in maize include WO 2005/004586 (line PK6) and SU921138. A recent related application (US 20070292951) "Methods of seed breeding using high throughput nondestructive seed sampling" (Monsanto) describes a mechanical seed sorting method for identifying haploid seed in large population of seed. This process was developed for use in maize and could presumably be linked to the use of compatible phenotypic makers.

Overexpression of various regulatory genes as a means of inducing embryogenesis is also an area of considerable patent activity and there are several relevant patents and applications. For example, US Patent 7151170 claims the use of BNM3, an AP2 domain transcription factor isolated from *Brassica napus*. Similarly, the granted patent US 7148402 claims the overexpression of a Plant Growth Activator 37 PGA37 gene (identical to transcription factor MYB118) to create haploid plants, which can be used to produce dihaploid plants. It is suggested that this can be achieved by stably transforming a plant cell or tissue with a MYB118 gene under the control of a tissue-specific promoter that is active in a haploid cell or tissue, and expressing the MYB118 gene therein, or by introducing the MYB118 gene into a plant tissue or cell under the control of an inducible promoter and applying the inducer to cause expression of the MYB118 gene. In a preferred example, the MYB118 gene is under the control of a promoter that is both haploid-tissue-specific and inducible. In this case, a MYB118 gene linked to an inducible pollen-specific promoter may be used to induce embryogenesis in haploid pollen or ovule cells. A similar application (US 20050071898) makes the same claims for the use of the *Wuschel* gene, which encodes a homeodomain protein that was previously characterized as a key regulator for specification of meristem cell fate.

A particularly specific use of these embryo-inducing genes (e.g. *Wuschel*) is claimed in application WO 2006/128707. This describes a method for producing haploid plant embryos, first by transient expression of cell division inducing genes in microspores or pollen; secondly, by pollinating an embryo sac cell, in particular an egg cell, with the transformed microspores or pollen; and thirdly by allowing the microspores or pollen to secrete the cell division inducing proteins into or in the vicinity of the embryo sac cell, in particular the egg cell, to trigger its division to produce a haploid embryo.

Chromosome Doubling

Two US patents relate to methods for chromosome doubling in haploids. In the first (US 5770788) fertile maize plants were produced by culturing anthers or pollen in the presence of colchicine which was added to an otherwise conventional anther culture medium after a pre-culture phase in the absence of the colchicine. The colchicine induced doubling of the chromosome numbers in regenerated plants. The second patent (US 7135615) also involves maize and claims a method of obtaining a plant with a doubled chromosome number by treating a haploid plant with nitrous oxide gas at a pressure of about 500–700 kPa for 24–144 hours during the 3–8 leaf stage of development, at which time formation of the floral primordium takes place. This treatment induces doubled sectors within the floral primordium and the plant remains amenable to self pollination. After self pollination a progeny plant with a doubled chromosome number is selected among the progeny.

In addition, two US applications (20040210959, 20050289673) (Monsanto) describe a novel system for generating transformed dihaploid maize plants from haploid cells and tissues without the use of chromosome doubling agents. The method comprises: obtaining haploid sporophytic tissue; transforming the haploid sporophytic tissue via host cell-mediated transformation (*Agrobacterium* or equivalent); and regenerating a transformed dihaploid plant from the transformed haploid tissue in the absence of any chromosome doubling agent.

Transformation of Haploids

Patents in this section cover both direct and indirect methods for transformation of plant cells. Some make generic claims. For example US Patent 5840557 "A method for producing transgenic seeds and plants" comprises the isolation of microspores in their uni-nucleate stage and removing anther tissue in which they are embedded, culturing these microspores in a nutrient solution, transferring foreign genetic material into them to obtain transformed microspores, bringing about complete maturation of the transformed microspores to obtain transformed pollen grains *in vitro*, pollinating receiver plants with the transformed pollen grains and obtaining seeds from these plants.

However, most of the methods, including those described above in two applications (US 20040210959, US 20050289673), involve the use of *Agrobacterium* mediated methods. One such example, the granted US Patent 5929300 describes a culture medium that supports pollen germination and pollen tube growth in the presence of *Agrobacterium* as part of a method for the genetic transformation of plants. The method comprises the steps of obtaining pollen from a selected plant, applying a lawn of Agrobacteria to a solid pollen culture medium, applying the pollen to the solid medium, allowing the pollen to germinate and grow on the medium, whereby the *Agrobacterium* attaches to the pollen tube or mediates transfer of the heterologous

gene sequence to the germinating pollen to obtain transgenic pollen, applying the transgenic pollen to the stigma of a second plant, obtaining transgenic seed from this plant and germinating the transgenic seed to obtain a transgenic plant. It is claimed that the method is particularly suited for use with so-called “dry stigma” pollen.

A second example, US patent 6316694 “Transformed embryogenic microspores for the generation of fertile homozygous plants”, relates to transformed, embryogenic microspores and their progeny, characterized by being transformed by *Agrobacterium tumefaciens*, capable of leading to non-chimeric transformed haploid or doubled haploid embryos that develop into fertile homozygous plants within one generation. Application WO 92/14828 concerns cocultivation of barley microspores with *Agrobacterium*.

Patents describing direct methods for DNA introduction include the US Patent 5272072 awarded to Sapporo Breweries. This claims a transformed plant of the Gramineae can be produced by culturing an anther in a callus induction medium and, at a stage immediately before the microspore begins to divide or during the initial division, introducing DNA into the microspore through a pore formed by a laser pulse. According to this invention, it is unnecessary to prepare protoplasts and therefore, time and resources required for transformation can be greatly reduced. Since haploid cells are transformed, the introduced trait is stably inherited by the subsequent generations.

In a second example described in US Patent 5629183, plants are transformed with foreign DNA by introducing the DNA into pollen grains by a technique such as electroporation, using such pollen in a cross and selecting for the transformed germplasm. The germinating pollen, resultant seed, and the progeny can each be screened for expression of the foreign gene.

A third example, US application 20070107077 (Pioneer), relates particularly to the genetic manipulation of Brassica plants. Specifically, methods are described for producing such plants by the introduction of a DNA construct by microprojectile bombardment into pre-incubated microspores, microspore-derived embryos and microspore-derived hypocotyls.

In a less specific example, US Patent 5066594 describes a method for the *in vitro* stabilization and manipulation of pollen from flowering plants. In detail, it comprises stabilizing germinating pollen in an aqueous solution; the growth of the pollen tube may be resumed by suspending the stabilized pollen in germination medium. Such stabilized pollens may be used as vectors for the transfer of exogenous DNA into plants or in gametophyte selection.

Miscellaneous

There are two cases in which specific gene sequences are claimed. The first (WO 2004/042066) describes the development of a male sterile systems by the use of a tobacco gene, the expression of which is increased in stressed microspores. A second case (US Patent 6861576) concerns a microspore-specific promoter and its use

for obtaining plants with gametophytic male sterility with inducible fertility. It also describes a method for obtaining hybrid plants.

An interesting example, US Patent (5547866), concerns the use of haploid cell cultures for production of valuable chemical compounds. The invention describes haploid cells and their derivatives which are cultured *in vitro* to produce taxanes and other metabolites that evolve from the isoprenoid biosynthesis pathway.

The only example concerning non-flowering plants, Chinese application CN1080114, describes the cultivation of seaweed, and relates to the problem of artificial culture of *Undaria pinnatifida*. The invention is for the rapid propagation, in large amounts, of a haploid clone of this species by the control of temperature, illumination and nutrient components.

Conclusion

The summary provided above demonstrates the range of subjects within the area of plant haploids that have been claimed in patents and application. It is important to note that this summary does not represent nor provide formal legal advice about the validity of any of these documents. Such advice should be obtained from qualified patent agents.

Another outcome from this brief survey relates to exploitation of science for commercial gain and the consequences of applying IPR systems in plant breeding and the seed industry (Eaton 2007; Kingston 2007; Kock 2007; Lence et al. 2002; Llewelyn and Adcock 2006; Louwaars et al. 2005; Morris et al. 2006). Although the 1906 conference on "Genetics "(Anon 1907) did receive commercial support and there were 20 pages of adverts in the proceedings, this funding was restricted to horticulture and associated gardening items. Bateson, in his after dinner speech to foreign guests, concluded "I expect a century must elapse before the … complete union of Science and Practice will be achieved". More than a century has now elapsed and indeed the value of genetics in agricultural and horticultural biotechnology has been proven. In particular many of the major breeding programmes in hybrid crops such as maize are now based on haploid methods. However, it is less easy to quantify the exact role played by patent protection in this process or to predict the future shape of the patent landscape over the coming years.

References

Adcock M (2007) Intellectual property, genetically modified crops and bioethics. Biotechnol J 2:1088–1092

Allyn RS (1933) Plant patent queries. A patent attorney's views on the law. J Hered 24:55–58

Anon (1900) In: W. Wilks (ed), Hybrid Conference Report 1900. J Roy Hort Soc 24:47

Anon (1906) The relations between science and industry. Nature 74:386–387

Anon (1907) "Copyright" for raisers of novelties. In: W. Wilks (ed), Report of the third international conference 1906 on Genetics; hybridisation (the cross-breeding of genera or species),

the cross-breeding of varieties and general plant breeding. Royal Horticultural Society, London. pp 474–475
Barrons KC (1936) A defense of basic plant patents. From the plant breeder's point of view. J Heredity 27:475–478
Binenbaum E, Nottenburg C, Pardey PG, Wright BD, Zambrano P (2003) South-north trade, intellectual property jurisdictions, and freedom to operate in agricultural research on staple crops. Econ Develop Cult Change 51:309–335
Brennan M, Pray C, Naseem A, Oehmke JF (2005) An innovation market approach to analyzing impacts of mergers and acquisitions in the plant biotechnology industry. AgBioForum 8:89–99
Brown WM (2003) Intellectual property law: a primer for scientists. Mol Biotechnol 23: 213–224
Cahoon RS (2000) Property rights and agricultural biotechnology. In: Hrazdina G (ed) Use of agriculturally important genes in biotechnology. IOS Press, Amsterdam. pp 203–215
Cantley M (2004) How should public policy respond to the challenges of modern biotechnology? Curr Opin Biotechnol 15:258–263
Chan HP (2006) International patent behavior of nine major agricultural biotechnology firms. AgBioForum 9:59–68
Cook RC (1931a) The first plant patent. J Hered 22:313–319
Cook RC (1931b) Three more plant patents. J Hered 22:369–372
Cook RC (1933) Other plant patents. J Hered 24:49–54
Cook RC (1936) Plant patent 110 declared invalid. J Hered 27:394–400
Donnenwirth J, Grace J, Smith S (2004) Intellectual property rights, patents, plant variety protection and contracts: a perspective from the private sector. IP Strategy Today 9:19–34
Dunwell JM (2007) 100 years on: a century of genetics. Nat Rev Genet 8:231–235
Eaton D (2007) Intellectual property rights in plant breeding and biotechnology: a comparative institutional analysis. Presented at: 11th Ann Conf Int Soc New Instit Econ (ISNIE), Reykjavik
Eisenberg RS (2006) Biotech patents: looking backward while moving forward. Nat Biotech 24:317–319
Farnley S, Morey-Nase P, Sternfeld D (2004) Biotechnology – a challenge to the patent system. Curr Opin Biotechnol 15:254–257
Fay AE (1937) Are plant patents "inventions"? J Hered 28:261–262
Fleck B, Baldock C (2003) Intellectual property protection for plant-related inventions in Europe. Nat Rev Genet 4:834–838
Forster BP, Thomas WTB (2005). Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88
Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375
Gaisford JD, Hobbs JE, Kerr WA (2007) Will the TRIPS agreement foster appropriate biotechnologies for developing countries? J Agr Econ 58:199–217
Garrison C (2006) Exceptions to patent rights in developing countries. International centre for trade and sustainable development (ICTSD). Issue Paper No. 17
Giannakas K (2001) The economics of intellectual property rights under imperfect enforcement: developing countries, biotechnology, and the Trips Agreement. EPTD Discussion paper No. 80. Int Food Policy Research Institute, Washington, DC
Graff GD, Cullen SE, Bradford KJ, Zilberman D, Bennett AB (2003) The public-private structure of intellectual property ownership in agricultural biotechnology. Nat Biotechnol 21:989–995
Hails R (2004) Bioethics for technology? Curr Opin Biotechnol 15:250–253
Heisey PW, King JL, Rubenstein KD (2005) Patterns of public-sector and private-sector patenting in agricultural biotechnology. AgBioForum 8:73–82
Helfer LR (2004) Intellectual property rights in plant varieties. International legal regimes and policy options for national governments. FAO Legislative Study 85
Huskisson FM (1996) Patents and biotechnology. In: Owen MRL, Pen J (eds) Transgenic plants: a production system for industrial and pharmaceutical proteins. Wiley, New York, pp 313–328
Kingston W (2007) Repairing incentives to invest in plant breeding. Int Prop Quart 10:294–311

Kock MA (2007) Essentially biological processes: the interpretation of the exception under Article 53(b) of the European Patent Convention. J Intell Prop Law Prac 2:286–297
Koo B, Nottenburg C, Pardey PG (2004) Intellectual property. Plants and intellectual property: an international appraisal. Science 306:1295–1297
Kryder RD, Kowalski SP, Krattiger AF (2000) The intellectual and technical property components of pro-vitamin A rice (GoldenRice™): a preliminary freedom-to-operate review. ISAAA Brief No 20. International service for the acquisition of agri-biotech resources, Ithaca, NY
Kukier KN (2006) Navigating the future(s) of biotech intellectual property. Nat Biotech 24:249–251
Léger A (2007) The role(s) of intellectual property rights for innovation: a review of the empirical evidence and implications for developing countries. Discussion papers 707. DIW Berlin. German Inst Econ Res
Lence SH, Hayes DJ, McCunn A, Smith S, Niebur B (2002) Welfare Impacts of Property Rights in the Seed Industry. http://www.econ.iastate.edu/department/seminar/ispw/Sergio-Dermot-seed-paper.pdf
Llewelyn M, Adcock M (2006) European plant intellectual property. Hart Publishing, Oxford/Portland
Locke SD (2007) Intellectual property for the botanist and the plant breeder: an overview of protection afforded by plant patents and plant variety protection certificates. Chicago-Kent J Intellect Prop 6:198–212
Louwaars NP, Tripp R, Eaton D, Henson-Apollonio V, Hu R, Mendoza M, Muhhuku F, Pal S, Wekundah J (2005) Impacts of strengthened intellectual property rights regimes on the plant breeding industry in developing countries: a synthesis of five case studies. Wageningen UR
Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) (2003) Doubled haploid production in crop plants: a manual. Kluwer, Dordrecht
Merrill SA, Levin RC, Myers MB (2004) A patent system for the 21st century. The National Academies Press, Washington, DC
Ministry of Economic Development (2002) New issues in patentability 1: Biotechnological patents. In: Review of the Patents Act 1953: boundaries to patentability. Wellington, New Zealand, pp 29–40
Morris M, Edmeades G, Pehu E (2006) The global need for plant breeding capacity: what roles for the public and private sectors? Hortscience 41:30–39
Palmer, CE, Keller, WA and Kasha, KJ (eds) (2005) Haploids in crop improvement II, Biotechnology in agriculture and forestry Vol. 56. Springer, Heidelberg
Parayil G (2003) Mapping technological trajectories of the green revolution and the gene revolution from modernization to globalisation. Research Policy 32:971–990
Schimmelpfennig D, King J (2006) Mergers, acquisitions and flows of agbiotech intellectual property. In: Evenson RE, Santaniello V (eds) International trade and policies for genetically modified products. CAB International, Wallingford, pp 97–109
Schrell A, Bauser H, Brunner H (2007) Biotechnology patenting policy in the European Union-as exemplified by the development in Germany. Adv Biochem Eng Biotechnol 107:13–39
Shear RH, Kelley TE (2003) A researcher's guide to patents. Plant Physiol 132:1127–1130
Straus J (2007) The impact of the new world order on economic development: the role of the intellectual property rights system. Europ Rev 15:47–63
Tokgoz S (2003) R&D spillovers in agriculture: results from a trade model. Center for Agric Rural Devel, Iowa State University, Ames, Iowa. Working Paper 03-WP 344
Wright BD (2006) Plant genetic engineering and intellectual property protection. Agr Biotech in California Series. Publication 8186
Wright BD, Pardey PG (2006) The evolving rights to intellectual property protection in the agricultural biosciences. Int J Technol Globalis 2:12–29
Yerokhin O, Moschini G (2007) Intellectual property rights and crop-improving R&D under adaptive destruction. Center for Agric Rural Devel, Iowa State University, Ames, Iowa. Working Paper 07-WP 449

Chapter 8
Gene Expression Profiling of Microspore Embryogenesis in *Brassica napus*

M.R. Malik and J.E. Krochko

Abstract Isolated microspores from selected cultivars of *Brassica napus* readily form embryos in culture after mild heat stress treatments (32°C for 1–3 days). Transcript profiling methods were used to identify differentially-expressed genes as well as shifts in metabolism during the early stages of microspore embryogenesis. Approximately 20,000 expressed sequence tags (ESTs) from cDNA libraries representing freshly-isolated microspores (0 hours) and microspores cultured for 3, 5 or 7 days under embryogenesis-inducing conditions were prepared. *In silico* analyses of ESTs and semi-quantitative and real time reverse transcription-polymerase chain reaction (RT-PCR) based profiling identified differentially-regulated gene clusters and 16 genes that could be used as specific markers for microspore embryogenesis. These molecular marker genes also were expressed during zygotic embryogenesis, underscoring the common developmental pathways that function during zygotic and gametic embryogenesis. Future studies will focus on characterization of embryogenesis-related genes and development of fluorescently-labeled gene/protein probes to precisely mark and isolate early stages of microspore embryogenesis.

Keywords Embryogenesis, microspores, transcript profiling, *Brassica*, ESTs, differentially-expressed genes

M.R. Malik
Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada

J.E. Krochko(✉)
Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Sakatchewan, Canada
E-mail: joan.krochko@nrc-cnrc.gc.ca

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Microspore Embryogenesis in *Brassica napus* – A Model System for Revealing the Molecular Factors Underlying Cellular Totipotency

Microspore Embryogenesis in Brassica napus

In recent years there has been an increasing interest in understanding the molecular mechanisms underlying totipotency in living organisms. Notably, plants display this unique feature in a wide variety of starting materials, both reproductive and vegetative, and are more tractable models than their animal counterparts for studies of *in vitro* embryogenesis and totipotency/pluripotency. Isolated microspores of *Brassica napus*, collected from across a fairly wide developmental window surrounding the first asymmetric mitotic division (pollen mitosis I), can be induced to form embryos *in vitro* (Keller et al. 1987; Pechan and Keller 1988; Binarova et al. 1997). These microspore-derived embryos closely simulate all stages of zygotic embryogenesis, developing without an intervening callus phase, and therefore are an ideal system for studying *in vitro* embryo development. *B. napus* is the closest taxonomic relative of *Arabidopsis thaliana* (the plant model system with the largest collection of genomics resources, see http://www.arabidopsis.org) for which there are established methodologies for inducing microspore embryogenesis *in vitro*. At this time, there are no robust or reproducible published protocols for microspore embryogenesis in *Arabidopsis*. *B. napus* Topas DH4079 is highly embryogenic and more than 10% of the cultured microspores will form well-developed embryos (Ferrie 2003). For the data presented here, microspore collections and culture conditions for *B. napus* were as described by Ferrie and Keller (1995). There is an initial swelling and enlargement of microspores that occurs during early embryo induction, and in many species this has been correlated with the acquisition of embryogenic potential (Hoekstra et al. 1992; Touraev et al. 1996a, b); therefore, microspores from cultures at 3, 5 and 7 days were size-selected by sieving to enrich for cell clusters most advanced in embryogenic development and to discard the smaller physiologically unresponsive microspores.

Gene Expression Profiling During Microspore Embryogenesis

Subtractive hybridizations, microarray analysis and mRNA differential display have been used widely for uncovering changes in gene expression during microspore embryogenesis in species such as *Brassica napus*, barley, tobacco and wheat (Reynolds and Crawford 1996; Reynolds 1997; Vrinten et al. 1999; Boutilier et al. 2002; Maraschin et al. 2003; Fiers et al. 2004; Boutilier et al. 2005; Chan and Pauls 2007; Tsuwamoto et al. 2007). The most notable and well-characterized gene isolated by subtractive hybridization from early stage microspore cultures of *B. napus* is *BABY BOOM1* (*BBM1*, Boutilier et al. 2002). Over-expression of BBM1 induces somatic embryogenesis from vegetative tissues of young seedlings and occasionally

from the leaves of mature plants, underscoring its role in promoting cell division and morphogenesis (Boutilier et al. 2002). Ectopic or spontaneous embryogenesis phenotypes, similar to those observed with the over-expression of BBM1, also have been reported for the over-expression of the *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, and *WUSCHEL* (*WUS*) genes and for knockouts of *PICKLE* (*PKL*) in *Arabidopsis* (Lotan et al. 1998; Ogas et al. 1999; Stone et al. 2001; Zuo et al. 2002). Details of the cascade of molecular events by which the over-expression of these genes (or knockout, in the case of *PKL*) result in the formation of ectopic embryos are not known, nor are their precise roles in the induction of microspore embryogenesis.

Our lab has focused on gene expression changes during the early stages of microspore embryogenesis. Expressed sequence tag (EST) based information derived from sequencing of cDNA libraries, microarray hybridizations, and cDNA AFLP have been used to detail changes in gene expression during the first 7 days of microspore embryogenesis. Of these, the EST collections have been the more powerful tool for both gene discovery and for interpreting transcriptome activity during developmental transitions, for cDNA libraries provide both sequence information as well as estimates of transcript abundance. Gene expression patterns from 19,254 ESTs (deposited into GenBank), representing microspores at the time of initial collection (0 hours), following heat stress induction (3 days), early division (5 days), and globular or pre-globular embryos (with or without suspensors) (7 days), were analyzed. Singletons and contigs were identified, as well as clusters of genes with similar expression profiles (Malik et al. 2007). Semi-quantitative RT-PCR and real time RT-PCR techniques, with primers generated from the EST sequences, were used for further validation and for expression profiling of genes of interest and lowly-expressed genes, particularly transcription factors that were present only as singletons in the EST collections (Malik et al. 2007). Subsequent microarray hybridizations further validated the *in silico* analyses for many of these differentially expressed genes (Xiang et al. 2008; Malik et al. 2008).

EST Analyses Reveal Molecular Changes Associated with Developmental Transitions

There are major changes in gene expression during the induction stages of microspore embryogenesis in *B. napus*. Initially, the EST collections were examined for highly- and moderately-expressing genes, and these unigene contigs (genes represented by more than one EST) were compared across the developmental time-course of microspore embryogenesis. The microspores at 0 hours showed high transcript diversity (60.3%), and examination of the unigene contigs expressed at this stage revealed a large number of transcripts related to protein synthesis and function, particularly 40S and 60S ribosomal proteins and initiation and elongation factors, e.g. RPL28C, RPS17A, RPS8A, RPS15C, RPL26A, EIF-5A, EF-1α and EF-1β (Fig. 1). There was a very large decrease in total number of unigene contigs in 3 day heat-stressed microspores as compared to 0 hours microspores. The 3 day

induced microspores showed an increased number of ESTs for metabolism-related genes, particularly those related to cell wall and membrane-associated functions (Malik et al. 2007). As well, genes related to the control of cellular organization, cellular communication and signal transduction were abundant in the 3 day heat-stressed microspores. The 0 hour and 3 day microspores share only an 18% overlap in unigene contigs, underscoring the transcriptional re-programming that has occurred in response to heat stress. Genes related to pollen cell wall synthesis, for example, polygalacturonase, pectinesterase and pectate lyase, were abundant in the 3 and 5 day induced microspore cultures (Fig. 1). Notably, a large number of the transcripts in the 3 day microspores were pollen-related and some of these continued to be expressed until at least day 5. We have determined that the microspore cultures at 3 and 5 days are heterogeneous, comprised of both embryogenic microspores and enlarged oval-shaped microspores developing as pollen, and these are not easily separable (see Hause and Hahn 1998); however, there also is evidence that pollen- and embryo-specific gene expression may occur simultaneously within the same cell cluster (Malik et al. 2007), and this is being investigated further. The 5 and 7 day EST collections have greater than 30% overlap, suggesting that the 5 day stage represents a transition stage where many embryogenesis- and sporophyte-related genes are highly expressed along with a large number of persistent pollen-type

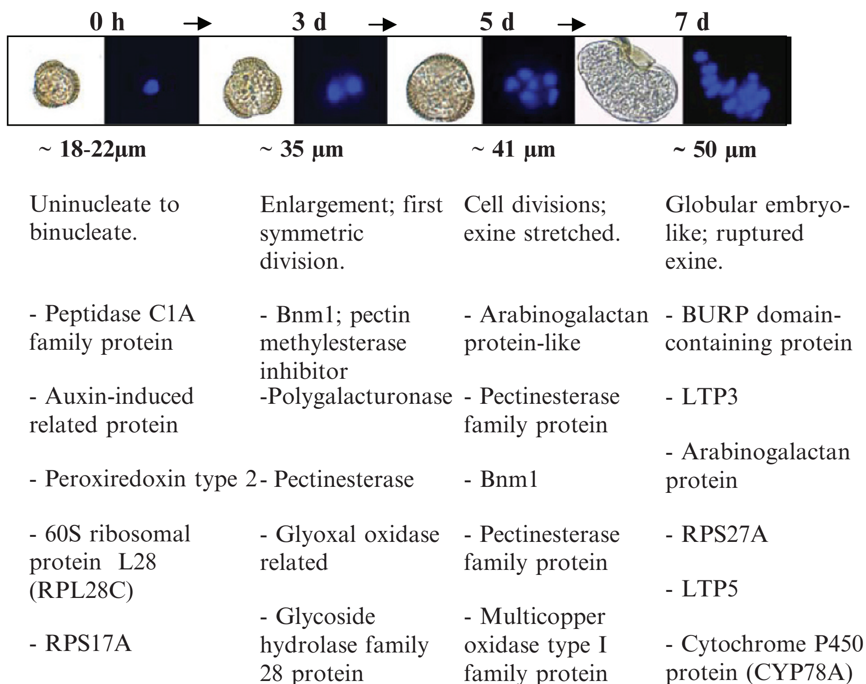


Fig. 1 Induction of microspore embryogenesis in *B. napus* Topas DH4079. Light micrographs and DAPI-stained microspores at 0 hours, 3, 5 and 7 days of development. Size, cellular organization and the five largest unigene contigs (greatest numbers of ESTs) are listed for each stage

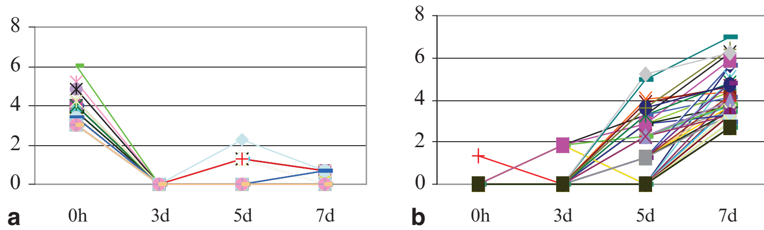


Fig. 2 K-means clustering (http://rana.lbl.gov/EisenSoftware.htm; Eisen et al. 1998) of the differentially expressed genes (from unigene contigs) identified by IDEG6 software (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/). **A**. Cluster of genes down-regulated during the induction of microspore embryogenesis, and **B**. Cluster of genes up-regulated during the commitment to microspore embryogenesis (adapted from Malik et al. 2007)

genes (Malik et al. 2007). The highly-expressed transcripts in the 7 day embryogenic microspores include lipid transfer proteins (LTP1, LTP3, and LTP5), a BURP domain-containing protein, arabinogalactan proteins and a cytochrome P450 gene (BnCYP78A) (Fig. 1). Transcripts for these embryo-related genes are also present in the 5 days libraries. These genes are associated with sporophytic development and are expressed at significant levels during both *B. napus* and *Arabidopsis* zygotic embryo development. In summary, we found *in silico* cDNA library comparison tools (for example, http://telethon.bio.unipd.it/bioinfo/IDEG6) to be very useful for defining shifts in metabolism and changing patterns of gene expression across various stages of microspore embryo development (Fig. 2). Examples of two of the clusters of co-regulated genes that were differentially regulated during the early stages of microspore embryogenesis are shown in Fig. 2.

In addition to the highly- and moderately-expressed EST contigs (see above), a large number of lowly-expressed embryogenesis-related genes also were sequenced from globular and pre-globular embryos at 7 days, for example, *LEAFY COTYLEDON1*, *LEAFY COTYLEDON2*, *BABY BOOM1*, *ABSCISIC ACID INSENSITIVE3* and *FUSCA3*. These gene expression data emphasize a strict commitment to embryogenesis in the 7 day induced microspores. Furthermore, PCR-based profiling detected these embryogenesis-related genes even earlier than the EST analyses, at 1–3 days of microspore culture, indicating that some important molecular changes associated with the transition to embryogenesis were initiated (or completed) during the 3 day heat stress treatment.

Molecular Markers for Microspore Embryogenesis

The analysis of EST abundance described in the previous section revealed many genes that were differentially expressed during embryogenesis. Detailed PCR-based expression profiling, across a range of tissue types, of 104 candidate genes selected from the sequenced ESTs revealed a set of 16 genes that are now considered

unequivocal markers for microspore embryogenesis (Malik et al. 2007). These molecular marker genes are listed in Table 1. The marker genes showed expression during the induction of microspore embryogensis, in microspore-derived embryos (MDEs) and during zygotic embryogenesis (Fig. 3). None of these genes were expressed in the microspores at the time of culture or in developing pollen in

Table 1 Molecular markers for microspore embryogenesis in *Brassica napus*

Gene name	Genbank accession No.	BLAST description
Embryo-specific		
BnFUS3	DY012343	Transcription factor
BnLEC2	DY021430	Transcription factor B3 family, Leafy Cotyledon2
BnLEC1	DY023102	CCAAT-box binding transcription factor, Leafy Cotyledon1
BnUP1	DY018256	Unknown protein, Unknown Protein 1
BnUP2	DY022815	Unknown protein, Unknown Protein 2
Embryo-expressed		
BnNAPIN	DY011967	Napin precursor (gNa)
BnBBM1	DY021802	AP2/EREBP transcription factor, Baby Boom1
BnFAD1	DY013003	Putative delta 9 desaturase
BnWOX9	DY023056	WUSCHEL-related homeobox 9
BnABI3	DY012041	Abscisic acid-insensitive protein 3 (ABI3)
BnATS1	DY022066	Embryo-specific protein 1 (ATS1)
Sporophyte-specific		
BnLRR1	DY012467	Leucine-rich repeat protein
BnCP1	DY017482	Cysteine proteinase homolog, similar to COT44
BnCYP78A	DY022203	Cytochrome P450 family protein
BnWOX2	DY017872	WUSCHEL-related homeobox 2
BnCYP81F	DY015952	Cytochrome P450 family protein

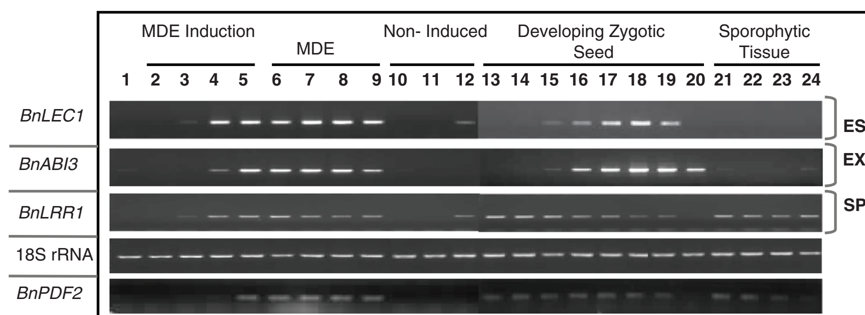


Fig. 3 Expression profiles of some molecular markers for microspore embryogenesis in *B. napus* Topas DH4079, as determined by semi-quantitative RT-PCR. These are classified as Embryo-Specific (ES), Embryo-Expressed (EX), or Sporophyte-Specific markers (SP). Lanes 1–9, stages of MDE development (0 hours, 1, 2, 3, 5, 7, 14, 21 and 28 days). Lanes 10 and 11, Non-induced microspores/pollen (3 and 5 days at 18°C). Lane 12, 3 day induced microspores of 'Garrison' (poorly embryogenic). Lanes 13 to 20, Postanthesis seed (0 hours, 3, 5, 7, 9, 14, 21 and 28 days). Lane 21, Young inflorescence. Lane 22, Leaf. Lane 23, Stem. Lane 24, Root (adapted from Malik et al. 2007)

the negative control cultures (at 18°C; Custers et al. 1994). All of the marker genes listed in Table 1 were first detected from 1 to 3 days of culture, i.e. they all showed expression by the time of completion of the inducing heat stress treatment (Fig. 3). The molecular markers were further classified as embryo-specific (ES), embryo-expressed (EX), or sporophyte-specific (SP) depending on the individual expression profiles in the tissues used for expression profiling (Table 1; Fig. 3). Embryo-specific genes were transcribed only in MDEs during the induction or development stages and in developing zygotic seeds. The embryo-expressed genes were similar to the embryo-specific group, except that some of these also showed expression in one (or more) of the other non-seed tissues also included in this study; for example, *BnBBM1* and *BnFAD1* showed strong root expression (Malik et al. 2007). The sporophyte genes showed expression in developing embryos and also in many other sporophytic tissues; nonetheless, these also reliably marked the transition in microspores from the gametophytic (pollen) developmental pathway to the sporophytic (embryogenic) pathway (Fig. 3; Malik et al. 2007). Similarly, expression profiling of *Protodermal Factor2* (*PDF2*), which in *Arabidopsis* is restricted to the L1 layer of vegetative shoot meristems and the outermost layer of embryos (Abe et al. 2003) showed that it was transcribed by 5 days in embryogenic microspores (Fig. 3). Future studies certainly will reveal many more such genes that clearly mark the developmental transition from gametophyte to microspore embryogenesis.

The expression patterns of *AtLEC1*, *AtLEC2*, *AtFUS3*, *AtABI3*, *AtATS1*, *AtWOX2*, and *AtWOX9* have already been described during seed development in *Arabidopsis* (Parcy et al. 1994; Lotan et al. 1998; Luerßen et al. 1998; Nuccio and Thomas 1999; Stone et al. 2001; Haecker et al. 2004). The identified *Brassica* orthologues have between 68% to 95% identity with their *Arabidopsis* counterparts at the protein level. Seven of the markers for microspore embryogenesis are transcription factors (Table 1; *LEC1*, *LEC2*, *FUS3*, *ABI3*, *BBM1*, *WOX2*, *WOX9*). LEC1, LEC2, ABI3, and FUS3 have been shown recently to be major interacting and redundant regulators of embryogenesis and seed development (To et al. 2006). BnWOX2 and BnWOX9 (Table 1) are WUS-like homeobox proteins involved in the specification of tissue domains during early embryogenesis (Haecker et al. 2004). Evidence for early transcription of *BnWOX2* and *BnWOX9* during microspore embryogenesis suggests that the gene expression programmes necessary for the establishment of polarity domains in the developing embryo have been initiated; nonetheless, at this time there is no direct evidence for the *de facto* establishment of polarity in the pre-globular stage MDEs. Three of the molecular markers listed in Table 1, namely *BnCYP78A, BnUP1* (Unknown Protein 1) and *BnUP2*, also have been confirmed to be differentially expressed through *in silico* EST analyses as well as microarray analyses (Malik et al., unpublished data); however, the specific functions of these proteins during embryogenesis are still unknown.

Some cultivars of *B. napus*, for example, Allons, Westar, and Garrison, are poorly embryogenic in tissue culture. Expression surveys of some of the marker genes, e.g. *BnLEC1*, *BnLEC2*, *BnBBM1* and *BnUP1*, in microspore cultures of these poorly embryogenic cultivars have revealed strong correlations between

embryogenic potential and quantitative expression of these genes (measured by qRT-PCR; Malik et al. 2007). Thus, the marker genes identified in this study also can be used to differentiate between highly embryogenic and poorly embryogenic cultures at early stages of microspore culture. These molecular markers will have a practical utility in shaping tissue culture protocols for improving embryogenesis in recalcitrant species and cultivars of the *Brassicaceae*.

Future Directions

One of the major challenges to developing precise molecular studies for early microspore embryogenesis is the continuing presence of both pollen-like (non-induced) and embryogenic cells in early cultures as a result of incomplete induction/development. These cell types can be difficult to distinguish and separate, and although some techniques have been successful in separating these subgroups at 3 days of culture (Schulze and Pauls 2002), there are no generally applicable protocols for refining tissue selections to specifically select embryogenic tissues. With the identification of molecular marker genes known to be expressed early in the induction phase of embryogenesis (during the first 3 days of heat stress; Malik et al. 2007), it will become easier to identify and subsequently separate embryogenic cells from mixed cultures. Our data have clearly established that transcription factors such as *LEC1*, *LEC2* and *ABI3* are transcriptionally active during early microspore embryogenesis in *B. napus*. We have initiated specific promoter-reporter (GFP:Green Fluorescent Protein) studies to develop high expressing transgenic reporter lines in order to distinguish between embryogenic and pollen-like (or non-induced) cells in early microspore cultures (Fig. 4). These GFP-expressing transgenic lines also enable the precise tracking of embryogenic microspores on a continuing basis as they undergo dedifferentiation and subsequent sporophytic development. Many of the recent refinements in micro-dissection techniques have made it possible to carry out specific fluorescence-based cell selection. These specific promoter-reporter expressing microspores can be used as source material for additional transcript profiling (that is facilitated by the availability of cost effective sequencing technologies and *B. napus* microarray chips covering large parts of the transcribed genome) during the very early stages of embryogenesis and stress induction (1–3 day period).

In addition to these revealing studies of transcriptional change during embryogenesis, the pluripotent microspores of *B. napus* also present an exciting single cell system for studying stress-related phenomena and epigenetic reprogramming during embryogenesis. Studies of the molecular mechanisms of pluripotency/totipotency in plants are particularly relevant now with many exciting advances in directing and understanding pluripotency in animal stem cells appearing in the recent literature (Takahashi and Yamanaka 2006; Maherali et al. 2007; Okita et al. 2007). Further studies of early embryogenesis, and particularly the induction of embryogenesis,

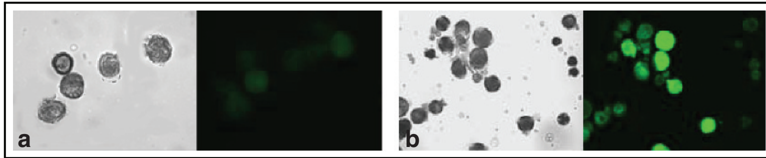


Fig. 4 Embryogenesis-specific promoter-driven GFP expression during microspore embryogenesis. A. Microspores of non-induced cultures (18°C) and, B. Embryogenic microspores in induced cultures (32°C)

would be particularly illuminating for addressing and revealing the basic differences in cellular plasticity between plants and animals (Costa and Shaw 2007).

References

Abe M, Katsumata H, Komeda Y, Takahashi T (2003) Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*. Development 130:635–643

Binarova P, Hause G, Cenklová V, Cordewener JHG, Van Lookeren Campagne MM (1997) A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex Plant Reprod 10:200–208

Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu CM, van Lammeren AA, Miki BL, Custers JB, Van Lookeren Campagne MM (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. Plant Cell 14:1737–1749

Boutilier K, Fiers M, Liu C-M, Van der Geest AHM (2005) Biochemical and molecular aspects of haploid embryogenesis. In: Palmer CE, Keller WA, Kasha KJ (eds) Haploids in crop improvement II. Springer, Berlin, pp 73–96

Chan J, Pauls KP (2007) *Brassica napus* Rop GTPases and their expression in microspore cultures. Planta 225:469–484

Costa S, Shaw P (2007) 'Open minded' cells: how cells can change fate. Trends Cell Biol 17:101–106

Custers JBM, Cordewener JHG, Nöllen Y, Dons HJM, Van Lookeren Campagne MM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep 13:267–271

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868

Ferrie AMR (2003) Microspore culture of *Brassica* species. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I, (eds) Doubled haploid production in crop plants. Kluwer, Dordrecht, pp 195–204

Ferrie AMR, Keller WA (1995) Microspore culture for haploid plant production. In: Gamborg OL, Philips GC (eds) Plant cell, tissue and organ culture: fundamental methods. Springer, Berlin, pp 155–164

Fiers M, Hause G, Boutilier K, Casamitjana-Martinez E, Weijers D, Offringa R, Van der Geest L, Van Lookeren Campagne M, Liu CM (2004) Mis-expression of the *CLV3/ESR*-like gene *CLE19* in *Arabidopsis* leads to a consumption of root meristem. Gene 327:37–49

Haecker A, Groß-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T (2004) Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131:657–668

Hause G, Hahn H (1998) Cytological characterization of multicellular structures in embryogenic microspore cultures of *Brassica napus* L. Bot Acta 111:204–211

Hoekstra S, Van Zijderveld MH, Louwerse JD, Heidekamp F, Van der Mark F (1992) Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci 86:89–96

Keller WA, Arnison PG, Cardy BJ (1987) Haploids from gametophytic cells – recent developments and future prospects. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) Plant tissue and cell culture. Alan R Liss, New York, pp 223–241

Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell 93:1195–1205

Luerßen H, Kirik V, Herrmann P, Miséra S (1998) *FUSCA3* encodes a protein with a conserved VP1/ABI3-like B3 domain which is of functional importance for the regulation of seed maturation in *Arabidopsis thaliana*. Plant J 15:755–764

Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1:55–70

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol 144:134–154

Malik MR, Wang F, Dirpaul JM, Zhou N, Hammerlindl J, Keller W, Abrams SR, Ferrie AMR, Krochko JE (2008) Isolation of an embryogenic line from non-embryogenic *Brassica napus* cv. Westar through microscope embryogenesis. J Exp Bot 59:2857–2873

Maraschin SF, Lamers GEM, Pater BS, Spaink HP, Wang M (2003) 14–3–3 isoforms and pattern formation during barley microspore embryogenesis. J Exp Bot 54:1033–1043

Nuccio M, Thomas T (1999) ATS1 and ATS3: two novel embryo-specific genes in *Arabidopsis thaliana*. Plant Mol Biol 39:1153–1163

Ogas J, Kaufmann S, Henderson J, Somerville C (1999) PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. Proc Natl Acad Sci USA 96:13839–13844

Okita K, Ichisakan T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J (1994) Regulation of gene expression programs during *Arabidopsis* seed development: roles of the ABI3 locus and of endogenous abscisic acid. Plant Cell 6:1567–1582

Pechan PM, Keller WA (1988) Identification of potentially embryogenic microspores of *Brassica napus* L. Physiol Plant 74:377–384

Reynolds TL (1997) Pollen embryogenesis. Plant Mol Biol 33:1–10

Reynolds TL, Crawford RL (1996) Changes in abundance of an abscisic acid-responsive, early cysteine-labeled metallothionein transcript during pollen embryogenesis in bread wheat (*Triticum aestivum*). Plant Mol Biol 32:823–829

Schulze D, Pauls KP (2002) Flow cytometric analysis of cellulose tracks development of embryogenic *Brassica* cells in microspore cultures. New Phytol 154:249–254

Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. Proc Natl Acad Sci USA 98:11806–11811

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

To A, Valon C, Savino G, Guilleminot J, Devic M, Giraudat J, Parcy F (2006) A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. Plant Cell 18:1642–1651

Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996a) Stress induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep 15:561–565

Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996b) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. Sex Plant Reprod 9:209–215

Tsuwamoto R, Fukuoka H, Takahata Y (2007) Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. Planta 225:641–652

Vrinten PL, Nakamura T, Kasha KJ (1999) Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (*Hordeum vulgare*) L. Plant Mol Biol 41:455–463

Xiang D, Datla R, Li F, Cutler A, Malik MR, Krochko JE, Sharma N, Fobert P, Georges F, Selvaraj G, Tsang E, Klassen D, Koh C, Deneault J-S, Nantel A, Nowak J, Keller W, Bekkaoui F (2008) Development of a *Brassica* seed cDNA microarray. Genome 51:236–242

Zuo J, Niu QW, Frugis G, Chua NH (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in *Arabidopsis*. Plant J 30:349–359

Chapter 9
Expression Profiles in Barley Microspore Embryogenesis

M. Muñoz-Amatriaín, J.T. Svensson, A.M. Castillo, L. Cistué, T.J. Close, and M.P. Vallés

Abstract In barley, microarray technology has allowed to study the gene expression profiling associated with the stress pretreatment phase of microspore embryogenesis, where the reprogramming of microspores takes place. Transcriptome analysis of anthers before and after 4 days of mannitol pretreatment, revealed changes in the expression of 2,673 genes. A thorough study of these differentially expressed genes indicated that microspores maintained their cell cycle machinery in a steady state during stress pretreatment and underwent a major reorganization of metabolic pathways accompanied by a multi-dimensional stress response. Up-regulation of transcription factors related to stress responses and changes in developmental programmes took place during the pretreatment. Preliminary studies have indicated that YABBY5, ZML2, CURLY LEAF and ICE1 transcription factors have a stress pretreatment specific induction and, therefore, could play a direct role in microspore reprogramming.

Keywords Barley, expression profiling, microarray, microspore embryogenesis, reprogramming, stress pretreatment, transcription factors

---

M. Muñoz-Amatriaín
Departamento de Genética y Producción Vegetal, Estación Experimental Aula Dei, CSIC, 50059, Zaragoza, Spain

J.T. Svensson, T.J. Close
Department of Botany and Plant Sciences, University of California Riverside, CA 92521, USA
Present address:
Department of Plant Biology, University of Copenhagen, DK-1871, Frederiksbergc, Denmark

A.M. Castillo, L.Cistué
Departamento de Genética y Producción Vegetal, Estación Experimental Aula Dei ,CSIC, 50059, Zaragoza, Spain

T.J. Close
Department of Botany and Plant Sciences, University of California Riverside, CA 92521, USA

M.P. Vallés(✉)
Departamento de Genética y Producción Vegetal, Estación Experimental Aula Dei, CSIC, 50059, Zaragoza, Spain
E-mail: valles@eead.csic.es

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

Introduction

Plant microspores can be diverted from their normal pollen developmental pathway towards an embryogenic route after exposure to a stress pretreatment. This process, termed microspore embryogenesis or androgenesis, has been widely used to produce doubled haploids for genetics and breeding programmes (Forster and Thomas 2005) and represents a suitable system to study plant cell totipotency and embryogenesis (Hosp et al. 2007).

Stress acts as a trigger to induce microspore embryogenesis and represents a key point where the reprogramming of microspores takes place. Although microspore embryogenesis can be induced by different stresses, in barley, higher regeneration efficiencies are obtained when microspores at the mid-late to late uni-nucleate stage are subjected to a combination of starvation and osmotic stress pretreatments, which is achieved by incubating anthers in a medium with mannitol (Hoekstra et al. 1992).

The first genes identified in barley as being differentially expressed in the early stages of microspore embryogenesis encoded a lipid transfer protein (ECLTP), a glutathione S-transferase (ECGST) and a previously unknown protein (ECA1, possibly an arabinogalactan-like protein) (Vrinten et al. 1999). In recent years, the availability of large sets of expressed sequence tags (ESTs) have triggered the development of macro- and microarray technology approaches that provide a rich source of information of the molecular processes that take place during microspore embryogenesis, specially at the microspore reprogramming phase (Maraschin et al. 2006; Muñoz-Amatriaín et al. 2006).

In this chapter we show an analysis of gene expression profiling associated with mannitol stress pretreatment, obtained by using the Barley1 GeneChip (Close et al. 2004).

Expression Profile Associated with Stress Pretreatment

Transcriptome analysis of anthers before and after 4 days of stress pretreatment resulted in a broad overview of the mechanisms underlying the developmental transition from gametogenesis to embryogenesis (Muñoz-Amatriaín et al. 2006). The stress pretreatment consisted of incubating the anthers in a medium containing 0.7 M mannitol, 40 mM $CaCl_2$ and 8 g/l agarose, for 4 days at 25°C in the dark (Cistué et al. 2003). The recently developed Barley1 GeneChip (Close et al. 2004) was used for this study, thus enabling the analysis of more than 22,000 genes simultaneously.

A stringent statistical approach was adopted (False Discovery Rate adjusted P-value cut-off of 0.01, followed by filtering for four-fold or greater changes) resulting in a total of 2,673 differentially expressed genes, which revealed large changes in the transcriptome as a consequence of the mannitol pretreatment applied (Fig. 1). From these genes, 887 were up-regulated after the pretreatment whereas 1,786 were down-regulated, indicating that these differences could be mainly due to a decrease in gametophytic information.

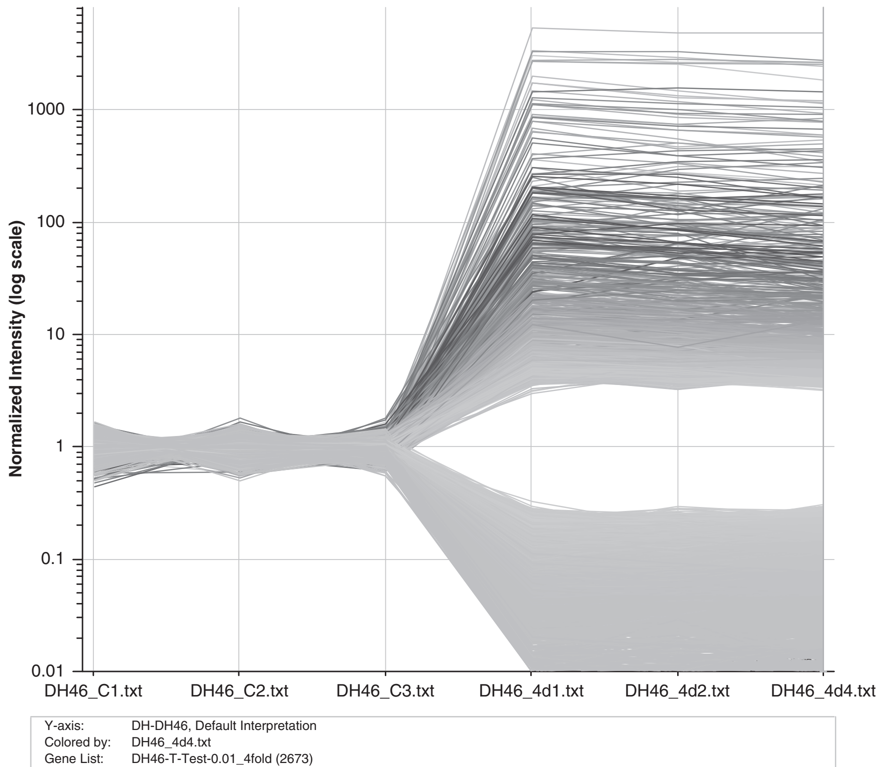


Fig. 1 Expression profile between zero and 4 days of mannitol pretreatment (False Discovery Rate adjusted P-value = 0.01, 4-fold filtering). Three replicates from each condition are shown: C1, C2 and C3 are control replicates, and 4d1, 4d2 and 4d4 are 4-days after mannitol pretreatment replicates

Functional classification of the differentially expressed genes was done using the MIPS database (http://mips.gsf.de/). Analysis revealed that 40% belonged to the category "unclassified proteins". Of the classified genes, more than 50% belonged to the categories "Metabolism" and "Energy", where most of the "metabolism" genes were involved in C-compound and carbohydrate metabolism whereas the "energy" genes were related to glycolysis and gluconeogenesis. A thorough examination of genes belonging to these categories indicated that catabolism of reserve compounds such as starch, sucrose and lipids, was predominant over their biosynthesis. These expression data confirmed the reorganization of central carbon metabolism during carbohydrate starvation towards the flexible use of carbon skeletons from different sources, as already described for sugar starvation situations (Yu 1999; Contento et al. 2004).

Around 11% of the differentially expressed genes belonged to the "Cell rescue, defence and virulence" category. A multi-dimensional stress response was observed, since genes related to water deficit, osmotic stress, oxidative stress, phosphate starvation, cold stress, and response to wounding and pathogens were found. This is in agreement with the existence of complex networks in abiotic and biotic stress

responses (Cheong et al. 2002). Among these stress-response genes, genes encoding glutathione S-transferases (GSTs) were over-represented, representing a total of 2% of the up-regulated genes. GSTs are involved in oxidative stress response, pathogen attack and heavy metal toxicity (Marrs 1996) and the induction of members of the GST family during the initial steps of microspore embryogenesis has been described (Vrinten et al. 1999; Maraschin et al. 2006). With regard to heat shock proteins (HSPs), which have been reported to be highly expressed in microspores of rapeseed and tobacco upon heat shock and starvation (Cordewener et al. 1995; Zarsky et al. 1995; Smycal and Pechan 2000), only minor changes in the expression of the *HSP* genes were observed in this study. This is in agreement with the study of Malik et al. (2007) in *Brassica*, who found very few transcripts for HSPs in cDNA libraries of 3 day heat stress treatment.

In relation to the cell cycle, most genes were constitutively expressed, which might reflect maintenance of the cell cycle machinery in a steady state during mannitol pretreatment. *CDC25*, involved in the control of transition from G2 to M phase (Dewitte and Murray 2003) was one of the few genes with an increased expression after stress pretreatment, suggesting that the cell cycle is not arrested in G2.

Transcription Factor Analysis

Developmental programmes are controlled largely at the level of transcriptional regulation. The Barley1 GeneChip targets 340 genes encoding putative transcription factors and, of these, 79 were down-regulated and 27 were up-regulated after mannitol pretreatment.

Almost all members of families of: ethylene insensitive 3-like (EIL), auxin-response factors (ARF) and auxin-regulated proteins (Aux/IAA) decreased their expression after the stress pretreatment. ARF and Aux/IAA proteins play a critical role in most growth responses, controlling auxin-response genes (Hagen and Guilfoyle 2002). EIL participates in the ethylene signalling pathway, and its inhibition has been associated with cell growth inhibition and accelerated senescence (Guo and Ecker 2004).

The up-regulated transcription factors were divided into two groups according to the processes they regulated: abiotic and biotic stress responses (ICE1, ATAF2-like, WRKY46, AtbZIP60 and HSF7) and changes in developmental programmes (YABBY5, ZML2 and CURLY LEAF). Interestingly, we observed an induction of CURLY LEAF, a repressor of floral homeotic genes (Goodrich et al. 1997; Katz et al. 2004) together with the down-regulation of the ABC model genes *APETALA1*, *APETALA3*, *AGAMOUS* and *AGAMOUS-LIKE 9 (AGL9)*. No transcription factors related to early embryo formation were found in this study.

The up-regulated transcription factors were compared to the barley reference experiment (Druka et al. 2006) to study their expression pattern in different tissues (Fig. 2). The expression patterns were very diverse: some transcription factors had a high level of expression in almost all tissues studied whereas others had very low level of expression in these tissues. 54% of the transcription factors up-regulated after mannitol pretreatment were highly expressed in anthers.

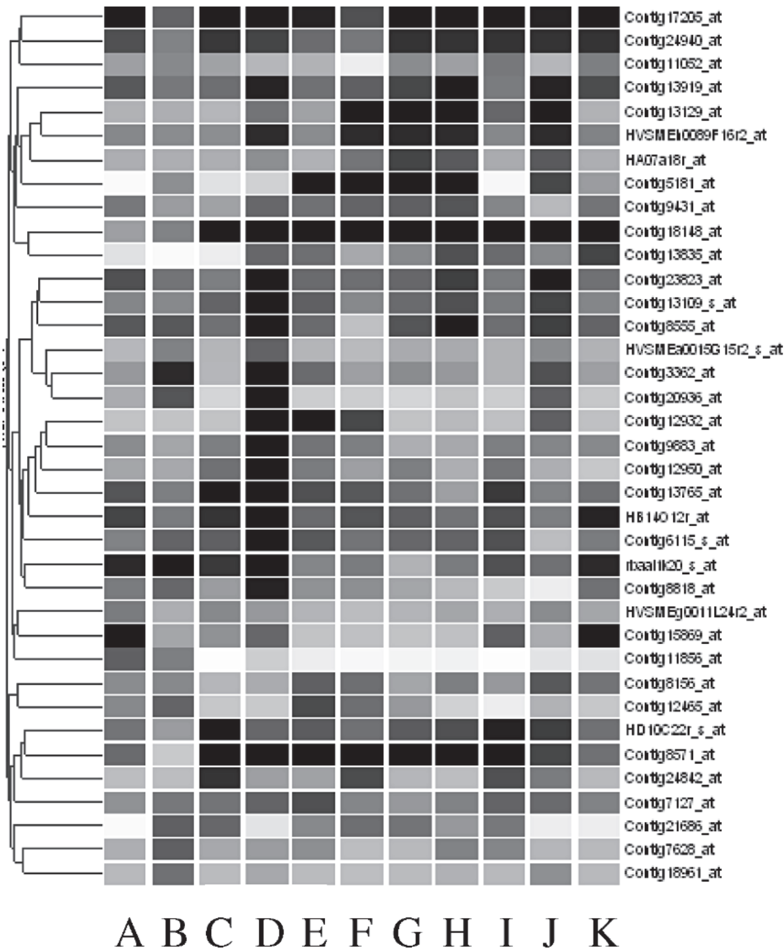


Fig. 2 Comparison of the Barley reference experiment versus the stress up-regulated transcription factors expression. Mannitol induced genes were compared to; (A) Root, (B) Leaf, (C) Inflorescence, (D) Anther, (E) Pistil (F) Caryopsis 5 DAP (Days After Pollination), (G) Caryopsis 10 DAP, (H) Caryopsis 16 DAP, (I) Embryo 22 DAP, (J) Endosperm 22 DAP and (K) Germinated seed

Although some transcription factors were up-regulated during mannitol stress pretreatment, no information related to the specificity of their expression in this phase was available.

Preliminary results have allowed us to compare the expression level of these transcription factors after zero, 4 days of mannitol pretreatment and 3 days of culture, where the sporophytic developmental pathway has started (Fig. 3). Out of the transcription factors down-regulated after pretreatment, 46.2% increased their expression at 3 days of culture. However, 72.9% of the up-regulated ones decreased their expression during culture. Thus, 52% of the transcription factors were differentially expressed at 4 days of stress pretreatment.

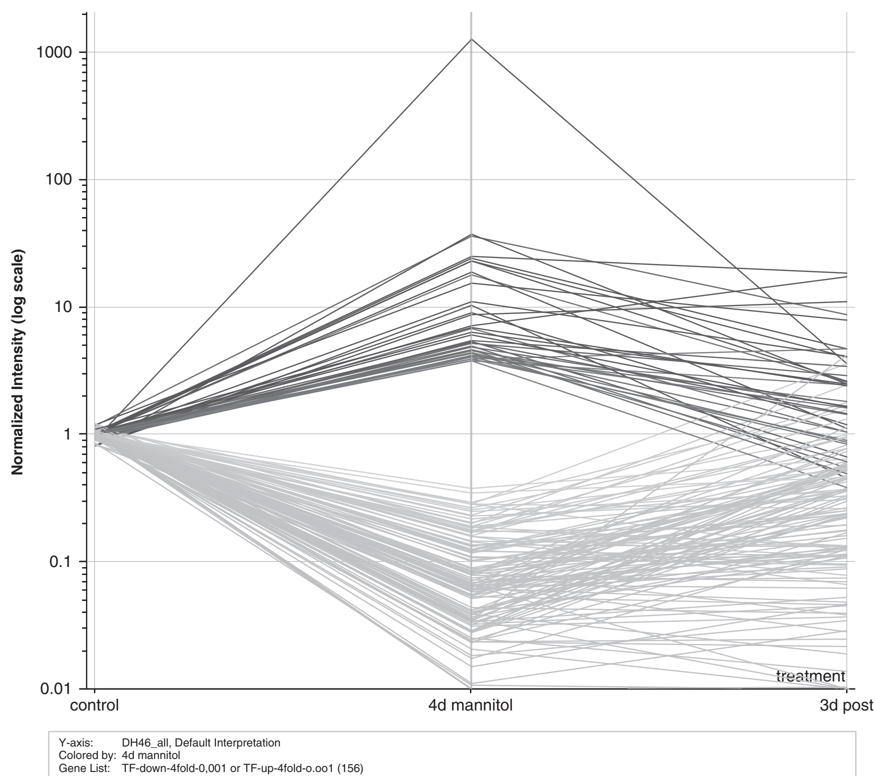


Fig. 3 Expression profile of the transcription factors between zero, 4 days of mannitol pretreatment and 3 days of culture (P-value = 0.01, 4-fold filtering)

The up-regulated transcription factors related to changes in developmental programmes as: YABBY5, with a role in meristem development and maintenance of stamens and carpels (Jang et al. 2004); ZML2, a C2C2 transcription factor associated with cell wall modifications during elongation (Shikata et al. 2004); and the repressor of floral homeotic genes CURLY LEAF, showed a stress pretreatment transient expression. Among those transcription factors related to abiotic and biotic stress responses, only ICE1, a MYC-like b-HLH transcriptional activator associated with cold and dehydration response (Chinnusamy et al. 2003), showed a stress pretreatment specific expression, whereas others as the HSF7 (Busch et al. 2005), maintained the same level of expression after 3 days of culture.

Conclusions and Perspectives

DNA microarray technology has allowed the analysis of expression profiles of a large number of genes, before and after the stress pretreatment phase of microspore embryogenesis in barley. Barley microspores were found to undergo a dramatic

change in their transcriptome as a consequence of the mannitol pretreatment, with major reorganization of metabolic pathways accompanied by a multi-dimensional stress response. Evidence of progression but not of arrest of the cell cycle was found. Transcription factor analysis indicated that the transcriptional control relied mainly on the protective roles to stress and suppression of the gametophytic developmental pathway, but without any evidence of embryogenesis initiation. In this way, after 4 days of mannitol stress pretreatment, barley microspores would be in a transitional de-differentiated phase.

Although, around 55% of the up-regulated transcription factors were highly expressed in anther tissue, few of them had an anther-specific expression. Up-regulated transcription factors related to development showed a mannitol pre-treatment specific induction, whereas only one of the transcription factors related to stress response was specifically induced by the pretreatment. Thus, YABBY5, ZML2, CURLY LEAF and ICE1 could play a direct role in microspore reprogramming. Previously in barley, Maraschin et al. (2006) revealed that genes encoding an alcohol dehydrogenase 3 (*ADH3),* and proteolytic genes as a metalloprotease FtsH, a cysteine protease precursor, an aspartic protease, and a 26S proteasome regulatory subunit, could represent bio-markers for the embryogenic potential of microspores. These results indicated that metabolic changes and proteolysis have a critical role in the de-differentiation phase of microspore embryogenesis.

Further research is needed to characterise candidate genes playing a role in microspore reprogramming. Recently, studies using macro- and microarray technology have been used to further understanding of the molecular processes that take place in embryogenic microspores and microspore-derived embryos in *Brassica* (Pauls et al. 2006; Joosen et al. 2007; Malik et al. 2007). Although microspore embryogenesis is highly dependent on the species, genotype, stress pretreatment and culture media, utilization of microarray techniques is providing new insights into the molecular mechanisms that control microspore embryogenesis.

References

Busch W, Wunderlich M, Schöffl F (2005) Identification of novel heat shock factor-dependent genes and biochemical pathways in *Arabidopsis thaliana*. Plant J 41:1–14

Cistué L, Vallés MP, Echávarri B, Sanz JM, Castillo AM (2003) Barley anther culture. In: Malupszynski M, Kasha K, Foster B (eds) Doubled Haploid Production in Crop Plants. FAO/IAEA Division, Wien, A Manual, pp 29–35

Cheong YH, Chang H-S, Gupta R, Wang X, Zhu T, Luan S (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress and hormonal responses in Arabidopsis. Plant Physiol 129:661–677

Chinnusamy V, Ohta M, Kanrar S, Lee B-H, Hong X, Agarwal M, Zhu J-K (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev 17:1043–1054

Close TJ, Wanamaker S, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiol 134:960–968

Contento AL, Kim SJ, Bassham DC (2004) Transcriptome profiling of the response of Arabidopsis suspension culture cells to Suc starvation. Plant Physiol 135:2330–2347

Cordewener JHG, Hause G, Görgen E, Busink R, Hause B, Dons HJM, Van Lammeren AAM, Van Lookeren Campagne MM, Pechan P (1995) Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196:747–755

Dewitte W, Murray JAH (2003) The plant cell cycle. Annu Rev Plant Biol 54:235–264

Druka A, Muehlbauer G, Druka I, Caldo R, Baumann U, Rostoks N, Schreiber A, Wise R, Close TJ, Kleinhofs A, Graner A, Schulman A, Langridge P, Sato K, Hayes P, McNicol J, Marshall D, Waugh R (2006) An atlas of gene expression from seed to seed through barley development. Funct Integr Genomics 6:202–211

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breeding Rev 25:57–88

Goodrich J, Puangsomiee P, Martin M, Long D, Meyerowitz EM, Coupland G (1997) A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature 386:44–51

Guo H, Ecker JR (2004) The ethylene signalling pathway: new insights. Curr Opin Plant Biol 7:40–49

Hagen G, Guilfoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49:373–385

Hoekstra S, van Zijderveld MH, Louwerse JD, Heidekamp F, van der Mark F (1992) Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci 86:89–96

Hosp J, Maraschin SF, Touraev A, Boutilier K (2007) Functional genomics of microspore embryogenesis. Euphytica 158:275–285

Jang S, Hur J, Kim SJ, Han MJ, Kim SR, An G (2004) Ectopic expression of OsYAB1 causes extra stamens and carpels in rice. Plant Mol Biol 56:133–143

Joosen R, Cordewener J, Supena EDJ, Vorst O, Lammers M, Maliepaard C, Zeilmaker T, Miki B, America T, Custers J, Boutilier K (2007) Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol 144:155–172

Katz A, Oliva M, Mosquna A, Hakim O, Ohad N (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. Plant J 37:707–719

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol 144:134–154

Maraschin SF, Caspers M, Potokina E, Wülfert F, Graner A, Spaink HP, Wang M (2006) cDNA array analysis of stress-induced gene expression in barley androgenesis. Physiol Plantarum 127:535–550

Marrs KA (1996) The functions and regulation of glutathione S-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol 47:127–158

Muñoz-Amatriaín M, Svensson JT, Castillo AM, Cistué L, Close TJ, Vallés MP (2006) Transcriptome analysis of barley anthers: effect of mannitol treatment on microspore embryogenesis. Physiol Plantarum 127:551–560

Pauls KP, Chan J, Woronuk G, Schulze D, Brazolot J (2006) When microspores decide to become embryos – cellular and molecular changes. Can J Bot 84:668–678

Shikata M, Matsuda Y, Ando K, Nishii A, Takemura M, Yokota A, Kohchi T (2004) Characterization of *Arabidopsis ZIM*, a member of a novel plant-specific GATA factor gene family. J Exp Bot 55:631–639

Smycal P, Pechan PM (2000) Stress, as assessed by the appearance of sHsp transcripts, is required but not sufficient to initiate androgenesis. Physiol Plantarum 110:135–143

Vrinten PL, Nakamura T, Kasha KJ (1999) Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (*Hordeum vulgare* L.). Plant Mol Biol 41:455–463

Yu SM (1999) Cellular and genetic responses of plants to sugar starvation. Plant Physiol 121:687–693

Zarsky V, Garrido D, Eller N, Tupy J, Vicente O, Schöffl F, Heberle-Bors E (1995) The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ 18:139–147

Chapter 10
Proteomics in Rapeseed Microspore Embryogenesis

J. Cordewener, F. van der Wal, R. Joosen, K. Boutilier, and T. America

Abstract This chapter reviews the proteomics research performed to elucidate the molecular processes associated with embryo initiation and development in *Brassica napus* (rapeseed) microspore-derived embryo cultures. Classical biochemical methods such as combined [^{35}S]-methionine labelling and 2-D gel electrophoresis (2-DE) have been used for the large-scale study of proteins differentially expressed during the inductive phase of embryogenesis. Immuno-cytological experiments have been carried out to study the (sub)cellular localization of putative marker proteins for embryo induction. Changes in post-translational modification of proteins (phosphorylation) have been detected during the induction phase, as have changes in the profile of proteins en peptides secreted by developing embryos. We also discuss recent developments in quantitative proteomics technologies such as fluorescence labelling of protein samples and stable isotope labelling by amino acids in cell cultures that enable more detailed expression analyses and the identification of low abundant proteins in complex mixtures.

Keywords Rapeseed (*Brassica napus*), microspores, proteomics, 2-D gel electrophoresis, DiGE, phosphorylation

Introduction

Most studies on the molecular events that regulate zygotic embryogenesis have been performed using *Arabidopsis thaliana* as a model plant. A great deal of success in the search for genes that play a major role in embryo development has been achieved through mutational screens, generating a number of pattern formation

---

J. Cordewener(✉)
Plant Research International, Postbus 16, 6700AA, Wageningen UR, The Netherlands
E-mail: jan.cordewener@wur.nl

F. van der Wal, R. Joosen, K. Boutilier, and T. America
Plant Research International, Postbus 16, 6700AA Wageningen UR, The Netherlands

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

mutants for which the corresponding genes have been identified by molecular tagging and mapping techniques. As compared to embryo pattern formation, much less is known about the genes and proteins controlling the initiation of plant embryogenesis. The dearth of knowledge on the molecular mechanisms underlying embryo initiation in plants is mainly due to the lack of experimental accessibility, particularly at the early stages of embryogenesis. The amount of starting material is often a limiting factor for expression analysis using transcriptomics or proteomics. Recently, important advances have being made in profiling the dynamics of gene expression in specific populations of cells or even individual cells by employing new techniques like laser micro-dissection (LM) combined with high-throughput technologies such as transcript analyses, proteomic analyses and metabolomic profiling (Casson et al. 2005; von Eggeling et al. 2007).

A specific feature of plants compared to mammalian species is that, besides zygotic embryos, plants can form embryos without fertilisation, either *in vivo* starting from somatic or egg cells of the ovule (apomixis), or *in vitro* from somatic explants (somatic embryogenesis) or gametophytic cells like microspores (androgenesis or microspore embryogenesis) and egg cells (gynogenesis). In the past efficient tissue culture systems were developed to study gene expression during somatic embryogenesis (carrot, alfalfa) and microspore embryogenesis (tobacco, rapeseed, maize). Under the appropriate culture conditions somatic and microspore embryos undergo the same developmental stages that are typical for zygotic embryos of that species. However, while zygotic embryogenesis starts with the fusion of an egg cell with a sperm cell, the initiation of somatic or microspore embryogenesis is less-well defined, and embryos may arise directly from individual cells or indirectly via a callus phase. Both types of *in vitro* systems require an induction phase to stimulate formation of embryo competent cells, for which there is no direct counterpart in zygotic embryogenesis.

Pechan et al. (1991) illustrated that microspores of rapeseed (*Brassica napus*) when isolated at a developmental stage surrounding the asymmetric first pollen mitosis can be irreversibly induced to embryogenesis when treated for at least 8 hours at 32°C. Later it was reported that the same population of cells when cultured at 18°C continued gametophytic development (Custers et al. 1994), indicating that the switch in development towards embryogenesis is controlled only by culture temperature. Additional data accumulated in rapeseed and other species (tobacco, wheat, barley) indicated that other stress treatments could also stimulate microspore embryogenesis, leading to a unified concept of stress-induced embryogenesis (Shariatpanahi et al. 2006). The next sections focus on the proteomics research that has been carried out in the last decades to study the process of embryo initiation and development in rapeseed microspores.

2-DE Analysis of [^{35}S]-Methionine Labelled Microspore Cultures

The first data on protein changes associated with the induction of rapeseed microspore embryogenesis were reported by Pechan et al. (1991) using silver-stained 2-D gels of microspores cultured at 25°C and 32°C for 8 hours. As silver-staining

is not a quantitative method for protein detection, we initiated a study using [^{35}S]-methionine labelling of the cultures to monitor changes in protein synthesis (Cordewener et al. 1994). Also, an 18°C culture was used as the non-embryogenic control instead of the usual 25°C culture, because at 18°C only pollen maturation occurs in the cultures. By comparing 2-D protein patterns of [^{35}S]-methionine labelled embryogenic (32°C) and non-embryogenic (18°C) cultures, six proteins could be identified that were exclusively synthesized under embryogenic conditions. Another 18 proteins were synthesized at a significantly higher rate under embryogenic conditions than the control culture, while one protein was 2-fold up-regulated in the control culture. The temporal expression of the 25 differentially synthesized proteins during later stages of culture under embryogenic conditions was investigated by radioactive labelling at day 2, 5 and 12 (Cordewener et al. 2000). Four out of six proteins that were specifically synthesized during the first 8 hour in a 32°C culture were not synthesized anymore at or after day 2. These proteins represent good candidates as markers for the induction phase of microspore embryogenesis. The other two proteins disappeared from a 32°C culture around day 2, but were synthesized again at later stages of embryo development, i.e. around day 12. None of the six above mentioned proteins were detected in 18°C cultures radioactive labelled at day 2 or 5 after the onset of culture. Due to the low expression of the six proteins during embryo initiation, and the relatively large amount of protein that was necessary at that time for *de novo* sequencing, we were not able to sequence these proteins.

The observed up-regulation of the above mentioned 18 proteins in embryogenic cultures does not imply *a priori* that these proteins are related to the process of embryo initiation in microspores. While at 18°C only gametophytic development of isolated microspores occurs, at 32°C both embryogenic and gametophytic development occurs side by side. This was visualized by the simultaneous appearance of a number of abundant, low M_r protein spots in the 2-D gels of both the 18°C and 32°C cultures at day 2 (Cordewener et al. 2000), indicating that many of the proteins synthesized in a 32°C culture originated from gametophytically developing microspores. Also, the peak in protein synthesis activity observed in embryogenic cultures at day 2, as measured by the rate of incorporation of [^{35}S]-methionine into proteins, is probably (partly) due the high metabolic activity of gametophytic developing microspores at 32°C (Cordewener et al. 2000). Furthermore, changes in protein synthesis observed in a 32°C culture might be induced either as a consequence of entry into embryogenic development, or exclusively as a consequence of the elevated temperature. A quantitative analysis of protein expression at the cellular level, for instance by immuno-cytological studies, can help to discriminate between these different possibilities.

