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par

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Les plantes haploïdes chez l'orge, avec extension au blé: méthodes d'obtention et relations avec l'organisation de leur génome

Haploid plants in barley with extension to wheat: production methods and relations with their genome organization

Soutenue le 30 Mars 1998 devant le jury:

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Je dédicace cette thèse à Brigitte, Audrey et Charles-Hubert en les remerciant pour leur soutien et leur patience.

I dedicate this thesis to Brigitte, Audrey and Charles-Hubert and I thank them for their support and their patience.

We have not inherited the Earth from our parents, but have only borrowed it from our children.

- Uraltes Indian prophecy -

Avant-Propos

Ces recherches sont le fruit d'une collaboration originale et enrichissante entretenue pendant plusieurs années entre des laboratoires français et étrangers. Elles m'ont permis de suivre l'évolution rapide des méthodes et techniques dans ce monde mouvant de la semence, de développer des recherches dans une science aussi captivante que la génétique mais aussi de créer des liens immuables avec de nombreux collègues et amis.

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Je remercie beaucoup Monsieur Philippe Vernet, Professeur à l'Université des Sciences et Technologies de Lille et Directeur du Laboratoire de Génétique et Evolution des Populations Végétales, pour l'intérêt qu'il a toujours manifesté à l'égard de mes recherches et pour les bons contacts que nous avons toujours eus ensemble.

J'adresse mes plus chaleureux remerciements aux Professeurs Andris Kleinhofs et Steven Ullrich de m'avoir permis de réaliser une part significative de cette étude dans leurs laboratoires à l'Université de l'Etat de Washington, Pullman, Etats Unis d'Amérique. Je remercie aussi mon collègue et ami, le Dr. Andrzej Kilian pour ses conseils et son aide précieuse ainsi que tous les chercheurs et techniciens de ces laboratoires en particulier le Dr. Liming Hou, Zhongxiang Huang, David Kudrna et Deborah Tinnemore.

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Enfin, je remercie profondément mes parents et ma famille pour tout ce qu'ils ont fait pour moi.

Preface

This research results from an original and beneficial collaboration maintained for several years between French and foreign laboratories. This enabled me to follow the rapid evolution of methods and techniques in the moving world of the seed, not only to search in such a captiving science as genetics but also to establish tight links with numerous colleagues and friends.

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I thank Professors Andris Kleinhofs and Steven Ullrich for allowing me to perform a significant part of this study in their laboratories at the Washington State University, Pullman, U.S.A. I thank my colleague and friend Dr. Andrzej Kilian for his advice and for this useful help and also all the researchers and technicians from these laboratories particularly Dr. Liming Hou, Zhongxiang Huang, David Kudrna and Deborah Tinnemore.

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The first steps in molecular markers were carried out with Dr. Hervé Thiellement and continued with Dr. Michel Zivy, Plant Genetics Station, La Ferme du Moulon, Gif sur Yvette. Thank you very much.

A close collaboration has been made with Dr. Tadeusz Adamski and his colleagues, Drs Maria Surma and Zygmunt Kaczmarek, Institute of Plant Genetics, Poznan, Poland. I thank them.

I am grateful to Mr Jacques Lefebvre, former barley breeder, Philippe Lonnet, the colleagues, the heads of departments and the staff of Florimond Desprez particularly those from the Biotechnology Laboratory including Professor Hongchao Li.

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Finally, I would like to thank my parents and my family for what they have done for me.

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PRÉSENTATION GÉNÉRALE DE LA THÈSE

Le mémoire de thèse est basé sur nos publications desquelles nous extrayons les faits essentiels, formant un 1er texte placé avant les publications, et la conclusion formant un 2ème texte, après les publications. Nous les avons écrits en français et en anglais. En conséquence, nous voudrions d'abord informer le lecteur sur la manière dont nous avons organisé le mémoire. L'introduction va, dans la version française, de la page 3 à la page 9, et dans la version anglaise de la page 10 à la page 15. Le corps de la thèse comporte ensuite deux parties: la partie I, en version française (pages 16 à 37) et anglaise (pages 38 à 58), en donne les arguments ordonnés extraits de nos publications; la partie II (pages 59 à 135) est constituée par les publications. La conclusion générale, en français (pages 136 à 138), et en anglais (pages 139 à 141) retrace les principales découvertes de cette recherche. Chaque chapitre de la partie I est divisé en deux sous-chapitres: le premier donne les références des publications-support, et le second est une synthèse ou un complément de celles-ci qui reprend essentiellement la conférence donnée à Saskatoon (Saskatchewan, Canada) en août 1996 (Devaux *et al* 1996). Enfin l'introduction et chaque chapitre de la partie I se terminent par les références bibliographiques dont certaines sont déjà données dans les articles-support, mais la majorité sont nouvelles, parues après l'édition des articles-support. L'ensemble de ces références sont réunies dans une liste finale à la fin du mémoire, pages 142 à 157.

GENERAL PRESENTATION OF THE THESIS

This thesis is based on our publications from which we have reported the main achievements. This yields part I and the conclusion which are located before and after the publications respectively. They are both in french and in english. Thus we would like first to advise the reader on how this thesis has been arranged. The french version of the introduction is included between pages 3 and 9, and the english version between pages 10 and 15. The core of the thesis has two parts: part I, in french (pages 16 to 37) and in english (pages 38 to 58), gives the arguments of the thesis which have been classified and a synopsis of our published papers. Part II (pages 59 to 135) provides the published papers. The general conclusion, in french (pages 136 to 138) and in english (pages 139 to 141) highlights the main accomplishments of this research. Each chapter of Part I is divided into two parts: the first one lists the related papers and the second one is a summary or a complement of the papers, and this text is mainly the conference given at Saskatoon (Saskatchewan, Canada) in august 1996 (Devaux *et al* 1996). Part of the references reported at the end of the introduction and of each chapter has been already given in our publications but most of them have been published after that. All references have been reported at the end of the thesis from pages 142 to 157.

INTRODUCTION

A. OBJET ET PLAN DE LA THÈSE

La superficie mondiale d'orge (*Hordeum vulgare* L.) récoltée est approximativement de 80 millions d'hectares avec une production moyenne annuelle, entre 1989 et 1993, de 169 millions de tonnes (Brophy 1996). Trois céréales, le blé, le maïs et le riz, présentent des superficies et des productions grain supérieures à l'orge. Sa production atteint globalement les deux cinquièmes de celle du blé.

L'orge n'est pas uniquement la quatrième céréale par ordre d'importance, mais elle est aussi un système expérimental pour les sélectionneurs, les biochimistes, les physiologistes, les généticiens et les biologistes moléculaires. En effet, l'orge possède une extraordinaire souplesse d'adaptation au milieu, et elle est une espèce diploïde facile à cultiver dans des conditions de laboratoire. Ses grains servent principalement à la nourriture animale et humaine et à fabriquer le malt utilisé en brasserie.

La création de variétés d'orge performantes a été la résultante d'un effort constant de sélection pour un nombre élevé de caractères différents tels que le rendement grain, la résistance à des stress abiotiques et biotiques, la qualité brassicole (Anderson and Reinbergs 1985, Sharp 1985). Etant autogames, les orges locales et les variétés contemporaines sont principalement homozygotes et plusieurs méthodes de sélection, adaptées aux espèces homozygotes comme les sélections généalogique, bulk, par rétrocroisements et filiation unipare, sont utilisées depuis de nombreuses années. La stérilité mâle génique a permis de pratiquer la sélection récurrente (Falk *et al* 1981). Bien que la stérilité mâle cytoplasmique et la restauration de la fertilité aient été découvertes à partir de croisements avec *H. jubatum* (Schooler 1967) et *H. spontaneum* (Ahokas 1978) et que plusieurs agents chimiques d'hybridation soient connus, l'orge hybride a connu peu de succès et d'après nos connaissances aucune variété hybride n'a été obtenue jusqu'à présent.

Le dernier développement important apporté aux méthodes de sélection chez l'orge a porté sur les moyens d'obtenir un grand nombre d'haploïdes doublés, chacun donnant en une seule génération une lignée pure distincte. Deux techniques ont été décrites au début des années 1970, à savoir celle de l'hybridation interspécifique entre *Hordeum vulgare* et *H. bulbosum* (Kasha et Kao 1970) et celle de la culture d'anthères (Clapham 1973). Scientifiques et sélectionneurs les ont rapidement adoptées, parce que les haploïdes doublés apportent certains avantages par rapport aux populations F_2 des méthodes de sélection classiques (Kasha et

Reinbergs 1975). L'objet principal de ma thèse de 3ème cycle, soutenue en 1983, (Devaux 1983) a été de sélectionner des clones d'*H. bulbosum* performants en nombre d'haploïdes extraits d'un éventail de lignées d'orge d'hiver. Cependant il s'est avéré que, si on voulait assurer un cahier des charges d'un programme de sélection en nombre d'haploïdes doublés à produire, l'exploitation d'un seul système, soit celui d'*H. bulbosum,* soit celui de la culture d'anthères, n'était pas satisfaisant, car des génotypes récalcitrants à l'un ou l'autre système ont été identifiés (Fouroughi-Wehr et al 1976, Pickering et Hayes 1976). En plus les deux techniques diffèrent par le rendement en production d'haploïdes et par la nature des gamétophytes devenus plantes haploïdes. Mais seulement trois études ont été menées sur quelques cultivars et hybrides pour comparer de ce point de vue les deux techniques (Fouroughi-Wehr *et al* 1981, Huang *et al* 1984, Friedt *et al* 1987).

Les travaux décrits dans cette thèse ont été initiés il y a plus de 10 ans, en vue d'adapter les méthodes d'*H. bulbosum* et de la culture d'anthères aux programmes de sélection dans leur efficacité à produire des plantes haploïdes. Cette partie méthodologie de notre travail nous a amené à analyser les relations entre les deux méthodes d'obtention des plantes haploïdes et l'organisation de leur génome dans les trois domaines suivants: la mutation et la méthylation de l'ADN, la cartographie génétique comparée et la ségrégation des allèles à un locus dans différentes populations d'haploïdes doublés. Il en découle quatre chapitres qui composent la partie I.

Le chapitre 1 est un aperçu (ou monographie) sur les haploïdes doublés chez l'orge. Nous présentons d'abord les méthodes principales d'obtention d'haploïdes doublés; nous les discutons et nous donnons certaines de leurs applications en sélection. Nous décrivons ensuite les recherches qui ont permis de déterminer la cause génétique de la faible nouaison dans les croisements de l'orge cultivée par *H. bulbosum,* et d'améliorer la méthode de la culture d'anthères sur les génotypes récalcitrants à cette méthode. Enfin, nous étendons, dans ce chapitre, l'analyse de la production d'haploïdes doublés d'orge à celle du blé tendre, et nous étudions spécialement l'aptitude du croisement blé x maïs à donner des plantes haploïdes à partir de plusieurs lignées F_1 de blé.

Le chapitre 2 expose les recherches sur les niveaux de variation de l'ADN des plantes haploïdes doublées, phénotypiquement normales, obtenues à partir du cultivar Igri par la méthode *H. bulbosum* et par la culture d'anthères.

Le chapitre 3 compare les fréquences de recombinaison sur les populations de plantes haploïdes doublées dans lesquelles sont intervenues les recombinaisons femelle (*H. bulbosum)* ou mâle (culture d'anthères) et qui ont été obtenues de l'hybride F_1(Steptoe x Morex), et nous interprétons les différences observées.

Le chapitre 4 expose des ségrégations déviées de plusieurs loci dans les populations de plantes haploïdes doublées obtenues à partir de gamétophytes mâle et femelle chez l'orge et, pour ce sujet, également chez le blé, et nous montrons que ce phénomène génétique nous permet d'identifier des gènes intervenant dans la production de plantes haploïdes.

Ces quatre chapitres sont finalement regroupés par thème en sous-parties A, B, et C de la partie I.

REFERENCES

Ahokas H (1978) Cytoplasmic male sterility in barley. I. Anther and pollen characteristics of *msm1*, restored and partially restored *msm1* genotypes. Z Pflanzenzüchtg 81:327-332

Anderson MK, Reinbergs E (1985) Barley breeding. In: Rasmusson DC (ed) Barley. Amer Soc Agron, Madison WI, pp231-268

Brophy M (1996) Global production and markets for barley in the 21st century. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp. University Extension Press, Saskatoon, Sask, pp37-43

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

Devaux P (1983) Haploïdes doublés, issus d'orges d'hiver, par la méthode *bulbosum*. Analyse cytogénétique et recherche de meilleures souches *d'Hordeum bulbosum*. Thèse de 3ème cycle, Université des Sciences et Techniques de Lille, 125p

Falk DE, Kasha KJ, Reinbergs E (1981) Presowing selection of genetic male sterile plants to facilitate hybridization in barley. In: Asher MJC, Ellis RP, Hayter AM, Whitehouse RNH (eds) Barley Genetics IV, Proc 4th Int Barley Genet Symp. Edinburgh University Press, Edinburgh, pp778-785

Foroughi-Wehr B, Mix G, Gaul H, Wilson HM (1976) Plant production from cultured anthers of *Hordeum vulgare* L. Z Pflanzenzüchtg 77:198-204

Foroughi-Wehr B, Pickering RA, Friedt W (1981) Related responses of barley cultivars to the '*bulbosum*' and anther-culture technique of haploid production. Barley Genet Newsl 11:54-59

Friedt W, Foroughi-Wehr B, Snape JW (1987) The significance of biotechnology for the evaluation of barley breeding methods. In: Yasuda S, Konishi T (eds) Barley Genetics V, Proc 5th Int Barley Genet Symp. Mazuren, Okayama, pp903-913

Huang B, Dunwell JM, Powell W, Hayter AM, Wood W (1984) The relative efficiency of microspore culture and chromosome elimination as methods of haploid production in *Hordeum vulgare* L. Z Pflanzenzüchtg 92:22-29

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

Kasha KJ, Reinbergs E (1975) Utilization of haploid in barley. In: Gaul H (ed) Barley Genetics III, Proc 3rd Int Barley Genet Symp, Garching, pp307-315

Pickering RA, Hayes JD (1976) Partial incompatibility in crosses between *Hordeum vulgare* L. and *H. bulbosum* L. Euphytica 25:671-678

Scooler AB (1967) A form of male sterility in barley hybrids. J Hered 58:206-211

Sharp EL (1985) Breeding for pest resistance. In: Rasmusson DC (ed) Barley. Amer Soc Agron, Madison WI, pp313-333

B. LABORATOIRES IMPLIQUÉS DANS LA PRÉPARATION DE LA THÈSE

Ces recherches ont été effectuées dans les laboratoires suivants:

- Florimond Desprez, Laboratoire de Biotechnologies, 59242 Cappelle en Pévèle, France
- Washington State University, Departments of Crop and Soil Sciences, and Genetics and Cell Biology, Pullman, WA 99164-6420, U.S.A.
- CNRS/URA 1492-INRA-UPS, Station de Génétique Végétale, La Ferme du Moulon, 91190 Gif-sur-Yvette, France
- Université des Sciences et Technologies de Lille Flandres Artois, Laboratoire de Génétique et Evolution des Populations Végétales, 59655 Villeneuve d'Ascq, France
- New Zealand Institute for Crop & Food Research Ltd, Private Bag 4704, Christchurch, New Zealand
- Polish Academy of Sciences, Institute of Plant Genetics, ul. Strzeszynska, 60-479 Poznan, Poland

INTRODUCTION

A. SCOPE OF THE THESIS

The world average annual harvested area of barley (*Hordeum vulgare* L.) is about 80 million hectares with a 1989-93 average annual production of 169 million tons (Brophy 1996). Only three cereals, wheat, maize, and rice, exceeded barley in area harvested and in grain production. Roughly, its production is two-fifths that of wheat.

Barley is not only the fourth most important cereal crop, but also an experimental system for breeders, biochemists, physiologists, geneticists and molecular biologists. The reasons for this are its extraordinary ecological adaptation, its inbred diploid nature and the ease of growth under laboratory conditions. It is mainly used as a feed and food grain and as malt for brewing.

The development of superior barley cultivars has been a continuing dynamic breeding process for many different characters including grain yield, resistance to environmental stresses, pest resistance, malting quality (Anderson and Reinbergs 1985, Sharp 1985). Being primary self-pollinated, barley landraces and modern cultivars have been mostly homozygous and several methods of breeding including pedigree, bulk, backcross, single-seed descent have been successful for many years. The availability of genetic male sterility has enabled recurrent selection to be performed (Falk *et al* 1981). Although cytoplasmic male sterility and fertility restoration were found in *H. jubatum* (Schooler 1967) and in *H. spontaneum* (Ahokas 1978) crosses and several chemical hybridization agents were reported, hybrid barley has received little attention and, to our knowledge, no hybrids have been released so far.

The newest and most important development in breeding methods of barley has been the possibility to recover high frequency of doubled haploid i.e. homozygous inbred lines in a single generation. For this, two techniques were reported in the early 1970s namely the interspecific hybridization of barley with *H. bulbosum* (Kasha and Kao 1970) and anther culture (Clapham 1973). Scientists and breeders rapidly adopted the method because the doubled haploids produced provide several advantages over standard F_2 populations (Kasha and Reinbergs 1975). The major aim of the candidate's 3rd cycle thesis defended in 1983 (Devaux 1983) was to select clones of *H. bulbosum* for higher haploid production efficiency over a range of breeders' winter barley lines. However to ensure production of doubled haploid plants from every possible hybrid in a breeding program, it has been demonstrated that a sole system i.e. either the *H. bulbosum* or the anther culture technique could not be

sufficient since recalcitrant genotypes to the two techniques have been identified (Foroughi-Wehr *et al* 1976; Pickering and Hayes 1976). In addition, the two techniques differ in several aspects that may influence their efficiencies and the nature of the recovered gametes. Only three studies (Foroughi-Wehr *et al* 1981, Huang *et al* 1984, Friedt *et al* 1987) compared the two techniques for haploid plant production efficiency using a few cultivars and hybrids.

This thesis describes work initiated more than 10 years ago which was aimed at the comparison of the *H. bulbosum* and anther culture methods to make the doubled haploid method more efficient for the breeding programs. To assess possible deviations of *H. bulbosum*- and anther culture-derived doubled haploids as a consequence of biological differences between them, investigations have been carried out in three areas. We have studied DNA mutation and methylation, comparative mapping and segregation distortions in different doubled haploid populations. Therefore the four following chapters make up Part I.

Chapter 1 is an overview of doubled haploids in barley. The main systems available for producing doubled haploids are presented and discussed. Several applications in breeding are given. Studies were conducted to determine the genetic basis of low seed set in crosses of barley and *H. bulbosum* and to improve anther culture response of recalcitrant barley genotypes. Doubled haploid production has been extended to wheat. The wheat x maize cross has been investigated over several breeding F_1 lines to assess the efficiency of the technique for doubled haploid production in wheat.

Chapter 2 is an investigation carried out to assess the level of DNA variation in phenotypically normal doubled haploid plants derived by the *H. bulbosum* and anther culture techniques from the barley cultivar Igri.

Chapter 3 compares the frequencies of recombination in female (*H. bulbosum*) and male (anther culture) recombination-derived doubled haploid populations obtained from an F_1 (Steptoe x Morex) hybrid and speculates about the differences observed.

Chapter 4 investigates the single locus distortions in female and male gametophyte-derived populations in barley and, for this matter, also in wheat and shows how this phenomenon may help to identify genes important for haploid plant production.

Finally these four chapters are arranged in sub-parties A, B and C of part I.

REFERENCES

Ahokas H (1978) Cytoplasmic male sterility in barley. I. Anther and pollen characteristics of *msm1*, restored and partially restored *msm1* genotypes. Z Pflanzenzüchtg 81:327-332

Anderson MK, Reinbergs E (1985) Barley breeding. In: Rasmusson DC (ed) Barley. Amer Soc Agron, Madison WI, pp231-268

Brophy M (1996) Global production and markets for barley in the 21st century. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp. University Extension Press, Saskatoon, Sask, pp37-43

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

Devaux P (1983) Haploïdes doublés, issus d'orges d'hiver, par la méthode *bulbosum*. Analyse cytogénétique et recherche de meilleures souches *d'Hordeum bulbosum*. Thèse de 3ème cycle, Université des Sciences et Techniques de Lille, 125p

Falk DE, Kasha KJ, Reinbergs E (1981) Presowing selection of genetic male sterile plants to facilitate hybridization in barley. In: Asher MJC, Ellis RP, Hayter AM, Whitehouse RNH (eds) Barley Genetics IV, Proc 4th Int Barley Genet Symp. Edinburgh University Press, Edinburgh, pp778-785

Foroughi-Wehr B, Mix G, Gaul H, Wilson HM (1976) Plant production from cultured anthers of *Hordeum vulgare* L. Z Pflanzenzüchtg 77:198-204

Foroughi-Wehr B, Pickering RA, Friedt W (1981) Related responses of barley cultivars to the '*bulbosum*' and anther-culture technique of haploid production. Barley Genet Newsl 11:54-59

Friedt W, Foroughi-Wehr B, Snape JW (1987) The significance of biotechnology for the evaluation of barley breeding methods. In: Yasuda S, Konishi T (eds) Barley Genetics V, Proc 5th Int Barley Genet Symp. Mazuren, Okayama, pp903-913

Huang B, Dunwell JM, Powell W, Hayter AM, Wood W (1984) The relative efficiency of microspore culture and chromosome elimination as methods of haploid production in *Hordeum vulgare* L. Z Pflanzenzüchtg 92:22-29

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

Kasha KJ, Reinbergs E (1975) Utilization of haploid in barley. In: Gaul H (ed) Barley Genetics III, Proc 3rd Int Barley Genet Symp, Garching, pp307-315

Pickering RA, Hayes JD (1976) Partial incompatibility in crosses between *Hordeum vulgare* L. and *H. bulbosum* L. Euphytica 25:671-678

Scooler AB (1967) A form of male sterility in barley hybrids. J Hered 58:206-211

Sharp EL (1985) Breeding for pest resistance. In: Rasmusson DC (ed) Barley. Amer Soc Agron, Madison WI, pp313-333

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- Polish Academy of Sciences, Institute of Plant Genetics, ul. Strzeszynska, 60-479 Poznan, Poland

PARTIE I

ARGUMENTS DE LA THÈSE

A. LA PRODUCTION D'HAPLOÏDES DOUBLÉS

CHAPITRE 1

AMÉLIORATION DES MÉTHODES D'OBTENTION D'HAPLOIDES DOUBLÉS CHEZ L'ORGE ET LE BLÉ

Ce chapitre se réfère aux publications citées ci-dessous; la première est une monographie qui inclut d'autres publications personnelles. L'ensemble est complété par des données plus récentes présentées au " V International Oat Conference & VII International Barley Genetic Symposium" qui s'est tenu à Saskatoon, Saskatchewan, du 30 juillet au 6 août 1996.

1. **Pickering[a] RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: P.R. Shewry (ed.) Barley: Genetics, Molecular Biology and Biotechnology. CAB Int. Publ., Wallingford, UK, pp511-539**

   [Pages 60 à 88]

2. **Devaux P, Adamski[b] T, Surma[b] M (1992) Inheritance of seed set in crosses of spring barley and *Hordeum bulbosum* L. Crop Sci 32:269-271**

   [Pages 89 à 91]

3. **Devaux P, Hou[c] L, Ullrich[c] SE, Huang[c] ZX, Kleinhofs[c] A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32-36**

[Pages 92 à 96]

4. Lefebvre[d] 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

[Pages 97 à 103]

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Pour assurer la production de plantes haploïdes doublées (HD) de chaque hybride d'un programme de sélection, le croisement interspécifique avec *Hordeum bulbosum* L., couramment appelé la méthode *H. bulbosum (Hb)*, et la culture d'anthères (CA) sont utilisées en parallèle car des génotypes récalcitrants aux deux techniques ont été trouvés (Foroughi-Wehr *et al* 1976; Pickering et Hayes 1976). L'interaction significative "génotype x technique" que nous avons montrée dans une étude préliminaire (Devaux 1987) a été confirmée à une échelle plus large. L'efficacité de production de plantes haploïdes, c'est à dire le nombre de plantes vertes viables obtenues par épi, a été déterminée, pour les deux techniques, sur 958 hybrides F_1 (785 de type hiver et 173 de type printemps). Sur les 785 de type hiver, 432 (52%) ont mieux répondu à la méthode CA qu'à la méthode *Hb,* tandis que, sur les 178 de type printemps, 132 (76%) ont été plus efficaces avec la méthode *Hb.* Ces résultats sont en accord avec ceux rapportés par Huang *et al* (1984) et Friedt *et al* (1987) qui utilisèrent respectivement un ensemble de 3 génotypes et de 6 hybrides. En plus de leurs réussites différentes en fonction des génotypes, les deux techniques exploitent des conditions environnementales pour la croissance de la plante donneuse distinctes. Dans la méthode *Hb,* la plante donneuse et la plante receveuse du pollen croissent à des températures au-dessus de 18° C pour garantir une bonne qualité du pollen *Hordeum bulbosum* et l'élimination des chromosomes *Hordeum bulbosum* des cellules des jeunes embryons. Dans la méthode CA, la température optimale est beaucoup plus basse, environ 12° C (Foroughi-Wehr et Mix 1979; Lyne *et al* 1986). C'est pourquoi la production d'HD avec la méthode *Hb* peut être réalisée en saison chaude, et celle par CA en saison fraîche avec un léger chevauchement. Il en découle une économie d'énergie et le travail continu du personnel.

Au moins 61 cultivars à l'échelle mondiale sont des HD (tableau 1). Presque tous ont été produits par la méthode *Hb,* mais cinq obtentions récentes (Anthere, Henni, Lyric, Tantangara et Tender) l'ont été par la CA. A notre connaissance, toutes les lignées HD ont été produites à partir d'hybrides F_1 et il n'y a aucun avantage à utiliser d'autres générations (Iyamabo et Hayes 1995). Chose remarquable, pour certains cultivars HD, le site de production est totalement différent du site de sélection et de culture, ce qui constitue un avantage potentiel reconnu antérieurement par Kasha et Reinbergs (1975). Par exemple, Gwylan et Jing Zhuo ont été produits en Europe, mais sélectionnés et cultivés respectivement en Nouvelle Zélande et en République Populaire de Chine.

Des progrès importants ont été obtenus dans la culture de microspores isolées et la régénération. Ainsi, Cistué *et al* (1995) rapportent une fréquence dépassant 17 plantes vertes par anthère chez le cultivar Igri. En exploitant également Igri, Hoekstra *et al* (1992, 1996) et Davies et Morton (1995) montrèrent que la culture de microspores isolées peut produire des plantes HD à une fréquence plus élevée que la culture d'anthères. Si ce résultat peut être atteint régulièrement avec un grand nombre de génotypes, il n'y a plus de doute que cette technique jouera un rôle prédominant dans la production d'HD de l'orge (Kasha 1996; Jensen, comm pers) et du blé (Touraev *et al* 1996).

En dépit de nombreux travaux menés pour augmenter l'efficacité de la CA (revue par Henry et Buyser 1990), l'utilisation de la technique pour des programmes d'amélioration du blé est restée marginale. L'écueil majeur à une exploitation plus large de la CA réside dans sa dépendance génotypique (Lazar *et al* 1984; Marsolais *et al* 1984; Fouroughi-Wehr et Zeller 1990). De ce point de vue, le croisement intergénérique entre blé et maïs a montré un avantage extraordinaire sur la CA, car des plantes haploïdes ont été régénérées de tous les 18 hybrides F_1 prospectés (Lefebvre et Devaux 1996). Nous n'avons rencontré aucun génotype récalcitrant à la production de plantes vertes haploïdes puisque l'hybride le plus difficile a donné 4,4 plantes haploïdes pour 100 fleurs traitées.

Tableau 1. Cultivars d'orge haploïdes doublés produits par la méthode *Hb* et par CA (*)

Société/Institut	Pays	Nom des cultivars	Références
Abed PBS	Danemark	Etna, Give, Loma, Loke, Riga, Rima, Verona, Paloma, Bereta, Aberdeen, Pondus, Perma, Tender*	Rasmussen comm pers
Agriculture Canada	Canada	DB202	Choo *et al* 1995
Canterbury Malting	N. Zélande	Valetta	Pickering comm pers
Crop & Food Res	N. Zélande	Gwylan	Coles 1986
Florimond Desprez	France	Michka, Lombard, Moka, Anka, Vodka, Gaelic, Gotic, Logic, ZF3642, Jing Zhuo, Douchka, Tattoo, Jerka, Lyric*, Distic, Celinka	Pickering et Devaux 1992 Lefebvre J comm pers
ICI seeds	GB	Waveney	NIAB 1988
IPG/PBS	Pologne	KA7/3	Adamski *et al* 1995
IPGG	ancient URSS	Istok, Odesskil115, Preria	Choo comm pers
NSW Agriculture	Australie	Tantangara*	Read 1995
Saaten-Union	Germany	Anthere*, Henni*	Jäger-Gussen comm pers
Semico	Canada	HD87-18.14, HD87-12.1	Choo *et al* 1995
WG Thompson	Canada	Mingo, Rodeo, Craig, Winthrop, Lester,Ontario, TB891-6, Prospect, Bronco, Sandrina, Beluga, McGregor, T090-017, T086-156, T081-009, T103-003, H30-11	Ho et Jones 1980 Campbell *et al* 1984 Shugar et Etienne 1994 Choo *et al* 1995 Shugar comm pers
WPBS	GB	Doublet, Pipkin	Jones *et al* 1985; 1986

REFERENCES

Adamski T, Kaczmarek Z, Surma M, Madejewski R, Madajewska M (1995) Efektywnosc hodowli jeczmienia metoda rodowodowa i metoda wykorzystujaca lilie podwojonych haploidow. Hod Rosl 2:16-19

Campbell KW, Brawn RI, Ho KM (1984) Rodeo barley. Can J Plant Sci 64:203-205

Choo TM, Shugar L, Bastien D (1995) *Bulbosum* method in Eastern Canada: an observation. Barley Newsl 39:111-112

Cistué L, Ziauddin A, Simion E, Kasha KJ (1995) Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss Org Cult 42:163-169

Coles GD (1986) Gwylan barley (*Hordeum vulgare* L.). New Zealand J Exp Agri 14:101-103

Davies PA, Morton S (1995) Barley doubled haploids from isolated microspore cultures. In: Proc 7th Aus Barley Tech Symp. Perth WA, pp195-199

Devaux P (1987) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploids in winter barley. I. Production of green plants. Plant Breed 98:215-219

Foroughi-Wehr B, Mix G, Gaul H, Wilson HM (1976) Plant production from cultured anthers of *Hordeum vulgare* L. Z Pflanzenzüchtg 77:198-204

Foroughi-Wehr B, Mix G (1979) *In vitro* response of *Hordeum vulgare* L. anthers cultured from plants grown under different environments. Environ Exp Bot 19:303-309

Foroughi-Wehr B, Zeller FJ (1990) *In vitro* microspore reaction of different German wheat cultivars. Theor Appl Genet 79: 77-80

Friedt W, Foroughi-Wehr B, Snape JW (1987) The significance of biotechnology for the evolution of barley breeding methods. In: Yasuda S, Konishi T (eds) Barley Genetics V, Proc 5th Int Barley Genet Symp. Mazuren, Okayama, Japan, pp903-913

Henry Y, De Buyser J (1990) Wheat anther culture: agronomic performance of doubled haploid lines and the release of a new variety 'Florin'. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry Vol 13. Springer, Berlin, pp285-352

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

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

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

Huang B, Dunwell JM, Powell W, Hayter AM, Wood W (1984) The relative efficiency of microspore culture and chromosome elimination as methods of haploid production in *Hordeum vulgare* L. Z Pflanzenzüchtg 92:22-29

Iyamabo OE, Hayes PM (1995) Effects of selection and opportunities for recombination in doubled-haploid populations of barley (*Hordeum vulgare* L.). Plant Breed 114:131-136

Jones DL, Jones DM, Clifford BC, Clothier RB, Cook R, Griffiths TER, Jones EWC, Jones IT (1985) Cereal breeding, barley. In: Annual Rep WPBS, pp65-66

Jones DM, Clothier RB, Griffiths TER (1986) Cereal breeding, barley. In: Annual Rep WPBS, pp74-75

Kasha KJ (1996) Biotechnology and cereal improvement. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp. University Extension Press, Saskatoon, Sask, pp133-140

Kasha KJ, Reinbergs E (1975) Utilization of haploid in barley. In: Barley Genetics III, Proc 3rd Int Barley Genet Symp, Garching, pp307-315

Lazar MD, Schaeffer GW, Baenziger PS (1984) Cultivar and cultivar x environment effects on the development of callus and polyhaploid plants from anther cultures of wheat. Theor Appl Genet 67:273-277

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

Lyne RL, Bennett RI, Hunter CP (1986) Embryoid and plant production from cultured barley anthers. In: Alderson PG, Withers LA (eds) Plant tissue culture and its agricultural applications. Butterworth Scientific Ltd, Guildford, pp405-411

Marsolais AA, Séguin-Swartz G, Kasha KJ (1984) The influence of anther cold pretreatments and donor plant genotypes on *in vitro* androgenesis in wheat (*Triticum aestivum* L). Plant Cell Tissue Organ Cult 3:69-79

NIAB (1988) Recommended varieties of cereals, farmers leaflet number 8. National Institute of Agricultural Botany, Cambridge, UK

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, Hayes JD (1976) Partial incompatibility in crosses between *Hordeum vulgare* L. and *H. bulbosum* L. Euphytica 25:671-678

Pickering RA, Devaux P (1992) Haploid production: approaches and use plant breeding. In: Shewry PR (ed) Barley: Genetics, Molecular Biology and biotechnology. CAB Int Publ, Wallingford, pp511-539

Read BJ (1995) Overview of doubled haploids in Australia breeding programs and opportunities for doubled haploid germplasm exchange. In: Proc 7th Aus Barley Tech Symp. Perth WA, pp218-219

Shugar L, Etienne M (1994) Doubled haploid barley progress report. Barley Newsl 38:105-106

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

B. CARACTÈRES MOLÉCULAIRES DU GÉNOME SOUS LA DÉPENDANCE DES TECHNIQUES DE PRODUCTION

CHAPITRE 2

MUTATION ET MÉTHYLATION DE L'ADN INDUITES DURANT LE PROCESSUS DE PRODUCTION D'HAPLOÏDES

Ce chapitre se réfère à la publication suivante:

Devaux P, Kilian[a] A, Kleinhofs[a] A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241:674-679

[Pages 104 à 109]

[a] Washington State University, Departments of Crop and Soil Sciences, and Genetics and Cell Biology, Pullman, WA 99164-6420, U.S.A.