Sub-Cellular Localisation of Heat Shock Proteins

In both prokaryotes and eukaryotes it is well established that the most important response to heat stress is the increased synthesis of a set of protective proteins, called molecular chaperones or heat-shock proteins (HSPs). As microspore

embryogenesis is initiated by heat stress, 2-D immunoblotting experiments with anti-HSP antibodies were performed to investigate whether the set of 18 proteins, mentioned above to be up-regulated in a 32°C culture, include HSPs. Three protein spots, showing a 3-, 6- and 10-fold increase in protein synthesis during the first 8 hour of culture under embryogenic conditions, reacted with antibodies specific to members of the 68, 70 and 17-kDa families of HSPs, respectively. Subsequent immuno-cytochemical research focused on the question whether the observed changes in protein synthesis could be ascribed to embryogenic microspores. Major differences in the sub-cellular localisation of HSP70 were observed in microspores cultured for 8 hour at 18°C and 32°C. While early bi-cellular pollen in an 18°C culture showed weak cytoplasmic distribution of HSP70, at 32°C HSP70 was clearly localized in the nucleus of the vegetative cell. It was suggested that the translocation of HSP70 to the nucleoplasm of the vegetative nucleus is associated with the re-entry of the vegetative cell into the cell cycle, an event that is required for the onset of embryogenesis (Binarova et al. 1993). These findings were supported by data of Binarova et al. (1997) who showed that rapeseed pollen isolated at the late bi-cellular stage can be induced to undergo embryogenesis by the application of a short, but more severe heat stress of 41°C. While late bi-cellular pollen cultured at 32°C were unable to enter embryogenic development (Pechan and Keller 1988) a 41°C treatment at the onset of culture resulted in the induction of DNA synthesis in the vegetative nucleus and subsequent sporophytic cell division. Again, this re-entry into the cell cycle was accompanied by the accumulation of HSP70 in the vegetative nucleus, confirming the tight correlation between the induction of sporophytic divisions and the nuclear translocation of HSP70.

Zhao et al. (2003) studied the role of HSP70 and HSP19 during the induction of microspore embryogenesis using colchicine instead of heat treatment (32°C) to trigger the developmental switch in rapeseed microspores. As 1-D immunoblots of protein extracts from microspores treated with colchicine showed no significant increase in HSP70 expression, as it had in the heat treatments, the authors concluded that increased HSP70 synthesis is not required for the induction of microspore embryogenesis. However, since 2-D immunoblots revealed that anti-HSP70 recognizes at least six isoforms (Cordewener et al. 1995), each with different expression levels, small changes in the expression of individual isoforms can not be excluded using 1-D immunoblots. Even more important when studying the possible role of HSP70 in the induction of embryogenesis is to investigate the subcellular localisation of HSP70 after colchicine treatment, as previous experiments showed this to be the most discriminating factor in determining the developmental fate of a microspore. It is worthwhile to mention in this respect that the overall level of HSP70 expression in microspores at different developmental stages declines progressively as the pollen matures (Zhao et al. 2003). One could speculate that in uni-cellular vacuolated microspores responsive to colchicine treatment, the level of HSP70 is high enough to initiate embryogenesis, while in later stages of microspores a heat treatment and an increase in HSP level is required to support embryo induction.

Phosphorylation Events During Microspore Embryogenesis

Protein phosphorylation is considered as being a key event in the regulatory and signal transduction pathways of biological systems. The extensive use of this control mechanism as a primary means of rapidly switching the activity of a cellular protein from one state to another is apparent by the large number of known kinases and phosphatases. The activation of cell division and transitions between different phases of the cell cycle are controlled by a family of cyclin-dependent protein kinases (CDKs). As the initiation of embryo development in microspores is characterized by major changes in cell cycle activity, we compared the phosphorylation events occurring under embryogenic and non-embryogenic conditions. Microspores were cultured at 18°C and 32°C in the presence of [^{32}P]-orthophosphate and analysed by 2-DE (Cordewener et al. 2000). Numerous protein spots were radioactively labelled under both conditions, except for two proteins that were only phosphorylated at 32°C and three spots that were only phosphorylated at 18°C.

One cluster of protein spots was more highly phosphorylated under embryogenic conditions than non-embryogenic conditions. Double labelling experiments with [^{35}S]-methionine and [^{32}P]-orthophosphate revealed that these highly phosphorylated protein spots co-localized with HSP70 isoforms. The phosphorylation of HSP70 in embryogenic microspores is consistent with previous work of Hause et al. (1995), showing that HSP70 cross-reacted with MPM-2, a monoclonal antibody which recognizes a mitosis-specific phosphorylated epitope. Although it is known for many years that HSPs can be phosphorylated, the role of phosphorylation in HSP70 functioning remains poorly understood.

Extra-Cellular Proteins

The involvement of extra-cellular signalling molecules in *in vitro* embryo formation has been reported for several plant cell culture systems (Boutilier et al. 2005). Already in 1980, Hari (1980) showed that non-embryogenic cultures of carrot became embryogenic when treated with conditioned medium of highly embryogenic cultures. Several components in the conditioned medium have been found to promote cell proliferation and somatic embryogenesis, including specific endochitinases and arabinogalactan proteins. In rapeseed microspore cultures, the highest embryo yields are obtained with a cell density of approx. 40,000 cells per ml. Medium conditioned by culturing microspores at optimum density improved embryogenesis in low density of cultures (Huang et al. 1990). Although the extracellular molecule(s) that enhance microspore embryogenesis has not been characterized, Tsuwamoto et al. (2007) suggested that phytosulfokine is a good candidate as this sulphated pentapeptide promotes cellular proliferation and was found to be present in the conditioned medium of *Asparagus officinalis*.

To examine the secretion of proteins into the medium by cultured microspores, [^{35}S]-methionine labelled extracellular proteins were analysed by 1-DE

(Cordewener et al. 2000). Within 2 days after initiation of the cultures clear differences in the patterns of extra-cellular proteins were visible between embryogenic and non-embryogenic conditions, and the differences were even more pronounced after 5 days of culture. Fiers (2007) focussed on small proteins when analysing the conditioned media from embryogenic and non-responsive microspore cultures of rapeseed. HPLC fractionation on a reverse-phase column revealed clear differences in elution profile (A_{214}) between both media. Six proteins could be identified by mass spectrometry (MS) as encoded by genes with predicted secretion peptides, indicating that this proteomic approach is an efficient and sensitive way to analyze secreted (signal) proteins in rapeseed culture media.

Difference Gel Electrophoresis (DiGE) Analysis of Microspore Cultures

Recently, data was published on the transcriptional profiling of cultured rapeseed microspores, along with protein expression data obtained by 2-DE (Joosen et al. 2007). The proteome analysis resulted in the identification of 30 proteins up-regulated in one or more of the embryogenic cultures compared to the pollen cultures (see Table 1, last column). A number of temporally co-expressed transcript-protein pairs were identified, providing a set of robust markers for different stages of microspore embryogenesis. Here an extension of this proteomic research is presented in which spectrally-resolvable cyanine (Cy) dyes instead of SyproRuby were used to visualize and quantify the proteins. Difference gel electrophoresis (DiGE) with Cy-dyes allows the run of two different stained samples in one gel and the inclusion of a reference sample, avoiding problems with matching protein spots from different runs. Protein extracts from microspores cultured for 2, 5 and 10 days at 32°C (2e, 5e and 10e, respectively) were compared with microspores harvested immediately (0 days) or cultured for 5 days at 18°C (5p). In addition to these conventional temperature treatments, microspores were cultured for 24 hours at 32°C, followed by transfer to 25°C. Under these conditions microspores first produce a suspensor, after which the distal cell forms an embryo proper around day 8 (8se) that develops further into a pre-globular stage embryo around day 10 (10se). These so-called suspensor-bearing embryos show a much better structural organisation than embryos from conventional cultures, and because they develop more slowly, the suspensor-bearing embryo cultures are less prone to contamination by co-developing pollen grains (Joosen et al. 2007). Protein extracts of all developmental stages were labelled with the Cy-dyes before 2-DE. Figure1 shows the 2-D protein pattern of a 10 day embryo culture labelled with Cy-5. The numbers correspond to the 56 proteins listed in Table 1, and are crossed-referenced with the protein spots previously identified by Joosen et al. (2007). The temporal expression pattern of these previously reported proteins, as deduced from the spot intensities after SyproRuby staining, was very similar to the expression levels observed by DiGE labelling. Nine spots out of 56 differentially expressed proteins were identified as

members of different families of chaperones, i.e. HSC70 (no. 26, 27), HSP70 (no. 29), HSP81 (no. 28), HSP90 (no. 43) and type I chaperonins (no. 30, 38, 45, 50). The expression pattern of the various molecular chaperones was quite different. For instance, HSP70 (no. 29), mitochondrial HSP60 (no. 30) and chloroplast HSP60 (no. 38) showed an optimum in expression at different time points during embryo development, namely after respectively 2, 5 and 10 days of culture at 32°C (Fig. 2a–c). Major anti-oxidative enzymes are expressed during the various stages of embryo development: L-ascorbate peroxidase (no. 5–7), glutathione-S-transferase (no. 44, 55), dehydroascorbate reductase (46, 52), thioredoxin (no. 24) and peroxiredoxin (no. 47). While one GST (no. 55) was specifically expressed around day 2 in an embryogenic culture, L-ascorbate peroxidase (no. 5–6) and peroxiredoxin (no. 47) appeared from day 5 on in embryogenic cultures, and were also abundantly present in suspensor-bearing embryos. Acting as scavenging enzymes of reactive oxygen species (ROS), these proteins play a role in regulating the cellular redox status. Several lines of experimental evidence suggest that ROS and redox status

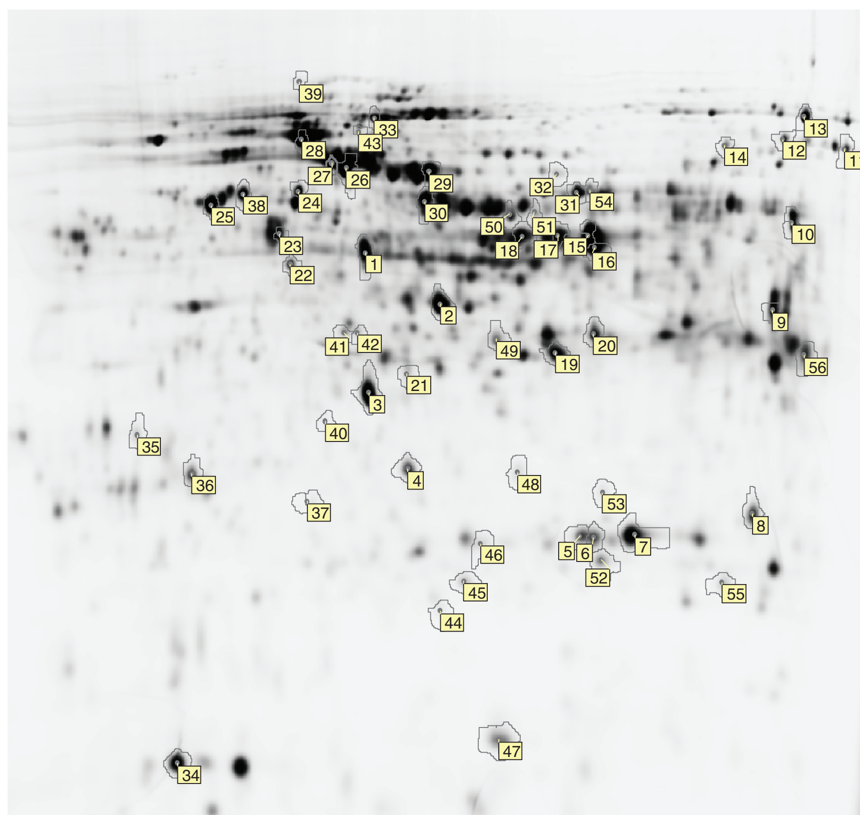


Fig. 1 Representative 2-DE image of a Cy5-labelled protein extract of a 10-day embryogenic microspore culture. The numbers assigned to the protein spots are those shown in Table 1

Table 1 Proteins identified in rapeseed microspore cultures by DiGE and mass spectrometry

No	Best-matching gene product (*Organism*)	ID	Matched	Coverage (%)	Spot no. in Joosen et al.
1	α-Tubulin *(Bn)*	gi34733239	10	25.1	
2	Actin *(Bn)*	gi4139264	7	22.5	
3	60 S acidic ribosomal protein *(At)*	gi21593316	5	13.6	
4	Lactoylglutathione lyase/glyoxylase I *(Bo)*	gi2494843	11	24.8	15
5	L-ascorbate peroxidase *(Bn)*	gi1890354	6	17.6	
6	L-ascorbate peroxidase *(Bn)*	gi1890354	9	27.2	
7	L-ascorbate peroxidase *(Bn)*	gi1890354	13	42.4	25
8	Glutathione-S-transferase *(At)*	gi11096018	4	13.9	29
9	Glutamate dehydrogenase *(Bn)*	gi15004984	3	8.8	
10	ATPase subunit 1 *(Bn)*	gi37591102	9	15.8	
11	Methionine synthase (putative) *(At)*	gi6006863	4	7.5	
12	Methionine synthase (cobalamin indep) *(At)*	gi47600741	1	1.8	
13	Elongation factor 2 *(At)*	gi6056373	3	3.4	
14	NADH ubiquinone reductase *(At)*	gi10177435	1	1.6	
15	Enolase *(Bn)*	gi34597332	8	21.1	
16	Adenosylhomocysteinase *(At)*	gi21553795	4	12.8	
17	F1-ATPase ß-subunit (mitochondrial) *(At)*	gi17939849	5	11.5	
18	F1-ATPase ß-subunit (mitochondrial) *(At)*	gi17939849	15	40.6	
19	Reversibly glycosylated polypeptide 1 *(At)*	gi2317729	2	7.0	
20	Phoshoglycerate kinase *(At)*	gi21536853	6	16.2	
21	Pyruvate dehydrogenase *(At)*	gi3507745	5	15.7	
22	ATPase subunit RPT5a (proteasome) *(At)*	gi23197864	3	11.6	
23	ß-Tubulin *(Bn)*	gi8050828	4	13.0	
24	Thioredoxin (putative) *(At)*	gi12323392	2	3.1	
25	Protein disulfide isomerase (putative) *(At)*	gi17104689	14	25.9	4
26	Heat shock cognate protein HSC70 *(Bn)*	gi2655420	17	28.7	
27	Heat shock cognate protein HSC70 *(Bn)*	gi2655420	5	10.2	
28	Heat shock protein 81-2 *(At)*	gi22136254	9	11.9	
29	Heat shock protein 70 like *(At)*	gi7270774	6	13.5	
30	Chaperonin hsp60 (mitochondrial) *(At)*	gi9294668	5	12.5	
31	Phosphoglycerate mutase *(At)*	At3g08590	4	8.5	
32	Succinate dehydrogenase α-subunit *(At)*	gi21700795	3	4.3	
33	Valosin-containing protein *(Gm)*	gi1705678	3	4.0	

(continued)

Table 1 (continued)

No	Best-matching gene product (*Organism*)	ID	Matched	Coverage (%)	Spot no. in Joosen et al.
34	Expressed protein (*At)*	At5g48480	3	16.8	1
35	Proliferating cellular nuclear antigen (*Bn)*	gi15222379	5	36.7	2
36	14-3-3 protein (*Bn)*	gi13447112	5	20.8	3
37	Hap5a subunit (*At)*	At1g08970	4	16.4	5
38	Cpn60-α; RuBisCO subunit binding protein (*Bn)*	gi464727	8	18.7	6
39	P-ribosylformylglycinamidine synthase (*At)*	At1g74260	2	2.1	7
40	Thiazole biosynthetic enzyme (*At)*	At5g54770	3	9.7	8
41a	40S ribosomal protein (*Bn)*	gi15214300	3	13.7	10a
41b	Glutamine synthetase (*Bn)*	gi1084350	3	9.5	10b
42	H + -transporting two-sector ATPase (*At)*	At3g28710	3	12.8	11
43	Heat shock protein HSP90 *(At)*	At2g04030	2	3.5	12
44	Glutathione S-transferase (*At)*	gi15218518	2	13.5	13
45	Cpn21 protein (chloroplast) (*At)*	At5g20720	5	21.3	14
46	Dehydroascorbate reductase (*Bj)*	gi22653413	3	11.7	16
47	Peroxiredoxin (*Br)*	gi4928472	6	41.3	18
48	Expressed protein (*At)*	At5g02240	10	19.3	19
49	Phosphoglycerate kinase (*At)*	gi21536853	10	23.2	20
50	Chaperonin Cpn60-like (*At)*	gi9294610	13	13.8	21
51	Acyltransferase (*At)*	gi10176861	2	6.4	22
52	Dehydroascorbate reductase (*Br)*	gi33285914	4	33.3	23
53	Expressed protein (*At)*	At5g02240	6	6.3	24
54	Phosphoglycerate mutase *(At)*	At3g08590	3	6.3	27b
55	Glutathione-S- transferase (GST6; *At)*	At2g47730	3	26.0	28
56	Glyceraldehyde-3-P dehydrogenase (*At)*	At1g13440	10	27.2	30

The number (No.) corresponds to the protein spot indicated in Fig. 1. "Matched peptides" gives the number of peptide sequences identified by LC-MS/MS that showed homology to the best matching gene product. The spots numbers given in the last column refer to the spots identified previously by Joosen et al. (2007). *At = Arabidopsis thaliana*, *Bj = Brassica juncea, Bn = Brassica napus*, *Bo = Brassica oleracea*, *Br = Brassica rapa*, *Gm = Glycine max*

may control development through their role in regulating cell growth (Gapper and Dolan 2006). In plants, an intimate relationship appears to exist between oxidative stress and heat stress, as many ROS-scavenging enzymes were found to be elevated by heat shock (Mittler et al. 2004). More research is required to provide a better understanding of the possible role of molecular chaperones and anti-oxidative enzymes during the various stages of embryo development.

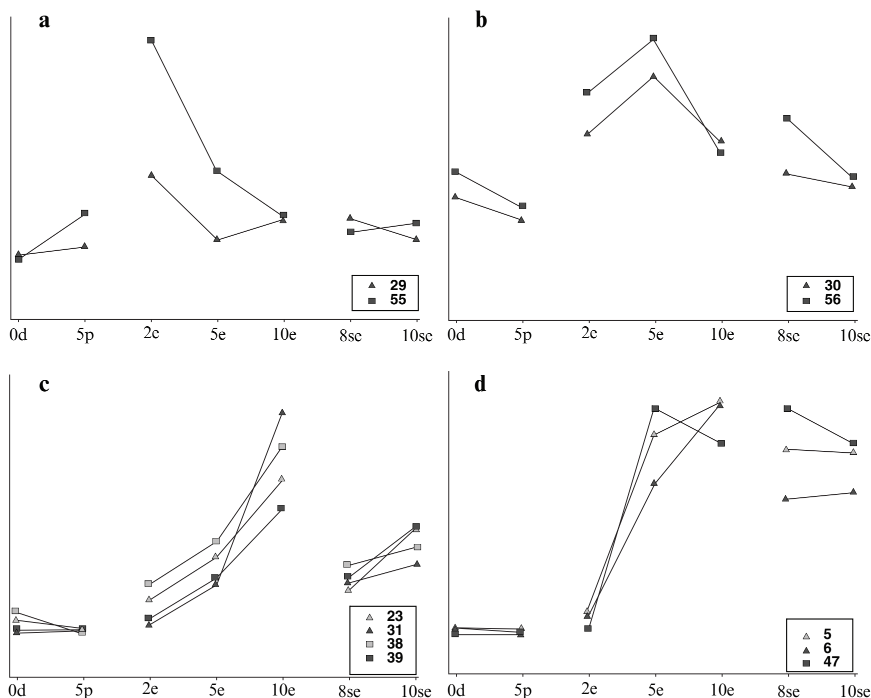


Fig. 2 Expression profiles of proteins that are differentially expressed in embryogenic and control, non-embryogenic microspore cultures. The numbers refer to the protein spots in Fig. 1 and the best-matching gene products of Table 1. Zero-day, starting microspore culture; 5p, 5-day pollen culture; 2e, 2-day embryo culture; 5e, 5-day embryo culture; 10e, 10-day embryo culture; 8se, 8-day suspensor-bearing culture; 10se, 10-day suspensor-bearing culture

Conclusions

Proteomics, like transcriptomics, is a powerful tool for studying complex developmental processes such as *in vitro* embryogenesis. While both techniques provide information about differential expression states, proteomics can provide additional information about the stability of the protein, post-translational modifications and sub-cellular localisation. The challenge now is to move proteomics into a more sensitive, high throughput realm so that large-scale data sets can be obtained from small amounts of starting material.

References

Boutilier K, Fiers M, Liu CM, van der Geest AHM (2005) Biochemical and molecular aspects of haploid embryogenesis. In: Palmer CE, Keller WA, Kasha KJ, (eds) Haploids in Crop Improvement II, Vol 56. Springer, Heidelberg, pp 73–95

Binarova P, Straatman K, Hause B, Hause G, Van Lammeren AAM (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. Theor Appl Genet 87:9–16

Binarova P, Hause G, Cenklova V, Cordewener JHG, Van Lookeren Campagne MM (1997) A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex Plant Reprod 10:200–208

Casson S, Spencer M, Walker K, Lindsey K (2005) Laser capture microdissection for the analysis of gene expression during embryogenesis of *Arabidopsis*. Plant J 42:111–123

Cordewener JHG, Busink R, Traas JA, Custers JBM, Dons JJM, Van Lookeren Campagne MM (1994) Induction of microspore embryogenesis in *Brassica napus* is accompanied by specific changes in protein synthesis. Planta 195:50–56

Cordewener JHG, Hause G, Görgen E, Busink R, Hause B, Dons JJM, Van Lammeren AAM, Van Lookeren Campagne MM, Pechan P (1995) Changes in synthesis and localization of the 70 kDa class of heat shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196:747–755

Cordewener JHG, Bergervoet J, Liu CM (2000) Changes in protein synthesis and phosphorylation during microspore embryogenesis in *Brassica napus*. J Plant Physiol 156:156–163

Custers JBM, Cordewener JHG, Nöllen Y, Dons JJM, Van Lookeren Campagne MM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep 13:267–271

Fiers M (2007) Peptide signaling in plant development: functional analysis of CLE ligands in *Arabidopsis*. PhD Thesis, Wageningen University, The Netherlands

Gapper C, Dolan L (2006) Control of plant development by reactive oxygen species. Plant Physiol 141:341–345

Hari V (1980) Effect of cell density changes and conditioned media on carrot cell embryogenesis. Z Pflanzenphysiol 96:227–231

Hause G, Cordewener JHG, Ehrmanova M, Hause B, Binarova P, Van Lookeren Campagne MM, Van Lammeren AAM (1995) Cell cycle dependent distribution of phosphorylated proteins in microspores and pollen of *Brassica napus* L. detected by the monoclonal antibody MPM-2. Protoplasma 187:117–126

Huang B, Bird S, Kemble R, Simmonds D, Keller W, Miki B (1990) Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. Plant Cell Rep 8:594–597

Joosen R, Cordewener J, Supena EDJ, Vorst O, Lammers M, Maliepaard C, Zeilmaker T, Miki B, America T, Custers J, Boutilier K (2007) Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol 144:155–172

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490–498

Pechan PM and Keller WA (1988) Identification of potentially embryogenic microspores in *Brassica napus*. Physiol Plant 74:377–384

Pechan PM, Bartels D, Brown DCW, Schell J (1991) Messenger-RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta 184:161–165

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiol Plant 127:519–534

Tsuwamoto R, Fukuoka H, Takahata Y (2007) Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. Planta 225:641–652
von Eggeling F, Melle C, Ernst G (2007) Microdissecting the proteome. Proteomics 7:2729–2737
Zhao J, Newcomb W, Simmonds D (2003) Heat-shock proteins 70 kDa and 19 kDa are not required for induction of embryogenesis of *Brassica napus* L. cv. Topas microspores. Plant Cell Physiol 44:1417–1421

Chapter 11
Programmed Cell Death and Microspore Embryogenesis

A.L. Varnier, C. Jacquard, and C. Clément

Abstract Programmed cell death (PCD) occurs in the anther during *in vivo* microspore/pollen development. The first hallmarks are detected in the tapetum at meiosis and consist in progressive degeneration of organelles such as mitochondria, as well as DNA degradation into multiple of 180 kpb fragments. This phenomenon radially extends in the anther sporophytic tissues and finally affects microspore/pollen at various stages of development depending upon species. PCD may infer with the process of microspore embryogenesis. Indeed, the inducing pre-treatment is performed using the whole anther during the process of microspore vacuolation. Thus the stress of pre-treatment may influence the triggering of PCD in both the anther tissues and the microspores and is likely involved in the competence of microspore to the process of microspore embryogenesis. These data are also discussed considering the regeneration of albino microspore derived plants in cereals.

Keywords Anther, microspore, plastids, pollen, pre-treatment, programmed cell death

Programmed cell death (PCD) is genetically controlled cell 'suicide'. PCD was first reported in animal cells and next progressively described in plant cells during the last decade. In plants PCD is triggered under two main circumstances: (i) in response to environmental variations resulting in biotic or abiotic stresses or (ii) during developmental processes when removing useless cells is required.

Following stress such as climatic change (Chen et al. 1999), toxic compounds (Yao et al. 2001), oxidative stress (Pennell and Lamb 1997) or pathogen attack (Hoeberichts et al. 2003), the plant kills a number of cells around the wounding

A.L. Varnier, C. Jacquard
Plant Stress Defence and Reproduction, University of Reims, P.O. Box 1039, 51687, Reims Cedex 2, France

C. Clément(✉)
Plant Stress Defence and Reproduction, University of Reims, P.O. Box 1039, 51687, Reims Cedex 2, France
E-mail: christophe-clement@univ-reims.fr

© Springer Science+Business Media B.V. 2009

area or pathogen penetration site, thus making a protection zone for inner tissues. During development PCD has been observed in several processes, including leaf shape modelling, disappearance of aleurone cells, shedding of root cap cells or differentiation of specialized cell types such as tracheary elements (Pennell and Lamb 1997; Kuriyama and Fukuda 2002).

Additionally, PCD is essential to ensure the achievement of sexual reproduction in angiosperms (Pennell and Lamb 1997; Wu and Cheung 2000). For example the process of self incompatibility is mediated through the triggering of PCD in the pollen tube by the female tissues (Rudd and Franklin-Tong 2003). In flowers PCD mainly consists of the deletion of cells or tissues with temporary functions, including both sterile and fertile organs (Buckner et al. 1998). In female organs, PCD occurs in restricted cells at precise stages in development. Apart from the 3 megaspores disappearing soon after meiosis (Buckner et al. 1998), PCD was mainly identified after fertilization in respect to embryo development, i.e. the disappearance of the suspensor (Pennell and Lamb 1997), endosperm (Young and Gallie 2000) or nucellus (Dominguez et al. 2001).

The situation is different in the male organs since most of the anther tissues are affected by PCD, which interact with the process of microspore embryogenesis. In this paper, we will first examine how PCD affects the anther tissues of angiosperms along pollen development and then gather the available information about the putative interference of this developmentally regulated PCD with microspore embryogenesis.

PCD in the Anther During Pollen Development

In the Sporophytic Tissues

All the anther diploid tissues are affected by a wave of PCD. The first hallmarks are detected in the tapetum when the sporogenous cells are at the pre-meiosis stage (Varnier et al. 2005). The occurrence of PCD is characterized by both the typical DNA degradation in 180 kbp fragments (or multiple) and by changes in the cytological traits of organelles, such as the invagination and the appearance of apoptotic like bodies from the nucleus or the alteration of mitochondria (Wang et al. 1999a; Varnier et al. 2005). Serine protease is a putative marker for PCD. It has been shown that its activity increases in the tapetum at precocious stages of pollen development being maximal at the tetrad stage. Afterwards, a progressive degeneration of the tapetum takes place (Varnier et al. 2005) until the final disappearance of the tapetal cell layer, more or less early during pollen development depending on the species.

The middle layers are the next to be affected by a degeneration process that corresponds to PCD. Progressively, the nucleus becomes invaginated and degenerative vacuoles develop within mitochondria. Soon after tapetum degradation, their cytoplasm disappears and middle layers only persist as cell walls (Varnier et al. 2005).

The endothecium has a special function in the anther. From meiosis up to the first pollen mitosis, the endothecium is mostly devoted to reserve accumulation and

mobilization. At the late vacuolated microspore stage, the metabolism of the endothecium turns to the development of cell wall thickenings that will represent the mechanical layer around the anther locule. During this latter process, all the cytological features of PCD can be detected in the cytoplasm, though the endothecium cells remain alive until anther dehiscence (Varnier et al. 2005). The expression of the PCD related *ZmMADS2* transcription factor is known to be related to PCD, and is strongly expressed in the endothecium at that time (Schreiber et al. 2004).

Apart from the synthesis of cell wall thickenings, the behaviour of the epidermis is the same as the endothecium regarding the timing of PCD development (Varnier et al. 2005).

Anther dehiscence is also a consequence of PCD. The process has been extensively studied in tobacco (Sanders et al. 2005). The first signs of cell hydrolysis in the dehiscence zone appear during late microspore vacuolation, in concomitance with PCD in the middle layers (Varnier et al. 2005). Again, all the cytological features coincide with the PCD process. The disappearance of the affected cells creates a fragile zone between adjacent anther locules that opens under the tension provided by the mechanical layer in the endothecium. It is of interest to note that male sterile mutants and those defective in anther dehiscence have defects in the PCD process (Sanders et al. 2005), indicating that PCD is required for normal pollen development, anther dehiscence and release of pollen grains at flower opening (Sanders et al. 2005).

The radial extension of PCD in the anther cell layers from the tapetum to the peripheral layers including the dehiscence zone suggests that a PCD hormonal signal is conveyed from the internal towards the peripheral cell layers (Kuriyama and Fukuda 2002). Both jasmonic acid and ethylene may be good candidates for the spread of PCD in the anther. Using mutants of *Arabidopsis thaliana* mutants a role for jasmonic acid signaling in controlling the time of anther dehiscence was revealed (Ishiguro et al. 2001). *Arabidopsis* mutants that are defective in either jasmonic acid biosynthesis (e.g., dde1, dde2, da1) or perception (e.g., coi1) are male sterile and have anthers that dehisce too late for successful pollination to occur (Ishiguro et al. 2001; Sanders et al. 2005). Other phytohormones, such as ethylene have also been shown to play a role in anther dehiscence. In *Nicotiana tabaccum* ethylene is involved in the final process of anther dehiscence and could act in synergy or in concurrence with jasmonic acid (Rieu et al. 2003).

When the pollen is released, all the remaining anther sporophytic tissues disappear rapidly. Indicating that during the whole of its development from microspore to pollen these gametic tissues are surrounded by cells that undergo PCD.

Gametophytic Tissues

There is only poor evidence that microspores or pollen grain are affected by PCD during development. In the pollen grain *in fine*, only the sperm cells have a long term fate through fertilization and zygotic embryogenesis. In the opposite, the vegetative cell, which fate is to build the pollen tube, die soon at fertilization.

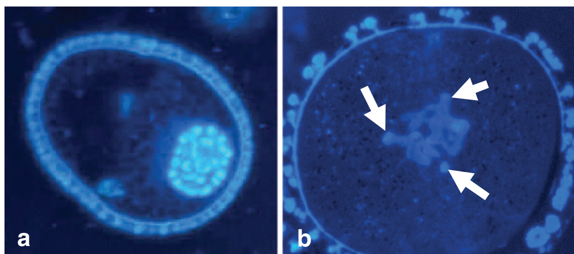


Fig. 1 Nucleus feature in a young microspore (**a**) and in the vegetative cell (**b**) of a pollen grain in *Lilium*. Note the invaginations of the nucleus in the vegetative cell and the formations of apoptotic-like bodies (arrows). Hoechst 33342 staining for DNA.

Microspores are temporarily affected by a wave of PCD-like traits during the early steps of vacuolation after meiosis. In barley microspores exhibit a positive reaction to the PCD specific tunnel staining (Wang et al. 1999a). At the same moment both endonuclease gene expression and activity are stimulated (Zaina et al. 2003), indicating that the genome undergoes some rearrangements. Most likely these features do not reflect PCD but rather chromatin re-modelling, following the transition from the diploid to the haploid state. This point is a key step for the re-programming of transcription associated with development and cell differentiation (Farrona et al. 2004). This is of importance for microspore embryogenesis because the microspore corresponds to the stage of sampling.

In the pollen grains after mitosis, the vegetative cell exhibits clear signs of PCD (Fig. 1). During pollen maturation its nucleus progressively invaginates, leading to the appearance of some nuclear bodies that separate from the nucleus (Varnier et al. 2005). Also, mitochondria are progressively hydrolysed as revealed by the loss of their DNA (Sato et al. 2004) and the appearance of internal degenerative vacuoles (Varnier et al. 2005). In the meantime, the presence of DNA laddering in pollen is an additional argument to conclude that PCD affects the vegetative cell during pollen maturation (Varnier et al. 2005). In the end, sperms are the sole cells in the pollen grains and in the whole anther that are not affected by PCD.

Later on, during pollen tube elongation, the expression of the *MADS box* gene indicates that PCD is still active in the pollen tube (Heuer et al. 2000), which is consistent with the fate of pollen tube elements.

Inference of PCD with Microspore Embryogenesis

At the Time of Sampling

In crops, the most suitable stage of sampling for microspore embryogenesis is the uni-nucleate microspore. As explained above, at that time the microspore remodels

its chromatin which may be wrongly understood as PCD. After pollen mitosis, the vegetative cell of the pollen grain undergoes PCD, which may explain why it is quite impossible to get microspore embryogenesis from pollen after mitosis.

However, one should keep in mind the fact that the microspores are surrounded by the tapetum which is fully affected by PCD at the same time. As a consequence, some information could transit from the tapetum through the locular fluid that may influence the microspore towards a PCD process. For example, plastids begin to degrade in the young microspores in some barley cultivars (Caredda et al. 2000), showing that organelle degeneration is initiated as early as the young microspore stage. This infers that the collected microspores for doubled haploid production are already affected by the PCD process at the time of sampling. The remaining question is then whether PCD can be reversed or not, which is indispensable for the microspore to behave like a zygote.

PCD and the Reorientation of the Microspore Following the Pre-Treatment

In the last few years, the transcriptome of microspore during anther pretreatment (Muñoz-Amatriaín et al. 2006) and the first steps of embryogenesis (Maraschin et al. 2006) have been analysed in details. These studies are the source of considerable information about the changes of microspore physiology related to the triggering of microspore embryogenesis.

The pretreatment is a key step in which process of microspore embryogenesis. It constitutes a stress on the microspores arrests their gametophytic development programme (Maraschin et al. 2006) and allows reorientation towards a sporophytic embryo programme. In barley anthers, up to 4,300 genes have their expression modified following the mannitol pre-treatment (Muñoz-Amatriaín et al. 2006). The stress pretreatment accelerates the course of PCD process in the sporophytic tissues of the anther but not in the microspores (Wang et al. 1999a, b).

In the Reoriented Microspores and Microspore Derived Multi-Cellular Structures

The competent reoriented microspores may develop into haploid embryos. Within these microspores, the induction of embryogenesis by stress is marked by the up regulation of transcripts involved in several metabolic pathways including the inhibition of PCD, especially the bax inhibitor *Bi1* gene (Maraschin et al. 2006). Such a regulation is specific of embryogenic microspores and does not occur in naturally developing pollen grains. This confirms that PCD is induced in developing pollen grains and indicates that the arrest of the PCD process is necessary to the embryogenic development of the microspore.

After pre-treatment, the reoriented microspores spend a few days before entering the first symmetric division whatever they are isolated or cultivated in the anther. In rapeseed, both transcriptome and proteome were recently analysed in detail and no sign of gene expression nor proteins related to PCD were identified in the embryogenic microspores and derived structures (Joosen et al. 2007; Malik et al. 2007). This may indicate that the PCD programme initiated during microspore/pollen development under natural conditions is arrested in the reoriented microspores that are capable of developing into an embryo. Nevertheless, obtaining an embryo from a microspore derived multi-cellular structure requires further cell death.

In barley, by combining viability studies with cell tracking, it was shown that release of embryo-like structures is preceded by a decrease of viability and next death of the cells positioned at the site of exine wall rupture. These results indicate that a position-determined cell death process marks the transition from a multicellular structure into an embryo-like structure (Maraschin et al. 2005). However, it is not yet determined whether this type of cell death is programmed or not.

PCD and Albinism in Cereals

One specific trait of microspore embryogenesis in cereals is the appearance of albino haploid plants that cannot be used for further selection. In albino plants plastids are devoid of internal membrane and chlorophyll (Caredda et al. 2000). This phenomenon may be linked to the precocious occurrence of PCD in the microspores. Indeed, plastids undergo degeneration during the whole of pollen development (Mogensen 1996). In genotypes that produce nothing but albino plants, plastids in the microspore lack internal membranes and are poor in DNA when compared to plastids in microspores of cultivars producing green plants (Caredda et al. 2000). It was then suggested that the triggering of PCD in microspores occurs more or less early depending on the cultivars and affects plastids as first targets. It further means that the pretreatment contributes to arrest PCD traits that are governed by the nucleus but not those affecting plastids.

Conclusion

PCD interferes with microspore embryogenesis because (i) it naturally occurs in all the tissues of the organ surrounding the microspores and (ii), microspores/pollen grains are also the site of PCD more or less early during development. PCD must then be overcome to allow the microspore to develop into an embryo. PCD may be an obstacle to microspore embryogenesis when the pretreatment does not arrest this programme in the microspore. PCD related genes specifically expressed in embryogenic microspores such as bax inhibitor, *Bi1* thus represent reliable ‘bio-markers’ (Maraschin et al. 2006) of the microspore competency to embryogenesis.

References

Buckner B, Janick-Buckner D, Gray J, Johal GS (1998) Cell-death mechanisms in maize. Trends Plant Sci 3:218–223

Caredda S, Doncoeur C, Devaux P, Sangwan RS, Clément C (2000) Plastid differentiation during androgenesis in albino and non-albino producing cultivars of barley (*Hordeum vulgare* L.). Sex Plant Reprod 13:95–104

Chen H, Yan C, Jiang X, Dai YR (1999) Hyperthermia-induced apoptosis and the inhibition of DNA laddering by zinc supplementation and withdrawal of calcium and magnesium in suspension culture of tobacco cells. Cell Mol Life Sci 55:303–309

Dominguez F, Moreno J, Cejudo FJ (2001) The nucellus degenerates by a process of programmed cell death during the early stages of wheat grain development. Planta 213:352–360

Farrona S, Hurtado L, Bowman JL, Reyes JC (2004) The *Arabidopsis thaliana* SNF2 homolog AtBRM controls shoot development and flowering. Development 131:4965–4975

Heuer S, Lörz H, Dresselhaus T (2000) The MADS box gene *ZmMADS2* is specifically expressed in maize pollen and during maize pollen tube growth. Sex Plant Reprod 13:21–27

Hoeberichts FA, Ten Have A, Woltering EJ (2003) A tomato metacaspase gene is upregulated during programmed cell death in *Botrytis cinerea*-infected leaves. Planta 217:517–522

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The *DEFECTIVE IN ANTHER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell 13:2191–2209

Jones A (2000) Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? Trends Plant Sci 5:225–230

Joosen R, Cordewener E, Supena EDJ, Vorst O, Lammers M, Maliepaard C, Zeilmaker T, Miki B, America T, Custers J, Boutilier K (2007) Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol 144:155–172

Kuriyama H, Fukuda H (2002) Developmental programmed cell death in plants. Curr Opin Plant Biol 5:568–573

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol 144:134–154

Maraschin SF, Vennik M, Lamers GEM, Herman P, Spaink AHP, Wang M (2005) Time-lapse tracking of barley androgenesis reveals position-determined cell death within pro-embryos. Planta 220:531–540

Maraschin SF, Caspers M, Potokina E, Wülfert F, Graner A, Spaink HP,Wang M (2006) cDNA array analysis of stress-induced gene expression in barley androgenesis. Physiol Plant 127:535–550

Mogensen HL (1996) The hows and whys of cytoplasmic inheritance in seed plants. Am J Bot 83:383–404

Muñoz-Amatriaín M, Svensson JT, Castillo AM, Cistué L, Close TJ, Valles MP (2006) Transcriptome analysis of barley anthers: effect of mannitol treatment on microspore embryogenesis. Physiol Plant 127:551–560

Pennell R, Lamb C (1997) Programmed cell death in plants. Plant Cell 9:1157–1168

Rieu I, Wolters-Arts M, Derksen J, Mariani C, Weterings K (2003) Ethylene regulates the timing of anther dehiscence in tobacco. Planta 217:131–137

Rudd JJ, Franklin-Tong VE (2003) Signals and targets of the self-incompatibility response in pollen of *Papaver rhoeas*. J Exp Bot 54:141–148

Sanders PM, Bui AQ, Le BH, Goldberg RB (2005) Differentiation and degeneration of cells that play a major role in tobacco anther dehiscence. Sex Plant Reprod 7:219–241

Sato H, Saito C, Handa H (2004) Mitochondrial DNA decreases during pollen development in rapeseed (*Brassica napus* L.), but mitochondrial linear plasmid encoded RNA polymerase persists in mature pollen. Protoplasma 224:79–185

Schreiber, DN, Bantin, J, Dresselhaus, T (2004) The MADS box transcription factor ZmMADS2 is required for anther and pollen maturation in maize and accumulates in apoptotic bodies during anther dehiscence. Plant Physiol 134:1069–1079

Varnier AL, Mazeyrat-Gourbeyre F, Sangwan RS, Clément C (2005) Programmed cell death progressively models the development of anther sporophytic tissues from the tapetum and is triggered in the pollen grain during maturation. J Struct Biol 152:118–228

Wang M, Hoekstra S, van Bergen S, Lamers GEM, Oppedijk BJ, van der Heijden MW, de Priester W, Schilperoort RA (1999a) Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. Plant Mol Biol 39:489–501

Wang M, van Bergen S, Lamers GEM, Oppedjik BJ, Schilperroort RA (1999b) Programmed cell death during androgenesis in *Hordeum vulgare* L. In: Clément C, Pacini E, Audran JC (eds) Anther and pollen: from biology to biotechnology. Springer Berlin/Heidelberg/Tokyo, pp 201–209

Wu HM, Cheung AY (2000) Programmed cell death in plant reproduction. Development 44:267–281

Yao N, Tada Y, Park P, Nakayashiki H, Tosa Y, Mayama S (2001) Novel evidence for apoptotoic cell response and differential signals in chromatin condensation and DNA cleavage in victorin-treated oats. Plant J 28:13–26

Young TE, Gallie DR (2000) Regulation of programmed cell death in maize endosperm by abscissic acid. Plant Mol Biol 42:397–414

Zaina G, Morassutti C, De Amicis F, Fogher C, Marchetti S (2003) Endonuclease genes up-regulated in tissues undergoing programmed cell death are expressed during male gametogenesis in barley. Gene 315:43–50

Chapter 12
Albinism in Microspore Culture

A.M. Torp and S.B. Andersen

Abstract Frequent formation of albino plants from *in vitro* cultured microspores is a particular problem for chromosome doubled haploid production in cereals and grasses. The phenomenon was first thought to be associated with maternal inheritance of plastids visualized by large deletions and rearrangements of plastid genomes in albino plants. Subsequently interests have changed to inactivation of plastid ribosomes, which has been shown to create albino phenotypes *in vitro*. A considerable knowledge on genetic regulation of the trait is used in this chapter to forward a hypothesis that the stressful *in vitro* conditions in these cultures make the plants fight their own plastids with antibiotic like compounds.

Keywords Albinism, plastid deficiency, haploids

Introduction

The discovery of the ability of plant microspores to develop directly into haploid plants *in vitro* (Guha and Maheshwari 1964; Nitsch and Nitsch 1969) spurred a great amount of optimism and experimental enthusiasm. The general application of the technique, however, met obstacles due to low *in vitro* response in most species and for some of the most important cereals, in addition, many completely white albino plants were regenerated (De Buyser and Henry 1979; Wang et al. 1974).

High frequency of albino plant formation in anther and microspore cultures is a general phenomenon in most cereals like wheat (Andersen et al. 1987), barley (Knudsen et al. 1989), rice (Guiderdoni et al. 1992), rye (Immonen 1999), oat

A.M. Torp
University of Copenhagen, Faculty of Life Sciences, Department of Agricultural Sciences, Plant and Soil Sciences, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

S.B. Andersen(✉)
University of Copenhagen, Faculty of Life Sciences, Department of Agricultural Sciences, Plant and Soil Sciences, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark
E-mail: sba@kvl.dk

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

(Kiviharju and Pehu 1998) and in several grasses like ryegrass (Olesen et al. 1988) and timothe (Abdullah et al. 1994). These white plants contain plastids arrested in development towards functional chloroplasts (Caredda et al. 1999, 2000; Sun et al. 1974) and therefore cannot grow without the sugar containing media of *in vitro* cultures (Sun et al. 1974).

An early hypothesis connected such albino plant formation with the maternal inheritance of plastids observed for most angiosperm plant species (Vaughn et al. 1980). Such ideas had strong support when it was demonstrated that albino plants from microspores of several different cereal species had deletions and reorganizations in their plastid (ptDNA) genomes (Day and Ellis 1984; 1985; Harada et al. 1991; Hofinger et al. 2001). Targeted inactivation of selected plastid genes of tobacco i.e. *rpo* genes encoding the plastid RNA polymerase, has been shown to generate albino phenotypes in tobacco (Allison et al. 1996; De Santis-Maciossek et al. 1999; Serino and Maliga 1998). Thus it is now well documented that such deletions of parts of the plastid genome can generate the albino phenotype *in vitro* at least in some plant species though the relationship with maternal inheritance has not been documented.

However, not all androgenic albino plants carry deletions in their ptDNA genome. Albino plants have been found which show restriction patterns indistinguishable from those of intact ptDNA genomes in wheat (Day and Ellis 1984; Hofinger et al. 2001) barley (Dunford and Walden 1991) and rice (Harada et al. 1991). Furthermore, Hofinger et al. (2001) observed transcription from all plastid DNA regions in these albino plants. Thus doubt has been raised as to what extend plastid genome deletions and rearrangments can indeed explain the high frequency of albino plant regenerants in many cereal microspore cultures (Hofinger et al. 2001).

Similar alterations in transcript pattern and translation levels have been found in plastids of albino plants with and without ptDNA deletions. Transcript levels of plastid encoded genes for photosynthetic proteins and ribosomal RNA were generally heavily reduced in albino plants relative to green plants (Ankele et al. 2005; Dunford and Walden 1991; Hofinger et al. 2001). In contrast, transcript levels of plastid encoded house-keeping genes were largely unaffected (Hofinger et al. 2001) or significantly elevated in such albino plants (Ankele et al. 2005). In addition, Sun et al. (1979) and Hofinger et al. (2001) found one of more plastid encoded photosynthetic or ribosomal proteins to be absent or strongly reduced in rice and wheat microspore derived albino plants. These results have led to the suggestion that the androgenic albino plants lack functional plastid ribosomes as this would explain both the translation deficiencies and the modified transcript pattern (Hofinger et al. 2001).

Deficiency of plastid ribosomes in cereals are non-lethal under *in vitro* conditions and can be caused by different factors. Recessive mutations in nuclear encoded genes leading to plastid ribosome deficiency in the affected tissue is known from both the barley mutant *albostrians* (Knoth and Hagemann 1977) and the maize mutants *iojap* (Shumway and Weier 1967; Thompson et al. 1983) and *ppr2* (Williams and Barkan 2003). *Ppr2* is thought to be involved in synthesis or assembly of one or more components of the plastid translation machinery (Williams and Barkan 2003), while the defect leading to loss of ribosomes in *iojap* and *albostrians* are unknown at present. It has been demonstrated that temperature stress can

inactivate plastid ribosomes in rye, wheat, barley and oat (Feierabend and Mikus 1977; Feierabend and Schrader-Reichhardt 1976). Finally treatment of seeds of brassica species with the antibiotic spectinomycin (Zubko and Day 1998) or seeds of barley and maize with spectromycin (Zubko and Day 2002) followed by propagation of bleached seedlings in the absence of antibiotics results in stable albino plants lacking plastid ribosomes. Thus it is now well documented that various physical stresses and in particular treatment with aminoglycoside antibiotics can inactivate the ribosomes in a major part of the cells of ordinary seed derived embryos of several cereals (Zubko and Day 2002). Upon further growth and cell division, cells are formed with only ribosome deficient plastids, which cannot recover because translation of genes for central functions like the plastid encoded RNA polymerase is prevented.

While the hypothesis of ribosome deficient plastids seems to be the best explanation for the albino phenotype of many plants regenerated from cereal microspore cultures it does not clearly indicate the reason for the frequent formation of such plants particularly in the cereal species. Some further hints about the nature of the phenomenon may be obtained from genetics of the tendency to form green or albino plantlets. Throughout the history of cereal anther and microspore culture, differences between plant lines in their ability to produce high frequencies of green versus albino plants in the cultures have been noted (Andersen et al. 1987; Holme et al. 1999; Knudsen et al. 1989). Such genotype differences are, however, not qualitative in nature because of both sampling error with limited plants regenerated per line in most experiments as well as considerable unknown environmental effects on the donor plants. These genetic differences between plant cultivars may at first be expected to be inherited by genes in the plastids, but studies of green plant formation from crosses in wheat and barley (Larsen et al. 1991; Tuvesson et al. 1989; Zhou and Konzak 1992) have mostly shown no reciprocal effects, indicating that the trait is controlled by chromosomally inherited genes.

A large number of quantitative trait locus (QTL) studies of the ability to form high frequencies of green plants from anther culture, particularly from wheat, barley and rice have identified many different genes affecting the trait. However, there are indications that in general genes on wheat homoeologous group 2 and 5 chromosomes may play a role. Torp et al. (2001) identified three QTLs for green plant percentage on wheat chromosomes 2AL, 2BL and 5BL in the Ciano × Walter doubled haploid (DH) mapping population as well as one QTL on chromosome 5BL in a second DH population Ciano × Benoist. The QTL on chromosome 2BL, which was the most important, were subsequently confirmed in a new F_2 population developed from two DH lines of the original mapping population (Torp et al. 2004). In Barley, Chen et al. (2007) identified QTLs for percentage and number of green plants on chromosome 5HL, while QTLs for number of albino and green plants respectively were identified on chromosome 2H and 6H. In rice, He et al. (1998) identified major QTLs for green and/or albino plant differentiation frequency on rice chromosomes 1 and 9, homoeologous to wheat group 3 and 5 chromosomes, respectively. Thus in spite of the relative complexity of genes affecting the trait there are indications of major genes and common mechanisms of action across the cereal species.

Genes affecting the green plant regeneration frequency from microspores may be expected to be selected for during the *in vitro* culture. This would mean that green chromosome doubled haploids derived from F_1 hybrids by such cultures should have an increased frequency of the green plant promoting allele. However, several studies of segregation of such genes using linked markers have indicated the existence of both genes where the green type allele is selected during the culture as well as genetic loci where green and albino type alleles are equally represented among offspring from the microspores (Chen et al. 2007; He et al. 1998; Manninen 2000; Torp et al. 2001; 2004). These results may indicate different modes of action of the genes affecting green plant formation. Genes selected during the culture probably act locally in each haploid structure in a gametophytic mode so that haploid structures with the green promoting allele have a higher chance of regenerating green plants than structures with the albino promoting allele. Mechanism of action of such genes could be mediated through large gene products or structural differences in ribosomes or else where, which will not be exchanged between cells. In contrast genes not selected during the microspore culture may exert their effects through small molecules which can diffuse and be exchanged between cells, so that haploid structures with the green type allele do not have a higher chance of forming green plantlets than structures with the albino promoting allele.

We try to link the hypothesis of ribosome deficient plastids with the genetic information about genes affecting green plant formation frequencies. This is done through the study of the major gene affecting the trait on wheat chromosome 2BL. There is no or little selection between the alleles of this gene in anther cultures from F_1 hybrids between high and low responding parents (Torp et al. 2001, 2004) indicating action through a low molecular weight compound. Furthermore the green plant promoting allele is highly recessive relative to the allele promoting albino plant regeneration. This dominance situation indicates a lack of function mutation in lines producing many green plants, which is further supported by the fact that such high responsive lines are generally rare.

A simple way to combine the genetics with the hypothesis of ribosome deficient plastids would be to assume that ordinary wheat lines with low frequency of green plant formation produce low molecular weight ribosome inactivating compounds before or during the *in vitro* culture, which will generally inactivate most plastid ribosomes. High responding lines then will have mutations in one or more genes in the synthetic pathway of such compounds so that many plastids escape with intact ribosomes. Plants are known to produce a multitude of such chemical compounds some of which are involved in defence against pathogens (D'Auria and Gershenzon 2005). Since some of these compounds are likely to play more general roles in plants in addition to their role in defence, it could be hypothesized that they may also be induced in microspores or in the anther wall upon stress treatment. Both barley and wheat are capable of producing highly potent antibiotic like compounds including hordatines (Burhenne et al. 2003; Jin et al. 2003; Stoessl 1967), which can inactivate ribosomes like streptomycin (Venis 1969). During the stressful *in vitro* culture these plants may simply be fighting their own plastids.

References

Abdullah AA, Pedersen S, Andersen SB (1994) Triploid and hexaploid regenerants from hexaploid timothy (*Phleum pratense* L.) via anther culture. Plant Breed 112: 342–345

Allison LA, Simon LD, Maliga P (1996) Deletion of rpoB reveals a second distinct transcription system in plastids of higher plants. EMBO J 15: 2802–2809

Andersen SB, Due IK, Olesen A (1987) The response of anther culture in a genetically wide material of winter wheat (*Triticum aestivum* L.). Plant Breed 99: 181–186

Ankele E, Heberle-Bors E, Pfosser MF, Hofinger BJ (2005) Searching for mechanisms leading to albino plant formation in cereals. Acta Physiol Plant 27 (4B): 651–664

Burhenne K, Kristensen BK, Rasmussen SK (2003) A new class of N-hydroxycinnamoyltransferases: purification, cloning and expression of a barley agmatine coumaroyltransferase (EC 2.3.1.64). J Biol Chem 278: 13919–13927

Caredda S, Devaux P, Sangwan RS, Clément C (1999) Differential development of plastids during microspore embryogenesis in barley. Protoplasma 208: 248–256

Caredda S, Doncoeur C, Devaux P, Sangwan RS, Clément C (2000) Plastid differentiation during androgenesis in albino and non-albino producing cultivars of barley (*Hordeum vulgare* L.). Sex Plant Reprod 13: 95–104

Chen X-W, Cistué L, Muñoz-Amatriaín M, Sanz M, Romagosa I, Castillo A-M, Vallés M-P (2007) Genetic markers for doubled haploid response in barley. Euphytica 158: 287–294

D'Auria JC, Gershenzon J (2005) The secondary metabolism of *Arabidopsis thaliana*: growing like a weed. Curr Opin Plant Biol 8: 308–316

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen. Possible basis for maternal inheritance of chloroplasts. Cell 39: 359–368

Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9: 671–678

De Buyser J, Henry Y (1979) Androgenèse sur des blés tendres en cours de sélection. I. L'obtension des plantes in vitro. Z Pflanzenzüchtung 83: 49–56

De Santis-Maciossek G, Kofer W, Bock A, Schoch S, Maier RM, Wanner G, Rüdiger W, Koop H-U, Herrmann RG (1999) Targeted disruption of the plastid RNA polymerase genes *rpoA*, *B* and *C1*: molecular biology, biochemistry and ultrastructure. Plant J 18: 477–489

Dunford R, Walden RM (1991) Plastid genome structure and plastid-related transcript levels in albino barley plants derived from anther culture. Curr Genet 20: 339–347

Feierabend J, Mikus M (1977) Occurrence of a high temperature sensitivity of chloroplast ribosome formation in several higher plants. Plant Physiol 59: 863–867

Feierabend J, Schrader-Reichhardt U (1976) Biochemical differentiation of plastids and other organelles in rye leaves with a high-temperature-induced deficiency of plastid ribosomes. Planta 129: 133–145

Guha S, Maheshwari SC (1964) In vitro production of embryos from anthers of *Datura*. Nature 204: 497

Guiderdoni E, Galinato E, Luistro J, Vergara G (1992) Anther culture of tropical Japonica × Indica hybrids of rice (Oryza-Sativa L). Euphytica 62: 219–224

Harada T, Sato T, Asaka D, Matsukawa I (1991) Large-scale deletions of rice plastid DNA in anther culture. Theor Appl Genet 81: 157–161

He P, Shen L, Lu C, Chen Y, Zhu L (1998) Analysis of quantitative trait loci which contribute to anther culturability in rice (*Oryza sativa* L.). Mol Breed 4: 165–172

Hofinger BJ, Ankele E, Gülly Ch, Heberle-Bors E, Pfosser MF (2001) The involvement of the plastid genome in albino plant regeneration from microspores in wheat. In: Bohanec B (Ed.), Biotechnological approaches for utilisation of gametic cells. COST 824 final meeting, Bled, Slovenia, 1–5 July 2000, pp. 215–228

Holme IB, Olesen A, Hansen NJP, Andersen SB (1999) Anther and isolated microspore culture response of wheat lines from northwestern and eastern Europe. Plant Breed 118: 111–117

Immonen S (1999) Androgenetic green plants from wither rye, Secale cereale L., of diverse origin. Plant Breed 118: 319–322

Jin S, Yoshida M, Nakajima T, Murai A (2003) Accumulation of hydroxycinnamic acid amides in winter wheat under snow. Bioscience Biotechnol and Biochem 67: 1245–1249

Kiviharju E, Pehu E (1998) The effect of cold and heat pretreatments on anther culture response of Avena sativa and A-sterilis. Plant Cell Tiss Org 54: 97–104

Knoth R, Hagemann R (1977) Struktur und Funktion der genetishen Information in den Plastiden. Biol Zbl 96: 141–150

Knudsen S, Due IK, Andersen SB (1989) Components of response in barley anther culture. Plant Breed 103: 241–246

Larsen ET, Tuvesson IKD, Andersen SB (1991) Nuclear genes affecting percentage of green plants in barley (*Hordeum vulgare* L.) anther culture. Theor Appl Genet 82: 417–420

Manninen OM (2000) Associations between anther-culture response and molecular markers on chromosomes 2H, 3H and 4H of barley (*Hordeum vulgare* L.). Theor Appl Genet 100: 57–62

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163: 85

Olesen A, Andersen SB, Due IK (1988) Anther culture response I perennial ryegrass (*Lolium perenne* L.). Plant Breed 101: 60–65

Serino G, Maliga P (1998) RNA polymerase subunits encoded by the plastid *rpo* genes are not shared with nucleus-encoded plastid enzymes. Plant Physiol 117: 1165–1170

Shumway LK, Weier TE (1967) The chloroplast structure of iojap maize. Am J Bot 54: 773–780

Stoessl A (1967) The antifungal factors in barley. IV Isolation, structure and synthesis of hordatines. Can J Chem 45: 1745–1760

Sun CS, Wang CC, Chu CC (1974) The ultrastructure of plastids in the albino pollen-plants of rice. Sci Sinica 17: 793–802

Sun CS, Wu SC, Wang CC, Chu CC (1979) The deficiency of soluble proteins and plastid ribosomal RNA in the albino pollen plantlets of rice. Theor Appl Genet 55: 193–197

Thompson D, Walbot V, Coe EH (1983) Plastid development in iojap- and chloroplast mutator-affected maize plants. Am J Bot 70: 940–950

Torp AM, Hansen AL, Andersen SB (2001) Chromosomal regions associated with green plant regeneration in wheat (*Triticum aestivum* L.) anther culture. Euphytica 119: 377–387

Torp AM, Bekesiova I, Holme IB, Hansen AL, Andersen SB (2004) Genetics related to doubled haploid induction *in vitro*. In: Mujib A, Cho M, Predieri S, Banerjee S (eds.), In vitro applications in crop improvement, Science Publishers Inc. Enfield (NH), USA, ISBN 1-57808-300-1, pp. 35–52

Tuvesson IKD, Pedersen S, Andersen SB (1989) Nuclear genes affecting albinism in wheat (*Triticum aestivum* L.) anther culture. Theor Appl Genet 78: 879–883

Vaughn KC, DeBonte LR, Wilson KG (1980) Organelle alteration as a mechanism for maternal inheritance. Science 208: 196–198

Venis MA (1969) Similarities in action between streptomycin and hordatine, an antifungal factor in barley. Phytochemistry 8: 1193–1197

Wang CC, Sun CS, Chu ZC (1974) On the conditions for the inductin of rice pollen plantlets and certain factors affecting the frequency of induction. Acta Bot Sin 16: 43–54

Williams PM, Barkan A (2003) A chloroplast-localized PPR protein required for plastid ribosome accumulation. Plant J 36: 675–686

Zhou HP, Konzak CF (1992) Genetic control of green plant regeneration from anther culture of wheat. Genome 35: 957–961

Zubko MK, Day A (1998) Stable albinism induced without mutagenesis: a model for ribosome-free plastid inheritance. Plant J 15: 265–271

Zubko MK, Day A (2002) Differential regulation of genes transcribed by nucleus-encoded plastid RNA polymerase, and DNA amplification, within ribosome-deficient plastids in stable phenocopies of cereal albino mutants. Mol Genet Genomics 267: 27–37

Chapter 13
Doubled Haploids in Breeding Winter Oilseed Rape

C. Möllers and M.C.M. Iqbal

Abstract The doubled haploid (DH) technique is routinely applied in winter rapeseed breeding programmes for the generation of completely homozygous lines. Although the method is quite old and perfected, there are still some problems that prevent a universal application of the technique in winter rapeseed breeding. Key problems remain the insufficient diploidisation rate, the low seed yield of primary haploids and the time-consuming and inadequate plantlet regeneration from the embryos. This contribution summarizes current applications and approaches to tackle above problems. Methods of early *in vitro* selection of microspore derived embryo genotypes are presented that appear promising for a further optimisation of the DH technology in rapeseed.

Keywords *Brassica napus,* microspore derived embryos, *in vitro* selection, hybrid breeding, diploidisation, marker assisted selection, canola

Introduction

It is generally accepted that the application of the doubled haploid (DH) technology to crop breeding has several advantages compared to conventional methods of producing homozygous inbred lines (Forster and Thomas 2005). Speeding-up of the breeding process is usually considered as the biggest benefit. Especially, increased selection efficiency can be achieved in early generations, which results from the greater proportion of additive genetic variation available for selection for quantitative traits due to homozygosity. Consequently, better discrimination

C. Möllers(✉)
Department of Crop Sciences, Plant Breeding, Georg-August-Universität,
Von-Siebold-Str. 8, 37075 Göttingen, Germany
E-mail: cmoelle2@gwdg.de

M.C.M. Iqbal
Institute of Fundamental Studies, Hantane Road,
Kandy, Sri Lanka

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Table 1 Minimum population size of an F_2 and a DH for the availability of a specific homozygous genotype in the case of unlinked loci ($\alpha = 0.95$; adapted from Jansen 1992)

No. of loci	F_2 segregation	F_2 plants (n)	DH segregation	DH plants (n)
1	1:3	11	1:1	5
2	1:15	47	1:3	11
3	1:63	191	1:7	23
5	1:1023	3067	1:31	95
7	1:16383	49081	1:127	383

between genotypes within crosses, better discrimination between crosses, and greater selection response across generations is possible (Snape 1997). Because of the reduced number of alternative genotypes for single and multiple alleles, smaller DH population sizes are needed compared to an F_2 population. Table 1 shows the minimum size of an F_2 and a DH population for the availability of a specific homozygous genotype in the case of unlinked loci (adapted from Jansen 1992). Clearly, DH technology requires considerably fewer genotypes compared to the F_2 when a large number of genes are involved in the inheritance of a trait. Nevertheless, a large number of DH lines need to be obtained from a cross segregating for a quantitative trait in order to obtain, with a high probability, the desired recombinant genotypes. Early simulation studies have shown that DH technology should be superior when selecting for quantitative traits with low heritability (Walsh 1974; Riggs and Snape 1977).

Theoretically, the use of DH lines should be less efficient in crosses where one of the parents is not adapted or lacks required traits, such as double low – canola – quality in rapeseed. In such materials, pedigree selection in early segregating generations is relatively easy, whereas an unselected DH population would contain a large proportion of unacceptable lines. Following a first yield testing, a large fraction of the material would be rejected, due to poor field performance. If, on the contrary, crosses among relatively narrow elite material are performed, visual selection in early generations of a pedigree programme is difficult and DH lines have the advantage that the genetic variance among them is higher than in any other generation.

There is still some debate on the question which generation should be used for the production of DH lines. In most cases F_1 derived DH lines are used to get a maximum gain in time, but DH production from F_2 or F_3 plants will allow for more recombination and for some pre-selection of the material (Snape 1997). The availability of only a single meiosis when applying the DH technology is a clear disadvantage, if undesirable linkages exist between traits under selection (Snape 1976). In this case, the pedigree or single seed descent (SSD) method has clear advantages. However, in many cases breeders have no prior knowledge of the degree of linkage between the genes under selection. Therefore, application of the DH method might be more suitable for crosses among adapted material and for species having a relatively large number of chromosomes, where a large part of the new variation would be released mainly as a result of re-assortment of chromosomes, and the importance of linkage would be relatively less than in species with fewer

chromosomes (Riggs and Snape 1977). Rapeseed has with n = 19 a comparatively large number of chromosomes. Zhao et al. (2006) detected 14 QTL for oil content in a rapeseed DH population and found that they were distributed over 12 linkage groups, indicating that for this trait, assortment of whole chromosomes, and not crossing over, would be the primary source of variation in DH populations. However, usually several quantitative traits are selected for simultaneously.

Tissue culture constraints can be another hindrance and in some cases it may be difficult to produce and diploidize haploids in sufficient numbers to exploit adequately the hybrid. Hence, actual importance of the advantages and disadvantages of the DH technology depends very much on the crop species and on the efficiency, with which large numbers of DH-lines can be produced.

Applying DH Technology in Winter Rapeseed Breeding Programmes

In rapeseed, homozygous lines are produced via the pedigree, the SSD and the DH method. With the SSD method, segregating generations are rapidly advanced in the glasshouse with no selection, each line being continued by a single seed in each generation. In spring rapeseed, the SSD method may be as quick as the DH method in producing nearly homozygous lines. However, in winter rapeseed, generation times are much longer due to its vernalisation requirement. Thus, application of the SSD method to winter rapeseed is not as fast as the DH method. The timetable of breeding winter rapeseed using conventional pedigree selection in comparison to DH is shown in Table 2. Using DH lines may shorten the breeding process by 1 or 2 years. However, the additional years required for pedigree selection can be used for a better evaluation of the year to year variation of the material in the field. Little is known regarding close linkage of traits relevant to cultivar breeding in rapeseed. Experimental comparisons showed that DH and SSD populations from the same crosses were very similar in mean and variance for agronomic and compositional traits (Charne and Beversdorf 1991).

Haploid embryos of *B. napus* were first obtained from anther culture (Thomas and Wenzel 1975) and later also from isolated microspores (Lichter 1982). Since

Table 2 Timetable using DH lines in comparison to conventional line breeding in winter rapeseed (modified after Paulmann and Frauen 1991; Frauen 1994)

Year	Conventional	DH-standard	DH-fast
1	$P_1 \times P_2$	$P_1 \times P_2$	$P_1 \times P_2$
2	F_1	DH line production	DH line production
3	F_2, observation plots	Multiplication	Observation plots
4	F_3, observation plots	Observation plots	Yield tests
5	F_4, yield test	Yield test	Yield tests
6	F_5, yield test	Yield test	Official yield trials
7	F_6, yield test	Official yield trials	
8	Official yield trials		

then, the method has been optimised. Presently, hundreds to thousands of microspore derived embryos (MDE) can be obtained from a single microspore preparation and the DH technology is widely applied in breeding programmes. A survey among eight companies having a winter rapeseed breeding programme in Germany showed that all of them use the DH method. On average the breeders applied the method to 33% of their crosses (Möllers 2007, unpublished results). However, the extent to which the companies applied the technology to their crosses, varied between 10% and 100%. Seven companies did not apply the DH technology to their complete breeding programme. Being asked for their reasons, they indicated that the DH technology is too expensive (57%), that there is a lack of sufficient glasshouse space (43%), that the success of obtaining sufficient DH lines is not certain (29%) and that there are bottleneck problems with other work loads of the conventional breeding programme (29%). Furthermore, the eight companies specified the following major problems in the application of the DH technology: low seed yield from primary DH (88%), insufficient diploidisation (75%) and plantlet regeneration rate (63%), followed by synchronisation problems with the vegetation period (50%). Ideally, primary DH-plants are planted in autumn directly into the field, to save labour and glasshouse space, to obtain a high seed yield per plant and to have a better first visual assessment of the plants performance. However, direct transfer to the field is only possible with plant material which is ready for transfer in August/September. Obviously, DH technology should become more efficient to enable an even wider application in winter rapeseed breeding. Key problems remain the insufficient diploidisation rate, the low seed yield of primary haploids and the time-consuming and inadequate plantlet regeneration from the embryos.

The Problem of Diploidisation and Low Seed Yield of Primary Haploids

Rapeseed shows a low spontaneous diploidisation rate of microspore derived haploid plants in the range of 10% to 30%. Hence, primary DH plantlets need to be treated with colchicine to restore the fertile diploid genome. Diploidisation success is genotype dependent and rarely exceeds 50–70%. Furthermore, colchicine treatment of haploid plantlets in the glasshouse is laborious, requires substantial amounts of colchicine and causes a developmental retardation of the treated plantlets in the range of several weeks. Consequently, flowering and maturation of plantlets in the glasshouse is delayed and not synchronized, and glasshouse space is occupied for a longer period. Spontaneously diploid plantlets flower and mature first and give the full seed yield. Colchicine treated haploid plantlets flower late and give only a low seed yield due to their haploid/diploid chimeric nature. This often requires a seed increase generation in the glasshouse (see Table 1. DH-standard), before sufficient seeds are available for quality analysis and for sowing in observation plots in the field. The *in vitro* treatment of freshly isolated *B. napus* microspores with colchicine or other potent mitotic inhibitors helps to

overcome several of the above mentioned problems. The treatment is performed with low dosages of colchicine or other mitotic inhibitors for a limited duration of up to 3 days. High diploidisation rates of up to 90% have been reported (Chen et al. 1994; Möllers et al. 1994; Hansen and Andersen 1996; Zhao et al. 1996). However, more recent results indicate considerable differences in the efficiency of diploidisation, which ranges between 30% and 95% (Zhou et al. 2002a; Weber et al. 2005; Möllers 2007, unpublished results from survey among companies). This may partly be explained by genotypic differences in the sensitivity towards colchicine. But there is also a wide range of colchicine concentrations (10 to 1,000 mg/l) and treatment durations (6 to 72 hours) applied. For practical applications, a further optimization of microspore protocols to obtain consistently high diploidisation rates with winter rapeseed breeding material is clearly desirable. This would render additional ploidy determination of regenerated embryos (Möllers et al. 1994) or plantlets unnecessary.

Diploidisation at the single cell microspore stage has additional advantages. *In vitro* colchicine treatment of microspores does not cause any developmental delay. On the contrary, colchicine treatment of microspores has been reported to improve embryogenic response by promoting symmetric division of the microspores (Zaki and Dickinson 1991; Iqbal et al. 1994). Regenerated plants are completely diploid, their development and maturation is synchronized and they give the normal seed yield one would expect from a diploid plant. This provides sufficient seeds for a direct testing of the material in observation plots in the field, making an additional seed increase generation unnecessary (see Table 2. DH-fast).

The Problem of Insufficient Conversion of Microspore Derived Embryos to Plants

Only a small fraction of the MDE converts directly into plantlets. The larger fraction tends to form secondary somatic embryos on the hypocotyl and cotyledons, from which eventually shoots may regenerate. The low frequency of direct conversion of MDE to shoots requires that a substantially larger number of MDE are sub-cultured to end up with the desired number of DH plants. Alternatively, several in vitro subcultures are required before normal plantlets are obtained, which can be transferred to the glasshouse. At present, very little is known about the endogenous factors that influence conversion of MDE to plantlets. Early colchicine treatment of isolated microspores in spring rapeseed, besides improving diploidisation and embryogenesis, also produced normal embryos developing directly into plantlets and avoiding cycles of secondary embryogenesis (Iqbal 1993; Zhou et al. 2002b). It has not been sufficiently investigated which of the different developmental stages of the MDE – torpedo, early, mid or late cotyledonary stage – is the best for transfer to solid medium to achieve direct plantlet regeneration. Results indicate that plantlet regeneration can be improved by adding phytohormones and vitamins to the regeneration medium (Tian et al. 2004), or by giving the MDE a cold or drought stress treatment

(Zhang et al. 2006). However, usually only very few genotypes were included in those studies and the effect of a combination of several factors on plantlet regeneration has yet not been tested. Again, for routine applications in winter rapeseed breeding programmes further improvement of existing protocols are very much desirable.