Des modifications génétiques sont apparemment induites par la culture de tissus (Larkin *et al* 1989; Karp 1991), et diverses formes d'altérations phénotypiques ont été décrites (Ullrich *et al* 1991; Baillie *et al* 1992). Des anomalies cytologiques et morphologiques ont été plus fréquentes sur les HD obtenus par la CA que par la méthode *Hb* (pour revue, voir Pickering et Devaux 1992). Nous avons analysé le niveau de variation de l'ADN sur 30 + 30 plantes HD phénotypiquement normales obtenues par CA et par la méthode *Hb* à partir du cultivar Igri (Devaux *et al* 1993). Aucun polymorphisme n'a été trouvé parmi les lignées HD pour 273 fragments RFLP et 89 amplifiés par PCR, ceci indiquant l'absence de mutation par délétion ou insertion. Le nombre de paires de bases (401.640), analysées par ces deux catégories de marqueurs moléculaires, indique que le taux de mutation ponctuelle est inférieur à $0{,}25 \times 10^{-5}$. Toutefois lorsque l'ADN a été digéré par les enzymes de restriction *Hpa*II et *Msp*I, sensibles aux nucléotides méthylés, des fragments RFLP distincts furent obtenus. Il est intéressant de noter que 96 % (49 sur 51) des polymorphismes totaux dûs à la méthylation ont été observés sur les HD obtenus par la CA. Ces modifications ont été probablement induites durant la phase de culture *in vitro* de la CA. Récemment, Smulders *et al* (1995) ont rapporté des polymorphismes dûs à des méthylations de l'ADN induites au cours de la culture *in vitro* chez la tomate. La durée plus longue de la culture et l'utilisation de substances de croissance dans la méthode CA par rapport à la méthode *Hb* pourrait expliquer le haut niveau de polymorphisme dû à la méthylation. Selon Müller *et al* (1990), chez le riz, des plantes régénérées à partir de cals maintenus en culture durant 67 jours ont montré une instabilité génétique plus élevée que ceux cultivés durant 28 jours. Néanmoins, nous ne pouvons exclure la possibilité que des tissus différents, à savoir des embryons immatures zygotiques (en *Hb)* d'une part, et les microspores (en CA) d'autre part, pourraient présenter des niveaux initiaux différents de la méthylation de l'ADN, comme ceci a été décrit chez la carotte (Palmgren *et al* 1991) et chez la tomate (Messeguer *et al* 1991).

REFERENCES

Baillie AMR, Rossnagel BG, Kartha KK (1992) Field evaluation of barley (*Hordeum vulgare* L.) genotypes derived from tissue culture. Can J Plant Sci 72:725-733

Devaux P, Kilian A, Kleinhofs A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241:674-679

Karp A (1991) On the current understanding of somaclonal variation. Oxf Surv Plant Mol Cell Biol 7:1-58

Larkin PJ, Banks PM, Bhati R, Brettell RIS, Davies PA, Ryan SA, Scowcroft WR, Spindler LH, Tanner GJ (1989) From somatic variation to variant plants: mechanisms and applications. Genome 31:705-711

Messeguer R, Ganal MW, Steffens JC, Tanksley SD (1991) Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. Plant Mol Biol 16:753-770

Müller E, Brown PTH, Hartke S, Lörz H (1990) DNA variation in tissue-culture-derived rice plants. Theor Appl Genet 80:673-679

Palmgren G, Mattsson O, Okkels FT (1991) Specific levels of DNA methylation in various tissues, cell lines, and cell types of *Daucus carota*. Plant Physiol 95:174-178

Pickering RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: Shewry PR (ed) Barley: genetics, molecular biology and biotechnology. CAB, Wallingford, pp511-539

Smulders MJM, Rus-Kortekaas W, Vosman B (1995) Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. Theor Appl Genet 91:1257-1264

Ullrich SE, Edmiston JM, Kleinhofs A, Kudrna DA, Maatougui MEH (1991) Evaluation of somaclonal variation in barley. Cereal Res Comm 19:245-260

CHAPITRE 3

CARTOGRAPHIE COMPARÉE DE POPULATIONS D'HAPLOÏDES DOUBLÉS ISSUS DES RECOMBINAISONS MÂLES ET FEMELLES

Ce chapitre se réfère à la publication suivante:

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

[Pages 110 à 118]

[a] Washington State University, Departments of Crop and Soil Sciences, and Genetics and Cell Biology, Pullman, WA 99164-6420, U.S.A.

La recombinaison méiotique est un phénomène crucial sur lequel sont basés les études génétiques et les programmes d'amélioration d'organismes. Le processus peut être affecté par des facteurs variés, y compris le sexe. Les lignées HD obtenues par la méthode *Hb* et par CA représentent des produits recombinés femelle et mâle, et sont, de ce fait, une excellente opportunité de cerner le rôle du sexe dans la recombinaison du génome de l'orge. Des distances cartographiques ont été déterminées sur 101 HD obtenus par la méthode *Hb* et 101 obtenus par CA à partir de l'hybride F_1(Steptoe x Morex) en utilisant des marqueurs moléculaires couvrant la plus grande partie du génome de l'orge (Devaux *et al* 1995). Les populations obtenues par la CA montrent globalement un accroissement du taux de recombinaison de 18 % par rapport aux populations obtenues par la méthode *Hb* pour chaque chromosome et pour la majorité des bras de chromosomes. Récemment, Groover *et al* (1995) et Benito *et al* (1996) ont rendu compte d'une fréquence de recombinaison significativement plus élevée du côté mâle que du côté femelle, respectivement chez *Pinus taeda* et *Secale cereale* L. Dans notre étude, huit distances entre loci individuels étaient significativement plus élevées dans la population obtenue par CA que dans la population obtenue par la méthode *Hb,* et une distance était plus élevée dans la population issue de la méthode *Hb* que dans celle issue de la CA. Trois des huit distances étaient localisées en région centromérique, les cinq autres en région télomérique, et parmi elles se trouvaient les deux distances significativement les plus différentes. Les intervalles les plus en position télomérique des 13 bras de chromosomes étaient de 43% plus longs dans la population issue de CA que dans la population issue de la méthode *Hb,* alors que les intervalles non télomériques ne l'étaient que de 12,5 %. Bien qu'on ait signalé dans d'autres systèmes aussi bien un accroissement qu'une diminution de la recombinaison mâle (voir revue dans Devaux *et al* 1995), la fréquence accrue des recombinaisons dans les régions télomériques au niveau mâle semble émerger comme un phénomène général.

Si nous nous référons à la cytogénétique humaine, la variation de la distance entre loci pourrait être la conséquence du processus d'appariement des homologues à la méiose, car l'appariement interstitiel est plus fréquent en méiose femelle qu'en méiose mâle (Bojko 1983). Rouyer *et al* (1990) font l'hypothèse que la distribution aléatoire des sites d'initiation de l'appariement, mieux assurée dans les méioses femelles que dans les mâles, pourrait expliquer une plus faible étendue de la carte génétique femelle pour les régions distales des chromosomes. Armour (comm pers 1996) a trouvé que la fréquence des mutations pour

certains minisatellites localisés en région télomérique était dix fois plus élevée chez les mâles que chez les femelles, et il a aussi rapporté que les fréquences de recombinaison étaient plus élevées côté mâle que côté femelle.

En outre il y a quelque évidence que les régions subtélomériques du génome de l'homme (Saccone *et al* 1992), des céréales (Moore *et al* 1993; Gill *et al* 1993; Gill *et al* 1996) et de la betterave à sucre (Schmidt comm pers 1996) sont plus riches en gènes que les régions proximales. C'est pourquoi la recombinaison mâle plus importante dans les régions télomériques pourrait être sélectivement plus avantageuse, car elle aboutit à un nombre accru d'assortiments de gènes dans les gamètes mâles plus abondants et soumis à une forte pression de sélection au moment de la pollinisation et/ou aux stades de la fécondation.

Il se dégage de cette discussion qu'on devrait s'attendre à une plus grande variabilité parmi les HD dérivés de la CA que de la méthode *Hb,* en absence de sélection *in vitro.*

REFERENCES

Benito C, Romero MP, Henriques-Gil N, Llorente F, Figueiras AM (1996) Sex influence on recombination frequency in *Secale cereale* L. Theor Appl Genet 93:926-931

Bojko M (1983) Human meiosis VIII. Chromosome pairing and formation of the synaptonemal complex in oocytes. Carlsberg Res Commun 48:457-483

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

Gill KS, Gill BS, Endo TR (1993) A chromosome region-specific mapping strategy reveals gene-rich telomeric ends in wheat. Chromosoma 102:374-381

Gill KS, Gill BS, Endo TR, Boyko EV (1996) Identification and high-density mapping of gene-rich regions in chromosome group 5 of wheat. Genetics 143:1001-1012

Groover AT, Williams CG, Devey ME, Lee JM, Neale DB (1995) Sex-related differences in meiotic recombination frequency in *Pinus taeda.* J Hered 86:157-158

Moore G, Abbo S, Cheung W, Foote T, Gale MD, Koebner R, Leitch A, Leitch I, Money T, Stancombe P, Yano M, Flavell R (1993) Key features of cereal genome organization as revealed by the use of cytosine-methylation sensitive restriction endonucleases. Genomics 15:472-482

Rouyer F, de la Chapelle A, Andersson M, Weissenbach J (1990) An interspersed repeated sequence specific for human subtelomeric regions. EMBO J 9:505-514

Saccone S, De Sario A, Della Valle G, Bernardi G (1992) The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. Proc Natl Acad Sci USA 89:4913-4917

C. LES LOIS DE L'HÉRÉDITÉ DE CARACTÈRES MOLÉCULAIRES SOUS LA DÉPENDANCE DES TECHNIQUES DE PRODUCTION

CHAPITRE 4

SÉGRÉGATIONS DÉVIÉES DANS LES POPULATIONS D'HAPLOÏDES DOUBLÉS

Ce chapitre se réfère aux cinq publications citées ci-dessous. Les ségrégations déviées sont mises en évidence dans les populations d'haploïdes et d'haploïdes doublés, aussi bien d'orge que de blé. Les lignées sont dérivées de croisements simples et obtenues par culture d'anthères ou par hybridation interspécifique.

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

   [Pages 119 à 124]

2. **Devaux P, Zivy[(a)] M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701-706**

[Pages 125 à 130]

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

[Pages 110 à 118]

4. **Devaux P, Lonnet[d] P, Jean[b] R (1990) Significant higher proportions of 1BL-1RS wheat-rye translocation lines among doubled haploid progenies derived from F1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp, HJJ, LHW Van der Plas and J. Van Aartrijk (eds.), Progress in plant cellular and molecular biology. Proc. VIIth Int. Cong. Plant Tissue and Cell Cult., Amsterdam, pp 422-426**

[Pages 131 à 135]

5. **Lefebvre[e] 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**

[Pages 97 à 103]

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Pour exploiter au mieux la variabilité génétique à l'intérieur d'un croisement, les HD doivent provenir d'un échantillon aléatoire de gamètes, c'est à dire qu'aucune sélection ne doit avoir lieu durant la période de culture *in vitro,* tout au plus peut-on admettre une très faible sélection. Des études basées sur des caractères morphologiques et sur l'utilisation de différents types de marqueurs n'ont pas permis de mettre en évidence des ségrégations significativement déviées de celles attendues dans les populations d'haploïdes doublés obtenues de sacs embryonnaires par la méthode *Hb* (Kjær *et al* 1990; Powell *et al* 1990; Schön *et al* 1990). Notre premier objectif fut de tester si des ségrégations déviées se produisaient dans un ensemble de lignées dérivées de microspores par la méthode CA. Dans ce but, nous avons mené une analyse en électrophorèse bidimensionnelle sur 28 gènes codant des protéines de structure de 62 individus obtenus par CA de la F_1(Kaskade x DH8293) (Zivy *et al* 1992). Des déviations significatives de la ségrégation 1:1 ont été mises en évidence à sept loci qui se répartissent en quatre groupes de liaison: cinq en deux groupes, et les deux autres loci appartenant chacun à un groupe; pour six loci, la déviation de ségrégation se fait le plus fréquemment en faveur de l'allèle de DH8293, et pour un locus, pour l'allèle de Kaskade. Nous qualifions ces allèles d'allèles +.

Nous avons voulu approfondir ce résultat en procédant au test d'aptitude à la CA sur 50 lignées HD, arrivées en deuxième génération, pour vérifier si les plantes portant les allèles + ont des aptitudes à la production de plantes haploïdes supérieures à celles ayant l'allèle de l'autre parent (Devaux et Zivy 1994). Dans ce test, les lignées sont classées suivant le nombre de plantes haploïdes obtenues pour cent anthères, en référence aux gènes ayant produit des ségrégations déviées. N'ayant pas de recombinés dans les groupes de liaison à plusieurs loci, nous nous référons aux groupes de liaison, qui sont au nombre de quatre comme nous l'avons vu ci-dessus, et nous considérons qu'un groupe est un gène que nous qualifions par l'allèle du parent. Les HD aux quatre allèles + se classent en premier. Pour les HD aux autres combinaisons d'allèles (avec trois, deux ou un allèle +), on constate que seulement deux allèles + sont des marqueurs d'aptitude à la CA, et les deux autres ne le sont pas (l'un provient de Kaskade). Ceci signifie que la déviation de ségrégation, dont ces derniers sont responsables, est l'effet du hasard. Mais deux autres marqueurs de gènes d'aptitude à la CA, qui n'étaient pas affectés par des ségrégations déviées, ont été identifiés. Pour expliquer cette discordance dans nos résultats, nous avançons plusieurs raisons dont une, qui nous paraît la plus judicieuse, est l'origine génétique des tissus de l'anthère: les ségrégations déviées ont été

analysées sur des plantes HD issues de microspores contenues dans les anthères de plante hybride, tandis que l'aptitude à la CA l'a été sur des anthères de plante HD.

Ainsi ces deux études révèlent que quatre loci (dont un est en réalité un groupe de trois gènes) sont liés à des gènes d'aptitude à la CA. En outre, nous montrons que deux loci interviennent dans la production d'embryons d'origine androgénétique et dans la régénération en plantes vertes, et les deux autres interviennent uniquement dans la régénération en plantes vertes. Parmi les quatre gènes vus sous leur aspect allèle d'origine, trois proviennent du parent DH8293, et un du parent Kaskade. D'ailleurs, dans le test d'aptitude à la CA des deux parents, DH8293 était meilleur que l'autre parent Kaskade.

Des recherches ultérieures ont été accomplies au moment où des populations dérivées de la voie mâle et femelle et des cartes à haute densité de liaison étaient disponibles (Devaux *et al* 1993). Kleinhofs *et al* (1993) signalent des loci déviés dans la population HD obtenue par la méthode *Hb* à partir de la F_1(Steptoe x Morex); cependant, l'importance de la déviation est beaucoup plus faible que celle observée dans la population issue du même hybride par CA (Devaux *et al* 1995) de sorte qu'elle peut être attribuée à l'effet du hasard. Deux segments à forte déviation ont été trouvés sur les chromosomes 1H et 5H dans les HD obtenus par la CA à partir de l'hybride F_1(Steptoe x Morex) (Devaux *et al* 1995). Parallèlement, des loci à ségrégation déviée ont été mis en évidence dans des populations obtenues par CA à partir d'autres croisements, sur les chromosomes 1H (Heun *et al* 1991; Kintzios *et al* 1994; Logue *et al* 1995), 5H (Graner *et al* 1991; Steffenson *et al* 1995), mais aussi sur tous les autres chromosomes, à savoir 4H et 6H (Thompson *et al* 1991), 2H et 7H (Graner *et al* 1991; Heun *et al* 1991; Logue *et al* 1995) et 3H (Graner *et al* 1991). Si dans la majorité des cas, la sélection des gamétophytes est la conséquence de la liaison entre les gènes marqués par la ségrégation déviée et les gènes intervenant dans l'aptitude à la CA, cela suggèrerait que le caractère est complexe et est contrôlé par de nombreux gènes répartis dans certains génotypes.

Chez le blé (*Triticum aestivum* L.), nous avons analysé l'hérédité du chromosome 1BL-1RS sur la production d'haploïdes obtenus par la CA et l'hybridation intergénérique avec le maïs (*Zea mays* L.). Les croisements comprenant un parent à bonne réponse à la CA, telle la lignée transloquée 1BL-1RS (Henry et Buyser 1985) permettent la production indirecte de plantes haploïdes de génotypes récalcitrants à la CA. Cependant, la fréquence des plantes régénérées était en faveur du parent transloqué à cause d'une sélection s'opérant au niveau des microspores ou des embryons qui en dérivent (Agache *et al* 1989; Devaux *et al* 1990). Dans le

but d'approfondir ce résultat, l'hérédité du chromosome 1BL-1RS a été analysée dans les descendances haploïdes et F_2 des mêmes croisements. Le chromosome 1BL-1RS a été trouvé à la proportion attendue dans la descendance F_2 et haploïde produite par le croisement blé x maïs, mais à une proportion différente de 1:1 dans la descendance haploïde produite par CA en faveur des plantes à chromosome transloqué (Lefebvre et Devaux 1996). Ceci confirme bien l'existence de gènes sur ce chromosome pour l'aptitude à la CA.

REFERENCES

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

Devaux P, Hou L, Ullrich SE, Huang ZX, Kleinhofs A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32-36

Devaux P, Lonnet P, Jean R (1990) Significant higher proportions of *1BL-1RS* wheat-rye translocation lines among doubled haploid progenies derived from F1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp HJJ, Van der Plas LHW, Van Aartrijk J (eds) Progress in plant cellular and molecular biology. Proc 7th Int Cong Plant Tissue Cell Cult. Kluwer, Amsterdam, pp422-426

Devaux P, Zivy M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701-706

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

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

Henry Y, De Buyser J (1985) Effect of the *1B/1R* translocation on anther culture ability in wheat (*Triticum aestivum* L). Plant Cell Rep 4:307-310

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34:437-447

Kintzios S, Islam RM, Fischbeck G (1994) Distorted segregation for mildew resistance in doubled haploid lines of spring barley. Plant Breed 112:248-251

Kjær B, Jensen HP, Jensen J, Jørgensen JH (1990) Associations between three *ml-o* powdery mildew resistance genes and agronomic traits in barley. Euphytica 46:185-193

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

Logue SJ, Oti-Boateng C, Karakousis A, Kretschmer JM, Manning S, Lance RCM, Langridge PJ (1995) Segregation analysis of DNA markers in anther culture-derived populations of barley (*Hordeum vulgare* L.) In: Proc 7th Aus Barley Tech Symp. Perth WA, pp210-217

Powell W, Ellis RP, Macauley M, McNicol J, Forster BP (1990) The effect of selection for protein and isozyme loci on quantitative traits in a doubled haploid population of barley. Heredity 65:115-122

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

Steffenson BJ, Jin Y, Rossnagel BG, Rasmussen JB, Kao K (1995) Genetics of multiple disease resistance in a doubled-haploid population of barley. Plant Breed 114:50-54

Thompson DM, Chalmers K, Waugh R, Forster BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L. Theor Appl Genet 81:487-492

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

PART I

ARGUMENTS OF THE THESIS

A. DOUBLED HAPLOID PRODUCTION

CHAPTER 1

IMPROVEMENT OF DOUBLED HAPLOID PRODUCTION METHODS IN BARLEY AND WHEAT

This chapter refers to the four following publications; the first one being an overall review which includes several papers of our own already published. It is complemented by more recent data presented at the V International Oat Conference & VII International Barley Genetic Symposium held at Saskatoon, Saskatchewan, July 30th to August 6th, 1996.

1. **Pickering[a] RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: P.R. Shewry (ed.) Barley: Genetics, Molecular Biology and Biotechnology. CAB Int. Publ., Wallingford, UK, pp511-539**

   [Pages 60 to 88]

2. **Devaux P, Adamski[b] T, Surma[b] M (1992) Inheritance of seed set in crosses of spring barley and *Hordeum bulbosum* L. Crop Sci 32:269-271**

   [Pages 89 to 91]

3. **Devaux P, Hou[c] L, Ullrich[c] SE, Huang[c] ZX, Kleinhofs[c] A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32-36**

   [Pages 92 to 96]

4. Lefebvre[d] 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

[Pages 97 to 103]

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To ensure production of doubled haploids (DHs) from every possible hybrid in a breeding program, the interspecific hybridization with *Hordeum bulbosum* L., commonly named the *H. bulbosum* method (*Hb*), and anther culture (AC) have been used in parallel since recalcitrant genotypes to the two techniques have been identified (Foroughi-Wehr *et al* 1976; Pickering and Hayes 1976). A preliminary study which had revealed a significant genotype x technique interaction (Devaux 1987) has now been confirmed on a larger scale. Haploid plant production efficiency i.e. the number of viable green plants per spike processed was assessed by the two techniques for 958 F_1 hybrids (785 winter and 173 spring types). Of the 785 winter types 432 (55%) responded better through AC than through *Hb*, while for spring types 132 (76%) were more efficient with the *Hb* method. These results are in accord with those reported by Huang *et al* (1984) and Friedt *et al* (1987) who used a survey of 3 genotypes and 6 hybrids, respectively. In addition to the genotype x technique interaction, the two techniques employ dissimilar environmental conditions for donor plant growth. In the *Hb* method the pollen donor and recipient plants are grown at temperatures above 18°C to insure good quality of *Hb* pollen and elimination of the *Hb* chromosomes from cells of young embryos (Pickering 1985). The optimal temperature in AC is much lower, ca. 12°C (Foroughi-Wehr and Mix 1979; Lyne *et al* 1986). Therefore, the *Hb* DH production can be carried out in the warm season and AC in the cool season with some overlap. As a consequence energy can be saved and permanent staff employed continuously.

At least 61 DH lines have been released as cultivars world wide (Table 1). Nearly all of them were produced by the *Hb* method but five recent releases (Anthere, Henni, Lyric, Tantangara and Tender) were by AC. To our knowledge all DH lines have been produced from F_1s and there are no advantages to using other generations (Iyamabo and Hayes 1995). Noticeable are several DH cultivars for which the sites where they were generated are very different from the site of selection and cultivation, a potential advantage of using DHs recognized early by Kasha and Reinbergs (1975). For example, Gwylan and Jing Zhuo were produced in Europe but selected and cultivated in New Zealand and China, respectively.

Much progress has been made in isolated microspore culture and regeneration. Thus, Cistué *et al* (1995) reported a frequency of over 17 green plants per anther of the cultivar Igri. Also using Igri, Hoekstra *et al* (1992, 1996) and Davies and Morton (1995) showed that higher frequencies of DHs can be produced by isolated microspore culture than with anther culture. If this could be consistently repeated with a range of genotypes, there is no doubt that this

technique will play a more predominant role for DH production of barley (Kasha 1996; Jensen pers comm) and wheat (Touraev *et al* 1996).

Despite intensive efforts to increase AC response (reviewed by Henry and de Buyser 1990), its use has remained marginal in wheat breeding programs. The major limitation to a broad exploitation of AC has been its genotypic dependency (Lazar et al 1984; Marsolais et al 1984; Foroughi-Wehr and Zeller 1990). In this respect, the intergeneric cross between wheat and maize has showed an extraordinary advantage over AC since haploid plants have been regenerated from all of the 18 F_1 hybrids investigated (Lefebvre and Devaux 1996). No recalcitrant genotype to green haploid plant production was encountered as the poorest responder yielded 4.4 haploid plants for 100 florets processed.

Table 1. Doubled haploid barley cultivars produced by the *Hbm* and by AC (*)

Company/Institute	Country	Name of cultivars	References
Abed PBS	Denmark	Etna, Give, Loma, Loke, Riga, Rima, Verona, Paloma, Bereta, Aberdeen, Pondus, Perma, Tender*	Rasmussen pers comm
Agriculture Canada	Canada	DB202	Choo *et al* 1995
Canterbury Malting	New Zealand	Valetta	Pickering pers comm
Crop & Food Res	New Zealand	Gwylan	Coles 1986
Florimond Desprez	France	Michka, Lombard, Moka, Anka, Vodka, Gaelic, Gotic, Logic, ZF3642, Jing Zhuo, Douchka, Tattoo, Jerka, Lyric*, Distic, Celinka	Pickering and Devaux 1992 Lefebvre J pers comm
ICI seeds	UK	Waveney	NIAB 1988
IPG/PBS	Poland	KA7/3	Adamski *et al* 1995
IPGG	former USSR	Istok, Odesskil115, Preria	Choo pers comm
NSW Agriculture	Australia	Tantangara*	Read 1995
Saaten-Union	Germany	Anthere*, Henni*	Jäger-Gussen pers comm
Semico	Canada	HD87-18.14, HD87-12.1	Choo *et al* 1995
WG Thompson	Canada	Mingo, Rodeo, Craig, Winthrop, Lester,Ontario, TB891-6, Prospect, Bronco, Sandrina, Beluga, McGregor, T090-017, T086-156, T081-009, T103-003, H30-11	Ho and Jones 1980 Campbell *et al* 1984 Shugar and Etienne 1994 Choo *et al* 1995 Shugar pers comm
WPBS	UK	Doublet, Pipkin	Jones *et al* 1985; 1986

References

Adamski T, Kaczmarek Z, Surma M, Madejewski R, Madajewska M (1995) Efektywnosc hodowli jeczmienia metoda rodowodowa i metoda wykorzystujaca lilie podwojonych haploidow. Hod Rosl 2:16-19

Campbell KW, Brawn RI, Ho KM (1984) Rodeo barley. Can J Plant Sci 64:203-205

Choo TM, Shugar L, Bastien D (1995) *Bulbosum* method in Eastern Canada: an observation. Barley Newsl 39:111-112

Cistué L, Ziauddin A, Simion E, Kasha KJ (1995) Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss Org Cult 42:163-169

Coles GD (1986) Gwylan barley (*Hordeum vulgare* L.). New Zealand J Exp Agri 14:101-103

Davies PA, Morton S (1995) Barley doubled haploids from isolated microspore cultures. In: Proc 7th Aus Barley Tech Symp. Perth WA, pp195-199

Devaux P (1987) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploids in winter barley. I. Production of green plants. Plant Breed 98:215-219

Foroughi-Wehr B, Mix G, Gaul H, Wilson HM (1976) Plant production from cultured anthers of *Hordeum vulgare* L. Z Pflanzenzüchtg 77:198-204

Foroughi-Wehr B, Mix G (1979) *In vitro* response of *Hordeum vulgare* L. anthers cultured from plants grown under different environments. Environ Exp Bot 19:303-309

Foroughi-Wehr B, Zeller FJ (1990) *In vitro* microspore reaction of different German wheat cultivars. Theor Appl Genet 79: 77-80

Friedt W, Foroughi-Wehr B, Snape JW (1987) The significance of biotechnology for the evolution of barley breeding methods. In: Yasuda S, Konishi T (eds) Barley Genetics V, Proc 5th Int Barley Genet Symp. Mazuren, Okayama, Japan, pp903-913

Henry Y, De Buyser J (1990) Wheat anther culture: agronomic performance of doubled haploid lines and the release of a new variety 'Florin'. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry Vol 13. Springer, Berlin, pp285-352

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

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

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

Huang B, Dunwell JM, Powell W, Hayter AM, Wood W (1984) The relative efficiency of microspore culture and chromosome elimination as methods of haploid production in *Hordeum vulgare* L. Z Pflanzenzüchtg 92:22-29

Iyamabo OE, Hayes PM (1995) Effects of selection and opportunities for recombination in doubled-haploid populations of barley (*Hordeum vulgare* L.). Plant Breed 114:131-136

Jones DL, Jones DM, Clifford BC, Clothier RB, Cook R, Griffiths TER, Jones EWC, Jones IT (1985) Cereal breeding, barley. In: Annual Rep WPBS, pp65-66

Jones DM, Clothier RB, Griffiths TER (1986) Cereal breeding, barley. In: Annual Rep WPBS, pp74-75

Kasha KJ (1996) Biotechnology and cereal improvement. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp. University Extension Press, Saskatoon, Sask, pp133-140

Kasha KJ, Reinbergs E (1975) Utilization of haploid in barley. In: Barley Genetics III, Proc 3rd Int Barley Genet Symp, Garching, pp307-315

Lazar MD, Schaeffer GW, Baenziger PS (1984) Cultivar and cultivar x environment effects on the development of callus and polyhaploid plants from anther cultures of wheat. Theor Appl Genet 67:273-277

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

Lyne RL, Bennett RI, Hunter CP (1986) Embryoid and plant production from cultured barley anthers. In: Alderson PG, Withers LA (eds) Plant tissue culture and its agricultural applications. Butterworth Scientific Ltd, Guildford, pp405-411

Marsolais AA, Séguin-Swartz G, Kasha KJ (1984) The influence of anther cold pretreatments and donor plant genotypes on *in vitro* androgenesis in wheat (*Triticum aestivum* L). Plant Cell Tissue Organ Cult 3:69-79

NIAB (1988) Recommended varieties of cereals, farmers leaflet number 8. National Institute of Agricultural Botany, Cambridge, UK

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, Hayes JD (1976) Partial incompatibility in crosses between *Hordeum vulgare* L. and *H. bulbosum* L. Euphytica 25:671-678

Pickering RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: Shewry PR (ed) Barley: Genetics, Molecular Biology and biotechnology. CAB Int Publ, Wallingford, pp511-539

Read BJ (1995) Overview of doubled haploids in Australia breeding programs and opportunities for doubled haploid germplasm exchange. In: Proc 7th Aus Barley Tech Symp. Perth WA, pp218-219

Shugar L, Etienne M (1994) Doubled haploid barley progress report. Barley Newsl 38:105-106

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

B. MOLECULAR CHARACTERS OF THE GENOME DEPENDING ON THE DOUBLED HAPLOID PRODUCTION METHODS

CHAPTER 2

DNA MUTATION AND METHYLATION INDUCED DURING HAPLOID PRODUCTION PROCESS

This chapter refers to the following publication:

Devaux P, Kilian[a] A, Kleinhofs[a] A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241:674-679

[Pages 104 to 109]

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Genetic changes are apparently induced by tissue culture (Larkin *et al* 1989; Karp 1991) and a variety of phenotypic alterations have been reported (Ullrich *et al* 1991; Baillie *et al* 1992). Cytological and morphological abberations have been more frequent in AC than in *Hb*-derived DHs (for review, see Pickering and Devaux 1992). We have studied the level of DNA variation in 30 + 30 phenotypically normal DH plants derived by AC and *Hb* from the cultivar Igri (Devaux *et al* 1993). Polymorphism was not found among the DH lines for 273 RFLP and 89 PCR-amplified fragments indicating absence of deletion/insertion mutations. The number of base pairs screened (401,640) by these two classes of molecular markers indicated that the point mutation rate was less than 0.25×10^{-5}. However when DNA was digested with the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I, RFLPs were obtained. Interestingly, 96% (49 out of 51) of the total methylation polymorphisms were from AC-derived DHs. These changes were probably induced during the *in vitro* culture phase of AC. Recently, Smulders *et al* (1995) reported tissue culture-induced methylation polymorphisms in tomato. The longer period in culture and the use of growth substances for the AC- *versus* the *Hb*-derived DHs might account for the higher level of methylation polymorphism observed. Müller *et al* (1990) reported higher genetic instability of the rice actin genes in plants regenerated from calli maintained for 67 days *versus* 28 days in culture. However, we could not exclude the possibility that different tissues i.e. immature zygotic embryos (*Hb*) *versus* microspores (AC) may have different initial levels of DNA methylation as reported for carrot (Palmgren *et al* 1991) and tomato (Messeguer *et al* 1991).

REFERENCES

Baillie AMR, Rossnagel BG, Kartha KK (1992) Field evaluation of barley (*Hordeum vulgare* L.) genotypes derived from tissue culture. Can J Plant Sci 72:725-733

Devaux P, Kilian A, Kleinhofs A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241:674-679

Karp A (1991) On the current understanding of somaclonal variation. Oxf Surv Plant Mol Cell Biol 7:1-58

Larkin PJ, Banks PM, Bhati R, Brettell RIS, Davies PA, Ryan SA, Scowcroft WR, Spindler LH, Tanner GJ (1989) From somatic variation to variant plants: mechanisms and applications. Genome 31:705-711

Messeguer R, Ganal MW, Steffens JC, Tanksley SD (1991) Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. Plant Mol Biol 16:753-770

Müller E, Brown PTH, Hartke S, Lörz H (1990) DNA variation in tissue-culture-derived rice plants. Theor Appl Genet 80:673-679

Palmgren G, Mattsson O, Okkels FT (1991) Specific levels of DNA methylation in various tissues, cell lines, and cell types of *Daucus carota*. Plant Physiol 95:174-178

Pickering RA, Devaux P (1992) Haploid production: approaches and use plant breeding. In: Shewry PR (ed) Barley: genetics, molecular biology and biotechnology. CAB, Wallingford, pp511-539

Smulders MJM, Rus-Kortekaas W, Vosman B (1995) Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. Theor Appl Genet 91:1257-1264

Ullrich SE, Edmiston JM, Kleinhofs A, Kudrna DA, Maatougui MEH (1991) Evaluation of somaclonal variation in barley. Cereal Res Comm 19:245-260

CHAPTER 3

COMPARATIVE MAPPING WITH MALE AND FEMALE RECOMBINATION-DERIVED DOUBLED HAPLOID POPULATIONS

This chapter refers to the following publication:

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

[Pages 110 to 118]

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Meiotic recombination is a crucial phenomenon on which genetic studies and improved organism breeding is based. The process may be affected by various factors including sex. The *Hb*- and AC-derived DH lines represent female and male recombinant products, providing an excellent opportunity to investigate the role of sex in barley genome recombination. Map distances were determined in 101 *Hb*- and 101 AC-derived DHs obtained from an F_1(Steptoe x Morex) hybrid using molecular markers covering most of the barley genome (Devaux *et al* 1995). The AC-derived population showed an 18% overall increase in the recombination rate. This was observed for every chromosome and for most of the chromosome arms. Recently, Groover et al (1995) and Benito *et al* (1996) also reported significantly higher male than female recombination frequency in *Pinus taeda* and in *Secale cereale* L, respectively. Eight pair-wise distances between individual loci were significantly higher in the AC population and one in the *Hb* population. Although three of the eight distances compared were centromeric, the two most significantly different distances were located in telomeric regions. The most telomeric intervals from 13 chromosomal arms were 43% longer in the AC than in the *Hb* population, while nontelomeric intervals were only 12.5% longer. Although overall increased and decreased male recombination has been reported in other systems (reviewed in Devaux *et al* 1995), increased recombination frequency at the telomeres in male meiosis seems to be emerging as a general phenomenon.