In Vitro Selection

Assuming that the parents of a cross are different for a larger number of loci contributing to the expression of relevant traits, it is clear that breeders need to test many DH-lines to identify the desired recombinant line with a high probability (see Table 1). However, breeders usually aim to obtain only between 50 and 200 DH lines per cross (Möllers, unpublished results from survey among companies), which clearly appears to be too few. As mentioned above, thousands of microspore derived embryos can be obtained from a single microspore preparation. Usually only a smaller fraction of up to 300 of these embryos are sub-cultured *in vitro* to regenerate plantlets which are then transferred to the glasshouse for seed production. The subset of MDE genotypes used for plantlet regeneration represents a random sample of the total number of regenerated MDE, without any knowledge of their quality traits and agronomic performance. Thus many undesired genotypes go through plantlet regeneration and the costly glasshouse process. It also implies that valuable rare recombinant genotypes may be discarded at an early stage of *in vitro* culture. Any method that could be applied to determine useful agronomic or seed quality traits at an early stage of *in vitro* culture would definitely increase the frequency of valuable genotypes among the total number of regenerated plants. Selection *in vitro* for seed oil quality traits is possible in segregating populations of MDE (Albrecht et al. 1995). Fatty acid composition was determined by gas liquid chromatography (GLC) from single cotyledons, dissected from MDE. The rest of the MDE were maintained *in vitro* and regenerated to plantlets. Unequivocal selection for zero, intermediate and high erucic acid (Albrecht et al. 1995) and oleic acid contents (Möllers et al. 2000) was possible in segregating MDE populations. However, the application of this early *in vitro* selection system is limited to those traits which can be rapidly and cost effectively analysed and for which a close correlation between the MDE and the seeds from the regenerated plants has been shown. Alternatively, marker-assisted selection (MAS) could be applied at the MDE stage to screen a larger population for desired recombinants. One of the two cotyledons of the MDE can be dissected and used simultaneously for oil quality analysis and for DNA extraction (Horn and Rafalski 1992; Nath et al. 2007). Currently, only few trait specific molecular markers are available in rapeseed, but it is foreseeable that their number will increase substantially during the next years. A good example is the practised marker assisted selection for the Ogura CMS restorer gene. The single restorer gene segregates one to one in a DH population, resulting in 50% of the lines being homozygous for the restorer gene. By applying MAS at the MDE stage, those 50% could be identified early and exclusively regenerated to plants.

DH Technology in Hybrid Breeding Programmes

A relatively new application for DH technology is the development of inbred lines for producing F_1 hybrid cultivars. Currently, mainly two male sterility systems are used for breeding hybrids in winter rapeseed. These are the cytoplasmic 'Ogura'-system and the genic 'MSL'-system. As mentioned above, restoration of fertility in the Ogura-system is achieved by a single restorer gene. In the MSL-system there is no specific restorer required, most of the genotypes being capable of restoration. Although it has been possible to obtain haploids from male sterile plants via microspore culture, they can not be used for breeding hybrid cultivars, because an isogenic maintainer has to be developed simultaneously by back-crossing. Consequently, DH technology can be meaningfully applied only to the male restorer gene pool. For the Ogura CMS-system, the restorer has to be incorporated into the gene pool. DH lines can be produced right away from F_1 plants or starting from later selfing generations, which allows for some early testing (Paulmann and Frauen 1991; Frauen 1994). Applying the DH technology will render repeated test crossing in later selfing generations unnecessary and simplifies maintenance breeding. Depending on the stage at which the DH technology will be applied it will not save time, but the selection for combining ability will be more efficient due to the larger variance among DH lines.

Outlook

It is anticipated that, in the future, further improvements of the microspore culture technique will allow a higher output of doubled haploid plantlets in practical breeding programmes. The number of PCR-based DNA markers for agronomically important traits are increasing and will allow for a more efficient marker assisted selection in segregating microspore derived embryo populations at an early developmental stage in the Petri dish.

References

Albrecht S, Möllers C, Röbbelen G (1995) Selection *in vitro* for erucic-acid content in segregating populations of microspore-derived embryoids of *Brassica napus*. Plant Breed 114:210–214

Charne DG, Beversdorf WD (1991) Comparison of agronomic and compositional traits in microspore-derived and conventional populations of spring *Brassica napus*. In: Proceedings of the 8th International Rapeseed Congress, Saskatoon, Canada (Vol 1: pp. 64–69)

Chen ZZ, Snyder S, Fan ZG, Loh WH (1994) Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breed 113:217–221

Forster BP and Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–881

Frauen M (1994) Use of biotechnology in breeding new varieties of winter oilseed rape in Germany. GCIRC Bull 10:16–20

Hansen NJP, Andersen SB (1996) *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in *Brassica napus* microspore culture. Euphytica 88:159–164

Horn P, Rafalski A (1992) Non-destructive RAPD genetic diagnostics of microspore-derived *Brassica* embryos. Plant Mol Rep 10:285–293

Iqbal MCM (1993) Methodological improvements in microspore culture of *Brassica napus* L. and investigations on *in vitro* glucosinolate biosynthesis. Doctoral dissertation, Faculty of Agriculture, University of Göttingen, Germany. Göttingen: Cuvillier Verlag

Iqbal MCM, Möllers C, Röbbelen G (1994) Increased embryogenesis after colchicine treatment of microspore cultures of *B. napus*. J Plant Physiol 143:222–226

Jansen RC (1992) On the selection for specific genes in doubled haploids. Heredity 69:92–95

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus* L. Z Pflanzenphysiol 105:427–434

Möllers C, Iqbal MCM, Röbbelen G (1994) Efficient diploidization of *Brassica napus* by colchicine treatment of microspores and regeneration of doubled haploid plants. Euphytica 75:95–104

Möllers C, Rücker B, Stelling D, Schierholt A (2000) *In vitro* selection for oleic and linoleic acid content in segregating populations of microspore derived embryos of *Brassica napus*. Euphytica 112:195–201

Nath UK, Iqbal MCM, Möllers C (2007) Early, non-destructive selection of microspore derived embryo genotypes in oilseed rape (*Brassica napus L.)* by molecular markers and oil quality analysis. Mol Breed 19:285–289

Paulmann W, Frauen M (1991) Einsatz von biotechnologischen Verfahren in der praktischen Rapszüchtung. In: Bericht 42. Arbeitstagung, Saatzuchtleiter, 26–28. Nov., Gumpenstein, Austria, pp. 173–182

Riggs TJ, Snape JW (1977) Effects of linkage and interaction in a comparison of theoretical populations derived by diploidized haploid and single seed descent methods. Theor Appl Genet 49:111–115

Snape JW (1976) A theoretical comparison of diploidized haploid and single seed descent populations. Heredity 36:275–277

Snape JW (1997) Application of doubled haploid lines in plant breeding and genetical research: current issues and approaches. In: Krajewski P, Kaczmarek Z (eds) Advances in Biometrical Genetics. Proceedings of the Tenth Meeting of the EUCARPIA Section Biometrics in Plant Breeding, Poznan, Poland, pp. 35–46

Thomas E, Wenzel G (1975) Embryogenesis from microspores of *Brassica napus*. Zeitschrift für Pflanzenzüchtung 74:77–81

Tian H, Yao CY, Sun MX (2004) High frequency conversion of microspore-derived embryos of *Brassica napus* cv. Topas by supplemental calcium and vitamins. Plant Cell Tiss Org Cult 76:159–165

Walsh EJ (1974) Efficiency of the haploid method in breeding of autogamous diploid species: a computer simulation study. In: Kasha KJ (ed) Haploids in Higher Plants, Advances and Potential. Proceedings of the First International Symposium on Haploids, University of Guelph, Canada, pp. 195–209

Weber S, Ünker F, Friedt W (2005) Improved doubled haploid production protocol for *Brassica napus* using microspore colchicine treatment *in vitro* and ploidy determination by flow cytometry. Plant Breed 124:511–513

Zaki MAM, Dickinson HG (1991) Microspore-derived embryoids in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex Plant Reprod 4:48–55

Zhang GQ, Zhang DQ, Tang GX, He Y, Zhou WJ (2006) Plant development from microspore-derived embryos in oilseed rape as affected by chilling, desiccation and cotyledon excision. Biol Plantarum 50:180–186

Zhao J, Simmond DH, Newcomb W (1996) High frequency production of doubled haploid plants of *Brassica napus* cv. Topas derived from colchicine-induced microspore embryogenesis without heat shock. Plant Cell Rep 15:668–671

Zhao J, Becker HC, Zhang D, Zhang Y, Ecke W (2006) Conditional QTL mapping of oil content in rapeseed with respect to protein content and traits related to plant development and grain yield. Theor Appl Genet 113:33–38

Zhou WJ, Tang GX, Hagberg P (2002a) Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regul 37:185–192

Zhou WJ, Hagberg P, Tang GX (2002b) Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspore in spring *Brassica napus*. Euphytica 128:27–34

Chapter 14
Anther Culture Derived Doubled Haploids in Oat

E.M. Kiviharju

Abstract In oat (*Avena sativa* L.) low production rates have limited the use of doubled haploids (DH). Alternative techniques for oat DH production are anther culture and wide hybridization. In this paper, progress in anther culture is summarized, concentrating specially on the work done in MTT Agrifood Research Finland. Up to 30 green plants per 100 isolated anthers have been regenerated from cultivated oat, but common recoveries are much below that. Genotype-dependency is also evident. Oat anther culture can be used to produce DH populations for genetic mapping, as well as material for other genetic research purposes. Application to the practical cultivar breeding may still demand improved DH production efficiency, as well as overcome of the genotypic restrictions.

Keywords Androgenesis, anther culture, Avena, DH, haploid

Introduction

Homozygous doubled haploid (DH) plants are useful for haploid breeding, as well as for genetic studies. In oat (*Avena sativa* L.) DHs have been produced by anther culture and by wide hybridization, but the efficiencies have been low with both techniques. However, progress has been made and green plant production rates are coming closer to a level acceptable for research and breeding applications. The initial progress in producing DHs in oat is reviewed by Rines et al. (1997) with an update for wide hybridizations in Rines (2003) and Sidhu et al. (2006). Oat anther culture work has been carried out at MTT Agrifood Research Finland since 1993 and is presented here.

E.M. Kiviharju(✉)
MTT Agrifood Research Finland, FI-31600; Jokioinen, Finland
E-mail: elina.kiviharju@mtt.fi

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

Development of the Oat Anther Culture Method

The first plants derived from the anther culture of cultivated oat were reported by Rines in 1983 and for cultivated naked oat in year 1991 by Sun et al. This is rather late compared to other cereals, including barley, wheat, and rice, due not only to the recalcitrance of oat in anther culture, but also to the smaller international interest in oat and consequently less financial input for its research.

Kiviharju et al. (1997) were able to regenerate green oat plants from the hexaploid wild red oat (*Avena sterilis* L.), later from the cross progeny between cultivated and wild red oat (Kiviharju et al. 1998) and more recently from hulled and hull-less cultivated oats (Kiviharju and Tauriainen 1999; Kiviharju et al. 2000). The anther culture protocol was improved and hundreds of regenerants were reported in Kiviharju et al. (2005). Recently, Ślusarkiewicz-Jarzina and Ponitka (2007) reported anther culture derived plants from the Polish oat cultivars.

The current anther culture protocol at MTT is as follows. The anther donor plants are grown in a glasshouse at low temperatures (approx. 16–18°C day, 12–14°C night with a 16/8 photoperiod) for 5–6 weeks, cut tillers are pre-treated with cold for 7 days at + 4°C and then surface-sterilized by wiping them with 70% EtOH. Anthers are isolated onto induction medium (Table 1) containing a solid and a liquid layer. Thirty anthers are plated onto 3.5 cm diameter Petri dishes (Falcon), then heat pre-treated at + 32 °C for 5 days before culture at + 28 °C in the dark. Developing embryo-like structures (ELSs) or calli are transferred onto solid regeneration medium (Table 1), 5 ELSs per 3.5 cm dish after 5, 6, 7 and 8 weeks of initiation of the anther culture. Regeneration cultures are incubated at the same temperature as induction cultures, under low light (approximately 40 µmol/m/s) with a 16-hour photoperiod. Regenerating plantlets are transferred onto rooting medium (MS salts with vitamins, 0.2 mg/l NAA, 2% sucrose, and 0.3% Phytagel, pH 5.8) into glass tubes or plastic chambers and cultured at 25°C. Ploidy level is determined by flow cytometry (Immonen et al. 1999) followed by doubling of the haploid genome by colchicine treatment if necessary.

The important steps in developing the protocol were:

- To give 5 days of heat stress at + 32°C for isolated anthers, promoting the induction of embryo-like structures (ELSs) (Kiviharju and Pehu 1998)
- To give a high enough 2,4-D concentration (3–5 mg/l) together with a low level of kinetin. Kinetin seemed to affect to the quality of the induced ELSs, promoting formation of regenerable structures, although higher concentrations of cytokinins often caused browning of the anthers
- To use 10% maltose instead of sucrose was suitable for most genotypes tested.
- To adopt W_{14} basic medium (Ouyang et al. 1989), although MS-medium (Murashige and Skoog 1962) was better for some genotypes.
- To use an induction medium containing both a solid phase and a liquid phase, to improve the availability of the nutrients and hormones. However, this seems not to be critical, since Ślusarkiewicz-Jarzina and Ponitka (2007) reported that the

Table 1 Media used for oat anther culture

Media components	Induction medium (mg/l)	Regeneration medium (mg/l)
W_{14} macro salts		
KNO_3	2,000	2,000
$NH_4H_2PO_4$	380	380
$MgSO_4 \times 7H_2O$	200	200
$CaCl_2 \times 2H_2O$	140	140
K_2SO_4	700	700
Iron source		
$FeSO_4 \times 7\ H_2O$	27.8	27.8
$NaEDTA \times 2\ H_2O$	37.3	37.3
W_{14} micro salts		
$MnSO_4 \times H_2O$	8.0	8.0
$ZnSO_4 \times 7H_2O$	3.0	3.0
H_3BO_3	3.0	3.0
KI	0.5	0.5
$CuSO_4 \times 5H_2O$	0.025	0.025
$CoCl_2 \times 6H_2O$	0.025	0.025
$NaMoO_4 \times H_2O$	0.005	0.005
W_{14} vitamins		
Thiamine HCl	2.0	2.0
Pyridoxine	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2.0	2.0
Other components		
Maltose $\times H_2O$	105,000	–
Sucrose	–	20,000
2,4-D	3–5	–
NAA	–	2.0
BAP	0.5	–
Kinetin	–	0.5
Ethephon	20	–
L-cysteine	50	–
Myo-inositol	500	–
pH	6.0	6.0
For solid medium		
Phytagel	3,000	3,000
For liquid medium		
Ficoll	100,000	–

highest ELS induction frequency was obtained on the solid medium and plants were regenerated only from the ELSs initiated on the solid medium.

- To add ethylene releasing component Ethephon and cysteine to improve induction of the regenerable-type ELSs.

The double layer induction medium is prepared as follows: To make 250 ml of the solid medium, Phytagel is added to 150 ml of sterilized water and autoclaved. The other components are dissolved into 100 ml of sterilized water, the pH adjusted, and the solution then sterilized by filtering through a 0.22 or 0.45 µm syringe filter

straight into the autoclaved Phytagel solution. The liquid part is prepared by dissolving all components except Ficoll to sterilized water and then adjusting the pH. Ficoll is added to the solution and the volume adjusted to 250 ml with water. Ficoll is dissolved by heating before sterilizing by autoclaving. We use 3.5 ml Petri dishes (Falcon) and add 2.5 ml of solid medium and 1 ml of liquid medium to each.

There are also other factors, which seem to have positive effects on either the induction or regeneration phase, but which have not been confirmed either due to the low induction levels, or just to lack of time or labour. For some genotypes, low light in the induction phase seemed to be better than darkness, this had been noticed earlier for recalcitrant genotypes of wheat (*Triticum aestivum* L.) (Bjørnstad et al. 1989). Selection of cytokinin may be critical, since use of BAP in the induction medium of cultivar Kolbu greatly increased the number of albinos compared to the same concentration of kinetin. We have also found preliminary evidence that the polyamine spermidine might increase the induction of the regenerable ELSs (unpublished). Anther culture response level of the control treatment varied between different donor plant sowings, although genotype was the same. This well known batch to batch variation can be assumed to be mainly dependent on seasonal variation in the growing conditions (early autumn and early to late spring being the best sowing times). However, other factors may also play a role, such as differences in the watering or fertilization of the plants, or possible disease infections during the growing period. Effect of these factors should be studied in the future experiments.

Efficiency and the Effect of Genotype

Over 400,000 oat anthers have been isolated in total since year 1993. In the best results 30 green plants per 100 anthers isolated were obtained from the progeny of a cross between Aslak and the hexaploid naked oat Lisbeth, both cultivars originate from Boreal Plant Breeding Ltd, Finland. Ten plants per 100 anthers were obtained from the cultivar Roope and about 8 per 100 anthers from Aslak and Robert. However, a more common result was 1 green plant per 100 anthers.

The high number of ELSs or calli induced did not necessarily correlate with high green plant regeneration rates of the tested genotypes. The key stage seems to be induction. For example, many ELS or micro-calli were induced from the responsive genotypes of cultivated oat in the hormone-free induction medium, as noticed earlier by Rines (1983), or in the induction medium containing only 2,4-D as a hormone. However, it was not possible to regenerate plants from these structures despite extensive trials of different regeneration media and treatments.

The majority of oat regenerants were haploid, as measured by flow cytometry, which necessitates colchicine treatment. Genotypic variation in ploidy was found. In many genotypes, spontaneous chromosome doubling occurs in only 10% of the regenerants; the highest rate (39%) was observed in an Aslak × Matilda DH population (148 DH plants). Production of albino plants varies between genotypes, but is generally low.

The choice of genotype is critical when this doubled haploid production technique is used for practical purposes, whether with oat or with other cereals. In anther culture of wheat, genotype differences are reported to account for 32–85.6% of the total variation in green plant regeneration; in barley (*Hordeum vulgare* L.) the level is 73% (Zhou 1996; Torp et al. 2001). Also in oat, the effect of genotype is evident: of 76 genotypes of cultivated oat tested (over 100 if the progeny of crosses are included) only 29 produced green plants. The responsive cultivars were Kolbu, Stout, Katri, Aslak, Freja, Kapp, Minerva, Diana, Elvy, Dumont, Robert, Tropicale, Poncho, Belote, Poney, Roope, and in addition eight breeding lines.

One possible strategy to improve anther culture response is to introgress desirable alleles by crossing from genotypes known to respond well in anther culture. This method has been used successfully to improve the anther culture ability of recalcitrant genotypes in other cereals, including wheat (Hu 1997) and barley (Ouédraogo et al. 1998). This was also found to be the case at MTT. If at least one cross parent had been regenerated in anther culture previously, nearly 90% (29/33) of the tested cross progenies regenerated plants.

Use of Oat DHs in Research and Breeding

The potential to establish totally homozygous lines in a single generation provides advantages to both breeding and research (Forster and Thomas 2005). The question has so far been: can as many DHs as needed be produced, and at a reasonable cost?

The efficiency of genetic mapping is increased by using a totally homozygous mapping population, because dominant markers function as if they were co-dominant. Oat anther culture can be used for production of DH mapping populations. For example, MTT has constructed a map for a population consisting of 137 Aslak × Matilda DH lines. Aslak is a Boreal Plant breeding Ltd cultivar, Matilda is from Svalöf Weibull AB. DH lines were produced in three different sets; the average efficiency was around one green plant per 100 anthers.

In cultivar breeding, cost efficiency is especially critical, in addition to genotypic limitations. What does the overall regeneration efficiency mean in practical terms of labour and time? Let's take a hypothetical case, where we want to have 100 DH lines from the progeny of a cross and assume that we will get 1 DH per 100 isolated anthers. That entails the isolation of 10,000 anthers, which a skilled technician can routinely accomplish in 5 days. With an efficiency of 5 DH plants per 100 isolated anthers, 1 day of work is needed. Compared to wheat and barley, the isolation of oat anthers is slower due to the structure of the panicle and spikelets. In total, the whole programme from donor plant sowing to the mature DH seeds, will however take over half a year: 5–6 weeks to grow the anther donor plants, 1 week for cold treatment, 5–8 weeks of induction culture, 2–4 weeks of regeneration culture and a few weeks on the rooting medium, finally followed by growing of the plantlets in the glasshouse for several months to get the seeds.

With these results, we can at least start to talk about the use of anther culture-derived oat DHs, not only for research, but also for practical aims. It is essential to first check the anther culture ability of the crossing parents. The efficiency in many cases may not be sufficient, but application of the method for an elite cross material may be worth already in efficiency of one or more plants per 100 isolated anthers, in order to produce around 100 DH lines needed.

Gametoclonal variation may affect to the quality of microspore-derived DH plants. Nevertheless, agronomic performance of microspore-derived wheat and barley DH lines is generally thought to be acceptable (Powell et al. 1992; Ma et al. 1999) and many crop cultivars are currently derived from DH techniques (about 50% of European barley cultivars for instance). The agronomic performance of some oat DH lines was compared with the performance of plants sown from commercial seeds of the same cultivar. Statistically, some differences were found in both the positive and the negative direction. In general, the MTT DH lines seemed to be uniform and pure and DNA-marker analyses of the anther culture-derived mapping population confirmed the homozygous status of the plants.

Future Prospects

It has been shown that oat DH production by anther culture has potential in terms of practical applications. However, more effort is needed to improve plant production efficiency and to overcome genotypic limitations. One approach would be to develop an isolated microspore culture method for oat, in order to relieve the laborious isolation of anthers. However, so far success in microspore culture has been humble. In the work at MTT, viability of the isolated microspores after a few days of culture was very low compared to that reported in wheat. Some micro-calli or ELSs initiation was observed, but no plants were regenerated by this method. To date there has not been success in the culture of the female gametophytes or protoplasts in oat either.

DNA markers and novel genomics technologies could be used to understand the genetic base and regulation of the anther culture response. Overall efficiency depends on the induction of ELS, the regeneration of plants from these structures and the ratio of green to albino plants regenerated, all controlled mainly by nuclear genes. DNA markers associated with good anther culture traits could be used to select responsive crossing parents and breeding lines. This approach would also support transfer of regeneration ability from responsive genotypes to recalcitrant ones through crossing. In this regard, some preliminary work has been carried out with the non-responsive oat cv. Puhti and responsive wild red oat acc. CAV 2648 and their cross progeny. Some putative random amplified polymorphic DNA (RAPD) markers associated with anther culture ability traits of oats were identified based on F_2 progeny analysis (Kiviharju et al. 2004).

References

Bjørnstad Å, Opsahl H-G, Aasmo M (1989) Effects of donor plant environment and light during incubation on anther cultures of some spring wheat (*Triticum aestivum* L.) cultivars. Plant Cell Tiss Org Cult 17:27–37

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88

Immonen S, Tauriainen A, Manninen O (1999) Assesment of green regenerants from rye and triticale anther cultures. In: Clement C, Pacini E, Audran JC (eds) Anther and pollen: from biology to biotechnology. Springer, Berlin, pp. 237–245

Hu H (1997) In vitro induced haploids in wheat. In: Jain SM, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, Vol 4: Cereals. Kluwer, Dordrecht, pp. 73–97

Kiviharju E, Pehu E (1998) The effect of cold and heat pretreatments on anther culture response of *Avena sativa* and *A. sterilis*. Plant Cell Tiss Org Cult 54:97–104

Kiviharju E, Tauriainen A (1999) 2,4-dichlorophenoxyacetic acid and kinetin in anther culture of cultivated and wild oats and their interspecific crosses: plant regeneration from *Avena sativa* L. Plant Cell Rep 18:582–588

Kiviharju E, Puolimatka M, Pehu E (1997) Regeneration of anther-derived plants of *Avena sterilis*. Plant Cell Tiss Org Cult 48:147–152

Kiviharju E, Puolimatka M, Saastamoinen M, Hovinen S, Pehu E (1998) The effect of genotype on anther culture response of cultivated and wild oats. Agric Food Sci Finl 7:409–422

Kiviharju E, Puolimatka M, Saastamoinen M, Pehu, E (2000) Extension of anther culture to several genotypes of cultivated oats. Plant Cell Rep 19:674–679

Kiviharju E, Laurila J, Lehtonen M, Tanhuanpää P, Manninen O (2004) Anther culture properties of oat × wild red oat progenies and a search for RAPD markers associated with anther culture ability. Agric Food Sci 13:151–162

Kiviharju E, Moisander S, Laurila J (2005) Improved green plant regeneration rates from oat anther culture and the agronomic performance of some DH lines. Plant Cell Tiss Org Cult 81:1–9

Ma H, Busch RH, Riera-Lizarazu O, Rines HW, Dill-Macky R (1999) Agronomic performance of lines derived from anther culture, maize pollination and single-seed descent in a spring wheat cross. Theor Appl Genet 99:432–436

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue culture. Physiol Plant 15:437–497

Ouédraogo JT, St-Pierre C-A, Collin J, Rioux S, Comeau A (1998) Effect of amino acids, growth regulators and genotype on androgenesis in barley. Plant Cell Tiss Org Cult 53:59–66

Ouyang JW, Jia SE, Zhang C, Chen X, Feng G (1989) A new synthetic medium (W14) for wheat anther culture. Annu Rep Inst Genet Acad Sin 1987–1988, Beijing, pp. 91–92

Powell W, Thomas WTB, Thompson DM (1992) The agronomic performance of anther culture derived plants of barley produced via pollen embryogenesis. Ann Appl Biol 120:137–150

Rines HW (1983) Oat anther culture: genotype effect on callus initiation and the production of a haploid plant. Crop Sci 23:268–272

Rines HW (2003) Oat haploids from wide hybridization. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants. Kluwer, Dordrecht, pp. 155–159

Rines HW, Riera-Lizarazu O, Nunez VM, Davis DW, Phillips RL (1997) Oat haploids from anther culture and from wide hybridizations. In: Jain SM, Sopory SK, Veilleux RE (eds) In Vitro Haploid Production in Higher Plants, Vol. 4. Kluwer, Dordrecht, pp. 205–221

Sidhu PK, Howes NK, Aung T, Zwer PK, Davies PA (2006) Factors affecting oat haploid production following oat × maize hybridization. Plant Breed 125:243–247

Ślusarkiewicz-Jarzina A, Ponitka A (2007) The effect of physical medium state on anther culture response in polish cultivated oat (*Avena sativa* L.). Acta Biol Craciviensia Ser Bot 49:27–31

Sun CS, Lu T-G, Söndahl MR (1991) Anther culture of naked oat and the establishment of its haploid suspension cell. Acta Bot Sin 33:417–420
Torp AM, Hansen AL, Andersen SB (2001) Chromosomal regions associated with green plant regeneration in wheat (*Triticum aestivum* L.) anther culture. Euphytica 199:377–387
Zhou H (1996) Genetics of green plant regeneration from anther culture in cereals. In: Jain SM, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, Vol. 2: Applications. Kluwer, Dordrecht, pp. 169–187

Chapter 15
Barley and Wheat Doubled Haploids in Breeding

J. Weyen

Abstract The application of doubled haploids is a routine in barley and wheat breeding today. While in barley anther and isolated microspore culture are efficient technologies in wheat wide crosses with maize and subsequent phytohormone treatment and embryo rescue are better established due to genotype dependency of wheat in androgenic technologies. Although a quite significant number of DH lines can be produced in breeding programmes there are still some limiting factors which are related to technical and genetic factors. The actual status of DH lines in barley and wheat breeding and some of the limiting factors are listed and described below. Furthermore some aspects of R&D programmes to circumvent those limitations are described, followed by an outlook.

Keywords Doubled haploids, cereals, breeding, anther culture, microspore culture, wide crosses

Introduction

First reports about the regeneration and production of doubled haploid plants in barley and wheat were published more than 30 years ago. The regeneration of green plantlets from excised anthers in barley was reported by Clapham (1973) and in wheat by Ouyang et al. (1973). Barclay (1975) reported high frequencies of haploid production in wheat by chromosome elimination. Early reports concerning successful protocols of isolated microspore culture in barley were published by Kao et al. (1991) and in wheat by Datta and Wenzel (1987) and Hunter (1988).

Since then significant improvements resulting in more or less efficient technologies were published by many authors and generally the different technologies of anther culture (AC), isolated microspore culture (IMC) and wide crosses (WC) have become "routine" in public and private breeding and research programmes today worldwide. A collection of the most recent protocols for doubled haploid production in a range of different species, including barley and wheat can be found

J. Weyen(✉)
Saaten-Union Resistenzlabor GmbH, Hovedisser Str. 92, 33818 Leopoldshoehe, Germany
E-mail: weyen@saaten-union-labor.de

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

in Maluszynski et al. (2003). Nevertheless there are still some obstacles and further R&D programmes are necessary to overcome constraints such as genotype dependency, albino regeneration and low spontaneous or incomplete fertility after induced doubling. As doubled haploid technologies *per se* become more and more efficient at the research laboratory level, the technologies and logistics in practical breeding programmes and the interaction with molecular marker technologies have to be aligned and adapted significantly. The production and deployment of fertile doubled haploid lines are still considered expensive and require speedy seed multiplication, multi locational field testing and a high quality data generation, collection and analysis system.

Barley

Doubled Haploid Technologies in Barley

Anther culture and the *Hordeum bulbosum* technologies were described earlier than isolated microspore culture in barley. Each of the technologies has major advantages and disadvantages. While *H. bulbosum* technology is relatively independent from high quality equipment both androgenic technologies require more sophisticated and stable glasshouse capacities (cooling, illumination) and also more attention to the issue of laboratory sanitary conditions. Nevertheless today the *H. bulbosum* technology is not used much, because the efficiency is significant lower than established AC and IMC programmes. One of the breakthroughs in barley androgenesis came with the patented use of maltose described by Hunter (1987, see also chapter by Dunwell, “Patents and haploid plants”) and further improvements by many other authors are being developed in many laboratories and breeding companies.

As a precondition for successful androgenic doubled haploidy technology, optimal growing regimes for the donor plants must be at hand. In addition to optimal protection against diseases such as mildew and insects (aphids, thrips, spider mites) optimal abiotic conditions must be provided. Quality and quantity of light, water supply, fertilization, growth substrates, air humidity and carbon dioxide concentration are important factors that need careful maintenance. The optimal stage of microspore development (mid to late uni-nucleate) is another prerequisite which is essential for success in AC and IMC. The most common pre treatments which were described in successful protocols are cold treatment of tillers (in water or wrapped in watered paper towels and foil) or in a 0,3 M mannitol solution for a varying number of days. Some of these and further detailed protocols are published in Maluszynski et al. (2003).

Necessary Improvements

Although there are many published improvements, the production of several hundreds of green plantlets per genotype in breeding programmes is only possible by

the adaption of those protocols (working often with only a minor range of genotypes) to the specific conditions of each barley breeding station. Working on several hundred crosses per year in a doubled haploid laboratory requires extensive work and reliable protocols. Conducting conventional breeding alongside doubled haploid (DH) systems is logistically difficult and expensive. Stable and reliable numbers of doubled haploid plants are a pre requisite for successful DH breeding. Despite the significant improvements described above there are still three major obstacles in barley AC and IMC. One is the very genotype dependent occurrence of albino plantlets and the second is the more technical problem of contamination by bacteria and fungi in isolated microspore culture. Contamination is not a significant problem in AC, because losses are not too dramatic under normal circumstances. Nevertheless the use of antibacterial and anti-fungal substances is possible, but is a rather expensive solution to the problem.

Albino plants are still a problem in many cereal species (see also chapter by Torp and Andersen on “Albinism in microspore culture”). It is clearly a genotype dependent trait and other factors may also have an influence (light quantity and quality, media composition, stress treatment, etc.). Oleszczuk et al. (2006) described a relatively lower number of albinos in barley ICM after increased temperatures in a low temperature pre-treatment of isolated anthers. Other components of media were also often reported to have an impact on the ratio of green/albino plantlets, but the physiological status of the donor plants and the exact stage of microspore development remain the most important influencing factors. The significant genetic variation between barley genotypes in this respect is still not explained sufficiently. Often spring barley seems to be more prone to albinism.

While in barley AC and IMC the spontaneous doubling rates are often acceptable they are one of the major disadvantages in wheat. In our experience the average doubling percentage in spring and in winter barley was between 66% in field planted DH populations. Nevertheless the data varied between 20–95% per genotype, which proves a genetic control and dependency also of this trait, which has a significant economical impact. The biological processes for spontaneous doubling in barley were described by Shim et al. (2006). Plants should be grown under optimal conditions in order to maximise doubled haploidy. Flow cytometry can be used to select and transfer haploid plantlets to a chemical induction procedure. This process could be automated, but the costs are prohibitive and, usually, the spontaneous doubling rate in barley is high enough for breeding purposes.

Further technological improvements require studies on the influence of carbon dioxide fertilization and air humidity on the response of barley in AC and/or ICM. The quality and quantity of data in this respect is very low due to the costs of such experiments. Plant physiological status is also dependent upon water and nutrient supply and culture substrate and these are all worthy for testing in future R&D programmes (see also chapter by Węzdony et al. on “Progress in doubled haploid technology in higher plants”).

DHs in Barley Breeding

Worldwide several hundred if not thousands of doubled haploid barley varieties are registered. Unfortunately a complete overview does not exist. Nevertheless on the COST 851 website (www.scri.ac.uk/assoc/COST851/Default.htm) a list of several spring and winter barley varieties is provided (see also chapter by Devaux and Kasha on “Overview of barley doubled haploid production”). It is quite obvious that every year an impressive number of new DH varieties are listed and registered worldwide (mainly in EU, Canada, US, Australia). One very good example of the usefulness of the DH technology is the successful breeding of varieties with different resistance genes against the Barley Mild Mosaic Virus/Barley Yellow Mosaic Virus (BaMMV/BaYMV) complex (Werner et al. 2000). Other pyramiding projects are running for BYDV resistance genes and significant efforts have been made in Australian breeding programmes to combine genes for quality, abiotic stresses and biotic stresses by combining doubled haploidy with molecular marker assisted breeding (P. Langridge 2008, personal communication).

Advantages of DH breeding programmes were often published and discussed worldwide. Accelerated breeding has been brought about by instant access to homozygote material, faster and easier recognition of quantitative traits and easier maintenance of material. These factors can act in concert with conventional programmes, but there are examples where the whole breeding programme has been converted to DH breeding. Quality data of quantitative traits such as malting and brewing quality are more reliable if they are generated with doubled haploid material as this allows repeated sampling and replication. After accelerated multiplication of DH progenies it is easy to test resistance and even yield data in replicated experiments on more than one location in a very short time interval. By this kind of breeding strategy the number of years for testing is replaced by the number of locations and often lines can be notified for the official testing procedures after 4–5 years of breeding (Forster and Thomas 2005).

Different authors compared the level and variation of yield, resistance and other agronomic traits in populations derived by SSD, DH and conventional breeding schemes. Some of them concluded the loss of genetic variation in DH populations out of crosses between parents with a significant genetic diversity, while crosses between similar genetic material were not affected. However such publications are rather old now and with the improved technologies and often excellent numbers of green plantlets per genotype, including exotic crosses in IMC, these problems seem to be of little concern to practical breeders. Nevertheless major disadvantages in respect to yield, yield stability, agronomic performance or resistance/tolerance to biotic and abiotic stresses were not described.

Traditional objections against doubled haploids as somaclonal variation, loss of genetic variation and lower stability of resistance to diseases are still under discussion. Nevertheless there are more and more winter and spring barley breeding programmes which are completely reliant upon doubled haploidy and these are becoming more and more successful on the markets worldwide.

Success on the seed markets was only possible by integrating DH laboratories with field breeding methodologies. This interaction is absolutely important in making DH technology a success. Issues here are mainly concerned with the timing of production and planting or culturing the donor plants and subsequent DH populations. Due to the fact that the culture and the maturing of DH populations (typically, up to 60,000 individual plants for a breeding programme per cycle) is not feasible (financially) under glasshouse conditions, field planting systems have to be established and optimized, and these vary considerably between the two, winter and spring barley, crop types. Optimal preparation of the field and further control of the DH nurseries against abiotic stresses (mainly drought, but also hail, wind, heavy rain showers), biotic stresses (fungi, soil borne and insect transmitted viruses, birds) are necessary. Due to, the often, intercalating time regimes of DH planting and their location (often separate or isolated) the populations can be very sensitive to insects and birds. Regular watering and pesticide applications should be provided when necessary, bird nets and protection against rabbits, mouse and deer are often necessary.

It should be noted that the use of doubled haploids is only a minor technical step in a total breeding procedure. The intellectual investment in planning a crossing programme and the quality of the breeding programme in total, the marketing and a good portion of luck in the official and unofficial field experiments are all essential inter-related factors.

Wheat

Doubled Haploid Technologies in Wheat

As in barley wheat doubled haploids were described early in the 1970s. Today in routine breeding programmes the maize pollination method in combination with a phytohormone treatment (dipping, spraying, injection etc.) and a subsequent embryo rescue and regeneration step is the method of choice. This is due to significant genotype dependency, high numbers of albinos and work load involved in AC and IMC in wheat. Nevertheless there are some institutions still working with AC, but the efficiencies remain quite low in comparison to the maize/wheat system. But AC remains still an option for specialized groups with a relatively uniform genetic pool and may take the lead in the future once more efficient methods are developed.

Significant improvements in wheat ICM are now possible by the application of inducer chemicals (Zheng et al. 2001), ovary co-culture (Mejza et al. 1993; Puolimatka et al. 1996) and arabinogalactanproteins (Letarte et al. 2006). Hansen and Andersen (1998a, b) described the use of antimitotic agents as colchicine, amiprophosmethyl and trifluralin in ICM and Navarro-Alvarez et al. (1994) described this approach in wheat anther culture too.

There are many publications that describe stress treatments such as temperature treatments, osmotic treatments and starvation, or even combinations thereof, of spikes, tillers or even isolated anthers or microspores. Touraev et al. (1996) reported

the successful regeneration after heat shock treatment on anthers and Shariatpanahi et al. (2006) reported successful regeneration without any stress pre-treatment and the use of a nutrient free induction medium.

Recently the number of publications concerning DH protocols for durum wheat has increased (Cistue et al. 2006; Labbani et al. 2007). The main improvements which are described by them are a mannitol pretreatment and the use of colchicine *in vitro*. The production of doubled haploids by maize durum technology is routine today, but unfortunately not very efficient and therefore expensive.

Necessary Improvements

Although there are wheat genotypes that exhibit a significantly higher response in anther or microspore technique, and sometimes there are also small pools of genotypes with satisfactory response levels, the reproducibility and the transfer of published protocols has not been reported to be successful to date with respect to breeding. Glasshouse conditions, laboratory specific technologies and the experience and the "green finger" are still very important factors to transfer published protocols. The exchange of knowhow by exchange of people/expertise seems one of the most promising and efficient ways to bring tissue culture successful to routine operations.

Isolated microspore cultures with the Bulgarian winter wheat variety 'Svilena' are often reported to be excellent. The identification of genomic regions which are responsible for this trait would be scientifically very interesting. There is, theoretically, also a possible exploitation for wheat breeders, but due to economic restrictions and strong competition such a breeding programme is very difficult to perform by a private plant breeding company.

Similarly it has not been possible to identify the signals or molecules which are emitted by, or exchanged between, wheat ovaries and wheat pollen *in vitro*. Although there are reports that certain arabinogalactanproteins (AGPs) might be enhancing factors, physiological and technical proof is lacking. The pursuit of genetic explanations is also required.

Spontaneous doubling in wheat haploid plantlets is normally low. While after isolated microspore culture higher values can be observed in some genotypes after wide crosses, the rate does not exceed 10%. Therefore the application of anti-mitotic agents on plantlets, microspores or even on the donor plants is necessary. *In vitro* application of colchicine, APM and trifluralin on freshly isolated microspores of wheat was described by Hansen and Andersen (1998a, b). The best doubling success was achieved after 48 hours application of 10 µM APM (74%) and with trifluralin (65%) and after 24 hours application of 1 mM colchicine (53%). The number of embryos was lower, but the number of green plantlets and doubled haploid plantlets per spike was higher than the control. Other anti-mitotic agents as caffeine (Thomas et al. 1997) have also been reported.

Normally the procedure of chemical induced doubling is performed on plantlets which were already potted in soil. Soil residues are removed from plantlets which are at the 3–5 leaf stage, roots are shortened and plantlets are dipped to a colchicine solution for some hours. By thorough washing with tap water the excess colchicine is removed and the plantlets are re-potted. After keeping the air humidity high in the first days the recovery rate can be increased. Normally only a small percentage of the plantlets die in this treatment. Nevertheless the doubling rate is very variable from year to year and chimeras often occur. Complete fertility is seldom observed due to the, often, chimeric nature of such plants. The doubling success is also very dependent on the environment in which the plantlets are cultured and matured.

The injection of colchicine, sometimes together with a phytohormone, to the internodes of spikes which are then subsequently pollinated with maize pollen has been described (Sood et al. 2003). Nevertheless due to the toxicity of colchicine such treatments are often difficult with respect to health and safety issue of workers in glasshouses and laboratories.

DH in Wheat Breeding

Many wheat doubled haploids were also listed by COST851 (http://www.scri.ac.uk/assoc/COST851/Default.htm). Exact data about the application of doubled haploid technologies in breeding programmes worldwide do not exist. It is estimated by the author that several hundred thousands of doubled haploid lines are produced per year worldwide (EU, Canada, Australia, China and elsewhere). It is most probable that the majority of the plantlets are produced by maize wheat pollination (J. Thomas 2007, P. Davies 2007, personal communication). Nevertheless from time to time there are interesting publications on wheat microspore culture, and it is not known what is happening in the biotechnology laboratories of the wheat breeding companies concerned.

The utilization of doubled haploids in wheat breeding is not as common as in barley. For example, most of the recently registered barley varieties in Germany are doubled haploids and 50% of the seed propagation acreage in barley was taken up with doubled haploid varieties in spring and in winter barley in Germany in 2006/07. Other data from other EU countries or worldwide are not known. In wheat these numbers are significantly lower in the EU. But exact data are not known.

Generally, it seems to be possible to apply for variety protection and registration after 4 or 5 years, also in winter types. Due to the lack of years the number of tested environments must be adjusted.

Specific examples for specific traits in registered varieties which were developed by a doubled haploid technology are not known by the author. Nevertheless interesting registered doubled haploid wheat varieties have been on the market for many years and the economical advantages of DH breeding in wheat is becoming more and more visible.

References

Barclay IR (1975) High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. Nature 256:410–411

Cistue L, Soriano M, Castillo AM, Valles MP, Sanz JM, Echavarri B (2006) Production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. Plant Cell Rep 25:257–264

Clapham D (1973) Haploid *Hordeum* plants from anthers *in vitro*. Mol Breed 69:142–155

Datta SK, Wenzel G (1987) Isolated microspore derived plant formation *via* embryogenesis in *Triticum aestivum* L. Plant Sci 48:49–54

Devaux P, Kasha KJ (2008) Overview of barley doubled haploid production. In: Touraev A, Forster BP, Mohan Jain S (eds) Advances in Haploid Production in Higher Plants (this volume)

Dunwell JM (2008) Patents and haploid plants. In: Touraev A, Forster BP, Mohan Jain S (eds) Advances in Haploid Production in Higher Plants (this volume)

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88

Hansen NJP, Andersen SB (1998a) *In vitro* chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). Euphytica 102:101–108

Hansen NJP, Andersen SB (1998b) Efficient production of doubled haploid wheat plants by *in vitro* treatment of microspores with trifluralin or APM. Plant Breed 117:410–405

Hunter CP (1987) Method of generating barley plants. European Patent EP0245898

Hunter CP (1988) Plant regeneration from microspores of barley, *Hordeum vulgare* L. Ph.D. Thesis, Wye College, University of London

Kao KN, Saleem S, Abrams M (1991) Culture conditions for induction of green plants from barley microspores by anther culture methods. Plant Cell Rep 9:595–601

Labbani Z, de Buyser J, Picard E (2007) Effect of mannitol pretreatment to improve green plant regeneration on isolated microspore culture in *Triticum turgidum* ssp. Durum cv. ‘Jennah Khetifa’. Plant Breed 126:565–568

Letarte J, Simion E, Miner M, Kasha KJ (2006) Arabinogalactans and arabinogalactan-proteins induce embryogenesis in wheat (*Triticum aestivum* L.) microspore culture. Plant Cell Rep 25:877–882

Maluszynski M, Kasha KJ, Forster BP, Szarejko I (2003) Doubled haploid production in crop plants: A manual. Kluwer, Dordrecht

Mejza SJ, Morgant V, DiBona DE, Wong JR (1993) Plant regeneration from isolated microspores of *Triticum aestivum*. Plant Cell Rep 12:149–153

Navarro-Alvarez W, Baenziger PS, Eskridge KM, Hugo M, Gustafson VD (1994) Addition of colchicine to wheat anther culture media to increase doubled haploid plant production. Plant Breed 112:192–198

Oleszczuk, S, Sowa S, Zimny J (2006) Androgenic response to preculture stress in microspore cultures of barley. Protoplasma 228:95–100

Ouyang T, Hu H, Chuang C, Tseng C (1973) Induction of pollen plants from anthers of *Triticum aestivum* L. cultured *in vitro*. Sci Sin 16:79–95

Puolimatka M, Laine S, Pauk J (1996) Induction of wheat microspore embryogenesis using ovary co-cultivation. In: Plant Embryogenesis Workshop. “From Single Cell to Plant – Progress Towards Understanding Zygotic, Androgenic and Somatic Embryogenesis”, Hamburg 12.–14.9.1996. Abstracts. Hamburg: Univ., Zentrum f. Angew. Pflanzen Molekular Biologie

Shariatpanahi ME, Belogradova K, Hessamvaziri L, Heberle-Bors E, Touraev A (2006) Efficient embryogenesis and regeneration in freshly isolated and cultured wheat (Triticum aestivum L.) microspores without stress pretreatment. Plant Cell Rep 22:449–456

Shim YS, Kasha KJ, Simion E, Letarte J (2006) The relationship between induction of embryogenesis and chromosome doubling in microspore cultures. Protoplasma 228:79–86

Sood S, Dhawan R, Singh K, Bains NS (2003) Development of novel chromosome doubling strategies for wheat x maize system of wheat haploid production. Plant Breed 122:493–496

Thomas J, Chen Q, Howes N (1997) Chromosome doubling of haploids of common wheat with caffeine. Genome 40:552–558

Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996) Efficient microspore embryogenesis in wheat (Triticum aestivum L.) induced by starvation at high temperature. Sex Plant Reprod 9:209–215

Wędzony M, Forster BP, Zur I, Golemięc E, Szechińska-Hebda M, Dubas E (2008) Progress in doubled haploid technology in higher plants. In: Touraev A, Forster BP, Mohan Jain S (eds) Advances in Haploid Production in Higher Plants (this volume)

Werner K, Friedt W, Ordon F (2000) Strategies for pyramiding resistance genes against the barley yellow mosaic virus complex based on molecular markers and DH-lines. – 8th International Barley Genetics Symposium, Adelaide, Australia. Volume II, pp. 203–206

Zheng MA, Liu W, Wenig Y, Polle E, Konzak CF (2001) Culture of freshly isolated wheat (Triticum aestivum L.) microspores treated with inducer chemicals. Plant Cell Rep 20:685–690

Chapter 16
Rice Doubled Haploids and Breeding

J. Pauk, M. Jancsó, and I. Simon-Kiss

Abstract Cell- and tissue culture methods in combination with conventional breeding process were suitable way to produce new varieties. These applications gave new breeding alternatives to release competitive genotypes in comparison with traditional ones. To the breeding of 'Risabell' (1997) DH lines were produced via anther culture from F_2 population of a single cross combination. The new variety was improved for resistance to blast disease, high milling and cooking quality and long grain type. In case of 'Janka' (2003) haploid cell cultures were developed and their vigorous regenerants were colchicine treated. One of the best fertile lines was released as 'Janka' has vigorous seedling growth, drought tolerance and good grain quality. The variety 'Ábel' (2005) was improved through somatic tissue culture regeneration followed by anther culture. Main characteristics of this variety are earliness, early stage cold tolerance and good performance in aerobic conditions. The breeding-processes of these State approved rice varieties demonstrate the successful integration of biotechnological methods and pedigree-breeding.

Keywords *Oryza sativa* L, anther culture, somatic tissue culture, pedigree breeding, variety improvement

Introduction

The first report of successful *in vitro* haploid induction in anther culture of rice (*Oryza sativa* L.) was published by Niizeki and Oono (1968) from Japan. They described a direct method of rice androgenesis (callus induction and plant regeneration on the same

J. Pauk(✉)
Cereal Research Non-Profit Company, Department of Biotechnology, Szeged
Alsó kikötö sor 9, H-6726, Hungary
E-mail: janos.pauk@gk-szeged.hu

M. Jancsó, I. Simon-Kiss
Research Institute for Fisheries, Aquaculture and Irrigation, Department of Aquaculture, Szarvas
Anna liget 8, H-5540, Hungary

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

medium) using Miller (1953) medium supplemented with 1 mg/l α-Naphthaleneacetic acid (NAA). Later, an indirect (callus induction and plant regeneration on different medium) method of rice androgenesis was published by Nishi and Mitsuoka (1969), Guha et al. (1970), Niizeki and Oono (1971) and Guha (1973). In their results, the induced calli were obtained using 2,4-Dichlorophenoxyacetic acid (2,4-D) in the induction medium and the plant regeneration was achieved through a combination of Indole-3-acetic acid (IAA) and Kinetin. Extensive research to find the most suitable developmental stage of microspores that are competent to become sporophytic was carried out by Chen (1977). In this paper the mid-uni-nucleated stage of microspore development was identified as having the highest callus initiation frequency in anther culture.

One of the most important improvements to the culture medium was published by Chu et al. (1975) who described optimization of nitrogen supplements. Chu (1978) reported that N6 medium was superior to MS medium for anther and pollen culture in rice. Twelve years later, Reiffers and Freire (1990) used N6 basic medium for callus induction supplemented with 1 mg/l NAA, but plant regeneration was carried out in a modified MS medium (3 mg/l Kinetin and 0.5 mg/l NAA). Their callus induction frequency varied between 0.22% and 29%, while plant regeneration frequency was recorded at 144.4%. In this research, a high portion of albinos were described (27% of regenerated plants). Lentini et al. (1995) reported the beneficial effects of maltose and silver nitrate on androgenesis of some recalcitrant rice genotypes.

After the first period of research, into *in vitro* androgenesis was taken up in several countries such as Korea (Harn 1969; Lee et al. 2003), China (Woo and Tung 1972), Hungary (Heszky and Pauk 1975) and recently in France (Guiderdoni et al. 1992; Alemanno and Guiderdoni 1994), in South-America (Lentini et al. 1995). High percent (61%) of albinos from shoot forming calli were reported even by Guiderdoni et al. (1992), while an average frequency of callusing anthers (18.7%) and a mean frequency (8.7%) of green plant regeneration was observed.

Colchicine was used effectively for chromosome doubling of haploids. In the protocol of Zapata-Arias (2003) haploid plants were immersed into 0.1% colchicine and 2% Dimethylsulphoxide (DMSO) solution for 4–5 hours to obtain doubled haploid lines. Chen et al. (2001) applied colchicine and oryzalin for chromosome doubling in a concentration of 1.25 mM and 25 μM for 12 hours, respectively. Colchicine can also be applied in the culture media where it can improve regeneration efficiency. Alemanno and Guiderdoni (1994) described a 1.5 to 2.5-fold increase in doubled haploid green plant production by using of colchicine supplemented media for callus induction.

Doubled haploids (DHs) have advantages in basic research. Polymorphism of AFLP markers was detected in an IR64/Azucena DH population; these markers can promote marker assisted backcrossing (Maheswaran et al. 1997). Androclonal variation was also detected in anther derived rice lines using RAPD markers (Afza et al. 2001). Genetic markers can be combined with DH populations to develop genetic maps and to identify genetic markers linked to traits of interest (Forster and Thomas 2005). In addition DHs have been used in transformation. Chair et al. (1996) reported successful transient GUS activity in rice microspore derived cell suspension protoplasts via PEG transformation system. Maize ubiquitin promoter was found to be the most active and selection was carried out by bar gene.

Anther culture is also a suitable target for mutagenesis and mutant isolation; it can be treated with chemical and physical mutagens and *in vitro* selection can be carried out (Chen et al. 2001; Lee and Lee 2002). From anther cultures treated with 0.5% Ethyl methanesulfonate (EMS) by the tenth day of culture the frequency of stable mutants was 20.7%. For the production of doubled haploid mutant lines by irradiation, 20 Gy gamma rays and 30 mg/l concentration of colchicine have been found to be effective.

The above mentioned advantages stimulated breeders to integrate the new results from *in vitro* androgenesis into their breeding programmes. Each year, more and more varieties (e.g. 'Tanfeng 1', 'Hua Yu No.1' and 'No.2', 'Xin Xiu') were released (Yin et al. 1976; Hu and Zheng 1984) using haploid induction. Other papers published new varieties originating from anther culture exhibiting higher quality and yield (Zhang 1982; Zapata et al. 1982; Hu 1985; Zhu et al. 1986; Swaminathan 1986; Heszky et al. 1989; Lapitan et al. 2004).

This paper summarises new improvements of rice breeding protocols in Hungary by integrating the *in vitro* haploid induction into the pedigree breeding method. These have led to the release of the varieties: 'Risabell', 'Janka', 'Ábel'.

Material and Methods

Plant Material and Breeding Method

In Hungary, the classic Pedigree method starts by crossing selected parents (P_1, P_2, … P_n) to obtain genetic variability for specific selections. Traditionally, from the F_2 segregated generation – and the following generations – elite panicle selections are carried out (conventional part of Fig. 1). From the $F_{4–5}$ generations performance testing of the selected lines is carried out in parallel with the elite head selections from the older generations. The biotechnological methods (anther culture, haploid cell culture, somatic tissue culture) were combined with our conventional breeding methods to develop new high yielding and improved quality varieties 'Risabell' (1997), 'Janka' (2003), 'Ábel' (2005).

The three rice varieties were derived from different basic materials. All the mentioned genotypes belong to *Japonica* subspecies. The 'Risabell' and 'Ábel' originated from different single cross combinations, Miara/No.3 mutant and Kaláris/Timis, respectively. During their breeding process the *in vitro* haploid technology and the pedigree breeding method were combined. 'Janka' was derived by combining haploid cell suspension (in N6 liquid medium supplemented with 1 mg/l 2,4-D), *in vitro* haploid technology and a traditional breeding programme. Its basic source was the Hungarian bred 'Nucleoryza' (an induced mutant of the French variety 'Cesariot').

The new lines were integrated into further breeding processes and the best selected lines were released as new varieties after 3 years of official national testing (National Institute for Agricultural Quality Control, OMMI).

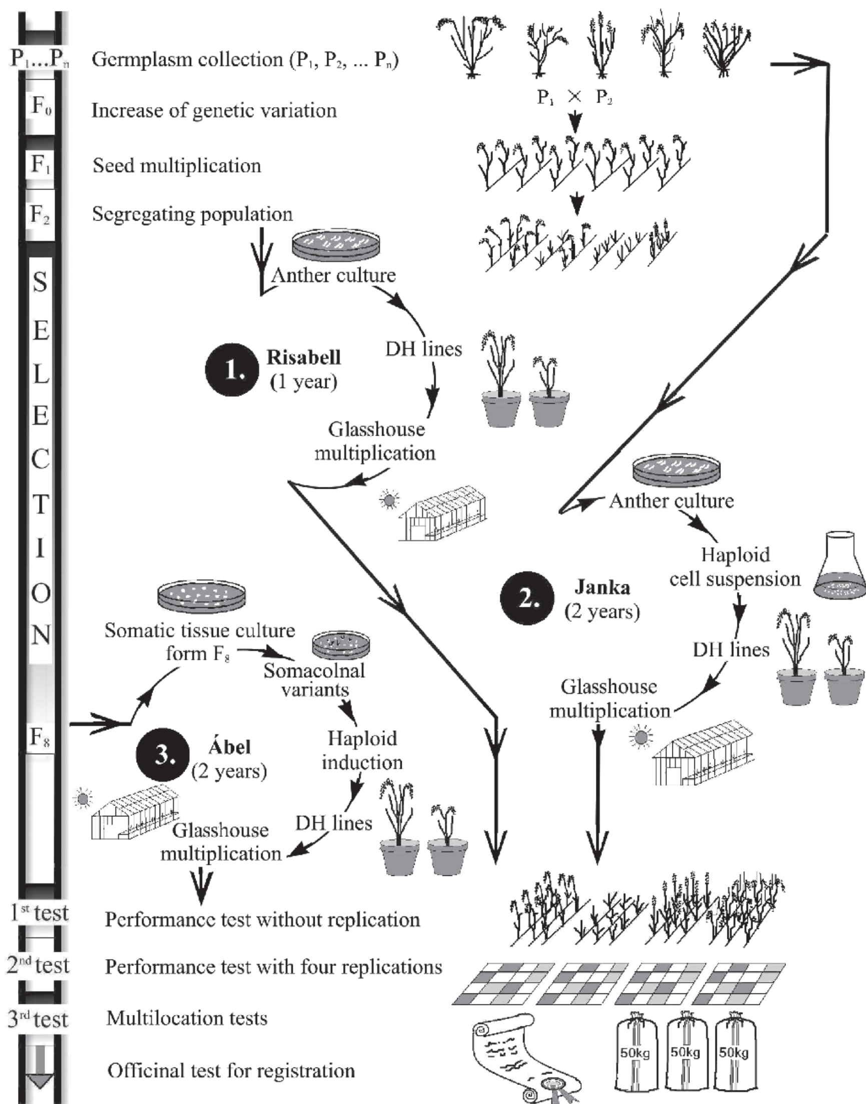


Fig. 1 Integration of *in vitro* haploid technology into the pedigree breeding method in Hungary

Tissue Culture- and Doubled Haploid Technology

The main tillers of donor elite plants were collected during the second part of July from a rice nursery in Szarvas. At this time the microspores were in early to middle vacuolated uni-nucleated developmental stage. The donor tillers were put into an Erlenmeyer flask with tap water and covered with PVC bags and cold treated at

5–6°C for about 2-weeks. Following the cold pretreatment the panicles were surface-sterilized with 50% commercial bleach (containing 2.5% NaO'Cl and 4–5 drops/l Tween) for 20 minutes on a shaker and then washed 2–3 times with sterilized distilled water. The disinfected panicles containing predominantly middle vacuolated uni-nucleated microspores were selected and isolated after a standard sterile isolation protocol. The microspore developmental stage was determined simply by crushing the anthers in a drop of water and inspecting using a light microscope.

The anthers from the florets were collected with fine forceps and tapped onto the rim of Petri dish (glass, normal size: 90 mm) so that the anthers lay on the semi-solid induction medium (250 anthers/dish onto 40 ml medium). N_6 basic medium was used (Chu et al. 1975). The one-step androgenesis system was used so that induction and regeneration were carried out in the N_6NAA medium (N_6 supplemented with 1 mg/l NAA, 30 g/l sucrose, 1.8 g/l Phytagel, pH 5.8 before autoclaving).

After isolation, the cultures were kept in a dark incubator at 28°C until the appearance of calli (about 4–5 weeks). For regeneration, the Petri dishes were transferred to 16/8 light/dark photoperiod conditions (20 μE/m^2/s irradiance) in a culture room at a constant 28°C temperature. The green regenerated plantlets were separated into individual culture tubes for rooting and tillering, keeping the plantlets in the same culture room. After about 4–5 weeks of incubation the well-rooted plantlets were transplanted into a glasshouse. The newly transplanted plantlets were covered with plastic bags which were removed after some days when the transplanted plantlets had become acclimatised to the autotrophic conditions.

The haploids were treated with 0.1% colchicine solution with 2% DMSO in tap water for 5 hours. The spontaneous and colchicine induced DH lines produced fertile seeds, which were propagated, tested for agronomical value and then integrated into a traditional breeding programme.

In the case of somatic (immature panicle) tissue culture in the Kalaris/Timis F_8 lines, the somatic tissue cultures were induced in dark on N_6 medium supplemented with 2 mg/l 2,4-D, 30 g/l sucrose, 1.8 g/l Phytagel, pH 5.8. The auxin content was reduced in stages (1, 0.5, 0.2 mg/l). The regeneration was carried out on auxin-free medium during the fifth month of subculture in a culture room. All of the chemicals used in haploid and somatic tissue cultures were bought from Sigma.

Results

Production of DH Lines via Anther Culture, 'Risabell' (1997)

The segregated F_2 generation of Miara/No.3 mutant single cross combination was used for an *in vitro* haploid induction experiment. Seventy-six percent of the regenerants were haploid and were treated with colchicine. Subsequently, from 27 colchicine treated haploids, DHs were obtained (Table 1). From the regenerants six were spontaneous diploids producing fertile seeds. Later, seedlings with doubled

Table1 Development details of three released rice doubled haploid varieties

Pedigree	Colchicine treated haploids	DH lines*	Selected DHs 1st year	Selected DHs 2nd year	Name (year of registration)
Miara/No.3 mutant	27	33	25	1	'Risabell' (1997)
Nucleoryza	11	20	9	2	'Janka' (2003)
Kaláris/Timis	20	31	12	1	'Ábel' (2005)

*Spontaneous and colchicine doubled.

chromosomes were placed in a glasshouse together with spontaneous diploids and propagated. Thirty-three DH lines were passed through nursery selection. After the first year of observation and propagation of DH lines 25 were selected for inclusion in four replicated performance tests. From the 25 DH lines the best were selected (Table 1) for official national test by OMMI. After the 3-year examination our DH line with name of 'Risabell', was released as a new variety. Main characteristics of 'Risabell' are resistance to blast disease, high milling and cooking quality and long grain type (long B). Together, the *in vitro* and glasshouse process took 1 year (Fig. 1).

Development of haploid cell suspension, regeneration and selection for fertility: 'Janka' (2003).

In the haploid cell suspension experiment the objective was to improve the regeneration capacity of cell suspension. We wanted to develop a high regenerating haploid cell suspension culture for protoplast-plant system. In the first step, androgenic calli were induced in anther culture of the variety 'Nucleoryza'. From the individual anther culture-derived calli, independent micro-suspensions were induced and the cells were propagated after a cell suspension protocol. The suspensions (over 100) were selected during the regeneration experiments and the regenerants were transplanted into the glasshouse. Eleven haploid plantlets with vigorous plant habit were colchicine treated to produce seeds. Nine plants produced seeds without colchicine treatment. Altogether 20 DH lines (colchicine induced and spontaneous diploids) were obtained from this experiment (Table 1). These lines were tested for agronomic parameters and after the first nursery selection only nine lines were found to have significantly better agronomic parameters then the control varieties. Three lines had an outstanding grain profile index when compared to the basic variety, 'Nucleoryza'. After the second year of nursery two lines were selected. From four replicated performance tests the best were selected for variety registration test by OMMI (Table 1). After 3 years of positive results this line was released as a new variety, named 'Janka'. Main characteristics of this variety are earliness, vigorous seedling growth, drought tolerance and good grain quality (long A). The haploid suspension, cell-level "selection", modifications and seed propagation of 'Janka' took 2 years (Fig. 1/2).

Selection for somatic regeneration, DH production, field abiotic stress selection: 'Ábel' (2005).

In the F_8 generation the somatic tissue-derived regenerants were tested for tissue culture ability to determine if there was a correlation between somatic tissue culture ability and abiotic stress resistance. The Kalaris/Timis single cross combination was carried out in a traditional (pedigree) breeding programme. Among the tested lines the Kalaris/Timis F_8 line showed the best somatic regeneration results. In the glasshouse significant differences were noted in the earliness and fertility in the somatic tissue-derived lines. Haploid induction was carried out on the lines selected (Fig. 1/3). From this experiment 20 colchicine treated haploids doubled and produced seeds and, together with spontaneous (11 lines) diploids, 31 DH lines proceeded to the nursery for abiotic stress (cold) selection. There were 3 years of nursery testing and seed propagation that focused on earliness and early stage cold tolerance. The best line with good agronomic value (Table 1) was subjected to official national tests in 2003, named 'Ábel' it was released in 2005. In the most recent experiments 'Ábel' has demonstrated good performance in aerobic cultivation systems too.

Discussion

In vitro haploid induction and somatic tissue culture improvements and traditional breeding steps were combined and used for practical goals to release modern rice genotypes for the Carpathian basin, particularly for Hungary. The rice breeding improvements summarised here were generated to last 16–17 years. These results confirmed the applicability of the *in vitro* induced lines, DH genotypes in breeding published earlier in rice (Heszky et al. 1996; Khush and Virmani 1996; Thomas et al. 2003; Lapitan et al. 2004) and in other crops like barley, rapeseed, wheat etc. (Forster and Thomas 2005; Cistué et al. 2004). Our results confirmed – in harmony with the previously cited papers – the importance of the new biotechnology methods in plant breeding. The new methods combined with traditional breeding processes could produce new released varieties with good agronomical values.

Acknowledgements This work was supported partially by three different grants (BIO-00140 KPI; NKTH 4 064 04; GVOP 0096/3.0).

References

Afza R, Xie J, Shen M, Zapata Arias FJ, Fundi HK, Lee K, Bobadilla-Mucino E, Kodym A (2001) Detection of androclonal variation in anther-cultured rice lines using rapds. In Vitro Cell Dev Biol Plant 37(5):644–647

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant regeneration from rice (Oryza sativa L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep 13(8):432–436

Chair H, Legavre T, Guiderdoni E (1996) Transformation of haploid, microspore-derived cell suspension protoplasts of rice (Oryza sativa L.). Plant Cell Rep 15(10):766–770

Chen CC (1977) In vitro development of plants from microspores of rice. In Vitro Cell Dev Biol Plant 13(8):484–489
Chen QF, Wang CL, Lu YM, Shen M, Afza R, Duren MV, Brunner H (2001) Anther culture in connection with induced mutations for rice improvement. Euphytica 120:401–408
Chu CC (1978) The N6 medium and its applications to anther culture of cereal crops. In: Proceedings of Symposium on Plant Tissue Culture. May 25–30, 1978, Science Press, Peking, pp. 43–50
Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Pi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668
Cistué L, Vallés MP, Echávarri B, Sanz M, Castillo AM (2004) Production of barley doubled haploids by anther and microspore culture. In: Munjib A, Cho MJ, Predieri S, Banerjee S (eds) In Vitro Application in Crop Improvement. Science Publishers, Enfield, pp. 1–17
Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88
Guha S (1973) Genotipic differences in the in vitro formation of embryoids from rice pollen. J Exp Bot 24:139–144
Guha S, Iyer RD, Gupta N, Swaminathan MS (1970) Totipotency of gametic cells and the production of haploids in rice. Curr Sci 39:174–176
Guiderdoni E, Galinato E, Luistro J, Vergara G (1992) Anther culture of tropical japonica x indica hybrids of rice (Oryza sativa L.). Euphytica 62(3):219–224
Harn D (1969) Studies on the another culture of rice. Korean J Breed 1:1–11
Heszky LE, Li SN, Simon-Kiss I, Lökös K, Gyulai G, Kiss E (1989) Organ-specific and ploidy-dependent somaclonal variation; a new tool in breeding. Acta Biol Hung 40(4):381–394
Heszky L, Pauk J (1975) Induction of haploid rice plants of different origin in anther culture. Il Riso 24(3):197–204
Heszky LE, Simon-Kiss I, Do Quangh B (1996) Release of rice variety 'DAMA' developed trough haploid somaclone breeding. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Volume 36 Somaclonal variation in Crop Improvement II. Springer Verlag, Berlin/Heidelberg/New York, pp. 46–54
Hu H, Zeng JZ (1984) Development of new varieties via anther culture. In: Ammirato PV, Evans DA, Sharp WR, Yamada Y (eds) Handbook of Plant Cell Culture, Volume 3. Macmillan, New York, pp. 65–90
Hu H (1985) Use of haploids in crop improvement. In: Biotechnology in International Agricultural Research. Proceedings of Inter-Seminar IARC's and Biotech. International Rice Research Institute College, Laguna, Philippines, pp. 763–772
Khush GS, Virmani SS (1996) Haploids in plant breeding. In: Jain MS, Sopory SK, Veilleux RE (eds) In Vitro Haploid Production in Higher Plants. Volume 1: Fundamental Aspects and Methods. Kluwer, Dordrecht, The Netherlands, pp. 11–33
Lapitan VC, Cayaban, EB, Roferos LT, San Valentin GO, Sebastian LS (2004) Utilization of anther culture technique for rice improvement in the Philippines. In: Fischer T, Turner N, Angus J, McIntyre L, Robertson M, Borrell A, Lloyd D (eds) New directions for a diverse planet: Proceedings of the 4th International Crop Science Congress, Brisbane, Australia, 26 Sept–1 Oct 2004, Published on CDROM
Lee JH, Lee SY (2002) Selection of sable mutants from cultured rice anthers treated with ethyl methane sulfonic acid. Plant Cell Tiss Org Cult 71(2):165–171
Lee SY, Lee JH, Kwon TO (2003) Selection of salt-tolerant doubled haploids in rice anther culture. Plant Cell Tiss Org Cult 74(2):143–149
Lentini Z, Reyes ZP, Martinez CP, Roca WM (1995) Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Sci 110:127–138
Maheswaran M, Subudhi PK, Nandi S, Xu JC, Parco A, Yang DC, Huang N (1997) Polymorphism, distribution, and segregation of AFLP markers in a doubled haploid rice population. Theor Appl Genet 94(1):39–45
Miller CO (1953) Reversible inhibition of cell division and enlargement in plant tissue by 2,6-diaminopurine. Proc Soc Expt Biol Med 83:561–565

Niizeki H, Oono K (1968) Induction of haploid rice plants from anther culture. Proc Jpn Acad 44:544–557
Niizeki H, Oono K (1971) Rice plants obtained by anther culture. Les Cultures de Tissus des Plantes. Colloq Int CNRS (Paris) 193:251–257
Nishi T, Mitsuoka S (1969) Occurrence of various ploidy plants from anther and culture of rice plant. Jpn J Genet 44:341–346
Reiffers I, Freire AB (1990) Production of doubled haploid rice plants (Oryza sativa L.) by anther culture. Plant Cell Tiss Org Cult 21(2):165–170
Swaminathan MS (1986) Plant research and world agriculture. Plant Mol Biol Rep 4(1):1–17
Thomas WTB, Forster BP, Gertsson B (2003) Doubled haploids in breeding. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer, Dordrecht, The Netherlands, pp. 337–349
Woo SC, Tung IJ (1972) Induction of rice plants from hybrid anthers of indica and japonica cross. Bot Bull Acad Sin 16:19–24
Yin KC, Hsu C, Chu CY, Pi FY, Wang ST, Liu TY, Chu CC, Wang CC, Sun CS (1976) A study of the new cultivar of rice raised by haploid breeding method. Sci Sin 19:227–242
Zapata FJ, Torrizo L, Romero RD, Alejar MS (1982) Androgenesis in Oryza sativa. In: Fujiwara A (ed) Plant Tissue Culture. Maruzen, Tokyo, pp. 531–532
Zapata-Arias FJ (2003) Laboratory protocol for anther culture technique in rice. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer, Dordrecht, The Netherlands, pp. 109–116
Zhang ZH (1982) Application of anther culture techniques to rice breeding. In: Proceedings on Rice Tissue Culture Planning Conference. IRRI, Los Banos, pp. 55–61
Zhu D, Pan X, Liu W (1986) Developing high quality rice via anther culture of hybrid rice. In: Abstract of the 6th International Congress on Plant Tissue and Cell Culture, Minnesota, p. 413

Chapter 17
Potato Haploids and Breeding

V.-M. Rokka

Abstract Potato (*Solanum tuberosum* L. ssp. *tuberosum*) was one of the first crop plants in which haploid techniques were used to improve cultivar breeding programmes. These new breeding tools were introduced towards the end of the 1950s but have not totally replaced the conventional breeding of potato at the tetraploid (2n = 4x) level. Generally (di)haploid (2n = 2x) lines are produced by pollination of cultivated potato and related *Solanum* species with specific haploid inducer clones of *S. phureja* or alternatively by anther culture *in vitro*. The resultant clones provide excellent material for the subsequent reconstitution of the polyploid hybrids having maximized heterozygosity levels. Therefore the haploids have a considerably significant role in the potato breeding programmes of quite a few companies. Certain valuable haploid techniques, such as anther culture and somatic hybridization, are quite complex and highly genotype dependent and thus are less readily put into practise. There is an important application for use of haploids in interspecific hybridization to overcome incompatibility barriers caused by the differences in ploidy levels and endosperm balance numbers. Thus, the gene pool of the potato can be broadened and certain valuable traits such as disease resistance characters from the wild solanaceous species can be more efficiently introgressed into cultivated potato.

Keywords 2n gamete, anther culture, diploid breeding, haploid, potato, solanaceous· species, *Solanum*, somatic hybrid

Introduction

There are more than 200 wild potato species named, but only a limited number of them have been utilised in breeding for potato germplasm development. Many solanaceous species are diploid (2n = 2x = 24), and therefore cannot readily cross

V.-M. Rokka(✉)
MTT Agrifood Research Finland, Biotechnology and Food Research,
Plant Genomics, FI-31600 Jokioinen, Finland
E-mail: veli-matti.rokka@mtt.fi

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

with tetraploid (2n = 4x = 48) cultivated potato (*Solanum tuberosum* L. ssp. *tuberosum*). Incompatibility is common, because certain tetraploid wild potato species, such as *S. acaule*, do not hybridise with cultivated potato due to differences in the endosperm balance numbers (EBNs). The overwhelming breeding barriers caused by ploidy level differences and EBN variations could, however, be partly overcome by induction of haploid lines. Therefore, haploids produced by anther culture or alternatively by *Solanum phureja* (haploid inducer) pollinations, can be utilised in hybridisations to broaden the gene pool of potato, and to increase the genetic diversity in terms of transferring valuable traits e.g. disease resistance genes. Haploids are an excellent tool for genetic studies such as the analysis of the polyploidy status of *Solanum* species and their interspecific hybrids, and for analysis of genetic basis of traits (Peloquin et al. 1991; Bouarte-Medina et al. 2002; Gavrilenko et al. 2001). Haploids can also be used for genetic mapping and in identifying genes which control disease or pest resistance (Pineda et al. 1993; Song et al. 2005).

Potato was actually one of the first crop plants in which haploid breeding techniques were used (Hougas and Peloquin 1958; Chase 1963). As an alternative to conventional breeding of tetraploid potato, breeding programmes can be carried out at the diploid level. In potato breeding, selection for polygenic traits should be oriented towards maximizing heterozygosity. With (di)haploids the efficient trait combination levels can be obtained through 2n gamete formation capacity to reconstitute the polyploid genome. Alternatively, protoplast fusion techniques can be utilised to combine the complete genomes (nuclear and cytoplasmic), including all the dominant characters of the haploid lines.

Induction of Haploid Lines from Potato and Related Solanaceous Species

Haploid Induction Techniques for Solanum Species

The first known haploid plant in nature was discovered in Jimson weed (*Datura stramonium*), which is noteworthy as it is a wild solanaceous species (Blakeslee et al. 1922). Subsequently, in terms of cultivated potato, certain interspecific crosses were demonstrated to induce haploids (Ivanovskaja 1939). Crossing efforts between hexaploid *S. demissum* wild species and diploid *Solanum* species also resulted in (tri)haploid (2n = 36) plants. These results were further utilised to enhance haploid induction frequencies in cultivated *S. tuberosum* lines by pollinations with specific clones of *S. phureja* (Hougas et al. 1958).

Anther culture is based on sporophytic development of microspores in anthers grown *in vitro*. Another solanaceous species, *Datura innoxia* was the first plant species in which embryo formation and shoot regeneration derived from isolated anthers was reported (Guha and Maheswari 1964). These initial experiments were soon followed by the successful *in vitro* haploid production in tobacco (Nitsch and

Nitsch 1969). The *Solanum* species were, however, more recalcitrant in attempts to induce androgenic response, but production of an anther-derived haploid plant from wild potato species *S. verrucosum* was reported by Irikura and Sakaguchi (1972). In cultivated potato, Kohlenbach and Geier (1972) were the first to demonstrate adventitious embryos from anther-derived callus cultures, but the embryos failed to undergo shoot regeneration. These studies were subsequently followed by *in vitro* produced entire (di)haploid plant from potato cv. Pentland Crown (Dunwell and Sunderland 1973). Despite this early work, the development of *in vitro* haploids from commercial clones has been limited. There have been a few tetraploids, which showed high androgenic efficiency (Uhrig and Salamini 1987), but still only a limited number of cultivars formed anther-derived shoots (Tiainen 1992). Compared to tetraploids, anther culture of diploid cultivated potato lines responded better (Uhrig 1985). Plant regeneration through isolated microspore culture was not reported by Sopory (1977) or Bugárová and Pretová (1996). Despite the previous restraints in haploid induction, in Finland we were able to improve the frequency of green shoot formation of tetraploid cultivated potato through gametic embryogenesis (Rokka et al. 1996; Rokka 2003). The same protocol (Rokka 2003) was subsequently applied in other countries, such as in the UK, Italy and Germany, and Schwarzfischer et al. (2002) reported that the highest response was obtained using that protocol in comparison to the other anther culture methods tested.

Gametic Embryogenesis in Potato – The Influence of Genetic and Environmental Factors

Androgenic plants are regenerated either through organogenesis (formation of microsporial callus) or embryogenesis (formation of embryo-like structures) from microspores of *in vitro* grown anthers. In general, shoot regeneration via gametic embryo formation is preferred over regeneration via organogenesis because the somaclonal variation frequencies are generally higher in plants that originate from callus (Sopory and Tan 1979). Therefore in Finland, we only accept shoots obtained through gametic embryogenesis for further development analyses (Rokka 2003).

In anther culture, plant development from microspores can be initiated from vegetative cells, generative cells, or their fused cells (Sunderland and Evans 1980). For successful culture of potato, anthers are isolated when the microspores are at their late tetrad or at early uni-nucleate stage (Calleberg 1996). The anther culture response is both environmentally and genotypically controlled. Calleberg (1996) stated four different factors that effect potato anther culture response: (1) environmental origin of the donor plants, (2) developmental stage of the microspores, (3) pretreatment of anthers, and (4) culture conditions including media components. In tetraploid potato, Wenzel et al. (1984) demonstrated that only 0.6% of cultured anthers responded by producing green shoots. It was hypothesised that the low haploid induction levels could be a result of the presence of sub-lethal alleles in the heterozygous anther donor plants (Wenzel and Uhrig 1981). Regardless of that, some breeding lines showed

superior androgenic efficiency (Uhrig 1985; Uhrig and Salamini 1987), the potential of which was also inheritable. Sopory (1977) and Singsit and Veilleux (1989) suggested that different developmental stages in embryo production and plant regeneration were each under independent genetic control, probably controlled by two (Meyer 1991) or even several genes (Sonnino et al. 1989).

Haploid Lines in Potato Breeding

Breeding with Diplogametes

Most of the dihaploids obtained from cultivated potato can be maintained under field conditions. However, their gross yield is only about 50% that of the tetraploid cultivars. Intense selection at the diploid level and the subsequent reconstitution of tetraploids are therefore essential. The major concern of using primary dihaploids in breeding is connected to their low fertility levels, especially in terms of male gametes (De Maine 1997). Therefore the production of further generation of dihaploids (i.e. inter-dihaploids) via 2x x 2x crosses is considerably difficult (De Maine 1997; Schwarzfischer et al. 2002). Only highly selected dihaploid clones with high fertility can be crossed sexually. That may result in both improved agronomic traits and also in improved fertility in generating more dihaploids.

Chase (1963) proposed and pioneered an analytical potato breeding procedure using diploid lines. This scheme was later used for subsequent breeding schemes (e.g. Wenzel et al. 1979). The main advantage of using dihaploid lines over traditional potato breeding at the tetraploid level is based on their capacity to form unreduced 2n gametes (Peloquin et al. 1991). The dihaploid potato clones form diplandroids (male 2n gametes) more frequently compared to their diplogynoid (female 2n gametes) frequencies (Hutten 1994).

At the diploid level, breeding using diplogametes, both unilateral 4x x 2x (or rarely 2x x 4x) and bilateral 2x x 2x crosses are used to generate tetraploid progeny to maximise the heterozygosity levels in the resulted tetraploid hybrids (Hutten 1994). It has been demonstrated that more than 80% of the heterozygosity of the dihaploid parent can be transmitted via 2n gametes to the tetraploid progeny (Peloquin et al. 1991). At least two commercial potato varieties have been released on the market as a result of breeding with diplogametes (Hutten 1994).

Interspecific Crosses at the Diploid Level

Haploid lines are an excellent tool for the genetic enhancement of cultivated potato because they can be utilised to overcome incompatibility boundaries between different species. As a result of an interspecific cross, the normal endosperm

development occurs only when there is a 2:1 maternal to paternal EBN (endosperm balance number) ratio in the hybrid endosperm (Johnston et al. 1980). The diploid hybrid progeny produced as a result of wild *Solanum* spp. x primary dihaploid hybridisation has shown improved characters such as higher fertility than in the primary dihaploids, and also improved vigour and yield with high breeding values and are therefore excellent material for introgression (Peloquin et al. 1991).

Somatic Hybridisation

Since dihaploid lines induced from cultivated potato often show low fertility, somatic hybridisation is an alternative technique to combine characters of both parental dihaploid clones. Introduction of the analytic-synthetic breeding scheme by Wenzel et al. (1979) was the start of potato breeding using a combination of both anther culture and subsequent protoplast fusion techniques to produce tetraploid potato hybrids with maximised heterozygosity levels. Through cell fusion, all of the dominant characters of the dihaploid lines are theoretically expressed in the resulting somatic hybrid, which may subsequently have a capacity for development as a commercial variety.

Although the first experiences in protoplast fusion between two dihaploid lines of cultivated potato were not very promising, the introduction of electrical cell fusion, optimisation of shoot regeneration protocols and development of more sophisticated molecular techniques for hybrid identification enabled high numbers of somatic hybrids, which were also found to have good agronomic traits. Certain hybrid combinations are still difficult or even impossible to obtain, which may be caused by differences in the cytoplasmic compositions of the parental dihaploid lines (Lössl et al. 1999). The fusion technology also enables the hybridisation of taxonomically distant species, i.e. it is a practical tool for the genetic enhancement programmes.

Related to that, haploid lines are valuable materials for interspecific somatic hybridisation, because the final ploidy levels and theoretical EBNs in the resulted hybrids can be estimated for successful backcrosses (Rokka et al. 2005).

Haploids in Practical Breeding

Many potato breeding companies actively use the techniques described in the current review. Based on a survey of relevant companies, the induction of primary dihaploids through *S. phureja* pollinations and the utilisation of unreduced gametes are the main techniques applied in breeding at the diploid level. As an example, HZPC in the Netherlands described that breeding at the diploid level has a significant position in the overall potato breeding work of the company, but currently new primary dihaploids are no longer induced. The diploid clones which were previously produced can now function as a bridge for the introgression of genetic

material from wild *Solanum* species. Another value for using haploids is their potential in gene stacking at the diploid level and to deliver them as a cassette into tetraploid clones. HZPC also carries out diploid breeding by diplogametes, mainly with 2x x 2x, or alternatively with 4x x 2x, which they call 'valention crosses' (Dr. R. Graveland 2008, personal communication). Similarly, a relatively intensive diploid breeding programme is carried out by Agrico Research, the Netherlands. The company produces spontaneously doubled 4x materials from their highly selected diploid clones to circumvent the lack of male fertility. The doubled 4x clones are further crossed with tetraploids. Also 4x x 2x crosses with the use of diplogamete formation capacity are made (if the diploid clones have sufficient male fertility). Primary dihaploids are induced through *S. phureja* pollinations, but only rarely at present. The somatic hybridisation programme has been ceased by Agrico Research (Dr. S. Allefs 2008, personal communication).

The Spanish company APPACALE also uses diploid breeding, but similar to HZPC and Agrico, no primary dihaploid lines are induced currently. The reconstitution of tetraploid genome is obtained through diplogametes (unilateral sexual 4x x 2x polyploidization) or via somatic hybridisation. In addition, introgression of valuable traits from 1EBN wild species is obtained through protoplast fusion (Dr. A.C. Perez 2008, personal communication).

NORIKA in Germany considers the use of unreduced gametes in pre-breeding programmes once the company finds interesting clones for breeding (Dr. H. Junghans 2008, personal communication). Europlant (Germany) described the use of *S. phureja* pollination for dihaploid induction and the company also carries out crosses with unreduced gametes. The somatic hybridisation programme run earlier in collaboration with Agrico Research has now been terminated, but field tests for approximately 500 somatic hybrids are still carried out (Dr. H.R. Hofferbert 2008, personal communication). LKF Vandel (Denmark) produces dihaploid lines with *S. phureja* pollination and diplogametes are the main tool used to reconstitute the tetraploid genome in the company (Dr. J.-P. Nepper 2008, personal communication).

Anther Culture or Pollination with *Solanum phureja*

Anther culture to induce primary haploids from cultivated potato clones and related species can now be applied in a range of solanaceous genetic resources. Although *S. tuberosum* was ranked previously as a recalcitrant species due to a poor androgenic capacity (Veilleux 1996), we have obtained plants in genetically diverse potato material through gametic embryogenesis (Rokka et al. 1996). Over 20 commercial cultivars have shown androgenic response with dihaploid green plant formation. However, the genotypic influence remains obvious, and Schwarzfischer et al. (2002) concluded that anther culture was very efficient in only one cultivar (cv. Assia). In addition, Asakavičiūtė et al. (2007) demonstrated that only three potato cultivars (cvs. Goda, Nida and Aista) responded by plant regeneration as a result of anther culture. The *S. phureja* haploid induction method was suitable to all

cultivated potato genotypes according to the studies of Schwarzfischer et al. (2002), but Hutten (1994) concluded that the genotypic differences in the frequency of successful pollinations and in the frequency of dihaploids obtained were also common as a result of *S. phureja* pollinations.

Schwarzfischer et al. (2002) described that both haploid techniques are very laborious in terms of plant induction, but in certain cases, *in vitro* anther culture may be preferable. Some of the advantages of anther culture over *S. phureja* pollinations are: (A) faster shoot formation rate without a seed dormancy stage, (B) relatively high frequency of plants with haploid genome constitution can be obtained, (C) no alien genetic material is found in the resulted dihaploids, (D) more vigorous plants with higher agronomic values can be obtained.

Unfortunately, reduction in ploidy to haploid level is not always certain as a result of anther culture (Rokka et al. 1996). Based on studies carried out in Finland, most of the anther-derived regenerated plants had reduced chromosome numbers, but the frequency of dihaploids was extremely dependent on the genotype. Schwarzfischer et al. (2002) concluded that the frequency of dihaploids derived from anther culture was much higher than the frequency obtained from *S. phureja* crosses. The major concern of *S. phureja* pollination is the formation of aneusomatic plants that contain sections of the wild *S. phureja* genome (Clulow et al. 1991). This indicates that *S. phureja* is strongly involved in the haploid formation and the actual mechanism in reduction of ploidy is related to chromosome elimination, rather than pseudogamy (Ercolano et al. 2004).

Despite considerable progress to date, improvements in haploid induction techniques are still urgently needed. Kopecký and Vagera (2005) recently reported the positive influence of chemomutagenic treatments on the anther culture response in *S. nigrum*. In addition, microspore culture may be preferred over anther culture, because inhibitors produced in anther wall cells can inhibit gametic embryogenesis (Weatherhead and Henshaw 1979). Regardless of some microspore-derived embryos obtained in potato cv. Albina, reports on microspore culture have been very limited to date (Bugárová and Pretová 1996; Millam 2001).

The superior growth habit of androgenic potato haploids over *S. phureja* induced clones was shown in a very detailed study by Lough et al. (2001), where anther-derived haploids had greater leaf size, greater height and even higher yields than their *S. phureja* induced counterparts. Similar observations have also been made in Finland. One concern may be linked to the genetic studies which have shown skewed segregation in the populations produced by anther culture (Rivard et al. 1996; Chani et al. 2002).

Conclusion

It is clear from the above review that haploids are an excellent tool for the improved introgression of specific traits originating from wild species into cultivated potato. Haploids can now be produced even from allopolyploid hybrids between genetically

distant species including potato, tomato and eggplant (Gavrilenko et al. 2001; Rizza et al. 2002; Ermishin et al. 2006). This opens up potential for the wider utilisation of solanaceous species genetic resources to overcome hybridisation barriers and to produce breeding lines with suitable EBNs for successful backcrosses with *S. tuberosum*. This may also provide us with completely new sources of resistance for disease control, such as in terms of potato late blight caused by *Phytophthora infestans*. Some examples of recently applied resources of durable resistance to late blight are originated from *S. bulbocastanum* (Helgeson et al. 1998) and *S. nigrum* (Zimnoch-Guzowska et al. 2003). Our recent study by Väänänen et al. (2005) has also shown that even completely novel compounds may be introduced by use of combined genomes of haploid clones.

Acknowledgements The author would like to acknowledge Dr. Steve Millam, Chichester College, Department of Horticulture, UK, and Mrs. Jill Middlefell-Williams, Scottish Crop Research Institute, Dundee, UK, for the critical review of the article.

References

Asakavičiūtė R, Clement C, Ražukas A (2007) The genetic aspect in anther culture of Lithuanian potato (*Solanum tuberosum* L.) cultivars. Biologija 18:19–22

Blakeslee AF, Belling J, Farnham ME, Bergner AD (1922) A haploid mutant in the Jimson weed,"*Datura stramonium*". Science 55:646–647

Bouarte-Medina T, Fogelman E, Chani E, Miller AR, Levin I, Levy D, Veilleux RE (2002) Identification of molecular markers associated with leptine in reciprocal backcross families of diploid potato. Theor Appl Genet 105:1010–1018

Bugárová Z, Pretová A (1996) Isolated microspore cultures in *Solanum tuberosum* cultivars. Biologia 51:411–416

Calleberg E (1996) Anther culture of potato. Ph.D. thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden

Chani E, Ashkenazi V, Hillel J, Veilleux RE (2002) Microsatellite marker analysis of an anther-derived potato family: skewed segregation and genome-centromere mapping. Genome 45:236–242

Chase SS (1963) Analytic breeding in *Solanum tuberosum* L. – A scheme utilizing parthenotes and other diploid stocks. Can J Genet Cytol 5:359–363

Clulow SA, Wilkinson MJ, Waugh R, Baird E, De Maine MJ, Powell W (1991) Cytological and molecular observations on *Solanum phureja*-induced dihaploid potatoes. Theor Appl Genet 82:545–551

De Maine MJ (1997) The male fertility of primary and further generation dihaploids of *Solanum tuberosum*. Potato Res 40:59–68

Dunwell JM, Sunderland N (1973) Anther culture of *Solanum tuberosum* L. Euphytica 22:317–323

Ercolano MR, Carputo D, Li J, Monti L, Barone A, Frusciante L (2004) Assessment of genetic variability of haploids extracted from tetraploid (2n = 4x = 48) *Solanum tuberosum*. Genome 47:633–638

Ermishin AP, Mahanko OV, Voronkova EV (2006) Application of somatic hybrids between dihaploids of potato *Solanum tuberosum* L. and wild diploid species from Mexico in breeding: generation and backcrossing of dihaploids of somatic hybrids. Rus J Genet 42:1414–1421

Gavrilenko T, Thieme R, Rokka V-M (2001) Cytogenetic analysis of *Lycopersicon esculentum* (+) *Solanum etuberosum* somatic hybrids and their androgenetic regenerants. Theor Appl Genet 103:231–239

Guha S, Maheswari SC (1964) *In vitro* production of embryos in *Datura*. Nature 204:497
Helgeson JP, Pohlman JD, Austin S, Haberlach GT, Wielgus SM, Ronis D, Zambolin L, Tooley P, McGrath JM, James RM, Stevenson WR (1998) Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor Appl Genet 96:738–742
Hougas RW, Peloquin SJ (1958) The potential of potato haploids in breeding and genetic research. Am Potato J 35:701–707
Hougas RW, Peloquin SJ, Ross RW (1958) Haploids of the common potato. J Heredity 49:103–106
Hutten RCB (1994) Basic aspects of potato breeding via the diploid level. Ph.D. thesis, University of Wageningen, The Netherlands
Irikura Y, Sakaguchi S (1972) Induction of 12-chromosome plants from anther culture in a tuberous *Solanum*. Potato Res 15:170–173
Ivanovskaja EV (1939) A haploid plant of *Solanum tuberosum* L. Comptes Rendus (Doklady) de l'Académie des Sciences de l'URSS 24:517–520
Johnston SA, den Nijs T, Peloquin SJ, Hanneman RE Jr (1980) The significance of genic balance to endosperm development in interspecific crosses. Theor Appl Genet 57:5–7
Kohlenbach HW, Geier T (1972) Embryonen aus *in vitro* kultivierten Antheren von *Datura meteloides* Dun., *Datura wrightii* Regel und *Solanum tuberosum* L. Zeitschrift Pflanzenphys 67:161–162
Kopecký D, Vagera J (2005) The use of mutagens to increase the efficiency of the androgenic progeny in *Solanum nigrum*. Biol Plant 49:181–186
Lössl A, Adler N, Horn R, Frei U, Wenzel G (1999) Chondriome-type characterization of potato: mt α, β, γ, δ, ε and novel plastid-mitochondrial configurations in somatic hybrids. Theor Appl Genet 98:1–10
Lough RC, Varrieur JM, Veilleux RE (2001) Selection inherent in monoploid derivation mechanisms for potato. Theor Appl Genet 103:178–184
Meyer RC (1991) Die genetische und zytologische Analyse der Kartoffel (*Solanum tuberosum* L.) nach Antherenkultur unter besonderer Berücksichtigung der Entwicklung reiner diploider Linien. Ph.D. thesis, University of Cologne, Germany
Millam S (2001) Strategies towards the application of microspore culture in potato. In: Bohanec, B. (ed) Biotechnological approaches for utilisation of gametic cells. COST 824. Office for Publications of the European Communities, Luxembourg, pp. 111–114
Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87
Peloquin SJ, Werner JE, Yerk GL (1991) The use of haploids in genetics and breeding. In: Tsuchiya T, Gupta PK (eds) Developments in Plant Genetics and Breeding 2B. Chromosome Engineering in Plants: Genetics, Breeding, Evolution. Part B. Elsevier, Amsterdam/Oxford/New York/Tokyo, pp. 79–92
Pineda O, Bonierbale MW, Plaisted RL (1993) Identification of RFLP markers linked to the *H1* gene conferring resistance to potato cyst nematode *Globodera rostochiensis*. Genome 36:152–156
Rivard SR, Cappadocia M, Landry BS (1996) A comparison of RFLP maps based on anther culture derived, selfed, and hybrid progenies of *Solanum chacoense*. Genome 39:611–621
Rizza F, Mennella G, Collonnier C, Sihachakr D, Kashyap V, Rajam MV, Presterá M, Rotino GL (2002) Androgenic dihaploids from somatic hybrids between *Solanum melongena* and *S. aethiopicum* group *gilo* as a source of resistance to *Fusarium oxysporum* f. sp. *melogenae*. Plant Cell Rep 20:1022–1032
Rokka V-M (2003) Anther culture through direct embryogenesis in a genetically diverse range of potato (*Solanum*) species and their interspecific and intergeneric hybrids. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Kluwer, Dordrecht, pp. 235–240
Rokka V-M, Pietilä L, Pehu E (1996) Enhanced production of dihaploid lines via anther culture of tetraploid potato (*Solanum tuberosum* L. ssp. *tuberosum*) clones. Am Potato J 73:1–12
Rokka V-M, Laurila J, Tauriainen A, Laakso I, Larkka J, Metzler M, Pietilä L (2005) Glycoalkaloid aglycone accumulations associated with infection by *Clavibacter michiganensis* ssp. *sepedonicus*

in potato species *Solanum acaule* and *Solanum tuberosum* and their interspecific hybrids. Plant Cell Rep 23:683–691

Schwarzfischer A, Song Y, Scholz H, Schwarzfischer J, Hepting L (2002) Haploidiezüchtung, Protoplastenfusion and Entwicklung von genetischen Markern zur gezielteren Sortenentwicklung bei Kartoffeln. Vorträge für Pflanzenzüchtung 54:123–130

Singsit C, Veilleux RE (1989) Intra- and interspecific transmission of androgenetic competence in diploid potato species. Euphytica 43:105–112

Song Y-S, Hepting L, Schweizer G, Hartl L, Wenzel G, Schwarzfischer A (2005) Mapping of extreme resistance to PVY (Ry_{sto}) on chromosome XII using anther-culture-derived primary dihaploid potato lines. Theor Appl Genet 111:879–887

Sonnino A, Tanaka S, Iwanaga M, Schilde-Rentschler L (1989) Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Rep 8:105–107

Sopory SK (1977) Development of embryoids in isolated pollen culture of dihaploid *Solanum tuberosum*. Zeitschrift für Pflanzenphysiol 84:453–457

Sopory SK, Tan BH (1979) Regeneration and cytological studies of anther and pollen calli of dihaploid *Solanum tuberosum*. Zeitschrift für Pflanzenzüchtung 82:31–35

Sunderland N, Evans LJ (1980) Multicellular pollen formation in cultured barley anthers. J Exp Bot 31:501–514

Tiainen TK (1992) The role of ethylene and reducing agents on anther culture response of tetraploid potato (*Solanum tuberosum* L.). Plant Cell Rep 10:604–607

Uhrig H (1985) Genetic selection and liquid medium conditions improve the yield of androgenetic plants from diploid potatoes. Theor Appl Genet 71:455–460

Uhrig H, Salamini F (1987) Dihaploid plant production from 4x-genotypes of potato by the use of efficient anther plants producing tetraploid strains (4x EAPP-clones) – Proposal of a breeding methodology. Plant Breed 98:228–235

Väänänen T, Ikonen T, Rokka V-M, Kuronen P, Serimaa R, Ollilainen V (2005) Influence of incorporated wild *Solanum* genomes on potato properties in terms of starch nanostructure and glycoalkaloid content. J Agric Food Chem 53:5313–5325

Veilleux RE (1996) Haploidy in important crop plants – Potato. In: Jain SM, Sopory SK, Veilleux RE (eds) In Vitro Haploid Production in Higher Plants, Vol. 3, Kluwer, Dordrecht. pp. 37–49

Weatherhead MA, Henshaw GG (1979) The induction of embryoids in free pollen culture of potatoes. Zeitschrift für Pflanzenphys 94:441–447

Wenzel G, Uhrig H (1981) Breeding for nematode and virus resistance in potato via anther culture. Theor Appl Genet 59:333–340

Wenzel G, Schieder O, Przewozny T, Sopory SK, Melchers G (1979) Comparison of single cell culture-derived *Solanum tuberosum* L. plants and model for their application in breeding programs. Theor Appl Genet 55:49–55

Wenzel G, Uhrig H, Burgermeister W (1984) Potato – A first crop improved by the application of microbiological techniques? In: Arber W, Illmensee K, Peacock WJ, Starlinger P (eds) Genetic Manipulation: Impact on Man and Society. The Garden City, Letchworth, pp. 127–137

Zimnoch-Guzowska E, Lebecka R, Kryszczuk A, Maciejewska U, Szczerbakowa A, Wielgat B (2003) Resistance to *Phytophthora infestans* in somatic hybrids of *Solanum nigrum* L. and diploid potato. Theor Appl Genet 107:43–48

Chapter 18
Current Status of Doubled Haploids in Medicinal Plants

A.M.R. Ferrie

Abstract Although herbs, spices, medicinal, and nutraceutical plants have been used in human health for millennia, there has been renewed interest over the past number of years. In the past, people have relied on landraces or "wild" plants as there has been very little breeding or genetics done on these species. This is changing as consumers are demanding scientific evidence for the medical claims (clinical trials) as well as uniformity in the products. Doubled haploidy would be one way in which to achieve homozygous, true-breeding lines. Haploidy response, i.e. callus, embryos, regenerated haploid/doubled haploid plants, have been reported in a number of medicinal/nutraceutical species, however the frequency of response is low compared to other species. This review will focus on a few of the major plant families with medicinal properties.

Keywords Apiaceae, Compositae, Labiatae, medicinal plants, nutraceuticals, Solanaceae

Introduction

There are many potential benefits for developing a doubled haploidy protocol in a species, as outlined in this book and many other reviews. This is especially so for the medicinal plants. Compared to crops like wheat, barley, *Brassica* species etc., there has been very little breeding or genetics done on the medicinal plants. This results in variation in plant populations which can cause problems if the plants are grown for commercial production to produce active compounds, conduct agronomic research, or do clinical trials. The development of doubled haploidy protocols

A.M.R. Ferrie(✉)
National Research Council/Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9, Canada
E-mail: alison.ferrie@nrc-cnrc.gc.ca

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© 2008 The National Research Council of Canada
© Springer Science+Business Media B.V. 2009

would yield uniform, true-breeding lines. These homozygous lines could be incorporated into a breeding program as starting material for varietal development or parental lines in F_1 hybrids. Doubled haploidy would also allow selection of lines yielding high levels of the important medicinal compound. However, there has been very little haploidy work done on the medicinal plants (Ferrie 2006; Ferrie et al. 2005), although micropropagation and *de novo* plant regeneration has been successful in some of these species. There are several methods available for development of doubled haploids, such as androgenesis (anther culture, isolated microspore culture), gynogenesis, and wide crosses as described in other chapters of this book. Most of the success has been with androgenesis, although this depends on the species. Table 1 is a listing of reports of production of callus, embryos or haploid/doubled haploid plants for species classified as having medicinal benefits.

It would be impossible to review all medicinally important species in this chapter, therefore the focus is made on a few plant families.

Compositae

The Compositae is the largest plant family and the most diverse. There are over 1,100 genera and 20,000 species. Many of the species are economically important as foods, oilseeds, ornamentals, industrial products, and medicinals (e.g. yarrow, chamomile, feverfew). The plants within this family contain many novel secondary metabolites, fatty acids, insecticides, and industrial chemicals. Milk thistle is known for the treatment of liver disorders. The active component is silymarin, which functions as an antioxidant. *Echinacea* is one of the top five selling medicinals and is known as an immune-stimulant. *Artemisia annua* contains the antimalarial compound, artemisinin.

The Compositae are considered recalcitrant in terms of doubled haploidy. Although anther culture, isolated microspore culture, and ovule culture protocols have been reported, there are no efficient, reliable protocols available for this family. Fifteen Compositae species were screened for microspore culture response using the *Brassica napus* microspore culture protocol (Ferrie et al. 2005). There was no response from 14 of the 15 species. Swollen microspores were observed in *Eupatorium perfoliatum* (boneset). Further experimentation has resulted in callus and haploid/doubled haploid plants in several other compositae species (Ferrie 2007).

Haploid plants were regenerated from anther culture of purple coneflower (*Echinacea purpurea* L.) (Zhao et al. 2006). The authors evaluated basal media (N6, MS) and growth regulators (BA, NAA, 2,4-D). Callus was observed after 2 weeks and reached maximum production after 4 weeks. Plants were regenerated from this callus. Of the 30 plants analyzed, 19 were haploid, indicating an androgenic origin.

Hieracium pilosella (mouse-ear hawkweed) is native to Europe and North Asia and is classified as a weed and as a noxious weed in some parts of the USA. This plant contains umbelliferone, which is used in sunscreens. An anther culture

Table 1 List of nutraceutical/medicinal plant species in which doubled haploidy response has been observed. (A = anther culture, G = gynogenesis, IMC = isolated microspore culture, C = callus, E = embryos, P = plant) (modified from Ferrie 2006)

Species	Common name	Method	Results	Reference
Aconitum carmichaeli	Chinese aconite	A	C	Hatano et al. (1987)
Allium sativum	Garlic	A	C, P	Suh and Park (1986)
Ammi majus	Laceflower	IMC	E, P	Ferrie et al. (2005)
Anemone sp.		A	E	Johansson et al. (1982)
Anethum graveolens	Dill	IMC	E, P	Ferrie et al. (2005)
Angelica archangelica	Angelica	IMC	C	Ferrie et al. (2005)
Atropa belladonna	Deadly nightshade	A	E, P	Zenkteler (1971)
Azadirachta indica	Neem	A	C, P	Chaturvedi et al. (2003)
Borago officinalis	Borage	IMC	C, E	Ferrie et al. (2005)
Boswellia serrata	Indian boswellia	A	C	Prakash and Chand (1999)
Bupleurum falcatum	Bupleurum	A	C, P	Shon and Yoshida (1997); Shon et al. 2004
Camellia sinensis	Tea	A	C	Seran et al. (1999)
Carum carvi	Caraway	IMC	E, P	Ferrie et al. (2005)
Catharanthus roseus	Madagascar periwinkle	A	C	George (1985)
Digitalis sp.	Foxglove	A	C, P	Perez-Bermudez et al. (1985);
			C	Corduan and Spix (1975)
				Diettrich et al. (2000)
				Badea et al. (1985)
Datura metel	Devil's trumpet	A	E, P	Iqbal and Wijesekara (2007)
Echinacea purpurea	Purple coneflower	A	C, P	Zhao et al. (2006)
Fagopyrum esculentum	Buckwheat	A	C, P	Bohanec et al. (1993)
		G	C, P	Bohanec (1997)
Foeniculum vulgare	Fennel	A	C	Matsubara et al. (1995)
		IMC	E, P	Ferrie et al. (2005)
Gingko biloba	Gingko	IMC	E	Laurain et al. (1993)
Hepatica nobilis	Sharp-lobed hepatica	A	E, P	Nomizu et al. (2004)
Hieracium pilosella	Mouse-ear hawkweed	A	C, P	Bicknell (1996)
Hyoscyamus sp.	Henbane	A	E, C, P	Raghavan and Nagmani (1989)
		G	E, P	Chand and Basu (1998)
Hypericum perforatum	St. John's wort	A	C, P	Schulte et al. (1996)
Levisticum officinale	Lovage	IMC	E, P	Ferrie et al. (2005)
Mentha spp.	Mint	A	C	Van Eck and Kitto (1990)
Oenothera hookeri	Evening primrose	A	C, P	Martinez and de Halac (1995)

(continued)

Table 1 (continued)

Species	Common name	Method	Results	Reference
Panax ginseng	Ginseng	A	C, P	Qiquan and Anscheng (1986)
Papaver somnifernm	Opium poppy	A	C, P	Dieu and Dunwell (1988)
Physalis ixocarpa		A	E, P	Bapat and Wenzel (1982)
Psoralea corylifolia	Scurf pea	G	C, P	Chand and Sahrawat (2007)
Pimpinella anisum	Anise	IMC	E, P	Ferrie et al. (2005)
Salvia sclarea	Clary sage	A	C	Bugara (1986)
Saponaria vaccaria	Cow cockle	IMC	E, P	Kernan and Ferrie (2006)
Sesamum indicum	Sesame	A	E	Govil and Singh (1982)
Silene latifolia	Bladder campion	A	E, P	Safarova et al. (2005)
Zingiber officinale	Ginger	A	C, P	Samsudden et al. (2000)

method was developed for an apomictic biotype of *H. pilosella* (Bicknell 1996). Callus developed from both the somatic tissue and the microspores. The uni-nucleate stage of microsporogenesis was the most responsive in terms of callus development. The regenerated plants showed segregation for apomixis as well as a range of ploidy levels.

Labiatae

The Labiatae (Lamiaceae) family is another large family with 224 genera and over 3,200 species. This family is known for their aromatic qualities. The essential oils found in many of the Labiatae are used for pharmaceutical and cosmetics purposes. The important commercially grown species include mint, basil, oregano, rosemary, sage, and thyme. Rosemary is used in food, cosmetics, and as a medicinal. The German government has approved internal use of rosemary for indigestion and as a supportive treatment for rheumatism. External use of rosemary is approved for circulation problems. Essential oils include cineole, B-pinene, camphor, and limonene. Lemon balm contains polyphenols and is used for insect repellents, cosmetics, foods, and medicines. Oregano is also used in foods and medicines. Oregano contains carvacrol and thymol, which have anti-helmintic and anti-fungal properties.

Tissue culture regeneration has been reported in some of the Labiatae species, however, no reliable doubled haploidy protocols are available for any of the species within this family. Fourteen Labiatae species were screened for microspore culture response (Ferrie et al. 2005); swollen microspores were observed in only three

[anise-hyssop (*Agastache foeniculum*), mint (*Mentha piperita*), marjoram (*Origanum marjorana*)] of the 14 species. Haploid embryos have been observed in *Salvia sclarea* (Bugara 1986) and callus was observed from anthers of *Mentha* species (Van Eck and Kitto 1990), but no plants were regenerated.

Apiaceae

The Apiaceae (carrot family) consists of vegetables, herbs, and spices. Although there are tissue culture protocols for the Apiaceae, this group of plants is also considered recalcitrant for doubled haploidy methodology. Nineteen Apiaceae species were screened for microspore culture response using the *Brassica napus* standard protocol (Ferrie et al. 2005). Response was observed and with further modifications to the protocol, microspore-derived embryos were observed in 10 of the 19 species, with haploid/doubled haploid plants generated in 8 of these. Haploid and doubled haploid plants were regenerated in dill (*Anethum graveolens*), caraway (*Carum carvi*), carrot (*Daucus carota*), fennel (*Foeniculum vulgare*), lovage (*Levisticum officinale*), parsnip (*Pastinaca sativa*), anise (*Pimpinella anisum*), and laceflower (*Amni majus*). Field evaluation of dill, caraway, and fennel doubled haploid lines has resulted in the identification of lines with different agronomic characteristics and beneficial biochemical profiles (Ferrie, in preparation).

Bupleurum falcatum is used in Chinese medicine as a painkiller, and as an anti-inflammatory, anti-allergy, and anti-pyretic. Anther culture studies have resulted in callus formation and haploid plantlet regeneration (Shon and Yoshida 1997; Shon et al. 2004). Differences in anther culture response were observed among the genotypes and the habitats in which the genotypes were grown.

Solanaceae

The Solanaceae (potato family) is an economically important family which includes herbs, shrubs, trees, fruits, vegetables, and medicinals. The first *in vitro* haploid embryos and plantlets derived from culturing anthers was observed in *Datura* in the 1960's (Guha and Maheshwari 1964; Guha and Maheshwari 1966). Several of the Solanaceae species have exhibited embryogenic properties (i.e. tobacco).

Atropa belladonna (deadly nightshade) is a member of the solanaceae that contains a number of alkaloids including hyoscyamine. This compound is used to dilate the eye pupils, as an antispasmodic for respiratory problems, and for rheumatic and muscular pain. Microspore-derived embryos and haploid plants have been produced from this species (Zenkteler 1971). Microspore-derived embryos from *A. belladonna* have also been cryopreserved and regenerated (Bajaj 1978). This could be beneficial in germplasm conservation.

Hyoscyamus (henbane) species contain tropane alkaloids. Some of the early developmental work was done on *H. niger* (Nagmani and Raghavan 1983; Raghavan 1978; Sunderland and Wildon 1979). Embryos of *H. niger* were produced, however plantlets were not obtained (Nagmani and Raghavan 1983; Raghavan 1978). Embryogenesis and plant regeneration was obtained from unpollinated ovaries of *H. muticus* L. (Chand and Basu 1998). Plant regeneration occurred from callus cultures.

Some *Physalis* species contain steroids that are of medicinal importance. Haploid plants have been regenerated via microspore embryogenesis in *P. ixocarpa* Brot. A cold treatment (3°C for 2 days) of the anthers was required. NN basal medium supplemented with IAA and Kinetin resulted in embryo formation. For further embryo development, coconut milk was essential. Haploid plants were regenerated from these embryos. Steroidal levels of the regenerated plantlets were not evaluated (Bapat and Wenzel 1982).

Other Medically Important Species

Opium poppy (*Papaver somniferum* L.) is a source of many alkaloids including morphine, codeine, and thebaine. Anther culture was reported (Dieu and Dunwell 1988) with differences observed among the genotypes evaluated. The authors also observed that growth regulators (2,4-D, NAA, Kinetin) were necessary for callus production and a cold treatment (7°C) of 7 days was beneficial. A total of 140 plants were regenerated, of those, 63 were analyzed for ploidy using chromosome counts. Of the 63 plants analyzed, only one was haploid, two had both haploid and diploid cells whereas 60 were diploid.

The Ranunculaceae (buttercup family) is found throughout the world. Many are used as ornamentals and as a source of alkaloids. Many species are highly toxic, whereas other have uses in traditional medicine (i.e. black cohosh). In some cases, overharvesting of these species for medicinal use has resulted in near extinction of the plant. Developing a doubled haploidy protocol would not only be beneficial for developing uniform lines but for germplasm preservation. *Hepatica nobilis* was once used as a medicinal herb to treat liver disorders, but is now marketed commercially as an ornamental. Anther culture techniques have been developed and have resulted in haploid plants (Nomizu et al. 2004). Activated charcoal was required for embryo induction although not for regeneration. A temperature shock of 35°C was also beneficial for embryo induction.

Catharanthus roseus L. (Madagascar periwinkle) belongs to the Apocynaceae family. This plant contains vincristine and vinblastine, which are extracted commercially from the leaves to be used as a cancer treatment, especially for leukemia and Hodgkin's disease. Anther culture has been reported (George 1985). Early globular stage embryos were observed, but no further development occurred.

The leaves of *Digitalis* species (e.g. *D. purpurea, D. lanata*) contain glycosides, especially digitoxin and digoxin. These compounds are used as standard medicines for treatment of heart disorders. Androgenesis studies have been conducted on these

species, however most response comes via callus proliferation and not direct embryogenesis (Perez-Bermudez et al. 1985; Corduan and Spix 1975; Badea et al. 1985). Very few plants have been obtained. Isozyme analysis has shown that callus/plants were obtained from the microspore (Corduan 1976). Cardenolides have also been analyzed from haploid *D. lanata* plants (Diettrich et al. 2000). Variation in total amount of cardenolides and in the levels of different cardenolides was observed.

Psoralea corylifolia (Papillionaceae) is used in Indian Ayurveda medicine for teeth care, diarrhoea, bronchitis, and inflammation. This species is also an endangered plant. Haploid plants were obtained via gynogenesis (Chand and Sahrawat 2007). Of the 13 regenerated plants that were evaluated, 11 were haploid, whereas 2 were diploid. There was no mixoploidy or albinism observed.

Conclusion

Over the past decade, there has been resurgence in the use of herbs, spices, and medicinal plants for prevention and treatment of medical problems. Consumers have many products from which they can choose. Studies have shown that uniformity of the product is not always reliable, which can result in conflicting clinical trial reports and perhaps less than optimum efficacy of the product. The majority of the medicinal plants used in product formulations are from wild plant populations which have inherent variability in the levels of active compounds. Very little plant breeding or genetics research has been conducted on these medicinal plant species. The development of plant breeding systems and protocols for doubled haploid plant production would be extremely beneficial for this group of plants.

As described in this chapter, there has been very little doubled haploid research conducted on these plants, although some progress has been made. Screening studies have been conducted, in which a number of species have been evaluated using standard microspore culture protocols. Optimization is then required in order to develop an efficient protocol that can be used for practical and basic research. Factors influencing embryogenesis include donor plant growth conditions, genotype, pretreatments, developmental stage of the explants, media composition, and culture conditions. Once an efficient protocol has been developed, breeders can use the methodology and the resulting doubled haploid lines for breeding, mutagenesis, and transformation. Doubled haploid plants would also be useful for studying biochemistry and physiology of the plants and the important biochemical pathways, similar to what the *Brassica, Hordeum*, and tobacco groups have been doing for years.

References

Badea E, Iordan M, Mihalea A (1985) Induction of androgenesis in anther culture of *Digitalis lanata*. Revue Roumaine de biologie. Serie de Biologie vegetale 30:63–71

Bajaj YPS (1978) Effect of super-low temperature on excised anthers and pollen-embryos of *Atropa*, *Nicotiana* and *Petunia*. Phytomorphology 2:171–176

Bapat VA, Wenzel G (1982) *In vitro* haploid plantlet induction in *Physalis ixocarpa* Brot. through microspore embryogenesis. Plant Cell Rep 1:154–156

Bicknell RABNK (1996) Isolation of reduced genotypes of *Hieracium pilosella* using anther culture. Plant Cell Tiss Org Cult 45:37–41

Bohanec B (1997) Haploid induction in buckwheat (*Fagopyrum esculentum* Moench). In Jain SM, Sopory SK, Veilleux RE (eds), *In Vitro* Haploid Production in Higher Plants. Kluwer, Dordrecht, 4:163–170

Bohanec B, Neskovic M, Vujicic R (1993) Anther culture and androgenetic plant regeneration in buckwheat (*Fagopyrum esculentum* Moench). Plant Cell Tiss Org Cult 35:259–266

Bugara AM (1986) Embryoidogenesis in anther culture of *Salvia sclarea*. Fiziologiia I Biokhimiia Kulturnykh Rastenii. Physiol Biochem Cultivated Plants 18:381–386

Chand S, Basu P (1998) Embryogenesis and plant regeneration from callus cultures derived from unpollinated ovaries of *Hyoscyamus muticus* L. Plant Cell Rep 17:302–305

Chand S, Sahrawat AK (2007) Embryogenesis and plant regeneration from unpollinated ovary culture of *Psoralea corylifolia*. Biologia Plantarum 51:223–228

Chaturvedi R, Razdan MK, Bhojwani SS (2003) Production of haploids of neem (*Azadirachta indica* A. Juss.) by anther culture. Plant Cell Rep 21:531–537

Corduan G (1976) Isozyme variation as an indicator for the generative or somatic origin of anther-derived plants of *Digitalis purpurea* L. Z. Pflanzenzuchtg 76:47–55

Corduan G, Spix S (1975) Haploid callus and regeneration of plants from anthers of *Digitalis purpurea* L. Planta 124:1–11

Diettrich B, Ernst S, Luckner M (2000) Haploid plants regenerated from androgenic cell cultures of *Digitalis lanata*. Planta Medica 66:237–240

Dieu P, Dunwell JM (1988) Anther culture with different genotypes of opium poppy (*Papaver somniferum* L.): effect of cold treatment. Plant Cell Tiss Org Cult 12:263–271

Ferrie AMR (2006) Doubled haploid production in nutraceutical species: a review. Euphytica 158:347–357

Ferrie AMR (2007) Developing double haploidy breeding methods for nutraceutical species. In Proceedings of The Natural Health Products Research Society of Canada and The Canadian Herb, Spice, and Natural Health Products Coalition Conference: Tradition to Technology, Saskatoon, Canada. May 10–13

Ferrie AMR, Bethune T, Kernan Z (2005) An overview of preliminary studies on the development of doubled haploid protocols for nutraceutical species. Acta Physiol Plant 27:735–741

George L (1985) Anther culture of *Catharanthus roseus* L. – development of pollen embryoids. Curr Sci 54:641–642

Govil CM, Singh VRR (1982) Induction of haploids in anther culture of *Sesamum indicum*. In Proceedings of the 5th International Congress Plant Tissue and Cell Culture. pp. 545–546

Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of *Datura*. Nature 204:497

Guha S, Maheshwari SC (1966) Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212:97–98

Hatano K, Shoyama Y, Nishioka I (1987) Somatic embryogenesis and plant regeneration from the anther of *Aconitum carmichaeli* Debx. Plant Cell Rep 6:446–448

Iqbal MCM, Wijesekara KB (2007) A brief temperature pulse enhances the competency of microspores for androgenesis in *Datura metel*. Plant Cell Tiss Org Cult 89:141–149

Johansson L, Andersson B, Eriksson T (1982) Improvement of anther culture technique: activated charcoal bound in agar medium and elevated CO2 concentration. Physiol Plant 54:24–30

Kernan Z, Ferrie AMR (2006) Microspore embryogenesis and the development of a double haploidy protocol for cow cockle (*Saponaria vaccaria*). Plant Cell Rep 25:274–280

Laurain D, Trémouillaux-Guiller J, Chénieux J (1993) Embryogenesis from microspores of *Ginkgo biloba* L., a medicinal woody species. Plant Cell Rep 12:501–505

Martinez LD, de Halac IN (1995) Organogenesis of anther-derived calluses in long-term cultures of *Oenothera hookeri* de Vries. Plant Cell Tiss Org Cult 42:91–96

Matsubara S, Dohya N, Murakami K (1995) Callus formation and regeneration of adventitious embryos from carrot, fennel and mitsuba microspores by anther and isolated microspore cultures. Acta Hortic 392:129–137

Nagmani R, Raghavan V (1983) Induction of embryogenic divisions in isolated pollen grains of *Hyoscyamus niger* in a single-step method. Z.Pflanzenphysiol 109:87–90

Nomizu T, Niimi Y, Han D-S (2004) Haploid plant regeneration via embryogenesis from anther cultures of *Hepatica nobilis*. Plant Cell Tiss Org Cult 79:307–313

Perez-Bermudez P, Cornejo MJ, Segura J (1985) Pollen plant formation from anther cultures of *Digitalis obscura* L. Plant Cell Tiss Org Cult 5:63–68

Prakash DVSSR, Chand S (1999) In vitro response from cultured anthers of *Boswellia serrata* Roxb. (Burseraceae). In Plant Tissue Culture and Biotechnology: Emerging trends. Universities Press, Hyderabad, India, pp. 226–231

Qiquan S, Anscheng L (1986) Somatic embryogenesis and plant regeneration from anther culture of *Panax quinquifolius* (Ginseng). Int Plant Biotech Network 6:2

Raghavan V (1978) Origin and development of pollen-embryoids and pollen calluses in cultured anther segments of *Hyoscyamus niger* (Henbane). Am J Bot 65:984–1002

Raghavan V, Nagmani R (1989) Cytokinin effects on pollen embryogenesis in cultured anthers of *Hyoscyamus niger*. Can J Bot 67:247–257

Safarova D, Kopecky D, Vagera J (2005) The effect of a short heat treatment on the *in vitro* induced androgenesis in *Silene latifolia* ssp. *alba*. Biol Plantarum 49:261–264

Samsudden K, Babu KN, Divakaran M, Ravindran PN (2000) Plant regeneration from anther derived callus cultures of ginger (*Zingiber officinale* Rosc.). J Hortic Sci Biotechnol 75:447–450

Schulte J, Büter B, Schaffner W, Berger K (1996) Gametic embryogenesis in *Hypericum* spp. In Pank F (ed) International Symposium on Breeding Research on Medicinal and Aromatic Plants. Quedlinburg, Germany, pp. 307–310

Seran TH, Hirimburegama K, Hirimburegama WK, Shanmugarajah V (1999) Callus formation in anther cultures of tea clones, *Camellia sinensis* (L.) O. Kuntze. J Natl Sci Found Sri Lanka 27:165–175

Shon T-K, Kim S-K, Acquah D, Lee S-C (2004) Haploid plantlet production through somatic embryogenesis in anther-derived callus of *Bupleurum falcatum*. Plant Prod Sci 7:204–211

Shon T-K, Yoshida T (1997) Induction of haploid plantlets by anther culture of *Bupleurum falcatum* L. Jpn J Crop Sci 66:137–138

Suh SK, Park HG (1986) Studies on the anther culture of garlic (*Allium sativum* L.). J Korean Soc Hortic Sci 27:89–95

Sunderland N, Wildon DC (1979) A note on the pretreatment of excised flower buds in float culture of *Hyoscyamus* anthers. Plant Sci Lett 15:169–175

Van Eck JM, Kitto SL (1990) Callus initiation and regeneration in *Mentha*. Hortscience 25:804–806

Zenkteler M (1971) In vitro production of haploid plants from pollen grains of *Atropa belladonna* L. Experientia 27:1087

Zhao F-C, Nilanthi D, Yang Y-H, Wu H (2006) Anther culture and haploid plant regeneration in purple coneflower (*Echinacea purpurea* L.). Plant Cell Rep 86:55–62

Chapter 19
Microspore Embryogenesis in Selected Medicinal and Ornamental Species of the *Asteraceae*

U. Bal and A. Touraev

Abstract Isolated microspore culture experiments were carried out to induce microspore embryogenesis in *Chamomilla recutita*, *Solidago virgaurea*, *Sanvitalia procumbens* of the *Asteracea*, and *Valeriana officinalis* of the *Valerianaceae*. The *Asteracea* is one the largest plant families of commercial significance for medicinal, aromatic, food and ornamental use. Availability of protocols for an efficient production of doubled haploids via microspore embryogenesis would facilitate breeding efforts in this family. Following the establishment of microspore culture protocols uninucleate microspores divided symmetrically in all the species studied. Additionally, in *Sanvitalia* multi-nucleate structures were observed, without further development. Swelling of the microspores was routinely achieved, and viability was maintained up to 2 weeks. Results were encouraging for further studies in microspore embryogenesis in the *Asteraceae*.

Keywords Symmetrical division, multi-nucleate structures, stress pre-treatment, *Chamomile, Solidago, Sanvitalia, Valeriana*

Introduction

There is a huge diversity in medicinal and ornamental plants which have importance for health and the quality of life. These plants are in demand throughout the world and have considerable market value. In order to meet demand some species are collected from the wild others are cultivated. Improvement of medicinal and

---

U. Bal
Namik Kemal University, Faculty of Agriculture, Department of Horticulture,
Tekirdag, 59030, Turkey

A. Touraev(✉)
Max F Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Plant Molecular Biology, Dr Bohrgasse 9/4, 1030 Vienna, Austria
E-mail: alisher.touraev@univie.ac.at

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

ornamental species for more favourable drug properties and attractive appearance would be beneficial both to producers and consumers of these species. While ornamental plants are improved mainly using conventional breeding methods a considerable number of medicinal plants are collected from the wild endangering several species (Lambert et al. 1997; Swallow & Ochola 2006). Conventional methods of breeding require time and effort, and since breeding materials are often poorly developed in these species response times to market demands are slow. However, use of doubled haploidy via microspore embryogenesis can help shorten breeding programmes or enhance their efficiency. The technique of isolated microspore culture is increasingly employed in the production of doubled haploids in a wide range of species with a special emphasis on major food crops (Maluszynski et al. 2003; Thomas et al. 2003). However, the technique has received little attention in the improvement of ornamental and medicinal species of the *Asteraceae* family. The second most abundant number of species of the flowering plants belongs to the *Asteracea* family and several of these are economically important for their food (lettuce, Jerusalem and globe artichoke, chicory), oil (sunflower, safflower), medicinal (chamomile) and ornamentals properties (Chrysanthemum, dahlia, marigold) (Pink & Keane 1993; Pérez-García et al. 2001; Wang et al. 2003; Phillips & Ruggio 2005). In order to induce microspore embryogenesis via microspore culture we have carried out experiments on selected species.

The Species Studied

A total of four species namely from the genera *Valeriana*, *Chamomilla*, *Solidago* and *Sanvitalia* were studied (Table 1). *Solidago virgaurea* L. ssp. Minuta (Golden Rod) is useful for the treatment of upper respiratory catarrh. Active components of Golden Rod include saponins, clerodane diterpenes, phenolic glucosides, flavonoids, acetylenes, cinnamates, hydroxybenzoates, polysaccharides, phenolic acids and tannins and many others. Flowers of chamomile (*Chamomilla recutita* (L.) Rauschert) are used as a source of various chemical constituents such as essential oil with chamazulene, bisabolol and apigenin. The importance of this species is characterised by an annual import of about 3,000 t of flowers into Europe from non-European countries. Valerian (*Valeriana officinalis*) is a well-known and frequently used medicinal herb. It is noted especially for its effect as a tranquilliser and nervine, particularly for those people suffering from nervous tension. The rhizome of valerian contains a variety of compounds

Table 1 Ornamental and medicinal plants studied for microspore embryogenesis

Species	*Common name*	*Family*	*The use of species*
Chamomilla recutita	Chamomile	Asteraceae	Medicinal
Solidago virgaurea	Golden rod	Asteraceae	Medicinal
Sanvitalia procumbens	Creeping zinnia	Asteraceae	Ornamental
Valeriana officinalis	Valerian	Valerianaceae	Medicinal

including valepotriates, valeric acid, and volatile oils. Currently, no publications exist on haploid induction of ornamental/medicinal species reported here. *Sanvitalia procumbens* is a perpetually flowering ornamental plant widely grown around the world.

Flower Bud/Capitula Collection, Sterilization, Microspore Isolation and Culture

General Considerations

As a general rule flower buds containing microspores at the uni-nucleate stage are of interest in microspore embryogenesis. Flower buds of many species contain a single ovary and anthers and develop in a predictable manner. Specific bud sizes can be used to stage microspore development in such species. Following collection of a particular bud size, specific to a species or genotype, uni-nucleate microspores can be collected for culture. Usually there is little need for purification of the microspores from such buds as there is usually sufficient inoculum of a homogeneous microspore population for culture. Asteraceous species however have a specialised flower structure and require a different treatment. The flower is compositum having several individual flowers i.e., florets, each of which is attached to the receptacle and accompanied by common petals and sepals of the flower. In general the florets are positioned in whorls, the outer whorl of florets developing earlier than inner whorls. At any given time, therefore, florets of varying maturity are present in a single flower bud. The central florets always contain microspores at earlier stages in comparison to the peripheral ones (Fig. 1). Therefore, microspore populations

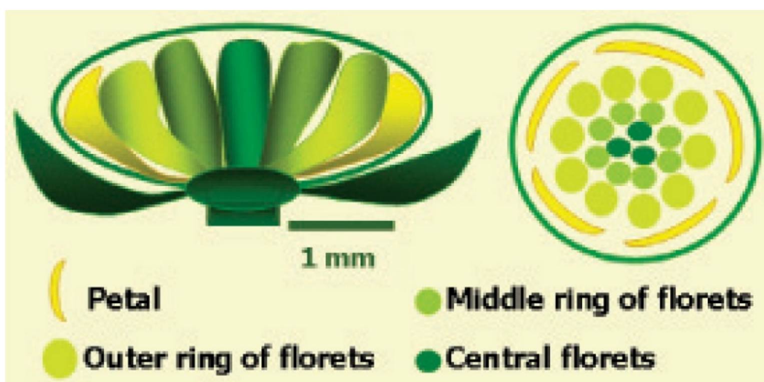


Fig. 1 The organisation of capitula and florets of *Sanvitalia procumbens.* Maturity of the florets begins at the periphery. Microspores in the central florets are at earlier stages and there is a radial cline in developmental rate

isolated from such buds are highly heterogeneous. Additionally, in asteraceous species with small bud size, such as 2–4 mm, manual dissection of anthers may be tedious and removal of a sufficient number of anthers may prove difficult. In such circumstances isolation of microspores via blender may be the only feasible alternative. Therefore, in the asteraceous species of *Sanvitalia*, *Chamomilla*, *Solidago* and in the *Valeraiana* of the *Valerianaceae* family (also with small buds) microspore isolation was carried out using a blender.

The following sizes were considered appropriate when collecting flower buds/capitula for microspore isolation: for both *Chamomile* and *Sanvitalia* the capitula diameter was 3–4 mm; for Solidago 2–3 mm capitula diameter; for *Valeriana* 2–3 mm bud diameter (Fig. 2). Although *Valeriana* is a non-asteraceous species, flower buds were treated in the same way as an asteraceous species because of the small bud size. Also, this approach allowed economy of time.

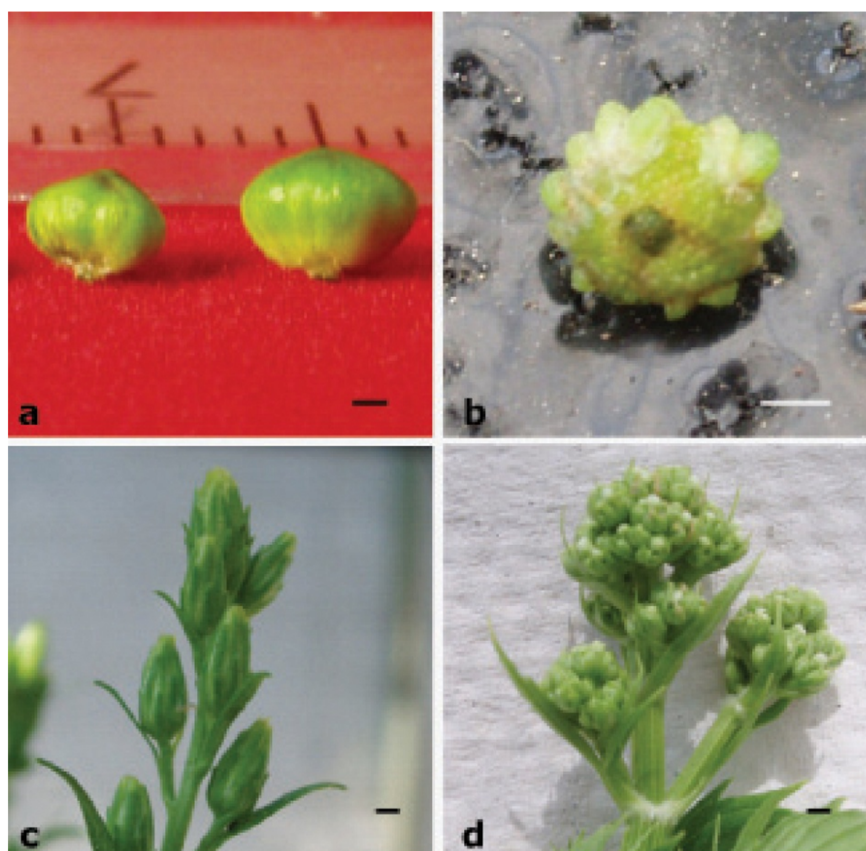


Fig. 2 Flower buds/capitula used for the induction of microspore embryogenesis in the species tested: (**a**) Sanvitalia, 3–4 mm diameter; (**b**) Chamomile, 3–4 mm diameter; (**c**) Solidago, 2–3 mm diameter; (**d**) Valeriana, 2–3 mm diameter (Bar = 1 mm)

Buds were sterilized using 15% H_2O_2 containing 1–3 drops of Tween 20 for 15–20 minutes. This was followed by washing with sterile distilled water several times. Once the buds were ready they were transferred to the container of a blender for microspore isolation.

Microspore Isolation and Culture

In all the species studied microspores were isolated using a commercial blender. This was due to the small bud and capitula therefore small anther sizes. Additionally robust capitula did not allow efficient microspore isolation using other methods. Following sterilization the capitula/buds together with the B-medium (Kyo & Harada 1986) were loaded into the blender cup. Then the blender was operated for 15–20 seconds at low speed. The suspension in the container was removed, sieved through 100, and then 40 µm mesh. Somatic tissues were removed by sedimentation via at least three centrifugation steps. Then the microspore suspension was subjected to density gradient centrifugation in 30% and 50% percoll gradients to separate the population into different stages of microspore development. The percoll gradient centrifugation was carried out between 150–200 g for 5–10 minutes. Microspores accumulated at the top in the middle and in a pellet, respectively, and were removed and washed with the isolation medium and cultured in the media (Fig. 3). The cultures were then subjected to stress pre-treatments such as cold and heat shock as well as other widely used and novel stress treatments (Shariatpanahi et al. 2006). Additionally effects of growth regulators, lactalbumine hydolysate and gum arabic were tested. Following the stress treatments the cultures were maintained at 25°C in the dark.

The above protocol for microspore isolation and separation via the gradient centrifugation allows testing of three different microspore populations, representing different developmental stages, separately. In all cases the uninucleate stage microspores were responsive to the culture conditions therefore only the results obtained from the uninucleate microspores are presented.

Isolation of the microspores was carried out in B-medium which consisted of KCl, 1.49 g/l; $MgSO_4$•$7H_2O$, 0.25 g/l; $CaCl_2$, 0.11 g/l and mannitol (0.3 M) 54.63 g/l and 1 mM phosphate buffer of pH 7. The pH of the medium was adjusted to 7.0 with a few drops of KOH (Kyo & Harada 1986). Miller macro salts contained H_2PO_4, 300 mg/l; KNO_3, 1,000 mg/l; NH_4NO_3, 1,000 mg/l, $Ca(NO_3)_2$•$4H_2O$, 500 mg/l and $MgSO_4$•$7H_2O$, 71.5 mg/l (Doerschug & Miller 1967). The tobacco maturation (TM) medium contained sucrose, 0.5 M (171.15 g); Glutamine 3 mM (0.438 g); Lactalbumine hydrolysate, 10.0 g/l; KNO_3, 10 mM (1.01 g); $Ca(NO_3)_2$•$4H_2O$, 1 mM (0.236 g); $Mg(SO_4)$•$7H_2O$ 1 mM (0.247 g); H_3BO_3, 0.16 mM (0.01 g); Uridine, 1 mM (0.244 g); Cytidine 0.5 mM (0.127 g); Phosphate buffer 1 mM with a pH of 7.0 (Tupy et al. 1991). B5 medium was prepared according to Gamborg et al. (1968). NLN micro elements and vitamins were according to Lichter (1981, 1982).

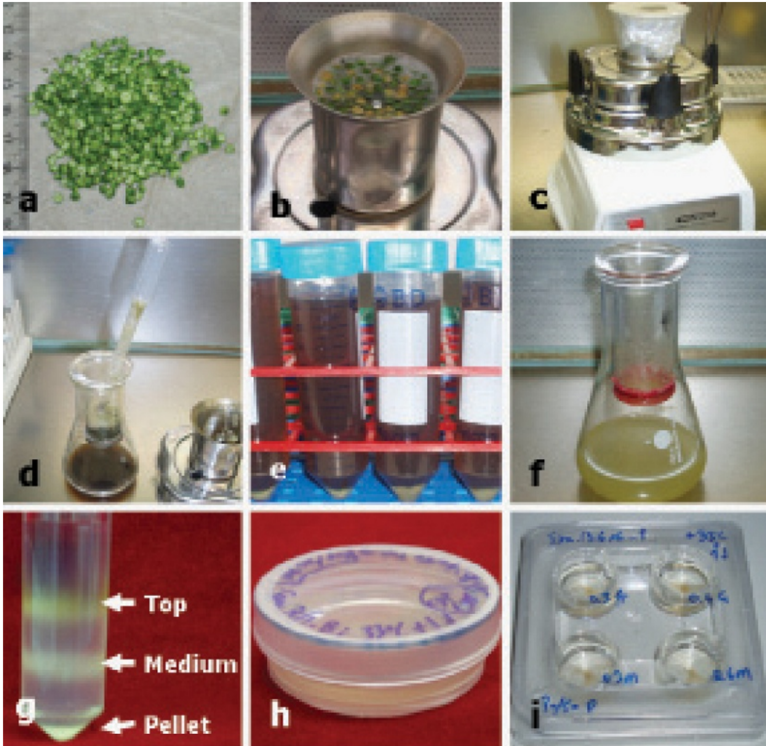


Fig. 3 Stages in the microspore isolation and culture of *Sanvitalia*, also used in the other species studied. Capitula collected (**a**), sterilized, washed and set in a blender container (**b**), the container covered with a sterile aluminium foil and the blender is run (**c**), microspore suspension is sieved (**d**), centrifuged (**e**), re-suspended in fresh isolation medium (**f**), microspore suspension is subjected to percoll gradient centrifugation (**g**), and each band obtained removed and cultured separately in Petri dishes or multi-well plates (**h, i**)

Results

In all the species tested induction of symmetrical division was achieved. Further development was observed only in *Sanvitalia* with multi-nucleate structures of four or more nuclei. In all experiments microspores survived not more than 2 weeks in culture.

Chamomile

In Chamomile microspores symmetrical nucleus division was induced from uninucleate microspores in B5 medium containing 0.25 M maltose with a 2-day pretreatment at 4°C (Fig. 4). Early bi-cellular microspores and later stages became swollen in the presence of 0.5 M sucrose and various pre-treatments without embryogenic development. No other factors tested were effective in further development.

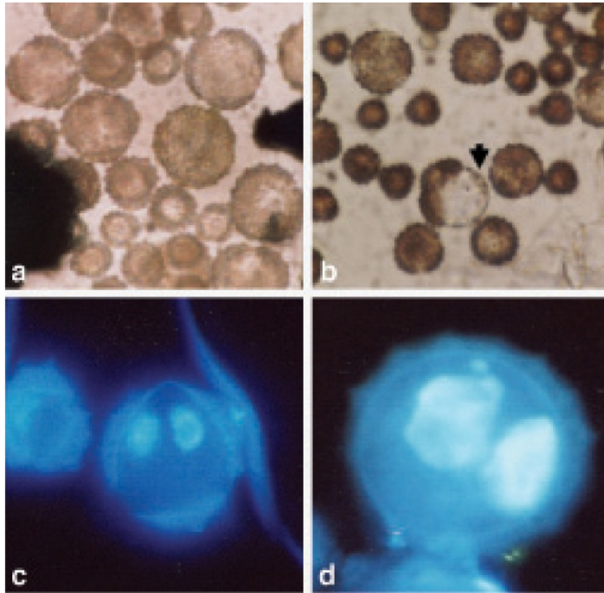


Fig. 4 Swelling of Chamomile microspores (**a**) and in some cases bursting out of the exine (**b**, arrow) were observed. Symmetrical division (**c, d**) was induced

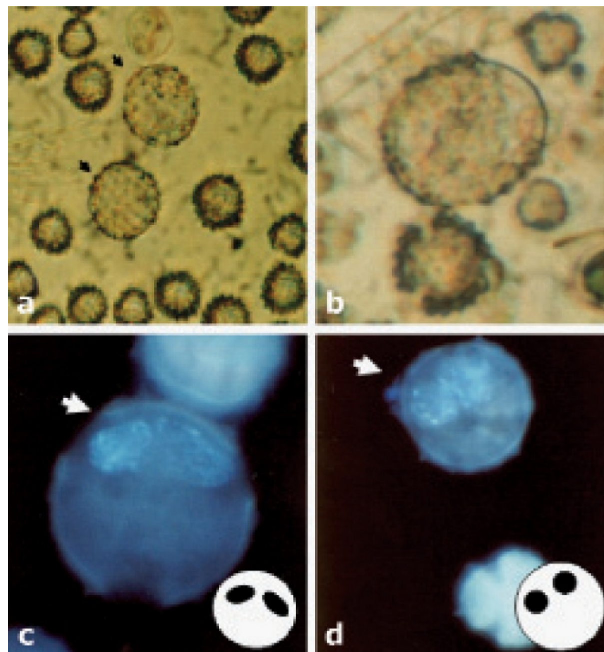


Fig. 5 Swollen microspores of *Solidago* (**a**), some of which burst out of the exine (**b**). Symmetrical nucleus division in Solidago microspores. (schematic representation of the nuclei are presented at the bottom right of the pictures (**c, d**)

Solidago

In *Solidago* symmetrical nucleus division was induced from uni-nucleate microspores in tobacco maturation medium (TM) containing 0.3 M maltose following a pre-treatment at 33°C for 1 day (Fig. 5). Immediately after culture the microspores became swollen and after about 2 weeks some swollen microspores were about to break out of the exine. When the microspores were checked later, during the exine rupture, no multi-nucleate structures were observed. After swelling and exine rupture symmetrical nucleus division could be observed.

Valeriana

In *Valeriana* microspores symmetrical nucleus division was induced from uni-nucleate microspores in medium with Miller macro salts, NLN micro elements and NLN vitamins and sucrose at 0.50 M. Microspores became swollen immediately after culture (Fig. 6). *Valeriana* microspores appeared to be very sensitive to *in vitro* manipulation.

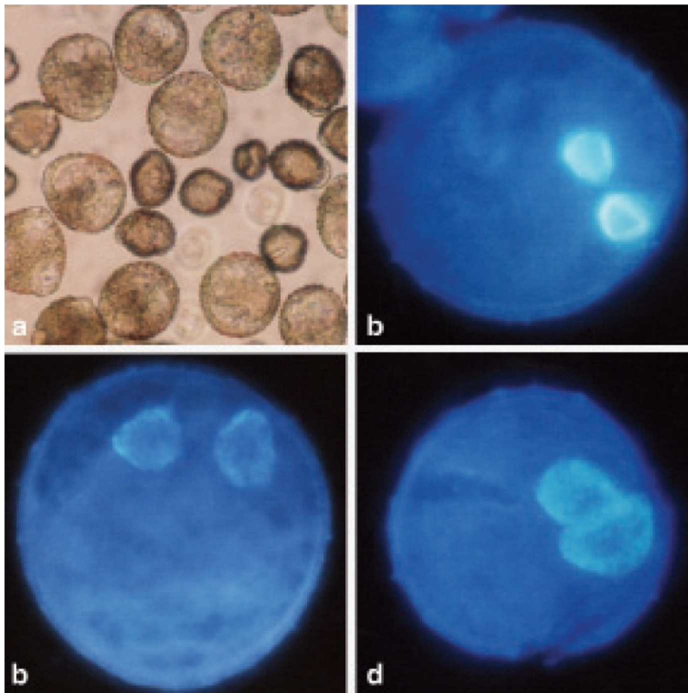


Fig. 6 Swollen *Valeriana* microspores in culture (**a**), and symmetrical division of microspore nucleus (**b–d**)

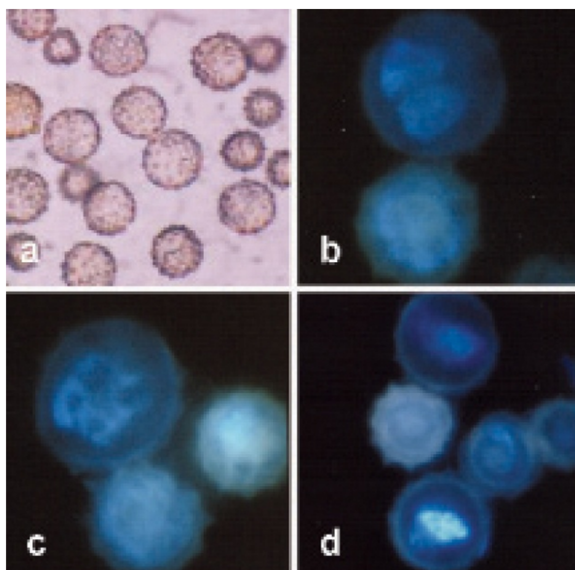


Fig. 7 Swollen microspores of *Sanvitalia* (**a**); symmetrical nucleus division (**b**) and multi-nucleated structures (**c, d**)

Sanvitalia

Symmetrical nucleus division and formation of multi-nucleate structures were induced from uni-nucleate *Sanvitalia* microspores in medium containing Miller macro salts, NLN micro elements and vitamins together with sucrose at 0.5 M following a pre-treatment at 33°C for 1 day (Fig. 7). However, further development was not observed following testing of additional factors. Early bi-cellular and tri-cellular stage microspores readily became swollen in the culture media surviving pre-treatments without further development.

Conclusions and Future Prospects

Several approaches were tested for microspore embryogenesis in the species of *Chamomilla recutita, Solidago virgaurea, Valeriana officinalis* and *Sanvitalia procumbens*. Symmetrical division of the microspore nucleus was induced in all the species. Additionally, in *Sanvitalia* multi-nucleate structures were observed, without further development. Swelling of the microspores was routinely achieved, and viability was maintained up to 2 weeks. Although the progress was limited it was encouraging and has initiated further studies. Isolated microspore culture is the most refined method used in the induction of haploidy and offers various advantages

over anther culture (Kasha et al. 2001). However, establishment of a regeneration protocol requires considerable time and effort. Successful regeneration protocols reported for tobacco, rapeseed and barley were developed by several researchers over many years.

Microspore embryogenesis research in the asteraceous species is in its infancy. The HaploTech project (Touraev 2006) is probably the first comprehensive research project undertaken using these species. The future work with the species of *Asteraceae*, especially those with small capitula, should take various challenges into consideration. A better understanding of the asteraceous species is necessary. In order to realize that and then adopt the results to the species of interest it may be more feasible to consider working with an asteraceae model plant. A model plant in question would have the characteristics of perpetual flowering preferably without the need of vernalization. The size of the capitula is another important factor affecting, directly, the amount of microspores that can be harvested to carry out experiments of an appropriate scale. Also the capitula should be free from any source of contamination thus precluding the use of antibiotics. Several species possessing such traits should be available in the *Asteraceae* as it is a large and varied group containing approximately 900 genera and 13,000 species. Once a model species is designated in the *Asteraceae* systematic experiments can be performed. Taking into consideration the history of microspore embryogenesis, it can be concluded that the efforts reported here represents the initial steps in the path to the production of haploid embryos in the *Asteraceae* through the culture of isolated microspores.