If we refer to human cytogenetics, this could be a consequence of the pairing process of homologs at meiosis since interstitial pairing is more frequent in female than in male meiosis (Bojko 1983). Rouyer *et al* (1990) hypothesized that more random initiation of pairing could explain the lesser expansion of the female genetic map in the distal regions of chromosomes. Interestingly, Armour (pers comm 1996) found that frequency of mutations for some minisatellites located in telomeric areas were 10-fold higher in males than in females and also reported higher male recombination frequencies than female. Moreover there is some evidence that subtelomeric regions of human (Saccone *et al* 1992), cereal (Moore *et al* 1993; Gill *et al* 1993; Gill *et al* 1996) and sugar beet (Schmidt pers comm 1996) genomes are much more gene-rich than proximal regions. Therefore increased male recombination at the telomeric regions may be selectively advantageous since it results in an increased number of gene assortments in the more abundant male gametes which are subjected to high selection pressure at the pollination and/or fertilization stages. Based on the above discussion, one

could speculate that more variability is likely to be expected among DHs derived from AC than from the *Hb* in absence of *in vitro* selection.

REFERENCES

Benito C, Romero MP, Henriques-Gil N, Llorente F, Figueiras AM (1996) Sex influence on recombination frequency in *Secale cereale* L. Theor Appl Genet 93:926-931

Bojko M (1983) Human meiosis VIII. Chromosome pairing and formation of the synaptonemal complex in oocytes. Carlsberg Res Commun 48:457-483

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

Gill KS, Gill BS, Endo TR (1993) A chromosome region-specific mapping strategy reveals gene-rich telomeric ends in wheat. Chromosoma 102:374-381

Gill KS, Gill BS, Endo TR, Boyko EV (1996) Identification and high-density mapping of gene-rich regions in chromosome group 5 of wheat. Genetics 143:1001-1012

Groover AT, Williams CG, Devey ME, Lee JM, Neale DB (1995) Sex-related differences in meiotic recombination frequency in *Pinus taeda*. J Hered 86:157-158

Moore G, Abbo S, Cheung W, Foote T, Gale MD, Koebner R, Leitch A, Leitch I, Money T, Stancombe P, Yano M, Flavell R (1993) Key features of cereal genome organization as revealed by the use of cytosine-methylation sensitive restriction endonucleases. Genomics 15:472-482

Rouyer F, de la Chapelle A, Andersson M, Weissenbach J (1990) An interspersed repeated sequence specific for human subtelomeric regions. EMBO J 9:505-514

Saccone S, De Sario A, Della Valle G, Bernardi G (1992) The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. Proc Natl Acad Sci USA 89:4913-4917

C. MENDELIAN INHERITANCE OF MOLECULAR MARKERS DEPENDING ON THE DOUBLED HAPLOID PRODUCTION METHODS

CHAPTER 4

SEGREGATION DISTORTIONS IN DOUBLED HAPLOID POPULATIONS

This chapter refers to the five following publications. Segregation distortions were assessed in haploid and doubled haploid progenies both in barley and wheat. The lines were derived from single crosses by anther culture and by interspecific hybridization.

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

   [Pages 119 to 124]

2. **Devaux P, Zivy[(a)] M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701-706**

   [Pages 125 to 130]

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

[Pages 110 to 118]

4. **Devaux P, Lonnet[d] P, Jean[b] R (1990) Significant higher proportions of 1BL-1RS wheat-rye translocation lines among doubled haploid progenies derived from F1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp, HJJ, LHW Van der Plas and J. Van Aartrijk (eds.), Progress in plant cellular and molecular biology. Proc. VIIth Int. Cong. Plant Tissue and Cell Cult., Amsterdam, pp 422-426**

[Pages 131 to 135]

5. **Lefebvre[e] 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**

[Pages 97 to 103]

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To fully exploit the genetic variability within a cross, DHs must be derived from a random sample of gametes i.e. no or little selection should occur during the *in vitro* culture phase. Studies using morphological traits and several kinds of markers failed to show any significant deviation from expected segregations in *Hb*-derived DH populations (Kjær *et al* 1990; Powell *et al* 1990; Schön *et al* 1990). Our primary aim was to check whether segregation distortions in doubled haploid lines derived from microspores occur. For this, segregation of 28 protein encoding genes was investigated by two dimensional electrophoresis in a population of 62 AC-derived DHs from the F_1(Kaskade x DH8293) (Zivy *et al* 1992). Significant deviations from the 1:1 segregation ratio were found at seven loci representing four linkage groups: five loci in two groups, each of the other two belonging to one group; segregation distortion was more frequent for the DH8293 allele at six loci, and for the Kaskade allele at one locus. We call them + alleles.

We investigated this further by assessing anther culturability of 50 DH lines of second generation to check whether plants having the + alleles showed significantly higher haploid plant production abilities than those having the allele from the other parent (Devaux and Zivy 1994). In this investigation, plants were classified according to the number of haploid plants obtained per 100 anthers, referring to the genes which have induced these distortions of segregation. As no recombinant within linkage groups has been tested, we refer to the four linkage groups and we consider a group as a gene which is identified by the allele of the parent. DH lines with four + alleles were the best responders. For those with other allele combinations (with three, two or one + alleles), we found that only two + alleles were markers for anther culturability but not the other two (one originated from Kaskade). This means that these deviations could have occurred by random chance. But two other alleles, not different from a 1:1 segregation ratio in the F_1-derived DH progeny, were markers for anther culturability. Several hypotheses can explain this discrepancy but this is most likely related to the genetic origin of anther tissues: distortions of segregation were assessed in DH plants derived from microspores inside anthers of hybrid plants while anther culture responses were assessed from anthers of DH plants.

These studies have shown that four loci (one is a group of three genes) were linked to genes involved in anther culturability. Two loci were found to be linked to genes involved in both embryoid production and green plant regeneration while the other two were linked to genes involved only in green plant regeneration. Three of the four most efficient alleles for anther

culturability originated from DH8293 which was actually the best AC responder of the two parents.

Further research was performed as male and female derived, doubled haploid populations (Devaux *et al* 1993) and high-density linkage maps become available. Kleinhofs *et al* (1993) reported some distorted loci in the Steptoe x Morex *Hb*-derived DH population, however, the magnitude of distortions was much smaller than that of AC-derived DHs from the same F_1 hybrid (Devaux *et al* 1995) and the frequency was low enough to be attributed to random chance. Two areas of strong distortion have been found on chromosomes 1H and 5H in AC-derived DHs from the F_1(Steptoe x Morex) (Devaux *et al* 1995). Similarly, distorted loci were reported in AC-derived DH populations from other crosses on chromosomes 1H (Heun *et al* 1991; Kintzios *et al* 1994; Logue *et al* 1995), 5H (Graner *et al* 1991; Steffenson *et al* 1995) but also on all the other chromosomes i.e. 4H and 6H (Thompson *et al* 1991), 2H and 7H (Graner *et al* 1991; Heun *et al* 1991; Logue *et al* 1995) and 3H (Graner *et al* 1991). If most of these biased gamete selections result from linkages with genes involved in AC culturability, this would suggest that the character is complex and controlled by many genes dispersed in several genotypes.

In wheat (*Triticum aestivum* L.), the inheritance of the 1BL-1RS chromosome was investigated in haploid progenies derived by anther culture and by intergeneric hybridization with maize (*Zea mays* L.). Crosses involving an anther culture-responsive parent such as a 1BL-1RS translocated line (Henry and de Buyser 1985) enabled the indirect recovery of haploid plants from recalcitrant genotypes. However, the frequency of regenerated plants were skewed in the direction of the translocated parent type by selective development of microspores or of the derived embryoids (Agache *et al* 1989; Devaux *et al* 1990). To investigate this further, haploid and F_2 progenies from the same crosses where analyzed for inheritance of the 1BL-1RS chromosome. The 1BL-1RS chromosome was found at the expected ratios in the F_2 and in the haploid progenies produced through the wheat x maize cross but deviated from the 1:1 ratio in the haploid progenies produced by anther culture in favor of plants carrying the translocated chromosome (Lefebvre and Devaux 1996). This confirms the presence of genes involved in AC response on this chromosome.

REFERENCES

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

Devaux P, Hou L, Ullrich SE, Huang ZX, Kleinhofs A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32-36

Devaux P, Lonnet P, Jean R (1990) Significant higher proportions of *1BL-1RS* wheat-rye translocation lines among doubled haploid progenies derived from F1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp HJJ, Van der Plas LHW, Van Aartrijk J (eds) Progress in plant cellular and molecular biology. Proc 7th Int Cong Plant Tissue Cell Cult. Kluwer, Amsterdam, pp422-426

Devaux P, Zivy M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701-706

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

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

Henry Y, De Buyser J (1985) Effect of the *1B/1R* translocation on anther culture ability in wheat (*Triticum aestivum* L). Plant Cell Rep 4:307-310

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34:437-447

Kintzios S, Islam RM, Fischbeck G (1994) Distorted segregation for mildew resistance in doubled haploid lines of spring barley. Plant Breed 112:248-251

Kjær B, Jensen HP, Jensen J, Jørgensen JH (1990) Associations between three *ml-o* powdery mildew resistance genes and agronomic traits in barley. Euphytica 46:185-193

Kleinhofs A, Kilian A, Kudrna D, Bollinger J, Hayes P, Chen FQ, Saghai Maroof MA, Lapitan N, Fenwick A, Blake TM, Kanazin V, Ananiev E, Dahleen L, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ, Knapp SJ (1993)

A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705-712

Logue SJ, Oti-Boateng C, Karakousis A, Kretschmer JM, Manning S, Lance RCM, Langridge PJ (1995) Segregation analysis of DNA markers in anther culture-derived populations of barley (*Hordeum vulgare* L.) In: Proc 7th Aus Barley Tech Symp. Perth WA, pp210-217

Powell W, Ellis RP, Macauley M, McNicol J, Forster BP (1990) The effect of selection for protein and isozyme loci on quantitative traits in a doubled haploid population of barley. Heredity 65:115-122

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

Steffenson BJ, Jin Y, Rossnagel BG, Rasmussen JB, Kao K (1995) Genetics of multiple disease resistance in a doubled-haploid population of barley. Plant Breed 114:50-54

Thompson DM, Chalmers K, Waugh R, Forster BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L. Theor Appl Genet 81:487-492

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

PARTIE II
PART II

PUBLICATIONS - SUPPORT DE LA THÈSE
PUBLISHED PAPERS SUPPORTING THE THESIS

25

Haploid Production: Approaches and Use in Plant Breeding

R.A. Pickering & P. Devaux

Haploids in plant breeding and related research have been used increasingly in recent years in many agricultural and horticultural crop plants. Their applications and development have been the subject of several recent reviews (e.g. Choo *et al.*, 1985; Dunwell, 1985; Kasha *et al.*, 1989) and conferences (Kasha, 1974; Davies & Hopwood, 1980; Maluszynski, 1989) and they are now attracting interest in the field of molecular biology. In the Gramineae, success rates have lagged behind species more amenable for tissue culture, but results are now becoming sufficiently promising to warrant investment in large breeding programmes. In barley, several cultivars have been released as doubled haploids, and there is no reason to doubt that many more will be bred using the techniques outlined in the following pages.

In vitro gynogenesis (ovary culture)

The possibility of producing barley haploid plants by unpollinated ovary culture was first reported by San Noeum (1976), and studies on factors which influence success rates have been undertaken (Yean-San, 1987). The most important of these are:

- the developmental stage of the embryo sac, which is optimal when almost mature. This corresponds to microspores having two nuclei in young pollen grains.
- cold pretreatment (5–14 days at 3°C) applied to barley spikes before ovary collection and plating.
- the culture medium composition such as a high concentration of sucrose (80–120 g l^{-1}), the presence of 2,4-D (2,4-dichlorophenoxyacetic acid) and the use of a combination of growth substances (Wang & Kuang, 1981).
- dorsal orientation of the ovaries on to the medium.

One striking phenomenon which occurs just a few days after inoculation, is that a high proportion of ovaries enlarge (Tang & Xie, 1987; Kasha, pers. comm.; Devaux, unpublished data). Unfortunately the inefficiency of this technique, usually less than one plantlet for 100 ovaries plated (Yean-San, 1987), makes its use limited in breed-

ing and insufficient data exist to draw conclusions about genotypic influence on success rates. However, ovaries or ovules may be suitable for transforming barley by microinjection of foreign DNA (Steinbiss *et al.*, 1985).

Pseudogamy (haploid initiator gene)

This system enables haploid plants to be directly produced by pseudogamy using a haploid initiator gene *hap* (Hagberg & Hagberg, 1980). Although normal development of the endosperm takes place after the polar nuclei fuse with one of the sperm, the other sperm fails to enter the egg which subsequently develops into a haploid embryo (Mogensen, 1982). The numbers of haploids produced in progeny from plants homozygous for the gene (*hap/hap*) ranged from 1 to 40% depending on genotype, and 0.1 to 10% in progeny from the heterozygote (Hagberg & Hagberg, 1987). However, some of the fertile diploid plants were spontaneously doubled haploids, and these occurred three times as frequently as normal haploids. To identify them, a strategy has been devised using marker genes, such as recessive mildew resistance alleles *mlo* (Hagberg & Hagberg, 1987).

The main advantage over other haploid systems is that an *in vitro* culture phase is not required, but the number of crosses needed is greater than with the 'bulbosum' technique, and limits its practical applications.

In vitro androgenesis (anther culture)

Research on barley anther culture was initiated by Clapham (1971) whose techniques and media have been used extensively since, but several important modifications (Table 25.1) have now enabled anther culture to be used in part of a breeding programmes. The following survey outlines the stages of the method and describes the developments which have contributed most to improving success rates, a sample of which is presented in Table 25.2.

In vivo anther development

Following meiosis and tetrad release the isolated microspore is enclosed in a thin membrane and the nucleus becomes centrally situated (Gaul *et al.*, 1976; Sunderland & Dunwell, 1977). A thick wall gradually forms with a single pore and the nucleus then migrates to the cell periphery during vacuolation. Before first pollen grain mitosis the nucleus increases in size, often being located opposite the pore at this stage. First pollen grain mitosis results in the formation of a large vegetative cell and a small genera-

Table 25.1. Recent progress in anther culture of barley.

Source of progress	Reference
Ficoll vs agar	Kao, 1981
Cold pretreatment of spikes	Xu *et al.*, 1981 Dunwell *et al.*, 1987
Agarose vs agar	Lyne *et al.*, 1986
Anther orientation on the medium	Shannon *et al.*, 1985 Hunter, 1985
Melibiose vs sucrose in starch medium	Sorvari & Schieder, 1987
Low [NH_4NO_3] + Glutamine vs High [NH_4NO_3]	Olsen, 1987
Maltose vs sucrose	Hunter, 1987
Pretreatment of anthers on mannitol	Roberts-Oehlschlager & Dunwell, 1990

Table 25.2. Regeneration rates of green plants from 100 cultured anthers, presented in chronological order, with examples of cultivar used.

Cultivar or line	Medium*	Maximum no. of green plants per 100 cultured anthers	Specific purpose of experiment	Reference
'Sabarlis'/'Akka' etc.	mod. LS	3.0	Media and albinism study	Clapham, 1973
'Amsel'	mod. LS	1.0	Genotype and cytological study	Malepszy & Grunewaldt, 1974
'Dissa'	mod. LS	8.0	Genotype influence	Foroughi-Wehr *et al.*, 1976
'Dissa'	mod. LS	1.5	Environmental influence	Foroughi-Wehr & Mix, 1979
'Dissa'	mod. LS	2.7	Genotype influence and heritability of response	Foroughi-Wehr *et al.*, 1982
Cvs + Hybrids	Kao, 1981	10.0	Ficoll study	Kao & Horn, 1982
'Igri'	mod. LS	2.4	Production of virus-resistant barley	Foroughi-Wehr & Friedt, 1984
'Sabarlis'	mod. LS	28.9	Anther orientation	Hunter, 1985
'Sabarlis'	mod. LS	4.2	Media and gelling agent comparisons	Lyne *et al.*, 1986
'Dissa'	mod. LS	2.0	Gelling agent comparisons	Sorvari, 1986
'Sabarlis' (S) 'Dissa' (D) S × (D × S)	mod. MS	0.6 0.3 1.0	Genetic study of response	Dunwell *et al.*, 1987
'Igri' 'Sabarlis'	mod. LS	590 122	Carbon source comparisons	Hunter, 1987
'Igri'	mod. LS	464	Media modifications	Olsen, 1987
'Arra' 'Dissa'	N6	43.3 34.8	Osmoticum study in medium	Sorvari & Schieder, 1987
–	Kao, 1981	40	Media development	Kao, 1988
'Doublet' 'Igri' 'Dissa' 'Sabarlis'	mod. MS	18.3 11.1 5.6 2.8	Anther orientation	Powell *et al.*, 1988
Hybrid	mod. BAC1	14.4	Media development	Szarejko & Kasha, 1988
'Tweed'	mod. LS (Hunter, 1987)	190	Carbon source comparison	Finnie *et al.*, 1989
'Igri'	mod. LS	18.6	Genotype and environmental effects on response	Knudsen *et al.*, 1989

Table 25.2. *cont.*

Cultivar or line	Medium*	Maximum no. of green plants per 100 cultured anthers	Specific purpose of experiment	Reference
'Igri'	mod. LS (Hunter, 1987; Kao, 1981; Sorvari, 1986)	10.4	Media comparisons	Kuhlmann & Foroughi-Wehr, 1989
Hybrid	mod. LS (Hunter, 1987)	11.3	Media comparisons	Luckett (pers. comm.)
'Igri'	mod. LS (Hunter, 1987)	69.4	Media development	Devaux, 1990
'Igri'	mod. LS (Hunter, 1987)	30.6	Anther pretreatment; inoculation density; anther orientation	Roberts-Oehlschlager & Dunwell, 1990
Hybrid	mod. LS (Olsen, 1987)	65	Spike pretreatment; environmental influence	Hou & Ullrich, 1990

*BAC1 = Marsolais & Kasha, 1985
LS = Linsmaier & Skoog, 1965
MS = Murashige & Skoog, 1962
N6 = Chu, 1978
mod. = modified

tive cell, the latter cut off by a wall. Starch formation begins before second pollen grain mitosis. To obtain the best results from anther culture, it is generally agreed that anthers with microspores at the mid-uninucleate stage (stage 4 – Gaul *et al.*, 1976; stage 2 – Sunderland, 1974) are most responsive when the nucleus is in G1 interphase (Sunderland, 1974; Sunderland & Huang, 1985).

Anomalous development

The previous description applies to most developing microspores. However, Sunderland (1974) observed pollen dimorphism in barley in which 15–20% non-staining grains were present amongst those which had developed normally. Dale (1975) subsequently observed that the proportions of non-staining pollen grains and pollen calluses were comparable. The frequency of these grains was also greater in the proximal anther region, where calluses were most often formed. Sunderland *et al.* (1979) and Sunderland & Evans (1980) reported further on these anomalies, calling the routes of development the A, B and C pathways, after those previously described in *Nicotiana tabacum* (Sunderland, 1974). The routes differ after first pollen grain mitosis in the presence or absence of cell wall formation and whether the products of division resemble generative and vegetative cells (nuclei) or two morphologically similar nuclei. Cell fusion may or may not occur resulting in haploid, diploid, polyploid or mixoploid multicellular structures. The ability to switch pollen development from the normal gametophytic into the sporophytic pathways

just outlined can be induced by an environmental stress applied as a treatment to spikes gathered at the appropriate developmental stage.

Spike pretreatment

The proportions of responsive pollen grains have been increased by pretreating spikes, with microspores at the mid-uninucleate stage, at various temperatures before anther culture. Huang & Sunderland (1982) obtained a positive anther response (as measured by callus production) by pretreating excised spikes kept in darkness in sealed Petri dishes at various temperatures. At 25°C the optimum duration of pretreatment was 2–4 days, but progressively greater responses were achieved by reducing the temperature to 4°C and increasing the treatment time to 28 days. Highest rates of green plant regeneration were obtained at 4°C for 35 days. Spike pretreatment has subsequently been used extensively to improve success of anther culture (Hunter, 1985; Shannon *et al.*, 1985; Dunwell *et al.*, 1987; Olsen, 1987). However, the technique has not been universally adopted (Kao, 1988) possibly because of differences in the environmental conditions under which donor plants are grown (Kasha, 1989).

The development of anthers from pretreated spikes differs from those *in vivo* in that the tapetal cells, and the fluid released from them into the anther cavity, disappear prematurely (Sunderland *et al.*, 1984). This disruption to the synchrony of microspore and tapetal development is thought to influence the switch from the gametophytic to the sporophytic phase (Sunderland & Huang, 1985).

Donor plant growth conditions

Pollen dimorphism is also influenced by the environment in which donor plants are raised. Sunderland (1978) observed that the proportion of pollen grains which undergo cell division after culture (E-type or embryogenic) was increased from around 3 to 20% when donor plants were grown at 10°C compared with 15°C; this could partly explain the increase in success using anthers from plants grown below 15°C. For example, Foroughi-Wehr & Mix (1979) using controlled environments compared several temperatures and reported that highest callus and green plant production was obtained from plants grown at 12/5°C, with progressively poorer results at higher temperatures. Lyne *et al.* (1986) also identified 12°C as being more suitable for green plant production than 15°C. Spikes harvested from the field and glasshouse at temperatures lower than those encountered at the height of summer were also more amenable to callus and green plant production (Clapham, 1973; Foroughi-Wehr *et al.*, 1976; Kuhlmann & Foroughi-Wehr, 1989).

Although 12°C is now frequently quoted as the optimum temperature for donor plant growth (Hunter, 1985; Olsen, 1987) a preference for 16–20°C, without a spike cold pretreatment, has been expressed (Kasha, 1989) which may be more appropriate for barley cultivars adapted to the Canadian environment. Operating costs may also preclude the use of controlled environment chambers and many research workers still raise their plants in glasshouses at varying temperatures (Devaux, 1987; Dunwell *et al.*, 1987; Lee & Chen, 1987; Powell *et al.*, 1988), although this is likely to result in seasonal fluctuations in success.

Genotypic influence

Variation in anther response among cultivars has been widely reported (Wilson *et al.*, 1977; Foroughi-Wehr & Friedt, 1984; Sorvari & Schieder, 1987; Powell, 1988). The first report illustrating a marked genotypic effect on callusing and plant production was that of Malepszy & Grunewaldt (1974) who found 'Amsel' the most responsive of eight cultivars tested, but 'Dissa' appeared to be even more suitable for anther culture in a later comparison involving 19 cultivars including 'Amsel' (Foroughi-Wehr *et al.*, 1976). The high callus and green

plant production of 'Dissa' was inherited partly from 'Amsel', one of its parents (Friedt & Foroughi-Wehr, 1981), although these two characteristics are thought to be controlled by independent genetic systems (Foroughi-Wehr *et al.*, 1982; Huang *et al.*, 1984). It has since been shown that ethylene plays a major role in embryogenesis, and as cultivars differ in their capacity to produce ethylene, this may explain some of the differences in anther response (Cho & Kasha, 1989). Although a cytoplasmic effect on green plant production has been implicated (Foroughi-Wehr *et al.*, 1982) this has not been confirmed (Foroughi-Wehr & Friedt, 1984; Dunwell *et al.*, 1987).

Many other investigations into anther culture methodology have involved single cultivars. Clapham (1971) in the earliest report on barley anther culture, carried out his research on 'Sabarlis', a spring cultivar which has since been used extensively by groups elsewhere (e.g. Huang & Sunderland, 1982; Hunter, 1985; Lyne *et al.*, 1986). The winter cultivar 'Igri' was subsequently found to be particularly responsive (Foroughi-Wehr & Friedt, 1984) and is now widely recommended for research purposes (Olsen, 1987; Hunter *et al.*, 1989; Devaux, 1990).

Spike and anther culture

There have been two reports of culturing intact barley spikes in liquid media. Wilson (1977) either stood excized tillers of 'Dissa' in water for 1–2 days before culture, or transferred freshly harvested spikes, with microspores at first pollen grain mitosis to a shaken liquid medium based on Linsmaier & Skoog (1965). Inconsistent callus formation occurred and of the 40 plants regenerated, only two were green. Datta (1987) pretreated spikes with early uninucleate microspores at 5°C for 12 days before culture in a medium containing Ficoll 400 at 100 g l^{-1}. Embryogenic callus was observed which may have been induced partly because of the addition of Ficoll to the medium, and 16 green plants were regenerated. All other published reports describe the culture of individual anthers. These are removed from spikes after surface sterilization of the enclosing last leaf sheath, or the spike itself, with ethanol or a dilute solution of sodium or calcium hypochlorite.

The orientation of anthers on solid media has been shown to be important for subsequent callus production. This was enhanced when anthers were placed with only one of the two loculi in contact with the medium (Hunter, 1985; Shannon *et al.*, 1985) and embryogenic callus formation was only observed in this situation, from which increased green plant production occurred (Hunter, 1985). Powell *et al.* (1988) using several cultivars, confirmed that anther orientation had a significant effect on green plant production, but not on the proportions of responding anthers over all cultivars. They attributed this result to possible differences in growth regulators in the medium although IAA (indole-3-acetic acid) and BAP (6-benzylaminopurine) were both included at the same concentrations as those used by Hunter (1985). A possible explanation for the influence of anther orientation on response is that the microspores in the upper loculus escape the inhibitory effects of sucrose (Wei *et al.*, 1986) in the medium, or its breakdown products (Hunter *et al.*, 1989).

Media

Carbon source and gelling agent

The use of maltose as carbon source is perhaps the most significant recent development for improving success rates in barley. Green plant production is greatly increased compared with sucrose (Hunter, 1987; Finnie *et al.*, 1989) probably because maltose is hydrolysed to glucose more slowly than sucrose thus preventing inhibitory concentrations of glucose from accumulating (Roberts-Oehlschlager *et al.*, 1990). Before this research, sucrose at 6–12% was the most frequently used carbon source in media and had been shown to raise the medium osmolality with increases in concentration. This resulted in the promotion of callus (Kao, 1981) and embryoid formation, in

contrast to plant regeneration via organogenesis from calluses on media with low osmolality (Sorvari & Schieder, 1987). In the same report, sucrose was replaced with melibiose as osmoticum in combination with barley starch. As a result, significant improvements in overall response and green plant production were obtained compared with the control medium containing sucrose.

Sorvari (1986) observed that embryo formation and green plant production could be increased by using starches from various sources in place of agar, and Lyne *et al.* (1986) obtained an improved anther response using sea-plaque agarose instead of agar. Other types of agarose may be as effective (Luckett, pers. comm.) and Ziauddin *et al.* (1988a) failed to confirm the superiority of sea-plaque agarose over agar in their BAC1 medium. Nevertheless it is known that agar has deleterious effects on embryo development (Kohlenbach & Wernicke, 1978) and agarose, a purer compound, may be preferable in some media. Kao (1981) omitted agar from the callus-inducing medium but added Ficoll 400 to support the anthers in an otherwise liquid medium. Anther response was significantly improved over media without Ficoll 400, and embryo formation was observed on calluses 2–3 mm in diameter, a feature not previously recorded on calluses derived from barley microspores. It appears, therefore, that to achieve embryo formation, which is preferable to organogenesis for plant regeneration (Lyne *et al.*, 1986), it is necessary to stabilize media osmolality by replacing sucrose with maltose, and to substitute starch (Kuhlmann & Foroughi-Wehr, 1989) or Ficoll 400 (Kao, 1981) for agar. To reduce the cost of media components, Kasha (1989) has obtained comparable results to Ficoll 400 with dextran and soluble starch both of which are cheaper than Ficoll 400, the cost of which may be prohibitive for routine haploid production.

Plant growth regulators

Auxins and cytokinins are incorporated into media to induce callusing from anthers, and their concentrations are reduced to promote plant regeneration after callus subculture. The most frequently used are 2,4-D (0.5–2 mg l^{-1}) and kinetin (0.25–0.5 mg l^{-1}) or IAA (indole-3-acetic acid) and BAP (6-benzylaminopurine) (both at 1 mg l^{-1}). NAA (L-naphthaleneacetic acid) (2 mg l^{-1}) with BAP (1 mg l^{-1}) has also been adopted (Devaux, 1987).

Media development

In almost 20 years of research, many media have been formulated, some of which will be outlined here. Clapham (1971) used the medium of White (1943) with 12% sucrose, 1 mg l^{-1} NAA, coconut milk and glutamine, and obtained multicellular structures and plantlets from anthers cultured at the uninucleate stage. Clapham (1973) subsequently reported that calluses were induced on a Linsmaier & Skoog (1965) medium modified by reducing the NH_4NO_3 concentration and adding IAA and BAP (both at 1 mg l^{-1}) and sucrose at 12%. Plants were regenerated on a similar medium with 3% sucrose. Foroughi-Wehr *et al.* (1976) further modified Clapham's medium by omitting $CoCl_2.6H_2O$, changing the iron source and reducing the sucrose concentration to 6%. Calluses were subcultured to this basal medium with reduced IAA, BAP (0.4 mg l^{-1}) and sucrose (3%). This medium has subsequently been used extensively but other groups have developed their own. Sunderland and co-workers at the John Innes Institute, UK, have employed media based on one devised for wheat which includes potato extract (Chuang *et al.*, 1978) and the N6 medium of Chu (1978) developed originally for rice anther culture. This has reduced ammonium ions compared with that of Miller (1963) on which it is based. Kao (1981) in Canada published details of a relatively complex medium for use with Ficoll 400. Because of a lack of success with the above media, Marsolais & Kasha (1985) at Guelph University, Canada, developed a defined medium – BAC1 – which also incorporated Ficoll 400. Both Kao (1988) and the Guelph group (Szarejko & Kasha, 1988) have since modified

their media by the addition of $KHCO_3$ in attempts to stabilize the pH, which tended to drop during culture from 5.5 to 4.5 (Kao, 1981).

The important role of inositol in anther response has been reported by Xu & Sunderland (1981) who observed a 20-fold increase in callus production over that obtained from the Chuang and N6 media without inositol, although maximum effect was only gained at an anther density of 40 per ml compared with 20 per ml of medium.

It is not possible to elaborate greatly on all the changes in media composition, and only the major ones have been described here. However, there have been several reports comparing the relative efficiencies of media. Kuhlmann & Foroughi-Wehr (1989) reported that for four different hybrids and 'Igri' a liquid medium based on the formulation of Foroughi-Wehr *et al.* (1976) produced better results than the N6 (Chu, 1978). Both media were modified by substituting maltose for sucrose, and Ficoll 400 plus barley starch for agar. Lyne *et al.* (1986) also obtained best overall response with the medium of Foroughi-Wehr *et al.* (1976) compared with those of Chu (1978), Chuang *et al.* (1978) and Kao (1981). However, the relative efficiencies of the media varied for the different stages during culture. Huang *et al.* (1984) did not find it possible to identify which of the two media - N6 (Chu, 1978) and that of Chuang *et al.* (1978) - was superior because of genotype and media interactions.

Although Wilson *et al.* (1977) obtained an increase in the proportions of green plants after filter sterilizing their medium, autoclaving has frequently been used for sterilization (Foroughi-Wehr *et al.*, 1976; Chuang *et al.*, 1978; Xu *et al.*, 1981; Sorvari, 1986; Lee & Chen, 1987). Other researchers either failed to report the sterilization method or autoclaved all nutrients except a few thermolabile components (Clapham, 1973; Xu & Sunderland, 1981; Shannon *et al.*, 1985; Devaux, 1987). Complete filter-sterilization of all components, except gelling agent, is preferred by several other groups (Kao, 1981; Marsolais & Kasha, 1985; Olsen, 1987; Hunter *et al.*, 1989).

Media preconditioning and inoculation density

To maximize callus production, Xu *et al.* (1981) advocated using conditioned media. This was effected by culturing anthers with microspores at the binucleate to starch formation stages for 7 days before their removal and culture of uninucleate anthers in the normal way for callus induction. Percentage anther response could not be assessed accurately, but it was clear that callus formation was greatly increased after conditioning, especially when anthers for callus induction were plated at densities of 20 compared with 10 per ml. A conditioning factor present in anthers is thought to be responsible for this effect, and its effect was synergistic with inositol in the medium. Preconditioning of media in this way reduced the need for high anther inoculation densities which, without media preconditioning, produced maximum response and calluses per anther at 60–120 anthers per ml (Xu & Sunderland, 1982). This positive effect of high inoculation density was not recorded when anthers were initially cultured on a medium containing 3.2% mannitol for 4 days at 25°C before re-culturing on a medium with maltose and incubation at 25°C (Roberts-Oehlschlager & Dunwell, 1990). Anther inoculation densities in other published reports range from 2–4 per ml (Dunwell *et al.*, 1987; Lee & Chen, 1987; Olsen, 1987; Powell, 1988) to 10–20 per ml (Kao, 1981, 1988; Marsolais & Kasha, 1985; Kuhlmann & Foroughi-Wehr, 1989) to 40–70 per ml (Shannon *et al.*, 1985).

Incubation

This is usually carried out at between 22 and 28°C in darkness, and embryos and/or calluses become visible around 21 days after culture. A reduced temperature of 4°C (Jing & Hu, 1981) or 15°C (Kao, 1981) for the first 3 or 5 days, respectively, may enhance

callus formation, and even an increased temperature of 28°C for up to 3 days followed by 25°C improved the response of 'Bruce' although temperatures greater than 28°C were deleterious for all cultivars tested (Ziauddin *et al.*, 1988a).

Regeneration

To promote plant regeneration, calluses greater than 1 mm in diameter are usually subcultured to a solid medium, for example B5 (Gamborg *et al.*, 1968), or, more frequently, one based on that described by Foroughi-Wehr *et al.* (1976), with 2–3% sucrose and either a reduced concentration of growth hormones or a change from 2,4-D plus kinetin to BAP plus IAA (Huang & Sunderland, 1982). Kao (1988) however, actually increased the sucrose level from 4.25 to 6% and used polyester floats on a liquid medium to support calluses. Calluses and embryos are incubated for 5–7 days in darkness until the start of plant regeneration. Further development takes place under lights, the daylength ranging from 10 h to continuous light (12–70 $\mu E\ m^{-2}\ s^{-1}$), at 20–26°C. Green plantlets are then either subcultured again to a medium without hormones (Lyne *et al.*, 1986; Olsen, 1987) or IAA only (Devaux, 1987), or transferred to potting compost.

Progeny from anther culture

Albinos

Albinism has been and remains a problem amongst regenerants, and is known to be strongly influenced by genotype (Dunwell *et al.*, 1987; Powell, 1988; Knudsen *et al.*, 1989). Clapham (1973) obtained 80–100% albino plants and these proportions could not be reduced by changes in media composition or culture conditions. Fewer albinos were, however, obtained from late-season plants (Clapham, 1973) an observation confirmed by other researchers (Foroughi-Wehr *et al.*, 1976; Foroughi-Wehr & Mix, 1979; Kuhlmann & Foroughi-Wehr, 1989). This may be attributed to a temperature effect, as Lyne *et al.* (1986) obtained greater proportions of green plants by growing donor plants at 12°C compared with 15°C. Significant improvements in green plant production have also been achieved as follows:

1. Spike pretreatment at 4°C (Huang & Sunderland, 1982);
2. Filter sterilization of media (Wilson, 1977);
3. Sea-plaque agarose (Lyne *et al.*, 1986) or starch (Sorvari, 1986) as gelling agents in place of agar;
4. Melibiose as osmoticum at 80–120 $g\ l^{-1}$ in sucrose-free medium (Sorvari & Schieder, 1987);
5. Substitution of maltose for sucrose in the medium (Hunter 1987; Hunter *et al.*, 1989). By way of confirmation, Devaux (1990) subsequently reported that 58, 72, and 79% of total plants regenerated were green on media containing sucrose, maltose or maltose plus glutamine respectively.