References

Doerschug, M.R. & Miller C.O. (1967). Chemical control of adventitious organ formation *in Lactuca sativa* explants. *Am J Bot, 54*, 410–413

Gamborg, O.L., Miller, R.A., & Ojima, K. (1968). Nutrient requirement suspension cultures of soybean root cells. *Exp Cell Res 50*, 151–158

Kasha, K.J., Simion, E., Oro, R., Yao, Q.A., Hu, T.C., & Carlson, A.R. (2001). An improved in vitro technique for isolated microspore culture of barley. *Euphytica 120*, 379–385

Kyo, M. & Harada, H. (1986). Control of the developmental pathway of tobacco pollen in vitro. *Planta 168*, 427–432

Lambert, J., Srivastava., J. & Vietmeyer, N. (1997). Medicinal plants, rescuing global heritage. World Bank Technical Paper 355. Washington, DC: World Bank

Lichter, B. (1981). Anther culture of *Brassica napus* in a liquid culture medium. *Z. Pflanzenphysiol 103*, 229–237

Lichter, B. (1982). Induction of haploid plants from isolated pollen of *Brassica napus. Z. Pflanzenphysiol 105*, 427–434

Maluszynski, M., Kasha, K.J., & Szarejko, I. (2003). Published doubled haploid protocols in plant species. In: M. Maluszynski, K.J. Kasha, B.P. Forster, & I. Szarejko (eds) *Doubled Haploid Production in Crop Plants, A Manual* (pp. 309–335). Dordrecht: Kluwer

Pérez-García, F., Marín, E., Adzet, T., & Cañigueral, S. (2001). Activity of plant extracts on the respiratory burst and the stress protein synthesis. *Phytomedicine 8*, 31–38

Phillips, K.M. & Ruggio, D.M. (2005). Ashraf-Khorassani M. Phytosterol composition of nuts and seeds commonly consumed in the United States. *J Agric Food Chem 53*, 9436–9445

Pink, D.A.C. & Keane, E.M. (1993). Lettuce *Lactuca sativa* L. In: G. Kalloo & B.O. Bergh (eds) Genetic Improvement of Vegetable Crops, Pergamon, Oxford, pp. 543–571

Shariatpanahi, M.E., Bal, U., Heberle-Bors, E., & Touraev, A. (2006). Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. *Physiol Plantarum 127*, 519–534

Swallow, B. & Ochola, S. (2006). Agroforestry, nutrition, and health: understanding the links between agriculture and health. Focus 13, Brief 11. International Food Policy Research Institute (IFPRI) 2020 vision briefs, Washington, DC

Thomas, W.T.B., Forster, B.P., & Gertsson, B. (2003). Doubled haploids in breeding. In: M. Maluszynski, K.J. Kasha, B.P. Forster, & I. Szarejko (eds) *Doubled Haploid Production in Crop Plants, a Manual* (pp. 337–349). Dordrecht: Kluwer

Touraev, A. (2006). Proposal Number: 508210 HAPLOTECH; Contract Number: COOP-CT-2003–508210; Programme: Sixth Framework Programme (2002–2006), Horizontal Activities for SMEs

Tupy, J., Rihova, L., & Zarsky, V. (1991). Production of fertile tobacco pollen from microspores in suspension culture and its storage for in situ pollination. *Sex Plant Reprod 4*, 284–287

Wang, M, Simon, J.E., Aviles, I.F., He, K., Zheng, Q-Y., & Tadmor, Y. (2003). Analysis of anti-oxidative phenolic compounds in Artichoke (*Cynara scolymus* L.). *J Agric Food Chem 51*, 601–608.

Chapter 20
Carrot Doubled Haploids

K. Górecka, D. Krzyżanowska, W. Kiszczak, U. Kowalska, and R. Górecki

Abstract Anther culture of over 20 carrot cultivars was studied. The uni-nucleate stage proved optimal for embryogenesis for which bud length was used as a surrogate. Large genotypic variation in culture response was found. Anther culture at 27°C in the dark without sub-culturing had a significant beneficial impact on embryogenesis. Embryos were obtained regardless of donor plant culture; however, plants grown in glasshouse conditions produced more embryos than those from the field. Secondary embryogenesis was induced in some cultures and in one case, 102 plants were obtained from one embryo (on B_5 medium without hormones) over a 12 week period. Regenerated plants were planted into peat and acclimatized to glasshouse and growth room conditions. Cytological and cytometric studies revealed that over 90% of regenerated plants possessed a doubled chromosome complement and isozyme analysis showed that 96–100% were homozygous. Anatomical studies confirmed that embryos had formed from microspores.

Keywords Anther culture, secondary embryogenesis, cytometric studies, isozyme analysis

Introduction

The carrot is a plant species of increasing economic importance in Poland and other parts of the world. It has great value in that it is available raw all year round. In addition to its use as a vegetable, carrot juices, or mixed juices in which carrot is one of the main ingredients, are becoming increasingly popular. Carrot is also a raw material for the pharmaceutical industry.

K. Górecka(✉)
Institute of Vegetable Crops, ul. Konstytucji 3 Maja 1/3, 96–100 Skierniewice, Poland
E-mail: kgoreck@inwarz.skierniewice.pl

D. Krzyżanowska, W. Kiszczak, U. Kowalska, and R. Górecki
Institute of Vegetable Crops, ul. Konstytucji 3 Maja 1/3, 96–100 Skierniewice, Poland

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Hybrids have become dominant in the carrot market and are replacing traditional cultivars. To breed hybrids, homozygous lines are necessary. However, obtaining these by conventional breeding methods is difficult and very time-consuming. The use of androgenesis has great potential to significantly shorten the production of pure lines and breeding (Bajaj 1990; Wang et al. 2000; Datta 2005). There are very few publications in the literature worldwide on the subject of *in vitro* androgenesis in carrot (see Andersen et al. 1990; Hu et al. 1993; Matsubara et al. 1995; Tyukavin et al. 1999; Ferrie et al. 2005).

At the Institute of Vegetable Crops in Skierniewice, wide-ranging experiments were carried out with the aim of developing a method of obtaining homozygous carrot plants from anther cultures. Detailed results of some of the stages of this work have already been published (Górecka et al. 2005a, b; Kiszczak et al. 2005), other results are in the process of being compiled or published (Górecka et al. in press). This summary article presents the most important and the most interesting results of the whole process of obtaining carrot plants in anther cultures and their subsequent evaluation.

Methodology

Carrot roots harvested in the field were stored in a chilling chamber at about +4°C (±1°C). After 3 months, the roots were placed (in pairs) into 10 L pots containing a mixture of sand and peat (2:1, v/v), with the addition of multi-component complex fertilizer Azofoska and microelements at 1.25 kg m^{-3} and chalk at 8.0 kg m^{-3}. The plants developed from the roots were kept in a glasshouse at about +20°C.

Production of Embryos from Anther Culture

Anthers were obtained from a range of umbel types (from one or more shoots, main shoot or from cut back re-growth) from donor plants grown in the glasshouse or field (Table 2). Flower buds were surface sterilized (70% ethanol, v/v) for 1–2 minutes and then rinsed twice with distilled water. Anthers were picked out under a dissecting microscope (16X) using forceps and needles. Only anthers without filaments were used (cells of the filament were found to form callus in culture). The isolated anthers were placed on modified B_5 medium (Gamborg et al. 1968) with 500 mg L^{-1} glutamine, 100 mg L^{-1} serine, 0.1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} NAA, 100 g L^{-1} sucrose and 6.5 g L^{-1} agar (Andersen et al. 1990). The medium pH was adjusted to 5.8. The anther cultures were kept in the dark at 27°C. The effect of various factors on the process of embryogenesis were studied.

Anthers were crushed and examined under a light microscope at 1,000X magnification. Microspore development was correlated with bud development (Table 1).

Table 1 The influence of microspore development stage and bud length on the effectiveness of androgenesis in two carrot cultivars (Feria F_1 and HCM)

Feria F_1				HCM			
Bud length (mm)	Microspore developmental stage in anther (%)		No. of embryos per 100 anthers	Bud length (mm)	Microspore developmental stage in anther (%)		No. of embryos per 100 anthers
0.7–0.9	Tetrads	25			Tetrads	0	6.9
	Uni-nucleate	64	63.1	0.7–0.9	Uni-nucleate	47	
	Early bi-nucleate	3			Early bi-nucleate	41	
	Late bi-nucleate	8			Late bi-nucleate	10	
	Unidentified	0			Unidentified	2	
1.0–1.3	Tetrads	4	10.2	1.0–1.3	Tetrads	3	2.5
	Uni-nucleate	71			Uni-nucleate	31	
	Early bi-nucleate	19			Early bi-nucleate	41	
	Late bi-nucleate	6			Late bi-nucleate	25	
	Unidentified	0			Unidentified	0	
1.5–1.8	Tetrads	0	17.5	1.5–1.8	Tetrads	0	0.3
	Uni-nucleate	53			Uni-nucleate	0	
	Early bi-nucleate	6			Early bi-nucleate	30	
	Late bi-nucleate	44				70	
	Unidentified	1			Late bi-nucleate	0	
					Unidentified		

Table 2 The effect of donor plant growth conditions and cultivar on androgenesis on carrot

Donor plant growth conditions	Cultivar	No. of cultured anthers	No. of embryos	No. of embryos per 100 anthers	No. of responding anthers	% of responding anthers
Greenhouse	Splendid F_1	833	4	0.5*	3	0.4c*
	HCM	3163	91	2.9b	62	2.0b
	Feria F_1	776	361	46.5a	89	11.5a
Field	Splendid F_1	80	0	0.0c	0.0	0.0c
	HCM	829	2	0.2c	2	0.2c
	Feria F_1	1836	615	33.5a	170	9.3a

*Numbers marked with the same letter within columns do not differ significantly at $\alpha = 0.05$

Anther culture response was compared among 22 cultivars selected by breeders. Individual plants from the same cultivar were also investigated. About 500 anthers from each genotype were cultured.

Two procedures of cultivating anther cultures were used. The first involved transferring anthers laid out on induction media onto fresh media after 2 weeks incubation in the dark at 27°C and placing them under continuous light, but at the same temperature. The second involved leaving the anthers on the first media in the dark at 27°C until embryos had formed.

Donor plants were grown in a greenhouse and in the field.

Once embryos had formed, cultures were transferred into the light, but temperature was maintained at 27°C. On greening up embryos were counted and transferred to regeneration media. First, B_5 medium with 20 mg L^{-1} kinetin and 20 g L^{-1} sucrose was used – the medium which had proved to be the best in our experiments on regenerating head cabbage and Brussels sprouts from androgenetic embryos. Next, MS medium (Murashige and Skoog 1962) containing 1 mg L^{-1} BA, 0.001 mg L^{-1} NAA and 20 g L^{-1} sucrose was used. Since mainly shoots formed on this medium. They were transferred onto rooting medium containing one auxin – IAA, NAA, or IBA, or a combination of two auxins. Other regeneration media were sought and, to this end, media based on B_5 and MS without hormones were introduced while maintaining a reduced concentration of sucrose at 20 g L^{-1}.

Plantlets were acclimatized to none sterile conditions in a glasshouse or growth chamber using various pot substrates: highmoor peat, peat substrate, sand, charcoal, brown coal, mineral wool, superabsorbent Alcosorb 400, Glomus inoculum with the addition of chalk and compound fertilizers. Adaptation was carried out under the conditions of 100% humidity using. small plastic tents.

Plant Evaluation

Ploidy was determined by nuclear DNA content using flow cytometry (Partec CA II) and by chromosome counts from root tip metaphase cells. Homozygosity was determined by isozyme analysis of PGI (phosphoglucose isomerise) and/or AAT (aspartate aminotransferase) which were known to be heterozygous in the donor plants.

Anatomical studies on carrot embryo development were carried out to confirm androgenic origin. Carrots anthers were sampled daily from cultures and embryogenesis monitored (Fig. 2a,b).

Results

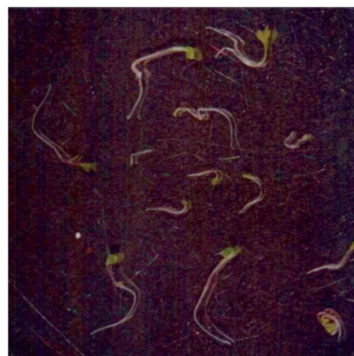
Harvested carrot anthers contained microspores at different stages in development, but one stage predominated. The uni-nucleate stage was found to be the most suitable for initiating embryogenesis and this could be determined by bud length (Table 1).

Very large differences were found between cultivars in their ability to form embryos in anther cultures. There were also clear differences in the ability to undergo androgenesis between individual donor plants of the same cultivar.

By comparing the different methods of cultivating anther cultures it was found that transferring them onto a fresh medium 2 weeks after they had been set up and subjecting them to continuous light produced much worse results (a statistically significant difference)

All embryos obtained from transfer onto B_5 medium with 20 mg L^{-1} kinetin and 20 g L^{-1} sucrose, died. On MS medium with cytokinin and at a low concentration of auxin shortened shoots (rosettes) were mainly formed, but very rarely roots. We were thus forced to carry out regeneration of carrot plants in two stages: (1) rosette production and (2) rooting on special media containing one or two auxins. Unfortunately, the rooting process took a long time and only a low percentage of shoots formed roots. In addition plants obtained as a result of the two-stage regeneration often died during the adaptation process despite the different growth substrates used. Work was therefore focused on the regeneration media and on the substrate for adaptation to glasshouse and growth room. On B_5 and MS media without hormones, which were then included in the experiments, secondary embryogenesis was found to take place with a subsequent conversion of embryos into plants (Fig. 1a, b). The conversion occurred faster on B_5 medium. These effects were very beneficial as they made it possible to eliminate the long-lasting and not very effective stage of root formation, and provided several plants from one embryo. Experiment on the effectiveness of regeneration showed that in the case of the most embryogenic cultivar 102 plants were obtained from one embryo over a 12 weeks period using B_5 medium without hormones. Moreover, the plants obtained via conversion survived the adaptation stage better. Over 80% of plants survived the removal from glassware to planting in pots. The best substrate proved to be the one containing larger amounts of highmoor peat and the addition of a compound fertilizer

a secondary embryogenesis,



b conversion of secondary embryos

Fig. 1 Regeneration of androgenic plant (**a**) Secondary embryogenesis, (**b**) conversion of secondary embryos

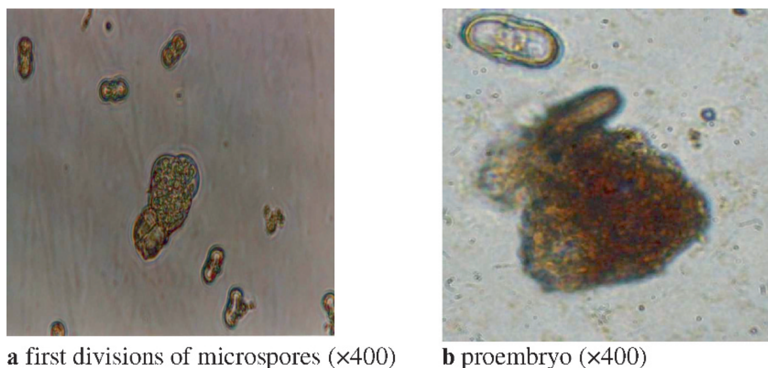


Fig. 2 Anatomical studies of embryo induction form micros pores (phase contrast microscopy) (**a**) First divisions of microspores (×400) (**b**) proembryo (×400)

and chalk. The results of adaptation were also favourably affected by transferring first to a growth chamber rather than a glasshouse.

The counting of chromosomes in the cells of root growth tips and the cytometric analyses both revealed that more than 90% of androgenetic plants had double the number of chromosomes.

The obtained androgenetic plants of the cultivar Narbonne F_1 were in 100% of cases homozygous in respect of the AAT isoenzyme and in 96% for PGI. In the case of the cultivar Kazan F_1, 96% of the androgenetic plants were homozygous for AAT, but only 4% for PGI.

Discussion

Andersen et al. (1990) found responding anthers, i.e. anthers forming callus or embryos, when microspores were cultured at the tetrad stage, either early-uni-nucleate or middle-uni-nucleate. Most success was gained, in both years of the experiments, when microspores were at the middle -uni-nucleate stage. Matsubara et al. (1995) obtained the largest number of embryos when anthers containing tetrads were cultured, which during culture reached the uni-nucleate stage. On the other hand, Tyukavin et al. (1999) were of the opinion that carrot microspores are able to change the path of their development and form either callus or embryos at the tetrad stage or bi-nucleate stage. In our experiments, we obtained the largest number of embryos when uni-nucleate microspores predominated in anthers.

In the experiments by Andersen et al. (1990), pre-embryos and calli, from which embryos formed, were obtained from only 2 out of the 15 cultivars studied. Our experiments also confirmed the effect of genotype on inducing androgenesis. One of the cultivars proved to be highly embryogenic, while five others did not produce any embryos at all. Like Arnison et al. (1990) for broccoli and our team for head cabbage (Górecka and Krzyżanowska 2004) and Brussels sprouts (Krzyżanowska

and Górecka 2004), we found very large differences between individual carrot plants of the same cultivar in their ability to undergo androgenesis.

Tyukavin et al. (1999) laid out whole flower buds on a medium for inducing androgenesis and placed them in the dark for 2–4 weeks. Then they isolated anthers and placed them onto the same medium under continuous light. Andersen et al. (1990) recommend transferring the cultured anthers after 2 weeks onto a fresh medium and exposing them to continuous light. In our experiments, we obtained significantly better results when anthers were left on the same medium in the dark until embryos appeared.

Andersen et al. (1990) and Matsubara et al. (1995) cultivated donor plants in the field, whereas Tyukavin et al. (1999) in a growth chamber. Ferrie et al. (2005) emphasize the importance of donor plant growth conditions for the effectiveness of isolated microspore cultures in the family *Apiaceae*. We compared this effectiveness in anther cultures set up from donor plants cultivated in a glasshouse and an open field. For each of the cultivars involved, we obtained better results from plants grown in glasshouse conditions.

Regeneration of embryos from anther culture was carried out by Tyukavin et al. (1999) by placing embryos on filter paper rafts on liquid MS medium containing 0.1 mg L^{-1} kinetin. Later they transferred the growing rosettes or plants onto liquid MS medium without hormones and again used the filter paper rafts. Initially, we carried out regeneration in two stages. On a medium with cytokinin and auxin, we obtained rosettes which were then placed on special rooting media with the addition of one or two auxins. However, the rooting process took a long time and its efficiency was very low. Later, on B_5 and MS media without hormones, we obtained a significant improvement. Secondary embryogenesis took place followed by embryo conversion. Whole plants were formed, which allowed us to skip the rooting stage. The plants were able to survive adaptation better. Moreover, the secondary embryogenesis made it possible to obtain a greater number of plants from one embryo. Tyukavin et al. (1999) also described the occurrence of secondary embryogenesis.

Andersen et al. (1990), Matsubara et al. (1995), and Tyukavin et al. (1999) all determined ploidy of regenerated plants by counting chromosomes in the cells of root tips. We also used this method, but compared the results with those obtained by the indirect method of determining the amount of nuclear DNA in a flow cytometer. Tyukavin et al. (1999) concluded that ploidy changed from 1n to 2n during secondary embryogenesis, embryo development and plant regeneration. They claimed that the changes in ploidy contributed to better adaptation to open environment. Smykalova et al. (2006) carried out determination of ploidy of caraway plants obtained in anther cultures by means of a flow cytometer. They found that 40% of the plants were haploid. In our experiments, using both methods, we found that more than 90% of carrot plants had a diploid number of chromosomes, which precludes the need for colchicine treatment.

The authors quoted here, who dealt with carrot anther cultures (Andersen et al. 1990; Hu et al. 1993; Matsubara et al. 1995; Tyukavin et al. 1999) did not carry out any studies to confirm homozygosity of the plants they obtained. Ferrie et al. (2005) also did not study this in respect carrot plants obtained by isolated microspore

culture. Smykalova et al. (2006) carried out an analysis of esterase isoenzyme in a population of caraway plants obtained in anther cultures (in order to confirm homozygosity). In our studies, we chose PGI and AAT because Westphal and Wricke (1989) had studied these isozymes in carrot. They investigated the inheritance of these enzyme systems and the linkage relationships among isozyme loci. Androgenic plants of the cultivars Kazan F_1 and Narbonne F_1 obtained from donor plants heterozygous in respect of AAT were found to be homozygous for this locus at a very high percentage. We obtained a similar result for the cultivar Narbonne F_1 for PGI. However, the androgenic plants of the cultivar Kazan F_1 were at a very high percentage of heterozygosity for PGI.

Nitsch and Norreel (1973) while carrying out anatomical studies of microspores during anther culture of *Datura innoxia*, observed pro-embryos. Smykalova et al. (2006) carried out observations of microspore development in caraway anther cultures under fluorescence microscopy. They found that, apart from an analysis of isozymes, this was a good method of confirming the androgenic origin of plants. In our observations, carried out by means of the phase contrast we saw the transformations of microspores into pre-embryos and embryos.

Conclusions

The experiments that we carried out resulted in the development of a technology for obtaining carrot plants by anther cultures. The plants were subsequently evaluated with respect of ploidy and homozygosity. We found that spontaneous doubling of the chromosome complement occurred at a very high rate. With respect to isozymes most of the plants tested were homozygous. The anatomical studies also confirmed that embryos developed from microspores. The plants cultivated by us were handed over to breeders and are in use in breeding.

References

Andersen, S.B., Christiansen, J., Farestveit, B. 1990. Carrot (*Daucus carota* L.): *In vitro* production of haploids and field trials. In: Y.P.S Bajaj (Ed), *Biotechnology in Agriculture and Forestry Vol. 12*. (pp. 393–402), Berlin/Heidelberg, Springer

Arnison, P.G., Donaldson, P., Jackson, A., Semple, C., Keller, W.A. 1990. Genotype-specific response of cultured broccoli /*Brassica oleracea* var. *italica*/ anthers to cytokinins. *Plant Cell Tissue and Organ Culture 20*: 217–222

Bajaj, Y.P.S. 1990. *In vitro* production of haploids and their use in cell genetics and plant breeding. In: Y.P.S Bajaj (Ed), *Biotechnology in Agriculture and Forestry, Vol. 12*. (pp. 3–44), Berlin/Heidelberg, Springer

Datta, S.K. 2005. Androgenic haploids: Factors controlling development and its application in crop improvement. *Current Science 89(11)*: 1870–1878

Ferrie, A.M.R., Bethune, T., Kernan, Z. 2005. An overview of preliminary studies on the development of doubled haploid protocols for nutraceutical species. *Acta Physiologiae Plantarum 27(4B)*: 735–741

Gamborg, O.L., Miller, R.A., Ojima, O. 1968. Nutrient requirements of suspension cultures of soybean root cell. *Experimental Cell Research 50*: 151–158
Górecka, K., Krzyżanowska, D. 2004. The influence of different factors on the effectiveness of embryogenesis in head cabbage anther culture. *Vegetable Crops Research Bulletin 61*: 5–13
Górecka, K., Krzyżanowska, D., Górecki, R. 2005a. The influence of several factors on the efficiency of androgenesis in carrot. *Journal of Applied Genetic 46(3)*: 265–269
Górecka, K., Krzyżanowska, D., Kiszczak, W., Górecki, R. 2005b. Embryo induction in anther culture. *Vegetable Crops Research Bulletin 63*: 25–32
Górecka, K., Krzyżanowska, D. Kiszczak, W., Kowalska, U. Recovery of plants from embryos obtained from carrot anther cultures (in press)
Hu, K.L., Matsubara, S., Murakami, K. 1993. Haploid plant production by anther culture in carrot (*Daucus carota* L.). *Journal of Japanese Society for Horticultural Science 62(3)*: 561–565
Kiszczak, W., Górecka, K., Krzyżanowska, D., Kowalska, U. 2005. Size of flower buds in carrot (*Daucus carota* L.) as an indicator of a stage of microsporogenesis and its suitability for induction of androgenesis. *Vegetable Crops Research Bulletin. 62*: 81–90
Krzyźanowska, D., Górecka, K. 2004. Characteristic of Brussels sprouts plants obtained by anther culture. *Vegetable Crops Research Bulletin 60*: 25–31
Matsubara, S., Dohya, N., Murakami, K., Nishio, T., Dore, C. 1995. Callus formation and regeneration of adventitious embryos from carrot, fennel and mitsuba microspores by anther and isolated microspore cultures. *Acta Horticulturae 392*: 129–137
Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum 15*: 473–497
Nitsch, C., Norreel, B. 1973. Histophysiologie Végétable. Effect d'un choc thermique sur le pouvoir embryogéne du pollen de *Datura innoxia* cultivé dan l'anthére ou isolée de l'anthére. *Les comptes rendus de l'Académie des sciences Paris D 276*: 303–306
Smykalova, I., Horacek, J., Smykal, P., Soukup, A., Gasmanova, N., Kubosiova, M., et al. 2006. Influence of some climatic factors in the period of donor plants detection of dihaploid status using isozyme analysis and fluorescent microscopy in caraway. Proceedings from the International Conference "Haploids in Higher Plants III" Vienna, Austria, February 12–15, 2006 (p. 43)
Tyukavin, G.B., Shmykova, N.A., Mankhova, M.A. 1999. Cytological study of embryogenesis in cultured carrot anthers. *Russian Journal of Plant Physiology 46(6)*: 876–884
Wang, M., van Bergen, S., van Dujin, B. 2000. Update on plant development: Insights into a key developmental switch and its importance for efficient plant breeding. *Plant Physiology 124*: 523–530
Westphal, L., Wricke, G. 1989. Genetic analysis of DIA, GOT and PGI isozyme loci in *Daucus carota* L. ssp. *sativus. Plant Breeding 102: 51–57*

Chapter 21
Haploids and Doubled Haploids in Fruit Trees

M.A. Germanà

Abstract Haploid and doubled haploid (DH) production provides a particularly attractive biotechnological tool in breeding of perennial crop species, such as fruit trees. Haploids (plants with a gametophytic chromosome number) and doubled haploids (haploids that have undergone chromosome duplication) reduce the time needed to produce homozygous lines compared to conventional breeding. In fruit crops, characterized by high heterozygosity, long generation cycle times, large size, and (often) self-incompatibility, there is no other way to produce homozygous breeding lines through conventional methods that involve several generations of selfing. Gametic embryogenesis, enables the development of haploids and double haploids from heterozygous parents in a single step, and is therefore increasingly the object of research for fruit breeders. This chapter provides the current status of research on doubled haploid production in many fruit crops: *Actinidia deliciosa*, *Annona squamosa, Eriobotrya japonica, Carica papaya*, *Citrus*, *Feijoa sellowiana*, *Malus domestica*, *Morus alba*, [*Musa balbisiana* (BB)], *Prunus armeniaca*, *Prunus avium*, *Prunus domestica*, *Prunus persica*, *Pyrus communis*, *Pyrus pyrifolia*, *Olea europaea, Opuntia ficus-indica*, *Vitis vinifera*.

Keywords Anther culture, gametic embryogenesis, gynogenesis, homozygosity, isolated microspore culture

Introduction

World fruit production amounted to over 524 million tonnes in 2006 (FAOSTAT, Database). Worldwide, the most cultivated fruit trees are citrus, bananas, grapes, apples, peaches, pears, plums, apricot and kiwis.

M.A. Germanà(✉)
Dipartimento S.EN.FI.MI.ZO, Sezione Frutticoltura Mediterranea,
Tropicalee Subtropicale, Università degli Studi di Palermo, Italy
E-mail: agermana@unipa.it

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Fruit tree breeding goals are focused on increasing the external and internal quality of the product, the resistance or tolerance of the scions and of the rootstocks to biotic and abiotic stresses, always keeping in mind the necessity of a more sustainable agriculture. To achieve such improvements, conventional (hybridization, selection, mutation) or biotechnological methods are available. Embryo culture, regeneration from protoplasts, somatic hybridization, *in vitro* mutant selection, genetic transformation and haploid production are all biotechnological tools that can greatly help fruit crop breeding. Among the biotechnological methods, haploid and doubled haploid production, through gametic embryogenesis, allows the single-step development of complete homozygous line from the heterozygous parents, increasing the efficiency of perennial crop breeding programmes. In fact, there is no way to produce homozygous breeding lines in fruit trees through conventional methods that involve several generations of selfing.

There are several methods available to obtain haploids (Hs) and doubled haploids (DHs) in fruit trees. This chapter deals with the current status of research on doubled haploid production in the main fruit crops.

Importance of Haploids and Doubled Haploids in Fruit Crops

A sporophytic (2n) and a gametophytic (n) generation constitute the living cycle of higher plants, where the gametes are products of meiosis and the sporophytic generation, usually diploid, develops by fertilization producing the zygote. The gametophyte size and lifespan have been progressively reduced during evolution, compared with those of the sporophytic generation. Heslop-Harrison (1979) termed this step of angiosperm life the "forgotten generation", because the influence of the gametophytic phase on the sporophyte is usually underestimated and gametes are only considered as vectors for the genome transmission to the next sporophytic generation.

In the last few decades, pollen biology and biotechnology has received much more attention, making the manipulation of pollen development and function a reliable tool for crop improvement (Mulchay 1986). The most important application of pollen biotechnology in breeding and genetic studies derives from the ability to obtain haploids and doubled haploids.

Haploid plants are sporophytes carrying the gametic chromosome number (n instead of 2n), and doubled haploids (DHs) are haploids that have undergone spontaneous or induced chromosome duplication. Already by the time of the first discovery of a natural haploid in *Datura stramonium* and *Nicotiana* (Blakeslee et al. 1922; Kostoff 1929), but long before techniques for producing haploids by *in vitro* gametic embryogenesis were available, the importance of haploids in plant breeding and genetic research was recognized.

Guha and Maheshwari (1964) discovered that, by *in vitro* culture of immature anthers of *Datura innoxia*, it is possible to obtain haploid embryoids, thus paving the way to further and more extensive research on anther culture.

Recent technological innovations, greater understanding of underlying control mechanisms and an expansion of end-user applications has brought about a resurgence of interest in haploids in higher plants (Forster et al. 2007). In fact, haploids and DHs mostly attract breeders, working with both annual and perennial crop species.

Application of DH systems in a conventional breeding programme saves many generations normally needed to produce pure breeding lines. In fact, haplodization through gametic embryogenesis allows the single-step development of complete homozygous lines from heterozygous parents. In a conventional breeding programme, pure lines are developed after several generations of selfing, but these are not 100% homozygous.

Haploid and DHs are important in the fields of genetic and developmental studies, as well as for plant breeding. In fact, they have a potential use in mutation research, selection, genetic analysis, transformation and in the production of homozygous cultivars also required to utilize as parental lines for F_1 hybrids (in exploiting heterosis).

DH technologies also enhance the effectiveness of selection of desired recombinants, especially when quantitative traits are evaluated (Forster and Thomas 2005). More than 280 new superior varieties produced with the use of various DH methods in many annual crops (http://www.scri.sari.ac.uk/assoc/COST851/, see also Forster and Thomas 2005).

The release of new fruit trees cultivars through DH technology is much more difficult and more time requiring, as several years are required for the *in vitro* regenerated plants to go through the juvenile period and begin flowering. Many haploids and DHs are under observations in many fruit trees such as citrus, apple, papaya and peach, but it takes considerable time to characterize them (Pooler and Scorza 1995a,b; Germanà et al. 2000a, 2005; Höfer et al. 2002; Yahata et al. 2005; Vanwynsberhe et al. 2005; Rimberia et al. 2007).

"Gametoclonal variation", i.e. the morphological, biochemical and genetic variation observed among plants regenerated from cultured gametic cells (Evans et al. 1984; Morrison and Evans 1987), is another reason to use haploids and DHs in crop improvement. It results from both meiotic and mitotic division other than from the *in vitro* tissue culture ("somaclonal variation", Larkin and Scowcroft 1981), and, because of their homozygousity, the gametoclones show the direct expression of both dominant and recessive mutations.

Haploid cells are also ideal for mutation induction and selection, because they facilitate screening for both recessive and dominant mutants, and avoid chimeras, thus shortening the breeding time. The most interesting aspect of the mutagenic treatments applied to haploid cells is the immediate expression of recessive mutations and the possibility to obtain, by doubling chromosomes, complete homozygous diploids (Howland and Hart 1977). The development of improved DH protocols now makes this a viable option for many species (Maluszynsky et al. 2003).

Doubled haploids have featured strongly in basic and applied genetic studies of crop plants and have an important role in the development and exploitation of structured mutant populations for forward and reverse genetics (reviewed by Forster and

Thomas 2005). DHs are also very useful for genome mapping, providing reliable information on the location of major genes and QTLs for economically important traits (Khush and Virmani 1996).

Methods in Obtaining Haploids and Doubled Haploids in Fruit Crops

Fruit crops characteristics, such as high heterozygosity, long generation cycle with a long juvenile period, large size, and (often) self-incompatibility, do not allow easy haploidization through conventional methods, i.e. via several generations of selfing. The single-step development of complete homozygous lines from heterozygous parents is possible through gametic embryogenesis, making feasible and shortening the time required to produce completely homozygous lines.

Considerable research has been carried out since the 1970s in obtaining haploids for fruit tree breeding through gametic embryogenesis, but they were not always successful (Ochatt and Zhang 1996; Germanà 2006). Doubled haploid techniques including anther and microspore cultures, wide hybridisation, ovary and ovule cultures have been well established in a range of economically important crop species, including major cereals and Brassicas (Wedzony et al. 2006).

Haploids can be mainly induced by regeneration from the male gamete (pollen embryogenesis) or from the female gamete (gynogenesis).

Haploids from the Female Gametes

In gynogenesis, haploid cells of the female gametophyte (usually the unfertilized egg cell) are stimulated to develop into an embryo in a process similar to parthenogenesis. Gynogenesis is mainly used in onion (*Allium cepa*) and sugarbeet (*Beta vulgaris*) as well as in some trees. Currently, gynogenesis is the least favoured technique because of the low efficiency, but the value of doubled haploids in species that do not respond to more efficient techniques makes the method worthwhile (Forster et al. 2007).

Development of Spontaneous Haploids

Spontaneous haploid recovery has been reported in over 100 angiosperm species (Kasha 1974). In fruit trees particularly, spontaneous and low viable haploid plants have been recovered in apple, pear, peach, plum, apricot, etc., but with a frequency too low for practical application in fruit breeding (Zhang et al. 1990).

The origin of spontaneously occurring haploids can be parthenogenesis (i.e., the production of an embryo from an egg cell without the participation of the male gamete) or apogamy (the production of an embryo from a gametophytic cell other than the ovum).

In situ parthenogenesis induced by irradiated pollen followed by *in vitro* embryo culture

Gynogenesis achieved by *in situ* pollination with irradiated pollen, has been successfully reported for fruit trees in *Malus domestica* (Zhang and Lespinasse 1991; Höfer and Lespinasse 1996), *Pyrus communis* (Sniezko and Visser 1987; Bouvier et al. 1993), *Actinidia deliciosa* (Pandey et al. 1990; Chalak and Legave 1997), *Citrus* (De Lange and Vincent 1988; Ollitrault et al. 1996; Froelicher et al. 2007) and *Theobroma cacao* (Falque et al. 1992). These methods rely on pollination with pollen irradiated by gamma rays from cobalt 60 followed by *in vitro* culture of immature seeds or embryos. The selection of an efficient radiation dose, the optimization of the pollination method, the seed collection time, the developmental stage and the cultural media and conditions are important factors affecting the success of this technique and the number of haploid embryos rescued through this technique.

Several studies showed that irradiated pollen can germinate on the stigma, grow within the style and reach the embryo sac, but cannot fertilize the egg cell and the polar nuclei (Musial and Przywara 1998). However, it can stimulate the division of the egg cell, inducing parthenogenesis or development of parthenocarpic fruit. Pollen response to irradiation is genotype and dose-dependent. Usually, low doses induce conventional mutational events, while at higher doses increased frequency of parthenogenetic embryos is recovered. This phenomenon has been explained by the 'Hertwig effect' (the production in frogs of maternal individuals with the use of male gametes treated with high doses of ionizing radiation) (Pandey and Phung 1982). Genetic and development aberrations, such as selective gene transfer, 'egg transformation' via incorporation of fragments of male DNA have been observed after high pollen irradiation doses (Pandey 1978, 1980). Pollination performed by irradiated pollen induced autonomous endosperm development in apple (James et al. 1985; Nicoll et al. 1987; Zhang and Lespinasse 1991) and cacao (Falque 1994).

In situ or *in vitro* parthenogenesis induced by pollen from triploid plants followed by *in vitro* embryo culture

Gynogenesis can also be induced by using pollen from triploid plants, that like irradiated pollen, germinates, but does not fertilize the egg, stimulating the development of haploid embryos from ovules. Gynogenic haploids have been rescued after pollinating *in situ* or *in vitro* pistils of mono-embryonic citrus varieties (Oiyama and Kobayashi 1993; Germanà and Chiancone 2001).

Haploids from the Male Gametes

The ability of obtaining haploids and DHs is one of the most important applications of pollen biotechnology in breeding and genetics. Regeneration from male gametes has been reported in about 200 species belonging to some families, such as *Solanaceae*, *Cruciferae* and *Gramineae* (Dunwell 1986; Hu and Yang 1986; Maluszynsky et al. 2003), while many members of *Leguminosae* family and many woody plants remain recalcitrant (Sangwan-Norrel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995).

In vitro pollen embryogenesis is affected by numerous factors: genotype, pretreatment applied to anthers or to floral buds, pollen developmental stage, donor plant growth conditions, culture media (macro and microelements, carbon source, and plant growth regulators), and conditions of incubation. Particularly referring to the donor plant growth conditions, they result a 'weak point' in the DH technology of woody plants that are perennial and cultivated in open-air in comparison to the annual species. In fact, the physiological and growth conditions of the donor plant influence the endogenous levels of hormones and the nutritional status of the anther tissues (Sunderland and Dunwell 1977), dramatically affecting the success of the embryogenic process. In spite of their importance, these factors can not be standardized in anther culture technique of perennial plants. The differences in the physiological conditions of the donor plant, affected by climatic (temperature, photoperiod and light), cultural (pruning, irrigation, fertilization, etc.) and pedological conditions (especially during flower induction and differentiation), could explain why the results of anther culture are season dependent, although the same conditions (genotype, pollen development stage, floral bud pretreatments, medium, light and temperature conditions of culture) are employed.

Although the application of pollen embryogenesis is widespread, the cellular, biochemical and molecular bases for the transformation of microspores into pollen embryoids are poorly understood. However, some results and factors are clear. For example, it is well known that in fruit trees the capacity to regenerate from a male gamete is genotype-dependent, and that the stage of microspore development is a critical factor for induction. Particularly, the fruit male gametes become competent to differentiate in a different way from the gametophytic pathway around the time of the first haploid mitosis (late uni-nucleate, vacuolate or early bi-cellular pollen stage). Usually, physical, thermal (heat, cold) or chemical (water stress, starvation) stresses are necessary to enable competent microspores to follow the sporophytic pathway instead of the gametophytic one. *In vitro* anther or isolated microspore culture are the most effective and widely used methods of producing haploids and DHs.

Haploidization by Anther Culture

Many years ago (1964), Guha and Maheshwari showed that it is possible, by *in vitro* culture of immature anthers of *Datura innoxia*, to induce a change in the normal gametophytic development of microspores into sporophytic development and that embryoids and plants with a haploid chromosome number can be produced.

Research on haploidization by anther culture has been carried out on several fruit trees, especially in pome and stone fruits and in citrus (Ochatt and Zhang 1996; Germanà 2006).

Anther Culture Technique

Floral buds, with the microspores at around the uni-nucleate stage of development, are collected from cultivated trees in the field. After pretreatment, the buds are

surface sterilized usually by immersion in 70% (v/v) ethanol, followed by immersion in sodium hypochlorite solution (about 1.5% active chlorine in water) containing a few drops of Tween 20, and finally rinsed three times for 5 minutes with sterile distilled water. Petals are aseptically removed with small forceps, and anthers are carefully dissected and placed into the medium. The stage of pollen development is commonly determined by staining one or more anthers per bud by acetocarmine, Schiff's reagent, or DAPI staining.

The diverse genotypes show very different basal medium, carbon sources and plant growth regulators requirements to induce pollen-derived plant formation. In some cases, mutagens (both physical and chemical) have been used to increase doubled haploid frequency in fruit tree anther culture technique such as apple (Maluszynsky et al. 1996). Particularly in *Malus* x *domestica* cv. Golden Delicious the application of 5–10 Gy gamma rays on flower buds before anther culture, induced the androgenic response (Zhang et al. 1992). It should be pointed out, however, that the stimulating effect of mutagenic treatment on androgenetic responses is highly genotype and dose specific (Szarejko and Forster 2007). For example, experiments carried out applying 100, 500, 1,000 and 2,000 Gy to flower buds of *Citrus clementina* Hort. ex Tan. just before anther culture, did not increase haploid embryos production (our unpublished result, 2005).

Haploidization by Isolated Microspore Culture

The technique of the isolated microspore culture, performed by removing somatic anther tissue, has several advantages over anther culture even if it requires better equipment and more skills compared to anther culture. In fact, it eliminates the unknown effects of the sporophytic anther tissue, allowing a greater control over the culture process of pollen embryogenesis. There are a few reports on isolated microspore cultures in fruit crops, such as apple (Oldani 1993; Höfer et al. 1999; Höfer 2004), citrus (Germanà et al. 1996) and olive (Bueno et al. 2004). Research is in progress on microspore culture of several fruits crops (prickly pear, cherry, olive, loquat, etc.) showing in some cases the first androgenic divisions (Germanà M.A. 2004, unpublished; Germanà et al. 2006).

Haploids and Doubled Haploids Obtained Through Gametic Embryogenesis in the Main Fruit Crops

Actinidia deliciosa (A. Chev.) C.F. Liang et A.R. Ferguson – Kiwifruit

Both gynogenesis and androgenesis have been used since the 1980's to obtain haploids in kiwifruit (an hexaploid species, $2n = 6x = 174$), however only gynogenesis has been successful (Fraser et al. 1991), while anther culture produced only somatic

embryogenesis (Fraser and Harvey 1988). Ploidy level reduction was obtained by pollinating several female kiwifruit cultivars with lethally irradiated pollen of different males and inconstant male (involving transitions between the male and female expressions) genotypes (Pandey et al. 1990). Irradiation doses of 500, 700 and 900 Gy did not affect pollen germination and seeds were regularly set. Seeds were germinated *in vitro*, after 3–6 months storage at 4°C and the ploidy level was estimated by measuring the size of nuclei of both embryos and endosperm cells with an image analyser (Musial and Przywara 1999). Fraser et al. (1991) reported to have two haploids plants from the Pandey breeding programme with half the chromosome number (87).

Chalak and Legave (1997) obtained parthenogenetic tri-haploids by pollinating the female cv. Hayward with pollen of two male genotypes, M1 and M2, irradiated with gamma rays at the dose of 200–1,500 Gy. Pollen irradiation little affected pollen germination, but decreased fruit set, fruit growth and the number of viable seeds produced per fruit, while the genotype of the pollen parent greatly influenced the ability to obtain both seedlings and tri-haploids (Chalak and Legave 1997). Seedling ploidy was assessed by flow cytometry and the analysis of isozymes and RAPD markers was carried out (Chalak 1995; Chalak and Legave 1997). Tri-haploid doubling was obtained by oryzalin treatment (Chalak and Legave 1996).

Annona squamosa L. – Anona

Haploid embryos from male gametes were produced through anther culture of *Annona squamosa* L. (Nair et al. 1983).

Carica papaya L. – Papaya

Haploid plantlets and pollen-derived embryos were obtained from papaya, a polygamous species, through anther culture (Litz and Conover 1978; Tsay and Sue 1985). Rimberia et al. (2005, 2006) investigating the effects of pre-cultural conditions and of hormonal medium conditions, greatly improved the induction rate up to about 4.0% (2005), and 13.8% (2006). They found that the pretreatment of 35°C was more efficient than 25°C and liquid media were more effective as pre-treatment than solid media (Rimberia et al. 2005). All the established plants were identified as female (by a sex specific SCAR marker for papaya), and almost all of them were triploids (Rimberia et al. 2006). Morphological and fruiting characteristics of papaya plants derived via anther culture have been evaluated (Rimberia et al. 2007). A sex-diagnostic PCR technique was also applied to the plantlets obtained.

Citrus spp. – Citrus

Citrus species, the largest produced fruit worldwide with over 107 million tonnes in 2006 (FAOSTAT database), are diploid with a monoploid number of chromosomes

(n = x = 9) (Frost 1925), although triploid and tetraploid forms of *Citrus* also exist. Haploid seedlings were first obtained by the application of gamma rays in *Citrus natsudaidai* (Karasawa 1971). One haploid embryo was obtained in an immature seed from a diploid (Clementine mandarin) x diploid (Pearl tangelo) cross (Esen and Soost 1972) and a haploid plant was obtained from the cross between ‘Banpeiyu’ pummelo and ‘Ruby Red’ grapefruit (Toolapong et al. 1996). Morphological characterization and reproductive potential of this fertile haploid pummelo has been reported (Yahata et al. 2005).

Nine haploid plantlets, which did not survive, and two embryogenic callus lines have been obtained in clementine (*Citrus clementina* Hort. ex Tan.), cv. SRA 63 after *in situ* parthenogenesis induced by pollen of Meyer lemon (*Citrus meyeri* Y. Tan.) irradiated at 300, 600 and 900 Gray (Gy) from a cobalt 60 source (Ollitrault et al. 1996). While *in vitro* pollen germination rate was not affected by irradiation up to 900 Gy, seed production and size were reduced at this dose. Ploidy level of the obtained plantlets was evaluated by flow cytometry and isozyme analyses have been carried out to characterize the regenerants.

Recently, haploid induction has been obtained in the mandarins ‘Fortune’ (*C. clementina* Hort. ex Tan. x *C. tangerina* Hort. ex Tan.) and ‘Ellendale’ (*C. reticulata* Blanco x *C. sinensis* L. Osb.) by Froelicher et al. (2007) through *in situ* gynogenesis by pollination with irradiated pollen of ‘Meyer’ lemon. Pollination was carried out with three genotypes of mandarin with four levels of gamma-ray irradiated pollen (150, 300, 600, and 900 Gy). Embryos dissected from the resulting small seeds were rescued *in vitro*. Ploidy level of plantlets, determined by flow cytometry, was haploid, diploid and triploid. Microsatellite analysis showed that diploid and triploid plants were the result of crosses between mandarin and lemon.

In situ or *in vitro* parthenogenesis was also induced in *Citrus* by pollen from triploid plants, followed by *in vitro* culture of embryos. Pollen from the triploid plant, like irradiated pollen, is able to germinate, is not able to fertilize, but stimulates the ovules to develop haploid embryoids. Three haploid plants were obtained from *in vivo* crosses of two mono-embryonic diploids (clementine and “Lee”) x a triploid hybrid of “Kawano natsudaidai” (*Citrus natsudaidai* Hayata) (Oiyama and Kobayashi 1993). Restriction endonuclease analyses of nuclear ribosomal DNA and of chloroplast DNA verified the maternal origin of these haploids.

Haploid plantlets have also been obtained through gynogenesis by *in vitro* pollination of pistils of *Citrus clementina* Hort. ex Tan., cv. Nules, with pollen of ‘Oroblanco’, a triploid grapefruit-type citrus (Germanà and Chiancone 2001).

With respect to anther culture in *Citrus* and their relatives, haploid plantlets have been recovered from *Poncirus trifoliata* L. Raf. (Hidaka et al. 1979) and *C. madurensis* Lour. (Chen et al. 1980); one doubled haploid plantlet has been obtained from the hybrid No. 14 of *C. ichangensis* x *C. reticulata* (Deng et al. 1992); haploid plantlets and highly embryogenic haploid calli of *C. clementina* Hort. ex Tan. (Germanà et al. 1994, 2000b, 2005; Germanà and Chiancone 2003); haploid, but albino embryos of ‘Mapo’ tangelo (*C. deliciosa* x *C. paradisi*) (Germanà and Reforgiato 1997); haploid and diploid calli, embryos and leafy structures but no green plants of *C. limon* L. Burm. f. (Germanà et al. 1991);

haploid embryos of *Clausena excavata* (Froelicher and Ollitrault 2000) have been also produced.

Anther-derived calli can be highly embryogenic, and they can maintain embryogenic potential for a long time. The morphogenic calli in citrus appear friable and white. Sometimes calli develop from two different lobes of an anther (Fig. 1A). The embryogenic calli differentiate into a clump of embryos.

The cytological development of embryos from microspores in *C. aurantium* and *P. trifoliata* has been described by Hidaka and Omura (1989) and morphological

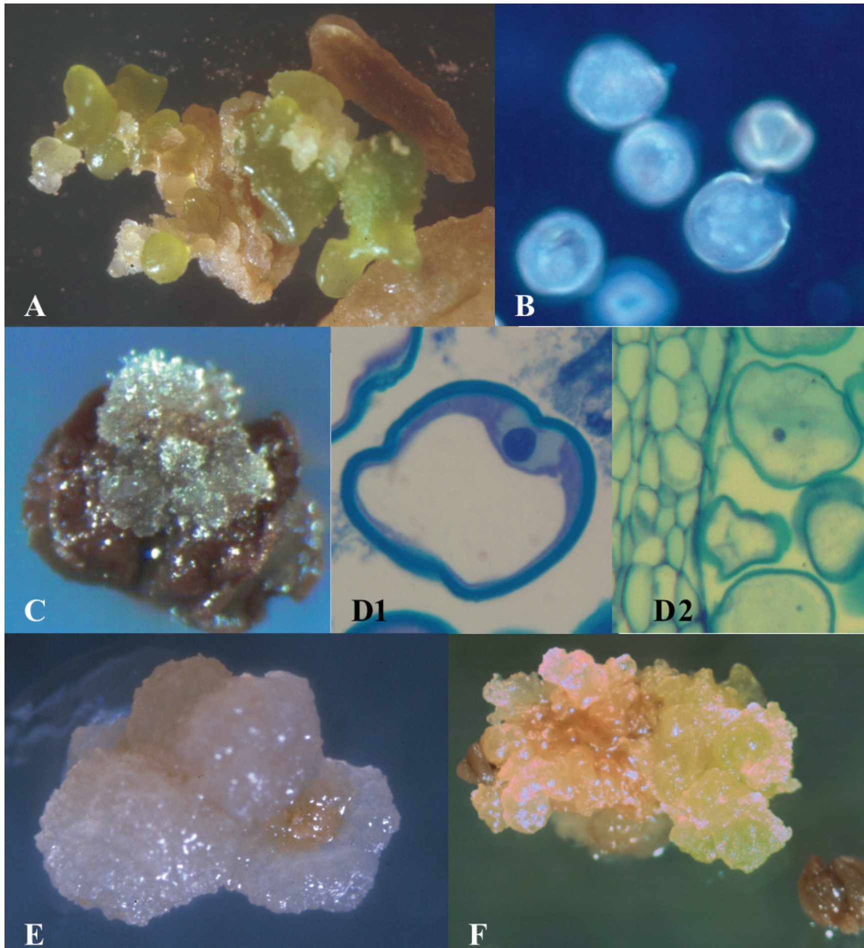


Fig. 1 **1A**. Calli developed from two different lebes of *Citrus clementina* Hort. ex Tan. anther **1B**. Multi-cellular pollen grains of *Eriobotrya japonica* Lindt. enclosed by the exine **1C**. Non-morphogenic callus from *Morus alba* I., cv. Florio, anther culture. **1D1** and **1D2**. Uni-nucleated (**D1**) and bi-cellular (**D2**) pollen grains in apricot anther culture (section stained with toluidine blue, photos taken the the laboratory of M.C. Risueño, C.S.I.S., Spain) **1E**. Callus from cherry anther culture. **1F**. Non-morphogenic callus from grape, cv Lacrima di Maria, anther culture.

and ultrastructural studies, at cellular and subcellular levels of early microspore embryogenesis in several embryogenic varieties of *Citrus clementina* have been carried out by Ramirez et al. (2003).

Isozyme analyses (Germanà et al. 1991, 1994, 2000a,b; Deng et al. 1992; Ollitrault et al. 1996; Germanà and Reforgiato 1997; Germanà and Chiancone 2001) and microsatellites (Germanà and Chiancone 2003; Germanà et al. 2005) have been employed to characterize regenerants obtained from citrus anther culture.

Triploids (very important in fruit crops such as *Citrus* or table grape because they are seedless and in demand by the fresh fruit market) are conventionally produced by 2x × 4x and 4x × 2x crosses. One of the most interesting applications of haploids in *Citrus* breeding is the possibility of obtaining triploid somatic hybrids by fusion between haploid and diploid protoplasts (Kobayashi et al. 1997; Ollitrault et al. 2000). Moreover, the ploidy analysis by flow cytometry of 94 regenerants from clementine anther culture, showed that about the 82% were tri-haploids, rather than haploids or doubled haploids as expected, indicating this technique to be a reliable method of obtaining new triploid varieties in clementine (Germanà et al. 2005).

Investigations of isolated microspore culture of several *Citrus* species (lemon, orange, clementine, sour orange, grapefruit) and a related genus (*Poncirus*) have been carried out (Germanà et al. 1996). Multi-nucleated structures, pseudo-bulbils and small proembryos, which failed to develop significantly, have been obtained.

For a complete review of haploidization in *Citrus* see Germanà (1997, 2003, 2006, 2007) and Germanà et al. (2005).

Eriobotrya japonica Lindl. – Loquat

Loquats originate from China and are grown mainly in sub-tropical and Mediterranean countries. The responses of nine of the most widely grown loquat cultivars to anther culture have been evaluated; four cultivars (El Buenet, Marchetto, Peluche and Sanfilippara) were subsequently selected due to their positive response (Germanà et al. 2006). The cellular changes promoted in the *in vitro* cultured anthers have been characterized through microscopical analysis and show the presence of pollen-derived multi-cellular structures that indicate pollen re-programming (Fig. 1B) (Germanà et al. 2006).

Feijoa sellowiana Berg. – Feijoa

Experiments regarding anther culture of feijoa have been reported with formation of multi-nucleated pollen grains (Canhoto and Cruz 1993).

Malus domestica (L.) Borkh. – Apple

Apples, with almost 64 million tonnes produced during 2006 (FAOSTAT database), are among the most produced fruits in the world, alongside citrus, bananas and grapes. Haploid production has been obtained in *Malus domestica* (L.) Borkh, 2n = 2x = 34, by various methods. Gynogenesis through *in vitro* culture of un-pollinated ovaries and ovules, without plant regeneration has been induced by Zhang and Lespinasse (1988). Haploid plants have been obtained through *in situ* parthenogenesis induced by pollination of several cultivars with pollen irradiated at different gamma rays from Cobalt 60, followed by *in vitro* embryo rescue (Zhang et al. 1987; Zhang 1988; Zhang et al. 1990; Zhang and Lespinasse 1991; Zhang et al. 1992; De Witte and Keulemans 1994; Verdoodt et al. 1998). Pollination by irradiated pollen affected fruit set, seed number and seed content and induced the formation of parthenogenetic embryos, mainly at high irradiation doses. There was an influence of the female genotype on the percentage of empty seeds, of embryo-containing seeds or seeds with only endosperm. Immature embryos were extracted 2–3 months after pollination and cultured *in vitro* after 2 months of cold treatment (3°C).

De Witte and Keulemans (2000a, b) carried out studies on the influence of pollination techniques and of plant growth regulators on the homozygous plant production by parthenogenesis *in situ*. James et al. (1985) and Nicoll et al. (1987) obtained, by pollination with irradiated pollen, the formation of seeds just with endosperm or with endosperm and embryos, observing that, only a single sperm nucleus is present in the tube of pollen grains irradiated at high dose levels, allowing the fertilization either of the egg cell or of the fused polar nuclei.

Zhang and Lespinasse (1991) observed that the pollination with gamma irradiated pollen in apple can promote, interrupting normal double fertilization, the production of parthenocarpic fruits and the development of parthenogenesis with the production of haploid and diploid embryos as well as endosperms with or without an embryo. They also stated that the optimum condition for inducing *in situ* parthenogenesis are: (1) pollen irradiation doses of 200 and 500 Gy; (2) collection of fruits 3 months after pollination; (3) *in vitro* culture of immature embryos and (4) cold treatment (3°C) of embryos for 2 months.

Several authors (Fei and Xue 1981; Xue and Niu 1984; Zarsky et al. 1986; Zhang et al. 1987; Höfer and Hanke 1990; Verdoodt et al. 1998; Höfer 1994, 2003) have reported pollen-derived plants from anther culture in this species, where the induction rate is still low and highly genotype-dependent (see also Höfer 1995, 1997). Genotype was found to have a much less marked effect on *in situ* parthenogenesis induced by irradiated pollen than on *in vitro* pollen embryogenesis (Zhang and Lespinasse 1992; Höfer and Lespinasse 1996). As in *Citrus*, triploids regenerated from anther culture have been reported in apple (Höfer 1994; Höfer et al. 2002).

Isozyme analyses, sequence characterized amplified regions (SCARs) (Höfer and Grafe 2000) simple sequence repeats (SSRs) (Kenis and Keulemans 2000; Höfer et al. 2002; Vanwynsberhe et al. 2005) and the single multi-allelic self-incompatibility gene (Verdoodt et al. 1998) have been used to genotype and to confirm the gametic origin of calli and plantlets in apple haploid technology.

Zhang (1988) observed three different routes in apple pollen embryogenesis: formation of two identical nuclei after an abnormal pollen mitosis; division of the vegetative nucleus after a normal pollen mitosis; and division of the generative nucleus after a normal pollen mitosis.

Pollen embryogenesis and plant formation through isolated apple microspores have been reported in the cultivar ‘Rene’ (Höfer et al. 1999) and later, with an improvement of the pretreatment, the concentration of carbon source and the microspore density, in the cultivars ‘Alkmene’, ‘Remo’, ‘Rene’ and ‘Realka’ (Höfer 2004).

Vanwynsberhe et al. (2005) reported that the use of homozygous androgenic genotypes from the cultivar Braeburn in apple breeding programmes is currently not a realistic approach, partly because of the low efficiency of anther culture, but mainly because of the reduced vigour and severe sterility of the androgenic genotypes produced.

Morus alba L. – Mulberry

Selfing to obtain haploids and homozygous plants is not possible in mulberry due to its dioecious nature. Haploids of a female clone of mulberry (*Morus alba* L. Cv.K-2) were produced through *in vitro* culture of un-pollinated ovaries (Dennis Thomas et al. 1999). Attempts to obtain haploids by anther culture (Fig. 1C) have not been successful in this genus (Sethi et al. 1992; Jain et al. 1996).

Musa spp. – Banana

Bananas production amounted to over 71 million tonnes during 2006 (FAOSTAT database). Kerbellec (1996) reported successful haploid (n = x = 11) plant regeneration in banana (*Musa acuminate,* AA) and 41 banana (*Musa balbisiana,* BB) haploid plants have been obtained by anther culture (Assani et al. 2003). The genotypes that responded were: Pisang klutuk, Pisang batu, Pisang klutuk wulung and Tani.

Olea europaea L. – Olive

Olive, a diploid (2n = 2x = 46), out-crossing, long-living species, is the most important oil-producing plants of the Mediterranean basin. It is characterized by a very long juvenile phase a large plant size and often by self-incompatibility. Isolated microspore culture of two olive cultivars (Arbequina and Picual) have been carried out resulting in induction of sporophytic division, multi-nucleate microspores and multi-cellular structures (Bueno et al. 2004). Pollen-derived multi-cellular structures and calli have been also obtained in two Sicilian olive cultivars, Nocellara Messinese and Tonda Iblea (Germanà et al. 2006 unpublished).

Opuntia ficus-indica (Mill.) – Prickly Pear

Opuntia ficus-indica L. Mill., native of Mexico, is cultivated for its fruits and as a forage crop for animals. Cleistogamy, nucellar embryony and low seed germination have been obstacles in the development of new cultivars in prickly pear (Chessa and Nieddu 2002; Chessa et al. 2000) and little innovative research has been carried out on *Opuntia* breeding.

Research has been carried out to study the correlation of sequential floral and male gametophyte development and to investigate the response to *in vitro* culture of anthers collected from flower buds of two different stages of development of the cv. "Gialla" of prickly pear (Gonzalez-Melendi et al. 2005). Because of the auto-fluorescence of the intricate exine of the pollen grain, *Opuntia* is not amenable to DAPI staining and light-microscopy observation of semi-thin sections has been deployed. These sections were stained with toluidine blue for general structure recognition and with I_2KI to study starch deposition. The appropriate starting material for anther cultures, which aim to induce pollen embryogenesis have been selected. The preliminary study on anther culture allowed the production of calli, however no regeneration was obtained thus far.

Prunus armeniaca L. – Apricot

Harn and Kim (1972) obtained callus formation from apricot anther culture, but ploidy level was not determined. Peixe et al. (2004) reported the formation of calli in cultured anthers of apricot 'Harcot', as well as the differentiation of nodular structures. The authors studied the influence of the temperature pretreatment and evaluated, by flow cytometry, the ploidy level of calli that ranged from haploid to octoploid (Fig. 1D1 and 1D2).

Prunus avium L. – Sweet Cherry

In situ parthenogenesis has been induced by pollination with irradiated pollen, followed by immature embryo and cotyledon culture in sweet cherry (*Prunus avium* L.), cultivar 'Altenburger', with the production of four homozygous lines (Höfer and Grafe 2003). The pollen parent used was *Prunus cerasiferal,* cv. 'Atropurpurea', carrying a marker gene R encoding anthocyanin synthesis in the whole plant. Pollen was irradiated by gamma rays from cobalt 60 at various doses, but the irradiation treatments did not significantly decrease *in vitro* germination, however, they markedly decreased the fruit set.

Haploid callus has been obtained also through anther culture (Fig. 1E) (Seirlis et al. 1979; Höfer and Hanke 1990; Germanà et al. 2004 unpublished).

The gametic origin of calluses has been confirmed by isozyme analyses (Höfer and Grafe 2003).

Prunus domestica L. – Plum

A study on the effects of different (0, 100, 200, 500 and 1,000 Gy) doses of irradiation with gamma rays on the viability of pollen of the cv. "Stanley" and subsequent fruit set and seed development after pollination were carried out on a European plum (*Prunus domestica* L.), cv. "Rainha Clàudia Verde" (Peixe et al. 2000). The formation of 2n endosperm and abnormal embryo development after pollination with gamma irradiated (200 Gy) pollen and the haploid induction in this genotype by *in situ* parthenogenesis were observed. Only heart-shaped embryos, that did not further developed, were produced, while many of the plants produced from the pollination with 100 Gy irradiated pollen were abnormal and all of them analysed by flow cytometry were hexaploid like the female parent. An uncompleted transmission of the male genome in the plant obtained from germination of seeds obtained by pollen irradiated at low doses has been hypothesized.

Prunus persica (L.) Batsch – Peach

In peach (*Prunus persica* L. Batsch) haploids (1x = 1n = 8) plants developed spontaneously (Pratassenja 1939; Hesse 1971; Toyama 1974). Michellon et al. (1974). Seirlis et al. (1979) and Hammerschlag (1983) obtained haploid callus from peach anther culture, but no plants were regenerated.

Haploid peach characteristics such as thin shoots, narrow leaves, weak vegetative growth and small non-fertile or less fertile flowers have been described by several authors (Hesse 1971; Toyama 1974; Pooler and Scorza 1995a). Moreover, Pooler and Scorza (1995a, 1995b), reported the occurrence of unreduced gametes in haploid trees recovered by Toyama and aberrant transmission of DNA markers, this is likely to be due to somatic rearrangements (bud sports).

Further studies carried out by Scorza and Pooler (1999) on the growth and yield of F_1 hybrid peaches developed from DHs showed productivity similar to those of standard cultivars, but F_1 hybrids offer advantages in the production of uniform seedling scion cultivars. This can be profitable, especially in high-density production systems, because it is much cheaper to plant non-grafted seedlings than to use grafted plants.

Pyrus communis L. – Pear

As long ago as 1987, Sniezko and Visser, using 50 krad irradiated pear pollen of 'Bonne Louise d'Avranches', to pollinate flowers of the cultivars 'Conference', 'Doyenné du Comice' and 'Gieser Wildeman' obtained parthenocarpic fruit set, presumably associated with endosperm rather than embryo development.

In situ parthenogenesis has been induced through irradiated pollen (P) or seedling selection (S) in three pear cultivars: 'Doyenné du Comice' (P and S), 'Williams'

(S) and 'Harrow Sweet' (S) (Bouvier et al. 1993). Among more than 10,000 seedlings, 12 resulted, by chromosome counting, haploids (2n = x = 17). Instead, by the induction of haploid plants by irradiated pollen, three plants from 'Doyenné du Comice' and one from 'Williams' resulted of maternal origin by isozymatic analyses (Bouvier et al. 1993).

Two embryos were produced by pear anther culture, cv. Le Lectier, but their origin was not established and plant regeneration was not obtained (Kadota et al. 2002). To obtain regeneration from embryos in pear, as well as in apple, a cold treatment (many weeks long) is necessary to break the dormancy and usually only weak shoots develop from embryos (Kadota et al. 2002). Problems of hyperhydricity are often present in these genotypes. Most DHs were obtained through doubling the chromosome number by oryzalin administered *in vitro* (200–300 µM), and ploidy level has been assessed by flow cytometry (Bouvier et al. 2002).

Isozyme analyses and microsatellites (SSRs) have been employed to assess homozygosis in pear, confirming the gametic origin of calluses and plantlets (Bouvier et al. 2002).

Pyrus pyrifolia Nakai – Japanese Pear

Nine embryos were obtained from anther culture of the diploid (2n = 2x = 34) Japanese pear *Pyrus pyrifolia* Nakai, cultivar Gold Nijisseiki (at 0.12% rate) and ten from cv. Shinko (at 0.13% rate), but plant regeneration was not established (Kadota et al. 2002). Later, Kadota and Niimi (2004) reported the production of triploid plants through anther culture of the cultivar Shinko. The effect of activated charcoal has been studied in anther culture of Japanese pear (Kadota and Niimi 2004).

Theobroma cacao L. – Cacao

Attempts to induce haploid production through *in situ* parthenogenesis by pollination with irradiated pollen have been carried out in *Theobroma cacao* L. Preliminary research regarded the influence of different irradiation doses (0, 50, 100, 200, 500 and 1,000 Gy gamma rays) on pollen viability and *in vitro* germination (Falque et al. 1992). While pollen viability and *in vitro* germination was not affected by irradiation, no fruit set was obtained using pollen irradiated over 100 Gy. In further experiments Falque (1994) carried out pollinations with irradiated (0, 50, 70 and 90 Gy), but no haploid was obtained. Pod size, ripening time and number of beans were affected by irradiation. However, morphological mutants were obtained and some of them had inherited paternal alleles for biochemical markers. Accidental self-fertilization of the two self-incompatible clones through mentor pollen effect, was observed in rare cases.

Vitis vinifera L. – Grapevine

In grapevine (2n = 2x = 38), that is one of the most cultivated plants in the world with almost 69 million tonnes produced during 2006 (FAOSTAT database), one haploid was reported by Zou and Li (1981) and haploid callus line production has been reported by Gresshoff and Doy (1974), Kim and Peak (1981) and Cersosimo (1986). Regeneration of plants has been obtained through anther culture by Rajasekaran and Mullins (1979), Bouquet et al. (1982), Hirabayashi and Akihama (1982), Mauro et al. (1986) and Cersosimo et al. (1990). In *Vitis,* anther culture (Fig. 1F) is usually employed to establish diploid somatic embryogenic cultures (Mauro et al. 1986; Cersosimo et al. 1990).

Androgenic development of the microspores in anthers of *Vitis rupestris* du Lot cultured *in vitro* has been observed by a histological study (Altamura et al. 1992). However, embryos did not develop into plants, probably due to the many deleterious genetic effects (Cersosimo 1996).

Cersosimo (1987) and Rajasekaran and Mullins (1979) have studied the effect of cultural conditions on grape anther culture.

Conclusions

In perennial plants particularly, where breeding is usually cumbersome and time-consuming due to their long reproductive cycle, high degree of heterozygosity and complex reproductive biology, the potential of gamete biotechnology constitutes a great advantage in comparison with conventional breeding methods.

A deeper understanding of the gametic embryogenesis process and of the factors affecting morphogenic competence and development, will enable the effective deployment of gametic embryogenesis and haploid technology in the improvement of fruit trees. This has already occurred in many annual crops such as *Cruciferae, Gramineae* and *Solanaceae*. The future of haploid and doubled haploid production as a powerful fruit tree breeding tool also requires the availability of reliable tissue culture protocols, which can overcome several problems such as low frequencies of embryo induction, albinism, plant regeneration, plant survival and the genotype- and season-dependent response. However, the increasing number of recent reports regarding haploid research in fruit trees, shows the great interest in this useful breeding tool, and encourages us to look with optimism at its future applications in these important crops.