There is still speculation about the causes of albinism in regenerants. Clapham (1973) observed proplastids and chloroplasts of almost normal appearance in barley albinos but Day & Ellis (1985) reported deletions in plastid DNA.

Ploidy level

Diploidization can occur during the first cell divisions after culture following cell fusion and endomitosis (Wilson *et al.*, 1978; Sunderland *et al.*, 1979; Sunderland & Evans, 1980; Chen *et al.*, 1984; Lee & Chen, 1987) resulting in callus with haploid, diploid and polyploid cells (Malepszy & Grunewaldt, 1974; Grunewaldt & Malepszy, 1975; Wilson, 1977) and successive subculturing of calluses leads to a gradual reduction in haploid cell numbers (Grunewaldt & Malepszy, 1975). Regenerated plants, both albino (Grunewaldt & Malepszy, 1975; Kao, 1981) and green (Mix *et al.*, 1978; Foroughi-Wehr *et al.*, 1982; Devaux, 1988) can be haploid, diploid, tetraploid, aneuploid or mixoploid. Spontaneous chromosome doubling results in fertility levels ranging

from just under 50% (Powell, 1988) to over 90% (Huang & Sunderland, 1982; Lyne *et al.*, 1986; Olsen, 1987). This may eliminate the need for colchicine treatment to induce chromosome doubling. However, should the breeder consider it necessary to determine the ploidy level of regenerants, several methods can be used: chromosome counting (Foroughi-Wehr *et al.*, 1976); phenotypic identification (Mix *et al.*, 1978); stomatal guard cell length (Tometorp, 1939); and flow cytometry (Brown, 1989). Flow cytometry may be the most promising as it is rapid, reliable and only requires small amounts of tissue. Some profiles of haploid, doubled haploid and tetraploid microspore-derived barley plants (MDP) analysed by flow cytometry are illustrated in Fig. 25.1.

As well as variation in ploidy level, chromosomal abnormalities have been reported in mixoploid regenerants such as chromosome breakage, ring chromosomes, fragments and bridges (Mix *et al.*, 1978) which may have resulted from translocations and inversions. Powell *et al.* (1986) also recorded that three out of 13 diploid regenerants had reduced chiasma frequency, and another was heterozygous for a translocation.

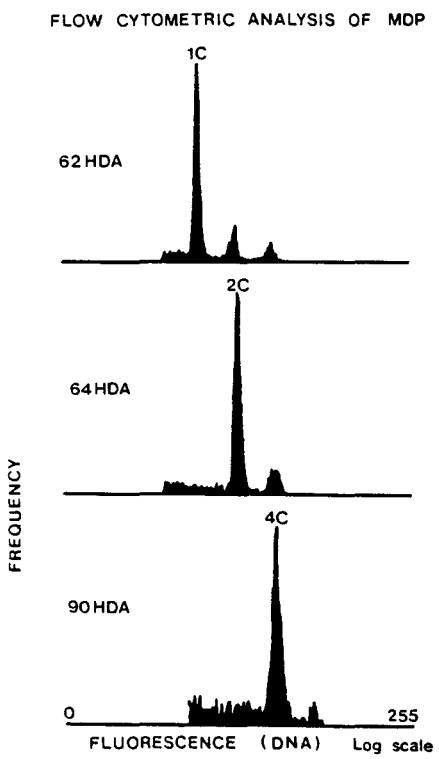

Fig. 25.1. Flow cytometric profiles of haploid (62HDA), diploid (64HDA) and tetraploid (90HDA) plants derived from anther culture (MDP). Analyses made at CNRS, Gif (France) in collaboration with S. Brown.

Changes in ploidy levels, recessive mutants (including albinos) and a maternally inherited mutant have also been observed in regenerants from calluses derived from haploid explants after crossing *H. vulgare* and *H. bulbosum* (Saalbach & Koblitz, 1977; Jensen, 1981; Pickering, 1989). It seems, therefore, that genetic and cytoplasmic changes do take place during callus induction regardless of explant source although regeneration via embryogenesis may reduce induced variation.

In addition to cytological aberrations, differences between regenerants and control barley cultivars have been reported for several quantitatively inherited traits (San Noeum & Ahmadi, 1982; Powell *et al.*, 1984). However, pre-existent heterogeneity within the parental cultivar may sometimes explain this variation (Snape *et al.*, 1988). Deviations from expected segregation ratios have also been recorded for simply inherited characters such as disease resistance (Foroughi-Wehr & Friedt, 1984; Friedt *et al.*, 1986) and row type (Foroughi-Wehr & Friedt, 1984; Kao, 1988) in regenerants derived from hybrid material. For example, an excess of six-rowed types over two-rowed has been obtained and, as the proportions of each could be modified by changes to the concentration of zeatin riboside in the medium, specific media for two-rowed versus six-rowed barley were developed (Kao, 1988).

Microspore culture

This technique, relying as it does on isolated microspore culture, is possibly more amenable than anther culture for research into transformation, mutations and *in vitro* selection. It eliminates the effect of diploid

anther tissue and competitive effects within the anther. Further advantages over anther culture are the potential regeneration of larger numbers of haploid plants without the interference of somatic callus derived from the anther wall although this problem has not arisen in barley anther culture. Despite these advantages less research has been carried out on microspore compared with anther culture.

Spike pretreatment

Spikes, with anthers containing uninucleate microspores, may be cold pretreated in a similar way to those used for anther culture (Huang & Sunderland, 1982). Olsen (pers. comm.) found 28 days at 4°C to be suitable, although Sunderland & Xu (1982) preferred 14 days at 7°C to obtain rapid anther dehiscence and microspore liberation. Datta & Wenzel (1988) on the other hand, obtained differential responses of spring and winter cultivars at 5°C for 8 and 12 days respectively. Köhler & Wenzel (1985) obtained greatest success by omitting this step altogether, using instead media preconditioned for 7 days with 10 ovaries per ml after the method of Xu *et al.* (1981). Sunderland & Xu (1982) also found a beneficial effect of preconditioning of media, most pronounced when microspores were liberated from anthers inoculated at a density of 80 per ml compared with 20 per ml. The positive effects on response of: (i) spike cold pretreatment; (ii) media preconditioned with anthers or ovaries; or (iii) inositol or mannitol in the initial culture medium (Sunderland & Xu, 1982; Wei *et al.*, 1986) led Ziauddin *et al.* (1989) to suggest that there may be a conditioning factor present in the anther for successful induction to occur.

Microspore isolation

There are two methods for isolating microspores, namely shed pollen and mechanical separation. Sunderland & Xu (1982) preferred the former for its simplicity and avoidance of damage to the pollen. In their method, anthers were cultured on drops of preconditioned medium with 1–2 g l^{-1} inositol (Sunderland & Xu, 1982; Köhler & Wenzel, 1985; Datta & Wenzel, 1988). Dehiscence took place 24 h later when the anthers could be removed after about half (1500) of the total number of microspores per anther had been liberated (Sunderland & Xu, 1982). Inoculation densities greater than 20 anthers per ml increased productivity (Sunderland & Xu, 1982; Köhler & Wenzel, 1985). Ziauddin *et al.* (1990) have also used the shed pollen technique by culturing anthers of 'Igri' on 0.3 M mannitol for 3 days at 25–28°C. The liberated microspores were then separated by centrifuging in mannitol, water or medium depending on cultivar (Ziauddin *et al.*, 1989), and recultured on fresh ovary-conditioned medium based on those of Olsen (1987) and Hunter (1987). Ficoll 400 was found to be inhibitory (Ziauddin *et al.*, 1988b) and may result in the collapse of microspores (Thörn, pers. comm.). A faster response and embryo formation (18 days) was obtained using this method compared with mechanical separation (3–4 weeks) (Ziauddin *et al.*, 1989).

Sunderland & Xu (1982) also observed greater productivity from shed pollen compared with isolated pollen released mechanically by gentle tapping. In addition to this latter method of mechanical separation (which has been used to great effect by Hunter, 1987), Wei *et al.* (1986) released microspores, by means of an internal pestle or injector, into a 0.3 M mannitol solution before filtering, rinsing, centrifuging and inoculating on to medium (Xu & Sunderland, 1981). Other methods of mechanical separation have been compared (Olsen, pers. comm.), namely maceration by Teflon rod (Nitsch, 1974) and microblending (Swanson *et al.*, 1987). Microblending released more microspores and had a gentler action as assessed by frequencies of large viable microspores (20% vs 10%). A cold spike pretreatment may assist the mechanical separation of microspores because tapetal degeneration enables them to be

squeezed out more easily (Sunderland *et al.*, 1984).

Media

For callus induction, liquid or semiliquid media of similar composition to those used for anther culture have been found to be satisfactory, for example, Foroughi-Wehr *et al.* (1976), Chuang *et al.* (1978) and Chu (1978). *m*-Inositol at 1 or 2 g l^{-1} was found to be beneficial and reduced the need for media preconditioning (Sunderland & Xu, 1982; Köhler & Wenzel, 1985). More recently, microspore response and/or embryo formation have been improved by the addition of phenyl acetic acid to the medium in place of more commonly used growth hormones (Kasha, pers. comm.).

Sucrose at 6–9% used to be the usual carbon source in media, but as with anther culture, when maltose was substituted for sucrose a greatly increased response was obtained (Hunter, 1987).

After a period of incubation which may include 1–3 days initially at 4–5°C (Thörn, pers. comm.), calluses 1 mm or more in diameter can be subcultured on to media with reduced auxin concentration and finally to solid media for plant regeneration (e.g. Köhler & Wenzel, 1985; Wei *et al.*, 1986). A simpler method has been adopted by R.B. Jørgensen (pers. comm.) in which microspores are isolated for initial culture in droplets of media. After plating out on a solid medium (Hunter, 1987) embryo development and plant regeneration occur without subculture.

Response

Köhler & Wenzel (1985) obtained 1.2 plants per 100 cultured anthers of 'Dissa', but 93% were albino. Datta & Wenzel (1988) improved success rates with this cultivar to 14 green plants per 100 cultured anthers, by modifying the medium (e.g. increased *m*-inositol; addition of Ficoll 400) and cold-pretreating the spikes. Wei *et al.* (1986) had previously obtained up to 60% plant regeneration from inoculated calluses of 'Sabarlis', and 37.5% of regenerants were green. Hunter (1987), Olsen (pers. comm.) and Ziauddin *et al.* (1990), all using 'Igri', regenerated 608, 94 and 292 green plants per 100 anthers cultured respectively and Thörn (pers. comm.) has obtained 330 green plants per 100 anthers with the spring cultivar 'Alva'.

If these regeneration rates can be consistently repeated with a range of cultivars, microspore culture could become a more appropriate technique than anther culture for haploid production in a breeding programme and in research.

Interspecific hybridization

Selective chromosome elimination has been reported in crosses within the Triticeae, frequently resulting in haploids of one of the parental species (e.g. Bothmer *et al.*, 1983). Barley dihaploids and/or haploids have been produced from crosses of *Hordeum vulgare* L. × *Psathyrostachys fragilis* (Boiss.) Nevski (Bothmer *et al.*, 1984) and *H. vulgare* × *Secale cereale* L. (Fedak, 1977). However, the numbers of plants obtained were very low, and the following description of barley haploid production will be concerned only with *H. vulgare* × *H. bulbosum* crosses. As a measure of success rates, the numbers of green haploid plants per 100 pollinated florets are presented in Table 25.3.

Hordeum bulbosum L. is a perennial outcrossing species found in the Mediterranean region, and is thought to be closely related to *H. vulgare* (Bothmer *et al.*, 1983). It occurs as two cytotypes, tetraploid ($2n = 4x = 28$) and diploid ($2n = 2x = 14$), and has been crossed frequently with barley in attempts to transfer desirable characters from the wild into the cultivated species.

Successful crosses between *Hordeum vulgare* L. and *H. bulbosum* L. were first performed by Kuckuck (1934), who obtained one sterile triploid hybrid from *H. vulgare* ($2n = 2x = 14$) × *H. bulbosum* ($2n = 4x = 28$). In later studies Konzak *et al.*

Table 25.3. Regeneration rates of green haploid plants per 100 pollinated florets of *H. vulgare* x *H. bulbosum*, presented in chronological order.

Cultivar	Maximum no. of green haploid plants per 100 pollinated florets	Reference
F_1 hybrid	46.7	Jensen, 1976
'York'	26.8	Kasha *et al.*, 1978
F_1 hybrid	ca. 18–20	Pickering, 1980b
Hybrid	12.6	Simpson *et al.*, 1980
Hybrid	16	Simpson & Snape, 1981
'Sabarlis'	28.9	Pickering, 1983a
F_1 hybrid	15.4	Huang *et al.*, 1984
F_1 hybrid	ca. 11.5	Devaux, 1987
F_1 hybrid	41.6	Chen & Hayes, 1989
'Schuyler'	47.8	Hayes & Chen, 1989
F_1 hybrid	13.2	Devaux *et al.*, 1990
Hybrid	35.0*	Guerrero, pers. comm.
Hybrid	ca. 15* (7.5–10 average)	Jestin, pers. comm.
Hybrid	10 (average)	Shugar, pers. comm.
Hybrid	ca. 21	Torp, pers. comm.

*Doubled haploids.

(1951) and Morrison *et al.* (1959) amongst others also produced hybrids. Kao & Kasha (1969) obtained progeny resembling *H. vulgare* from similar hybridizations and proposed that the *H. bulbosum* chromosomes had been eliminated from the developing embryo after fertilization. This was confirmed by Kasha & Kao (1970) after hybridizations of *H. vulgare* ($2n = 2x = 14$) with *H. bulbosum* ($2n = 2x = 14$) resulted in the production of haploid *H. vulgare* plants. These had been regenerated after rescuing embryos to a nutrient medium. Haploid formation is known to depend on genetic factors (Ho & Kasha, 1975), genome ratios (Subrahmanyam & Kasha, 1973; Subrahmanyam, 1980) and temperature after fertilization (Pickering, 1985). Subrahmanyam (1982) proposed a hierarchical system in *Hordeum* based on species dominance to explain differences between crosses whereby chromosomes of the non-dominant species are eliminated. Several haploids and polyhaploids obtained after interspecific hybridization have been meiotically analysed to obtain data about pairing regulatory mechanisms and genome homologies. For example, Subrahmanyam (1978) and Bothmer & Subrahmanyam (1988) have presented evidence from which it was concluded that the polypoid species examined were alloploid with some genome homology.

The potential value of interspecific hybridization between *H. vulgare* and *H. bulbosum* in breeding new cultivars faster than conventional methods was predicted by Kasha & Kao (1970). To use this interspecific cross for successful doubled haploid production, the technique has been developed further by making improvements at each stage of the process.

Plant growth conditions

Diploid *H. bulbosum* plants are vernalized at 4°C for 6–8 weeks (8 h daylength) before repotting and transfer to a suitable environment for use as pollinator with *H. vulgare*. The environmental requirements for crossing, although important, are probably not as stringent as those for anther culture, and a heated glasshouse maintained at 14–24°C is satisfactory. Continuous temperatures

lower than 18°C during the first 5 days of embryo development can, however, result in higher proportions of hybrids, which retain both sets of parental chromosomes (Pickering, 1985).

To make use of field-grown material for crossing, Kasha *et al.* (1978) recommended placing detached tillers into nutrient solution + GA_3 (gibberellic acid) and maintaining them in a controlled environment. Although inconsistent results are frequently obtained with this technique (Pickering, 1980a), Chen & Hayes (1989) and Hayes & Chen (1989) reported regenerating 30.6 and 13.5 haploid plants per 100 pollinated florets (respectively). Unlike anther culture, where large spike to spike variation in response occurs (Dunwell *et al.*, 1987) no significant differences in success rates were obtained between pollinated spikes from the main shoot and first and second tillers (Pickering, 1983a).

Hybridization

Emasculation of barley florets is carried out conventionally, and freshly collected *H. bulbosum* pollen is applied to receptive stigmas 2–3 days later. Some researchers prefer to use pollen from individual clones of *H. bulbosum* (Guerrero, pers. comm.) whereas others use mixtures of pollen collected from selected clones (Devaux & Jean, 1985; Chen & Hayes, 1989; Jestin, pers. comm.). A possible disadvantage of pollen mixtures is that one of the components may preferentially effect fertilization (Pickering, 1984). It may therefore be more effective to pollinate individually, with the two best *H. bulbosum* clones available, on opposite sides of the same spike, but this is a decision each breeder must make individually.

Seed setting

Seed sets on most barley cultivars pollinated with *H. bulbosum* are over 80%, but after crossing certain cultivars with *H. bulbosum*, reduced seed setting occurs. This was recorded on 'Vada' and found to be controlled by a single dominant gene located on barley chromosome 7 (Pickering, 1983b). By preparing sections of pollinated ovaries of 'Vada' × *H. bulbosum*, it was observed that pollen tubes successfully penetrated the stigma branches, but were subsequently arrested in the stylar transmitting tract as a result of inflation and bursting of tube tips (Pickering, 1981). Similar manifestations of incompatibility have been observed in wheat × rye (e.g. Jalani & Moss, 1980) and wheat × *H. bulbosum* (Snape *et al.*, 1980; Sitch & Snape, 1987), crossabilities of which are governed by two dominant genes on wheat chromosomes 5A and 5B (Riley & Chapman, 1967). As this chromosome is homoeologous with barley chromosome 7 (Islam & Shepherd, 1981) these three incompatibility systems, as well as crossability within the Triticeae generally, may be closely related (Thomas *et al.* 1981; Fedak & Jui, 1982; Pickering, 1984).

Other cultivars possibly unrelated to 'Vada' have also exhibited incompatibility in the form of reduced seed setting, for example 'Firlbeck III' (Pickering, 1980b), 'Agneta' (Bjørnstad, 1986) and 'Apex' (Devaux *et al.*, 1990). Unlike 'Vada', the incompatibility in 'Agneta' is strongly influenced by environment and appears not to be dominantly inherited (Bjørnstad, 1986). Winter cultivars of barley, especially six-rowed types (e.g. 'Vogelsanger Gold', Thörn pers. comm.), tend to be more strongly incompatible with *H. bulbosum* (Simpson *et al.*, 1980; Chen & Hayes, 1989) which partly explains the reduced overall success rates with winter compared with spring barleys (Torp, pers. comm.; Devaux, 1986).

The problem has only been overcome to some extent by screening and selecting amongst different *H. bulbosum* accessions (Pickering, 1980b). Changes in technique and post-pollination sprays of growth hormones such as IAA + GA_3 did not achieve any marked improvements, although an application of 200 g l^{-1} proline at the time of emasculation tended to increase seed setting (Navolotsky, pers. comm.).

Seed quality

The major advance in improving longevity of developing seeds in intergeneric and interspecific hybrids in the Gramineae was the application of GA_3 to florets after fertilization (Larter & Chaubey, 1965). Without this plant growth hormone developing seeds degenerate before embryo rescue. GA_3 (75 mg l^{-1}) is therefore routinely applied as a spray to florets 1–2 days after pollination to enhance seed development and embryo size (Kasha *et al.*, 1978; Adamski, 1979). Parental genotype of the pollinator does not appear greatly to affect seed quality but there are differences between barley cultivars in this respect (Pickering, 1983a; Bjørnstad, 1986). Chen & Hayes (1989) in comparisons with the detached tiller technique of Kasha *et al.* (1978) have obtained improvements in the numbers of green seeds by culturing individual pollinated florets before embryo excision. This had previously been used effectively in wheat × maize crosses to prolong seed development (Laurie & Bennett, 1988). Although the procedure creates an additional step in the scheme, the advantages of greater success rates may offset the extra labour required.

Embryo differentiation

In order to regenerate haploid plants, embryos are cultured at an optimum length of 1–1.5 mm (Adamski, 1979; Pickering, 1980a). This is reached 8.5 to 28 days after pollination at temperatures ranging from 25.5 to 10°C respectively. Apart from small globular embryos obtained from *H. vulgare* × *H. bulbosum* Cb 2951 (selected from P.I.240163 by Pickering), there are three other classes of embryo:

1. Well-differentiated haploid;
2. Undifferentiated, often cuneiform, haploid;
3. Well-differentiated hybrid.

The frequencies of the various types of embryo are influenced by male and female genotype (Jensen, 1976; Pickering, 1983a) and season (Pickering, 1980a; Devaux & Jean, 1985). For example, type 3 embryos (hybrids) are more frequently encountered during winter months at suboptimal temperatures (Pickering, 1985). To improve success rates *H. bulbosum* genotypes have been selected to produce higher proportions of 'type 1' embryos (Pickering & Rennie, 1990).

For embryo culture, the medium most favoured is that 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 & Kao, 1970). Incubation is generally carried out in darkness at 20–22°C, and once the coleoptile is about 1 cm in length, the culture vials are put under lights (16 h daylength) at a similar temperature. These conditions are not critical, and cultured embryos, plantlets, and even pollinated spikes, can be maintained at 2–4°C to slow down growth and smooth out work schedules.

Plantlets at the two-leaf stage are removed directly to potting compost, and the weaker ones covered with clear plastic beakers to maintain high humidity. Around 50–60% of those embryos classified as type 1 will regenerate viable haploid plantlets after embryo culture compared with only 10–15% of type 2 embryos (Kasha *et al.*, 1978; Pickering, 1980a).

Chromosome doubling with colchicine

Despite reports of mutations having been induced by colchicine treatment in barley (Gilbert & Patterson, 1965) we have not observed their occurrence in our breeding programmes. Finch & Bennett (1979) did, however, record a slight reduction in chiasma frequency in colchicine-treated barley seedlings compared with a 'once-grown' doubled haploid control, but the differences were not considered important. Although Snape *et al.* (1988) detected 'gametoclonal variation' in 'bulbosum-derived' wheat doubled haploids, which may have been attributable to cochicine treatment, no such variation was reported in barley doubled haploids using this technique. Thus, unlike anther culture, gameto-

clonal variation has not proved to be a problem and its effects can be disregarded in breeding and research. Furthermore, segregation ratios amongst doubled haploid progenies derived from early generation material are generally as expected (for example, Simpson & Snape, 1979; Doll *et al.*, 1989), and therefore random gamete selection takes place for simply inherited characters.

Regarding colchicine treatment, all hybrid plants can usually be identified morphologically and discarded at tillering (Jensen, 1976). Haploid plants are removed from compost when they have produced three or four tillers; they are then washed and their roots trimmed. Incisions can be made at each of the stem bases before immersion to a depth of 5 cm in an aerated 0.05% aqueous solution of colchicine + 2% dimethyl sulphoxide (Jensen, 1976; Thiebaut *et al.*, 1979; Pickering, 1980a). The plants are treated for 5 h at 25–30°C then washed for 5 min in tap water and replanted into compost. Chromosome doubling rates of >80% are commonly achieved, although there are significant differences in the proportions and fertility of doubled haploid plants obtained (Adamski, 1979; Pickering, 1983a; Devaux, 1989a).

Comparisons between anther culture, 'bulbosum' technique and conventional breeding programmes

The 'bulbosum' method has been shown to be more efficient for haploid production than *in vitro* androgenesis (Huang *et al.*, 1984) but in a similar comparison, Friedt *et al.* (1987) reported greater success with anther culture for winter barley hybrids. Using seven F_1 winter barley hybrids, Devaux (1987) found that the response and the time required to produce doubled haploids from the two techniques were similar, but it was proposed to use both systems since a highly significant genotype × technique interaction was obtained. Furthermore, as the two methods demand dissimilar environmental conditions for donor plant growth, they can be carried out at different seasons to obtain maximum success.

The time taken to process a spike is about 30 min from emasculation or spike excision to transfer of green plants to soil with both techniques (Devaux, 1987) and in each case, 7–9 months are needed from the time of sowing donor spring barley plants to harvesting doubled haploid progeny (Choo *et al.*, 1985). On this basis, both methods offer similar returns of doubled haploids for the amount of labour expended (cf. Tables 25.2 and 25.3) although it must be remembered that recent improvements in anther culture success have been obtained with a limited range of hybrids and responsive cultivars (e.g. 'Igri').

A scheme for rapidly obtaining spring barley doubled haploids from an F_1 hybrid is presented in Table 25.4. Only one year is required to enter doubled haploids in field nurseries from the time of sowing F_1 seeds. By using off-season nurseries for multiplication, the subsequent testing process can be accelerated even further. Time-savings are greater when the system is used with winter barley, because every selfed generation in a conventional breeding programme requires vernalization to induce flowering.

A refinement to the breeding programme is the use of recurrent selection whereby superior doubled haploids from a diallel are used as parents in another round of crossing before further doubled haploid production (Choo *et al.*, 1979; Patel *et al.*, 1985). By accumulating desirable genes, gradual improvements in the performance of the progeny should be achieved more efficiently following the use of haploids to obtain increased additive genetic variance (Kasha & Reinbergs, 1981).

Because of the very rapid production of populations of homozygous lines which have similar yield potential, and in some cases means and variances, to those produced conventionally, the 'bulbosum' technique has been compared favourably with single seed descent (SSD) pedigree and bulk breeding methods (Park *et al.*, 1976; Song *et al.*, 1978; Choo *et al.*, 1985). The doubled

Table 25.4. Current scheme for obtaining spring barley doubled haploid lines from two parents A and B.

Year 0	15 Feb.	Sowing of the two parents A & B (glasshouse)
	15 April	Crossing A × B
	5 May	Sowing of F_1 grains AB
	15–20 June	Anther culture and/or crossing F_1 plants with *H. bulbosum*
	20–30 July	Transfer of gametophyte-derived plants into soil (glasshouse)
	10 August	Colchicine treatment of haploid plants
	Oct.–Dec.	Harvest of grains from doubled haploids
Year 1	March	Sowing of doubled haploids in field nurseries, for first multiplication and selection

haploids have also shown similar stability compared with control cultivars over a range of environments (Reinbergs *et al.*, 1978). At Florimond Desprez, comparisons have also been made between breeding methods for several agronomic traits. Grain yields of F_5 pedigree-bred and 'bulbosum'-derived doubled haploids were assessed 4 years after making the initial cross. Mean yield was higher and standard deviation lower for the pedigree lines compared with the doubled haploids (60.13 ± 4.98 dt ha^{-1} and 56.88 ± 6.75 dt ha^{-1} respectively). This probably reflects the lack of selection pressure on the doubled haploids derived from an F_1 hybrid.

Regarding anther culture derived lines, Friedt & Foroughi-Wehr (1983) obtained no differences in mean yield between doubled haploid lines and their mid-parent values, but there was a shift in the frequency distribution towards lower yields in the doubled haploids. In a later study, however, identical means, variances and frequency distributions were obtained for yield from *H. bulbosum*-derived and anther culture-derived doubled haploids (Friedt *et al.*, 1987). In another investigation, mean values of anther culture-derived doubled haploid lines for yield and its components, were inferior to those lines obtained by the 'bulbosum' technique and pedigree and SSD systems (Rossnagel *et al.*, 1987). Selection of superior lines among segregating populations during pedigree breeding would account for their improved attributes as the anther culture-derived lines were obtained directly from F_1s. Despite their poorer overall performance, it was possible to identify lines from anther culture equal in performance to those selected conventionally (Rossnagel *et al.*, 1987). A summary of the main points to consider when comparing the two techniques is presented in Table 25.5, but in the future, greater improvements in success will more likely come from anther or isolated microspore culture than the 'bulbosum' technique. This forecast is based on the significant increases recently obtained in plant numbers regenerated from single anthers by changes in media and cultural conditions.

Optimum F generation for doubled haploid production

Although doubled haploid production from small numbers of F_1 hybrids offers great savings in time over conventional breeding programmes, to increase efficiency and break linkages it may be more effective to produce fewer doubled haploids from many more agronomically selected F_2 or F_3 plants (Simpson & Snape, 1981). One result of linkage disequilibrium present in hybrid material is that differences in means (if epistasis is present) and variances can be detected between doubled haploid populations derived from the F_1 and F_2 generations (Snape & Simpson, 1981). However, except for 'Lester', all of the doubled haploid

Table 25.5. A summary of the differences in requirements and results between the 'bulbosum' technique and anther culture (for spring barley).

Requirements and results	'Bulbosum' technique	Anther culture
No. of donor plants needed	*H. bulbosum* *H. vulgare*	*H. vulgare*
Labour requirements	similar	
Environmental requirements:		
vernalization chamber	✓	
heated glasshouse	✓	✓
growth room		(✓)
Callus stage		✓
Regenerants:		
albinos	–	+
spontaneously chromosome doubled	–	+
gametoclonal variation	–	+
random gamete selection	✓	Some deviations from expected
Success rates	Becoming comparable, but anther culture more genotype-dependent	

cultivars currrently registered or recommended have been obtained using the 'bulbosum' technique with F_1 hybrids (Table 25.6), or selected F_1s from first backcross ('Pipkin'; Habgood, pers. comm.). This may reflect the more usual practice in breeding programmes of using F_1s as starting material (Foroughi-Wehr *et al.*, 1982; Foroughi-Wehr & Friedt, 1984; Kao, 1988; Chen & Hayes, 1989; Devaux, 1989a; Kuhlmann & Foroughi-Wehr, 1989). However, Luckett (pers. comm.) prefers F_2s for anther culture having obtained a better *in vitro* response compared with F_1s and with the 'bulbosum' technique, selected F_2s and/or F_3s have also been used (Pickering,

Table 25.6. Doubled haploid barley cultivars currently available.

Name	Institution/country	Reference
'Mingo' (S)	Ciba-Geigy, Canada	Ho & Jones, 1980
'Rodeo' (S)	Ciba-Geigy, Canada	Campbell *et al.*, 1984
'Doublet' (S)	WPBS, UK	Jones *et al.*, 1985
'Gwylan' (S)	WPBS, UK*; DSIR, NZ**	Coles, 1986
'Pipkin' (W)	WPBS, UK	Jones *et al.*, 1986
'Waveney' (W)	ICI Seeds, UK	NIAB, 1988
'Craig' (S)	Thompson, Canada	Shugar, 1989
'Winthrop' (S)	Thompson, Canada	Shugar (pers. comm.)
'Michka' (S)	Desprez, France	Devaux, 1989b
'Odessky 115' (S)	Institute of Plant Breeding, Odessa, USSR	Navolotsky (pers. comm.)
'Lester' (S)†	Thompson, Canada	Shugar (pers. comm.)
'Lombard' (S)†	Desprez, France*; Italy**	Devaux, 1989b

S, spring; W, winter cultivar.

* Country where the doubled haploid has been produced.

** Country where the doubled haploid has been selected and cultivated.

† In course of registration.

1980a; Simpson & Snape, 1981). Doubled haploid production from later generation material (e.g. F_4, F_5) is a means to achieve homozygosity to satisfy the requirements of Plant Variety Rights (Griffin, pers. comm.; Jestin, pers. comm.).

Applications of haploids

The use of haploids in breeding new cultivars has been described briefly, and some examples of other applications in breeding techniques will now be outlined.

Identification of superior hybrid combinations

Plant breeders need to predict the potential value of crosses in breeding programmes at the earliest opportunity to discard inferior combinations. As few as 20 doubled haploids produced from early generation hybrids were required for this, after assessing yield in hill plots (Reinbergs *et al.*, 1976). More doubled haploids could then be produced from superior hybrid combinations. However, Bjørnstad & Aastveit (1990) pointed out that larger numbers of doubled haploids would be needed to obtain this result in cases where there are negative pleiotropic effects on the mean of a character.

Linkage analysis

Doubled haploids have been valuable for detecting linkage associated with quantitatively inherited characters, and can also be used for calculating recombination values between linked genes. The production of haploids from a heterozygous F_1 (assuming random gamete selection), reflects the genotype of the egg ('bulbosum' technique) or sperm (anther culture) and is equivalent to backcrossing the F_1 to the double recessive. Any significant deviation from expected ratios is therefore a result of linkage. The method has been successfully used to determine linkage and recombination values between genes on chromosome 7 (Thomas *et al.*, 1984) and chromosome 5 (Shewry *et al.*, 1980). In the latter case, the test involved analysing seed storage proteins so homozygous triploid endosperm tissue present in the doubled haploids was advantageous.

Pleiotropic effects of specified genes

The production of doubled haploids from heterozygous F_1s enables the researcher to study the effects of particular genes on quantitatively inherited characters. The procedure entails separating the doubled haploids into two groups according to which allele is present, and studying the effect on the quantitative characters under investigation. Any significant differences between the means of the two groups can then be considered to be a pleiotropic effect of the major gene. Snape & Simpson (1981) reported a deleterious effect of a dwarfing gene on several characters. Bjørnstad (1987), Bjørnstad & Aastveit (1990) and Kjaer *et al.* (1990) also recorded pleiotropic effects on several agronomic characters of the gene on chromosome 7 controlling grain lysine content and the *mlo* powdery mildew resistance genes on chromosome 4.

Restriction fragment length polymorphisms (RFLPs)

Many genes governing morphological characters and isozymes have been mapped on the barley genome (Tsuchiya, 1986; Søgaard & von Wettstein-Knowles, 1987), and multiple marker stocks have been produced for accurately locating and assigning genes to particular chromosomes (Franckowiak, 1987). The location of polygenes controlling quantitative characters such as yield, has not been easily carried out but could be aided by extracting doubled haploids from crosses between parents with contrasting marker genes (Choo, 1983). There is now, however, increasing interest in constructing a genome map based on molecular markers by RFLP analysis. In the same

way that doubled haploids can assist the research into conventional polygene mapping, homozygotes should therefore prove invaluable as an aid in linking polygenes to the molecular markers. Such a programme is being developed in barley in the United States (Hayes, pers. comm.) using the 'bulbosum' technique partly to avoid any variants which may arise with anther culture. The programme is only in its early stages and few data are available as yet.

Genetic engineering and mutations

These topics are discussed elsewhere in this volume, but haploid tissues could be used in biotechnology programmes. For example, after mutagenic treatment, recessive mutations are likely to be expressed in haploid regenerants without being masked by the dominant allele, and homozygotes obtained after chromosome doubling. Gaj (pers. comm.) has produced up to 21.7% mutants in the MII generation after treating haploid embryos (from *H. vulgare* × *H. bulbosum* crosses) with 1 mM *N*-methyl-*N*-nitrosourea for 3 h. As well as chlorophyll deficiencies most of the other mutants which arose were associated with isozyme variations but two semidwarfs were also identified from the same treatment. Immature haploid embryos have also been successfully cultured on media to induce callus formation and then regenerate plants after subculture of the calluses to auxin-free media. Regeneration was reported to be higher than from diploid embryo-derived calluses and the technique was subsequently used to assess the frequency of somaclonal variation, but unfortunately, very few useful variants were obtained (Pickering, 1989).