References

Altamura MM, Cersosimo A, Majoli C, Crespan M (1992) Histological study of embryogenesis and organogenesis from anthers of *Vitis rupestris* du Lot cultured *in vitro*. Protoplasma 171: 134–141

Assani A, Bakry F, Kerbellec F, Haicour R, Wenzel G, Foroughi-Wehr B (2003) Production of haploids from anther culture of banana [*Musa balbisiana* (BB)]. Plant Cell Rep 21: 511–516
Bajaj YPS (1990) *In vitro* production of haploids and their use in cell genetics and plant breeding. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry: Haploids in Crop Improvement. Berlin: Springer, Vol. 12. Part I. pp. 1–44
Blakeslee AF, Belling J, Farnham ME, Bergner AD (1922) A haploid mutant in the Jimson weed, *Datura stramonium*. Science 55: 646–647
Bouquet AB, Piganeau B, Lamaison AM (1982) Influence du génotype sur la production de cals, d'embryons et de plantes entières par cultures d'anthères *in vitro* dans le genre Vitis. CR Acad Sci Paris 259: 569–574
Bouvier L, Zhang YX, Lespinasse Y (1993) Two methods of haploidization in pear, *Pyrus communis* L.: Greenhouse seedling selection and *in situ* parthenogenesis induced by irradiated pollen. Theor Appl Genet 87: 229–232
Bouvier L, Guérif P, Djulbic M, Durel CE, Chevreau E, Lespinasse Y (2002) Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers. Euphytica 123: 255–262
Bueno MA, Pintos B, Prado MJ, Gomez A, Manzanera JA (2004) Androgenesis: A tool for woody plant breeding. Rec Devel Genet Breed 1: 365–383
Canhoto JM, Cruz GS (1993) Induction of pollen callus in anther cultures of *Feijoa sellowiana* Berg. (*Myrtaceae*). Plant Cell Rep 13: 45–48
Cersosimo A (1986) Coltura *in vitro* di antere in *Vitis* sp. (I contributo). Rivista di Viticoltura e di Enologia di Conegliano 39(12): 520–531
Cersosimo A (1987) Coltura *in vitro* di antere in *Vitis* sp. (II contributo). Agr Ric, 75–76: 55–60
Cersosimo A (1996) Haploidy in vitis. In: Jain SM, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants. Dordrecht: Kluwer, Vol. 3, pp. 211–222
Cersosimo A, Altamura MM, Crespan M, Paludetti G (1990) Embryogenesis, organogenesis and plant regeneration from anther culture in *Vitis*. Acta Hort 280: 307–314
Chalak L (1995) Haploidisation et cultures cellulaires chez le kiwi (*Actinidia deliciosa* cv. Hayward). Characterisation préliminaire de matériels obtenus. Ph.D. Thesis, Université Montpellier, France 2, 218
Chalak L, Legave JM (1996) Oryzalin combined with adventitious regeneration for an efficient chromosome doubling of trihaploid kiwifruit. Plant Cell Rep 16: 97–100
Chalak L, Legave JM (1997) Effects of pollination by irradiated pollen in Hayward kiwifruit and spontaneous doubling of induced parthenogenetic trihaploids. Sci Hort 68: 83–93
Chen Z, Wang H, Liao H (1980) The induction of *Citrus* pollen plants in artificial media. Acta Genet Sin 7: 189–192
Chessa I, Nieddu G (2002) Investigations on variability in the genus *Opuntia* as fruit crop for genetic improvement. Acta Hort 575: 345–353
Chessa I, Russu C, Nieddu G (2000) Embrionia nucellare ed allevamento dell'embrione zigotico in *Opuntia ficus-indica* (Mill.). Atti IV Congresso Nazionale su Biodiversità "Germoplasma locale e sua valorizzazione" 2: 649–652
De Lange JH, Vincent AP (1988) Studies on *Citrus* pollination using gamma irradiated pollen. S Afr J Bot 54: 257–264
Deng XX, Deng ZA, Xiao SY, Zhang WC (1992) Pollen derived plantlets from anther culture of *Ichang papeda* hybrids No.14 and Trifoliate orange. Proceedings of the International Society of Citriculture. Acireale, Italy, pp. 190–192
De Witte K, Keulemans J (1994) Restriction of the efficiency of haploid plant production in apple cultivar 'Idared', through parthenogenesis *in situ*. Euphytica 77: 141–146
De Witte K, Keulemans J (2000a) Influence of pollination techniques, plant growth regulators for fruit set and *ex vitro* germination on homozygous plant production by parthenogenesis *in situ* in apple. In: Geibel M, Fischer M, Fischer C (eds) Proceedings of the Eucarpia Symposium on Fruit Breeding and Genetics. Acta Horticulturae. 538 ISHS, pp. 309–314
De Witte K, Keulemans J (2000b) Homozygous plant production in apple by parthenogenesis *in situ*: Improvement of the pollination stimulus for parthenogenic egg cell development. In:

Geibel M, Fischer M, Fischer C (eds) Proceedings of the Eucarpia Symposium on Fruit Breeding and Genetics. Acta Horticulturae. 538 ISHS, pp. 315–320

Dennis Thomas T, Bhatnagar AK, Razdan MK, Bhojwani SS (1999) A reproducible protocol for the production of gynogenic haploids of mulberry, *Morus alba* L. Euphytica 110: 169–173

Dunwell JM (1986) Pollen, ovule and embryo culture, as tools in plant breeding. In: Withers LAP, Alderson G (eds) Plant Tissue Culture and Its Agricultural Applications. London: Butterwoorths, pp. 375–404

Esen A, Soost RK (1972) Unexpected triploids in citrus: Their origin, identification, and possible use. J Hered 62: 329–333

Evans DA, Sharp WR, Medina-Filho HP (1984) Somaclonal and gametoclonal variation. Am J Bot 759–774

Falque M (1994) Pod and seed development and phenotype of the M1 plants after pollination and fertilization with irradiated pollen of cacao (*Theobroma cacao*). Euphytica 75(1–2): 19–25

Falque M, Kodia AA, Sounigo O, Eskes AB, Charrier A (1992) Gamma-irradiation of cacao (*Theobroma cacao*) pollen: Effect on pollen grain viability, germination and mitosis and fruit set. Euphytica 64: 167–172

FAOSTAT Database (2006) http://faostat.fao.org/default.aspx

Fei KW, Xue GR (1981) Induction of haploid plantlets by anther culture *in vitro* in apple. cv. “Delicious”. Sci Agric Sin 4: 41–44

Fraser LG, Harvey CF (1988) Somatic embryogenesis from anther derived callus in two *Actinidia* species. Sci Hort 29: 335–346

Fraser LG, Harvey CF, Kent J (1991) Ploidy manipulations of kiwifruit in tissue culture. Acta Hort 297: 109–114

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25: 57–88

Forster BP, Herberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12(8): 368–375

Froelicher Y, Ollitrault P (2000) Effects of the hormonal balance on *Clausena excavata* androgenesis. In: Goren R, Goldschmidt EE (eds) Proceedings of the First International Symposium on *Citrus* Biotechnology. Eilat, Israel. Acta Horticulturae 535, pp. 139–146

Froelicher Y, Bassene JB, Jedidi-Neji E, Dambier D, Morillon R, Bernardini GG, Costantino G, Ollitrault P (2007) Induced parthenogenesis in mandarin for haploid production: Induction procedures and genetic analysis of plantlets. Plant Cell Rep 26: 937–944

Frost HB (1925) The chromosomes of *Citrus*. J Wash Acad Sci 15: 1–3

Germanà MA (1997) Haploidy in citrus. In: Jain SM, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants, Dordrecht: Kluwer, Vol. 5, pp. 195–217

Germanà MA, Chiancone B, Padoan D, Barany I, Risucño MC, Testillano PS (2008) Induction of Pollan embryogenesis in seven cultivars of *Prunats armeniaca* L. In: Xiloyannis C (ed) Proceeding of the XIV International Symposium on apricot Breeding and culture, Matera, Italy, Acta-Horiculturae, (in press)

Germanà MA (2003) Haploids and doubled haploids in *Citrus spp*. In: Maluszynsky M, Kasha KJ, Forster BP, Szaejko I (eds) Doubled Haploid Production in Crop Plants: A Manual FAO-IAEA, Dordrecht: Kluwer, pp. 303–308

Germanà MA (2006) Doubled haploid production in fruit crops. Plant Cell Tiss Org Cult 86: 131–146

Germanà MA (2007) Haploidy. In: Khan I (ed) Citrus Genetics, Breeding and Biotechnology. Wallingford: CAB International, UK, pp. 167–196

Germanà MA, Reforgiato G (1997) Haploid embryos regeneration from anther culture of ‘Mapo’ tangelo (*Citrus deliciosa* x *C. paradisi*). Adv Hort Sci 11: 147–152

Germanà MA, Chiancone B (2001) Gynogenetic haploids of *Citrus* after *in vitro* pollination with triploid pollen grains. Plant Cell Tiss Org Cult 66: 59–66

Germanà MA, Chiancone B (2003) Improvement of the anther culture protocol in *Citrus clementina* Hort. ex Tan. Plant Cell Rep 22: 181–187

Germanà MA, Crescimanno FG, De Pasquale F, Wang YY (1991) Androgenesis in 5 cultivars of *Citrus limon* L. Burm. f. Acta Hort 300: 315–324

Germanà MA, Wang YY, Barbagallo MG, Iannolino G, Crescimanno FG (1994) Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and *Citrus reticulata* Blanco. J Hort Sci, 69(3): 473–480

Germanà MA, Scarano MT, Crescimanno FG (1996) First results on isolated microspore culture of *Citrus*. Proc Inter Soc Citriculture (South Africa). 2: 882–885

Germanà MA, Crescimanno FG, Reforgiato G, Russo MP (2000a) Preliminary characterization of several doubled haploids of *Citrus clementina* cv. Nules. In: Goren R, Goldschmidt EE (eds) Proceedings of the First International Symposium on Citrus Biotechnology. Eilat, Israel. Acta Horticulturae 535, 183–190

Germanà MA, Crescimanno FG, Motisi A (2000b) Factors affecting androgenesis in *Citrus clementina* Hort. ex Tan. Adv Hort Sci 14: 43–51

Germanà MA, Chiancone B, Lain O, Testolin R (2005) Anther culture in *Citrus clementina*: A way to regenerate tri-haploids. Aust J Agric Res 56: 839–845

Germanà MA, Chiancone B, Levy Guarda N, Testillano PS, Risueño MC (2006) Development of multicellular pollen of *Eriobotrya japonica* Lindl. through anther culture. Plant Sci 171: 718–725

Gonzalez-Melendi P, Germanà MA, Levy Guarda N, Chiancone B, Risueño MC (2005) Correlation of sequential floral development and male gametophyte development and preliminary results on anther culture in *Opuntia ficus-indica*. Acta Physiol Plant 27(4B): 687–694

Gresshoff PM, Doy CH (1974) Derivation of a haploid cell line from *Vitis vinifera* and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. Z. Pflanzenphysiol 73: 132–141

Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of *Datura*. Nature 204: 497–498

Harn C, Kim MZ (1972) Induction of callus from anthers of *Prunus armeniaca*. Korean J Breed 4: 49–53

Hammerschlag FA (1983) Factors influencing the frequency of callus formation among cultivated peach anthers. HortScience 18: 210–211

Heslop-Harrison J (1979) The forgotten generation: Some thoughts on the genetics and physiology of angiosperm gametophytes. In: Davies DR, Hopwood DA (eds) The Plant Genome. 4th J. Innes Symposium, Norwich, pp. 1–14

Hesse CO (1971) Monoploid peaches, *Prunus persica* (L.) Batsch: Description and meiotic analysis. J Am Soc Hort Sci 96: 326–330

Hidaka T, Omura M (1989) Origin and development of embryoids from microspores in anther culture of *Citrus*. Jpn J Breed 39: 169–178

Hidaka T, Yamada Y, Shichijo T (1979) *In vitro* differentiation of haploid plants by anther culture in *Poncirus trifoliata* (L.) Raf. Jpn J Breed 29: 248–254

Hirabayashi T, Akihama H (1982) *In vitro* embryogenesis and plant regeneration from the anther-derived callus of *Vitis*. In: Fujiwara A (ed) Plant Tissue Culture. Tokyo: Maruzen, pp. 547–548

Höfer M (1994) *In vitro* androgenesis in apple: Induction, regeneration and ploidy level. In: Schmidt H, Kellerhals M (eds) Progress in Temperate Fruit Breeding. Dordrecht/Boston/London: Kluwer, pp. 193–197

Höfer M (1995) In-vitro-Androgenese bei Apfel. Gartenbauwissenschaft 60: 12–15

Höfer M (1997) *In vitro* androgenesis in apple: Optimization of the Anther Culture. Acta Hort 447: 341–343

Höfer M (2003) *In vitro* androgenesis in apple. In: Maluszynsky M, Kasha KJ, Forster B, Szaejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. FAO-IAEA. Dordrecht: Kluwer, pp. 287–292

Höfer M (2004) *In vitro* androgenesis in apple – improvement of the induction phase. Plant Cell Rep 22: 365–370

Höfer M, Grafe Ch (2000) Preliminary evaluation of doubled haploid-material in apple. Acta Hort 538: 587–592

Höfer M, Grafe Ch (2003) Induction of doubled haploids in sweet cherry (*Prunus avium* L.). Euphytica 130: 191–197

Höfer M, Hanke V (1990) Induction of androgenesis *in vitro* in apple and sweet cherry. Acta Hort 280: 333–336

Höfer M, Lespinasse Y (1996) Haploidy in apple. In: Jain SM, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants. Dordrecht: Kluwer, Vol. 3, pp. 259–274

Höfer M, Touraev A, Heberle-Bors E (1999) Induction of embryogenesis from isolated apple microspores. Plant Cell Rep 18: 1012–1017

Höfer M, Gomez A, Aguiriano E, Manzanera JA, Bueno MA (2002) Analysis of simple sequence repeat markers in homozygous lines in apple. Plant Breed 121: 159–162

Howland GP, Hart RW (1977) Radiation biology of cultured plant cells. In: Reinert J, Bajaj YPS (eds) Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture. Berlin/Heidelberg/New York: Springer. pp.732–756

Hu H, Yang HY (eds) (1986) Haploids in Higher Plants *in Vitro*. Beijing/Berlin: China Academic Publishers/Springer

Jain AK, Sarkar A, Datta RK (1996) Induction of haploid callus and embryogenesis *in vitro* cultured anthers of Mulberry (*Morus indica*). Plant Cell Tiss Org Cult 44: 143–147

James DJ, Passey AJ, Mackenzie KAD, Menhinick EC (1985) The effect of pollen irradiation on the development of the post-fertilization ovule of apple (*Malus pumilia* Mill.). In: Chapman et al. (eds) Experimental Manipulation of Ovule Tissue. London: Longman, pp. 210–224

Kadota M, Niimi Y (2004) Production of triploid plants of Japanese pear (*Pyrus pyrifolia* Nakai) by anther culture. Euphytica 138: 141–147

Kadota M, Han DS, Niimi Y (2002) Plant regeneration from anther-derived embryos of apple and pear. HortScience, 37(6): 962–965

Karasawa K (1971) On the occurrence of haploid seedlings in *Citrus natsudaidai* Hayata. Bull. Sakushingakuin Junior Col Woman 1: 1–2

Kasha KJ (ed) (1974) Haploids in Higher Plants: Advances and Potential, Canada: University of Guelph Press, p. 421

Kenis K, Keulemans J (2000) The use of microsatellites to investigate the homozygous status of apple plants obtained by anther culture and parthenogenesis in situ. Acta Hort, 538: 581–585

Kerbellec F (1996) Establissement d'une technique d'androgenèse pour l'amélioration génétique du bananier (*Musa* ssp.) Ph.D. Thesis, Ecole Nationale Agronomique de Rennes, France

Khush GS, Virmani SS (1996) Haploids in plant breeding. In: Jain SM, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants, Dordrecht: Kluwer, Vol. 1, pp. 11–33

Kim SK, Peak KY (1981) Study on anther culture of grape. 1. Varietal differences in callus formation. J Korean Soc Hort Sci 22(2): 89–91

Kobayashi S, Ohgawara T, Saito W, Nakamura Y, Omura M (1997). Production of triploid somatic hybrids in *Citrus*. J Jpn Soc Hort Sci 66(3–4): 453–458

Kostoff D (1929) An androgenic *Nicotiana* haploid. Z Zellforsch Mikrosk Anat 9: 640–642

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60: 197–214

Litz RE, Conover RA (1978) Recent advances in papaya tissue culture. Proc Florida State Hort Soc 91: 180–182

Maluszynsky M, Szrejko I, Sigurbjornsson B (1996) Haploidy and mutation techniques. In: Jain SM, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants. Dordrecht: Kluwer, Vol. 1, pp. 67–93

Maluszynsky M, Kasha KJ, Szrejko I (2003) Published protocols for other crop plant species. In: Maluszynsky M, Kasha KJ, Forster BP, Szaejko I (eds) Doubled Haploid Production in Crop Plants: A Manual FAO-IAEA. Dordrecht: Kluwer, pp. 309–335

Mauro MC, Nef G, Fallot J (1986) Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet Sauvignon. Plant Cell Rep 5: 377–380

Michellon RJ, Hugard J, Jonard R (1974) Sur l'isolément de colonies tissulaires de pêcher (*Prunus persica* Batsch., cultivars Dixired and Nectared IV) et d'amandier (*Prunus amygdalus* Stokes, cultivar Ai) a partir d'anthères cultivées *in vitro*. C R Acad Sci Serie D 278: 1719–1722

Morrison RA, Evans DA (1987) Gametoclonal variation. Plant Breed Rev 5: 359–391
Mulchay DL (1986) Preface in recognition of the forgotten generation. Biotechnology and ecology of pollen. Proceedings of the International Conference on the Biotechnology and Ecology of pollen 1985. Amherst, MA: University of Massachusetts, pp. 6–11
Musial K, Przywara L (1998) Influence of irradiated pollen on embryo and endosperm development in kiwifruit. Ann Bot 82: 747–756
Musial K, Przywara L (1999) Endosperm response to pollen irradiation in kiwifruit. Sex Plant Reprod 12: 110–117
Nair S, Gupta PK, Mascarenhhas AS (1983) Haploid plants from *in vitro* anther culture of *Annona squamosa* L. Plant Cell Rep 2: 198–200
Nicoll MF, Chapman GP, James DJ (1987) Endosperm responses to irradiated pollen in apples. Theor Appl Genet 74: 508–515
Ochatt SJ, Zhang YX (1996) *In vitro* haploidization of fruit trees. In: Jain S, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants. Dordrecht: Kluwer, Vol. 3, pp. 193–210
Oiyama II, Kobayashi S (1993) Haploid obtained from diploid X triploid crosses of Citrus. J Jpn Soc Hort Sci 62(1): 89–93
Oldani C (1993) Cultures de microspores isolées: un outil nouveau pour l'haploidisation du pommier (*Malus* X *domestica* Borkh.). Mémoire de D.E.A. de Biologie du Développement des Plantes, Université P. and M. Curie (Paris VI)
Ollitrault P, Allent V, Luro F (1996) Production of haploid plants and embryogenic calli of clementine (*Citrus reticulata* Blanco) after *in situ* parthenogenesis induced by irradiated pollen. Proc Int Soc Citric South Africa, 2: 913–917
Ollitrault P, Vanel F, Froelicher Y, Dambier D (2000) Creation of triploid *Citrus* hybrids by electrofusion of haploid and diploid protoplasts. In: Goren R, Goldschmidt EE (eds) Proceeding of the First International Symposium on Citrus Biotechnology, Eilat, Israel. Acta Horticulturae, Vol. 535, pp. 191–198
Pandey KK (1978) Gametic gene transfer in *Nicotiana* by means of irradiated pollen. Genetics 49: 56–69
Pandey KK (1980) Further evidence for egg transformation in *Nicotiana*. Heredity 45: 15–29
Pandey KK, Phung M (1982) 'Hertwig Effect' in plants: Induced parthenogenesis through the use of irradiated pollen. Theor Appl Genet 62: 295–300
Pandey KK, Przywara L, Sanders PM (1990) Induced parthenogenesis in kiwifruit (*Actinidia deliciosa*) through the use of lethally irradiated pollen. Euphytica 51: 1–9
Peixe A, Campos MD, Cavaleiro C, Barroso J, Pais MS (2000) Gamma-irradiated pollen induces the formation of 2n endosperm and abnormal embryo development in European plum (*Prunus domestica* L., cv. "Rainha Clàudia Verde"). Sci Hort 86: 267–278
Peixe A, Barroso J, Potes A, Pais MS (2004) Induction of haploid morphogenic calluses from *in vitro* cultured anthers of *Prunus armeniaca* cv. 'Harcot'. Plant Cell Tiss Org Cult 77: 35–41
Pooler MR, Scorza R (1995a) Aberrant transmission of RAPD markers in haploids, doubled haploids, and F1 hybrids of peach: Observations and speculation on causes. Sci Hort 64: 233–241
Pooler MR, Scorza R (1995b) Occurrence of viable eggs in haploid peach. Fruit Varieties J, 49(4): 239–241
Pratassenja GD (1939) Production of polyploid plants, haploid and triploids in *Prunus persica*. Camp. Rend. Acad. Sci. (Doklady) URSS, 22: 348–351
Raghavan V (1990) From microspore to embryo: Faces of the angiosperm pollen grain. In: Nijkamp HJJ, van der Plas LH, J van Hartrigik (eds) Progress in Plant Cellular and Molecular Biology. I.A.P.T.C., Dordrecht: Kluwer, pp. 213–221
Rajasekaran K, Mullins MG (1979) Embryos and plantlets from cultured anthers of hybrid grapevines. J Exp Bot 30: 399–407
Ramirez C, Chiancone B, Testillano PS, Garcia-Fojeda B, Germanà MA, Risueño MC (2003) First embryogenic stages of *Citrus* microspore-derived embryos. Acta Biol Cracov Bot 45: 53–58
Rimberia FK, Sunagawa H, Urasaki N, Ishimine Y, Adaniya S (2005) Embryo induction via anther culture in papaya and sex analysis of the derived plantlets. Sci Hort 103: 199–208

Rimberia FK, Adaniya S, Takeomi E, Yukio I (2006) Sex and ploidy of anther culture derived papaya (*Carica papaya* L.) plants. Euphytica 149: 53–59
Rimberia FK, Adaniya S, Ishimine Y, Etoh T (2007) Morphology of papaya plants derived via anther culture. Sci Hort 111: 213–219
Sangwan-Norrel BS, Sangwan RS, Pare J (1986) Haploïdie et embryogenèse provoquée *in vitro*. Bull Soc Bot Fr 133, Actual Bot 4: 7–39
Scorza R, Pooler MR (1999) Growth and yield of F1 hybrid peaches developed from doubled haploids. HortScience 34(5): 928–931
Seirlis G, Mouras A, Salesses G (1979) Tentatives de culture *in vitro* d'anthères et de fragments d'organes chez Prunus. Ann Am Plant 29: 145–161
Sethi M, Bose S, Kapur A, Rangaswamy NS (1992) Embryo differentiation in anther cultures of mulberry. Indian J Exp Biol 30: 146–148
Sniezko R, Visser T (1987) Embryo development and fruit-set in pear induced by untreated and irradiated pollen Euphytica 1: 287–294
Sunderland N, Dunwell JM (1977) Anther and pollen culture. In: Street HE (ed) Plant Tissue and Cell Culture. Oxford: Blackwell, pp. 223–265
Szarejko I, Forster BP (2007) Doubled haploidy and induced mutation. Euphytica 158: 359–370
Toolapong P, Komatsu H, Iwamasa M (1996) Triploids and haploids progenies derived from small seeds of 'Banpeiyu' pummelo, crossed with 'Ruby Red' grapefruit. J Jpn Soc Hort Sci 65: 255–260
Toyama TK (1974) Haploidy in peach. HortScience 9(3): 187–188
Tsay HS, Sue CY (1985) Anther culture of papaya (*Carica papaya* L.). Plant Cell Rep 4: 28–30
Vanwynsberhe L, De Witte K, Coart E, Keulemans J (2005) Limited application of homozygous genotypes in apple breeding. Plant Breed 124: 399–403
Verdoodt L, Van Haute A, Goderis IJ, De Witte K, Keulemans J, Broothaerts W (1998) Use of the multi-allelic self-incompatibility gene in apple to assess homozygosity in shoots obtained through haploid induction. Theor Appl Genet 96: 294–300
Wedzony M, Zur I, Golemiec E, Szechin'ska-Hebda M, Dubas E (2006) Progress in doubled haploid technology. Euphytica DOI: 10.1007/s10681-006-9241-1
Wenzel G, Frei U, Jahoor A, Graner A, Foroughghi-Wehr B (1995) Haploids – an integral part of applied and basic research. In: Terzi M et al. (eds) Current Issues in Plant Molecular and Cellular Biology. Dordrecht: Kluwer, pp. 127–135
Yahata M, Harusaki S, Komatsu H, Takami K, Kunitake H, Yabuya T, Yamashita K, Toolapong P (2005) Morphological characterization and molecular verification of a fertile haploid pummelo (*Citrus grandis* Osbeck). J Am Soc Hort Sci 130(1): 34–40
Xue GR, Niu JZ (1984) A study on the induction of apple pollen plants. Acta Hort Sin 11: 161–164
Zarsky V, Tupy J, Vagera J (1986) In-vitro-culture of apple anther. In: Moet-Henessy Conference Fruit Tree Biotechnology, Moet Henessy, Paris, October, p. 75
Zhang YX (1988) Recherche *in vitro* de plantes haploïdes chez le pommier cultivé (*Malus* x *domestica* Borkh.): androgenèse, gynogenèse, parthénogenèse *in situ* induite par du pollen irradié. Ph.D. Thesis, Université de Paris-Sud, Centre d'Orsay, Paris
Zhang YX, Lespinasse Y (1988) Culture *in vitro* d'ovules non fécondés et d'embryons prélevés 8 jours après pollinisation chez le pommier cultivé (*Malus* x *domestica* Borkh.). Agronomie 8: 837–842
Zhang YX, Lespinasse Y (1991) Pollination with gamma-irradiation pollen and development of fruits, seeds and parthenogenetic plants in apple. Euphytica 54: 101–109
Zhang YX, Lespinasse Y (1992) Haploidy. In: Hammerschlang FA, Litz RE (eds) Biotechnology of Perennial Fruit Crops. Wallingford: CAB International, pp. 57–75
Zhang YX, Boccon-Gibod J, Lesinasse Y (1987) Obtention d'embryons de pommier après culture d'anthères. Comptes rendus de l'Académie des Sciences, Serie III 305: 443–448
Zhang YX, Lespinasse Y, Chevreau E (1990) Induction of haploidy in fruit trees. Acta Hort 280: 293–304
Zhang YX, Bouvier L, Lespinasse Y (1992) Microspore embryogenesis induced by low gamma dose irradiation in apple. Plant Breed 108: 173–176
Zou CJ, Li PF (1981) Induction of pollen plants of grape (*Vitis vinifera* L.). Acta Bot Sin 1: 79–81

Chapter 22
Haploidy in Tef

L. Gugsa and J. Kumlehn

Abstract Newly developed gynogenesis and androgenesis methods are described for tef. The diminutive floral structures of tef are problematic and have necessitated the development of spikelet culture and more effective treatments. Media composition has been optimised. In the case of anther culture, radiation treatments of seed of donor plants enhances the level of androgenesis. Various ploidy levels are found in the regenerants: haploids, doubled haploids, tetraploids and octoploids. The regenerated plants show variation for useful agronomic traits. Doubled haploidy in tef offers opportunities for improved genetic studies, accelerated breeding, the development of useful mutants and the development of new ploidy levels for agricultural exploitation.

Keywords Tef, *Eragrostis tef,* androgenesis, gynogenesis, polyploidy, mutants variation

Introduction

Tef [*Eragrostis tef* (Zuccagni) Trotter] is endemic and unique to Ethiopia and its diversity is only found in that country. Tef is one of the most important cereal crops of Ethiopia, in 2005/06 for instance, tef was grown by 5.2 million holders on 2.3 million hectares with a national yield of 2.2 million tonnes (Central Statistical

---

L. Gugsa
Leibniz Institute of Plant Genetics and Crop Plant Research, Plant Reproductive Biology, Corrensstrasse 3, D-06466 Gatersleben, Germany
Present address:
EARO (Holetta), P.O. Box 2003, Addis Ababa, Ethiopia

J. Kumlehn(✉)
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Corrensstrasse 3, D-06466 Gatersleben, Germany
E-mail: kumlehn@ipk-gatersleben.de

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Agency 2006). It is an annual small grained cereal crop (0.3 mg/grain), self pollinated and allotetraploid (2n = 4x = 40). It has a panicle type inflorescence with three or more florets per spike. The flowers are hermaphrodite with each floret consisting of three anthers, two stigmas and two lodicules (Moffet and Hurcombe 1949; Jones et al. 1978; Ebba 1975; Ketema 1997).

Most Ethiopian farmers use 'farmer's varieties' distributed in different climatic and edaphic zones at elevations of 1,000 to 2,500 m. Tef is considered to be well adapted to both drought and water-logged conditions. Recently its importance has been elevated and once a neglected crop, it is now considered an important staple partly due to its hardy nature. Despite the importance of the crop, the average yield in Ethiopia (less than 1 t/ha) is far below the crop's potential under well-managed systems devoid of lodging, for instance. All the varieties currently being grown by farmers in Ethiopia are susceptible to lodging making the breeding for lodging resistance a prime objective in the genetic improvement programmes for the crop (Assefa et al. 1998; Ketema 1997). The overall loss in grain yield due to lodging is estimated to range between 10% and 22% annually (Mulat 1999).

The small size of the floret, its autogamous nature and its pollination habit (pollination occurs only during the early hours of the day) are some of the major problems that constrain the improvement of this staple cereal through hybridization. Improved varieties have been produced by cross-pollination and line selection, but landraces are still widely grown. To date, little attention has been focused on the application of biotechnology to tef improvement. Another hindrance to conventional genetic improvement of the crop is the paucity of genetic variation within the gene pool for traits of interest (such as sturdy stalks for lodging resistance), a situation that makes the crop a candidate for other improvement strategies. The adoption of other genetic improvement strategies, especially those that would involve *in vitro* techniques, are however hampered by the lack of information on tef biotechnology. The development of an efficient and routine method of *in vitro* plant regeneration is therefore a pre-requisite for a successful alternative breeding programme such as one that could involve induction of new variation, a strategy that is clearly required in order to overcome the lack of adequate exploitable genetic variation.

Some studies in cell and tissue culture have been reported using embryo-rescue, zygotic embryos (Cheverton 1985), somatic embryogenesis from cultured leaf explants (Mekbib 1991), spikelet culture (Tefera and Chapman 1992; Tefera et al. 1999; Afza et al. 2000) and more recently, somatic embryogenesis and plant regeneration in seed derived callus (Assefa et al. 1998). Haploid technologies such as anther and ovary culture have proved to be a very useful tool in genetic studies and practical breeding (Yi 1991; Forster and Thomas 2005). The main advantage of these techniques is that homozygous inbred lines can be obtained rapidly. It has been used to study gametoclonal variability, to reveal useful recessive characters and to develop recombinant genotypes that are eliminated in ordinary crosses (Hu and Huang 1987).

Gynogenesis

In vitro culture of excised embryos or ovules has not been attempted to date, presumably because of the small size of these organs in tef. A facility to easily generate haploids can be used to significantly accelerate the conventional breeding process in self-pollinating species, and so would represent a useful technology for tef improvement. However, until recently, no method to allow for the production of haploid or doubled haploid tef has been elaborated, although Gugsa et al. (2006) have shown recently that gynogenesis can be induced from unpollinated inflorescence explants. This alternative to embryogenic pollen has been pioneered for a number of other cereal crop species (barley – San Noeum 1976; Castillo and Cistué 1993; rice – Zhou and Yang 1980; Asselin De Beauville 1980; wheat – Zhu et al. 1981; maize – Ao et al. 1982; Truong-André and Demarly 1984).

Initial attempts to generate haploids or doubled haploids in tef were based on the culture of immature anthers or immature pollen. However, only a small number of embryogenic calli could ever be obtained, and mostly these failed to undergo embryogenesis and thus any subsequent plant regeneration. Only a single albino plantlet was regenerated from isolated microspores. Thus other explant types have been investigated including the pre-anthesis inflorescence, with a view to exploiting the gynogenic route for the regeneration of haploid and doubled haploid progeny. Here we report that immature spikelets of tef, which are still attached to panicle segments, represent a suitable source of explant for efficient gynogenic plant regeneration. Using flow cytometry, we further show that the regenerated plants vary with respect to their ploidy level, and that unusual ploidy levels can have conspicuous effects on phenotype. The potential implementation of this technology for tef improvement is discussed.

Gynogenic Development from Flower Explants

When cultivated *in vitro*, tef pistils excised from flowers at the bi-cellular pollen stage proceeded to enlarge, eventually forming friable amorphous calli. In contrast to what is possible with its hexaploid relative *E. mexicana*, roots (but no shoots) could be successfully regenerated from this tissue. When spikelets or panicle segments were cultured, accessory floret development was common. *In planta* spikelets typically bear up to ten florets, but *in vitro* this number reached as high as 35, with an average of 17 per spikelet. Additionally, a few of the *in vitro* florets carried twin and even triplet pistils, some pistils bore three rather than the wild type of two stigmas, and as many as seven (instead of the wild-type of three) anther primordia per floret were observed. The development of accessory florets commenced a few days after culture initiation, and continued until the basal spikelets had senesced (Gugsa et al. 2006). Analogous secondary floret growth in spikelet or immature inflorescence cultures has been described in the literature for sorghum

(Brettell et al. 1980), tef (Tefera and Chapman 1992), the purple amaranth (*Amaranthus paniculatus*) (Arya et al. 1993), and durum wheat (Benkirane et al. 2000).

In excised pistil culture ovary enlargement appeared to be longitudinal, while the pistils of cultured spikelets and panicle segments enlarged in a more spherical fashion. The styles were shortened, but still bore normal looking feathery stigmas. The enlarged ovaries were frequently surrounded by a white spongy cellular mass, presumably derived from proliferating epidermis. The expanded pistils were as much as 15 times larger than those grown *in planta*. However, anther development was retarded, and did not proceed beyond a pale, primordial structure. In the secondary florets, however, some anthers developed normally with respect to size and colour, but microscopic examination revealed that they contained no viable pollen (Gugsa et al. 2006).

After about 10 days of spikelet or panicle segment culture, embryonic tissue emerged from the micropylar end of the enlarged ovaries, often entirely overgrowing the pistil over the following 14 days. Embryogenic callus formation appeared to be direct, rather than involving an intermediate phase of complete de-differentiation. In responding spikelets, pistil enlargement and gynogenic tissue formation characteristically occurred from the fourth floret onwards. In spikelets undergoing accessory floret formation, gynogenic development was observed even in florets beyond the 15th. Shoots were initiated 10–15 days after transfer of the entire explant to a shoot induction medium. When transferred to a regeneration medium, these shoots also formed roots. After transfer to soil, the regenerants established easily, and grew into normal plants, the majority of which set seed. A small number of sterile or only partially fertile individuals were also observed (Gugsa et al. 2006). Since tef is a strictly self-pollinating species, the seeds were most probably derived via self-fertilization. Similarly, self-incompatibility is unlikely to have been the basis of the occasional sterility observed.

Explants at a range of developmental stages (from early microspore to tri-cellular pollen) were tested for their response to either spikelet or panicle segment culture. The majority of spikelets in panicles which had emerged by 14–17 cm beyond the flag leaf sheath contained bi-cellular pollen. Only explants from this stage were amenable to gynogenesis. A maximum of four florets per spikelet taken from panicles of this stage enlarged and went on to form embryonic tissue (Gugsa et al. 2006).

The induction of gynogenic development proved more efficient from panicle segments than from excised spikelets. In rice, gynogenic development was also reported to be most efficient when entire florets (with intact pistils, stamens and glumes attached to a piece of receptacle) were cultured as a unit, while dissected pistils proved unresponsive (Zhou and Yang 1981). Furthermore, in tef the central third of the panicle was significantly more efficient with respect to gynogenesis than either the top or the basal thirds. Based on a set of optimization experiments, a nutrient medium 2,4-dichlorophenoxyacetic acid concentration of 18.4 μM proved most effective for inducing gynogenesis from panicle segment explants. The protocol established by Gugsa et al. (2006) was efficient for three tef genotypes, one of which was the landrace Fesho, and the other two both widely grown commercial varieties (DZ-CR-37 and DZ-01-196). Of the spikelets attached to cultivated

panicle segments of DZ-01-196, 38.6% formed embryonic tissue, and the regeneration efficiency, i.e. the number of regenerated plants per 100 cultured spikelets, reached 12.2%. About 25% of the regenerated plantlets were single regenerants per floret, whereas each of the remaining regenerable florets produced two to eight plantlets. Multiple plant regeneration per floret is most likely to have resulted from somatic embryogenesis observed at the surface of the proliferating tissue. Furthermore, twin or triplet pistils, which are only rarely seen *in planta*, but occurred frequently in the cultivated panicle segments, might have contributed to this phenomenon.

Several lines of evidence suggest that the embryogenesis seen in cultures of immature spikelets and panicle segments could not have been an outcome of unintended pollination, even though most of the florets used as explants were not emasculated. Firstly, it has been shown that when pollinated spikelets are cultured *in vitro*, seed development proceeds normally (Tefera and Chapman 1992), a result which we have been able to verify in our laboratory. Secondly, anther development was aborted *in vitro*, and no anthers carrying viable pollen were observed within the cultured spikelets. Thirdly, when emasculated spikelets were included in a few experiments they formed embryonic tissue in the same manner as did the non-emasculated ones. Fourthly, the most mature inflorescence stage tested (which would be expected to be the one most likely able to form functional pollen *in vitro*) was substantially less embryogenic than were some of the less mature stages. Fifthly, pollination *in planta* is followed by stigma degeneration, a response which was never observed in any of the explants sampled from any stage prior to anthesis. Although the observed pistil enlargement and embryonic tissue formation are most unlikely to have resulted from self-pollination, the occurrence of rare pollination events cannot be entirely ruled out.

Ploidy Level of Regenerated Plants

Ayele et al. (1996) reported that no deviation from the tetraploid level was found among plants of 32 landraces and three commercial varieties of *E. tef* including the landrace Fesho and the cvs. DZ-01-196 (Manga) and DZ-CR-37 which were used in the experiments presented here. However, flow-cytometric analysis of plants regenerated through culture of immature panicle segments revealed that 2.7% were (di)haploid, 1.1% triploid, 95.6% tetraploid and 0.5% octoploid (Gugsa et al. 2006). The range of ploidy levels is represented in Fig. 1 showing the flow-cytometric histograms of a di-haploid and an octoploid, along with that of a DZ-01-196 control plant.

In some species, e.g. barley some 90% of the regenerated plants are spontaneously doubled during microspore culture (Li and Devaux 2003; Kumlehn et al. 2006). However, spontaneous diploidisation is rather rare in gynogenesis-based regeneration systems, especially in the cereals (Castillo and Cistué 1993). A significant level of spontaneous diploidisation following gynogenesis has, however, been observed in both sugar beet (Lux et al. 1990) and onion

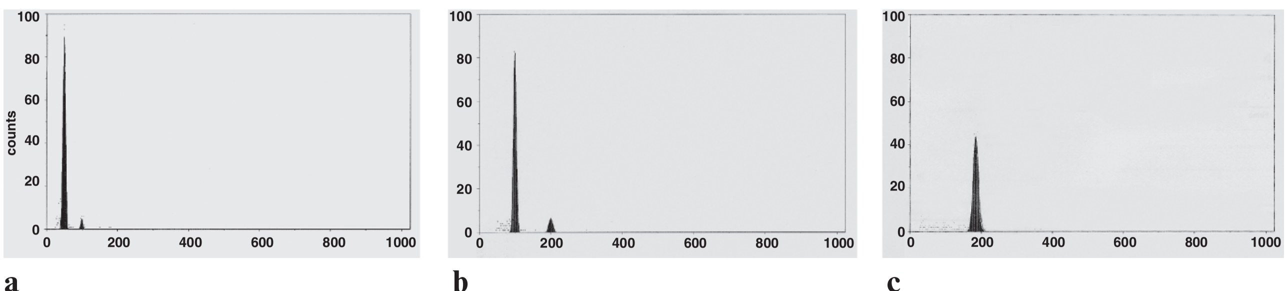


Fig. 1 Flow-cytometric analysis of plants regenerated from immature panicle segments of tef cv. DZ-01-196; **(A)** di-haploid plant; **(B)** tetraploid DZ-01-196 control plant; **(C)** octoploid plant. The x-axis represents the relative chromatin content of isolated nuclei. The chromatin content of tetraploid wild-type tef nuclei at the G1 phase of the cell cycle was set to 100

(Michalik et al. 1997). The identification of a number of haploids among the plants regenerated in the present study does suggest, however, that the overwhelming proportion of tetraploids obtained arose via spontaneous genome doubling. However, neither the cellular origin nor the zygosity of these plants has been elucidated. Since tef is an inbreeding species, its natural homozygosity will make it difficult, if not impossible, to distinguish between whether regenerated tetraploids are doubled haploids, or whether they were derived from maternal somatic tissue. Currently, there is no molecular marker system available that provides sufficient resolution to detect allelic segregation within a single tef line. However AFLP analysis in tef (Bai et al. 1999) has shown some promising potential in this context.

Phenotypic Characterisation of Regenerated Plants

The plant population obtained through culture of immature panicle segments was grown in the glasshouse at about 20°C. Under these conditions the plants showed huge diversity with respect to agronomically relevant morphological traits (Table 1). Di-haploid plants had comparatively thin leaves and culms, as well as small panicles, florets and grains (Fig 2A and B, Table 2). The majority of di-haploids did not set seed, yet one of the haploids unexpectedly produced grains. While the initially developed branches of the main tiller of this plant prematurely died, several new, comparatively vigorous branches emerged, which formed partially fertile panicles, i.e. one to two grains were produced per spikelet. These grains contained embryos, but sometimes no or only partially developed endosperm (Fig. 3A). Both grain size and weight of this haploid plant proved significantly reduced compared to regenerated tetraploids and octoploids (Fig. 3, Table 2). Also the grain

Table 1 Variation of agronomically relevant morphological traits of di-haploid, tetraploid and octoploid tef plants regenerated by panicle segment culture compared to cv. DZ-01-196 plants. For all measurements only panicles from main tillers were used

	Regenerated plants (1n = 2x through 4n = 8x)		DZ-01-196 (2n = 4x, Ctrl)	
Trait	min	max	min	max
Plant height (cm)	24	284	165	218
Flag leaf length (cm)	29	71	30	56
Panicle length (cm)	7	77	48	62
Panicle weight (g)	0.3	2.26	0.7	0.8
No. spikelets per panicle	26	685	189	366
No. florets per spikelet	5	19	7	10
Grain yield per panicle (g)	0.02	2.26	0.4	0.6
Culm thickness (cm)	0.1	0.45	0.25	0.3

Fig. 2 Morphological characteristics of tef plants obtained from cultures of immature panicle segments; **(a)** leaves from di-haploid (left), octoploid (central) and tetraploid (right) plants derived from cv. DZ-01-196; **(b)** spikelets from di-haploid (left), octoploid (central) and tetraploid (right) plants derived from DZ-01-196; **(c)** formation of abnormal spikelets in a triploid plant derived from cv. DZ-CR-37

Table 2 Comparison of panicle segment culture-derived tef plants of different ploidy levels compared to wild-type plants regarding agronomically relevant morphological traits. For all measurements only panicles from main tillers were used. Values followed by the same letter do not differ significantly from each other (P<0.01) as calculated by ANOVA

	Regenerated plants			Wild-type
Trait	1n = 2x	2n = 4x*	4n = 8x	2n = 4x (Ctrl)
Plant height (cm)	173.1 ± 1.3c	241.0 ± 2.2a	166.4 ± 2.0cd	199.4 ± 1.0b
Panicle length (cm)	34.6 ± 0.1c	64.2 ± 0.1a	47.6 ± 0.5b	59.8 ± 0.3a
Panicle weight (g)	0.30 ± 0.2d	1.48 ± 0.2b	1.55 ± 1.2a	0.78 ± 0.2c
Grain yield per panicle (g)	0.2 ± 1.2d	1.29 ± 0.7a	0.91 ± 1.2b	0.47 ± 0.2c
No. spikelets per panicle	219.0 ± 3.2c	421.8 ± 2.1a	298.5 ± 1.7b	297.8 ± 1.3b
No. florets per spikelet	9.0 ± 0.3b	12.5 ± 0.2a	10.2 ± 0.1ab	9.5 ± 1.5b
grain weight (g/1,000)	0.27 ± 0.2c	0.49 ± 0.5b	0.53 ± 1.7a	0.36 ± 1.1c
Fertility (%)	0.2	90	40	98

* Only the 10% highest yielding regenerated tetraploids were analysed.

Fig. 3 Grains of tef plants regenerated from cultures of immature panicle segments; **(a)** grains from a di-haploid plant (note the limited endosperm formation); **(b)** grains from a tetraploid; **(c)** grains from an octoploid plant derived from DZ-01-196

colour appeared to be influenced by the ploidy level. More specifically, the haploid plant produced nearly bright white grains containing white embryos, whereas tetraploids had more pale white grains with yellowish red embryos, and the grains and embryos of the octoploid plant were even more intensely pigmented than those of the tetraploids. Interestingly, white-grained varieties are considered as high-quality tef in the Ethiopian society and by selling grains of such quality farmers receive comparatively high market prices (Assefa et al. 2000). About 90% of the grains obtained from the fertile haploid plant germinated, and its progeny plants grew vigorously and were fertile. Flow-cytometric analysis of four successive generations derived from the partially fertile haploid plant revealed that all progeny plants were tetraploid, indicating spontaneous duplication of the di-haploid genome *in vivo*. This observation suggests that the cytological instability of the di-haploid genome might have caused the vast majority of the regenerated plants to be tetraploids. And as a further consequence, it supports our earlier assumption that the regenerated tetraploids are likely to be doubled haploids which have undergone spontaneous genome duplication during their *in vitro* or *in vivo* development.

With regard to the vegetative development, the triploid plants were morphologically largely comparable to the wild-type. However, the triploids typically showed abnormal panicle morphology as well as underdeveloped florets and malformed reproductive organs (Fig. 2C), and all triploid plants proved entirely sterile.

Many of the tetraploids obtained by culture of immature panicle segments possessed interesting features compared to their wild-type counterparts. Promising observations were made with regard to yield components such as the number of spikelets per panicle, the number of florets per spikelet, and the thousand-grain weight (Table 2). The octoploid plant obtained from culture of immature panicle segments had comparatively wide, deep green leaves, exceedingly thick culms, yet remained shorter than wild-type cv. DZ-01-196 plants. Furthermore, this plant showed significantly larger grains and higher thousand-grain weight than tetraploids (Fig. 3; Table 2). However, its fertility was reduced to about 40%, maturity was delayed and the majority of grains were shed before full maturity. The vigorous and compact growth, the strong culms and large seed size represent promising features with regard to the yield potential of tef. Octoploid lines may thus represent a suitable basis for the future development of lodging-resistant and high-yielding tef lines. Moreover, large grains are preferred for sale both in local markets as well as for export.

Androgenesis

A. Tadesse, H. Tefera, M. Guzmann, F.J. Zapata, A. R. Afza* and C. Mba

Ionising radiation is a physical stress and besides its ability to induce genetic variation, is thought to be helpful in improving the efficiency of anther culture and consequently leading to increased efficiency for green plant regeneration (Aldemita and Zapata 1991). Doubled haploidly, with its ability to achieve homozygousity of alleless in one generation has been recognized as a valuable tool in induced crop mutations. Recessive mutations are therefore fixed with one cycle of doubled haploidly (Van Harten 1998). There is little and in some cases, contradictory information on the effect of radiation treatments on tef. Brunner (1990) reported doses between 600 to 1,200 Gy of gamma rays for mutation induction (based on a study using eight genotypes) but Ketema (1993) recommended a dose of 1,500 Gy. But as in many other crops, significant intraspecific variation can be expected (El-Keredy and Abd-Alla 1976; Kowyama et al. 1987a, b).

In preliminary studies, isolation of tef anthers proved to be a slow and laborious process due to the small size of tef anthers (0.5 mm, Fig. 4B); the teasing out of the anthers has to be performed under a stereo-microscope. From preliminary reports (Ayele 2007, personal communication), unlike other crops such as rice, where isolation of thousands of anthers is possible in a working day, in tef, only a maximum of 350–400 anthers were isolated over the same period of time.

Somatic embryogenesis had been reported in tef (Bekele et al. 1995; Kebebew et al. 1998; Mekbib et al. 1997) but there are no reported cases of the regeneration of viable tef plants through androgenesis. The aim of this study was therefore to develop a routine anther culture procedure for tef. The irradiation of seeds, as a stimulatory agent for androgenesis response (calli induction and green plant regeneration) was also studied, using three well adapted and widely grown tef varieties (DZ-01-354, DZ-CR-37, and DZ-01-196) from Ethiopia. We report here the successful regeneration of viable plants through tef androgenesis. The regenerants induced albinos, haploids and fertile spontaneous disploids.

Irradiation of Seeds

Seeds of the varieties DZ-01-354, DZ-CR-37, and DZ-01-196 were obtained from the Debre Zeit Agricultural Research Center, Debre Zeit, Ethiopia. Seeds were dried in a desiccator containing 60% glycerol for 1 week to adjust seed moisture content to 11–14% prior to irradiation treatment. Seeds were irradiated in a gamma-radiation facility (^{60}Cobalt), with doses of: 200, 400, 600, 800, 1,000, 1,200, 1,400, 1,600, 1,800

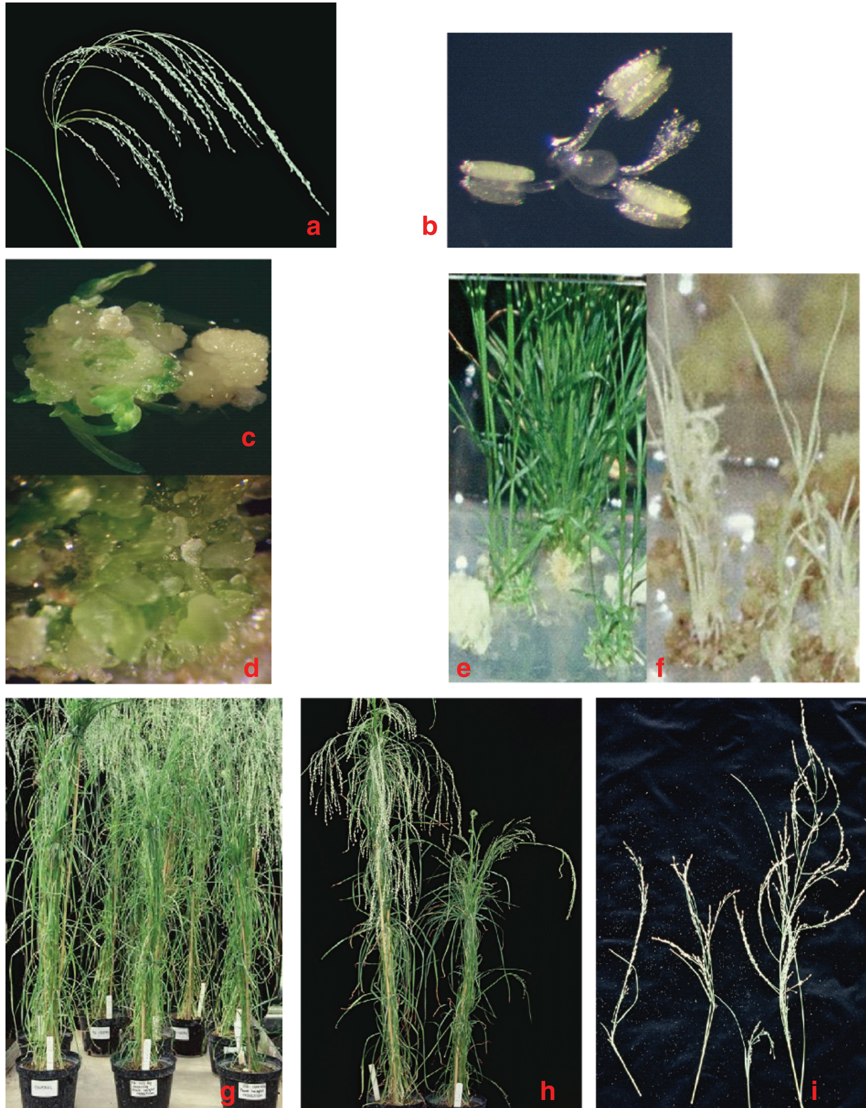


Fig. 4 **(a)** Typical tef spikelet; **(b)** Freshly teased out tef anthers; **(c)** Initiation of regeneration from induced calli from anthers of DZ-CR-37; **(d)** Initiation of regeneration from induced calli from anthers of DZ-01-354; **(e)** Viable spontaneous doubled haploid plants; **(f)** Lethal albino haploid plants; **(g)** Potted fertile anther culture derived plants with inflorescence; **(h)** Spontaneous doubled haploid (left) showing inflorescence and sterile haploid plant (right) showing absence of inflorescence; **(i)** Panicles of varying lengths from different anther culture derived plants showing variations in this trait

and 2,000 Gy at a dose rate of 979.671 rad min^{-1}. Five replicates of 20 seeds for each radiation dose – by – variety combination (including the non-irradiated control) were used for the study. These seeds were sown in 20-seed rows in plastic trays containing soil in a glasshouse, with each row corresponding to a treatment combination (dose – by – variety) and separated from the next row by a space of 8 cm. Seeds of varieties from different dose treatments including the control were sown randomly in the tray. Data on percentage of germination, plant height and survival rates were collected. Survival rate was scored 1 month after mutagenic treatment. Surviving plants from irradiated seeds were transferred to pots and were grown as donor plants for the anthers to be cultured.

In Vitro Culture of Anthers

Panicles from each variety and dose were harvested between 8:30 and 9:00 am, when the panicle was emerging from the flag leaf (when the emerged inflorescence was not more than 5 cm in length), and before anther dehiscence. Panicles were cold pre-treated for 8–10 days, (wrapped in a moist paper towel and kept in darkness at 8°C). In order to carry out a preliminary determination of pollen stage, anthers were fixed in acetic acid – ethanol (1:3) with 2% $FeCl_3$ for 24 hours, and stained with 2% aceto-carmine. This procedure revealed that all stages of pollen development were present in harvested panicles.

Panicles were surface-sterilised with 70% ethanol and soaked for 15 minutes in 20% commercial bleach (5.25% NaOCl), and rinsed three times with sterile distilled water. Anthers from each variety – by – irradiation dose treatment combination were harvested under sterile conditions and plated on both liquid and semi-solid N6 (Chu 1978) callus induction media. An average of 35 anthers per treatment were randomly plated in Petri dishes (5 cm diameter) containing 5ml of the liquid N6 callus induction media. For anthers plated on semi-solid media, an average of 40 anthers were plated per Petri dish (10 cm diameter) containing approximately 30 ml of media. After plating the anthers, Petri dishes were sealed with parafilm. Cultures were kept in the dark, at 25°C ± 2°C. Anther response was evaluated after 3 weeks and every week thereafter.

In plant regeneration studies, micro-calli and anthers with microspore-derive calli were transferred to three MS based media (Murashige and Skoog 1962). Magenta vessels (Magenta, Co.) containing 50 ml of medium were used. Calli were exposed to 66 αE m^{-2}s light with 12 hours photoperiod provided by 36 W Day Light Philips fluorescent lamps. Temperatures were maintained at 25°C ± 2°C. After 3–6 weeks, plant regeneration was examined and data were collected on the frequency of calli producing single or multiple green plants on regeneration media. Calli producing multiple green shoots were sub-cultured every 1 to 2 weeks after data collection. Green regenerated plants with developed roots were transplanted to pots in the glasshouse and grown to maturity.

Calli Induction Media

The liquid and semi-solid variants of the N6 (Chu 1978) media, both containing salts and vitamins, were evaluated for the purposes of comparing their efficacies in the induction of callusing from the anthers in tef. Media were supplemented with 200 mg l^{-1} Myo-inositol, 1.5 mg l^{-1} Biotin, 9.2 αM (2 mg l^{-1}) 2,4-dichlorophenoxy-acetic acid (2,4-D) and 5% maltose. In the semi-solid media, 0.45% agarose (Sigma, Type I-A: A-0169), was used.

For the regeneration of plantlets, the following 3 modifications of the MS media (Murashige and Skoog 1962) all supplemented with 3% sucrose were evaluated for efficacy in the regeneration of plantlets from calli:

- MS-1: N-19 media (Zapata et al. 1982) solidified with 0.15% Gelrite i.e. no hormone;
- MS-2: 4.4 αM (1 mg l^{-1}) BAP, 2.8 αM (0.5 mg l^{-1}) NAA, 200 mg l^{-1} myo-inositol, and 0.45% agarose; and
- MS-3: 8 αM (2 mg l^{-1}) BAP, 5.6 αM (1 mg l^{-1}) NAA, 9.2 αM Kinetin (2 mg l^{-1}) and 0.45% agarose.
- The pH of the media was adjusted to 5.8 prior to autoclaving (121°C for 20 minutes).

Optimal Radiation Dosage

It has been established that a quick method to determine the effect of a mutagenic seed treatment is to measure germination percentage, seedling height and survival rates in relation with doses increase (Brunner 1977). These three parameters were used in the evaluation of seedlings from the irradiated seeds and it was shown that relative to the control, expectedly, seedling height and percentage of survival decreased with an increase in gamma ray dose. Also, as has been previously reported for low doses in other plants (Aldemita and Zapata 1991), the irradiation of tef seeds at 200 Gy was found to have a stimulatory effect on plant height for DZ-01-354 though seedling height was reduced in other varieties at the same dosage, indicating a probable variable genotypic response to irradiation in tef. In general, based on these three parameters, DZ-01-196 and DZ-CR-37 were the most radiosensitive and radio-resistant varieties, respectively. In order to obtain a more valid inference on radiosensitivity in the genus however, a more detailed study will have to be carried out.

With sparsely ionising radiation, the optimal irradiation doses in cereals will normally cause 30 to 50% reduction in seedling growth tests relative to the control samples. Our results are in accord with some preliminary studies (unpublished) indicating that in comparison with many other seed propagated crops, tef is highly radio-tolerant. Irradiation doses ranging between 300 and 600 Gy would be suggested for a mutation induction programme, but differences in genotype responses should be considered.

Similar to plant height, the percentage of survival decreased with an increase in the dose. No seedling survived beyond 1 month at gamma ray dose of 1,800 Gy and above. Germination was not strongly affected by increasing gamma irradiation dose, an observation that was made for all three genotypes used in this study.

Calli Induction in Anther Culture

Most calli induction was achieved for anthers plated on semi-solid media with the addition of maltose in two of the genotypes, DZ-CR-37 (Fig. 4C) and DZ-01-196 (Table 3). The majority of the anthers plated in liquid medium became necrotic. The relatively lowest callusing found in the variety DZ-01-354 could be related to the physiological stage of the donor plants at the moment of harvesting as the onset of flowering was the latest in this variety. It is probable therefore that photoperiod and temperature conditions could be influencing anther response in the culture media resulting therefore in the production of low amount of calli in this variety. Marked differences in calli induction (e.g. in rice) have been noticed among plants grown during different months of the year with differences in temperature and photoperiod (Tsai and Lin 1977; Hu et al. 1978; Chaleff and Stolarz 1981).

Table 3 The average percentages of callus induction in seeds of three Ethiopian tef varieties exposed to eight different doses of gamma irradiation and plated on different callus induction media

		Anther response (%) of the variety		
Medium	Doses used (Gy)	DZ-CR-37	DZ-01-354	DZ-01-196
Solid medium	0Gy	6.84	0.85	1.98
	200	25.32	15.38	1.16
	400	43.42	19.86	22.35
	600	12.69	4.53	23.08
	800	7.88	9.77	0
	1,000	2.77	0	0
	1,200	2.6	0	0
	1,400	0	0	0
	1,600	0	0	0
Liquid medium	0Gy	7.76	0	1.06
	200	10.28	26.09	13.33
	400	4.05	0	0
	600	7.49	0	0
	800	11.47	18.29	0
	1,000	6.38	0	0
	1,200	4.63	0	0
	1,400	0	0	0
	1,600	0	0	0

Many authors have demonstrated the positive effects of maltose in calli induction and the relative advantages of its use as a carbon source for promoting androgenesis in other cereals such as rice, wheat and barley (Finnie et al. 1989; Navarro-Alvarez et al. 1994; Lentini et al. 1995; Laxmi and Reddy 1997). In a preliminary evaluation of the feasibility of the N6 medium using sucrose as carbohydrate source (both liquid and semi-solid state), calli induction was lower than 5% in the most responsive tef genotype and irradiation treatment combination (preliminary data). This result explained the functionality of sugars in culture media not just as a carbon source but as an osmotic regulator that (combined with the state of the media) increased the pollen response. Considering the tiny size of the tef anthers, small changes in osmolarity, induced by the use of sucrose in liquid medium could inhibit anthesis, or constrain anther metabolism and subsequent calli formation.

For the effect of radiation treatment on callus development, the anthers from the seeds originally irradiated at 200 and 400 Gy gave the most calli suggesting that these two doses, relatively low for this genus, had the most stimulatory influence on callus production from tef anthers. At the irradiation doses of 200 and 400 Gy, the average callus induction percentage sharply peaked at 12.5% ± 1.2% and 18.6% ± 1.25%, respectively and dropped off sharply with any further increases in the doses.

Plant Regeneration

Differences in green plant regeneration (Fig. 4E) efficiencies were found between the three genotypes (Table 4) but no differences were observed among the three regeneration media evaluated in terms of the number of calli producing single or multiple green plants. Nevertheless, the MS-1 media (N-19; Zapata et al. 1982) and MS-2 produced the highest number of green shoots per calli. The highest green plant regeneration was observed from calli which had been cultured on semi-solid medium and originating from anthers from donor plants of the variety DZ-CR-37 whose seeds had been irradiated at 400 Gy. Moreover, calli induction at this dose increased green plant regeneration efficiency and the production of multiple shoots from one callus was observed in many culture vessels. Green plant regeneration was strongly inhibited in calli originating from seeds that had been irradiated at doses higher than 800 Gy in all the genotypes.

Table 4 Average percentage plantlet regeneration from anther derived callus cultures on various regeneration media

	Percentage regeneration on three different media		
Genotype	MS-1	MS-2	MS-3
DZ-01-354	16	26	4
DZ-CR-37	17	26	15
DZ-01-196	100	0	0

Acclimatization of Regenerants and Variation Among Regenerated Plants

Regenerated anther culture derived plants were acclimatized and transferred to the green house (Fig. 4G). As is common in other cereals, albino and haploid plants were observed amongst the regenerated plantlets. On account of the lethality of this mutation, these plantlets were not transplanted to the soil. Figure 4F shows the albino regenerated plantlets growing on the culture media. The highest albino frequencies were detected amongst plants regenerating from callus induced from anthers harvested from seedlings whose seeds had been irradiated at 800 Gy.

An example of the sterile, and hence, haploid plants regenerated is shown in Fig. 4H. In addition to the absence of inflorescence the haploid plants displayed the characteristic shorter stature (relative to the spontaneous doubled haploid) usually associated with haploid plants. Other than haploid plants, other variations were observed amongst the regenerated plants. These included variations in panicle lengths and the total biomass of the regenerants. The rate of recovery of doubled haploid plants using the combination of the culture media and irradiation described, while being an improvement over the use of culture media alone, is still low and therefore not yet amenable to high throughput routine production of spontaneous doubled haploids from tef. More work still needs to be done to fine-tune the protocols.

Perspectives for Tef Doubled Haploidy

The implementation of doubled haploids has significantly accelerated the breeding process in several crop species, notably barley and rape seed. It is likely that tef breeding could also greatly benefit from this technology. Tef is a new species to doubled haploidy procedures and has shown positive results to both gynogenesis and androgenesis culture methods. As a first outcome, the wide phenotypic diversity of the lines obtained through both panicle segment culture and irradiation mediated anther culture will substantially contribute to tef germplasm collections. However, the specific cellular origin and the homozygous nature of the regenerated tetraploid plants obtained by the gynogenesis method still awaits experimental validation. Furthermore, the response of tef haploids to chemically-induced chromosome doubling has to be investigated, and a reliable protocol established. Synthetic polyploids often display agronomically interesting phenotypes, but characteristically are cytologically unstable. Thus both the field performance and the chromosomal stability of the octoploid line generated during this study will provide interesting topics for follow-up research. In light of their overwhelming diversity regarding agronomically relevant traits, the implementation of the putatively true-breeding tetraploids in the tef breeding process appears to be especially promising.

Acknowledgements In the gynogenesis work the excellent technical assistance of Ingrid Otto as well as the helpful comments and suggestions of Dr. Manfred Gahrtz are duly acknowledged. The authors express their particular thanks to the German Academic Exchange Service (DAAD) and the Ethiopian Agricultural Research Organisation (EARO) for the fellowship given to L.G.

References

Afza R, Shen M, Zapata-Arias FJ, Xie J, Fundi HK, Lee KS, Bobadilla-Mucino E, Kodym A (2000) Effect of spikelet position on rice anther culture efficiency, Plant Sci 153:155–159

Aldemita RR, Zapata FJ (1991). Anther culture of rice: Effects of radiation and media components on callus induction and plant regeneration. Cereal Res Commun 19:9–31

Ao GM, Zhao SX, Li GH (1982) *In vitro* induction of haploid plantlets from unpollinated ovaries of corn (*Zea mays* L.). Acta Genet Sin 9:281–283

Arya ID, Chakravarty TN, Sopory SK (1993) Development of secondary inflorescences and *in vitro* plantlets from inflorescence cultures of *Amaranthus paniculatus*. Plant Cell Rep 12:286–288

Assefa K, Gaj MD, Maluszynski M (1998). Somatic embryogenesis and plant regeneration in callus culture of tef, *Eragrostis tef* (Zucc.) Trotter. Plant Cell Rep 18:154–158

Assefa K, Tefera H, Gugsa L, Hundera F, Kefyalew T (2000) *In Vitro* manipulation of tef. In: Tefera H, Belay G, Sorrells M (eds) Narrowing the rift. Tef research and development. Proc Inter Workshop on tef Genetics and Improvement, October16–19, Debre Zeit, Ethiopia, pp. 131–143

Asselin De Beauville M (1980) Obtention d'haploides in vitro a partir d'ovaires non fécondés de riz, Oryza sativa L. C.R. Acad Sci 296: 489–492

Ayele M, Dolezel J, Vav Duren M, Brunner H, Zapata-Arias FJ (1996) Flow cytometric analysis of nuclear genome of the Ethiopian cereal tef [*Eragrostis tef* (Zucc.) Trotter]. Genetica 98:211–215

Bai G, Tefera H, Ayele M, Nguyen HT (1999) A genetic linkage map of tef [Eragrostis tef (Zucc.) Trotter] based on amplified fragment length polymorphism. Theor Appl Genet 99:599–604

Bekele E, Klock G, Zimmermann U (1995) Somatic embryogenesis and plant regeneration from leaf and root explants and from seeds of *Eragrostis tef* (*Gramineae*). Hereditas 123:183–189

Benkirane H, Sabounji K, Chlyah A, Chlyah H (2000) Somatic embryogenesis and plant regeneration from fragments of immature inflorescences and coleoptiles of durum wheat. Plant Cell Tiss Org Cult 61:107–113

Brettell RIS, Wernicke W, Thomas E (1980) Embryogenesis from cultured immature inflorescences of *Sorghum bicolor*. Protoplasma 104:141–148

Brunner H (1977) Radiosensitivity of a number of crop species to gamma and fast neutron radiation. In: Manual on Mutation Breeding, IAEA, Tech Rep Ser 119. 2nd edn, pp. 44–45

Brunner H (1990) Radiosensitivity of crop species to gamma and fast neutron radiation and estimated useful doses for mutation breeding. In: Darkalov S (ed) Mutation Breeding for Crop Plants, Academy of Science, Sofia, Bulgaria, pp. 43–46

Castillo AM, Cistué L (1993) Production of gynogenic haploids of *Hordeum vulgare* L. Plant Cell Rep 12:139–143

Central Statistical Agency (2006) Agricultural sample survey 2005/2006 (1998 E.C), Volume I. Report on area and production of crops (private peasant holdings, Meher season), Statistical Bulletin 361, Addis Ababa, Ethiopia

Chaleff RS, Stolarz A (1981) Factors influencing the frequency of callus formation among cultured rice (*Oryza sativa*) anthers. Physiol Plant 51:201–206

Cheverton MK (1985) Breeding tef: Lodging, weed control and other aspects of *Eragrostis tef*. M.Sc. Thesis, Wye College, University of London, UK

Chu CC (1978) The N6 medium and its application to anther culture of cereal crops. In: Proceedings of Symposium on Plant Tissue Culture, Science Press, Beijing, pp. 43–50
Ebba T (1975) Tef (*Eragrostis tef*) cultivars. Morphology and classification. Part II. Agric Expt Sta Bull No 66. Addis Ababa University, College of Agriculture, Dire Dawa, Ethiopia
El-Keredy MS, Abd-Alla SA (1976) A study in the differences in the radiosensitivity of some rice varieties. J Genet Cytol 5:48–57
Finnie SJ, Powell W, Dyer AF (1989) The effect of carbohydrate composition and concentration on anther culture response in barley. Plant Breed 103:110–118
Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88
Gugsa L, Sarial AK, Loerz H, Kumlehn J (2006) Gynogenic plant regeneration from unpollinated flower explants of *Eragrostis tef* (Zuccagni) Trotter. Plant Cell Rep 25:1287–1293
Hu C, Huang SC, Ho CP, Liang HC, Chuang CC, Peng LP (1978) On the inductive conditions of rice pollen plantlets in anther culture. In: Proceedings of Symposium on Plant Tissue Culture. Science Press, Beijing, pp. 87–95
Hu H, Huang B (1987) Application of pollen derived plants to crop improvement. Int Rev Cytol 107: 292–313
IAEA – International Atomic Energy Agency (1977) Technical Reports Ser No. 119. Manual on Mutation Breeding. Second ed. Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, pp. 87–165
Jones BMG, Ponti J, Tavassoli A, Dixon PA (1978) Relationships of the Ethiopian cereal tef [*Eragrostis tef* (Zucc) Trotter]: Evidence from morphology and chromosome number. Ann Bot 42:1369–1373
Kebebew A, Gaj MD, Maluszynski M (1998) Somatic embryogenesis and plant regeneration in callus culture of tef (*Eragrostis tef* [Zucc.] Trotter). Plant Cell Rep 18:156–158
Kowyama Y, Saito M, Kawase T (1987a) Absence of storage effects on radiation damage after thermal neutron irradiation of dry rice seeds. Jpn J Breed 37:301–310
Kowyama Y, Saito M, Kawase T (1987b) Intervarietal variations in radiosensitivity of dry rice seeds irradiated with sparsely and densely ionizing radiation. Environ Exp Bot 27:431–440
Kumlehn J, Serazetdinova L, Hensel G, Becker D, Loerz H (2006) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol J 4:251–261
Li H, Devaux P (2003) High frequency regeneration of barley doubled haploid plants from isolated microspore culture. Plant Sci 164:379–386
Lux H, Herrmann L, Wetze C (1990) Production of sugar beet (*Beta vulgaris* L.) by culturing unpollinated ovules. Plant Breed 104:177–183
Mekbib F (1991) *In vitro* regeneration, somatic embryogenesis study in tef. *Agrobacterium*-mediated transformation of tef and yam. M.Sc. Thesis, Wye College, University of London, UK
Mekbib F, Mantell SH, Buchanan-Wollaston V (1997) Callus induction and *in vitro* regeneration of tef [*Eragrostis tef* (Zucc.) Trotter] from leaf. J. Plant Physiol 151:368–372
Michalik B, Adamus A, Nowak E (1997) Induction of haploid plants in polish onion cultivars. Acta Hort 447:377–378
Moffet AA, Hurcombe R (1949) Chromosome number of South African grasses. Heredity 3:369–373
Mulat D (1999) Agricultural Technology, Economic Viability and Poverty Alleviation In Ethiopia presented to the Agricultural Transformation policy Workshop, Nairobi, Kenya, pp. 1–51
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
Laxmi G, Reddy GM (1997) Anther culture of indica rice: Technical improvements in callus induction and green plant regeneration. J Genet Breed 51:295–301

Lentini Z, Reyes P, Martinez CP, Roca WM (1995) Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Sci 110:127–138

Navarro-Alvarez W, Baenziger PS, Eskridge KM, Shelton DR, Gustafson VD, Hugo M (1994) Effect of sugars in wheat anther culture media. Plant Breed 112: 53–62

Ketema S (1993) Tef (*Eragrostis tef*): Breeding, genetic resources, agronomy, utilisation and role in the Ethiopian agriculture. Institute of Agricultural research, Addis Ababa, Ethiopia

Ketema S (1997) Tef [*Eragrostis tef* (Zucc.) Trotter]. Promoting the conservation and use of under-utilised and neglected crops, Institute of Plant Genetics and Crop Plants Research, Gatersleben/International Plant Genetic Resource Institute, Rome, Italy p. 47

San Noeum LH (1976) Haploïdes d'*Hordeum vulgare* L. par culture *in vitro* d'ovaires non fécondés. Ann Amélior Plantes 26:751–754

Tefera H, Chapman GP (1992) *In vitro* normal and variant development of tef (*Eragrostis tef*) spikelets. Plant Cell Tiss Org Cult 31:233–237

Tefera H, Zapata-Arias FJ Afza R, Kodym A (1999) Responses of tef genotypes to anther culture. Agritopia 14:8–9

Truong-André I, Demarly Y (1984) Obtaining plants by *in vitro* culture of unfertilized maize ovaries (*Zea mays* L.) and preliminary studies on the progeny of a gynogenic plant. Z. Pflanzenzuechtung 92:309–320

Tsai SC, Lin MH (1977) Production of rice plantlets by anther culture. J Agric Res China 26:100–112

Van Harten AM (1998) Mutation breeding in seed propagated crops. In: Mutation Breeding: Theory and applications. Cambridge University Press, Cambridge, pp. 204–251

Yi FP (1991) Breeding new rice strains through anther culture. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 14. Rice, Springer, Berlin, pp. 216–229

Zapata FJ, Torrizo L, Romero RD, Alejar MS (1982) Androgenesis in *Oryza sativa*. In: Fujiwara A (ed) Plant Tissue Culture, Maruzen, Tokyo, pp. 531–532

Zhou C, Yang HY (1980) *In vitro* induction of haploid plantlets from unpollinated young ovaries of *Oryza sativa*. Acta Genet Sin 7:287–288

Zhou C, Yang HY (1981) *In vitro* embryogenesis in unfertilized embryo sacs of *Oryza sativa* L. Acta Bot Sin 23:176–180

Zhu ZC, Wu HS, An QK, Liu ZY (1981) Induction of haploid plantlets from unpollinated ovaries of *Triticum aestivum* cultured *in vitro*. Acta Genet Sin 8:386–389

For tef gynogenesis

Gugsa, L.
Leibniz Institute of Plant Genetics and Crop Plant Research
Plant Reproductive Biology
Correcnsstrasse 2
D-06466 Gatersleben
Germany
Present address:
EARO (Holetta)
P.O. Box 2003
Addis Ababa
Ethiopia

Kumlehn*, J.
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)
Plant Reproductive Biology
Correcnsstrasse 3
D-06466 Gatersleben
Germany
E-mail:kumlehn@ipk-gatersleben.de

For Androgenesis

M. Guzmann
International Atomic Energy Agency, 2444 Vienna/Seibersdorf, Austria

C. Mba
Plant Breeding Unit, IAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, International Atomic Energy Agency, 2444 Seibersdorf, Austria

R. Afza
Plant Breeding Unit, IAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, International Atomic Energy Agency, 2444 Seibersdorf, Austria
E-mail: r.afza@iaea.org

A. Tadesse
Plant Breeding Unit, IAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, International Atomic Energy Agency, 2444 Seibersdorf, Austria
Current address:
Debre Zeit Agricultural Research Center, Ethiopian Institute of Agricultural Research, P.O. Box 32, Debre Zeit, Ethiopia

H. Teferas
Debre Zeit Agricultural Research Center, Ethiopian Institute of Agricultural Research, P.O. Box 32, Debre Zeit, Ethiopia

F.J. Zapata
Plant Breeding Unit, IAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, International Atomic Energy Agency, 2444 Seibersdorf, Austria
Current address
Bashati Vila, Apartment 5A, House 25, Road 68/A, Gulshan 1212, Dhaka, Bangladesh

Chapter 23
A Novel and Reversible Male Sterility System Using Targeted Inactivation of Glutamine Synthetase and Doubled Haploidy

A. Ribarits, A.N.K. Mamun, S. Li, T. Resch, M. Fiers, E. Heberle-Bors, C.-M. Liu, and A. Touraev

Abstract F_1 hybrid seeds are produced by controlled hybridization of homozygous inbred lines. Male sterility avoids tedious emasculation procedures of female parental plants but must be reversible easily in order to propagate and maintain the male-sterile lines. Doubled haploid plants provide complete homozygosity and therefore may be used as parental lines to breed F_1 hybrids. An environment-friendly F_1 hybrid breeding technology combining reversible male sterility and doubled haploid production was established through controlled starvation from glutamine to developing pollen. To this end, cytosolic glutamine synthetase was inactivated in tobacco anthers and microspores by a dominant-negative mutant approach, resulting in male sterility. Fertility was restored by exogenous application of glutamine. Pollen in transgenic tobacco plants aborted after the first pollen mitosis, which allowed to culture viable microspores *in vitro* to produce a non-segregating population of male sterile doubled haploid plants. The design of this novel system virtually precluded the release of transgenic pollen.