Calluses or embryoids derived either from haploid embryos or anther/microspore culture may provide a source of tissue for transformation research without the disadvantages associated with diploid cultures. For example, embryoids from anther culture have been microinjected with two plasmid constructs and the progeny screened for the gene encoding neomycine phosphotransferase (Olsen, pers. comm.). Sixteen plants which appeared to have increased resistance to kanamycin are being analysed further. Protoplasts derived from similar sources have also been successfully transformed using polyethylene glycol treatment and the reporter gene encoding chloramphenicol acetyl transferase (Olsen, pers. comm.).

Pathogen and *in vitro* screening

In addition to assisting with linkage studies described earlier, haploid production has been used effectively to regenerate recombinants rapidly from F_1 hybrids derived from crosses between cultivars resistant and susceptible to barley yellow mosaic virus (Foroughi-Wehr & Friedt, 1984). *In vitro* screening using haploid embryos or microspore callus has, however, rarely been reported, although diploid embryos have proved to be suitable explants for selecting biochemical mutants (Bright *et al.*, 1979) and plants with increased tolerance to herbicides (Gifford *et al.*, 1986). Anthers from a cross between salt tolerant and sensitive cultivars have been cultured on liquid media containing various concentrations of Na_2SO_4. All (six) green plants regenerated from the 'high salt' medium were more tolerant than the sensitive parent in subsequent germination tests (Ye *et al.*, 1987).

Regarding plant pathogens, the results of direct inoculation of powdery mildew (*Erysiphe graminis* f. sp. *hordei*) on to microspore calluses of barley have been unsuccessful (Franzone *et al.*, 1982). However, as it is known that certain plant pathogens produce phytotoxins which interfere with the host metabolism and are capable of reproducing some or all of the disease symptoms associated with the pathogen [e.g. *Drechslera* spp. (Shotwell & Ellis, 1976); *Rhynchosporium secalis* (Auriol *et al.*, 1978)] a more successful approach has been the incorporation of the toxin in tissue culture media before *in vitro* selection. Regeneration of green plants tolerant to *Drechslera* spp. has been carried out in maize (Gengenbach *et al.*, 1977), barley and wheat (Chawla & Wenzel, 1987), but the technique was not successful in identifying

any resistance to rhynchosporoside, a toxin produced by *R. secalis* (Branchard, 1982).

Although there is only a limited number of barley pathogens for which these screening techniques would be suitable for haploid explants, the savings in labour, materials and space would be considerable.

Conclusions

In this chapter we have outlined the history of doubled haploid barley production over a 20-year period. The techniques of anther culture and interspecific hybridization are now used extensively for cultivar production, genetic analysis and molecular biological research. Improvements in success rates should continue to be achieved in the 1990s and provide us with a greater understanding of the mechanisms involved in albinism, chromosome elimination and incompatibility systems. Furthermore, the regeneration of green barley plants from protoplasts (e.g. Jähne *et al.*, 1990) makes it clear that haploid cells and explants will increasingly be sought after for genetic manipulation experiments such as biolistics and microinjection. We can therefore look forward to an exciting decade in which haploid barley will play a key role in maintaining the momentum of agricultural research and development.

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References

Adamski, T. (1979) The obtaining of autodiploid barley lines using haploids from the cross *Hordeum vulgare* L. × *Hordeum bulbosum* L. *Genetica Polonica* 20, 31–42.

Auriol, P., Strobel, G., Beltran, J.P. & Gray, G. (1978) Rhynchosporoside, a host-selective toxin produced by *Rhynchosporium secalis*, the causal agent of scald disease of barley. *Proceedings of the National Academy of Sciences (USA)* 75, 4339–43.

Bjørnstad, Å. (1986) Partial incompatibility between Scandinavian six-rowed barleys (*Hordeum vulgare* L.) and *Hordeum bulbosum* L., and its genetical basis. *Hereditas* 104, 171–91.

Bjørnstad, Å. (1987) The role of linkage and pleiotropy in doubled haploid lines from a barley cross. *Hereditas* 106, 155–68.

Bjørnstad, Å. & Aastveit, K. (1990) Pleiotropic effects of the *ml-o* mildew resistance gene in barley in different genetical backgrounds. *Euphytica* 46, 217–26.

Bothmer, R. von & Subrahmanyam, N.C. (1988) Assessment of chromosome associations in haploids and their parental accessions in *Hordeum*. *Genome* 30, 204–10.

Bothmer, R. von, Flink, J., Jacobsen, N., Kotimäki, M. & Landström, T. (1983) Interspecific hybridization with cultivated barley (*Hordeum vulgare* L.). *Hereditas* 99, 219–44.

Bothmer, R. von, Jacobsen, N., Jørgensen, R.B. & Linde-Laursen, I. (1984) Haploid barley from the intergeneric cross *Hordeum vulgare* × *Psathyrostachys fragilis*. *Euphytica* 33, 363–7.

Branchard, M. (1982) *In vitro* culture of barley: a method to study *Rhynchosporium* scald disease and select plants resistant to the toxin, Rhynchosporoside. In: Earle, E.D. & Demarly, Y. (eds), *Variability in Plants Regenerated from Tissue Culture*. Praeger, New York, USA, pp. 343–50.

Bright, S.W.J., Norbury, P.B. & Miflin, B.J. (1979) Isolation of a recessive barley mutant resistant to *S*-(2-aminoethyl) L-cysteine. *Theoretical and Applied Genetics* 55, 1–4.

Brown, S. (1989) Sondes fluorescentes, analyse et tri de cellules par cytofluorométrie. *Le Selectionneur Français* 40, 21–30.

Campbell, K.W., Brown, R.I. & Ho, K.M. (1984) Rodeo barley. *Canadian Journal of Plant Science* 64, 203–5.

Chawla, H.S. & Wenzel, G. (1987) *In vitro* selection of barley and wheat for resistance against *Helminthosporium sativum*. *Theoretical and Applied Genetics* 74, 841–5.

Chen, F.Q. & Hayes, P.M. (1989) A comparison of *Hordeum bulbosum*-mediated haploid production efficiency in barley using *in vitro* floret and tiller culture. *Theoretical and Applied Genetics* 77, 701–4.

Chen, C.C., Kasha, K.J. & Marsolais, A. (1984) Segmentation patterns and mechanisms of genome multiplication in cultured microspores of barley. *Canadian Journal of Genetics and Cytology* 26, 475–83.

Cho, U.H. & Kasha, K.J. (1989) Ethylene production and embryogenesis from anther cultures of barley (*Hordeum vulgare*). *Plant Cell Reports* 8, 415–17.

Choo, T.M. (1983) Doubled haploids for locating polygenes. *Canadian Journal of Genetics and Cytology* 25, 425–9.

Choo, T.M., Christie, B.R. & Reinbergs, E. (1979) Doubled haploids for estimating genetic variances and a scheme for population improvement in self-pollinating crops. *Theoretical and Applied Genetics* 54, 267–71.

Choo, T.M., Reinbergs, E. & Kasha, K.J. (1985) Use of haploids in breeding barley. *Plant Breeding Reviews* 3, 219–52.

Chu, C.C. (1978) The N_6 medium and its applications to anther culture of cereal crops. In: *Proceedings of Symposium on Plant Tissue Culture*. Science Press, Beijing, China, pp. 43–50.

Chuang, C.C., Ouyang, T.W., Chia, H., Chou, S.M. & Ching, C.K. (1978) A set of potato media for wheat anther culture. In: *Proceedings of Symposium of Plant Tissue Culture*. Science Press, Beijing, China, pp. 51–6.

Clapham, D. (1971) *In vitro* development of callus from the pollen of *Lolium* and *Hordeum*. *Zeitschrift für Pflanzenzüchtung* 65, 285–92.

Clapham, D. (1973) Haploid *Hordeum* plants from anthers *in vitro*. *Zeitschrift für Pflanzenzüchtung* 69, 142–55.

Coles, G.D. (1986) 'Gwylan' barley (*Hordeum vulgare* L.). *New Zealand Journal of Experimental Agriculture* 14, 101–3.

Dale, P.J. (1975) Pollen dimorphism and anther culture in barley. *Planta* 127, 213–20.

Datta, S.K. (1987) Plant regeneration by pollen embryogenesis from cultured whole spikes of barley (*Hordeum vulgare*). *Theoretical and Applied Genetics* 74, 121–4.

Datta, S.K. & Wenzel, G. (1988) Single microspore derived embryogenesis and plant formation in barley (*Hordeum vulgare* L.). *Archiv für Züchtungsforschung, Berlin* 18, 125–31.

Davies, D.R. & Hopwood, D.A. (eds) (1980) *Proceedings of the Fourth John Innes Symposium the Plant Genome and Second International Haploid Conference*. The John Innes Charity, Norwich, England.

Day, A. & Ellis, T.H.N. (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. *Current Genetics* 9, 671–8.

Devaux, P. (1986) Yield of haploid production through the bulbosum method in a winter barley breeding programme. *Cereal Research Communications* 14, 273–9.

Devaux, P. (1987) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploids in winter barley. I. Production of green plants. *Plant Breeding* 98, 215–19.

Devaux, P. (1988) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploids in winter barley. II. Variation of chromosome number and seed set in the progeny. *Plant Breeding* 100, 181–7.

Devaux, P. (1989a) Variations in the proportions of fertile colchicine-treated haploid plants derived from winter barley hybrids. *Plant Breeding* 103, 247–50.

Devaux, P. (1989b) Haplodiploidisation chez l'orge. In: *Biotechnology for Major Crops*. Salon International des Techniques et Energies du Futur, Toulouse, France, pp. 12–18.

Devaux, P. (1990) Individual and combined effects of glutamine and maltose on anther culture response of the winter barley cultivar 'Igri'. *Bulletin de la Société Botanique de France, Actualitiés Botaniques* 137, 142–3.

Devaux, P. & Jean, R. (1985) Influence du mois et de la technique de castration dans la réussite de l'haploid diploidisation de l'orge par la méthode *bulbosum*. *Agronomie* 5, 795–800.

Devaux, P., Adamski, T. & Surma, M. (1990) Studies on low crossabilities encountered with the *Hordeum bulbosum* method for haploid production of barley, *Hordeum vulgare* L. *Plant Breeding* 104, 305–11.

Doll, H., Haahr, V. & Søgaard, B. (1989) Relationship between vernalization requirement and winter hardiness in doubled haploids of barley. *Euphytica* 42, 209–13.

Dunwell, J.M. (1985) Anther and ovary culture. In: Bright, S.W.J. & Jones, M.J.K. (eds), *Cereal Tissue and Cell Culture*. Martinus Nijhoff/Dr W. Junk, Dordrecht, The Netherlands, pp. 1–44.

Dunwell, J.M., Francis, R.J. & Powell, W. (1987) Anther culture of *Hordeum vulgare* L.: a genetic study of microspore callus production and differentiation. *Theoretical and Applied Genetics* 74, 60–4.

Fedak, G. (1977) Haploids from barley × rye

crosses. *Canadian Journal of Genetics and Cytology* 19, 15–19.

Fedak, G. & Jui, P.Y. (1982) Chromosomes of Chinese Spring wheat carrying genes for crossability with Betzes barley. *Canadian Journal of Genetics and Cytology* 24, 227–33.

Finch, R.A. & Bennett, M.D. (1979) Meiotic stability in control and newly colchicine-induced dihaploid barley. *Canadian Journal of Genetics and Cytology* 21, 33–5.

Finnie, S.J., Powell, W. & Dyer, A.F. (1989) The effect of carbohydrate composition and concentration on anther culture response in barley (*Hordeum vulgare* L.). *Plant Breeding* 103, 110–18.

Foroughi-Wehr, B. & Friedt, W. (1984) Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture. *Theoretical and Applied Genetics* 67, 377–82.

Foroughi-Wehr, B. & Mix, G. (1979) *In vitro* response of *Hordeum vulgare* L. anthers cultured from plants grown under different environments. *Environmental and Experimental Botany* 19, 303–9.

Foroughi-Wehr, B., Mix, G., Gaul, H. & Wilson, H.M. (1976) Plant production from cultured anthers of *Hordeum vulgare* L. *Zeitschrift für Pflanzenzüchtung* 77, 198–204.

Foroughi-Wehr, B., Friedt, W. & Wenzel, G. (1982) On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. *Theoretical and Applied Genetics* 62, 233–9.

Franckowiak, J.D. (1987) Multiple recessive marker stocks for mapping male-sterile genes in barley. In: Yasuda, S. & Konishi, T. (eds), *Barley Genetics V* (Proceedings of the 5th International Barley Genetics Symposium). Maruzen, Okayama, Japan, pp. 209–12.

Franzone, P.J., Foroughi-Wehr, B., Fischbeck, G. & Friedt, W. (1982) Reaction of microspore callus, androgenetic albino plantlets and roots of barley to *Erysiphe graminis* f.sp. *hordei*. *Phytopathologische Zeitschrift* 105, 170–4.

Friedt, W. & Foroughi-Wehr, B. (1981) Anther culture of barley (*Hordeum vulgare* L.): plant regeneration and agronomic performance of homozygous diploid progenies. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 690–8.

Friedt, W. & Foroughi-Wehr, B. (1983) Field performance of androgenetic doubled haploid spring barley from F_1 hybrids. *Zeitschrift für Pflanzenzüchtung* 90, 177–84.

Friedt, W., Braun, J., Züchner, S. & Foroughi-Wehr, B. (1986) Comparative value of androgenetic doubled haploid and conventionally selected spring barley lines. *Plant Breeding* 97, 56–63.

Friedt, W., Foroughi-Wehr, B. & Snape, J.W. (1987) The significance of biotechnology for the evolution of barley breeding methods. In: Yasuda, S. & Konishi, T. (eds), *Barley Genetics V* (Proceedings of the 5th International Barley Genetics Symposium). Maruzen, Okayama, Japan, pp. 903–13.

Gamborg, O.L., Miller, R.A. & Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50, 151–8.

Gaul, H., Mix, G., Foroughi-Wehr, B. & Okamoto, M. (1976) Pollen grain development of *Hordeum vulgare*. *Zeitschrift für Pflanzenzüchtung* 76, 77–80.

Gengenbach, B.G., Green, C.E. & Donovan, C.M. (1977) Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. *Proceedings of the National Academy of Sciences (USA)* 74, 5113–17.

Gifford, S.C., Collin, H.A. & Putwain, P.D. (1986) Enhancement of asulam resistance in barley. *Weed Research* 26, 389–96.

Gilbert, S.K. & Patterson, F.L. (1965) Colchicine-induced mutants in Decatur barley. *Crop Science* 5, 44–7.

Grunewaldt, J. & Malepszy, S. (1975) Observations on anther callus from *Hordeum vulgare* L. In: Gaul, H. (ed.), *Barley Genetics III* (Proceedings of the 3rd International Barley Genetics Symposium). Verlag Karl Thiemig, Munich, Germany, pp. 367–73.

Hagberg, A. & Hagberg, G. (1980) High frequency of spontaneous haploids in the progeny of an induced mutation in barley. *Hereditas* 93, 341–3.

Hagberg, A. & Hagberg, G. (1987) Production of spontaneously doubled haploids in barley using a breeding system with marker genes and the 'hap'-gene. *Biologisches Zentralblatt* 106, 53–8.

Hayes, P.M. & Chen, F.Q. (1989) Genotypic variation for *Hordeum bulbosum* L.-mediated haploid production in winter and facultative barley. *Crop Science* 29, 1184–8.

Ho, K.M. & Jones, G.E. (1980) Mingo barley. *Canadian Journal of Plant Science* 60, 279–80.

Ho, K.M. & Kasha, K.J. (1975) Genetic control of chromosome elimination during haploid formation in barley. *Genetics* 81, 263–75.

Hou, L. & Ullrich, S.E. (1990) Effect of genotype and donor plant environment on anther culture of barley. *14th American Barley Research Workshop*, 13–17 May 1990, Sacramento, USA (abstract).

Huang, B. & Sunderland, N. (1982) Temperature-stress pretreatment in barley anther culture. *Annals of Botany* 49, 77–88.

Huang, B., Dunwell, J.M., Powell, W., Hayter, A.M. & Wood, W. (1984) The relative efficiency of microspore culture and chromosome elimination as methods of haploid production in *Hordeum vulgare* L. *Zeitschrift für Pflanzenzüchtung* 92, 22–9.

Hunter, C.P. (1985) The effect of anther orientation on the production of microspore-derived embryoids and plants of *Hordeum vulgare* cv. Sabarlis. *Plant Cell Reports* 4, 267–8.

Hunter, C.P. (1987) Plant generation method. *European Patent Application*, No. 87200773.7.

Hunter, C.P., Loose, R.W., Clerk, S.P. & Lyne, R.L. (1989) Maltose – the preferred carbon source for barley anther culture. In: *International Congress by EUCARPIA, Genetic Manipulation in Plant Breeding – Biotechnology for the Breeder.*

Islam, A.K.M.R. & Shepherd, K.W. (1981) Wheat–barley addition lines: their use in genetic and evolutionary studies of barley. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 729–39.

Jähne, A., Lazzeri, P.A., Jäger-Gussen, M. & Lörz, H. (1990) Anther-culture-derived suspension cultures of barley (*Hordeum vulgare*); establishment, plant regeneration and protoplast culture. In: *VIIth International Congress on Plant Tissue and Cell Culture*, Amsterdam 24–29 June 1990 (abstract).

Jalani, B.S. & Moss, J.P. (1980) The site of action of the crossability genes (Kr_1,Kr_2) between *Triticum* and *Secale*. I. Pollen germination, pollen tube growth, and number of pollen tubes. *Euphytica* 29, 571–9.

Jensen, C.J. (1976) Barley monoploids and doubled monoploids: techniques and experience. In: Gaul, H. (ed.), *Barley Genetics III* (Proceedings of the 3rd International Barley Genetics Symposium). Verlag Karl Thiemig, Munich, Germany, pp. 316–45.

Jensen, C.J. (1981) Regenerating monoploid barley plants from cells, tissues and organs from various origins. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 677–85.

Jing, J. & Hu, H. (1981) Anther and immature inflorescence culture in *Hordeum vulgare*. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 945–8.

Jones, D.L., Jones, D.M., Clifford, B.C., Clothier, R.B., Cook, R., Griffiths, T.E.R., Jones, E.W.C. & Jones, I.T. (1985) Cereal breeding, barley. In: *Annual Report of the Welsh Plant Breeding Station for 1984*, pp. 65–6.

Jones, D.M., Clothier, R.B. & Griffiths, T.E.R. (1986) Cereal breeding, barley. In: *Annual Report of the Welsh Plant Breeding Station for 1985*, pp. 74–5.

Kao, K.N. (1981) Plant formation from barley anther cultures with Ficoll media. *Zeitschrift für Pflanzenphysiologie* 103, 437–43.

Kao, K.N. (1988) *In vitro* culture in barley breeding. In: *Semi-Dwarf Cereal Mutants and their Use in Cross-Breeding III* (Proceedings of the Final Research Coordination Meeting on Evaluation of Semi-Dwarf Cereal Mutants for Cross-Breeding). International Atomic Energy Agency, Vienna, Austria, pp. 183–91.

Kao, K.N. & Horn, D.C. (1982) A method for induction of pollen plants in barley. In: Fujiwara, A. (ed.), *Plant Tissue Culture* (Proceedings of the 5th International Congress of Plant Tissue and Cell Culture). Japanese Association of Plant Tissue Culture, Tokyo, Japan, pp. 529–30.

Kao, K.N. & Kasha, K.J. (1969) Haploidy from interspecific crosses with tetraploid barley. In: Nilan, R.A. (ed.), *Barley Genetics II* (Proceedings of the 2nd International Barley Genetics Symposium). Washington State University Press, Pullman, USA, pp. 82–8.

Kasha, K.J. (ed.) (1974) *Haploids in Higher Plants: Advances and Potential* (Proceedings

of the 1st International Symposium). University of Guelph, Guelph, Canada.

Kasha, K.J. (1989) Production of haploids in cereals. In: Maluszynski, M. (ed.), *Current Options for Cereal Improvement: Doubled Haploids, Mutants and Heterosis*. (Proceedings of the First FAO/IAEA Research Co-ordination Meeting on 'Use of Induced Mutations in Connection with Haploids and Heterosis in Cereals'. Kluwer, Dordrecht, The Netherlands, pp. 71–80.

Kasha, K.J. & Kao, K.N. (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). *Nature* 225, 874–6.

Kasha, K.J. & Reinbergs, E. (1981) Recent developments in the production and utilization of haploids in barley. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 655–65.

Kasha, K.J., Subrahmanyam, N.C. & Ali, A. (1978) Effect of gibberellic acid treatment, and nutrient supply through detached tillers, upon haploid frequency in barley. *Theoretical and Applied Genetics* 51, 169–75.

Kasha, K.J., Ziauddin, A. & Cho, U.H. (1989) Haploids in cereal improvement: anther and microspore culture. In: *XIXth Stadler Genetics Symposium*, pp. 213–36.

Kjaer, B., Jensen, H.P., Jensen, J. & Jørgensen, J.H. (1990) Associations between three *ml-o* powdery mildew resistance genes and agronomic traits in barley. *Euphytica* 46, 185–93.

Knudsen, S., Due, I.K. & Andersen, S.B. (1989) Components of response in barley anther culture. *Plant Breeding* 103, 241–6.

Kohlenbach, H.W. & Wernicke, W. (1978) Investigations on the inhibitory effect of agar and the function of active carbon in anther culture. *Zeitschrift für Pflanzenphysiologie* 86, 463–72.

Köhler, F. & Wenzel, G. (1985) Regeneration of isolated barley microspores in conditioned media and trials to characterize the responsible factor. *Journal of Plant Physiology* 121, 181–91.

Konzak, C.F., Randolph, L.F. & Jensen, N.F. (1951) Embryo culture of barley species hybrids. Cytological studies of *Hordeum sativum* × *Hordeum bulbosum*. *Journal of Heredity* 42, 124–34.

Kuckuck, H. (1934) Artkreuzungen bei Gerste. *Der Züchter* 6, 270–3.

Kuhlmann, U. & Foroughi-Wehr, B. (1989) Production of doubled haploid lines in frequencies sufficient for barley breeding programs. *Plant Cell Reports* 8, 78–81.

Larter, E. & Chaubey, C. (1965) Use of exogenous growth substances in promoting pollen tube growth and fertilization in barley–rye crosses. *Canadian Journal of Genetics and Cytology* 7, 511–18.

Laurie, D.A. & Bennett, M.D. (1988) The production of haploid wheat plants from wheat × maize crosses. *Theoretical and Applied Genetics* 76, 393–7.

Lee, F.M. & Chen, C.C. (1987) Nuclear fusion in cultured microspores of barley. *Plant Cell Reports* 6, 191–3.

Linsmaier, E.M. & Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum* 18, 100–27.

Lyne, R.L., Bennett, R.I. & Hunter, C.P. (1986) Embryoid and plant production from cultured barley anthers. In: Withers, L.A. & Alderson, P.G. (eds), *Plant Tissue Culture and its Agricultural Applications, University of Nottingham*. Butterworths, Guildford, UK, pp. 405–11.

Malepszy, S. & Grunewaldt, J. (1974) Ein Beitrag zur Erzeugung von Haploiden bei *Hordeum vulgare* L. *Zeitschrift für Pflanzenzüchtung* 72, 206–11.

Maluszynski, M. (ed.) (1989) *Current Options for Cereal Improvement: Doubled Haploids, Mutants and Heterosis* (Proceedings of the First FAO/IAEA Research Co-ordination Meeting on 'Use of Induced Mutations in Connection with Haploids and Heterosis in Cereals'). Kluwer, Dordrecht, The Netherlands.

Marsolais, A.A. & Kasha, K.J. (1985) Callus induction from barley microspores. The role of sucrose and auxin in a barley anther culture medium. *Canadian Journal of Botany* 63, 2209–12.

Miller, C.O. (1963) Kinetin and kinetin-like compounds. In: Linskens, H.F. & Tracey, M.V. (eds), *Moderne Methoden in der Pflanzenanalyse*, Vol. VI. Springer, Berlin, Germany, pp. 194–202.

Mix, G., Wilson, H.M. & Foroughi-Wehr, B. (1978) The cytological status of plants of *Hordeum vulgare* L. regenerated from microspore callus. *Zeitschrift für Pflanzenzüchtung* 80, 89–99.

Mogensen, H.L. (1982) Double fertilization in barley and the cytological explanation for haploid embryo formation, embryoless

caryopses, and ovule abortion. *Carlsberg Research Communications* 47, 313–54.

Morrison, J.W., Hannah, A.E., Loiselle, R. & Symko, S. (1959) Cytogenetic studies in genus *Hordeum* II. Interspecific and intergeneric crosses. *Canadian Journal of Plant Science* 39, 375–83.

Murashige, T. & Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* 15, 473–97.

NIAB (1988) *Recommended Varieties of Cereals, Farmers Leaflet Number 8*. National Institute of Agricultural Botany, Cambridge, UK.

Nitsch, C. (1974) Pollen culture - a new technique for mass production of haploid and homozygous plants. In: Kasha, K.J. (ed.), *Haploids in Higher Plants: Advances and Potential* (Proceedings of the 1st International Symposium). University of Guelph, Guelph, Canada, pp. 123–35.

Olsen, F.L. (1987) Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. *Carlsberg Research Communications* 52, 393–404.

Park, S.J., Walsh, E.J., Reinbergs, E., Song, L.S.P. & Kasha, K.J. (1976) Field performance of doubled haploid barley lines in comparison with lines developed by the pedigree and single seed descent methods. *Canadian Journal of Plant Science* 56, 467–74.

Patel, J.D., Reinbergs, E. & Fejer, S.O. (1985) Recurrent selection in doubled-haploid populations of barley (*Hordeum vulgare* L.). *Canadian Journal of Genetics and Cytology* 27, 172–7.

Pickering, R.A. (1980a) Use of the doubled haploid technique in barley breeding at the Welsh Plant Breeding Station. In: *Annual Report of the Welsh Plant Breeding Station for 1979*, pp. 208–26.

Pickering, R.A. (1980b) Attempts to overcome partial incompatibility between *Hordeum vulgare* L. and *H. bulbosum* L. *Euphytica* 29, 369–77.

Pickering, R.A. (1981) Pollen tube-stylodium interaction in *Hordeum vulgare* L. × *H. bulbosum* L. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 666–76.

Pickering, R.A. (1983a) The influence of genotype on doubled haploid barley production. *Euphytica* 32, 863–76.

Pickering, R.A. (1983b) The location of a gene for incompatibility between *Hordeum vulgare* L. and *H. bulbosum* L. *Heredity* 51, 455–9.

Pickering, R.A. (1984) Crossability relationships between certain species in the Hordeae. *Barley Genetics Newsletter* 14, 14–17.

Pickering, R.A. (1985) Partial control of chromosome elimination by temperature in immature embryos of *Hordeum vulgare* L. × *H. bulbosum* L. *Euphytica* 34, 869–74.

Pickering, R.A. (1989) Plant regeneration and variants from calli derived from immature embryos of diploid barley (*Hordeum vulgare* L.) and *H. vulgare* L. × *H. bulbosum* L. crosses. *Theoretical and Applied Genetics* 78, 105–12.

Pickering, R.A. & Rennie, W.F. (1990) The evaluation of superior *Hordeum bulbosum* L. genotypes for use in a doubled haploid barley breeding programme. *Euphytica* 45, 251–5.

Powell, W. (1988) Diallel analysis of barley anther culture response. *Genome* 30, 152–7.

Powell, W., Hayter, A.M., Wood, W., Dunwell, J.M. & Huang, B. (1984) Variation in the agronomic characters of microspore-derived plants of *Hordeum vulgare* cv. Sabarlis. *Heredity* 52, 19–23.

Powell, W., Borrino, E.M., Allison, M.J., Griffiths, D.W., Asher, M.J.C. & Dunwell, J.M. (1986) Genetical analysis of microspore derived plants of barley (*Hordeum vulgare*). *Theoretical and Applied Genetics* 72, 619–26.

Powell, W., Borrino, E.M. & Goodall, V. (1988) The effect of anther orientation on microspore-derived plant production in barley (*Hordeum vulgare* L.). *Euphytica* 38, 159–63.

Reinbergs, E., Park, S.J. & Song, L.S.P. (1976) Early identification of superior barley crosses by the doubled haploid technique. *Zeitschrift für Pflanzenzüchtung* 76, 215–24.

Reinbergs, E., Song, L.S.P., Choo, T.M. & Kasha, K.J. (1978) Yield stability of doubled haploid lines of barley. *Canadian Journal of Plant Science* 58, 929–33.

Riley, R. & Chapman, V. (1967) The inheritance in wheat of crossability with rye. *Genetical Research* 9, 259–67.

Roberts-Oehlschlager, S.L. & Dunwell, J.M. (1990) Barley anther culture: pretreatment on mannitol stimulates production of micro-

spore derived embryos. *Plant Cell, Tissue and Organ Culture*, 20, 235–40.

Roberts-Oehlschlager, S.L., Dunwell, J.W. & Foulks, R. (1990) Changes in the sugar content of barley anthers during culture on different carbohydrates. *Plant Cell, Tissue and Organ Culture* 22, 77–85.

Rossnagel, B.G., Sariah, M.A. & Kao, K.N. (1987) Field evaluation of anther culture derived breeding materials compared to materials developed by the pedigree and single seed descent methods in barley (*Hordeum vulgare* L.). In: Yasuda, S. & Konishi, T. (eds), *Barley Genetics V* (Proceedings of the 5th International Barley Genetics Symposium). Maruzen, Okayama, Japan, pp. 997–1004.

Saalbach, G. & Koblitz, H. (1977) Karyological instabilities in callus cultures from haploid barley plants. *Biochemie und Physiologie der Pflanzen* 171, 469–73.

San Noeum, L.H. (1976) Haploïdes d'*Hordeum vulgare* L. par culture *in vitro* d'ovaires non fécondés. *Annales de l'Amélioration des Plantes* 26, 751–4.

San Noeum, L.H. & Ahmadi, N. (1982) Variability of doubled haploids from *in vitro* androgenesis and gynogenesis in *Hordeum vulgare* L. In: Earle, E.D. & Demarly, Y. (eds), *Variability in Plants Regenerated from Tissue Culture*. Praeger, New York, USA, pp. 273–83.

Shannon, P.R.M., Nicholson, A.E., Dunwell, J.M. & Davies, D.R. (1985) Effect of anther orientation on microspore-callus production in barley (*Hordeum vulgare* L.). *Plant Cell, Tissue and Organ Culture* 4, 271–80.

Shewry, P.R., Faulks, A.J., Pickering, R.A., Jones, I.T., Finch, R.A. & Miflin, B.J. (1980) The genetic analysis of barley storage proteins. *Heredity* 44, 383–9.

Shotwell, O.L. & Ellis, J.J. (1976) *Helminthosporium, Drechslera*, and *Bipolaris* toxins. *Advances in Chemistry* 149, 318–43.

Shugar, L.P. (1989) Commercialization of the bulbosum method in barley (*Hordeum vulgare* L.). In: Maluszynski, M. (ed.), *Current Options for Cereal Improvement: Doubled Haploids, Mutants and Heterosis* (Proceedings of the 1st FAO/IAEA Research Co-ordination Meeting on 'Use of Induced Mutations in Connection with Haploids and Heterosis in Cereals'). Kluwer, Dordrecht, The Netherlands, pp. 161–70.

Simpson, E. & Snape, J.W. (1979) Differences between erect and prostrate genotypes of spring barley. *Barley Genetics Newsletter* 9, 97–8.

Simpson, E. & Snape, J.W. (1981) The use of doubled haploids in a winter barley programme. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 716–20.

Simpson, E., Snape, J.W. & Finch, R.A. (1980) Variation between *Hordeum bulbosum* genotypes in their ability to produce haploids of barley, *Hordeum vulgare*. *Zeitschrift für Pflanzenzüchtung* 85, 205–11.

Sitch, L.A. & Snape, J.W. (1987) Factors affecting haploid production in wheat using the *Hordeum bulbosum* system. I. Genotypic and environmental effects on pollen grain germination, pollen tube growth and the frequency of fertilization. *Euphytica* 35, 483–96.

Snape, J.W. & Simpson, E. (1981) Uses of doubled haploid lines for genetical analysis in barley. In: Asher, M.J.C., Ellis, R.P., Hayter, A.M. & Whitehouse, R.N.H. (eds), *Barley Genetics IV* (Proceedings of the 4th International Barley Genetics Symposium). Edinburgh University Press, Edinburgh, UK, pp. 704–9.

Snape, J.W., Bennett, M.D. & Simpson, E. (1980) Post-pollination events in crosses of hexaploid wheat with tetraploid *Hordeum bulbosum*. *Zeitschrift für Pflanzenzüchtung* 85, 200–4.

Snape, J.W., Sitch, L.A., Simpson, E. & Parker, B.B. (1988) Tests for the presence of gametoclonal variation in barley and wheat doubled haploids produced using the *Hordeum bulbosum* system. *Theoretical and Applied Genetics* 75, 509–13.

Song, L.S.P., Park, S.J., Reinbergs, E., Choo, T.M. & Kasha, K.J. (1978) Doubled haploid vs the bulk plot method for production of homozygous lines in barley. *Zeitschrift für Pflanzenzüchtung* 81, 271–80.

Sorvari, S. (1986) The effect of starch gelatinized nutrient media in barley anther cultures. *Annales Agriculturae Fenniae* 25, 127–33.

Sorvari, S. & Schieder, O. (1987) Influence of sucrose and melibiose on barley anther cultures in starch media. *Plant Breeding* 99, 164–71.

Steinbiss, H.H., Stabel, P., Töpfer, R., Hirtz, R.D. & Schell, J. (1985) Transformation of

plant cells by microinjection of DNA. In: Chapman, G.P., Mantell, S.H. & Daniels, R.W. (eds), *The Experimental Manipulation of Ovule Tissues*. Longman, New York, USA, pp. 64–75.

Subrahmanyam, N.C. (1978) Meiosis in polyhaploid *Hordeum*: hemizygous ineffective control of diploid-like behaviour in a hexaploid? *Chromosoma* 66, 185–92.

Subrahmanyam, N.C. (1980) Haploidy from *Hordeum* interspecific crosses. 3. Trihaploids of *H. arizonicum* and *H. lechleri*. *Theoretical and Applied Genetics* 56, 257–63.

Subrahmanyam, N.C. (1982) Species dominance in chromosome elimination in barley hybrids. *Current Science* 51, 28–31.

Subrahmanyam, N.C. & Kasha, K.J. (1973) Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. *Chromosoma* 42, 111–25.

Sunderland, N. (1974) Anther culture as a means of haploid induction. In: Kasha, K.J. (ed.), *Haploids in Higher Plants: Advances and Potential* (Proceedings of the 1st International Symposium). University of Guelph, Guelph, Canada, pp. 91–122.

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

Sunderland, N. & Dunwell, J.M. (1977) Anther and pollen culture. In: Street, H.E. (ed.), *Plant Tissue and Cell Culture*, 2nd edn. Blackwell, Oxford, pp. 223–65.

Sunderland, N. & Evans, L.J. (1980) Multicellular pollen formation in cultured barley anthers. *Journal of Experimental Botany* 31, 501–14.

Sunderland, N. & Huang, B. (1985) Barley anther culture – the switch of programme and albinism. *Hereditas* supplement 3, 27–40.

Sunderland, N. & Xu, Z.H. (1982) Shed pollen culture in *Hordeum vulgare*. *Journal of Experimental Botany* 33, 1086–95.

Sunderland, N., Roberts, M., Evans, L.J. & Wildon, D.C. (1979) Multicellular pollen formation in cultured barley anthers. *Journal of Experimental Botany* 30, 1133–44.

Sunderland, N., Huang, B. & Hills, J. (1984) Disposition of pollen *in situ* and its relevance to anther/pollen culture. *Journal of Experimental Botany* 35, 521–30.

Swanson, E.B., Coumans, M.P., Wu, S.C., Barsby, T.L. & Beversdorf, W.D. (1987) Efficient isolation of microspores and the production of microspore-derived embryos from *Brassica napus*. *Plant Cell Reports* 6, 94–7.

Szarejko, I. & Kasha, K.J. (1988) Media developments for barley anther culture. In: *University of Guelph, Department of Crop Science, Annual Report 1988*, pp. 119–21.

Søgaard, B. & von Wettstein-Knowles, P. (1987) Barley: genes and chromosomes. *Carlsberg Research Communications* 52, 123–96.

Tang, Z.K. & Xie, J.X. (1987) Investigations of *in vitro* induced parthenogenesis in barley (*Hordeum vulgare* L.). In: Yasuda, S. & Konishi, T. (eds), *Barley Genetics V* (Proceedings of the 5th International Barley Genetics Symposium). Maruzen, Okayama, Japan, pp. 1005–7.

Thiebaut, J., Kasha, K.J. & Tsai, A. (1979) Influence of plant development stage, temperature, and plant hormones on chromosome doubling of barley haploids using colchicine. *Canadian Journal of Botany* 57, 480–3.

Thomas, J.B., Kaltsikes, P.J. & Anderson, R.G. (1981) Relation between wheat–rye crossability and seed set of common wheat after pollination with other species in the Hordeae. *Euphytica* 30, 121–7.

Thomas, W.T.B., Powell, W. & Wood, W. (1984) The chromosomal location of the dwarfing gene present in the spring barley variety Golden Promise. *Heredity* 53, 177–83.

Tometorp, G. (1939) Cytological studies on haploid *Hordeum distichum*. *Hereditas* 25, 241–54.

Tsuchiya, T. (1986) List of genetic stocks with BGS numbers. *Barley Genetics Newsletter* 16, 81–121.

Wang, C.C. & Kuang, B.J. (1981) Induction of haploid plants from the female gametophyte of *Hordeum vulgare* L. *Acta Botanica Sinica* 23, 329–30.

Wei, Z.M., Kyo M. & Harada, H. (1986) Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. 'Sabarlis'. *Theoretical and Applied Genetics* 72, 252–5.

White, P.R. (1943) *A Handbook of Plant Tissue Culture*. Jacques Cattell, Lancaster, Pennsylvania, USA.

Wilson, H.M. (1977) Culture of whole barley spikes stimulates high frequencies of pollen calluses in individual anthers. *Plant Science Letters* 9, 233–8.

Wilson, H.M., Foroughi-Wehr, B. & Mix,

G. (1977) Haploids in *Hordeum vulgare* through anther culture: potential as starting material for genetic manipulation *in vitro*. In: Sanchez-Monge, E. & García-Olmedo, F. (eds), *Interspecific Hybridization in Plant Breeding* (Proceedings of the 8th Eucarpia Congress). Universidad Politecnica de Madrid, Madrid, Spain, pp. 243–52.

Wilson, H.M., Mix, G. & Foroughi-Wehr, B. (1978) Early microspore divisions and subsequent formation of microspore calluses at high frequency in anthers of *Hordeum vulgare* L. *Journal of Experimental Botany* 29, 227–38.

Xu, Z.H. & Sunderland, N. (1981) Glutamine, inositol and conditioning factor in the production of barley pollen callus *in vitro*. *Plant Science Letters* 23, 161–8.

Xu, Z.H. & Sunderland, N. (1982) Inoculation density in the culture of barley anthers. *Scientia Sinica B* 9, 961–8.

Xu, Z.H., Huang, B. & Sunderland, N. (1981) Culture of barley anthers in conditioned media. *Journal of Experimental Botany* 32, 767–78.

Ye, J.M., Kao, K.N., Harvey, B.L. & Rossnagel, B.G. (1987) Screening salt-tolerant barley genotypes via F_1 anther culture in salt stress media. *Theoretical and Applied Genetics* 74, 426–9.

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

Ziauddin, A., Kasha, K.J. & Szarejko, I. (1988a) Evaluation of factors in barley anther culture. In: *University of Guelph, Department of Crop Science, Annual Report 1988*, p. 118.

Ziauddin, A., Simion, E., Glover, K. & Kasha, K.J. (1988b) Microspore culture in cereals – barley and wheat. In: *University of Guelph, Department of Crop Science, Annual Report 1988*, pp. 122–3.

Ziauddin, A., Simion, E. & Kasha, K.J. (1989) Microspore culture in barley. In: *University of Guelph, Department of Crop Science, Annual Report 1989*, pp. 76–8.

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

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Inheritance of Seed Set in Crosses of Spring Barley and *Hordeum bulbosum* L.

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ABSTRACT

Seed set limits use of certain barley (*Hordeum vulgare* L.) genotypes in *H. bulbosum* L. doubled-haploid production. This study was conducted to determine the genetic basis of low seed set in the spring barley cultivar Apex crossed with *H. bulbosum*. Two hundred F_2 plants, all having Apex as one parent and either Havila, Krystal or Roland as the other parent, were crossed with one clone of *H. bulbosum*. Parameters of haploid production efficiency (seeds/100 florets, embryos/100 seeds, and plants/100 embryos), were measured. Segregation ratios in the three F_2 populations suggest that reduced seed set in Apex is governed by a single codominant gene. Haploid production efficiencies were intermediate between the two parents and were acceptable for applied plant breeding.

SINCE the discovery of the *H. bulbosum* method by Kasha and Kao (1970), probably more doubled haploids have been produced in barley than in any other crop. These DHs represent useful tools for applied breeding and genetic analyses (Kasha and Reinbergs, 1982; Choo et al., 1985) and they are now proving invaluable as an aid in linking genes governing agronomic traits, particularly quantitative trait loci, to RFLP markers (Kasha and Procunier, 1990; Knapp et al., 1990; Bridges and Knapp, 1990).

Large-scale barley DH production can be accomplished by both the *H. bulbosum* method and anther culture. In both cases, DH production efficiency depends on several factors, including the genotype of barley (Foroughi-Wehr et al., 1982; Pickering, 1983a; Bjornstad, 1986; Powell, 1988; Knudsen et al., 1989; Hayes and Chen, 1989).

In the *H. bulbosum* method, haploid production efficiency is determined by HPE = (seeds/100 florets) × (embryos/100 seeds) × (plants/100 embryos) (Hayes and Chen, 1989). Using several barley cultivars and F_1 hybrids between them, we reported low HPEs for cultivars Apex and Havila. Low HPE was a function of low seed set (seeds/100 florets) in Apex and of poor embryo quality (embryos/100 seeds) in Havila. By comparing the overall success rates of the different genotypes, we demonstrated that seed set is the major limiting factor of the *H. bulbosum* method (Devaux et al., 1990).

Low seed set in *H. vulgare–H. bulbosum* crosses was first reported by Pickering and Hayes (1976) with cultivars of *H. vulgare* having *H. distichum* var. *laevigatum* in their pedigree (e.g., Vada). One dominant gene is responsible for this reaction and seems to be linked with the gene controlling reaction to DDT on barley chromosome 7 (Pickering, 1983b). Other spring cultivars, possibly unrelated to Vada, were also found to exhibit low seed set (Pickering, 1980a). In this respect, Apex is known to have no *H. distichum* var. *laevigatum* in its ancestry (Devaux et al., 1990). But lowest HPEs were reported in six-rowed winter barleys (Simpson et al., 1980), in cultivars such as Vogelsanger Gold (Thörn and Jensen, 1985), Gaulois, and Express (Devaux, 1986, unpublished data), since reduced seed set is associated with poor embryo quality.

The purpose of this research was to determine the genetics of seed set in Apex, using three F_2 populations.

MATERIALS AND METHODS

Four spring barley cultivars (Apex, Havila, Krystal, and Roland) and F_2 populations derived from crosses of Havila × Apex, Krystal × Apex, and Roland × Apex were used in this investigation. Studies on HPE of the above barley genotypes and *H. bulbosum* were carried out in Poland at the Institute of Plant Genetics, Polish Academy of Sciences, Poznan, for the F_2 progenies of Krystal × Apex and Roland × Apex and their corresponding parents Apex, Krystal, and Roland. Studies of Havila, Apex, and the F_2 progeny were performed in France, at Florimond Desprez Seeds Co.

Plant crossing and in vitro procedures were performed as described by Devaux (1986), using a single clone of *H. bulbosum* ($2n = 2x = 14$). Experiments were carried out in both Poland and France from April to May 1989. The HPE was determined for 45 F_2 plants of Havila × Apex, 76 of Krystal × Apex, and 79 of Roland × Apex. Data were generated using two to five spikes of each plant and were averaged per plant.

Chi-square analyses were performed to determine segregation ratio for seed set with *H. bulbosum* of the F_2 hybrids. The hybrids were divided into the three following groups according to the Dunnet's test (Dunnet, 1955): (i) plants with a low seed set, not significantly different from Apex; (ii) plants with intermediate seed set, significantly different from their parents; and (iii) plants with high seed set, not significantly different from the high-seed-set parent (Havila, Krystal, or Roland).

RESULTS AND DISCUSSION

Seed set

Seed set in Apex was low (12.5). Seed set in the remaining parents ranged from 64 for Krystal to 71 for Roland. Average seed set in the three F_2 progenies was intermediate, but always less than the mid-parent value. Standard deviations in the F_2 progenies averaged more than twice as large as those for the parents, and are assumed to reflect the presence of segregating plants for this character.

In a previous paper, we reported low seed set of Apex with *H. bulbosum* across two locations, in Poland and in France (Devaux et al., 1990). Again in the present experiment, carried out a year later, sim-

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Abbreviations: DDT, dichlorodiphenyltrichloroethane; DH, doubled haploid; HPE, haploid production efficiency; QTL, quantitative trait loci; RFLP, restriction fragment length polymorphism.

Table 1. Means and standard deviations for seeds/100 florets (S/F), embryos/100 seeds (E/S), haploid plants/100 embryos (P/E) and haploid production efficiencies (HPE) for three F_2 progenies and their parents using the *Hordeum bulbosum* method.

Genotype	Trait			
	S/F	E/S	P/E	HPE
	no.			
Apex (A)	12.5 ± 5.7	16.0 ± 13.6	59.2 ± 11.6	1.2 ± 0.9
Krystal (K)	64.0 ± 10.5	20.2 ± 12.7	78.4 ± 28.9	10.1 ± 2.3
Roland (R)	71.0 ± 10.5	22.2 ± 10.3	62.7 ± 24.7	9.9 ± 2.0
Havila (H)	64.3 ± 12.3	21.7 ± 15.4	75.1 ± 19.7	10.5 ± 1.9
F2 (K × A)	32.8 ± 22.9	16.9 ± 14.2	85.2 ± 23.6	4.7 ± 1.3
F2 (R × A)	38.7 ± 28.1	24.0 ± 11.3	68.8 ± 20.5	6.4 ± 1.7
F2 (H × A)	27.5 ± 19.5	27.3 ± 19.4	58.0 ± 21.5	5.3 ± 1.5

ilar seed set rates were recorded with Apex. This indicates that the low seed set factor may not be strongly influenced by environment, since different conditions, especially in the growth of plants, would have occurred. Bjornstad (1986) found that the low seed set of the barley cultivar Agneta under greenhouse conditions could be overcome when plants were grown in a phytotron (i.e., under better regulated temperature and humidity regimes, and with a strong fluorescent light). In this respect, the poor seed set of Agneta seems to be different from that of Apex, but further experiments would be needed to confirm this.

Since determination of seed set was performed for each F_2 plant in the three progenies, all having Apex as one parent, study on segregation of the low seed set of Apex with *H. bulbosum* was undertaken. On the basis of the performed statistical analyses, the F_2 plants were divided into three classes. Numbers of plants in each class for the three F_2 hybrids are given in Table 2. As can be seen from χ^2 analyses, data fit a 1:2:1 ratio in the three F_2 progenies. This suggests a codominant gene controlling seed set.

A codominant gene for reduced seed set in Apex is preferred, rather than a fully dominant one, since heterozygous plants for this character have intermediate seed set between the two parents, resulting in better HPE. Bjornstad (1986) also reported partial dominance in an F_1 hybrid having the cultivar Agneta as one parent. To improve levels of seed set, the use of other *H. bulbosum* accessions or an application of 200 g L^{-1} of proline at the time of emasculation could be tried (Pickering, 1980b; V.D. Navolotsky, 1990, personal communication).

Other Parameters of Haploid Production Efficiency

Embryo quality appears to be less variable than seed set. Apex had the lowest embryo quality (16.0). The other cultivars ranged from 20.2 for Krystal to 22.2 for Roland; the F_2 progenies ranged from 16.9 to 27.3.

The high seed set and low embryo quality, found in the cultivar Havila (Devaux et al., 1990) were not confirmed in the present investigation. This may be due to different environmental conditions from one year to another, or because these characters are more subjective and were checked by different workers. But embryo quality of Apex remains the lowest among the tested cultivars across both studies. Significant improvements of embryo quality have been achieved by using in vitro floret culture (Chen and Hayes, 1989), which is recommended in the case of low HPE.

Table 2. Segregation for seed set in crosses of *Hordeum vulgare* × *H. bulbosum* in three F_2 populations involving Apex as one parent.

Cross	Seed set			χ^2§	*P*
	P1†	P1 < H < P2‡	P2†		
	no. plants				
F2 (K × A)	18	45	13	3.21	0.20
F2 (R × A)	20	43	16	1.03	0.50–0.70
F2 (H × A)	13	23	9	0.73	0.50–0.70
χ^2 (4) for homogeneity				0.90	0.90–0.95

†Number of plants not significantly different from P1 or P2.
‡Number of plants with seed set significantly different from P1 (Apex) and P2 (Krystal, Roland, or Havila); H = hybrid.
§Chi-square value for 1:2:1 ratio.

Numbers of haploid plants obtained for 100 embryos rescued for the cultivars and F_2 progenies did not differ much; however, significant differences arose for numbers of plants obtained for 100 florets pollinated (HPE). The lowest HPE value was obtained for Apex (1.2) and the highest for Havila (10.5). Other cultivars were almost as high as Havila, with 10.1 for Krystal and 9.9 for Roland. The F_2 progenies were intermediate between their corresponding parents, but two of them, Krystal × Apex and Havila × Apex, were inferior to mid-parent values, while the F_2 Roland × Apex (6.4) was superior (the mid-parent value of HPE between Roland and Apex was 5.55).

Haploid production efficiencies in the three F_2's having the incompatible cultivar Apex as one parent are acceptable, and good enough for barley breeding programs to use DHs. However, it remains important to rapidly identify the cause of low HPEs found in DH production programs and to try to overcome them using alternative procedures which should result in less time-consuming methods.

REFERENCES

Bjornstad, A. 1986. Partial incompatibility between Scandinavian six-rowed barleys (*Hordeum vulgare* L.) and *Hordeum bulbosum* L., and its genetical basis. Hereditas 104:171–191.

Bridges, W.C., and S.J. Knapp. 1990. Using molecular markers to estimate quantitative trait locus parameters: Quantitative genetic theory for replicated progeny. p. 194. *In* Agronomy abstracts. ASA, Madison, WI.

Chen, F., and P.M. Hayes. 1989. A comparison of *Hordeum bulbosum*-mediated haploid production efficiency in barley using in vitro floret and tiller culture. Theor. Appl. Genet. 77:701–704.

Choo, T.M., E. Reinbergs, and K.J. Kasha. 1985. Use of haploids in breeding barley. Plant Breed. Rev. 3:219–251.

Devaux, P. 1986. Yield of haploid production through the *bul-*

bosum method in a winter barley programme. Cereal Res. Comm. 14:273–279.
Devaux, P., T. Adamski, and M. Surma. 1990. Studies on low crossabilities encountered with the *Hordeum bulbosum* method for haploid production of barley, *Hordeum vulgare* L. Plant Breed. 104:305–311.
Dunnet, C.W. 1955. A multiple procedure for comparing several treatments with the control. J. Am. Stat. Assoc. 50:1096.
Foroughi-Wehr, B., W. Friedt, and G. Wenzel. 1982. On genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor. Appl. Genet. 62:233–239.
Hayes, P.M., and F.Q. Chen. 1989. Genotypic variation for *Hordeum bulbosum* L.–mediated haploid production in winter and facultative barley. Crop Sci. 29:1184–1188.
Kasha, K.J., and K.N. Kao. 1970. High frequency haploid production in barley (*Hordeum vulgare* L.). Nature (London) 225:874–875.
Kasha, K.J., and J.D. Procunier. 1990. RFLP studies in barley. Univ. of Guelph, Dep. of Crop Sci. Rep. for 1989–90. Ontario Agric. Coll., Guelph, ON.
Kasha, K.J., and E. Reinbergs. 1982. Recent developments in the production and utilization of haploids in barley. p. 655–665. *In* M.J.C. Asher. et al. (ed.) Barley Genetics IV. Proc. Int. Genet. Symp., 4th, Edinburgh. 22–29 July 1981. Univ. Press, Edinburgh, Scotland.
Knapp, S.J., W.C. Bridges, Jr., and D. Birkes. 1990. Mapping quantitative trait loci using molecular marker linkage maps. Theor. Appl. Genet. 79:583–592.
Knudsen, S., I.K. Due, and S.B. Andersen. 1989. Components of response in barley anther culture. Plant Breed. 103:241–246.
Pickering, R.A. 1980a. Use of the doubled haploid technique in barley breeding at the Welsh Plant Breeding Station. Rep. Welsh Plant Breed. Stn. (Aberystwyth, Wales) [1979]:208–226.
Pickering, R.A. 1980b. Attempts to overcome partial incompatibility between *Hordeum vulgare* L. and *H. bulbosum* L. Euphytica 29:369–377.
Pickering, R.A. 1983a. The influence of genotype on doubled haploid barley production. Euphytica 32:863–876.
Pickering, R.A. 1983b. The location of a gene for incompatibility in crosses between *Hordeum vulgare* L. and *Hordeum bulbosum* L. Heredity 51:455–459.
Pickering, R.A. and J.D. Hayes. 1976. Partial incompatibility in crosses between *Hordeum vulgare* L. and *Hordeum bulbosum.* Euphytica 25:671–678.
Powell, W. 1988. Diallel analysis of barley anther culture response. Genome 30:152–157.
Simpson, E., J.W. Snape, and R.A. Finch. 1980. Variation between *Hordeum bulbosum* genotypes in their ability to produce haploids of barley, *Hordeum vulgare*. Z. Pflanzenzuecht. 85:205–211.
Thörn, E., and C.J. Jensen. 1985. Haploid barley induction and the influence of embryo size and development on plant production. Hereditas 3:152–153.

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Factors affecting anther culturability of recalcitrant barley genotypes

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Abstract. One major problem encountered with cereal anther culture is that some genotypes are low or non-responders to the technique. The objective of this study was to improve anther culture efficiency of recalcitrant barley (*Hordeum vulgare* L.) genotypes. Reciprocal F_1s between the two low responsive cultivars, Morex and Steptoe, were used. These were chosen because doubled haploids (DH) were required from these genotypes for the North American Barley Genome Mapping project. Ficoll 400 at 200 g l^{-1} in the induction medium significantly increased green plant production compared to four other media formations containing different gelling/viscosity modifying agents. Cold pretreatment of donor spikes of 28 *vs* 14 d resulted in an increase in embryoid, total plant and green plant production. Anther culture response in these experiments was little influenced by donor plant growth conditions. Indole-3-acetic acid (1 mg l^{-1}) or 1-naphthaleneacetic acid (2 mg l^{-1}) in the induction medium did not affect anther culturability or plant regeneration. Based on this research, the negative genotypic effect for doubled haploid production could be diminished, which is desirable for practical application.

Abbreviations: BAP = 6-benzylaminopurine - IAA = Indole-3-acetic acid - LS = Linsmaier & Skoog -NAA = 1-naphthaleneacetic acid - DH = doubled haploid

Introduction

Production of doubled haploids (DHs) over a wide range of genotypes is a prerequisite for their use in both plant breeding and genetic analyses (Snape, 1981). Anther culture researchers have reported genotypic influences for microspore-derived barley plant production (for review, see Pickering and Devaux 1992) with some genotypes appearing recalcitrant to the technique. Using a survey of 18 popular barley cultivars, Knudsen et al. (1989) obtained less than one green plant per 100 cultured anthers from 11 of them while two did not produce any green plants at all. From a complete diallel among 7 barley cultivars, Powell (1988) was unable to regenerate green plants from 14 of 49 genotypes examined. Twenty-two of the 35 F_1 hybrids tested by Luckett and Smithard (1992) yielded less than one green plant per 100 anthers, and no green plants were recovered from half of these 22 hybrids. Since the first anther-derived barley plants were regenerated by Clapham (1973), two approaches have been adopted to enhance practical application of the technique: the incorporation of anther-responsive genotypes into breeding programs (Foroughi-Wehr and Friedt, 1984), or the use of hybrids with a least one responsive parent; and improvement through the manipulation of environmental and physiological factors (Huang and Sunderland 1982; Olsen 1987; Hunter 1988; Kuhlmann and Foroughi-Wehr 1989).

A linkage map of barley based on a population of 150 DHs derived from Steptoe/Morex F_1s by the *Hordeum bulbosum* method was constructed through the North American Genome Mapping Project (Kleinhofs et al., 1993). We are comparing this map with one based on anther culture-derived DHs to determine if the DH production technique affects meiotic recombination. This required a sufficient number (~100) of DHs to be produced by anther culture. Preliminary results in our laboratory revealed that both Morex and Steptoe had poor anther culturability, and it was thought unlikely that the anther culture performance of F_1 hybrid plants between the two cultivars would be high. The aim of the presentstudy was to check if four of the environmental and physiological factors which have proven to be successful when applied to anther culture responsive genotypes can improve anther culturability of low

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responders. Apart from this particular mapping application, it was hoped that the findings would also give practical information for those involved in DH production for barley breeding, who routinely find recalcitrant genotypes among the many hybrids they handle each year.

Materials and Methods

Plants from the spring 6-row cultivars Morex and Steptoe and the F_1's from reciprocal crosses between them were used as donor plants. Seeds for donor plant production were sown in 15 cm pots filled with commercial greenhouse potting mix. In experiments 1 and 3 plants were grown in a greenhouse with a 16 h photoperiod at 16 ± 2°C. Natural light was supplemented with artificial sodium lighting as required, to maintain a photon flux density of 200 - 300 $\mu E\ m^{-2}s^{-1}$ at the soil surface. In experiments 2 and 4, plants were grown in a growth chamber at constant 12°C (Lyne et al., 1986) with a photon flux density of 340 $\mu E\ m^{-2}s^{-1}$ at the soil surface supplied by a 3:1 mixture of fluorescent and incandescent lights.

The first three to five spikes from each plant were collected when the majority of the microspores in the anthers of central florets were at the mid-uninucleate stage, which was determined and monitored by cytological observation. The sampled spikes were surface sterilized with 75% ethanol, leaf sheaths and awns were removed and spikes were cold pretreated at 4• C for 28 d (Huang and Sunderland 1982) unless otherwise stated.

Modified LS induction medium (Hunter 1988) was used. It contained 6% maltose as the osmoticum and sugar source, 750 mg l^{-1} glutamine as a supplement to the inorganic nitrogen source (Olsen 1987), 1 mg l^{-1} BAP (Luckett and Smithard 1992) and 1 mg l^{-1} IAA as growth regulators, unless otherwise stated. Different gelling/viscosity modifying agents were added to induction media for comparison purposes. The regeneration medium was the same as the induction medium except that the BAP concentration was decreased to 0.4 mg l^{-1}, and 3% sucrose was used as the sugar source. It was solidified with 0.2% gelrite. Anthers were plated on 60x15 mm Petri dishes with about 10 ml per dish induction medium and incubated at 25• C in the dark. Embryoids 2 mm in diameter or greater were transferred for plant regeneration to 100x15 mm Petri dishes containing regeneration medium. Gelling/viscosity modifying agents and sugar were autoclaved with 890 ml of double distilled water, and the remaining medium components were filter-sterilized in a volume of 110 ml and added to the autoclaved portion at about 50• C.

Four experiments were included in this study:

Experiment 1. Gelling/viscosity modifying agents and two periods of cold pretreatment were compared for anther culture response of the F_1s from reciprocal crosses. The concentrations of ficoll 400 (liquid), gelrite (solid), agarose (solid) (BRL, Ultra-Pure), dextran (liquid) (MW 5,000,000 to 40,000,000) were 200, 2, 8 and 100 g l^{-1}, respectively (Kao 1981; Flehinghaus et al. 1991; Olsen 1987; Sorvari 1986; Zhou 1990). Preparation of media with ficoll was as described by Olsen (1987). The medium with gelrite + ficoll (solid/liquid bilayer) was prepared by adding about 2 ml of ficoll medium on top of 10 ml gelrite-solidified medium. Anthers from each 14 or 28 d cold pretreated spike were randomly plated on the five media to eliminate spike effects. Forty-four replications for each medium and genotype with a range of 25-56 anthers per Petri dish were used totaling 16,500 anthers.

Experiment 2. The gelrite + ficoll induction medium was further compared with ficoll medium using the reciprocal Morex/Steptoe F_1 hybrids as in experiment 1. There were 45 replications per medium and genotype (8,498 anthers).

Experiment 3. The effect of the two growth regulators, IAA (1 mg l^{-1}) and NAA (2 mg l^{-1}) in the induction medium were compared with the reciprocal F_1 Morex/Steptoe hybrids. There were 40 replications for each medium (4,698 anthers).

Experiment 4. The anthers from 15 spikes (28 d cold-pretreated) each of Morex and Steptoe were plated on induction medium containing 200 g l^{-1} ficoll 400 to assess anther culturability of the two parents, under optimum conditions as determined by the previous 3 experiments.

For all experiments, variables evaluated were number of embryoids induced, number of total plants regenerated and number of green plants regenerated, all calculated per 100 anthers plated. Statistical analyses were conducted using the Generalized Linear Model procedure in the Statistical Analysis System (SAS, 1988). Mean separations were tested by Duncan's Multiple Range Test due to the unbalanced nature of most experiments. Values of variables were reported separately for each F_1 hybrid (Steptoe/Morex vs Morex/Steptoe) only when a significant reciprocal effect was found. Leaf samples (70 mg fresh weight) of 230 regenerants derived from the F_1 hybrids were placed in a plastic bag with a piece of wet filter paper and sent to Florimond Desprez, France via express air mail to check the ploidy level by flow cytometry (Brown et al. 1991).

Results and Discussion

Experiment 1

a. Effects of gelling/viscosity modifying agents using reciprocal crosses between Morex and Steptoe

The media with ficoll and the gelrite + ficoll bilayer were equally effective producing 71.9 and 67.2 embryoids per 100 anthers, respectively (Table 1), while media containing gelrite, dextran and agarose were inferior (21.0, 17.7 and 16.8 embryoids per 100 anthers, respectively). Although the highest total plant regeneration per 100 anthers was obtained for embryoids produced on medium containing dextran (29.8), the highest green plant regeneration per 100 anthers was from embryoids produced on the medium containing ficoll (2.1). Green plant regeneration was similar for the four other media investigated, ranging from 0.1 to 0.8 per 100 anthers.

The beneficial effect of ficoll on anther culture response has been reported in barley (Kao 1981; Olsen 1987), wheat (Henry and De Buyser 1981; Fadel and Wenzel 1990; Devaux 1992) and triticale (Charmet and Bernard 1984). In the present experiment, ficoll was confirmed to enhance both quantity and quality of embryoids and consequently green plant regeneration. However, the high cost of ficoll (about US $150 per liter medium) makes it impractical for doubled haploid plant production in breeding programs. A previous report showed gelrite (about US $0.50 per liter medium) as promising for

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barley anther culture (Hou et al. 1993). Here, using low anther culture response genotypes, gelrite could not effectively replace ficoll. In an attempt to reduce the cost of the ficoll medium, the bilayer of ficoll medium on top of the gelrite solidified medium was investigated. Only 2 ml of ficoll medium is required compared with normally 10 ml of ficoll medium per Petri dish. However, the bilayer medium yielded significantly fewer green plant regenerates than the ficoll medium. The bilayer medium was further evaluated due to its ability to induce embryogenesis in this experiment.

Table 1. Comparison of gelling/viscosity modifying agents in the induction medium for anther culture response of Morex/Steptoe F_1 hybrids.

Compound	Embryoids	Total plants	Green plants
	- - - No. per 100 anthers - - -		
Ficoll	71.9a[1]	17.7b	2.1a
Gelrite + Ficoll	67.2a	13.0bc	0.8b
Gelrite	21.0b	9.4c	0.5b
Dextran	17.7b	29.8a	0.4b
Agarose	16.8b	11.0c	0.1b

[1] Means within a column followed by the same letter were not significantly different at $p \leq 0.05$ by Duncan's multiple range test

b. Effect of cold pretreatment using reciprocal crosses between Morex and Steptoe

In order to accelerate doubled haploid production, 14 *vs* 28 d cold pretreatment was investigated. However, the results indicated that 28 d cold pretreatment was much more efficient regarding the three parameters studied (Table 2). Green plant regeneration per 100 anthers was 8 times higher in 28 *vs* 14 d cold pretreatment. These results were consistent with those of Hou et al. (1993), but Luckett and Darvey (1992) investigating Australian F_2 breeding lines reported genotype dependency for optimal duration of cold pretreatment.

Table 2. Comparison of 28 vs 14 d cold pretreatment of donor spikes for anther culture response of Morex/Steptoe F_1 hybrids.

Period of cold pretreatment	Embryoids	Total plants	Green plants
	- - - - - - No. per 100 anthers - - - - - -		
28 d	85.7a[1]	27.9a	1.6a
14 d	4.9b	2.2b	0.2b

[1] Means within a column followed by the same letter were not significantly different at $p \leq 0.05$ by Duncan's multiple range test

Experiment 2

a. Comparison of gelrite and gelrite + ficoll media using reciprocal crosses between Morex and Steptoe

The gelrite + ficoll bilayer medium produced a significantly higher number of embryoids compared to the medium with ficoll alone (323.7 *vs* 115.1 embryoids per 100 anthers), Table 3, although the two media produced a similar number of total plants regenerated (33.6 *vs* 22.8). However, the quality of the embryoids from the bilayer medium was lower compared with the ficoll medium, as demonstrated by a lower number of green plants regenerated (2.1 *vs* 3.4 green plants per 100 anthers).

Table 3. Comparison of ficoll and gelrite + ficoll bilayer induction media on anther culture response of Morex/Steptoe F_1 hybrids.

Compound	Embryoids	Total plants	Green plants
	- - - - - - No. per 100 anthers - - - - - -		
Ficoll	115.1b[1]	22.8a	3.4a
Gelrite + Ficoll	323.7a	33.6a	2.1b

[1] Means within a column followed by the same letter were not significantly different at $p \leq 0.05$ by Duncan's multiple range test

b. Genotype effects (Morex/Steptoe vs Steptoe/Morex)

Reciprocal effects were not observed in experiment 2 for any of the traits examined (Table 4). In order to compare data from experiment 1 with those of this experiment, genotype means for experiment 1 were recalculated to include only the 28 d cold pretreatment and the ficoll and ficoll + gelrite media. Thus, apart from the fact that donor plants were grown in different environments: greenhouse *vs* growth chamber, all the experimental conditions were identical. Data calculated from experiment 1 revealed reciprocal differences between the two F_1 hybrids (Table 4). This suggests that the conflicting observations were due to the different donor plant environments. Any maternal/cytoplasmic effects suggested in experiment 1 was not confirmed in experiment 2 leaving this matter open. The non-reproductibility of apparent reciprocal effects of crosses on anther culture response is in agreement with Larsen et al. (1991). Although these two experiments were carried out independently, anther culture response overall of the two F_1 hybrids was not greatly affected by donor plant environment. However, the highest green plant regeneration occurred from greenhouse grown donor plants. Foroughi-Wehr and Mix (1979) and Lyne et al. (1986) identified 12°C as being more suitable for green plant production than higher temperatures, while a preference for 16-20°C has been expressed by Kasha

(1989). However, Hou (1992) did not find differences in efficiency between growth chamber and field grown donor plants among 16 genotypes investigated.

Table 4. Comparison of donor plant environment on anther culture response of Morex (M)/Steptoe (S) reciprocal F_1 hybrids.

Environment	Genotype	Embryoids	Total plants	Green plants
		No. per 100 anthers		
Experiment 1:				
Greenhouse	M/S	240.9a[1]	69.6a	4.8a
	S/M	108.2b	34.2b	2.1b
Experiment 2:				
Growth chamber	M/S	245.6a	30.5a	2.7a
	S/M	194.8a	24.9a	2.8a

[1] Means within a column and within an experiment followed by the same letter were not significantly different at $p \leq 0.05$ by Duncan's multiple range test

Experiment 3: Comparison of IAA and NAA

IAA (1 mg l^{-1}) has been observed to be beneficial for embryoid formation and plant regeneration (Hou 1992). NAA (2 mg l^{-1}) in the induction medium has been adapted to winter barley anther culture (Devaux 1992). No significant difference between IAA *vs* NAA was obtained for the three traits investigated: 193 *vs* 159 embryoids, 18 *vs* 15 total plants regenerated and 1.0 *vs* 0.8 green plants regenerated per 100 anthers.