Keywords F_1 hybrid breeding, male sterility, doubled haploids, tapetum, microspore, glutamine synthetase, dominant-negative mutant

---

A. Ribarits, A.N.K. Mamun, T. Resch; and E. Heberle-Bors
Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Plant Molecular Biology, Dr. Bohrgasse 9/4, 1030 Vienna, Austria

S. Li, C.-M. Liu; and M. Fiers
Plant Research International, P.O. Box 16, 6700 AA, Wageningen, The Netherlands

A. Touraev(✉)
Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Plant Molecular Biology, Dr. Bohrgasse 9/4, 1030 Vienna, Austria
E-mail: alisher.touraev@univie.ac.at

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Introduction

During the last decades, F_1 hybrid varieties have gained increasing importance for both farmers and breeders (Górecka et al., this volume). F_1 hybrid seeds are produced by crossing two homozygous inbred lines and, due to an effect termed heterosis, the progeny from such crosses typically out-perform their parents (Hochholdinger and Hoecker 2007; Lippman and Zamir 2007). Farmers benefit from growing F_1 hybrids, as plants grown from hybrid seeds offer higher yields and better resistance than those from open-pollinations and represent homogeneous populations and products. Importantly for breeders, the seed is more valuable and can be protected by Plant Breeders' Rights. F_1 hybrid seed production requires selected homozygous inbred lines, or doubled haploids (DHs) with defined traits, and a reliable method to ensure hybridization between the selected parentals (Duvick 2001). Pollination control is usually achieved by tedious manual emasculation of the female parent. This can be relieved by a reliable male sterility system that avoids such laborious and expensive logistics (Perez-Prat and van Lookeren Campagne 2002).

The integration of DH plants into plant breeding programmes significantly speeds up the breeding processes, especially when it is combined with marker assisted selection (Forster and Thomas 2005). As yet, DH production from male sterile plants has not been attempted, and was frequently precluded by early abortion of microspores. In an innovative technology that applied controlled starvation from glutamine to developing pollen, the early death of pollen grains was overcome and reversible male sterility and DH production were combined (Ribarits et al. 2007).

Male Sterility and Doubled Haploid Production in Plant Breeding

Male sterility allows selected and precise crossing without laborious emasculation procedures of female (seed bearing) plants. Plant breeders are therefore keen to seek out and exploit new sources of male sterility (Budar and Pelletier 2001). However, the maintenance and propagation of male sterile lines is problematic and specialized systems are needed to generate the material (Perez-Prat and van Lookeren Campagne 2002). The commercial deployment of male sterile plants in plant breeding and F_1 hybrid production requires rigor, sterility needs to be complete, reliable and independent of environmental factors. Ideally, pollen viability is recovered by simple and readily accessible methods that avoid laboratory procedures or further cross, e.g. with near isogenic lines. Crossing male sterile plants with fertility restorer lines inevitably leads to segregation in the progeny. Markers may facilitate the selection of desired individuals but their application adds to cost. Moreover, some methods cannot easily be applied on a large scale (reviewed by Perez-Prat and van Lookeren Campagne 2002).

Several naturally occurring nuclear mutations causing male sterility have been discovered (Chaudhury 1993), but they are of limited practical value. A widely used system is cytoplasmic male sterility (CMS), which is based on incompatibility between nuclear and mitochondrial genomes; mitochondrial genes determining CMS have been identified in many plant species (Pelletier and Budar 2007; Chase 2007). Although CMS is used routinely in breeding it remains laborious and the maintenance of CMS lines requires the availability of and crossing to restorer lines. As transformation technologies have advanced, transgene-induced CMS has emerged as an interesting alternative (Chase 2006; Khan 2005). Ruiz and Daniell (2005) achieved CMS by transforming the bacterial gene β-ketothiolase into tobacco chloroplasts. The sterility achieved was, to some extent, reversible by continuous illumination. However, to date effective plastid transformation is restricted to selected plant species (Daniell et al. 2005). As nuclear transformation is successful throughout the plant kingdom, various experimental approaches have been undertaken to engineer nuclear male sterility by introducing genes from different sources (Perez-Prat and van Lookeren Campagne 2002; Takada et al. 2005). Among them are important achievements such as reversible male sterility caused by targeted expression of the cytotoxic ribonuclease *barnase* in the tapetum of tobacco and oilseed rape (Mariani et al. 1990). Fertilization with pollen carrying the ribonuclease inhibitor *barstar* restored fertility (Mariani et al. 1992). In this system, crossing male sterile with male fertile lines leads to segregation in the progeny. The unwanted fertile plants are eliminated by herbicide treatments (Reynaerts et al. 1993; Perez-Prat and van Lookeren Campagne 2002). However, it does mean that excess seeds are sown and this raises environmental concerns, consequently, the system has been subject to further development (Bisht et al. 2004; Burgess et al. 2002; Ray et al. 2007).

Successful pollen maturation and germination depends on coordinated sequences of environmental influences, developmental processes and metabolic pathways, ensuring timely supply of microspores and pollen with essential metabolites made available by a complex network of enzymatic actions (McCormick 1993; McCormick 2004; Scott et al. 2004). This complexity opens many options to regulate pollen fate directly. Transcription factors (Steiner-Lange et al. 2003; Li et al. 2007; Yang et al. 2007) or hormone production and signalling (Huang et al. 2003; Al-Ahmad and Gressel 2005; Ishimaru et al. 2006) have been targeted to induce male sterility. More frequently, promotion or inactivation of enzymatic actions have been tried to control pollen development (e.g. van der Meer et al. 1992; Napoli et al. 1999; Goetz et al. 2001; Park et al. 2002; Ruiz and Daniell 2005). Pollen impaired by metabolic deficiencies may abort at various stages, depending on the pathway affected. In transgenic lines, aberrant developmental processes have been observed shortly after the release from tetrads (Mariani et al. 1990; Zheng et al. 2003; Li et al. 2007), around the first pollen mitosis (Goetz et al. 2001; Ribarits et al. 2007), during anther dehiscence (Steiner-Lange et al. 2003; Yang et al 2007) or at pollen germination (Mo et al. 1992; Napoli et al. 1999).

Doubled haploid plants are produced by different approaches, among which isolated microspore culture offers the highest potential (Forster and Thomas 2005;

Forster et al. 2007). Evidently, early abortion of microspores precludes the combination of male sterility and doubled haploids, as well as fertility restoration by exploiting *in vitro* pollen maturation. Doubled haploid plants guarantee immediate homozygosity, and may therefore significantly speed up the breeding process as compared to conventional repeated self-pollinations (Baenziger 1996; Forster and Thomas 2005). Owing to their homozygous state, doubled haploids offer a number of advantages to plant breeders, including the rapid evaluation of important traits, and they may also be used as parental lines in F_1 hybrid breeding (Forster et al. 2007).

Controlled fertilization and homozygosity are essential requirements for hybrid seed production (Duvick 2001). Consequently, both male sterility and doubled haploid technologies may facilitate F_1 hybrid production considerably. However, evaluation of existing male sterility systems reveals that incomplete sterility, lack of efficient restoration strategies, as well as the early abortion of microspores in male sterile plants impedes the successful simultaneous use of both technologies. The ultimate target is to develop a technology that would reverse male sterility easily and there is potential to do this in the viable microspores of male sterile plants, offering an additional benefit in the generation of homozygous lines via microspore embryogenesis.

Fertility Control by Metabolic Engineering of Glutamine

Glutamine, Pollen Maturation and Microspore Embryogenesis

Several studies show that glutamine plays a significant role in pollen development. Starvation from nitrogen sources, in particular glutamine, effectively suppresses maturation of isolated microspores *in vitro*, thereby supporting their conversion from the gametophytic to the sporophytic pathway (Kyo and Harada 1986). In contrast, upon culture in a glutamine-containing medium, microspores mature into fertile pollen leading to seed set if applied on to receptive stigmas of previously emasculated plants (Benito Moreno et al. 1988). In addition, localized application of glutamine synthetase (GS) inhibitors leads to male sterility (Kimura et al. 1994; Yasuor et al. 2007).

Metabolic Engineering of Glutamine Biosynthesis

Glutamine synthetase (GS, E.C. 6.3.1.2.) is a well-conserved enzyme throughout the plant kingdom. It catalyzes the detoxification of ammonia, and its product glutamine represents a pivotal point in plant metabolism, being the major source of many amino acids (Miflin and Habash 2002; Forde and Lea 2007). Two enzymatic isoforms, cytoplasmic GS1 and plastid GS2, have been identified in plants.

The GS2 isoform is encoded by one gene and a small family of up to five genes are involved in GS1 production (Forde and Lea 2007). GS inhibitors, including widely used herbicides, cease the production of glutamine by impeding the action of the enzyme (Forlani et al. 2006; Yasuor et al. 2007). Plants targeted by these chemicals die soon after the treatment. Severe growth defects have also been observed in barley GS2 mutants, whereas a range of phenotypes related to growth rate or kernel size and number was observed in rice and maize GS1 mutants (Forde and Lea 2007). In plants, the GS enzyme is an octamer, offering the possibility to disturb its function by mutant sub-units (Herskowitz 1987; Miflin and Habash 2002). Mutations in the target genes of herbicides have been shown to influence functional properties of the enzymes and have therefore been employed to create herbicide-tolerant crops (Tan et al. 2006).

Due to the importance of GS for plant metabolism, specifically acting promoters are a pre-requisite to precisely restrict the down-regulation of glutamine synthetase to target tissues, e.g. to the male reproductive organs. The time-course of promoter activity is crucial to achieve a defined starvation in the selected organs or tissues. The specificity of the tapetum-specific promoter *TA29* (Koltunow et al. 1990) has clearly been documented; expression of the cytotoxic gene *barnase* driven by the *TA29* promoter has been shown to lead to male sterility without any side effects on plant growth (Mariani et al. 1990). Besides, RNAi-mediated silencing of the *TA29* promoter caused male sterility (Nawaz-ul-Rehman et al. 2007) and the same promoter was successfully used in several studies on male sterility (e.g. Kriete et al. 1996; Huang et al. 2003; Yui et al. 2003; Ribarits et al. 2007). Similar specificity was demonstrated for the microspore-specific *NTM19* promoter (Oldenhof et al. 1996; Custers et al. 1997).

Targeted Inactivation of Glutamine Synthetase Causes Pollen Abortion after the Formation of Viable Microspores

Glutamine starvation was anticipated to lead to male sterility and therefore key sites in GS1 and GS2 amino acid sequences were modified to develop dominant-negative mutants (DNM) of both *GS1* and *GS2*, which were fused to the specific promoters *TA29* and *NTM19* (Ribarits et al. 2007). The *TA29* promoter led to 100% male sterile plants in all generations, whereas the *NTM19* promoter caused T_0 plants with a single insertion to be male fertile due to segregation of transgenic and non-transgenic pollen. In the doubled haploid state the plants were completely male sterile. Male sterile homozygous doubled haploid plants carrying constructs driven by either promoter can be used for crosses with male fertile inbred lines for F_1 hybrid seed production (Fig. 1). Hybrids containing the *NTM19* DNM GS are male fertile as the non-transgenic fertile pollen is sufficient for seed set, which permits their use in seed production. In contrast, 100% sterility is observed in hybrids derived from a parental line bearing a *TA29* DNM GS construct and these plants may be valuable in vegetative, foliage crops.

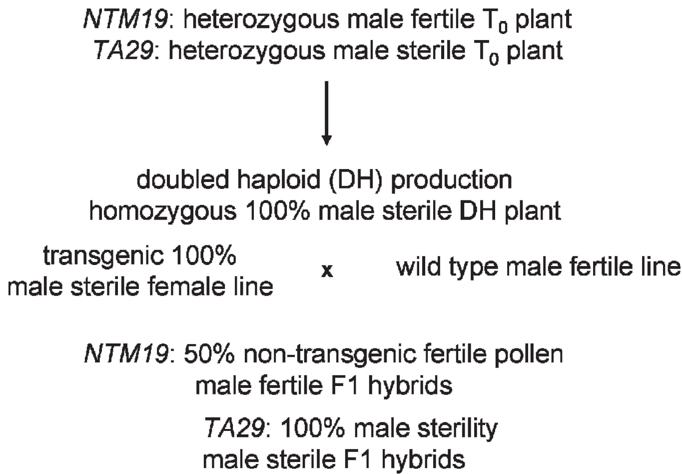


Fig. 1 The use of doubled haploids produced from male sterile plants for F_1 hybrid production. After crossing with a male fertile inbred line the F_1 hybrids carrying the *NTM19* promoter are male fertile due to segregating non-transgenic pollen. F_1 hybrid plants containing the *TA29* promoter are sterile. In both cases no transgenic pollen is released

Doubled Haploid Production from Male Sterile Lines

Pollen abortion in GS DNM begins after the first pollen mitosis, and microspores isolated from these plants were subjected to microspore embryogenesis as described by Touraev and Heberle-Bors (1999). All doubled haploid progeny generated from male sterile plants were again male sterile, proving that transmission of the trait was via this developmental pathway. A novel combination of reversible male sterility and doubled haploid production was demonstrated (Ribarits et al. 2007). The male sterile doubled haploid plants are completely homozygous, and hence might be used as female parental lines in F_1 hybrid seed production as depicted in Fig. 1. A large number of microspores can be isolated from any anther, therefore microspore embryogenesis represents an efficient method to maintain and propagate the male sterile lines. In species in which microspore embryogenesis has not yet been established, anther culture may be used as an alternative in generating doubled haploid plants.

Fertility Restoration by Exogenous Glutamine

If the sterility is caused by the lack of defined metabolites, it is reasonable that the application of the missing nutrient may restore fertility. Such an approach has been used successfully by Mo et al. (1992) who applied kaempferol to

stigmas of male sterile mutant plants, and thus restored the ability of pollen to germinate. Another attempt was to restore pollen fertility in carbohydrate-deficient lines by culturing isolated microspores in a sugar-containing medium (Goetz et al. 2001).

Similarly, it was postulated that providing glutamine-deficient lines with glutamine should restore their fertility. Fertility restoration was successfully accomplished using two different approaches: first, *in vitro* pollen maturation in a glutamine-containing medium and subsequent fertilization of the male sterile lines, and second, spraying of male sterile plants with glutamine (Ribarits et al. 2007).

An Environmentally Friendly and Reversible Male Sterility System

Complete absence of fertile pollen ensures transgene containment (reviewed by Daniell 2002). As a further benefit male sterile plants avoid tedious procedures for pollination control. Male sterility caused by the lack of glutamine does not employ toxic substances to induce sterility, nor to restore fertility, and is based solely on plant sequences. Fertility restoration is achieved by spraying glutamine, which is an innocuous metabolite. Moreover, laborious laboratory procedures are avoided. The commonly experienced segregation after crosses with near isogenic restorer lines is circumvented by self-pollination with rescued pollen. The possibility to generate male sterile DH plants represents an efficient method to maintain and propagate the male sterile lines. Besides, due to their complete homozygosity, such plants are valuable resources in F_1 hybrid breeding.

Conclusion and Future Prospects

The remarkable complexity of pollen and anther development as well as recent advances in transformation technologies have opened up numerous possibilities to engineer male sterility. However, due to the mechanisms of fertility restoration the male sterile lines frequently cannot be maintained in a homozygous state. This bottleneck was overcome by the production of DH plants from male sterile lines, and allowed for the development of a reversible male sterility system offering several technological advances as compared to established methods. Given the use of an appropriate tissue-specific promoter, metabolic engineering of glutamine through the inactivation of GS is expected to cause similar phenotypes in many other plant species since GS activity is found in most higher plants (Forde and Lea 2007). As a further benefit, the technology is designed to virtually preclude the release of transgenic pollen to the environment. The stability of the male sterility as well as the reliability of fertility restoration under different environmental conditions, however, awaits future surveys.

Acknowledgements The research on reversible male sterility and doubled haploids was supported by the European Commission-funded HybTech project (QLK5-CT-1999-30902).

References

Al-Ahmad H, Gressel J (2005) Transgene containment using cytokinin-reversible male sterility in constitutive, gibberellic acid-insensitive (Δ*gai*) transgenic tobacco. J Plant Growth Regul 24:19–27

Baenziger P (1996) Reflections on doubled haploids in plant breeding. In: Jain SM, Sopory S, Veilleux R (eds) *In Vitro* Haploid Production in Higher Plants, Kluwer, Dordrecht, pp. 35–48

Benito Moreno R, Macke F, Hauser M, Alwen A, Heberle-Bors E (1988) Sporophytes and male gametophytes from *in vitro* cultured, immature tobacco pollen. In: Cresti M, Jori P, Pacini E (eds) Sexual Reproduction in Higher Plants, Springer, Berlin, pp. 137–142

Bisht NC, Jagannath A, Gupta V, Burma PK, Pental D (2004) A two gene – two promoter system for enhanced expression of a restorer gene (*barstar*) and development of improved fertility restorer lines for hybrid seed production in crop plants. Mol Breed 14:120–144

Budar F, Pelletier G (2001) Male sterility in plants: occurrence, determinism, significance and use. C R Acad Sci III 324:543–550

Burgess DG, Ralston EJ, Hanson WG, Heckert M, Ho M, Jenq T, Palys JM, Tang K, Gutterson N (2002) A novel, two-component system for cell lethality and its use in engineering nuclear male-sterility in plants. Plant J 31:113–125

Chase CD (2006) Genetically engineered cytoplasmic male sterility. Trends Plant Sci 11:7–9

Chase CD (2007) Cytoplasmic male sterility: a window to the world of plant mitochondrial–nuclear interactions. Trends Genet 23:81–90

Chaudhury AM (1993) Nuclear genes controlling male fertility. Plant Cell 5:1277–1283

Custers JB, Oldenhof MT, Schrauwen JA, Cordewener JH, Wullems GJ, van Lookeren Campagne MM (1997) Analysis of microspore-specific promoters in transgenic tobacco. Plant Mol Biol 35:689–699

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20:581–586

Daniell H, Ruiz ON, Dhingra A (2005) Chloroplast genetic engineering to improve agronomic traits. Methods Mol Biol 286:111–138

Duvick DN (2001) Biotechnology in the 1930s: the development of hybrid maize. Nat Rev Genet 2:69–74

Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot 58:2339–2358

Forlani G, Obojska A, Berlicki L, Kafarski P (2006) Phosphinothricin analogues as inhibitors of plant glutamine synthetases. J Agric Food Chem 54:796–802

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev 25:57–88

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375

Goetz M, Godt DE, Guivarc'h A, Kahmann U, Chriqui D, Roitsch T (2001) Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proc Natl Acad Sci USA 98:6522–6527

Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. Nature 329:219–222

Hochholdinger F, Hoecker N (2007) Towards the molecular basis of heterosis. Trends Plant Sci 12:427–432

Huang S, Cerny RE, Qi Y, Bhat D, Aydt CM, Hanson DD, Malloy KP, Ness LA (2003) Transgenic studies on the involvement of cytokinin and gibberellin in male development. Plant Physiol 131:1270–1282

Ishimaru K, Takada K, Watanabe S, Kamada H, Ezura H (2006) Stable male sterility induced by the expression of mutated melon ethylene receptor genes in *Nicotiana tabacum*. Plant Sci 171:355–359

Khan MS (2005) Plant biology: engineered male sterility. Nature 436:783–785

Kimura Y, Shimada A, Nagai T (1994) Effects of glutamine synthetase inhibitors on rice sterility. Biosci Biotechnol Biochem 58:669–673

Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB (1990) Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2:1201–1224

Kriete G, Niehaus K, Perlick AM, Puhler A, Broer I (1996) Male sterility in transgenic tobacco plants induced by tapetum-specific deacetylation of the externally applied non-toxic compound *N*-acetyl-L-phosphinothricin. Plant J 9:809–818

Kyo M, Harada H (1986) Control of the developmental pathway of tobacco pollen *in vitro*. Planta 168:427–432

Li SF, Iacuone S, Parish RW (2007) Suppression and restoration of male fertility using a transcription factor. Plant Biotechnol J 5:297–312

Lippman ZB, Zamir D (2007) Heterosis: revisiting the magic. Trends Genet 23:60–66

Mariani C, de Beuckeleer M, Truttner J, Leemans J, Goldberg RB (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature 347:737–741

Mariani C, Gossele V, de Beuckeleer M, de Block M, Goldberg RB, de Greef W, Leemans J (1992) A chimaeric ribonuclease inhibitor gene restores fertility to male-sterile plants. Nature 257:384–387

McCormick S (1993) Male gametophyte development. Plant Cell 5:1265–1275

McCormick S (2004) Control of male gametophyte development. Plant Cell 16 Suppl:S142–S153

Miflin BJ, Habash DZ (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J Exp Bot 53:979–987

Mo Y, Nagel C, Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc Natl Acad Sci USA 89:7213–7217

Napoli CA, Fahy D, Wang HY, Taylor LP (1999) *White anther*: a petunia mutant that abolishes pollen flavonol accumulation, induces male sterility, and is complemented by a chalcone synthase transgene. Plant Physiol 120:615–622

Nawaz-ul-Rehman MS, Mansoor S, Khan AA, Zafar Y, Briddon RW (2007) RNAi-mediated male sterility of tobacco by silencing *TA29*. Mol Biotechnol 36:159–165

Oldenhof MT, de Groot PF, Visser JH, Schrauwen JA, Wullems GJ (1996) Isolation and characterization of a microspore-specific gene from tobacco. Plant Mol Biol 31:213–225

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J 31:1–12

Pelletier G, Budar F (2007) The molecular biology of cytoplasmically inherited male sterility and prospects for its engineering. Curr Opin Biotechnol 18:121–125

Perez-Prat E, van Lookeren Campagne MM (2002) Hybrid seed production and the challenge of propagating male-sterile plants. Trends Plant Sci 7:199–203

Ray K, Bisht N, Pental D, Burma PK (2007) Development of *barnase/barstar* transgenics for hybrid seed production in Indian oilseed mustard (*Brassica juncea* L. Czern & Coss) using a mutant acetolactate synthase gene conferring resistance to imidazolinone-based herbicide 'Pursuit'. Curr Sci 93:1390–1396

Reynaerts A, Van de Wiele H, De Sutter G, Janssens J (1993) Engineered genes for fertility control and their application in hybrid seed production. Sci Hort 55:125–139

Ribarits A, Mamun ANK, Li S, Resch T, Fiers M, Heberle-Bors E, Liu CM, Touraev A (2007) Combination of reversible male sterility and doubled haploid production by targeted inactivation of cytoplasmic glutamine synthetase in developing anthers and pollen. Plant Biotechnol J 5:483–494

Ruiz ON, Daniell H (2005) Engineering cytoplasmic male sterility via the chloroplast genome by expression of β-ketothiolase. Plant Physiol 138:1232–1246

Scott RJ, Spielman M, Dickinson HG (2004) Stamen structure and function. Plant Cell 16 Suppl:S46–S60

Steiner-Lange S, Unte US, Eckstein L, Yang C, Wilson ZA, Schmelzer E, Dekker K, Saedler H (2003) Disruption of *Arabidopsis thaliana MYB26* results in male sterility due to non-dehiscent anthers. Plant J 34:519–528

Takada K, Kamada H, Ezura H (2005) Production of male sterile transgenic plants. Plant Biotechnol 22:469–476

Tan S, Evans R, Singh B (2006) Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. Amino Acids 30:195–204

Touraev A, Heberle-Bors E (1999) Microspore embryogenesis and *in vitro* pollen maturation in tobacco. Methods Mol Biol 111:281–291

van der Meer IM, Stam ME, van Tunen AJ, Mol JN, Stuitje AR (1992) Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell 4:253–262

Yang C, Xu Z, Song J, Conner K, Vizcay Barrena G, Wilson ZA (2007) *Arabidopsis MYB26/MALE STERILE35* regulates secondary thickening in the endothelium and is essential for anther dehiscence. Plant Cell 19:534–548

Yasuor H, Abu-Abied M, Belausov E, Madmony A, Sadot E, Riov J, Rubin B (2007) Glyphosate-induced anther indehiscence in cotton is partially temperature dependent and involves cytoskeleton and secondary wall modifications and auxin accumulation. Plant Physiol 141:1306–1315

Yui R, Iketani S, Mikami T, Kubo T (2003) Antisense inhibition of mitochondrial pyruvate dehydrogenase E1α subunit in anther tapetum causes male sterility. Plant J 34:57–66

Zheng Z, Xia Q, Dauk M, Shen W, Selvaraj G, Zou J (2003) Arabidopsis *AtGPAT1*, a member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is essential for tapetum differentiation and male fertility. Plant Cell 15:1872–1887

Chapter 24
Embryogenic Pollen Culture: A Promising Target for Genetic Transformation

J. Kumlehn

Abstract As the immediate product of meiosis, the microspore represents the first stage of pollen development. The haploid chromosome set in addition to embryogenic competence renders microspores one of the most attractive cellular targets for the transfer and stable integration of recombinant DNA into plant genomes. However, the development of methods of plant genetic transformation through gene transfer to embryogenic pollen has turned out to be extremely difficult. In this chapter, diverse gene transfer approaches based upon embryogenic pollen cultures are critically reviewed with regard to target cell type, cell cycle stage, method of gene transfer as well as experimental evidence of transgene integration and homozygosity. Typical constraints and potential pitfalls are discussed and possible strategies to resolve these problems are suggested. Recent demonstrations of the successful generation of instantly true-breeding transgenic plants via gene transfer to embryogenic pollen cultures in both dicotyledonous and monocotyledonous species may encourage further development and broad applications of such methods in research and biotechnology.

Keywords Chimera, genetic engineering, homozygous, gene transfer, microspore, transgenic

Introduction

Embryogenic pollen is held to be a particularly appropriate cellular target for the stable integration of recombinant DNA into plant genomes. Apart from their high regenerability, the specific advantage of embryogenic pollen grains lies in their

---

J. Kumlehn(✉)
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany
E-mail: kumlehn@ipk-gatersleben.de

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

haploid state, inherited also to their mitotically derived cell descendants. As a result, when a transformed regenerant is genome-doubled, it will be homozygous and therefore true-breeding for the transgene. In contrast, the identification of transgene homozygotes derived from procedures based on diploid explants that have to be selected in segregating populations bred from hemizygous transgenic plants requires the production of two additional plant generations. The particular benefit of homozygous transgenic plants lies in the comparatively high transgene dosage that often results in more conspicuous phenotypes, and the ready availability of genetically fixed plant materials required for a proper phenotypic evaluation over multiple generations and environments. Any such transgenic lines which are found to display exceptional performance can then readily be multiplied for commercial exploitation.

The purpose of this review is to document and compare published gene transfer methods as applied to embryogenic pollen cultures. Various cell types and cell cycle stages present in pollen cultures are considered, and the specific consequences of their use discussed. It is concluded that neither cell cycle stage at the point of gene transfer, nor chimerism of the primary transgenic plants represent major bottlenecks for the formation or identification of homozygous transgenic plants. However, it is known that genome doubling can occur prior to transgene integration, and this does represent an undesirable outcome. Some possible strategies have been elaborated to circumvent precocious doubling in embryogenic pollen cultures, and the use of these control measures should allow embryogenic pollen cultures to make a significant contribution to the ongoing efforts to optimise the process of genetic modification in both model and crop plant species.

Gene Transfer Methods

Although a good deal of technical progress in gene transfer methods for haploid plant cells has been made in recent years, there is still demand for further enhancements in both model and crop plant species. A number of technical obstacles remain to be overcome. Immature pollen cells are enveloped by the exine, a particularly impervious cell wall, and this presents a significant physical barrier for transgene introduction, irrespective of the mode of gene transfer employed. In addition, the viability of immature pollen is easily compromised by abiotic stress, which is a problem given that the initiation of embryogenesis typically requires the imposition of stress to interrupt the maturation process, and to pave the alternative pathway of pollen embryogenesis. Thus during both inductive treatment and culture initiation, immature pollen cells need to tread close to the margin between life and death. The gene transfer process itself also requires the imposition of additional stress to the target cells. Therefore, the overall process can easily result in excessive cell death and even to total loss of the culture. A further complication is that the development of embryogenic pollen cultures relies on some cellular cross-talk, achieved by signalling molecules delivered either by the cell colonies and/or embryos, or by exogenous provision of heterologous feeder

systems. In the light of the typically high, and often rather highly variable mortality rate associated with all gene transfer procedures, the population density necessary to provide a sufficient abundance of signal molecules can be very difficult to achieve. Most target cells are not successfully transformed, and any heterologous feeder cells present are themselves non-transgenic. As a result, the imposition of post gene transfer *in vitro* selection will inevitably reduce the effective cell population density, and hence the quality of the inter-cellular communication which is indispensable for the well-being of the transgenic cells. This combination of circumstances has mitigated against the development of appropriate, generic and robust protocols for the generation of transgenic plants from embryogenic pollen. Nevertheless, a number of systems based upon embryogenic pollen cultures have been reported in the literature.

Direct DNA-Transfer

The earliest report of gene transfer to embryogenic pollen cultures was given by Neuhaus et al. (1987), who micro-injected purified DNA-molecules into rape seed pollen-derived cell colonies. The 12-cell stage was reported to be optimal with respect to both the micro-injection procedure itself and the post-injection survival of the cells. Dot blot analysis was used to demonstrate the presence of the *NptII* marker transgene in almost half of all regenerants. As the targets were multi-cellular, the majority of primary transgenics were, as expected, chimeric, since derived secondary somatic embryos segregated for the transgene. Despite numerous further attempts in rape seed (Miki et al. 1989; Jones-Villeneuve et al. 1995), barley (Bolik and Koop 1991; Olsen 1991) and maize (Gaillard et al. 1992), this technique has unfortunately proven to be only poorly reproducible, and as a result, the efforts have been unsuccessful to either enhance the protocol, or to extend it to other plant species, either in the context of basic research or biotechnology. Alternative transfer approaches have included polyethylene glycol-mediated poration and electroporation in isolated barley (Kuhlmann et al. 1991; Vischi and Marchetti 1997), maize (Fennell and Hauptmann 1992) and rape seed (Jardinaud et al. 1993) microspores. Although transient expression of reporter genes was obtained in some cases, embryogenic development was not reported in any of these studies. However, Salmenkallio-Marttila et al. (1995) did succeed in regenerating a few transgenic barley plants by applying non-selective *in vitro* conditions following the electroporation of protoplasts isolated from 3 to 4 week old embryogenic pollen cultures. However, all three transgenic plants emerging from 16.5×10^6 electroporated protoplasts were concluded to have been derived from a single gene transfer event, and as the plants proved to be hemizygous, spontaneous doubling of the haploid pollen genome must have taken place prior to the integration of the transgene.

Techniques based on the introduction of accelerated DNA-coated particles have enjoyed some success in a range of plant species. Transgenic plants regenerated

following the bombardment of freshly isolated microspores have been reported in barley (Jaehne et al. 1994; Yao et al. 1997; Carlson et al. 2001), rape seed (Fukuoka et al. 1998) and tobacco (Aionesei et al. 2006). Biolistic gene transfer has also resulted in transgenic plant formation by first applying an inductive treatment for the initiation of embryogenic development in bi-cellular tobacco pollen grains, followed by the gene transfer procedure (Nishihara et al. 1995; Aionesei et al. 2006). The kinetic energy required to penetrate the exine typically results in severe damage to the highly vacuolated embryogenically competent immature pollen cells. Consequently, more advanced developmental stages have been preferred in subsequent efforts to generate transgenic barley (Wan and Lemaux 1994), wheat (Folling and Olesen 2001) and tobacco (Stoeger et al. 1995). Nonetheless, biolistic transformation efficiency has remained very low (ca. one transgenic plant per 10^6–10^8 bombarded immature pollen grains). In most of these studies, no attempt was made to analyse the zygosity state of the transgenes. Although Jaehne et al. (1994) reported that homozygous transgenics can be readily obtained via this route; insufficient evidence was provided to validate this claim. Specifically, only one of the four marker gene-expressing primary transgenic plants was genetically analysed. This individual carried at least three transgene copies, but the molecular characterisation of only four T1-plants was reported, leading to a questionable level of reliability in the segregation analysis. Both Fukuoka et al. (1998) and Aionesei et al. (2006), however, were able to regenerate successfully haploid primary transgenics from, respectively, rape seed and tobacco, and recovered seed fertility by colchicine-induced chromosome doubling. No segregation was found in the T1-generation, indicating homozygosity of the transgene.

Despite numerous reports claiming the successful generation of transgenic plants through biolistic DNA-transfer to embryogenic pollen cultures, none of these protocols has yet been adopted for either functional analysis of genes-of-interest or molecular plant improvement. This failure probably reflects the low transformation efficiency compared to alternative transformation methods, and the fact that published protocols have proven to be at best poorly reproducible.

Agrobacterium-Based Gene Transfer

In contrast to direct gene transfer methods, transformation mediated by *Agrobacterium tumefaciens* relies on a naturally evolved mechanism, in which a defined DNA-segment is first excised from a bacterial plasmid, and directed into the plant nucleus, where it becomes integrated intact into the host genome (Tinland 1996; Tzfira and Citovsky 2006). In contrast to biolistic-based approaches, *Agrobacterium*-mediated gene transfer characteristically results in transgenic products carrying a comparatively low number of transgene copies (Cheng et al. 1997; Travella et al. 2005; Kumlehn et al. 2006) and has been associated with a high probability of both transgene integrity and integration sites within actively

transcribed chromosomal regions (Barakat et al. 2000). The generation of transgenic rape seed plants emerging from the co-cultivation of immature pollen with *A. tumefaciens* followed by embryogenic development was first described by Pechan (1989), and later was protected by a patent filed by Dormann et al. (2001). While enhanced resistance to *in vitro* selective conditions and transgene expression in plant tissue have been demonstrated in these reports, no direct evidence for transgene integration has been forthcoming, neither have any data been provided which characterize either the frequency of instant homozygosity or the sexual transmission of transgenes. To date there have been no published examples of the application of this, in principle, promising method in rape seed. As argued elsewhere (Potrykus 1990; Langridge et al. 1992) marker gene expression and resistance of host tissue to selective conditions can also be determined by persistence of *Agrobacterium*. An unambiguous validation of the transgenic status of the plant, using molecular analysis, is clearly indispensable before any transformation method can be considered truly reliable.

As has also been attempted for direct gene transfer, the possibility of targeting later developmental stages of embryogenic pollen cultures has been explored. In rape seed, co-cultivation with *A. tumefaciens* of immature pollen-derived cotyledonary embryos, followed by proliferation of resistant tissue sectors, has led to the generation of transgenic plants (Swanson and Erickson 1989; Huang 1992). Similar results have been obtained for both *Datura innoxia* and tobacco (Sangwan et al. 1993). Shanjun et al. (2005) reported that transgenic shoots could be obtained from *Agrobacterium*-infected hypocotyl explants of *Hyoscyamus niger* plantlets regenerated from embryogenic pollen cultures. However, no experiments were performed in the latter study, either to explore the ploidy level of the primary transgenic plants, or to reveal the sexual inheritance of the transgene.

More recently, an *Agrobacterium*-based gene transfer method was developed using embryogenic barley pollen cultures after 6–11 days of pre-culture (Kumlehn et al. 2006). This temporal window coincides with the time when the pollen wall bursts in response to cell proliferation, a result which is consistent with the idea that the pollen exine represents a significant barrier for *Agrobacterium* entry (Stoeger et al. 1995). The ultimate success of this system rests on optimizing both the gene transfer activity and the control of *Agrobacterium* proliferation during the co-cultivation period. Some of the derived primary transgenic lines were homozygous for the transgene. However, the method described still requires further analysis of the number of transgene integration sites along with the segregation among the sexual progeny, to allow the identification of the homozygous transgenic lines, which cannot immediately be distinguished from the hemizygous ones. Nonetheless, the transformation efficiency achieved (approximately one transgenic plant per 10^5 isolated pollen grains) was superior to prior embryogenic pollen culture-based methods, and was even on a par with the levels obtained using alternative barley transformation methods. Based on its efficiency and reproducibility, it represents the first, and as yet only, method of gene transfer to embryogenic pollen cultures to be exploited for functional gene analyses (Stein et al. 2005; Radchuk et al. 2006).

Target Cell Type and Cell Cycle Stage at the Time Point of Transgene Integration

The microspore is the immediate product of male meiosis, representing the first stage of pollen development. The developmental window over which immature pollen is amenable to *in vitro* embryogenic development extends from the microspore to the bi-cellular pollen, a stage generically referred to as "immature pollen". The embryogenic competence of immature pollen of many species is highly correlated with the appearance of a large central vacuole at the mid microspore stage. This structure expands to a maximum volume around the time of the first pollen mitosis, and then shrinks until its eventual disappearance during the maturation of bi-cellular pollen. Either microspores or bi-cellular pollen can be utilised as target material for gene transfer, either before or after a pre-treatment aimed at the initiation of embryogenesis. Embryogenic bi-cellular pollen grains can originate from either asymmetrical or symmetrical division of the microspore. In the former, the vegetative cell is the likely source of further embryogenic development, as the generative cell can give rise to only a small number of daughter cells. The bi-cellular pollen vegetative cell therefore represents a potential uni-cellular origin of embryogenesis and a suitable target for gene transfer, as does the microspore. In contrast, both cells present in symmetrical bi-cellular pollen grains can contribute to embryogenic development and plant regeneration. Nonetheless, this sort of pollen, as well as more advanced cell colonies or embryos, also represent valuable gene transfer targets, provided that the recipient cell type is still haploid and regeneration-competent.

Cell Cycle Stage

In the ideal transformation method, the gene transfer event should take place in a single haploid embryo progenitor cell at the G1-phase of its cell cycle. Transgene integration during G1 ensures the formation of two identical chromatids during the S-phase, both of which carry a copy of the transgene. However, G1-phase microspores are at best poorly responsive to treatments aimed at inducing embryogenesis, and are also not particularly amenable to *in vitro* maturation to improve their response to treatment post transformation. Nevertheless, some attempts have been made to establish relevant protocols (Hu and Kasha 1999; Shim and Kasha 2003; Aionesei et al. 2006). However, the time elapsed between particle bombardment or *Agrobacterium* inoculation and transgene integration can be as long as several days. In addition, T-DNA integration typically occurs during S-phase (Villemont et al. 1997), concomitant with a noticeable increase in transgene expression at G2 (Nagata et al. 1986). Thus there appear to be significant constraints to an efficient and reliable transgene integration in G1-phase microspores. A common assumption is that transgene integration during the S or G2-phase of haploid cells will result in

the generation of hemizygous transgenic lines. However, though the chromatid that carries the transgene sequence is inherited to only one of the two daughter cells, chimerism rather than hemizygosity will be the result. In addition, the non-transgenic cells will not participate in regeneration under sufficiently stringent *in vitro* selection. Thus the importance of the cell cycle stage at the point of transgene integration has probably been over-stated. Note, however, that ample experimental evidence has been provided to show that the major mode of spontaneous genome doubling in barley pollen embryogenesis occurs via an incomplete cytokinesis, followed by nuclear fusion (González-Melendi et al. 2005). In the event that two nuclei formed during the mitosis that directly follows transgene integration at S or G2 immediately fuse again, a diploid hemizygous transgenic cell can be generated possibly giving rise to a hemizygous plant. However, whether this sort of pathway is involved to any significant degree in any particular transformation method has yet to be elucidated.

Chimerism

Following gene transfer to bi-cellular or multi-cellular targets, both transgenic and non-transgenic cells can participate in regeneration, although the imposition of *in vitro* selection ensures that transgenic cells are favoured. However, since resistance to selective agents is commonly effected by a detoxification process specified by the selectable marker gene product, non-transgenic cells in the neighbourhood of transgenic ones can experience a reduced selective pressure. As a result, chimeras can be created. From a practical point of view, chimerism among primary transgenics does not constitute a serious problem, as this character will not be sexually transmitted. However, the formation of a zygote following fusion between transgenic and non-transgenic gametes of a chimeric plant, albeit a rather unlikely circumstance, can in theory lead to the generation of hemizygous progeny. If this scenario were at all common, the major advantage of choosing haploid target cells for gene transfer would be lost. Therefore, reliable methods of genetic transformation using embryogenic pollen cultures must include sufficiently stringent selective conditions.

Time Point of Genome Doubling

A highly critical aspect of gene transfer to haploid target cells surrounds the question as to whether transgene integration occurs before or after genome doubling. Homozygous transgenic plants can only be obtained when the event takes place before (either spontaneous or induced) chromosome doubling. Even where all the target cells are haploid at the moment of the gene transfer treatment, it can take up to several days before the transgene has been integrated within the host genome.

This delay allows for the possibility of spontaneous genome doubling occurring before integration, leading to the chance that some of the transgenic products will be hemizygous. Although hemizygosity in itself is not a major impediment to successful transgenesis, the whole purpose of targeting haploid cells for transformation is to generate homozygous outcomes. The discrimination of hemizygous from homozygous regenerants requires the determination of the number of insertion sites along with either a quantitative PCR assay, or a segregation analysis in the T1 generation. Note however that the identification of homozygotes derived from gene transfer to diploid explants nonetheless needs one more generation compared to an unknown mix of homozygous and hemizygous transgenic plants immediately obtained from embryogenic pollen cultures.

Two possible approaches are proposed to resolve the problem of hemizygote contamination among the transformation products obtained from haploid explants. The first requires that the gene transfer procedure be conducted prior to inducing the immature pollen to become embryogenic. During exposure to this inductive treatment, the immature pollen cells are arrested in their development, allowing them to undergo no more than one round of mitosis (Touraev et al. 2001). This should abolish any possibility of genome doubling, and permit sufficient time for complete transgene integration before releasing the block to proliferation and spontaneous genome doubling. However, as yet no appropriate protocol has been developed and validated on a sufficiently large scale. An even more reliable alternative approach exploits flow cytometry to sort haploid from diploid regenerants, and then applies the genome doubling treatment to only the haploid transgenics. In principle, this should allow the production of a population of exclusively homozygous transgenic plants. Experimental protocols along this line have been published for *D. innoxia* and tobacco (Sangwan et al. 1993; Aionesei et al. 2006), rape seed (Fukuoka et al. 1998) and barley (Kumlehn et al. 2004). Interestingly, in most of these cases, cultures were maintained to an advanced stage of embryogenic development prior to gene transfer effected by *A. tumefaciens*.

Conclusions

Driven by the sustained interest of plant researchers and breeders in haploid technology, regeneration methods based on embryogenic pollen cultures have continued to develop and be refined. Several experimental model and crop plant species are now amenable to pollen embryogenesis and the efficiency of the various protocols employed is steadily improving (Maluszynski et al. 2003; Ferrie and Keller 2007). This process should finally pave the way for the establishment of routine genetic transformation systems based on embryogenic pollen cultures. The reproducible transfer of recombinant DNA to microspores via biolistic transformation has been established for some time, but its efficiency has not kept pace with other available alternative applied transformation methods. The potential for further improvement in biolistic gene transfer to immature pollen appears rather limited, mainly because

of the mechanical damage unavoidably associated with bombardment by accelerated metal particles. More robust transformation methods have targeted instead multicellular pro-embryos and even certain later stages of pollen embryogenesis. While the cell cycle stage of the target cells and the generation of chimeras are both of only minor relevance to the formation and identification of homozygous transgenic plants, it remains of great importance to ensure that transgene integration generally occurs prior to spontaneous genome doubling. The present most convenient and reliable means to achieve this is by the use of flow cytometry, aiming to separate haploid from diploid transgenic plants. The perfection of this strategy should result in the formation of homogeneous populations of homozygous primary transgenic plants, thereby avoiding any need to eliminate hemizygous transgenic individuals.

Transgenic derivatives from embryogenic pollen cultures have recently been exploited for the functional analysis of transgenes in barley. Since true-breeding transgenic plants produce the most reliable phenotypic data relating to transgene expression, the development of methods permitting the rapid generation and identification of homozygous transgenic plants derived from embryogenic pollen cultures will be highly appreciated by the plant science and biotechnology community.

References

Aionesei T, Hosp J, Voronin V, Heberle-Bors E, Touraev A (2006) Methotrexate is a new selectable marker for tobacco immature pollen transformation. Plant Cell Rep 25: 410–416

Barakat A, Gallois P, Raynal M, Mestre-Ortega D, Salland C, Guiderdoni E, Delseny M, Bernardi G (2000) The distribution of T-DNA in the genomes of transgenic Arabidopsis and rice. FEBS Lett 471: 161–164

Bolik M, Koop HU (1991) Identification of embryogenic microspores of barley (*Hordeum vulgare* L.) by individual selection and culture and their potential for transformation by microinjection. Protoplasma 162: 61–68

Carlson AR, Letarte J, Chen J, Kasha KJ (2001) Visual screening of microspore-derived transgenic barley (*Hordeum vulgare* L.) with green-fluorescent protein. Plant Cell Rep 20: 331–337

Cheng M, Fry JE, Pang SZ, Zhou HP, Hironaka CM, Duncan DR, Conner TW, Wan YC (1997) Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiol 115: 971–980

Dormann M, Wang HM, Oelck M (2001) Transformed embryogenic microspores for the generation of fertile homozygous plants. US Patent 6,316,694 B1

Fennell A, Hauptmann R (1992) Electroporation and PEG delivery of DNA into maize microspores. Plant Cell Rep 11: 567–570

Ferrie AMR, Keller WA (2007) Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the Brassicaceae. In Vitro Cell Dev Biol – Plant 43: 348–355

Folling L, Olesen A (2001) Transformation of wheat (*Triticum aestivum* L.) microspore-derived callus and microspores by particle bombardment. Plant Cell Rep 20: 629–636

Fukuoka H, Ogawa T, Matsuoka M, Ohkawa Y, Yano H (1998) Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L.) and the production of fertile transgenic plants. Plant Cell Rep 17: 323–328

Gaillard A, Matthys-Rochon E, Dumas C (1992) Selection of microspore derived embryogenic structures in maize related to transformation potential by microinjection. Bot Acta 105: 313–318

González-Melendi P, Ramírez C, Testillano PS, Kumlehn J, Risueno MC (2005) Three dimensional confocal and electron microscopy imaging define the dynamics and mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis. Planta 222: 47–57

Hu T, Kasha KJ (1999) A cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum* L.) cv. Chris. Genome 3: 432–441

Huang B (1992) Genetic manipulation of microspores and microspore-derived embryos. In Vitro Cell Dev Biol – Plant 28P: 53–58

Jaehne A, Becker D, Brettschneider R, Loerz H (1994) Regeneration of transgenic, microspore-derived, fertile barley. Theor Appl Genet 89: 525–533

Jardinaud M-F, Souvré A, Alibert G (1993) Transient GUS gene expression in *Brassica napus* electroporated microspores. Plant Sci 93: 177–184

Jones-Villeneuve E, Huang B, Prudhomme I, Bird S, Kemble R, Hattori J, Miki B (1995) Assessment of microinjection for introducing DNA into uninuclear microspores of rapeseed. Plant Cell Tiss Org Cult 40: 97–100

Kuhlmann U, Foroughi-Wehr B, Graner A, Wenzel G (1991) Improved culture system for microspores of barley to become a target for DNA uptake. Plant Breed 107: 165–168

Kumlehn J, Broeders S, Valkov V (2004) Exclusive generation of true-breeding transgenic plants via Agrobacterium-mediated transformation of barley pollen cultures. Czech J Genet Plant Breed 40: 83

Kumlehn J, Serazetdinova L, Hensel G, Becker D, Loerz H (2006) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with Agrobacterium tumefaciens. Plant Biotechnol J 4: 251–261

Langridge P, Brettschneider R, Lazzeri P, Loerz H (1992) Transformation of cereals via Agrobacterium and the pollen pathway: a critical assessment. Plant J 2: 631–638

Maluszynski M, Kasha KJ, Szarejko I (2003) Published protocols for other crop plant species. In: Maluszynski M (ed) Doubled Haploid Production in Crop Plants: A Manual. Kluwer Academic Publishers, Dordrecht, Boston, London pp. 309–336

Miki BL, Huang B, Bird S, Kemble R, Simmonds D, Keller W (1989) A procedure for the microinjection of plant cells and protoplasts. J Tiss Cult Methods 12: 139–144

Nagata Z, Okada K, Takebe I (1986) Strong dependency of the transformation of plant protoplast on cell cycle. In: Kado C (ed) Fallen Leaf Lake conference on Agrobacterium and crown gall. University of California, Davis, p. 9

Neuhaus G, Spangenberg G, Mittelsten G, Schmid O, Schweiger HG (1987) Transgenic rape seed plants obtained by the microinjection of DNA into microspore-derived embryoids. Theor Appl Genet 75: 30–36

Nishihara M, Seki M, Kyo M, Irifune K, Morikawa H (1995) Transgenic haploid plants of *Nicotiana rustica* produced by bombardment-mediated transformation of pollen. Transgenic Res 4: 341–348

Olsen FL (1991) Isolation and cultivation of embryogenic microspores from barley (*Hordeum vulgare* L.). Hereditas 115: 255–266

Pechan PM (1989) Successful cocultivation of *Brassica napus* microspores and proembryos with Agrobacterium. Plant Cell Rep 8: 387–398

Potrykus I (1990) Gene transfer to cereals: an assessment. Biotechnology 8: 535–542

Radchuk V, Borisjuk L, Radchuk R, Steinbiss HH, Rolletschek H, Broeders S, Wobus U (2006) *Jekyll* encodes a novel protein involved in the sexual reproduction of barley. Plant Cell 18: 1652–1666

Salmenkallio-Marttila M, Aspegren K, Akerman S, Kurten U, Mannonen L, Ritala A, Teeri TH, Kauppinen J (1995) Transgenic barley (*Hordeum vulgare* L.) by electroporation of protoplasts. Plant Cell Rep 15: 301–304

Sangwan RS, Ducrocq C, Sangwan-Norreel B (1993) Agrobacterium-mediated transformation of pollen embryos in *Datura innoxia* and *Nicotiana tabacum* – production of transgenic haploid and fertile homozygous dihaploid plants. Plant Sci 95: 99–115

Shanjun T, Sangwan RS, Raghavan V, Verma DPS, Sangwan-Norreel BS (2005) Transformation of pollen embryo-derived explants by Agrobacterium tumefaciens in *Hyoscyamus niger*. Plant Cell Tiss Org Cult 81: 139–148

Shim YS, Kasha KJ (2003) The influence of pretreatment on cell stage progression and the time of DNA synthesis in barley (L.) uninucleate microspores. Plant Cell Rep 21: 1065–1071

Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in Hordeum vulgare (L.). Plant J 42: 912–922

Stoeger E, Fink C, Pfosser M, Heberle-Bors E (1995) Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep 14: 273–278

Swanson EB, Erickson LR (1989) Haploid transformation in *Brassica napus* using an octopine-producing strain of Agrobacterium tumefaciens. Theor Appl Genet 78: 831–835

Tinland B (1996) The integration of T-DNA into plant genomes. Trends Plant Sci 1: 178–184

Touraev A, Pfosser M, Heberle-Bors E (2001) The microspore: a haploid multipurpose cell. Adv Bot Res 35: 53–109

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. Plant Cell Rep 23: 780–789

Tzfira T, Citovsky V (2006) Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol 17: 147–154

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel BS (1997) Role of the host cell cycle in the Agrobacterium-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160–172

Vischi M, Marchetti S (1997) Strong extracellular nuclease activity displayed by barley (*Hordeum vulgare* L.) uninucleate microspores. Theor Appl Genet 95: 185–190

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104: 37–48

Yao QA, Simion E, William M, Krochko J, Kasha KJ (1997) Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). Genome 40: 570–581.

Chapter 25
Immature Pollen as a Target for Gene Targeting

T. Resch, E. Ankele, R. Badur, B. Reiss, E. Herberle-Bors, and A. Touraev

Abstract Immature pollen grains were used for gene targeting (GT) to evaluate the potential of higher plant male gametophyte as a target for gene targeting experiments. The artificial B18/4 target locus inserted to tobacco genome has been used to assess gene targeting in tobacco mid-bi-cellular pollen. In this system, a neomycin-phosphotransferase (*npt*II) gene, exclusively expressed in seeds, was converted into a constitutive *npt*II gene by insertion of the CaMV 35S promotor between the HMW seeds-specific promotor and a functional restored *npt*II gene at the target locus. The tobacco mid-bi-cellular pollen isolated from the B18/4 target locus plants was transformed by the biolistic approach with the repair construct. GT experiments led to the recovery of seven kanamycin resistant plants. Southern analysis confirmed that in one transgenic line an ectopic GT event occurred arising by modification of the repair construct by the target locus and subsequent integration elsewhere in the tobacco genome.

Keywords *Nicotiana tabacum,* gene targeting, immature pollen, particle bombardment, kanamycin selection, haploid

T. Resch, E. Ankele, and E. Herberle-Bors
Max F. Perutz Laboratories, University Departments at the Vienna Biocenter,
Dr. Bohrgasse 9/4, A-1030 Vienna, Austria

R. Badur
BASF Plant Science GmbH, Carl Bosch Straße 38, 67056 Ludwigshafen, Germany

B. Reiss
Max-Planck-Institut für Züchtungsforschung, Carl-von-Linne-Weg 10, 50829 Köln, Germany

A. Touraev(✉)
Max F. Perutz Laboratories University Departments at the Vienna Biocenter,
Dr. Bohrgasse 9/4, A-1030 Vienna, Austria
E-mail: alisher.touraev@univie.ac.at

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

© Springer Science+Business Media B.V. 2009

Introduction

Gene targeting (GT) by homologous recombination is a genetic tool that allows precise integration of genes at predetermined genomic positions, it also allows the production of specific and predictable changes in the host genome (Reiss 2003). Homologous recombination (HR) and gene targeting occurs efficiently in bacteria and budding yeast and also became available as a powerful tool in some higher eukaryotes such as *Drosophila* (Rong and Golic 2000), mouse (Capecchi 1989), and human somatic cells (Hanson and Sedivy 1995). In flowering plants GT technology is still inefficient and has limited reproducibility. In higher plants, most gene targeting systems have used artificially modified exogenous genes introduced before experimentation, homologous recombination (HR) between T-DNA and plant DNA has been reported to occur at a very low frequency (Risseeuw et al. 1995; Puchta and Hohn 1996). Several reports have claimed successful targeted modification of transgenic targets (Paszkowski et al. 1988; Offringa et al. 1990; Risseeuw et al. 1995). Targeting events have also been reported using a gene targeting system in which a *npt*II gene exclusively expressed in seeds is converted into a constitutive *npt*II gene by insertion of a CaMV 35S promotor between a seed-specific promotor and a functional restored *npt*II gene at the artificial target locus (Reiss et al. 2000). A few examples are available on the targeted disruption of an endogenous gene by HR (Kempin et al. 1997; Hanin et al. 2001; Shaked et al. 2005). A positive/negative selection method was used to obtain specific substitution mutants of the targeted rice *Waxy* gene by homologous recombination (Terada et al. 2002).

The successful gene targeting in rice (Terada et al. 2002) and moss (Schaefer and Zryd 1997) was attributed to the use of cells, which are competent to homologous recombination and gene targeting. In the moss *Physcomitrella* gene replacement occurs efficiently in the chloronemal cells; these represent the gametophytic generation, are haploid and are arrested in the G2 phase of the cell cycle prior to gene transfer (Schaefer and Zryd 1997; Reski 1998).

The plant male gametophyte as a target for gene targeting has been proposed earlier in th e light of success of gene targeting in moss (Puchta 1998). Higher plant microspores and immature pollen similar to moss are haploid, gametophytic cells which, experimentally, can be arrested in the G_2 phase of the cell cycle. They may, for these reasons, also offer high HR frequencies and provide the best cell type for gene targeting experiments. Tobacco microspores are highly synchronous in development, can be isolated in large quantities, cultured in a rich medium *in vitro* to maturity or can be re-programmed towards the sporophytic pathway by externally applied stress. In addition, two alternative methods to transform tobacco microspores and mid-bi-cellular pollen grains have been developed (Stöger et al. 1995; Touraev et al. 1997). One method, called male germ line transformation (MAGELITR) is based on the delivery of foreign DNA into microspores, culturing transformed microspores to maturity *in vitro*, pollination of receptive stigma with transformed pollen and selection of transgenic seeds (Touraev et al. 1997). Another method is based on transformation of embryogenic immature pollen and recovery of transgenic haploid embryos and plants which could be diploidized spontaneously or after treatment

with anti-mitotic drugs (Stöger et al. 1995; Aionesei et al. 2006). Thus, the tobacco male gametophyte can serve as an attractive target for gene targeting in higher plants.

In the present work, tobacco embryogenic immature pollen grains were evaluated as a target for gene targeting experiments at the artificial target locus, B18/4. The work in our laboratories was supported by EU Grant No. QLK-CT-2000-00365.

Materials and Methods

Plant Material

N. tabacum L. cv."Petit Havana" SR1 (Maliga et al. 1973) and transgenic *N. tabacum* L. cv."Petit Havana" B18/4 target locus (Reiss et al. 2000) plants were grown in the glasshouse at 25°C with a 16 hours light/8 hours dark regime with regular supply of fertilizers and routine watering.

Biolistic DNA Transfer into Mid-Bi-Cellular Pollen

The helium-driven PDS-1000/He particle delivery system (Bio-Rad, USA) was used for biolistic transformation experiments. Tobacco mid-bi-cellular pollen grains were bombarded immediately after isolation or after starvation in medium B (Kyo and Harada 1986). Plasmid was precipitated onto gold particles with an average diameter of 1.1 µm as described by Aionesei et al. 2006. Pollen suspension was dropped into the middle of a 6 cm wet Petri dish (IWAKI, Japan) to make a circle of evenly distributed cells on the bottom surface of the Petri dish without any support material.

Transformation and Culture of Embryogenic Bi-Cellular Tobacco Pollen

Mid-bi-cellular tobacco pollen grains were isolated aseptically from 18–19 mm flower buds in medium B (Kyo and Harada 1986) as described by Aionesei et al. 2006. The pollen suspension was re-suspended in medium B at a density of 5×10^4 pollen ml^{-1} for starvation or was used immediately for transformation. In transformation experiments performed after a starvation period, the population of viable embryogenic pollen obtained after incubation in medium B for 7 days were bombarded and then enriched by Percoll gradient centrifugation. Finally, the embryogenic pollen grains were re-suspended in medium AT3, pH 7 (Touraev and Heberle-Bors 1999)

at a density of 1.5×10^5 pollen ml^{-1} and cultured at 25°C in the dark until the formation of well-developed cotyledons.

Plasmid

The plasmid pMN 125 used for gene targeting experiments contained the *dhfr* gene encoding *dihydrofolate reductase* for methotrexate resistance under control of the CaMV35S promoter and a non-functional *npt*II gene, caused by the D42 deletion driven also by the CaMV35S promoter (Reiss et al. 2000; Beck et al. 1982; details see in Results).

Selection of Transgenic Embryos and Regeneration of Plants

Well-developed cotyledons formed after 6–8 weeks of incubation were placed onto a solid medium containing MS-salts and vitamins (Murashige and Skoog 1962), 0.27% phytagel, 1% sucrose and kanamycin (100 mg/l) for the recovery of transformants or, for the control transformations in SR1, on methotrexate (100 µg/l). After the transfer, the cotyledons were incubated at 25°C ± 1°C in a 16 hours light (2000 lux)/8 hours dark photoperiod for the formation of seedlings. Chromosome doubling was done prior to the transfer of the plants to the glasshouse or after the transfer of plantlets to soil as described by Aionesei et al. 2006.

PCR Amplification and Southern Blot Analysis

Kanamycin-resistant plants obtained after gene targeting experiments at the B18/4 target locus were analysed by PCR as described in Solomon and Puchta (1998). The sequences of the primers hmw2404 (pCTCCACAATT TCATCATCACC), b18rb-in (pAGAAGGATCG TTCATGTTGAG), 35 (CATCGTTGAAGATGCCTCTGC), 37 (CGTCAAGAAGGCGATAGAAGG) used for PCR are shown in Fig. 1.

For Southern blot analysis, tobacco genomic DNA was isolated according to Dellaporta et al. (1983). Genomic DNA (10–15 µg) was digested and separated on a 0.8% agarose gel, transferred to nylon Hybond-N filters and UV-crosslinked. Different probes have been used for detecting the sequence containing the *npt*II gene. The probe A hybridizes to a 610 bp DNA fragment situated upstream of the D42 deletion of *npt*II gene. The probe B hybridizes to a 73 bp DNA fragment situated in the 3′ deleted end of *npt*II gene. The labeling was performed using [α-^{32}P] dCTP according to the instructions of RadPrime DNA Labeling System (Invitrogen, USA) and hybridization has been done at a temperature of 65°C. Results of hybridization were visualized by exposure of the membranes to Kodak BIOMAX MR film at −80°C for several days.

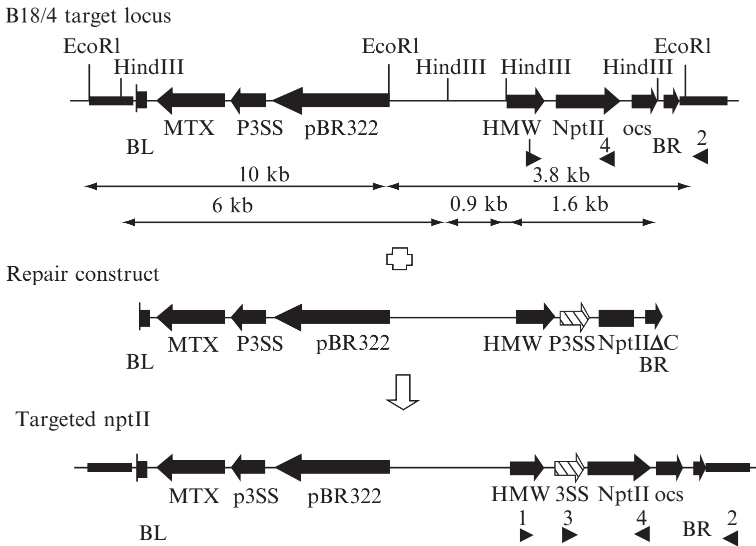


Fig. 1 Schematic physical maps of the B18/4 target locus, the pMN125 repair construct, and the recombination product obtained after precise gene replacement. Relevant genes, promoters, and polyadenylation sites are shown as large arrows (MTX, methotrexate resistance gene dhfr; pBR322, part of pBR322, including the origin of replication and the Ampicillin resistance gene; ocs, octopine synthase polyadenylation signal; P35S, CaMV 35S promoter; nptII ΔC, carboxyl-terminal deletion mutant of nptII), the T-DNA left (BL) and right (BR) borders as small arrows, and characterized flanking tobacco DNA sequences as small boxes. The primers used in the PCR analysis of recombination events at the B18/4 locus are shown schematically (1,hmw2404; 2,b18rbin; 3, 35; 4, 37). The size of fragments obtained after restriction of B18/4 target locus DNA with EcoRI and HindIII are indicated. The map is not drawn to scale (after Reiss et al. 2000)

Flow Cytometry Analysis

The ploidy level of transgenic tobacco plants was estimated by flow cytometry. Nuclei were isolated from the leaf of *Nicotiana tabacum* using *Secale cereale* “Dankovske” as an internal standard (Baranyi and Greilhuber 1996; Aionesei et al. 2006). For the measurement procedure a PARTEC Ploidy Analyser II (Partec, Münster, Germany) flow cytometer equipped with the Ca3 software (Partec, Münster, Germany) was used.

Results

Tobacco Transgenic Plants with the B18/4 Artificial Target Locus

The artificial B18/4 target locus inserted into the tobacco genome (Reiss et al. 2000) contains a methotrexate resistance gene (Mtx^R encoded by *dhfr*) driven by CaMV 35S promoter, a pBR322 replicon and a neomycin-phosphotransferase

(*npt*II) gene under control of a seed-specific HMW promoter. The pMN125 repair construct differs from the B18/4 target locus by the presence of a CaMV 35S promoter between the seed-specific HMW promoter and a defective *npt*II gene, caused by the D42 deletion (Reiss et al. 2000). The neomycin-phosphotransferase (*npt*II) gene of the B18/4 target locus, under control of HMW seed-specific promoter, is exclusively expressed in seeds. In contrast, the *npt*II of the repair construct, due to the D42 deletion, is non-functional (Reiss et al. 2000; Beck et al. 1982). The repair construct provides 6,693 bp of homology from the 35S promoter insertion to the left border and 752 bp of homology from the insertion to the other border of the target locus (Fig. 1). Targeted replacement results in the conversion of a neomycin-phosphotransferase (*npt*II) gene exclusively expressed in seeds into a constitutive *npt*II gene by insertion of the CaMV 35S promotor between the HMW seeds-specific promotor and a functional restored *npt*II gene at the B18/4 target locus (Reiss et al. 2000).

Gene Targeting in Mid-Bi-Cellular Tobacco Pollen

Tobacco pollen isolated from transgenic plants for the B18/4 target locus were bombarded in medium B with the pMN125 repair construct at two different time points: immediately after isolation and after 7 days of starvation. Mid-bi-cellular pollen bombarded with particles without DNA was used as control. Approximately 65% of the bombarded embryogenic pollen developed into multi-cellular structures and embryos in embryogenesis medium AT3 without a selectable agent. After 6 to 8 weeks embryos with well-developed cotyledons were transferred onto media containing kanamycin (100 mg/l) for further growth of plantlets and to select for the targeting event.

In total, seven independent kanamycin-resistant plants were obtained from 86 bombardments using 3.7×10^5 pollen grains per bombardment. Five kanamycin-resistant plants resulted from transformation of freshly isolated bi-cellular pollen and two kanamycin-resistant plants were recovered from experiments in which the bombardment was performed after a starvation period (Table 1). These plants were analyzed further by genetic and molecular methods. To determine gene targeting efficiency in relation to general transformation efficiency, wild type tobacco mid-bi-cellular pollen were transformed with the pMN 125 plasmid.

Table 1 Summary of gene targeting

Experiment	Number of bombarded pollen (10^6)	Number of kanamycin resistant plants	Number of recombinants	Type of targeting	
				Gene targeting	Ectopic event
Before starvation	20.3	5	1	–	1
Before starvation	11.5	2	–	–	–
Total	31.8	7	1	–	1

Molecular Analysis of Plants Obtained After Gene Targeting

The kanamycin-resistant plants obtained were analysed further by PCR and Southern blot analysis. The PCR analysis showed that all plants were not true gene targeting events because PCR with primers complementary to the HMW promoter sequences (primer hmw2404) and tobacco genomic sequences flanking the B18/4 T-DNA insert at its 3′end (primer b18rbin) detected a fragment representing the intact target. Furthermore, the plant DNA was digested with restriction enzymes that release fragments either from within the B18/4 T-DNA insert (*Hind*III) or from the B18/4 T-DNA insert, including sequenced regions of the flanking tobacco genomic DNA (*Eco*RI). Probe A hybridized to a fragment situated upstream of the D42 deletion of *npt*II gene. Hybridization of the blot with this probe detected in one plant (ET1) a fragment representing the unmodified B18/4 target locus (*Eco*RI, a 3.8-kb fragment; *Hind*III, a 1.6-kb fragment), as well as a strongly hybridising 18 kb additional fragment, which was the sign for the presence of a supplementary *npt*II gene (Fig. 2A).

Probe B, which should hybridize to a DNA sequence situated in the 3′ deleted end of *npt*II gene was used to find out whether the kanamycin resistance in the ET1 plant was the result of a homologous recombination or a random integration event. Hybridization of the blot with probe B revealed in the ET1 plant a fragment representing the unmodified B18/4 target locus as well as a restored kanamycin gene as

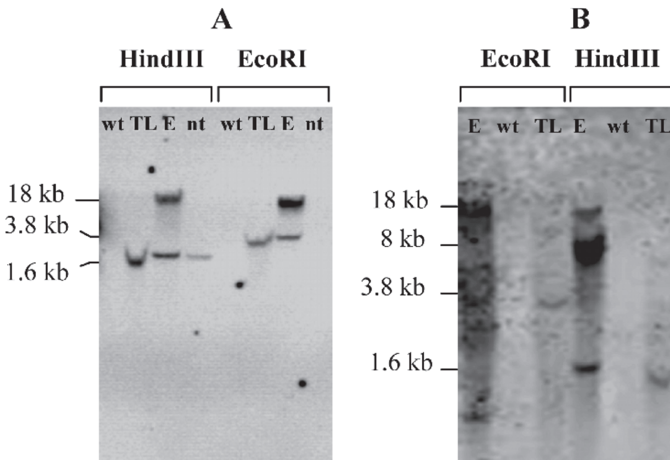


Fig. 2 Southern blot analysis of B18/4 events. DNA, digested by restriction enzymes indicated at the panels, separated by gel electrophoresis, and blotted. (A) DNA hybridized with probe A. (B) DNA hybridized with an nptII-specific probe B (wt, wild type SR1; TL, B18/4 target locus; E, ectopic gene targeting event; nt, untransformed plant)

demonstrated by the occurrence of the 18-kb kanamycin-specific *Hind*III and *Eco*RI fragment. Unspecific hybridization due to the small probe B size (73 bp) could explain the additional fragments observed in the Fig. 2B. The additional restored kanamycin fragment was bigger than the predicted increase in size of the fragment after correction of the repair construct by the B18/4 target locus. It seems that genomic sequences located in the B18/4 target locus were copied into the repair construct by homologous interaction and that the modified repair construct, with sequences incorporated from the target locus and may be flanking genomic DNA, integrated elsewhere in the genome.

Genetic Analysis of Plants Obtained After Gene Targeting

A colchicine treatment was performed to induce chromosome doubling for the plants recovered after gene targeting experiments. After colchicine treatment the recombinant plant ET1 was fertile and diploid, as proven by the flow cytometric analysis. The T1 seeds produced after self-pollination of T0 plants were grown on kanamycin containing media and resistant and sensitive seedlings were counted. The ET1 plant expressed 100% transmission of kanamycin resistance to the progeny indicating the homozygous nature of the T0 recombinant. In the other plants recovered after gene targeting experiments kanamycin resistance could not be observed in the progeny.

Discussion

Successful gene targeting in rice (Terada et al. 2002) and moss (Schaefer and Zryd 1997) was attributed to the use of cells which are competent to homologous recombination (HR) and gene targeting (GT). In the present work, higher plant immature pollen, or haploid gametophytic cells, were used as a target for gene targeting experiments to find out whether they might offer high HR frequencies and thus be “GT-competent” plant cells. After gene targeting experiments at the artificial B18/4 target locus seven kanamycin resistant plants were obtained from 86 bombardments by using 3.7×10^5 pollen grains per bombardment. Only one transgenic line (ET1) showed an ectopic gene-targeting event, while with the other six lines we could not trace the origin of kanamycin resistance. Since the B18/4 target locus remained unchanged and no intact *npt*II gene was found, mechanisms acting *in trans* are likely to be responsible for the ectopic activation of *npt*II in these plants (Reiss et al. 2000).

In several reports about alleged homologous recombination events, the majority of plants were not the result of a simple conversion of the target locus by the transforming DNA, but showed additionally rearrangements of the target locus or conversion of the T-DNA followed by its integration elsewhere in the genome (Risseeuw et al. 1995; Hanin et al. 2001). Southern analyses confirmed in the

recombinant line ET1 that the genomic sequences located in the B18/4 target locus were copied into the repair construct by non-reciprocal homologous recombination and the modified repair construct, with sequences incorporated from the target locus, integrated elsewhere in the genome. This type of event has been described, in previous work, as an ectopic gene targeting event and represents an alternative outcome of the gene targeting process (Offringa et al. 1993; Risseeuw et al. 1995; Reiss et al. 2000). It has been shown that highly asymmetric constructs used for gene targeting experiments decreased the recombination efficiency (Thomas et al. 1992; Risseeuw et al. 1995). In the B18/4 artificial target locus, the right arm had a length of only 752 bp, while the left arm has a length of 6,693 bp, which might have been a limiting factor for gene targeting in our experiments.

The current data show that in the ET1plant the additional kanamycin fragment (~18 kb) was bigger than the predicted increased size of the fragment after correction of the repair construct by the B18/4 target locus. It seems that a defective *npt*II gene present on the repair construct was restored by homologous recombination (gene conversion) with the B18/4 target locus, and the corrected repair construct with flanking genomic DNA integrated randomly in the genome. In plants, a comparable situation in which homologous recombination resulted in a restored marker gene and an unmodified target locus in the expected manner have also been characterized (Offringa et al. 1993; Risseeuw et al. 1995; Wang et al. 2001; Hanin et al. 2001). In mammalian cells too, sequences beyond the region of homology were copied from the target locus to the incoming construct and the corrected vector integrated elsewhere into the genome (Aratani et al. 1992). After gene targeting experiments at the B18/4 target locus, Reiss et al. (2000) reported that in two out three ectopic gene targeting events obtained sequences flanking the target locus were also included after correction of the repair construct. These events consisted of a precise partial duplication of the target locus at the end that has engaged in homologous recombination and a genuine T-DNA border junction at the other end (Reiss 2003). This end had integrated into repetitive DNA with no sequence homology to the sequences flanking the target locus.

In various gene targeting experiments using different plants and transformation systems, a wide range of GT frequencies was recorded from 10^{-3} to 10^{-5} (Vergunst and Hooykaas 1999). In Arabidopsis one targeting event out of 750 transformants was obtained by knocking out the AGL5 MADS-box gene (Kempin et al. 1997). By using a gene targeting system based on endogenous protoporphyrinogen oxidase (PPO) gene of Arabidopsis, a frequency of 7.2×10^{-4} targeting events per transformant has been reported (Hanin et al. 2001). In the present report immature pollen grains were used as target cells for gene targeting experiments at the B18/4 artificial target locus, and one plant showed a recombinant event. We could not estimate the GT frequency, since a classical gene-replacement event could not be confirmed in our analysis. However, one recombinant plant out of seven trans formants provides a good starting point for further optimization of GT technology in immature pollen.

The event, described here, represents the first example of a homologous recombination event by using tobacco embryogenic immature pollen as target for gene

targeting experiments. The immature pollen transformation protocol for the targeted introduction of genes into the genome of higher plants might have, in case of success, an impact on the biological safety of transgenic plants and on the quality and stability of expression of transgenes. For practical breeding this method might provide an efficient and less time consuming alternative by obtaining doubled haploid transgenic plants in one generation.