Experiment 4: Anther culturability of Morex and Steptoe

Using the optimal conditions determined in the three previous experiments, embryoid (310 *vs* 268) and total plant production (61 *vs* 51) per 100 anthers were similar for Morex and Steptoe, respectively. Morex showed better green plant regeneration ability (3.8 *vs* 1.6 for Steptoe, significant at $p \leq 0.05$). Comparing these results to those reported by Hou et al. (1993) for Pacific Northwest USA barley genotypes, Morex and Steptoe showed poor anther culturability. Up to 49 green plants per 100 anthers with the cultivar Hazen were obtained in the earlier work using the same experimental conditions (Hou et al., 1993).

Ploidy level of regenerants

The ploidy level determination was performed 2 to 3 d after the leaf tissues were collected but this did not cause any technical problem for flow cytometry analyses. Among the 246 regenerants, 45% were found to be haploid, 48% diploid and 7% tetraploid. The proportion of spontaneous chromosome doubling was inferior to the 59% reported previously (Devaux 1992) while the proportion of tetraploids was similar.

Variation in chromosome doubling of microspore-derived regenerants has been reported previously. Olsen (1987) found 94% of diploid plants among 50 regenerated green plants cytologically examined while Finnie et al. (1989) reported more haploid than diploid regenerants from anther culture performed on five barley genotypes. Finnie et al. (1989) concluded that the culture techniques they used favored the development of haploid embryos. Subsequently Finnie et al. (1991) reported that ploidy level of regenerated plants is under genetic control in barley.

In conclusion, it was shown that anther culture response of recalcitrant genotypes can be improved by environmental factors. One of these factors is the nature of the gelling/viscosity modifying agent used in the induction medium. Significant improvement was obtained by the use of ficoll in the induction medium. Although ficoll cannot be used for routine production because of its high cost, it can be limited to the recalcitrant genotypes when DHs have to be produced from them. Another factor which was important for the four genotypes studied was the length of time of cold pretreatment. Cold pretreatment of donor spikes for 28 d resulted in better anther culture response (*vs* 14 d). Combining these two factors (ficoll and 28 d cold pretreatment) may help to improve the response in other recalcitrant barley genotypes. Additional anther culture methodology research seems warranted. However, we were able to produce 246 anther culture-derived green plants from the Steptoe/Morex crosses by utilizing the information gained from this study, and 102 of them were used in the mapping study described in the introduction.

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References

Brown SC, Devaux P, Marie D, Bergounioux C, Petit PX (1991) Biofutur, Le Technoscope 105: 1-16
Charmet G, Bernard S (1984) Theor Appl Genet 69: 55-61
Clapham D (1973) Z Pflanzenzuchtg 69: 142-155
Devaux P (1992) In: Dattee Y, Dumas C, Gallais A (eds) Reproductive Biology and Plant Breeding. Springer-Verlag, Berlin, pp 139-151
Fadel F, Wenzel G (1990) Plant Breed 105: 278-282
Finnie SJ, Forster BP, Chalmers KJ, Dyer AF, Waugh R, Powell W (1991) Genome 34:923-928
Finnie SJ, Powell W, Dyer AK (1989) Plant Breed 103: 110-118
Flehinghaus T, Deimling S, Geiger HH (1991) Plant Cell Rep 10: 397-400
Foroughi-Wehr B, Mix G (1979) Env Exp Bot 19: 303-309
Henry Y, De Buyser J (1981) Theor Appl Genet 60: 77-79
Hou L (1992) PhD Dissert., Washington State Univ, Pullman

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Hou L, Ullrich SE, Kleinhofs A, Stiff CM (1993) Plant Cell Rep 12:334-338
Huang B, Sunderland N (1982) Ann Bot 49: 77-88
Hunter CP (1988) Plant regeneration from microspores of barley, *Hordeum vulgare* L. PhD Thesis, Wye College, Univ of London
Kasha KJ (1989) In: Maluszynski M (ed) Current Options for Cereal Improvement: Doubled Haploids, Mutants and Heterosis. Kluwer, Dordrecht, pp 71-80
Kao KN (1981) Z Pflanzenphysiol 103: 437-443
Kleinhofs A, Kilian A, Saghai Maroof MA, Biyoshev RM, Hayes PM, Chen F, Lapitan N, Fenwick A, Blake TK, Kanazin A, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) Theor Appl Genet 86:705-712
Knudsen S, Due IK, Andersen SB (1989) Plant Breed 103: 241-246
Larsen ET, Tuvesson KD, Andersen SB (1991) Theor Appl Genet 82:417-420
Luckett DJ, Darvey NL (1992) Aus J Bot 40:807-828
Luckett DJ, Smithard RA (1992) Aus J Agric Res 43: 67-78
Lyne RL, Bennett RI, Hunter CP (1986) In: Withers LA, Alderson PG (eds) Plant Tissue Culture and its Agricultural Applications. Univ of Nottingham, Butterworth, Guildford, pp 405-411
Olsen FL (1987) Carlsberg Res Comm 52: 393-404
Pickering RA, Devaux P (1992) In: Shewry PR (ed) Barley: Genetics, Molecular Biology and Biotechnology. CAB Internat, Wallingford, pp 511-539
Powell W (1988) Genome 30: 152-157
SAS (1988) SAS Institute Inc, Cary, NC
Snape JW (1981) In: Broertjes C (ed) Induced variability in plant breeding. Pudoc, Wageningen, pp 52-58
Sorvari S (1986) Ann Agric Fenn 25: 249-254
Zhou H (1990) Influence of the physical condition of induction media on the culture response of wheat (*Triticum aestivum* L.) anthers. M.S. Thesis. Washington State Univ, Pullman

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Doubled haploids of wheat from wheat × maize crosses: genotypic influence, fertility and inheritance of the 1BL-1RS chromosome

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Abstract The wheat × maize cross as a technique for haploid induction in wheat was evaluated in a replicated block design comprising 18 wheat F_1 hybrids and five *Zea mays* L. parents. Haploid plants were regenerated at an average of 9.1 (4.4–14.7) plants per 100 florets processed. Genotypic differences for haploid production efficiency were recorded for both wheat and *Zea mays* L. Interaction between parents was significant for number of plants/100 florets. All 610 of the 1,703 regenerated plantlets that were analyzed by flow cytometry were haploid. At maturity, 70% (60–81%) of the colchicine-treated haploid plants were fertile, but the frequency of fertile and sterile plants was not consistent over the wheat hybrids from which they were derived. Flow cytometry performed using the first tiller which arose following colchicine treatment enabled prediction of fertility. The 1BL-1RS chromosome was found at the expected ratios in the F_2 and in the haploid progenies produced through the wheat × maize cross but deviated from the 1:1 ratio in the haploid progenies produced by anther culture.

Key words Wheat · Wheat × maize cross · Haploid · Doubled haploid · Distortion of segregation

Introduction

Unlike barley, very few wheat cultivars have been released as doubled haploids (Devaux 1992). Cultivar release is suitable to assess the efficiency of haploid production in self-pollinated crop species such as barley and wheat, for which techniques have been available for many years. Despite intensive efforts to increase anther culture response (reviewed by Henry and de Buyser 1990), its use has remained marginal in wheat breeding programs. The major limitation to a broad exploitation of anther culture has been its genotypic dependency (Lazar et al. 1984; Marsolais et al. 1984; Foroughi-Wehr and Zeller 1990). Crosses involving an anther culture-responsive parent such as a 1BL-1RS translocated line (Henry and de Buyser 1985) enabled the indirect recovery of haploid plants from recalcitrant genotypes. However, regenerated plants were skewed in the direction of the translocated parental type by the selective development of microspores or the derived embryoids (Agache et al. 1989; Devaux et al. 1990).

Wide crosses followed by elimination of the genome of one parent have been an alternate method for inducing haploid zygotic embryos and subsequent plants. The production of haploid plants from crosses between wheat and maize was first reported by Laurie and Bennett (1988). Refinements of the technique (Suenaga and Nakajima 1989; Laurie et al. 1990; Comeau et al. 1992) enabled haploid plants to be produced from many commercial wheat cultivars (Laurie and Reymondie 1991; Riera-Lizarazu et al. 1992) and hybrids with the aim of obtaining homozygous recombinant lines resistant to Russian wheat aphid (Kisana et al. 1993).

The objectives of the study presented here were (1) to assess doubled haploid (DH) production efficiency through wheat × maize or teosinte crosses over a range of wheat F_1 hybrids; this implies the regeneration of haploid plantlets and successful chromosome doubling to restore fertile DH plants; (2) to select superior maize genotypes that could increase haploid production efficiency; and (3) to compare the inheritance 1BL-1RS chromosome in haploids obtained through the maize method (MM) and by anther culture (AC) and hence to assess whether random gamete sampling takes place.

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Material and methods

Plant material

The 18 F_1 hybrids of winter wheat (*Triticum aestivum* L.) used in this study were numbered for later reference as follows: (1) 'Rossini'/FD91147, (2) 'Texel'/FD91091, (3) 'Trémie'/'Avital', (4) 'Sidéral'/'Eridane', (5) FD91072/'Soissons', (6) 'Fertil'/FD91072, (7) FD91072/FD91147, (8) FD0830/'Corsaire', (9) 'Vivant'/'Rialto', (10) 'Ritmo'/FD627, (11) Rialto/Trémie, (12) Texel/Sidéral, (13) FD5566/FD502, (14) FD90050/FD627, (15) 'Contra'/Soissons, (16) 'Cordial'/Soissons, (17) Ritmo/Sidéral, (18) Soissons/FD91143.

The male parents consisted of the two F_1 *Zea mays* L. hybrids cvs 'Saviero' (M1) and 'Earlibelle' (M2); FL, an early-type population of maize (M3); and one genotype of teosinte (*Zea mays* ssp. *mexicana*) (T4). A pollen mixture of the above four male parents (M5) were also used in this study.

Doubled haploid production

After germination, seedlings of wheat were transferred to a vernalization room held at a constant temperature of 4°C for 8 weeks, then planted out in a glasshouse with a temperature regime of approximately 20°/16°C (day/night). A 16-h photoperiod was supplied by Philips SON-T400 high pressure sodium lights when necessary. Emasculations and pollinations were as described in Laurie and Bennett (1986). The 18 wheat F_1 hybrids and the five *Zea mays* parents were compared in a seven-replicate randomized complete-block design. Wheat spikes were considered to be replicates in cross-combinations with *Zea mays*. The 2,4-D tiller and floret treatment was the same as that described in Laurie and Reymondie (1991). Fifteen days after pollination, spikes were collected and embryos were excised and cultured in vials containing B5 medium (Gamborg et al. 1968). Embryos were incubated in the dark at 22°C for 5–10 days and then transferred to a 16-h light regime at the same temperature. Rooted seedlings were transplanted into the glasshouse. At the three to five tiller stage, plants were treated with a 0.1% aqueous solution of colchicine according to Pickering (1980), and at maturity seeds were collected from each fertile plant.

Statistical analyses were performed using Statistix® 4.0 (1992) and Biom© (1989) analytical software packages.

Ploidy level determination

Of the regenerated plants 610 were checked for ploidy level by flow cytometry. Briefly, 40 mg of leaf tissue was chopped with a razor blade in 2 ml of buffer (Bergounioux et al. 1986) and 16 µl of a filter-sterilized solution of 4', 6-Diamidino-2-Phenylindole (DAPI, Sigma D-9542) at a concentration of 250µg/ml. Samples were analyzed using a CA II flow cytometer (Partec GmbH, 4400 Münster, Germany).

Since the CA II flow cytometer was not precise enough to detect chromosome abnormalities such as aneuploids, root-tip cell chromosome counts were carried out for 4 plants which had a peculiar growth habit, i.e. many and small tillers, narrow leaves and reduced growth speed.

To assess chromosome doubling efficiency, flow cytometry was performed on 28 colchicine-treated haploid plants. For this purpose, leaf samples were cut off from the first tiller which had arisen a few weeks following colchicine treatment. Flow cytometry profiles were analyzed using the Dpac© 2.1 software provided by Partec.

Inheritance of the 1BL-1RS translocated chromosome

Among the wheat cultivars or advanced lines which had been used as parents, only 'Rialto' possessed a homozygous 1BL-1RS translocated chromosome. 'Rialto' was the parent of the 2 F_1 hybrids 9 and 11. As demonstrated by Ainsworth and Gale (1987), the glucose phosphate isomerase (GPI) system can be used as an indicator of the presence of the 1BL-1RS translocation in wheat lines, and therefore was used in the present study. Enzyme extraction from leaves of young plantlets, gel preparation, electrophoresis and enzyme visualization were the same as described in Wendel and Weeden (1989). Inheritance of the 1BL-1RS chromosome was investigated in the haploid progenies produced by the MM and by AC and in the F_2 progeny of the 2 hybrids 9 and 11. The AC method was the same as described in Devaux (1992). As a control, GPI analysis was carried out using the three parents, 'Rialto', 'Vivant' and 'Trémie', as well as the IR 'Chinese Spring' addition line (CS + 1R), Ditelo 1BL 'Chinese Spring' (D1BL) and cv 'Gabo', which has a 1BL-1RS chromosome. (CS + 1R, D1BL and 'Gabo' were provided by Dr. R. M. D. Koebner (IPSR Cambridge Laboratory, Norwich, UK).

Results

Haploid plant production

From the 18,716 wheat florets which were processed during this experiment, 15,342 (82%) of the ovaries enlarged after fertilization and 2,4-D treatment to reach a caryopses size similar to that of parental selfs of the same age. However, only 3,843 (25%) of the expanded ovaries contained an embryo. Attempts to identify those caryopses having an embryo by X-ray radiography were unsuccessful. A total of 1,703 plants (44.3% of the embryos) were regenerated. The number of embryos (%EMB/FL) and plants (%PL/FL) per 100 florets ranged from 26.1 (wheat hybrid no 1: whl) to 14.4 (whl8) and from 14.7 (whl) to 4.4 (whl8), respectively (Fig. 1). The effect of wheat genotype on the two characters was highly significant (Table 1). The *Zea mays* pollinator had a significant effect for %EMB/FL (highest value: 24.1 for M2, lowest: 15.2 for M3) and %PL/FL (highest value: 10.5 for M2, lowest: 6.8 for M3) (Fig. 1). The interaction between wheat and *Zea mays* genotypes was significant at the 0.01 level for %PL/FL (Table 1). The

Fig. 1 Effect of wheat and maize genotypes on haploid production of wheat. *Horizontal lines* represent homogeneous groups for number of plants per 100 florets ($P < 0.05$)

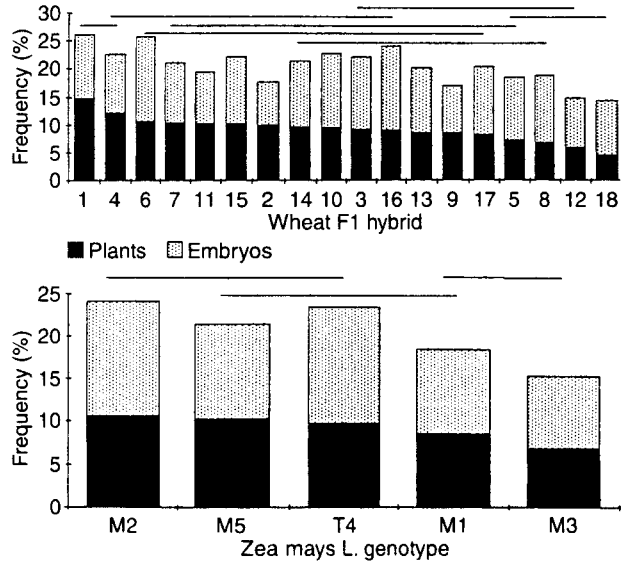

Table 1 Degrees of freedom and mean squares from analysis of variance for haploid embryo and plant production in wheat

Source	*df*	Embryos/100 florets MS	Plants/100 florets MS
Maize (M)	4	1708.5***	297.3***
Wheat (W)	17	380.9***	190.4***
M*W	68	145.4	84.0**
Error	540	115.1	51.9

,* Significant effect at $\alpha = 0.01$ and 0.001, respectively

highest %PL/FL (24.4) was obtained for wh1 crossed with M2, and the poorest (2.2) for wh18 with M3.

Heterogeneity *G*-tests (Sokal and Rolf 1981) for plant regeneration, i.e. the number of plants obtained from 100 embryos (%PL/EMB), were significant for both wheat ($G = 67.71$, $df = 17$) and *Zea mays* ($G = 9.74$, $df = 4$) genotypes at the 0.001 and 0.05 levels, respectively. The Pearson correlation coefficient between %EMB/FL and %PL/FL was $r = +0.630$ and reflects the difference between genotypes for regeneration ability. Of the cultured embryos 55.7% failed to establish themselves as plantlets; they just enlarged slightly or produced a coleoptile and/or roots.

Ploidy level and fertility of regenerants

The 610 plants which were checked for ploidy level by flow cytometry appeared to be haploid. This indicates that maize chromosome elimination was a consistent phenomenon in wheat × maize crosses and that no chromosome doubling had occurred during the process. Root-tip squashes revealed that the 4 plants which had a peculiar growth habit (see above) had 21 chromosomes. Noticeable differences existed in the proportion of diploid nuclei when tillers that developed after colchicine treatment were analyzed by flow cytometry (Fig. 2). The relative percentage of diploid over total nuclei, %DN/TN, of colchicine-treated haploid plants varied from 36 to 68 and revealed differences in chromosome doubling efficiency. % DN/TN was partially correlated to the number of grains collected from the whole plant at maturity ($r = +0.616$). All of the plants which had a % DN/TN of less than 42 were sterile. This suggests that a threshold exists under which sterility would be complete.

At maturity, 1,192 (70%) of the colchicine-treated haploid plants were fertile or partially fertile. The range was from 60% to 81% depending on the wheat hybrid from which the plants were derived. The contingency G

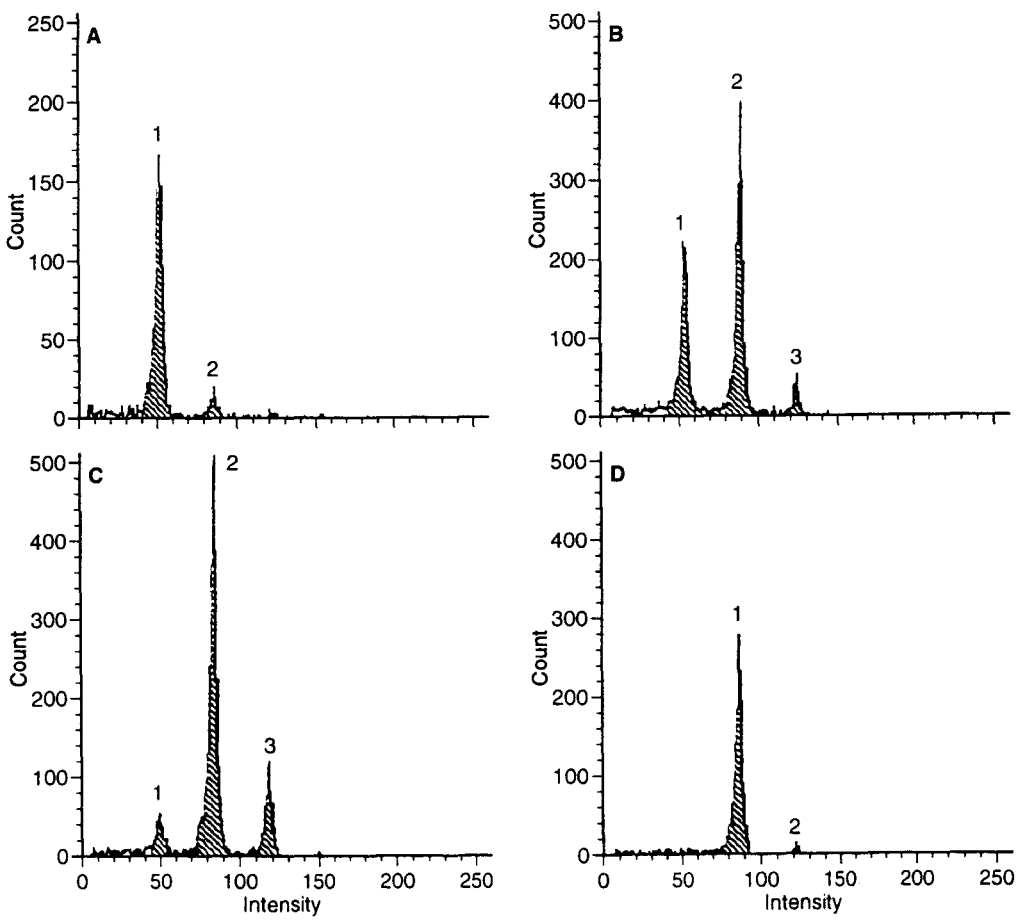

Fig. 2A–D Flow cytometric profiles of wheat plantlets derived from wheat × maize crosses. **A** haploid, **B** and **C** colchicine-treated haploids; **D** doubled haploid after one cycle of multiplication

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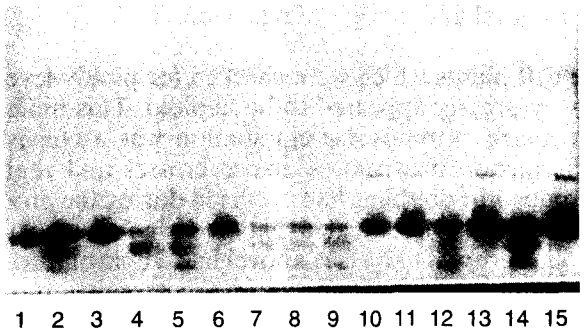

Fig. 3 Glucose phosphate isomerase (GPI) polymorphism of wheat. *Lanes 1–6* seedlings of various accessions and the parents of crosses 9 ('Vivant'/'Rialto') and 11 ('Rialto'/Trémie); *lanes 7–15* haploid plantlets derived from the cross 9 (*lanes 7–11*) and 11 (*lanes 12–15*) through wheat × maize hybridization. *1* Ditelo 1BL 'Chinese Spring', *2* 1R 'Chinese Spring' addition line, *3* cv 'Gabo' possessing a 1BL-1RS chromosome, *4* cv 'Vivant', *5* 'Trémie'. *6* 'Rialto'

value ($G_{(17)} = 30.86$) was significant at the, 0.05 level, indicating that the frequency of fertile and sterile plants was not consistent over genotypes.

Inheritance of the 1BL-1RS chromosome

Polymorphism for GPI between lines having the 1RS translocated chromosome and those with a normal 1B chromosome can be seen in Fig. 3. In 'Rialto', D1BL and 'Gabo', the two highest pI isozymes are missing. Segregation of alleles at the GPI locus did not deviate significantly from the 1:1 ratio in the haploid progenies derived from hybrids 9 and 11 by MM, while it did deviate in those produced by AC (Table 2). In the F_2 progenies derived from the same F_1 hybrids by self-fertilization, homozygous lines for the 1BL-1BS chromosome could not be distinguished without ambiguity from the structural heterozygotes 1BL-1RS/1BL-1BS through the GPI system. However, the segregation of parental phenotypes did not deviate from the 3:1 ratio (Table 2). This indicates that no selection pressure for the 1BL-1RS chromosome had occurred through self-fertilization or through fertilization with maize as it did for the regeneration of plants from microspores.

Discussion

In our study haploid plants were regenerated from all of the F_1 hybrids of wheat that were investigated at an average of 9.1 plants per 100 florets. This rate is quite acceptable with respect to the numerous advantages that haploids and derived DHs can bring into genetic studies and plant breeding (Kasha and Reinbergs 1982). Moreover, no recalcitrant genotype to green haploid plant production was encountered since the poorest responder yielded 4.4 haploid plants from 100 florets processed. In these respects, the MM showed an extraordinary advantage over AC for which recalcitrant and poor responder genotypes have been frequent (Ziegler et al. 1990; Devaux 1992; Orlov et al. 1993). However, genotypic differences for haploid production efficiency were found for both the wheat and *Zea mays* parents. In crop species for which the interspecific hybridization system has been exploited extensively for haploid production or for gene transfer (reviewed by Jiang et al. 1994), the influence of the cultivated target species used as the female parent has been reported in crosses of barley with *Hordeum bulbosum* (Pickering 1983; Bjørnstad 1986; Hayes and Chen 1989; Devaux et al. 1992), of potato with *Solanum phureja* (Hougas et al. 1964) and of bread and durum wheats with maize (Suenaga et al. 1991; Oury et al. 1993; Inagaki and Tahir 1990; Sarrafi et al. 1994). The genotype of the pollinator has also been reported to have a significant effect and, therefore, superior pollinators have been selected in *H. bulbosum* (Simpson et al. 1980; Pickering and Rennie 1990) and in *S. phureja* (Kotch and Peloquin 1987). Very little research has been devoted to the influence of the male parent component in the MM. Ushiyama et al. (1991) investigated over 39 maize genotypes and reported the superiority of one teosinte accession for haploid production. Inagaki and Tahir (1990) reported that differences between the pollen source were reflected in the frequencies of plant regeneration. It is clear from

Table 2 Inheritance of the 1BL-1RS chromosome in haploids produced by wheat × maize crosses (MM), anther culture (AC) and in F_2 progeny derived from two structural heterozygotes 1BL-1RS/1BL-1BS

Wheat cross	Group	1BL-1RS	1BL-1BS	1BL-1RS/1BL-1BS and 1BL-1BS/1BL-1BS[a]	χ^{2b}
9	MM	61	53	–	0.56
	AC	12	4	–	4.00*
	F_2	27	–	88	0.14
11	MM	65	74	–	0.58
	AC	59	30	–	9.45**
	F_2	20	–	77	0.99

[a] Homozygous lines for the 1BL-1BS chromosome could not be distinguished without ambiguity from the structural heterozygotes 1BL-1RS 1BL-1BS

[b] χ^2 test for deviation 1:1 ratio in haploids by MM and by AC, and from 3:1 in F_2

*,** Significant effect at $\alpha = 0.05$ and 0.01, respectively

the present study that there are superior genotypes of *Zea mays* and that these can be identified and used to improve the haploid production efficiency of wheat. However, the teosinte accession that was used was not different from the two best maize pollinators. Interactions between the two parent components were significant for plant production indicating that more than one *Zea mays* source should be used for the highest efficiency. As a pollen mixture of several *Zea mays* genotypes did not increase success rates, however, differences in fertilization efficiencies of *Zea mays* may exist, as in *H. bulbosum* (Pickering 1984).

At the time the spikes were collected for embryo excision, a high proportion (82%) of the wheat ovaries had enlarged. Suenaga and Nakajima (1989) reported that 2,4-D injected into Japanese wheat tillers after pollination with maize promoted ovary growth and embryo development. Similarly, 2,4-D was a prerequisite to the initiation of embryogenic callus or derivate suspension cultures (De Vries et al. 1988; Vasil et al. 1990). An average of 74.2% expanded ovaries was reported by Suenaga et al. (1991), this is similar to the rate found in our investigation. However, only a very small proportion of the expanded ovaries contained an embryo. In crosses between hexaploid wheat cv 'Chinese Spring' with Seneca 60 maize, Laurie et al. (1990) reported that 28% of the florets were fertilized. Since an average of 20.5 embryos per 100 florets were obtained, it is likely that most of the fertilized egg cells gave rise to an embryo. This is in agreement with the results reported in Laurie and Reymondie (1991). Laurie et al. (1990) identified different events as the causes of the fertilization failure. Further cytological observations of tetraploid wheat ovaries showed that maize pollen tubes exhibited various aberrations beyond the top of the ovule (O'Donoughue and Bennett 1994).

The reason why some of the cultured embryos failed to develop into plantlets is still unknown; however their frequency may be influenced by in vitro culture parameters. Comeau et al. (1992) showed differences between media can result in differences in the recovery efficiency of wheat haploid embryos. In carrot, the synthesis of extracellular glycoproteins promoting somatic embryogenesis is inducible by culture conditions (De Vries et al. 1988; Van Engelen and De Vries 1992). Plant embryogenesis is a very complex phenomenon which requires the expression of numerous genes (Lindsey and Topping 1993), and embryogenic-lethal mutants have been generated for these for example, in *Arabidopsis* (Meinke 1985) and maize (Sheridan and Clark 1993). It is well-established that growth regulators such as 2,4-D may induce chromosome alterations and mutations (Turkula and Jalal 1985; Lee and Phillips 1988; D'Amato 1990). Besides its beneficial effects, 2,4-D may induce mutations in the modifier or regulatory genes that control embryogenesis and seedling development; such mutants have been identified in *Arabidopsis* (Mayer et al. 1991). An alternative is that the dedifferentiating effects of 2,4-D that are so useful for inducing callus may be inhibiting proper embryo development. The failure of embryos to develop beyond the production of a coleoptile or roots may be due to the fact that the meristems are not properly formed.

The fertility of colchicine-treated haploid plants, a trait which can be assessed only after heading, is a critical and obviously an economical criterion for successful DH production. From this study, it can be predicted by an average approximation by flow cytometric analysis of the first tiller that arises after colchicine treatment. Further investigations would be necessary to increase the reliability of this predictor, especially by analyzing subsequent tillers. However, a threshold of %DN/TN seemed to exist under which there is no chance for a plant to be fertile. Consequently, those plants could be re-treated with colchicine for maximum efficiency.

Distortions in the segregation of marker alleles in anther culture-derived progenies have been reported. In barley, the most frequent allele found at some loci resulted in a significantly higher anther culture response (Devaux and Zivy 1994). Wheat cultivars which possess the 1BL/1RS translocated chromosome have been shown to have a higher anther culture regeneration ability than those with a normal 1B chromosome (Agache et al. 1989), and plants having this translocated chromosome are more frequent in populations derived from structural heterozygotes by AC. In contrast, this distortion of segregation did not occur in the MM-derived and selfed progenies of the same crosses, although Henry et al. (1993) reported that the IBL-IRS chromosome was transmitted through 45% of the egg cells. Further investigations are necessary to check for other markers spread over the genome. In this respect, Kleinhofs et al. (1993) reported distorted segregation for restriction fragment length polymorphism markers at several locations in the genome among barley DH lines derived by interspecific hybridization with *H. bulbosum*.

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References

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

Ainsworth C, Gale MD (1987) Enzyme structural genes and their exploitation in wheat genetics and breeding. In: Kruger JE, Staufer CE, Lineback DR (eds) Enzymes and their role in cereal technology. Amer Assoc Cereal Chem, St Paul, pp 53–82

Bergounioux C, Perennes C, Miège C, Gadal P (1986) Male sterility effect upon protoplast division in *Petunia hybrida*. Protoplasma 130:138–144

Biom (1989) A package of statistical programs. Applied Biostatistics, Setauket, N.Y.

1272

Bjϕrnstad Å (1986) Partial incompatibility between Scandinavian six-rowed barleys (*Hordeum vulgare* L.) and *Hordeum bulbosum* L., and its genetical basis. Hereditas 104:171–191

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

D'Amato F (1990) Somatic nuclear mutations *in vivo* and in *vitro* in higher plants. Caryologia 43:191–204

Devaux P (i992) Haploidy in barley and wheat improvement. In: Dattée Y, Dumas C, Gallais A (eds) Reproductive biology and plant breeding. (Proc 8th Eucarpia Congress) Springer Berlin Heidelberg New York, pp 139–151

Devaux P, Zivy M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701–706

Devaux P, Lonnet P, Jean R (1990) Significant higher proportions of 1BL-1RS wheat-rye translocation lines among doubled haploid progenies derived from F_1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp NJJ, Van der Plas LHW, Van Aartrijk J (eds) Progress in plant cellular and molecular biology. (Proc 7th Int Cong Plant Tissue Cell Cult.) Kluwer, Amsterdam, pp 422–426

Devaux P, Adamski T, Surma M (1992) Inheritance of seed set in crosses of spring barley and *Hordeum bulbosum* L. Crop. Sci 32: 269–271

De Vries SC, Booij H, Janssens R, Vogels R, Saris L, Lo Schiaro F, Terzi M, Van Kammen A (1988) Carrot somatic embryogenesis depends on the phytohormone-controlled presence of correctly glycosylated extracellular proteins, Genes Dev 2:462–476

Dpac (1994) Data pool application software for CA-II, version 2.1. Partec, Münster, Germany

Foroughi-Wehr B, Zeller FJ (1990) In Vitro microspore reaction of different German wheat cultivars. Theor Appl Genet 79:77–80

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

Hayes PM, Chen FQ (1989) Genotypic variation for *Hordeum bulbosum* L.-mediated haploid production in winter and facultative barley. Crop Sci 29:1184–1188

Henry Y, De Buyser J (1985) Effect of the 1B/1R translocation on anther culture ability in wheat (*Triticum aestivum* L.) Plant Cell Rep 4:307–310

Henry Y, De Buyser (1990) Wheat anther culture: agronomic performance of doubled haploid lines and the release of a new variety 'Florin'. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 13. Springer, Berlin Heidelberg New York, pp 285–352

Henry Y, Bernard S, Bernard M, Gay G, Marcotte JL, De Buyser J (1993) Nuclear gametophytic genes from chromosome arm 1RS improve regeneration of wheat microspore-derived embryos. Genome 36:808–814

Hougas RW, Peloquin SJ, Gabert AC (1964) Effect of speed-parent and pollinator on frequency of haploids in *Solanum tuberosum*. Crop Sci 4:593–595

Inagaki M, Tahir M (1990) Comparison of haploid production frequencies in wheat varieties crossed with *Hordeum bulbosum* L. and maize. Jpn J Breed 40:209–216

Jiang J, Friebe B, Gill BS (1994) Recent advances in alien gene transfer in wheat. Euphytica 73:199–212

Kasha KJ, Reinbergs E (1982) Recent developments in the production and utilization of haploids in barley. In: Asher MJC, Ellis RP, Hayter AM, Whitehouse RNH (eds) Proc 4th Int Barley Genet Symp. Edinburgh University Press, Edinburgh, pp 655–665

Kisana NS, Nkongolo KK, Quick JS, Johnson DL (1993) Production of doubled haploids by anther culture and wheat × maize method in a wheat breeding programme. Plant Breed 110:96–102

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

Kotch GP, Peloquin SJ (1987) A new source of haploid germplasm for genetic and breeding research. Am Potato J 64:137–141

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

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

Laurie DA, Reymondie S (1991) High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. Plant Breed 106:182–189

Laurie DA, O'Donoughue L, Bennett MD (1990) Wheat × maize and other wide sexual hybrids: their potential for genetic manipulation and crop improvement. In: Gustafson JP (ed) Gene manipulation in plant improvement II. Plenum, New York, pp 95–126

Lazar MD, Schaeffer GW, Baenziger PS (1984) Cultivar and cultivar × environment effects on the development of callus and polyhaploid plants from anther cultures of wheat. Theor Appl Genet 67: 273–277

Lee M, Phillips RL (1988) The chromosomal basis of somaclonal variation. Annu Rev Plant Physiol Plant Mol Bio 39: 413–437

Lindsey K, Topping JF (1993) Embryogenesis: a question of pattern. J Exp Bot 44: 359–374

Marsolais AA, Séguin-Swartz G, Kasha KJ (1984) The influence of anther cold pretreatments and donor plant genotypes on in vitro androgenesis in wheat (*Triticum aestivum* L) Plant Cell Tissue Organ Cult 3:69–79

Mayer U, Torres Ruiz RA, Berleth T, Misera S, Jurgens G (1991) Mutations affecting body organization in the *Arabidopsis* embryo. Nature 33:402–407

Meinke DW (1985) Embryo-lethal mutants of *Arabidopsis thaliana*: analysis of mutants with a wide range of lethal phases. Theor Appl Genet 69:543–552

O'Donoughue LS, Bennett MD (1994) Comparative responses of tetraploid wheats pollinated with *Zea mays* L. and *Hordeum bulbosum* L. Theor Appl Genet 87: 673–680

Orlov PA, Mavrishcheva EB, Palilova AN (1993) Estimation of the response to anther culturing in 60 genotypes of different wheat species. Plant Breed 111:339–342

Oury FX, Pichon M, Rousset M (1993) Une comparison entre 2 méthodes d'haplodiploïdisation chez le blé tendre: l'androgenèse in vitro et le croisement interspécifique avecle maïs. Agronomie 13: 95–103

Pickering RA (1980) Use of the doubled haploid technique in barley breeding at the Welsh Plant Breeding Station. Rep Welsh Plant Breed Stn for 1979, Aberystwyth, pp 208–226

Pickering RA (1983) The influence of genotype on doubled haploid barley production. Euphytica 32:863–876

Pickering RA (1984) Crossability relationships between certain species in the Hordeae. Barley Genet Newsl 14:14–17

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

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

Sarrafi A, Amrani N, Alibert G (1994) Haploid regeneration from tetraploid wheat using a maize pollen. Genome 37:176–178

Sheridan WF, Clark JK (1993) Mutational analysis of morphogenesis of the maize embryo. Plant J 3:347–358

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

Sokal RR, Rolf FJ (1981) Biometry, 2nd edn. W.H. Freeman and Co., New York

Statistix (1992) Analytical software version 4.0. Statistix, St. Paul, Minn.