References

Aionesei T, Hosp J, Voronin V, Heberle-Bors E, Touraev A (2006) Methotrexate is a new selectable marker for tobacco immature pollen transformation. Plant Cell Rep 25:410–416

Aratani Y, Okazaki R, Koyama H (1992) End extension repair of introduced targeting vectors mediated by homologous recombination in mammalian cells. Nucl Acids Res 20:4795–4801

Baranyi M, Greilhuber J (1996) Flow cytometric and Feulgen densitometric analysis of genome size variation in *Pisum*. Theor Appl Genet 92:297–307

Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327–336

Capecchi MR (1989) Altering the genome by homologous recombination. Science 244:1288–1292

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Hanin M, Volrath S, Bogucki A, Briker M, Ward E, Paszkowski J (2001) Gene targeting in *Arabidopsis*. Plant J 28 (6):671–677

Hanson KD, Sedivy JM (1995) Analysis of biological selections for high-efficiency gene targeting. Mol Cell Biol 15:45–51

Kempin SA, Liljegren SJ, Block LM, Rounsley SD, Yanofsky MF, Lam E (1997) Targeted disruption in *Arabidopsis*. Nature 389:802–803

Kyo M, Harada H (1986) Control of the developmental pathway of tobacco pollen *in vitro*. Planta 168:427–432

Maliga P, Breznovitz A, Marton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nat New Biol 244:29–30

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Offringa R, de Groot MJ, Haagsman HJ, Does MP, van den Elzen PJ, Hooykaas PJ (1990) Extra chromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium*-mediated trans-formation. EMBO J 9:3077–3084

Offringa R, Dijk MEIF, Groot MJAD, Elzen PJMVD, Hooykaas PJJ (1993) Nonreciprocal homologous recombination between Agrobacterium transferred DNA and a plant chromosomal locus. Proc Natl Acad Sci USA 90:7346–7350

Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. EMBO J 7:4021–4026

Puchta H (1998) Towards targeted transformation in plants. Trends Plant Sci 3:77–78

Puchta H, Hohn B (1996) From centMorgans to base pairs: homologous recombination in plants. Trends Plant Sci 1:340–348

Reiss B (2003) Homologous recombination and gene targeting in plant cells. Intern Rev Cytol 28:85–139

Reiss B, Schubert I, Köpchen K, Wendeler E, Schell J, Puchta H (2000) RecA stimulates sister chromatid exchange and the fidelity of double-strand break repair, but not gene targeting, in plants transformed by *Agrobacterium*. Proc Natl Acad Sci USA 97:3358–3363

Reski R (1998) Physcomitrella and Arabidopsis: the David and Goliath of reverse genetics, Trends Plant Sci 3(6):209–210

Risseeuw E, Offringa R, Franke-van Dijk MEI, Hooykaas PJJ (1995) Targeted recombination in plants using *Agrobacterium* coincides with additional rearrangements at the target locus. Plant J 7:109–119

Rong YS, Golic KG (2000) Gene targeting by homologous recombination in *Drosophila*. Science 288:2013–2018

Schaefer DG, Zryd J-P (1997) Efficient gene targeting in the moss *Physcomitrella patens*. Plant J 11:1195–1206

Shaked H, Melamed-Bessudo C, Levy AA (2005) High-frequency gene targeting in *Arabidopsis* plants expressing the yeast *RAD54* gene. Proc Natl Acad Sci USA 102:12265–12269

Solomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J 7:6086–6095

Stöger E, Fink C, Pfosser M, Heberle-Bors E (1995) Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep 14:273–278

Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20:1030–1034

Thomas KR, Deng C, Capecchi MR (1992) High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. Mol Cell Biol 12:2919–2923

Touraev A, Heberle-Bors E (1999) Microspore embryogenesis and *in vitro* pollen maturation in tobacco. In: Hall R (ed) *Plant Cell Culture Protocols* 111, Humana Press, Totowa, NJpp. 281–291

Touraev A, Stöger E, Voronin V, Heberle-Bors E (1997) Plant male germ line transformation. Plant J 12:949–956

Vergunst AC, Hooykaas PJJ (1999) Recombination in the plant genome and its application in biotechnology. Crit Rev Plant Sci 18:1–31

Wang HX, Viret J-F, Eldridge A, Perera R, Signer ER, Chiurazzi M (2001) Positive–negative selection for homologous recombination in *Arabidopsis*. Gene 272:249–255

Chapter 26
Induction of Semi-Dwarf, Salt Tolerant Rice Mutants from a Tall Salt Tolerant *Indica* Landrace

R. Afza, M. Guzmann, F.J. Zapata, E. Tumimbang-Raiz, G. Gregorio, B.P. Forster, and C. Mba

Abstract Doubled haploid production has become an important tool in cereal breeding and has helped in accelerating the development of improved cultivars. Genetic variation for salt tolerance is rare in contemporary semi-dwarf (*sd-1*) rice germplasm (cultivars and breeding lines). Some rice landraces exhibit greater tolerance to salt, but are agronomically unacceptable because of their tall stature. One such landrace, Pokalli was subjected to anther culture in an attempt to induce

---

R. Afza
Plant Breeding Unit, Joint FAO/IAEA Agriculture and Biotechnology Laboratory, International Atomic Energy Agency Laboratories, A-2444 Seibersdorf, Austria

M. Guzmann
Plant Breeding and Genetics Section, Joint FAO/IAEA Division, International Atomic Energy Agency, Vienna, Austria

F.J. Zapata
Plant Breeding Unit, Joint FAO/IAEA Agriculture and Biotechnology Laboratory, International Atomic Energy Agency Laboratories, A-2444 Seibersdorf, Austria
Current address:
Bashati Vila, Apartment 5A, House 25, Road 68/A, Gulshan 1212, Dhaka, Bangladesh

E. Tumimbang-Raiz
International Rice Research Institute, Los Baños, Laguna, Philippines

G. Gregorio
International Rice Research Institute, Los Baños, Laguna, Philippines
Current address:
African Rice Center (WARDA), c/o International Institute of Tropical Agriculture, Oyo Road, PMB 5320, Ibadan, Nigeria

B.P. Forster
Plant Breeding Unit, IAEA Laboratories, Seibersdorf, Austria
Current address:
BioHybrids International Ltd, Po Box 2411, Earley, Reading, RG6 5FY, UK.

C. Mba(✉)
Plant Breeding Unit, Joint FAO/IAEA Agriculture and Biotechnology Laboratory, International Atomic Energy Agency Laboratories, A-2444 Seibersdorf, Austria
e-mail: c.mba@iaea.org

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

gametoclonal variation for agronomically important traits such as plant height. Over 100 green plants were regenerated from 2,000 cultured anthers. Among these, two doubled haploid lines (DHs) exhibited semi-dwarf stature. Mutations (induced during the culture procedure) in the semi-dwarfing gene *sd-1* were confirmed by PCR using locus specific primers. The semi-dwarf DH lines were multiplied and checked for response to salinity in hydroponics (10 dS m^{-1}) and field tests at IRRI, Philippines. The work demonstrates the effectiveness of combining doubled haploid protocols with mutation detection.

Keywords Rice, semi-dwarf mutant, gametoclonal variation, salt tolerance

Introduction

Rice is pre-eminent as a world crop species providing food for about 3,000 million people (FAO:AGCP; Evans 1998). Production however is threatened by environmental factors, particularly salinity and drought. Salinity is a significant limiting factor to agriculture productivity, impacting on about 900×10^6 ha of land (Flowers 2004). The existing problem is becoming more acute as a result of poor quality irrigation water. It is estimated that one third of the potential irrigated rice production area is damaged by salt (Subhashini and Reddy 1989; Szabolcs 1992; Ghassemi et al. 1995; Flower et al. 1997). In the past, relatively unproductive lands were not required for agriculture, but the unprecedented increases in human population in recent years have led to heightened food security concerns. These dictate the need for increasing available land for agriculture. It is estimated that the global population will increase from about 6 billion in 2001 to more than 9 billion in 2050 (http://www.unfpa.org/swp/2001; Wollenweber and Luebberstedt 2005), but will not be matched by food production owing in part to diminishing areas of good agricultural land. It is therefore likely that more marginal land will be brought into agriculture.

Variation for salt tolerance is available in tall landrace rice material e.g. Pokkali and Nona-bokra, (Akbar 1986; Khan et al. 1987; Xie et al. 2000), which are traditional pure line selections that are well adapted to local stress conditions. However they have not been bred for improved grain yield or short stature. Developing improved varieties that can withstand salt affected soil remains a recalcitrant breeding objective for this crop. Breeding for salinity tolerance in rice is difficult. Considerable conventional breeding effort to increase salt tolerance has been made (Moeljopawiro and Ikehasi 1981; Gregorio and Senadhira 1993), but the progress in developing tolerant rice has been slow because of the complex nature of the physiological mechanisms involved (Flowers 2004). The genetics of salt tolerance is also complex showing heterosis, dominance and additive effects (Shanon 1985) and is governed by two or more quantitative genes (Akbar and Yabunu 1977) that significantly interact with the environment. As a consequence salt tolerance exhibits low heritability with values as low as 19% (Gregorio and Senadhira 1993; Lee 1995). A project was therefore set up to induce a mutation for semi-dwarf stature

in a salt tolerant landrace that would provide a more acceptable genetic variant for use by rice breeders. The project took advantage of the fact that semi-dwarfism is a relatively common phenotype arising from induced mutation and would, therefore, be relatively easy to obtain (Ahloowalia et al. 2004; Mandal et al. 1999). In this work, we attempted to induce a semi-dwarf mutation through somaclonal variation using anther culture. An additional advantage here is that the doubled haploid (DH) plants regenerated are completely homozygous, i.e. any mutation would be fixed immediately. Somaclonal variation is observed in many plants regenerated through tissue culture, especially those with a prolonged callus phase. In general, viable and fully fertile variants do not appear to involve gross changes in their genetic make-up, rather somaclones are usually characterised by one or a few altered traits compared to the donor plant phenotype (Mandal et al. 1999; Bouharmont et al. 1999). Here we report the development of semi-dwarf mutants derived from the salt tolerant landrace Pokkali via anther culture. Since the callus from which plants were regenerated originated from uni-nucleate gametic cells, microspores, the variants regenerated are referred to as gametoclonal variants.

Materials and Methods

Pokkali

Pokkali is a tall Indian landrace known for its tolerance to salt. Seeds were obtained from the Plant Breeding, Genetics and Biochemistry Division of IRRI, Los Baňos, Philippines. Plants were raised in soil-filled pots in a lit glasshouse at the IAEA Laboratories, Seibersdorf, Austria, with a photoperiod of 8–11 hours (min–max), an irradiance of 220–860 $\alpha mol^{-1}\,s^{-1}$ during the months December–April (1996/1997), day/night temperature of 33°C/18°C and a relative humidity of 80%. The panicles were harvested at the booting stages with the distance between the sub tending leaf and flag leaf being 7–10 of 8–12 cm. Light microscopy investigations showed that this stage contained microspores at the mid to late uni-nucleate stage. Harvested panicles were subjected to a cold pre-treatment of 8°C for 8–10 days, after which they were surface sterilized by immersion in 70% ethanol for less than a minute, then 10% Clorox (commercial bleach, with a sodium hyperchlorite, NaOCl, concentration of 5.2% w/v) for 10 minutes and washed three times with sterile distilled water before removing anthers for culturing.

Anther Culture

Anthers were excised and cultured on liquid N6 medium (Chu et al. 1975) supplemented with 2 mg l^{-1} 2,4-D and 5% maltose for callus induction, cultures were kept in the dark at 25°C. After 6–7 weeks in culture, calli with a size of 2 mm were transferred to a regeneration medium: modified MS (Murashige and Skoog 1962)

with 1 mg/l BAP, 0.5 mg/l NAA, 200 mg l^{-1} myo-inositol, abscisic acid and solidified with 0.45% agarose (Sigma, Type I-A: A-0169). Regenerating cultures were incubated in the light at 25°C under a 12h light photoperiod supplied by cool white fluorescent lamps (66 umol m^{-2} s^{-1}). Regenerated plantlets were labelled with respect to callus origin. Generally one callus produced one plantlet, but occasionally multiple regenerants were recorded. Once regenerated and large enough with both roots and shoots, plants were transferred to soil-filled pots in glasshouse conditions.

Glasshouse Evaluation

Regenerated plants were grown in pots to maturity and assigned a line number. Out of 125 plantlets, 59 plantlets were spontaneous doubled haploids (DH). DHs are assayed by phenotypic observation of plant stature. There was no attempt to use colchicine to induce chromosome doubling in the other lines as three semi-dwarf types were found in the 59 sub-set. The three semi-dwarf gametoclonal variants originated from two calli (GVP3 and GVP35) showing plant height reduction were identified in the first generation (DH_1). Semi-dwarf DH lines were selfed and the progeny re-evaluated for height reduction and other agronomic traits in subsequent generations (Table 1). Seed were also sent to IRRI for field testing to confirm glasshouse phenotypic assessments.

Rapid Seedling Screen for Salt Tolerance

Salinity tolerance of the gametoclones was assessed in the glasshouse using a seedling hydroponics test developed by IRRI (Gregorio et al. 1997) and further modified for large scale screening of mutant populations by the Plant Breeding Unit, FAO/IAEA Agriculture & Biotechnology Laboratory in Seibersdorf, Austria (Afza et al. 1999) (Fig. 1). Seedlings of the DH_2, DH_3 and DH_4 generations of the three gametoclonal lines (GVP3 and GVP35) were tested. Seeds were germinated on a seedling mesh plate suspended over the hydroponics solution. Salinity was introduced into the solution 3 days after germination by adding 640 mg^{-1} NaCl (10 dS m^{-1}) to the nutrient solution as described by Yoshida et al. (1976). The pH was adjusted daily to 5.5. Salinity injury rating was based on visual symptoms (1 – tolerant and 9 – sensitive) at seedling stage, according to the modified Standard Evaluation System of IRRI Gregorio et al. (1997): five evaluation grades were used: 1 Normal growth, no leaf damage; 3. Nearly normal growth, but lowest leaf is desiccated, 5. Growth is severely retarded, and the two basal leaves are desiccated; 7. Complete cessation of growth, only the young, apical leaf survives; 9. Glasshouse conditions consisted of day/night temperature of 30°C/20°C with 70% relative humidity.

Table 1 Agronomic traits of gametoclonal rice mutants and parental landrace, Pokkali

Lines	Conditions	Days to heading	Plant height	Number of tillers	Number of productive tillers	Number of filled grain
GVP3	Non-saline	67	114	19	19	2008
	Saline	72	102	13	13	798
GVP35	Non-saline	66	115	25	25	2828
	Saline	74	110	14	13	1161
Pokkali	Non-saline	61	168	17	16	1215
	Saline	59	187	15	15	1259

Fig. 1 Seedling hydroponics test with a salt treatment of 10 dS m^{-1} for 21 days

Table 2 Salt tolerance screening of gametoclonal seedlings at the DH_4 generation

Variety/line	Number of seedlings tested	Salinization score after	
		8 days	16 days
GVP3	665	3–5	5–7
GVP35	549	3–5	5–7
Pokkali Tolerant control	44	3	7
IR29 Susceptible control	56	7–9	9

In addition to the semi-dwarf GVP lines, the salt tolerant tall landraces, Pokkali and Nona-bokra, and a salt susceptible semi-dwarf cultivar (IR-29) were included as checks for salt tolerance and salt susceptibility, respectively, as well as plant height. Results are given in Table 2.

Genotyping for sd-1 Mutations

The semi-dwarf GVP DH lines exhibited a similar stature to standard *sd-1* cultivars. Since *sd-1* has been cloned (Monna et al. 2002; Spielmeyer et al. 2002) and sequenced (Ellis and Speilmeyer 2002) it was possible to check for mutations in the GVP lines. DNA was extracted (Doyle and Doyle 1987) from leaves of GVP3, and GVP35 and portions of the *sd-1* sequence amplified by PCR using primers targeted at specific regions in the *sd-1* locus. PCR reactions were carried out in 15 αl containing 50 ng DNA, 1.5 αl PCR buffer, 0.75 αl 1 mM dNTP, 2 αl of each of primers (10 mM) forward (Sd1-F: 5'-CAC GCA CGG, GTT CTT CCA GGT G-3) reverse (Sd1-R: 5'-AGG AGA ATA GGA GAT GGT TTA CC-3) and 0.3 αl of taq polymerase (5u/αl)was prepared to amplified the *sd-1* gene from selected population. The PCR profile was as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds; elongation at 72°C for 50 seconds and a final elongation step at 72°C for 7 minutes. After amplification, the PCR products were separated by agarose gel electrophoresis (at 100 volts for 45 minutes); the gel was stained in ethidium bromide and visualised using UV light. DNA profiles were compared to a standard *sd-1* cultivar (IR8) and a none *sd-1*, tall type (Pokkali). Amplification products from lines with deletions in the *sd-1* gene were expected to be of a lower molecular weight than the Pokkali amplification product.

Allelism Testing

To study the mode of inheritance and allelism, the GVP3 line was crossed reciprocally onto standard *sd-1* cultivars, IR8, IR64 and parental lines. The F_1 with the *sd-1* parents and subsequent selfed generations were all short, indicating allelism with *sd-1*. The F_1 with the tall parent (Pokkali) were all tall and segregation for height was observed in subsequent generations.

Results and Discussion

Of the 125 plants regenerated from anther culture, three DHs showed a semi-dwarf stature: GVP3 and GVP35 (Fig. 2). Since the semi-dwarf phenotype was stable over several DH generations the phenotype can be attributed to a stable genetic change. The two gametoclonal variants identified originated in the first DH generation and exhibited plant height reduction (Table 1). The three plantlets originated from two calli clusters, with GVP3 arising from the same callus, they may therefore be clones of each other. The DH_2 generation from self pollinated panicles was evaluated for tolerance to salt; GVP lines retained the salt tolerance characteristics of their tall landrace parental line, Pokkali (Table 2). As salt tolerance exhibits low heritability in breeding salt tolerant varieties, GVPs could be used in cross breeding programmes for the improvement of plant stature of wild salt tolerant varieties.

Fig. 2 Height comparison between rice gametoclones (GVP3 left, GVP35 right) and landrace Pokkali (centre)

Plant dwarfism is one of the most important traits used in plant breeding, particularly in rice, and the semi-dwarfing gene, *sd-1* is one of the most important genes deployed in rice. The properties of *sd-1* are consistent with a semi-dwarf phenotype that results from a partial block in gibberellin biosynthesis (Spielmeyer et al. 2002; Sesaki et al. 2002; Weitzen 2002; Monna et al. 2002). The *sd-1* gene was first identified in the Chinese variety Dee-geo-woo-gen (DGWG) and crossed in the early 1960s with Peta, a tall variety to develop the semi-dwarf IR8 which produced record yields. Since the 1960 *sd-1* has been the predominant semi-dwarfing gene in rice cultivation. (Ellis and Speilmeyer 2002; Spielmeyer et al. 2002).

The *sd-1* gene in *indica* rice from Dee-geo-woo-gen (DGWG) was found to contain a 383 bp deletion in the genomic sequence of Os200x2, a gene responsible for the production of a growth hormone, gibberellin. In the present study, the GVP lines were tested and confirmed as being *sd-1* deletion mutants by genotyping at the *sd-1* locus and by allelism testing. Using PCR, a full length gene product (721 bp) was amplified in the target region from Pokkali; in contrast, IR8 and GVP lines produced a smaller DNA fragment revealing a deletion of 383 bp within the *sd-1* sequence. The semi dwarf *sd-1* gene is the most common height reducing gene in rice and the presence of a functional *sd-1* gene is normally determined by phenotypic assessment of final plant height and is therefore influenced by environmental factors throughout the life cycle. Furthermore, *sd-1* is recessive and in heterozygous plants are masked in the tall stature phenotype, and can only be evaluated retrospectively by studying their progeny. The PCR genotyping assay, therefore provides a rapid assay for the presence of non-functional *sd-1* alleles regardless of zygosity or environmental conditions.

Crosses were also made in this project with advanced semi-dwarf lines, e.g. IR58 and IR 64, which served to test allelism and to initiate the first steps in an introgression programme to improve the salt tolerance of semi-dwarf rice cultivars. Segregation of seed shape was observed in F_2 generation when crossed with semi-dwarf varieties, but there was no segregation for height, i.e. all of them were dwarf. The F_1 progency of the cross between the semi-dwarf somaclonal variants and the tall parent (Pokkali) were all tall and segregation for height was observed in subsequent generations.

The project demonstrates the relative ease with which semi-dwarf mutations can be induced in tall rice lines via gametoclonal variation. This approach has potentials for rice breeding and may be used to target other traits found in rice landraces for introgression into, and assessment in, semi-dwarf cultivars. The use of a DH system has advantages in that mutations are fixed immediately in the homozygous (DH) lines generated. Mutation rates may also be increased by the application of mutagenic treatments at various stages in the anther culture process. For example, gamma ray irradiation of anthers has been used to develop salt tolerance DH mutants in wheat (Afza et al. 2006). The development of molecular markers for *sd-1* would also provide breeders with an option to test for the presence of short or tall alleles rapidly, in heterozygous material and at any growth stages. The semi-dwarf lines produced here are to be evaluated for salt tolerance in field conditions in other parts of the world, e.g. Asia, Africa and Latin America.

Acknowledgements The authors are acknowledging Drs. Pierre Lagoda and Brad Till of the IAEA for their critical reading of the manuscript.

References

Afza R, Zapata-Arias FJ, Zwiletitsch F, Berthold G, Gregorio G (1999) Modification of a rapid screening method for rice mutants to NaCl tolerance using liquid nutrient culture. Mutation Breeding, News Letter No.144. International Atomic Energy Agency, Vienna, Austria

Afza R, Jain SM, Shu Q, Guzmann M, Zapata FJ, Tumimbang E, Greogorio G, Mba C (2006) Doubled haploidy and induced mutation in breeding for salt tolerance in rice and wheat. Book of Abstracts, The International Conference on “Haploids in Higher Plants III”, Vienna, Austria. February 12–15

Ahloowalia BS, Maluszynski M, Nichterlien K (2004). Global impact of mutation derived varieties, Euphytica 138:187–204

Akbar M (1986) Breeding for salinity tolerance in rice. In: Ahmed R, San Pietro A (eds), Prospect of Biosaline Research Proceedings of US–Pakistan Biosaline Research Workshop Karachi, Pakistan, pp. 37–55

Akbar M, Yabunu T (1977) Breeding saline – resistance varieties of rice. IV Inheritance of delayed type of panicle sterility induced by salinity. Jpn J Breed 27: 237–240

Bouharmont J, Dekeyser A, Van Sint Jan V, Dogbe YS (1999) Application of somaclonal: variation and in vitro selection to rice improvement. In ‘Rice Genetics 11’. pp. 271–277. (International Rice Research Institute)

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of efficient medium for anther culture of rice through comparative experiments on nitrogen sources. Sci Sin 18:659–668

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf. Phytochem Bull 19, 11–15
Ellis MH, Speilmeyer W (2002) Perfect markers for the semi-dwarfing gene sd1 in rice: IRRN 27.2, December 13–14
Evans LT (1998) Feeding 10 billion: Plants and Population growth. Cambridge University press, Cambridge
FAO:AGCP:CropGroup:Field-FoodCrops:Rice
Flowers TJ (2004) Improving crop salt tolerance. J. Exp Bot 55(396):307–319
Flowers TJ, Gracia A, Koyama M, Yeo AR (1997). Breeding for salt tolerance in crop plants – the role of molecular biology. Acta Physiol Plant 19(4):427–433
Ghassemi F, Jakeman AJ, Nix HA (1995) Salinisation of land and water resources. CAB International, Wallingford, p. 526
Gregorio GB, Senadhira D (1993) Genetic analysis of salinity tolerance in rice. Theor Appl Genet 86:333–338
Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. IRRI Discussion Paper Series. IRRI, P. O. Box 933, 1099 Manila, Philippines
http://www.fao.org/ag/agp/agpc/doc/crops/4a-1.html
IRRI (1994) Standard Evaluation Score for Rice (SES). IRRI, P. O. Box 933, 1099 Manila, Philippines
Lee KS (1995) Variability and genetics of salt tolerance in japonica rice (*Oryza sativa* L.) Ph.D. Thesis, University of Philippines, Los Banos
Khan AA, Akbar M, Shesu DV (1987) Ethylene as an indicator of salt tolerance gene in rice. Crop Sci 27:1242–1247
Mandal AB, Pramanik SC, Chowdhury B, Bandyopadhyay AK (1999) Salt tolerance Pokkali somaclones: performance under normal and saline soils in Bay Island 1999. Field Crops Res 61:13–21
Moeljopawiro S, Ikehasi H (1981) Inheritance of salt resistance in rice. Euphytica 30:291–300
Monna L, Kitazawaa N, Yoshino R, Suzuki J, Masuda H, Maehera Y, Tanji M, Sato M, Nasu S, Minobe Y (2002) Positional cloning of the rice semi-dwarfing gene, sd-1: rice “green revolution” encodes a mutant enzyme involved in gibberellic biosynthesis. DNA Res 9:11–17
Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–479
Sesaki A, Ashikari M, Ueguchi-Tanaka M, Itoh M, Nishimura A, Swapon D, Ishiyama K, Saito T, Kobayashi GS, et al. (2002) Green revolution: a mutant the gibberellin-synthesis gene in rice. Nature 416:701–702
Shanon MC (1985) Principles and strategies in breeding for higher salt tolerance. Plant and Soil 89: 227–241
Spielmeyer W, Ellis MH, Chandler PM (2002) Semidwarf (sd-1), “green revolution” rice contains a defective gibberellin 20 oxidase gene. Proc Natl Acad Sci 99(13):9043–9048
Subhashini K, Reddy GM (1989) *In vitro* selection for salinity and regeneration of plants in rice. Curr Sci 58(10):584–586
Szabolcs I (1992) Salinisation of soils and water and its relation to desertification. Desertification Cont Bull 12:32–37
Weitzen JB (2002) Green revolution, mutation of a gene in the gibberellin metabolic pathway played a part in the green revolution. Scientist 3(1):419–501
Wollenweber B, Luebberstedt JR (2005) Need for multidisciplinary research towards second green revolution: Curr Opin Plant Biol 8:337–341
Xie JH, Zapata-Arias FJ, Shen M, Afza R (2000) Salinity tolerant performance and genetic diversity of four rice varieties. Euphytica 116:105–110
Yoshida S, Forno DA, Cock JH, Gomez KA (1976) Laboratory manual for physiological Studies of rice. IRRI, Las Banos, Philippines, p. 83

Chapter 27
Chromosome Doubling in Monocots

A.M. Castillo, L. Cistué, M.P. Vallés, and M. Soriano

Abstract The development of efficient chromosome doubling protocols is essential for the useful application of doubled haploid (DH) plants in breeding programs, since frequency of spontaneous doubling is most of the time too low. Chromosome doubling has been traditionally applied to plantlets, being the colchicine the most widely anti-microtubule agent used *in vivo* and *in vitro*. However during the last 15 years, protocols have been developed for the incorporation of different anti-microtubule agents during the early stages of androgenesis or gynogenesis. Factors affecting frequencies of spontaneous and induced chromosome doubling are summarized. For a successful chemical induction, a compromise between toxicity (which can result from high concentration and/or long time of application) and genome doubling efficiency should be adopted in order to obtain the highest number of green DH plants.

Keywords Monocots, doubled haploid, chromosome doubling, anti-microtubule agents

Introduction

Haploid plants are highly valuable in breeding programmes, genetic and mutation analysis. Great achievements have been obtained in the production of haploid plants in monocots by androgenesis, interspecific or intergeneric crosses and gynogenesis

A.M. Castillo(✉)
Dpto. Genética y Producción Vegetal, Estación Experimental de Aula Dei (CSIC), Apdo. 202. 50080 Zaragoza, Spain
E-mail: amcast@eead.scis.es

L. Cisué, M.P. Vallés, and M. Soriano
Dpto. Genética y Producción Vegetal, Estación Experimental de Aula Dei (CSIC), Apdo. 202. 50080 Zaragoza, Spain

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

during the last 20 years. However, the successful utilization of haploid plants depends not only on the production of a large number of plants, but also on the development of efficient chromosome doubling protocols. Chromosome doubling can occur spontaneously or be induced by the application of duplication agents. There are three excellent previous reviews on chromosome doubling: Jensen (1974), Rao and Suprasana (1996) and Kasha (2005). In this chapter we update and summarize the information on spontaneous and induced doubling, and more specifically the research performed to induce chromosome doubling at early stages of gametic embryogenesis.

Spontaneous Doubling

Spontaneous doubling can occur through somatic diploidization, but it is known to be rare in nature. On the contrary, the meiotic chromosome doubling resulting from the production of 2n gametes (unreduced gametes), has played a major role in the evolution of plant species by the production of autopolyploids and allopolyploids (Harlan and De Wet 1975), and especially in the Poaceae family, which has a preponderance of allopolyploids. Meiotic restitution has also given rise to seed in haploid plants of oat (Rines et al. 1997), bread and durum wheat (Jauhar et al. 2000; Jauhar 2007).

Different rates of spontaneous doubling are found among the methods used for obtaining DH plants. No or low doubling (10–15%) is produced in interspecific or intergeneric crosses and gynogenesis, respectively (Maluszynski et al. 2003). Androgenesis has the potential for spontaneous chromosome doubling during the first divisions of microspores, thus producing completely doubled and fertile plants. The frequency of doubling varies depending on the species. Averages of 70–90% have been reported in barley, 25% to 70% in bread wheat, 50–60% in rice, 50–90% in rye (Maluszynski et al. 2003), 20% in maize (Martin and Widholm 1996) and 70% in durum wheat (Cistue et al. 2006).

Factors Affecting Chromosome Doubling in Androgenesis

The genotype, the developmental stage of the microspores at the time of culture, the type of pretreatment, and the pathway of nuclear development also influence percentage of doubling. In bread wheat, frequencies of doubling varied from 25% to 68% in winter cultivars from Central and Eastern Europe (Barnabás 2003). Spontaneous doubling rates of wheat microspores at late uni-nucleate to early bi-nucleate stage (54–66%) were higher than that of mid to late uni-nucleate (33%) (Soriano et al. 2007).

A mannitol starvation or a cold pretreatment have given consistently high percentage of chromosome doubling (Kasha et al. 2001). Furthermore, a mannitol pretreatment applied with a cold or a heat pretreatment seemed to enhance doubling efficiency in wheat (Indrianto et al. 1999) and barley (Li and Devaux 2003). As suggested

by Kasha et al. (2001), mannitol may act in the disruption of microtubules. In this sense it has been proposed that the term "spontaneous doubling" produced by mannitol should be considered as "induced doubling" (Kasha 2005). For an extensive review on how the pathway of microspore development influences chromosome doubling see Kasha (2005).

Mechanism of Chromosome Doubling in Androgenesis

Nuclear fusion, after both a symmetric or an asymmetric division, has been proposed to be the main mechanism of spontaneous doubling after mannitol or cold stress pretreatment in barley (Kasha et al. 2001; González-Melendi et al. 2005; Shim et al. 2006) and maize (Testillano et al. 2004) microspore embryogenesis. Dynamics and mechanism of diploidization in barley microspore embryogenesis after cold pretreatment was studied by confocal and electron microscopy, showing that diploidization can start after the first embryogenic division and continues in multinuclear pro-embryos during culture (González-Melendi et al. 2005). Similar results have also been described in maize (Testillano et al. 2004).

Induced Chromosome Doubling

Frequencies of spontaneous doubling are (most of the time) too low to be useful in plant breeding, and thus justify the application of duplication agents. Doubling procedures should allow the handling of a large number of plants, from a wide range of genotypes, with a safe and simple technique, and render stable DH plants efficiently without any genetic change. Different duplication agents have been applied to several tissues *in vivo* (plantlets) or *in vitro*. The most frequently used doubling agents an explants, with special attention to the gametic cells or gametic embryos, are presented below (for a more extensive revision see Jensen 1974 and Rao and Suprasana 1996).

Anti-Microtubule Agents

Among the different anti-microtubule agents used successfully, colchicine has been the most widely used *in vivo* and *in vitro*. Herbicides such as amiprophos-methyl (APM), oryzalin, trifluralin, and pronamide, have also been used *in vitro*. All these compounds, with the exception of pronamide, inhibit spindle formation by binding to tubulin, disrupting the normal polar segregation of sister chromatids and results in doubling chromosome number (C-mitosis) (Levan 1938; Morejohn and Fosket 1984; Bartels and Hilton 1973). Pronamide apparently causes shortening of the

microtubule (Vaughan and Vaughn 1987). Colchicine has a much lower affinity to plant tubulins than anti-microtubule herbicides, and therefore these are used at micromolar concentrations to induce the same effect as colchicine at millimolar concentrations (Bartels and Hilton 1973; Morejohn and Fosket 1984).

Chromosome Doubling *In Vivo* (to Plantlets)

Treatments for chromosome doubling are traditionally applied to plants, colchicine being the most widely used agent. Colchicine is applied to plants at the 3–4 tillering stage by the capping technique (Bell 1950) or at the 3–4 leaves stages by the immersion method (Jensen 1974). Generally, 0.05–2% colchicine is applied for 3–5 hours in the immersion method and 1–3 days in the capping method in combination with DMSO (0.1–4%) (for review see Maluszynski et al. 2003).

Besides the factors mentioned above, the efficiency of chromosome doubling with colchicine varies between species (80% in barley, 60–70% in bread wheat, 40–70% in durum wheat, 50–80% in triticale, 40% in rice, 40% in maize) and even between genotypes (Eder and Chalyk 2002; Maluszynski et al. 2003; Ballesteros et al. 2005). Chromosomal doubling in onion and maize plantlets is difficult due to the inaccessibility of the meristem, and the lack of tillering and, in the case of maize, the separation of female and male flowers.

The application of colchicine to plants has several disadvantages such as: (a) the high concentration and solution volume needed (b) the high rate of mortality, and (c) production of mixoploids or chimeric plants, that can lead to production of low seed set and therefore requiring an additional growth cycle for seed multiplication before evaluation in the field. In interspecific or intergeneric crosses, some of these problems can be avoided by colchicine treatment after pollination through tiller injections (Sood et al. 2003).

Chromosome Doubling at Early Stages of Gametic Embryogenesis

In order to minimize the problems of colchicine application *in vivo* described above, colchicine, pronamide, APM trifluralin and/or oryzalin have been successfully incorporated in the induction medium of anthers, microspores or callus and in the regeneration medium of embryos (Table 1). Percentage of chromosome doubling at early stages of gametic embryogenesis varies with the genotype, the anti-microtubule agent, the concentration and the period of application. Genotypic dependence has been described in wheat (Soriano et al. 2007) and maize (Barnabas et al. 1999). The differences among genotypes in their responses to colchicine might be related to the kinetics of mitotic division in culture (Zaki and Dickinson 1991). Comparison studies among duplication agents showed different results depending on the specie.

Table 1 Chromosome doubling at early stages of gametic embryogenesis. Highest chromosome doubling percentages produced with anti-microtubular agents and its effect on culture phases

					Effects on culture parameters			
Species	Expl	Appl	Agent/µM/ days	Doubling (%)	Ind	Reg	Green %	References
Wheat	Ant	Ind	Col/500/3	69	+	No	+	Barnabás et al. (1991)
	Ant	Ind	Col/500/3	72	–	No	No	Navarro-Alvarez et al. (1994)
	Ant	Ind	Col/250/5	100	–	+/No	+/No	Redha et al. (1998)
	Ant	Ind	Col/751/3	0–100	+/–	–/No	+	Zamani et al. (2000)
	Ant	Ind	Col/751/2	50–58	No	No	No	Soriano et al. (2007)
	Callus (Ant)	Ind	Col/375/4	79–91	+	–	–	Ouyang et al. (1994)
	Callus (Ant)	Ind	Col/313/2 Trif/10/3 Oryz/10/3	50–100 0–36 0–50		nd nd nd	nd nd nd	Hassawi and Liang (1991)
	Emb (Ant)	Reg	Col/1250/1	0–100		–	–	Mentewab and Sarrafi (1997)
	Mic	Ind	Col/1000/2	53	No	No	–	Hansen and Andersen (1998a)
	Mic	Ind	Trifl/10/2 APM/10/2	74 65	– –	No No	– –	Hansen and Andersen (1998b)
	Mic	Ind	Col/751/2	73–88	+/No	No	+/No	Soriano et al. (2007)
	Mic	Pret	Col/751/2	69	No	No	No	Soriano et al. (2007)
Maize	Ant	Ind	Col/626/7	56	–	–/No	nd	Saisingtong et al. (1996)
	Ant	Ind	Col/750/3	80–100	No	No	No	Barnabáas et al. (1999)
	Ant	Ind	Col/500/3	20 (ns)	+(ns)	nd	nd	Obert and Barnabas (2004)
	Ant	Pret	Col/1250/7	38(ns)	+	No	nd	Antoine-Michard and Beckert (1997)
	Ant	Ind	Col/50/4 Prona/1/4 Oryz/1-5/4	43 0 0	– – –			Martin and Widholm (1996)
	Callus (Ant)	Ind	Oryza/10/2 APM/15/3 Pron/5/3 Trif/5/3	78 67 73 100		– + + –	nd nd nd nd	Wan et al. (1991)

(continued)

Table 1 (continued)

					Effects on culture parameters			
Species	Expl	Appl	Agent/μM/days	Doubling (%)	Ind	Reg	Green %	References
Rice	Ant	Ind	Col/626/2	78	No	No	No	Alemanno and Guiderdoni (1994)
Onion	Emb (Gyno)	Elon	APM/50/3	70		–		Jakse and Bohanec (2000)
			Oryz/50/3	90		–		
	Emb (Gyno)	Elon	APM/50/2	37		–		Jakse et al. (2003)
	Emb (Gyno)	Reg	APM/50/3	35	No	No		Grzebelus and Adamus (2004)
			Oryz/50/2	47	–	–		
			Trif/50/3	47	–	–		
			Col/313/3	35	No	–		

Col = colchicine; APM = amyprosphos methyl; Trif = trifluralin; Oryz = Oryzaline; Pron = pronamide; + = induction effect; − = inhibition effect; No = no effect; nd = not determined; ns t = no significant.

Colchicine induced higher duplication rates than anti-microtubular herbicides in wheat and maize (Hassawi and Liang 1991; Martin and Widholm 1996). However, trifluralin, oryzalin or APM produced higher doubling efficiencies than colchicine in gynogenic embryos of onion (Grzebelus and Adamus 2004).

The concentration of duplication agent and period of application that rendered the highest percentage of chromosome doubling in different monocots are shown in Table 1. These agents affect not only the percentage of doubling but also the whole androgenetic or gynogenetic process. The best concentration and time of application for chromosome doubling can have negative effects on embryogenesis, regeneration and/or percentage of green plants (Table 1). Positive effects of the anti-microtubule agents have also been described. Different actions of colchicine have been proposed to explain the positive effects on embryogenesis, as an increase of symmetrical divisions, the depression in the synthesis of pollen specific tubulins, and cytoskeletal restructuration (for review see Shariatpanahi et al. 2006). Colchicine could also cause a reduction of chloroplast DNA abnormalities (Aubry et al. 1989) or a selective elimination of microspores having abnormalities (Barnabas et al. 1991), increasing the percentage of green plants.

It has been suggested that any microtubule disrupting agent exhibiting a symmetric first mitotic division in microspores would also lead to both embryo induction and spontaneous chromosome doubling (Kasha 2005). In *Brassica*, the application of colchicine produced high frequency of embryogenesis and chromosome doubling (Zhou et al. 2002), and could even substitute a heat

stress pretreatment (Zhao et al. 1996). However, studies performed in maize, with a wide range of colchicine concentrations, indicated that although colchicine can induce embryogenesis, optimal concentration for embryogenesis and chromosomal doubling are not the same (Saisingtong et al. 1996; Obert and Barnabas 2004).

Application of colchicine during cold or mannitol stress pretreatment has only been studied in maize (Antoine-Michard and Beckert 1997) and wheat (Soriano et al. 2007), respectively. In the first study, increased rate of embryogenesis but no effect on doubling was observed. In the second one, the colchicine effect on green plant production and percentage of doubling was genotype dependent.

Anti-microtubule agents at early stages of gametic embryogenesis have been applied at the Agricultural Research Institute of the Hungarian Academy of Science (Dr. Barnabás), with maize and bread wheat genotypes that have low rates of spontaneous doubling, and at the Center for Plant Biotechnology and Breeding, University of Ljubljana (Dr. Bohanec) with gynogenetic embryos in onion (Maluszynski et al. 2003).

Conclusion and Further Perspectives

Chromosome doubling induced to plantlets is most common in monocots. During the last 15 years protocols have been developed for the application of anti-microtubule agents *in vitro* to microspores of maize and wheat, and embryos of onion. The use of both strategies combined can maximize the recovery of DH plants. Efforts in order to improve the protocols for application to isolated microspores, in the induction medium in gynogenesis and immediately after pollination in wide crosses could further increase the production of DH plants.

Successful chemical induction of chromosome doubling *in vivo* and *in vitro* depends on a compromise between toxicity and genome doubling efficiency. The concentration and time of application should be optimized to obtain the highest number of DH plants. Anti-microtubule herbicides have been used *in vitro* successfully as an alternative to colchicine in onion due to their lower toxicity. The possibility of using these agents in other monocot species should be studied further.

Microscopy studies can shed light on of how different types of stress pretreatment can affect cytoskeleton and produce chromosome doubling. Application of duplication agents at the time of stress pretreatment should be investigated further. Since genotype is one of the main factors affecting chromosome doubling, molecular approaches would be necessary to gain knowledge about the genes, and their regulation, involved in chromosome doubling.

Acknowledgements We are grateful for financial support from the Plan Nacional de Recursos y Tecnologías Agroalimentarias (AGL2002-04139-C02-02 and AGL2005-07195-C02-01).

References

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant-regeneration from rice (*Oryza-sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep 13(8):432–436

Antoine-Michard S, Beckert M (1997) Spontaneous versus colchicine-induced chromosome doubling in maize anther culture. Plant Cell Tiss Org Cult 48(3):203–207

Aubry CJ, De Buyser J, Hartmann C, Henry Y, Rode A (1989) Changes in molecular organization of the mitochondrial genome in albino tissue cultures derived from wheat pollen embryos and in plants regenerated from theses cultures. Plant Sci 65:103–110

Ballesteros J, Castillo AM, Cistué L, García-Lamas C, Martín A, Vallés MP (2005) Doubled-haploid technique in durum wheat breading. In: Royo C, Nachit MM, Di Fonzo N, Araus JL, Pfeiffer WH, Slafer GA (eds) Durum Wheat Breading, Current Approaches and Future Strategies, Food Products Press, Binghamton, New York, pp. 217–228

Barnabás B (2003) Protocol for producing doubled haploid plants from anther culture of wheat (*Triticum aestivum* L). In: Malupszynski M, Kasha KJ, Foster B, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual, FAO/IAEA Division, Wien, pp. 65–70

Barnabas B, Pfahler PL, Kovacs G (1991) Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum-aestivum* L). Theor Appl Genet 81(5):675–678

Barnabas B, Obert B, Kovacs G (1999) Colchicine, an efficient genome-doubling agent for maize (*Zea mays* L.) microspores cultured in anthero. Plant Cell Rep 18(10):858–862

Bartels PG, Hilton JL (1973) Comparison of triflularin, oryzalin, pronamide, propham, and colchicine treatments on microtubules. *Pesticide Biochem Physiol* 3:462–472

Bell GDH (1950) Investigations in the Triticineae. I. Colchicine techniques for chromosome doubling in interspecific and intergeneric hybridization. *J Agric Sci* 40:9–20

Cistue L, Soriano M, Castillo AM, Valles MP, Sanz JM, Echavarri B (2006) Production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. *Plant Cell Rep* 25(4):257–264

Eder J, Chalyk S (2002) In vivo haploid induction in maize. Theor Appl Genet 104:703–708

González-Melendi P, Ramírez C, Testillano PS, Kumlehn J, Risueño MC (2005) Three-dimensional confocal and electron microscopy imaging define the dynamics and mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis. Planta 222:47–57

Grzebelus E, Adamus A (2004) Effect of anti-mitotic agents on development and genome doubling of gynogenic onion (*Allium cepa* L.) embryos. Plant Sci 167(3): 569–574

Hansen NJP, Andersen SB (1998a) In vitro chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). Euphytica 102(1):101–108

Hansen NJP, Andersen SB (1998b) Efficient production of doubled haploid wheat plants by *in vitro* treatment of microspores with trifluralin or APM. Plant Breed 117(5):401–405

Harlan JR, De Wet JMJ (1975) On Ö. Winge and prayer: the origins of polyploidy. Bot Rev 41:361–390

Hassawi DS, Liang GH (1991) Antimitotic agents effects on double haploid production in wheat. Crop Sci 31(3):723–726

Indrianto A, Heberle-Bors E, Touarev A (1999) Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. Plant Sci 143:71–79

Jakse M, Bohanec B (2000) Studies of alternative approaches for genome doubling in onion. In: Bohanec B (ed) Biotechnological approach: utilization of gametic cells. COST 824 final meeting. Bled, Slovenia, pp. 101–104

Jakse M, Havey MJ, Bohanec B (2003) Chromosome doubling procedures of onion (*Allium cepa* L.) gynogenic embryos. Plant Cell Rep 21(9):905–910

Jauhar PP (2007) Meiotic restitution in wheat polyhaploids (Amphihaploids): a potent evolutionary force. J Hered 98(2):188–193

Jauhar PP, Dogramaci-Altuntepe M, Peterson TS, Almouslem AB (2000) Seedset on synthetic haploids of durum wheat: cytological and molecular investigations. Crop Sci 40(6):1742–1749

Jensen CJ (1974) Chromosome doubling techniques. In: Kasha KJ (ed) Haploids in Higher Plants: Advances and Potential, University of Guelph, Canada, pp. 153–190

Kasha KJ (2005) Chromosome doubling and recovery of doubled haploid plants. In: Palmer CE, Keller WA, Kasha KJ (eds) Biotechnology in Agriculture and Forestry 56. Haploids in Crop Improvement II. Springer, Berlin/Heidelberg, pp. 123–152

Kasha KJ, Hu TC, Oro R, Simion E, Shim YS (2001) Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. J Exp Bot 52(359):1227–1238

Levan A (1938). The effect of colchicine on root mitosis in *Allium*. Hereditas 24:471–486

Li H, Devaux P (2003) High frequency regeneration of barley doubled haploid plants from isolated microspore culture. Plant Sci 164:379–386

Maluszynski M, Kasha KJ, Forster BP, Szarejko I (2003) Doubled haploid production in crop plants: A manual. Kluwer. FAO/IAEA Division, Wien

Martin B, Widholm JM (1996) Ploidy of small individual embryo-like structures from maize anther cultures treated with chromosome doubling agents and calli derived from them. Plant Cell Rep 15(10):781–785

Mentewab A, Sarrafi A (1997) Androgenic ability and chromosome doubling by different colchicine treatments in anther culture of hexaploid wheat genotypes (*Triticum aestivum* L.). Cereal Res Commun *25*(4):897–903

Morejohn LC, Fosket DE (1984) Inhibition of plant microtubule polymerization in vitro by the phosphoric amide herbicide amiprophos-methyl. Science 224:874–876

Navarro-Alvarez W, Baenziger PS, Eskridge KM, Hugo M, Gustafson VD (1994) Addition of colchicine to wheat anther culture media to increase doubled haploid plant-production. Plant Breed 112(3):192–198

Obert B, Barnabas B (2004) Colchicine induced embryogenesis in maize. Plant Cell Tiss Org Cult 77(3):283–285

Ouyang JW, Liang H, Jia SE, Zhang C, Zhao TH, He LZ, Jia X (1994) Studies on the chromosome doubling of wheat pollen plants. Plant Sci 98(2):209–214

Rao PS, Suprasana P (1996) Methods to double haploid chromosome numbers. In: Mohan SJ, Sopory SK, Veilleux RE (eds) Current Plant Science and Biotechnology in Agriculture. In Vitro Haploid Production in Higher Plants, Vol. 1, Kluwer, Dordrecht

Redha A, Attia T, Buter B, Saisingtong S, Stamp P, Schmid JE (1998) Improved production of doubled haploids by colchicine application to wheat (*Triticum aestivum* L.) anther culture. Plant Cell Rep 17(12):974–979

Rines HW, Riera-Lizarazu O, Nunez VM, Davis DW, Phillips RL (1997) Oat haploids from anther culture and from wide hybridizations. In: Jain SM, Sopory SK, Veilleux RE (eds), Current Plant Science and Biotechnology in Agriculture. In Vitro Haploid Production in Higher Plants. Vol. 4, Kluwer, Dordrecht

Saisingtong S, Schmid JE, Stamp P, Buter B (1996) Colchicine-mediated chromosome doubling during anther culture of maize (*Zea mays* L). Theor Appl Genet 92(8):1017–1023

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiol Plant 127(4):519–534

Shim YS, Kasha KJ, Simion E, Letarte J (2006) The relationship between induction of embryogenesis and chromosome doubling in microspore cultures. Protoplasma 228(1–3):79–86

Sood S, Dhawan R, Singh K, Bains NS (2003). Development of novel chromosome doubling strategies for wheat x maize system of wheat haploid production. Plant Breed 122(6):493–496

Soriano M, Cistué L, Vallés MP, Castillo AM (2007) Effects of colchicine on anther and microspore culture of bread wheat (*Triticum aestivum* L.). Plant Cell Tiss Org Cult 91:225–234

Testillano P, Georgiev S, Mogensen HL, Coronado MJ, Dumas C, Risueño MC, Mathys-Rochon E (2004) Spontaneous chromosome doubling results from nuclear fusion during *in vitro* maize induced microspore embryogenesis. Chromosoma 112(7):342–349

Vaughan MA, Vaughn KC (1987) Pronamide disrupts mitosis in a unique manner. Pesticide Biochem Physiol 28:182–193

Wan Y, Duncan DR, Rayburn AL, Petolino JF, Widholm JM (1991) The use of antimicrotubule herbicides for the production of doubled haploid plants from anther-derived maize callus. Theor Appl Genet 81(2):205–211

Zaki M, Dickinson H (1991) Microspores derived embryos in Brassica: the significance of division symmetry on pollen mitosis I to embryogenic development. Sex Plant Reprod 4:48–55

Zamani I, Kovacs G, Gouli-Vavdinoudi E, Roupakias DG, Barnabas B (2000) Regeneration of fertile doubled haploid plants from colchicine-supplemented media in wheat anther culture. Plant Breed 119(6):461–465

Zhao JP, Simmonds DH, Newcomb W (1996) High frequency production of doubled haploid plants of *Brassica napus* cv Topas derived from colchicine-induced microspore embryogenesis without heat shock. Plant Cell Rep 15(9):668–671

Zhou WJ, Hagberg P, Tang GX (2002) Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspores in spring *Brassica napus*. Euphytica 128(1):27–34

Chapter 28
Tracking Gene and Protein Expression During Microspore Embryogenesis by Confocal Laser Scanning Microscopy

P.S. Testillano and M.C. Risueño

Abstract Confocal Laser Scanning Microscopy (CLSM) technology and bioimaging are powerful tools for three-dimensional (3D) and colocalization molecular analysis of the microspore embryogenesis. Strategies with fluorescent-labelled probes for *in situ* hybridization and immunofluorescence have provided unique images of the spatial and temporal pattern of the expression of genes and proteins, and of the sub-cellular rearrangements that accompany the microspore embryogenesis. Various signalling and stress proteins were differentially expressed in reprogrammed microspores and young embryos, and specific endosperm and embryo genes were expressed at different stages, supporting the existence of an endosperm-like domain, in cereals. Specific features such as changes in cell wall components and pectin esterification, presence of callose in special walls, and different behaviour of Cajal nuclear bodies were found in embryogenic microspores and young embryos, constituting early embryogenic markers. The 3D analysis of the nuclear dynamics at early stages of microspore embryogenesis has proved that the nuclear fusion was the mechanism of the spontaneous diploidization.

Keywords Microspore embryogenesis, confocal laser scanning microscopy, 3D analysis, bioimaging, gene and protein expression, tracking, FISH, immunofluorescence, stress, signalling

---

P.S. Testillano
Plant Development and Nuclear Architecture, Biological Research Centre, CIB, CSIC, Ramiro de-Maeztu 9, 28040 Madrid, Spain

M.C. Risueño(✉)
Plant Development and Nuclear Architecture, Biological Research Centre, CIB, CSIC, Ramiro de-Maeztu 9, 28040 Madrid, Spain
E-mail: risueno@cib.csic.es

A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,
© Springer Science+Business Media B.V. 2009

Introduction

Functional plant cell biology and genomics are now benefitting from modern bio-imaging approaches and especially from the use of confocal laser scanning microscope (CLSM) technology. Strategies with fluorescent-labelled probes for single and multi-coloured *in situ* hybridization and immuno-cytochemistry followed by confocal analysis have provided unique images of the spatial and temporal pattern of the expression of genes and their products at cellular and sub-cellular levels. *In situ* molecular identification techniques are of special interest to analyse plant developmental processes occurring asynchronously in different cell types and populations which grow together in a plant organ/tissue or in a *in vitro* culture system. They permit the identification of the presence and distribution of defined molecules in individual cells by bio-imaging technology of their architecture and corresponding developmental stages.

Microspore embryogenesis constitutes an intriguing system in which a cell is reprogrammed from its genetically controlled gametophytic programme towards an embryogenic pathway (Fig. 1). This process can be induced *in vitro* by different stress pretreatments in anther and microspore culture protocols. In both systems only a limited proportion of cells are able to respond to the inductive treatment and switch their developmental programme. The rest of the cells in the culture are

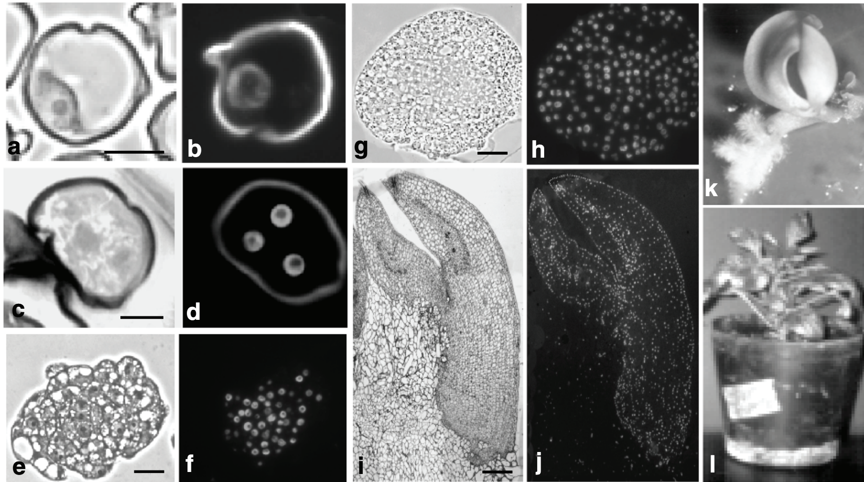


Fig. 1 Confocal monitoring of microspore embryogenesis (a–j) and regeneration of microspore-derived embryos and plants (k, l), in pepper. The same sections are observed under phase contrast (a, c, e, g, i) and DAPI fluorescence (b, d, f, h, j). a, b: Vacuolate microspore at the beginning of culture. c, d: Multicelular proembryo still surrounded by the exine, 3–4 days after stress treatment, e, f: Multicellular proembryo after the release of the exine, 10–12 days after the stress. g, h: Globular embryo, 15–16 days after the stress. i, j: Torpedo embryo, 20–25 days after the stress. k: Plantlet with cotyledons and root, 35–40 days of culture. L: 80-day old regenerated plant from anther culture. Bars represent 20 µm (a–f) and 50 µm (g–j)

mostly dead, have arrested development or follow a limited gametophytic-like pathway. The heterogeneous cell population of microspore and anther cultures, and the difficulty of separating the embryogenic responsive cells from others largely limit the biochemical and molecular analysis of early events of microspore embryogenesis induction. *In situ* approaches and bio-imaging techniques, including confocal analysis, have provided us with unique data on the cellular changes in gene expression, protein localization and wall components and distribution as well as the rearrangements of sub-cellular compartments which accompany the cell reprogramming. This approach has thus unveiled some of the cellular mechanisms involved in the embryogenic induction and cellular changes during the early stages of microspore embryogenesis. A significant contribution of confocal microscopy technology in understanding the microspore embryogenesis process has been three dimensional (3D) confocal analysis of the nuclear dynamics at early stages. The results have proved, unequivocally, that spontaneous diploidization occurred by nuclear fusion and revealed the timing of the process in barley.

Tracking Gene and Protein Expression During Early Microspore Embryogenesis

Signalling and Stress Proteins Are Differentially Expressed in Reprogrammed Microspores and Young Embryos

Mitogen-activated protein kinases (MAPKs) are involved in the signalling of extracellular stimuli in eukaryotes, including plants. Different MAPKs have recently been shown to be expressed during plant cell proliferation and developmental processes such as pollen development and embryogenesis. Extra-cellular signal regulated kinases (ERKs) of the MAPK family are involved in both cell proliferation and differentiation in mammals, through phosphorylation pathways. Confocal analysis of immunofluorescence assays with antibodies to ERK1/2 and the active phosphorylated forms of MAPKs (P-MAPKs) indicated that in developing microspores, P-MAPKs and ERKs were mostly cytoplasmic (Coronado et al. 2002). After microspore embryogenesis induction an increase in the immunofluorescence signals was observed for both P-MAPKs (Fig. 2c, d) and ERKs, the latter showing higher nuclear signal intensity. These results indicate the entrance into the nucleus of MAPKs accompanying a change in proliferative activity (Coronado et al. 2002; Ramírez et al. 2004; Testillano et al. 2005; Seguí-Simarro et al. 2005). *In situ* localizations have revealed that the entrance into the nucleus of signalling molecules from the cytoplasm is coincident with the switch to embryogenesis in various herbaceous plants (Coronado et al. 2002; Testillano et al. 2005; Seguí-Simarro et al. 2005) as well as some woody species (Ramírez et al. 2004). It is thus conceivable that there is a role for these ERKs, similar to that of mammalian ERKs, in the activation of nuclear targets such as transcription factors that specifically trigger the proliferative stages of embryogenic development. (Silva et al. 2004).

The expression of stress proteins such as heat-shock protein 70 (HSP70) and 90 (HSP90) immediately after embryogenesis induction has been reported in *Brassica napus* L. and *Capsicum annuum* L. (Barany et al. 2001; Seguí-Simarro et al. 2003). Immuno-fluorescence with anti-HSP70 and anti-HSP90 antibodies have revealed a low signal in microspores and pollen, corresponding to a very low level of the constitutive proteins. In very young microspore-derived structures an intense signal in the nucleus was observed for HSP70 (Fig. 2a, b). The cellular translocation of the induced HSP70 to the nucleus occurred in both embryogenic and non-embryogenic microspore in culture; however, the translocation was greater in embryogenic microspores, suggesting a possible additional role for HSP70 in the switch to embryogenesis (Seguí-Simarro et al. 2005). In contrast, HSP90 increase was similar

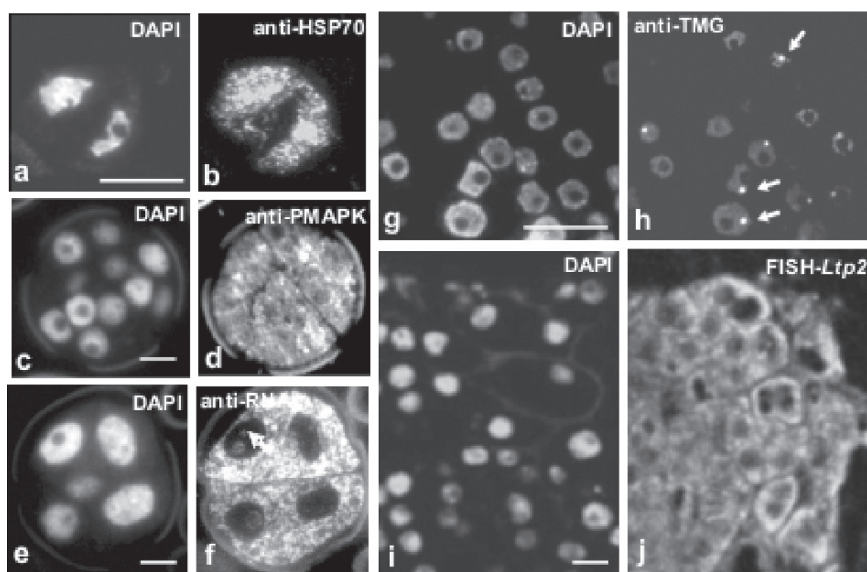


Fig. 2 Tracking gene and protein expression and revealing early markers in microspore-derived embryos by confocal microscopy. The figure shows the same embryo sections stained by DAPI which reveals the nuclei (a, c, e, g, i) and by specific fluorescence-labelled antibodies and probes (b, d, f, h, j). a, b: Two-celled microspore pro-embryo showing two nuclei specifically stained by DAPI (a), and the same pro-embryo section with anti-HSP70 immuno-fluorescence (IF) which labelled both nuclei and cytoplasms (b), Brassica napus. c, d: Multicellular proembryo still surrounded by the exine, stained by DAPI to reveal the nuclei fluorescent (c), and anti-phospho-MAPK IF providing specific signal to cytoplasms and nuclei (d), Nicotiana tabacum. e, f: Multicellular proembryo still surrounded by the exine, showing its nuclei specifically stained by DAPI (e), and anti-RNA IF in which the labelling is found in cytoplasms, nucleoli and Cajal bodies (arrow) (f), Nicotiana tabacum. g, h: Multicellular embryo without exine showing numerous nuclei stained by DAPI, the nucleoli appearing as small dark spots (g), and anti-TMG IF providing a diffuse fluorescence to the nuclei and highly brilliant nuclear spots (arrows) probably corresponding to Cajal bodies (h), Brassica napus. i, j: 30-day old multicellular embryo stained by DAPI for the nuclei (i) and by Fluorescence In Situ Hybridization, FISH, to revealing *Ltp2* gene expression and localizing Ltp2 mRNAs in cytoplasms (j), Zea mays. Bars: 10 µm (a–h), 50 µm (i–j)

in all microspores, occurring faster than for HSP70 and suggesting a more specific role for HSP90 in the stress response.

Expression of Embryo and Endosperm-Specific Genes in Young Microspore-Derived Embryos of Maize

The development of embryos from microspore in most systems closely resembles that of the normal zygotic embryogenesis pathway. However, very little information has been provided on the parallels between these two embryogenic programmes, especially at initial stages. In maize, a few genes have been reported to have specific spatial and temporal expression during early zygotic embryogenesis. *Esr*, *ZmAE1* and *ZmAE3* genes are expressed at the micropilar end of the endosperm, *Ocl3* at the suspensor, and *Ltp2*, at the embryo protoderm. A RT-PCR molecular approach has been applied to screen for the expression of these known maize embryo- or endosperm-specific genes at various developmental stages of microspore embryogenesis. Fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) studies with confocal laser microscopy using embryo- or endosperm-specific probes have provided relevant information about the expression and translation of the selected genes in the developing microspore-derived structures (Massonneau et al. 2005). Results showed that the endosperm genes (*Esr, ZmAE1, ZmAE3*) were expressed at early stages, from day 5 in proembryos (which are still enveloped by an exine) and have a transitory expression pattern at later stages. In contrast, embryo genes (*Ltp2, Ocl3*) were not expressed in 5 day old proembryos, but were highly expressed in the multi-cellular microspore embryos formed after exine breakdown (Fig. 2i, j). The spatial and temporal expression pattern of the embryogenesis-specific genes provide additional evidence of two domains in young microspore maize embryos as they display similar features of the embryo and endosperm (Testillano et al. 2002). They also support the existence of an endosperm-like function during early microspore embryogenesis and its parallelism with zygotic embryogenesis.

Early Markers of Microspore Embryogenesis in Sub-Cellular Rearrangement

The search for molecular and cellular markers during early stages of microspore embryogenesis is important as once found these may provide a means to monitoring the processes involved in the induction, and also to identify cells committed to the new developmental programme. In several species the process is accompanied by defined changes affecting various cell activities and structural organization of sub-cellular compartments which have been described as markers of the pollen reprogramming process (Ramírez et al. 2004; Barany et al. 2005; Satpute et al. 2005; Seguí-Simarro et al. 2006).

Cajal Nuclear Bodies – a Marker for Early Microspore Embryogenesis

Cajal bodies (CBs) are small rounded nuclear substructures which have been found in numerous differentiated and proliferating animal and plant cells. They have been reported to store constituents of the pre-mRNA and pre-rRNA processing machinery, like snRNPs, snoRNPs, nucleolar proteins and the p80-coilin. Immunofluorescence using antibodies recognizing the specific 2,2,7-trimethyl-guanosine (TMG) cap of the snRNAs, showed a pattern of distribution of splicing factors in nuclear speckles, these splicing factors accumulating in Cajal bodies were revealed as small bright spots (Fig. 2h). The increase in the number of CBs and the proportion of cells containing them has been closely correlated with transcriptional activation and cell cycle progression in various mammalian cells.

The presence of Cajal bodies during representative stages of gametophytic and haploid embryogenic development was analysed in isolated microspore and anther cultures of several systems, *Brassica napus* L., *Nicotiana tabacum* L., and *Capsicum annuum* L. (Testillano et al. 2005; Seguí-Simarro et al. 2006). Results showed that in most cases developing microspores showed no Cajal bodies when stained by anti-TMG immunofluorescence, exhibiting a disperse signal on the extra-nucleolar area, only the vacuolated microspore stage showed a few CBs. When the microspore was induced towards embryogenesis and proliferation, each nucleus of the multi-cellular microspore-derived structures displayed one or more bright spots, corresponding to CBs (Fig. 2g, h). Anti-RNA antibodies also labelled CBs in the vacuolate microspores and proembryos, as well as the abundant ribosomal population in cytoplasms of embryo cells (Fig. 2e, f), a typical feature of proliferating cells. These results indicated that Cajal bodies increase during the early stages of microspore embryogenic development suggesting that they have a role in the transcriptionally active, proliferative stages that characterise early microspore embryogenic development. Consequently they are a candidate as an early marker of the process.

Changes in Cell Wall Components and Pectin Esterification in Embryogenic Microspores and Young Microspore Embryos

During developmental processes the structures and components of cell walls change. Many molecular markers for somatic embryogenesis and organogenesis have been found in cell walls. Differences in the presence and abundance of various cell wall components have been reported during microspore differentiation and embryogenesis in some herbaceous and woody species (Ramírez et al. 2004; Barany et al. 2005) and during proliferation activity, cell wall growth and maturation (Willats et al. 2001). The modifications in pectin residues of the cell wall have been reported as being responsible for initiating cell responses in relation to cell fate and development. Immunocytochemistry with JIM7 and JIM5 antibodies (recognizing esterified and non-esterified pectins) has been performed in various systems

to compare cell walls of microspore-derived proembryos and pollen grains. In proliferative systems, such as root meristems and very young microspore-derived proembryos, the high proportion of esterified pectins in cell walls has been found to be associated with proliferative activity (Ramírez et al. 2004). In contrast, in differentiating microspores and late microspore-derived embryos, the proportion of non-esterified pectins in cell walls was found to be high. The de-esterification of pectins within the cell wall matrix results in the cross-linking of homogalacturonan residues with calcium, this process contributes to cell wall stiffness and could provide additional rigidity to the cell wall. The results obtained in different systems suggested that the proportion of esterified and non-esterified pectins and their distribution in the cell wall is related to proliferation and differentiation events after microspore reprogramming to embryogenesis.

Presence of Callose in Cell Walls of Young Microspore Derived Embryos

The 5–7 day old multicellular maize microspore derived embryos display two domains, one embryo-like the other endosperm-like and each has specific structural and molecular features (Magnard et al. 2000; Testillano et al. 2002; Massonneau et al. 2005). Cell wall structure was different between both domains and occasional wavy/sinuous and incomplete walls appeared in one of them. As β-1,3-glucan (callose) is an unusual polysaccharide which is specially abundant in the so-called “growing-cell walls” of the endosperm, anti-β-1,3-glucan antibodies recognizing callose were used for immunofluorescence. Specific immune-fluorescence was observed in certain walls of the large endosperm-like domain, although no signal appeared in the small embryo-like domain (Testillano et al. 2002). Labeling was especially high in thick regions of the wavy walls, and occurred frequently near a free end and revealed the presence of this unusual polysaccharide in the walls of the endosperm-like domain of young microspore derived multi-cellular structures of maize. Incomplete cell walls have also been found in young microspore proembryos of other systems of cereals, such as barley (Ramírez et al. 2001) and woody species, such as cork oak (Ramírez et al. 2004), suggesting a more general occurrence of this special feature.

Chromosome Doubling Mechanisms and Nuclear Dynamics

Nuclear Fusion Is the Mechanisms of Spontaneous Chromosome Doubling and Occurs Shortly After Embryogenesis Induction

Confocal microscopy has provided us with a unique method of approaching the analysis of the spontaneous diploidization of microspore embryos. González-Melendi et al. (2005) developed a new method to determine the ploidy level of

individual nuclei within microspore-derived proembryos of barley by using specific DNA fluorescent probes and confocal analysis. The method avoided the overlapping of the fluorescence signal in multi-nuclear proembryos, which cannot be studied using cytophotometric methods based on other types of fluorescence microscopes. The identification of haploid and diploid nuclei permitted the determination of the timing of diploidisation at early stages throughout microspore embryogenesis. Reconstruction of confocal 3D-images of entire proembryos and the observation of cross and longitudinal sections across the stacks of optical sections together with correlative light and electron microscopy provided unambiguous snapshots of nuclear fusion as the mechanism of genome doubling in the process of microspore-derived embryogenesis in barley (González-Melendi et al. 2005).

In maize, a multi-disciplinary study was carried out involving the determination of the chromosome number from squashed cells, measurement of DNA content by flow cytometry and ultra-structural analysis of the microspore derived proembryos (Testillano et al. 2004). Results showed the occurrence of a nuclear fusion process at early stages, such as the 5/7 day stage (Testillano et al. 2004).

Acknowledgments This work was supported by projects of the Spanish Ministry of Education and Science, MEC BFU2005-01094, AGL2005-05104, BFU2008-00203 and AGL200804255.

References

Bárány I, Testillano PS, Mityko J, Risueño MC (2001) The switch of the microspore developmental program in *Capsicum* involves HSP70 expression and leads to the production of haploid plants. Inter J Dev Biol 45(Suppl.1): 39–40

Bárány I, González-Melendi P, Mityko J, Fadón B, Risueño MC, Testillano PS (2005) Microspore-derived embryogenesis in *Capsicum annuum*: subcellular rearrangements through development. Biol Cell 97: 709–722

Coronado MJ, González-Melendi P, Seguí JM, Ramírez C, Bárány I, Testillano PS, Risueño MC (2002) MAPKs entry into the nucleus at specific interchromatin domains in plant differentiation and proliferation processes. J Struct Biol 140: 200–213

González-Melendi P, Ramírez C, Testillano PS, Kumlehn J, Risueño MC (2005) 3D confocal and electron microscopy imaging define the dynamics and mechanisms of diploidization at early stages of barley microspore-derived embryogenesis. Planta 222: 47–57

Magnard JL, Le Deunff E, Domenech J, Rogowsky PM, Testillano PS, Rougier M, Risueño MC, Vergne P, Dumas C (2000) Genes normally expressed in the endosperm are expressed at early stages of microspore embryogenesis in maize. Plant Mol Biol 44: 559–574

Massonneau A, Coronado MJ, Audran A, Bagniewska-Zadworna A, Mol R, Testillano PS, Goralski G, Dumas C, Risueño MC, Matthys-Rochon E (2005) Multicellular structures that develop during in vitro maize pollen embryogenesis express both endosperm- and embryo-specific genes: which is which? Eur J Cell Biol 84: 663–675

Ramírez C, Testillano, PS, Castillo AM, Vallés MP, Coronado MJ, Cistué L, Risueño MC (2001) The early microspore embryogenesis pathway in barley is accompanied by concrete ultrastructural and expression changes. Inter J Dev Biol 45(Suppl.1): 57–58

Ramírez C, Testillano PS, Pintos B, Moreno MA, Bueno MA, Risueño MC (2004) Changes in pectins and MAPKs related to cell development during early microspore embryogenesis in *Quercus suber* L. Eur J Cell Biol 83: 213–225

Satpute GK, Long, H, Seguí-Simarro JM, Risueño MC, Testillano PS (2005) Cell architecture during gametophytic and embryogenic microspore development in *Brassica napus* L. Acta Physiol Plant 27: 665–674

Seguí-Simarro JM, Testillano PS, Jouannic S, Henry Y, Risueño, MC (2005) MAP kinases are developmentally regulated during stress-induced microspore embryogenesis in *Brassica napus*. Histochem Cell Biol 123: 541–551

Seguí-Simarro JM, Testillano PS, Risueño MC (2003) Hsp70 and Hsp90 change their expression and in situ localization after microspore embryogenesis induction in *Brassica napus* cv. Topas. J Struct Biol 142: 379–391

Seguí-Simarro JM, Bárány I, Suárez R, Fadón B, Testillano PS, Risueño MC (2006) Nuclear bodies domain changes with microspore reprogramming to embryogenesis. Eur J Histochem 50: 35–44

Silva MS, Fortes AM, Testillano PS, Risueño MC, Pais S (2004) Differential expression and cellular localization of ERKs during organogenic nodule formation from internodes of *Humulus lupulus* var. Nugget. Eur J Cell Biol 83: 425–433

Testillano PS, Ramírez C, Domenech J, Matthys-Rochon E, Risueño MC (2002)Young microspore-derived maize embryos show two domains with defined features also present in zigotic embryogenesis. Int J Dev Biol 46: 1035–1047

Testillano PS, Georgiev S, Mogensen L, Coronado MJ, Dumas C, Risueño MC, Matthys-Rochon E (2004) Spontaneous chromosome doubling results from nuclear fusion during in vitro maize induced microspore embryogenesis. Chromosoma 112: 342–349

Testillano PS, González-Melendi P, Coronado MJ, Seguí JM, Moreno MA, Risueño MC (2005) Differentiating plant cells switched to proliferation remodel the functional organization of nuclear domains. Cytogenet Genome Res 109, 166–174

Willats WG, McCartney L, Mackie W, Knox JP (2001) Pectin: cell biology and prospects for functional analysis. Plant Mol Biol 47: 9–27.