Suenaga K, Nakajima K (1989) Efficient production of haploid wheat (*Triticum aestivum*) through crosses between Japanese wheat and maize (*Zea mays*). Plant Cell Rep 8:263–266

Suenaga K, Tamaki M, Nakajima K (1991) Influence of wheat (*Triticum aestivum*) and maize (*Zea mays*) genotypes on haploid wheat production in crosses between wheat and maize. Bull Natl Inst Agrobiol Resour 6:131–142

Turkula TE, Jalal SM (1985) Increased rates of sister chromatid exchanges induced by the herbicide 2,4-D. J Hered 76:213–214

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 Engelen FA, De Vries SC (1992) Extracellular proteins in plant embryogenesis. Trends Genet 8:66–70

Vasil V, Redway F, Vasil IK (1990) Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Bio/Technology 8: 429–434

Wendel JF, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Dioscorides Press, Portland, Ore., pp 5–45

Ziegler G, Dressler K, Hess D (1990) Investigations on the anther culturability of four German spring wheat cultivars and the influence of light on regeneration of green vs. albino plants. Plant Breed 105:40–46

Mol Gen Genet (1993) 241:674–679

Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation

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Abstract. Anther culture and *Hordeum bulbosum*-derived doubled haploid (DH) lines of barley (*Hordeum vulgare* L.) were analyzed for RFLP and RAPD polymorphisms. Polymorphisms were not detected in the anther culture-or *H. bulbosum*-derived DH lines among 273 RFLP and 89 polymerase chain reaction (PCR)-amplified DNA fragments assayed. It was calculated that base substitution or small deletion/insertion mutations had not been induced among 401 640 bp screened. Large deletion/insertion mutations were not observed among 33 Mb screened. Polymorphisms were observed when DNA was digested with the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I: these RFLPs originated primarily from the anther culture-derived doubled haploids. The data indicate that heritable DNA methylation changes had occurred during DH production, particularly with the anther culture method.

Key words: Anther culture – *Hordeum bulbosum* method – Doubled haploid – Mutation frequency – DNA methylation

Introduction

Doubled haploid (DH) lines are used for plant breeding, genetic analyses and to construct molecular marker maps (Kasha and Reinbergs 1982; Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993). In barley (*Hordeum vulgare* L.), DHs can be produced by *H. bulbosum* (Hb) and anther culture (AC) methods. Both techniques require an in vitro culture phase, but they differ in several aspects. (1) Length of time in culture: 3 weeks for Hb versus 2 months for AC. (2) Composition of the media: growth hormones are not used for the development of haploid embryos in the Hb technique while an auxin, indole acetic acid or naphthalene acetic acid, and a cytokinin, kinetin or benzylaminopurine, are commonly used in AC (for review see Pickering and Devaux 1992). (3) Regeneration mechanism: plants are obtained through the development of zygotic embryos in the Hb technique but via organogenic and embryogenic structures in AC.

It is well established that the in vitro culture phase can induce genetic and cytoplasmic changes in regenerated plants (Larkin and Scowcroft 1981; Evans and Sharp 1983; Day and Ellis 1985; Pickering 1989; Ullrich et al. 1991). Moreover the conditions of the culture phase influence the frequency of these variations (for review see Karp 1991). Phenotypic changes such as albinism, variation in ploidy level, and chromosomal abnormalities have been reported in AC-derived regenerants (Clapham 1973; Mix et al. 1978; De Buyser et al. 1985; Charmet et al. 1986). Most of the gametoclonal variations negatively affect the agronomic performance of DHs (Baenziger et al. 1983; Powell et al. 1984; Snape et al. 1988; Marburger and Jauhar 1989). Snape et al. (1988) reported variation in Hb-derived DHs of wheat for three quantitative traits but did not find variation in barley Hb-derived DHs among seven quantitative characters studied.

Although genetic alterations induced by AC and Hb methods are well documented, there are very few reports on gametoclonal variation at the DNA level. Day and Ellis (1985) showed changes in the chloroplast DNA of wheat and barley albino plants derived by the AC method. Reports on genomic DNA are limited to the analysis of repetitive sequences (De Paepe et al. 1983; Rode et al. 1987). The purpose of this investigation was to assess the level of DNA variation in phenotypically normal DH barley plants derived by the Hb and AC techniques from one cultivar. Two classes of molecular markers were

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applied [restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD)] to analyze for base substitution and insertion/deletion events.

Materials and methods

Plant material. Seeds of the winter barley cultivar Igri were kindly provided by Mr. Darrozés (Serasem – Pérenchies, France). Haploid and spontaneous DH plants were produced through AC and Hb techniques according to Devaux (1987); the haploid plants were treated with colchicine to induce chromosome doubling (Pickering 1980). Before anthesis, all spikes were bagged to avoid cross pollination. At maturity, grains were collected from spontaneous and colchicine-treated DH plants and propagated in a field nursery. Two to four spikes per DH were bagged and collected at maturity. Five grains each of 60 randomly chosen DHs (30 from AC and 30 from Hb techniques) as well as seeds from the original lot of Igri were sown in a greenhouse. Three to four weeks later, the aerial parts of the seedlings were harvested, plunged into liquid nitrogen and lyophilized. Dried tissues were stored at −20° C until DNA extraction.

DNA extraction. Genomic DNA was isolated as described in Kleinhofs et al. (1993). Briefly, total DNA was extracted from powdered lyophilized tissues using the cetyltrimethylammonium bromide and phenol-chloroform procedure and precipitated with 2-propanol. After dissolution in TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8), the DNA was treated with RNAase A, extracted with chloroform, precipitated with ethanol and finally dissolved in distilled water at about 0.5 µg/µl.

RFLPs. About 10 µg of genomic DNA were digested with 30 units of restriction enzyme according to the manufacturer's recommendations. The 6 bp recognition site enzymes *Bam*HI, *Eco*RI and *Hin*dIII and the 4 bp recognition site enzymes, *Hpa*II and *Msp*I, were used. The latter two enzymes are isochizomers, but respond differently to methylation of the second C in the 5′-CCGG-3′ recognition sequence. Restricted DNA fragments were separated by electrophoresis and transferred from the gels to MSI (Micron Separations, Westborough, MA) membranes. The probes Adh1, Amy2, Cab, Chs, Glb1, Hor1, Isa1, Mub2, Pox, ABG307, BCD351, BG123, SBC218, pHv294T, Akp6, ksuA1, ksuA3, ksuF2, 21B9, and 25H2 were used to detect polymorphisms with the restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII. The probes ABG471, ABC174, BCD351, BG123, ksuA3, 21B9, and 25H2 were used to detect polymorphisms with the restriction enzymes *Hpa*II and *Msp*I. The probes are from various sources described in Kleinhofs et al. (1993).

RAPDs. About 50 ng of genomic DNA were used as template for amplification with 11 random decamer primers as described by Kleinhofs et al. (1993).

Results

Assessment of mutation

Polymorphisms were not detected between the AC and Hb-derived DHs among 273 RFLP fragments revealed by 18 probes and using 3 restriction enzymes. The patterns obtained were identical to those of cv Igri. This indicates that base-substitution mutations, insertions, and deletions were not detected. The total number of base pairs (bp) screened for base substitutions and small (a few bp) insertion/deletion events was 294 840 and corresponds to the product of: (1) the number of DHs investigated – 60; (2) the total number of restriction fragments screened – 273; and (3) the number of bp screened per fragment – 18 [this corresponds to the 6 bp at the two ends of each fragment screened for loss of 6-base recognition sites plus the mean number of bp in a ca. 4 kb fragment screened for mutation to create the same restriction site (Kilian and Gale 1991)].

To estimate the number of bp screened for large insertions/deletions (detectable by agarose gels used to separate restriction fragments) a value of 4096 should be used instead of 18 in the above formula. This number corresponds to an expected average size of RFLP fragment generated by restriction enzymes with 6 bp recognition sites and gives a value of ca. 60 Mb analyzed for detectable insertions/deletions. This value was reduced by a factor of 2 to 30 Mb to correct for overlapping fragments, which were produced by different enzymes and detected by the same probe; the estimate is therefore likely to be conservative. In addition, no variation in fragment number, size, or intensity of hybridization was found using the ribosomal DNA intergeneric spacer probe, pHv294T (Gerlach and Bedbrook 1979) or the telomeric probe, Akp6 (Kilian and Kleinhofs 1992).

All electrophoretic patterns of PCR-amplified DNA fragments of the 60 DHs and cv Igri were identical, indicating that no mutation had occurred in annealing sites of the primers. The number of bp checked for nucleotide substitutions and small insertions/deleitons was 106 800. This number is based on the number of DHs

Table 1. Polymerase chain reaction (PCR)-amplified bands detected with 11 decamer primers used in this study

Primer	Sequence	No. of bands
CS19	5′-TAC GGC TGG C-3′	10
CS21	5′-CCC TAC CGA C-3′	8
CS31	5′-CTC GAC ACT G-3′	9
CS34	5′-GAT AGC CGA C-3′	9
CS35	5′-AGT CGC TCA T-3′	9
CS42	5′-CCC AGA ACA C-3′	8
CS45	5′-CAC GTC GGA G-3′	9
CS47	5′-TTG CCG TGT T-3′	8
CS50	5′-CCC AAA CTA G-3′	7
I04	5′-CCG CCT AGT C-3′	6
L20	5′-TGG TGG ACC A-3′	6
Total		89

investigated (60) multiplied by 20 (i.e. 2×10 bases) and by the 89 fragments screened (Table 1). The molecular basis for RAPD polymorphism is still obscure (Yu et al. 1993), so it is difficult to estimate the number of bp scanned for mutation using this technique. Since there is indirect evidence suggesting that a single bp mismatch between primer and template may result in a lack of product amplification (Williams et al. 1990), we used a value of 20 bp as a multiplication factor (2×10 bp, each annealing site) to calculate the number of bp screened for mutation. It is possible that it is an overestimate for loss of the RAPD band but, on the other hand, we did not take into account amplification of a new product due to a mutation event. If an average RAPD band size (600 bp) is used as a multiplication factor (instead of 20 bp) the formula gives ca. 3 Mb of DNA analyzed for insertion/deletion events.

Frequency of mutation

From these experiments, we conclude that no mutation had occurred in 401 640 (294 840 by RFLPs and 106 800 by RAPDs) bp screened for small insertion/deletion events and nucleotide substitutions. Since the barley genome is approximately 5.5×10^9 bp (Bennett and Smith 1976), this represents 0.0073% of the barley genome scored for point mutations. Since no mutations were found in 401 640 bp that were screened, the frequency of mutation per bp due to the in vitro culture is less than 0.25×10^{-5}. There were no large insetions/deletions detected in ca. 33 Mb screened, corresponding to more than 0.5% of the barley genome.

Changes in DNA methylation

DNA digested with the methylation-sensitive restriction enzyme *Hpa*II yielded RFLPs, suggesting that DNA methylation changes had occurred during DH production (Fig. 1A and B). These RFLPs originated primarily from AC-derived DHs: 49 aut of 51 variable fragments detected with seven probes. Most of the variations were confirmed using the *Hpa*II isochizomer, *Msp*I. The recognition sequence 5′-CCGG-3′ is cleaved by *Msp*I when the internal C is methylated, but not if the 5′-C is methylated, while the isochizomer *Hpa*II does not cleave when either C is methylated. Thus in most cases methylation had occurred at least at the 5′-C in the recognition sequence.

Methylation polymorphisms appear to be randomly distributed among the DHs, but nonrandomly distributed among the probes. Most of the 30 AC-derived DHs showed polymorphism with at least one probe, but only three DHs were different from the control with all three of the probes accounting for most of the observed polymorphisms. These probes, BCD351, ksuA3, and 21B9, revealed 48 out of the 49 RFLPs in the Ac-derived DHs suggesting nonrandom distribution of methylation changes among the probes. Polymorphisms were due to

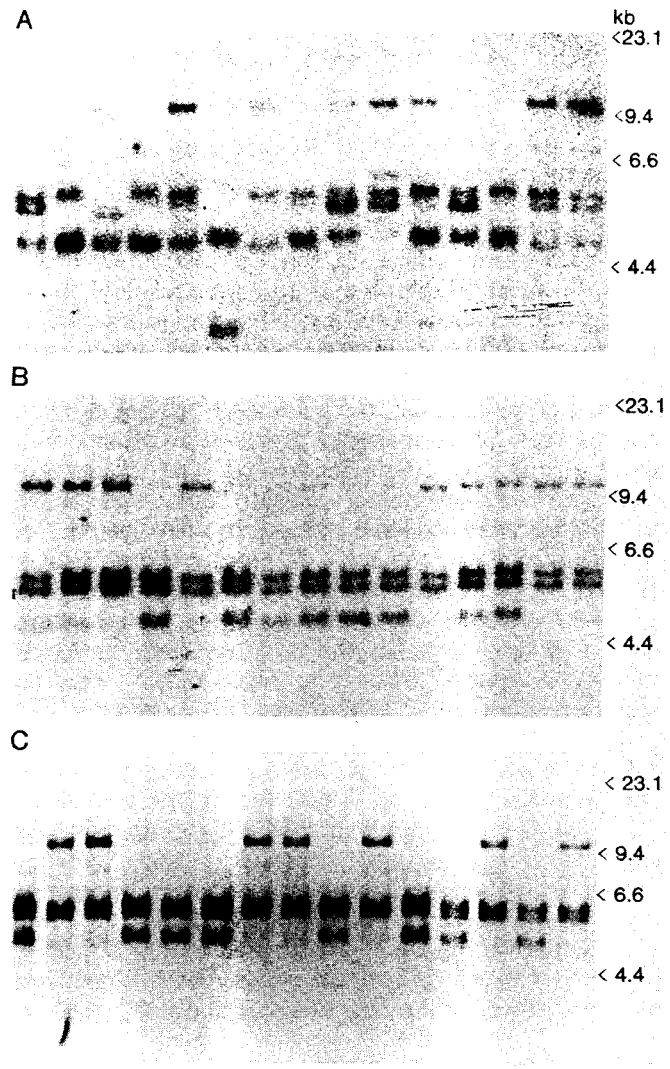

Fig. 1A–C. Doubled haploid- and individual seed-derived cv Igri line DNA digested with *Hpa*II and hybridized with probe 21B9. **A** Anther culture-derived doubled haploids (DHs); **B** *Hordeum bulbosum*-derived DHs; **C** Seed-derived lines. Note the large variation in the size and intensity of the individual bands in the anther culture-derived doubled haploids (**A**) and the distinct segregation pattern for the highest and lowest molecular weight bands in the seed-derived lines (**C**). The *H. bulbosum*-derived doubled haploids (**B**) show some variation in the hybridization intensity of the highest and lowest mol. wt. bands

increased methylation (37 out of 51 cases) or decreased methylation (14 out of 51 cases), as indicated by higher or lower molecular weight DNA fragments hybridizing, respectively.

To determine whether DNA methylation heterogeneity existed in the original lot of Igri, the DNA of 16 individual plants was digested with *Hpa*II and hybridized with the probes BCD351, ksuA3, 21B9, and BG123. Polymorphisms were not found among these 16 plants except with the probe 21B9, which hybridized with three fragments in all lines (Fig. 1C). Two bands were identical among all plants tested; the third band was polymorphic

with seven plants showing a higher and nine plants a lower molecular weight band. The same polymorphism was observed in the DH progeny with similar frequencies (ca. 1:1 ratio). This polymorphism was presumed to have preexisted in the parents and was not included in the overall summary of the methylation polymorphism.

Discussion

RLFP and RAPD techniques were employed to evaluate the level of gametoclonal variation induced in barley by AC and Hb systems of doubled haploid production. Both techniques are capable of detecting point mutations and larger rearrangements of DNA, although only the former allows discrimination between the two types of genetic alterations. Since the majority of restriction enzyme recognition sites (in RFLP) and oligonucleotide priming sites (in RAPD) are probably outside of the coding sequences, these techniques detect genetic variation not subjected to phenotypic selection. Snape et al. (1988) suggested that barley has lower levels of gametoclonal variation than wheat due to its diploid nature causing negative selection of deleterious mutations. Employing selection-neutral markers in comparative analyses of gametoclonal variation in these two species would facilitate critical evaluation of this hypothesis.

Point or deletion/insertion mutations were not detected in Hb or Ac-derived DHs by RFLP or RAPD even though a substantial length of DNA was analyzed. Subtelomeric heterochromatin and rDNA sequences, reported to be particularly sensitive to somaclonal variation (Larkin et al. 1984; Breiman et al. 1987; Rode et al. 1987; Karp et al. 1992), did not show any detectable changes. The apparently high genetic stability of barley AC-derived doubled haploids seems to be in contrast to the results of Ullrich et al. (1991) who reported an average of 17.2% and 7.8% somaclonal variation among immature embryo callus-derived R2 plant rows and R2 head rows, respectively. It is possible that changes in the methylation pattern detected with high frequency in the AC population may account for this discrepancy, since methylation may play an important role in gametoclonal variation (Brown 1989).

Molecular studies of gametoclonal variation in doubled haploids are limited to analyses of the plastid genome (Day and Ellis 1985) and repetitive sequences (De Paepe et al. 1983; Rode et al. 1987) of AC-derived plants. However mapping of the barley genome using DH populations provides numerous opportunities for observing non-parental RFLP bands. Putative mutations were not detected in an AC-derived population from the cross Steptoe × Morex (P. Devaux et al. in preparation). Mutations were also not reported for Igri × Franka or Proctor × Nudinka mapping with AC-derived populations (Graner et al. 1991; Heun et al. 1991). Extensive mapping of a Steptoe × Morex Hb-derived DH population (Kleinhofs et al. 1993) provided no evidence for mutational variation induced by this technique. Although mapping may not be influenced by rare, mutation-generated RFLP bands, we believe that if such bands had occurred in significant frequencies they would have been reported. These data indicate that AC- and Hb-derived DHs have very low levels of induced DNA changes in barley.

The level of DNA methylation was altered in AC and to a much smaller extent, in Hb-derived DHs. The most common methylated base, 5-methylcytosine, accounts for up to 30% of C in plant DNA (Gruenbaum et al. 1981; Adams and Burdon 1985) depending on the genome size (Adams 1990). Barley, with a genome size of 5.5×10^9 bp, has 25.7% of the C residues methylated (Amasino et al. 1990).

Brown et al. (1990) reported high levels of RFLPs in the R1 generation of protoplast-derived rice (*Oryza sativa* ssp *japonica*) plants both with methylation sensitive and insensitive restriction enzymes. Higher genetic instability of the actin genes was found in rice plants regenerated from calli maintained for 67 days versus 28 days in culture (Müller et al. 1990). In our study, 96% (49 out of 51) of the total methylation polymorphism was from AC-derived DHs. These changes in the DNA methylation pattern were probably induced during the in vitro culture of the microspores. The longer period in culture for the AC-versus the Hb-derived DHs might account for the higher level of C methylation polymorphism observed. The two variants observed in the Hb-derived DHs could be related to the fact that a few haploid embryos gave rise to calli prior to regeneration and, therefore, had to be maintained longer (up to 6 weeks) in vitro (Pickering and Devaux 1992).

Apart from the tissue culture conditions, AC and Hb systems start with different tissues: microspores versus immature zygotic embryos. Different tissues may have different levels of DNA methylation as reported for tomato (Messeguer et al. 1991). We cannot exclude the possibility that different methylation levels that could be heritable preexisted in the material used to initiate the DH development. However the relationships between different methylation patterns and their heritability are obscure.

Dramatic differences among the probes in the level of methylation pattern polymorphism detected indicates that DNA methylation changes do not occur at random throughout the genome. This is in agreement with the results of Brown (1989). The differences among the cDNA probes may be attributed to the differences in gene activity during the culture phase among the corresponding genes. It is unlikely that the observed changes in methylation pattern could have been induced by colchicine since a high proportion of spontaneous DHs are usually recovered from AC while no spontaneous doubling occurs in the Hb system (Pickering and Devaux 1992).

The heterogeneity detected by the probe 21B9 among the plants of cv Igri provides an example of hidden polymorphism present in barley cultivars. Kilian and Gale (1991) reported similar RFLP polymorphism in the population derived from cv Triumph. Polymorphism for the nicotinamide adenine dinucleotide (NADH) nitrate

reductase gene in cv Steptoe was observed in our laboratory and shown to be due to the presence of different alleles in the parent lines (Jeter 1987). It was not possible to verify the source of Igri polymorphism since the parents were not available. However the 1:1 ratio observed for the two bands suggests that these bands are inherited from parental genotypes.

An interesting aspect of cv Igri polymorphism comes from the fact that it was detected with a methylation-sensitive enzyme *Hpa*II, while being absent for three methylation-insensitive enzymes. Since *Hpa*II is a "4-cutter" and both alternative bands are several kb in size, we may assume that there is a methylation pattern polymorphism rather then differences in the distribution of the recognition sequence. If our assumptions about the source and nature of this polymorphism are correct, it may represent an example of high stability of methylation pattern polymorphism. Interestingly the same probe (21B9) detected very high levels of methylation pattern polymorphism in AC-derived DHs. The fact that nearly half of the tested probes detected very high levels of polymorphism among AC-derived DHs together with proven stability of methylation changes (Brown 1989; Brettell et al. 1991) and their Mendelian inheritance (Messeguer et al. 1991) suggests the possibility of using this system for generating novel RFLPs. This could find numerous applications both in theoretical and applied RFLP research in highly non-polymorphic populations.

References

Adams RLP (1990) DNA methylation. The effect of minor bases on DNA-protein interactions. Biochem J 265:309–320

Adams RLP, Burdon RH (1985) Molecular biology of DNA methylation. Springer, New York

Amasino RM, John MC, Klaas M, Crowell DN (1990) Role of DNA methylation in the regulation of gene expression in plants. In: Clawson GA, Willis DB, Weissbach A, Jones PA (eds) Nucleic acid methylation. Wiley-Liss, New York, pp 187–198

Baenziger PS, Wesenberg DM, Schaeffer GW, Galun E, Feldman M (1983) Variation among anther culture derived doubled haploids of 'Kitt' wheat. In: Sakamoto S (ed) Proceedings 6th International Wheat Genetics Symposium, Kyoto University, Japan, pp 575–582

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Phil Trans R Soc London (Biol) 274:227–274

Breiman A, Rotem-Abarbanell D, Karp A, Shaskin H (1987) Heritable somaclonal variation in wild barley *(Hordeum spontaneum)*. Theor Appl Genet 74:104–112

Brettell RIS, Dennis ES (1991) Reactivation of a silent *Ac* following tissue culture is associated with heritable alterations in its methylation pattern. Mol Gen Genet 229:365–372

Brown PTH (1989) DNA methylation in plants and its role in tissue culture. Genome 31:717–729

Brown PTH, Kyozuka J, Sukekiyo, Kimura Y, Shimamoto K, Lörz H (1990) Molecular changes in protoplast-derived rice plants. Mol Gen Genet 223:324–328

Charmet G, Bernard S, Bernard M (1986) Origin of aneuploid plants obtained by anther culture in triticale. Can J Genet Cytol 28:444–452

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

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, Taleb G (1985) Wheat androgenesis: cytological analysis and agronomic performance of doubled haploids. Z Pflanzenzüchtg 95:23–34

De Paepe R, Prat D, Huguet T (1983) Heritable nuclear DNA changes in doubled haploid plants obtained by pollen culture of *Nicotiana sylvestris*. Plant Sci Lett 28:11–28

Devaux P (1987) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploid in winter barley. I. Production of green plants. Plant Breeding 98:215–219

Evans DA, Sharp WR (1983) Single gene mutations in tomato plants regenerated from tissue culture. Science 221:949–951

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

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

Gruenbaum Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley *(Hordeum vulgare)*. Genome 34:437–447

Jeter JM (1987) Molecular analysis of the *Nar1* locus in barley. MS Thesis, Washington State University, Pullman

Karp A (1991) On the current understanding of somaclonal variation. Oxf Surv Plant Mol Cell Biol 7:1–58

Karp A, Owen PG, Steele SH, Bebeli PJ, Kaltsikes PJ (1992) Variation in telomeric heterochromatin in somaclones of rye. Genome 35:590–593

Kasha KJ, Reinbergs E (1982) Recent developments in the production and utilization of haploids in barley. In: Asher MJC, Ellis RP, Hayter AM, Whitehouse RNH (eds) Proceedings 4th International Barley Genetics Symposium. Edinburgh, UK, pp 655–665

Kilian A, Gale MD (1991) Induction of RFLPs by mutagenesis. Cereal Res Commun 19:119–127

Kilian A, Kleinhofs A (1992) Cloning and mapping of telomere-associated sequences from *Hordeum volgare* L. Mol Gen Genet 235:153–156

Kleinhofs A, Kilian A, Kudrna D, Bollinger J, Hayes P, Chen FQ, Saghai Maroof MA, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ, Knapp SJ (1993) A molecular, isozyme and morphological map of the barley *(Hordeum vulgare)* genome. Theor Appl Genet (in press)

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Larkin PJ, Ryan SA, Brettell RIS, Scowcroft WR (1984) Heritable somaclonal variation in wheat. Theor Appl Genet 67:433–455

Marburger JE, Jauhar PP (1989) Agronomic, isozyme, and cytogenetic characteristics of 'Chris' wheat doubled haploids. Plant Breeding 103:73–80

Messeguer R, Ganal MW, Steffens JC, Tanksley SD (1991) Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. Plant Mol Biol 16:753–770

Mix G, Wilson HM, Foroughi-Wehr B (1978) The cytological status of plants of *Hordeum vulgare* L. regenerated from microspore callus. Z Pflanzenzüchtg 80:89–99

Müller E, Brown PTH, Hartke S, Lörz H (1990) DNA variation in tissue-culture-derived rice plants. Theor Appl Genet 80:673–679

Pickering RA (1980) Use of doubled haploid technique in barley breeding at the Welsh Plant Breeding Station. Report Welsh Plant Breeding Station for 1979, pp 208–226

Pickering RA (1989) Plant regeneration and variants from calli derived from immature embryos of diploid barley (*Hordeum vulgare* L.) and *H. vulgare* L. × *H. bulbosum* L. crosses. Theor Appl Genet 78:105–112

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

Powell W, Hayter AM, Wood W, Dunwell JM, Huang B (1984) Variation in the agronomic characters of microspore-derived plants of *Hordeum vulgare* cv. Sabarlis. Heredity 52:19–23

Rode A, Hartmann C, Benslimane A, Picard E, Quetier F (1987) Gametoclonal variation detected in the nuclear ribosomal DNA from double haploid lines of spring wheat (*Triticum aestivum* L., cv 'Cesar'). Theor Appl Genet 74:31–37

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

Ullrich SE, Edmiston JM, Kleinhofs A, Kudrna DA, Maatougui MEH (1991) Evaluation of somaclonal variation in barley. Cereal Res Commun 19:245–260

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Yu KF, Deynze AV, Pauls KP (1993) Random amplified polymorphic DNA (RAPD) analysis. In: Glick B, Thompson JE (eds) Methods in plant molecular biology and biotechnology. CRC Press, Boca Raton, (in press)

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ORIGINAL PAPER

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Comparative mapping of the barley genome with male and female recombination-derived, doubled haploid populations

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Abstract Male (anther culture) and female (*Hordeum bulbosum*) derived, doubled haploid populations were used to map the barley genome and thus determine the different recombination rates occurring during meiosis in the F1 hybrid donor plants. The anther culture-derived (male recombination) population showed an 18% overall increase in recombination rate. This increased recombination rate was observed for every chromosome and most of the chromosome arms. Examination of linkage distances between individual markers revealed eight segments with significantly higher recombination in the anther culture-derived population, and one in the *Hordeum bulbosum*-derived population. Very strong distortions of single locus segregations were observed in the anther culture-derived population, but map distances were not affected significantly by these distortions. There were 1.047 and 0.912 recombinations per chromosome in the anther culture and *Hordeum bulbosum*-derived doubled haploid populations, respectively.

Key words Recombination frequency · *Hordeum vulgare* · Segregation distortion · Selection

Introduction

Meiotic recombination is a crucial phenomenon on which genetic studies and improved organism breeding are based. The process may be affected by a large number of factors, such as chemicals (Sinha and Helgason 1969), temperature (Powell and Nilan 1963; Jensen 1981), age (Zetka and Rose 1990) genetics (Reeves et al. 1990; Sall et al. 1990; Sved et al. 1991) and sex (Shiroishi et al. 1991). Sex-related differences are expressed as higher recombination frequencies during either female meiosis (Weitkamp et al. 1982; Donis-Keller et al. 1987; De Vicente and Tanksley 1991) or male meiosis (Sokoloff 1964; Robertson 1984). However, these differences do not appear to be distributed uniformly over the genome and may differ in polarity on different chromosomes (Donis-Keller et al. 1987).

Doubled haploid (DH) lines are used extensively in barley breeding programs to reduce the time required to obtain pure lines and increase breeding efficiency (Kasha and Reinbergs 1982; Devaux 1992). They are produced by either the interspecific crossing of barley (*Hordeum vulgare*), as the female parent, with *Hordeum bulbosum* (Hb), or by anther culture (AC). These methods differ in three major aspects: (1) the Hb and AC-derived DH lines arise from female and male recombinant products, respectively; (2) the optimal donor plant growth conditions differ (Pickering and Devaux 1992) and (3) the in vitro culture phases are distinct: microspores evolve into embryoids that give rise to plantlets, while in the Hb method, the plantlets develop from zygotic embryos. The recombination frequency is likely to be affected by the first two features, while the in vitro culture phase may influence the representation of recovered gametes through gametophytic and/or sporophytic selection.

The availability of high-density linkage maps of barley (Graner et al. 1991; Kleinhofs et al. 1993; Kleinhofs 1994) now makes it possible to compare the recombination frequencies in Hb and AC-derived DH lines across most of the barley genome. Here we report the results of an experiment comparing map distances observed in Hb and AC-derived DH lines obtained from an F_1 (Steptoe × Morex) hybrid, using molecular markers

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covering most of the barley genome at regular intervals. This investigation compares the frequencies of recombination in female and male gamete development, compares the single locus distortions in AC and Hb mapping populations, examines the prospects for merging genetic maps based on Hb and AC-derived DH lines, and provides useful information for geneticists and breeders using barley DH lines in their programs.

Materials and methods

Genetic and molecular methods

Doubled haploid plants were produced from the reciprocal F1 hybrids between cvs. Morex and Steptoe by the *Hordeum bulbosum* method (Chen and Hayes 1989), as described in Kleinhofs et al. (1993), and by the anther culture (Devaux et al. 1993) technique. Before anthesis, all spikes were bagged to avoid cross pollination and, at maturity, grains were harvested. One to five grains per plant were sown in a growth chamber and the aerial parts were cut, frozen in liquid nitrogen and lyophilized for 3 days. In the case of AC-derived plants, plant parts from 16 direct regenerants (11 haploid and 5 diploid) were also collected for DNA isolation and handled as described above. Dried samples were stored at − 20° C until DNA extraction.

DNA was isolated as described in Kleinhofs et al. (1993). Briefly, total DNA was extracted from powdered, lyophilized tissues using the cetyl triammonium bromide and phenol-chloroform procedure and precipitated with 2-propanol. After dissolution in TE (10 mM TRIS-HCl, 1 mM EDTA pH8.0), the DNA was treated with RNase A, extracted with chloroform, precipitated with ethanol and finally dissolved in distilled water at about 0.5 µg/µl.

DNA was digested with *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII or *Xba*I, depending on which restriction enzyme was required to reveal polymorphism with the probes used. Restricted DNA fragments were separated by electrophoresis and transferred from the gels to charged nylon membranes (GeneScreen Plus). Sixty-four probes detecting 86 loci, selected from a previous RFLP map (Kleinhofs et al. 1993) to cover 94% of the barley genome, were mapped with 101 AC-derived DH lines.

Maps were constructed using the Macintosh version of MAPMAKER (Lander et al. 1987) and GMENDEL (Liu and Knapp 1990). Statistical evaluation of single-locus distortions and heterogeneity of allele segregations between the two mapping populations were done using GMENDEL. The significance of differences in map distances between two loci was determined using contingency χ^2 test.

Results

Map distances comparison

The *Hordeum bulbosum*-derived DH recombination values were recalculated for 101 lines from previously published data (Kleinhofs et al. 1993). The gene order was the same and the genetic linkage distances were also very similar, but somewhat lower.

The anther culture method yielded 77 diploid (57 spontaneously doubled and 20 obtained after colchicine treatment) and 9 tetraploid plants. Plant material for DNA isolation was also harvested from 16 primary regenerants, yielding a total of 102 lines. All lines, except one, were homozygous for all the loci investigated confirming their gametophytic origin. However, one diploid direct regenerant was heterozygous at 31 loci and homozygous at 37 loci (the remaining loci were not scorable without ambiguity). Data from this plant were not used for the map construction.

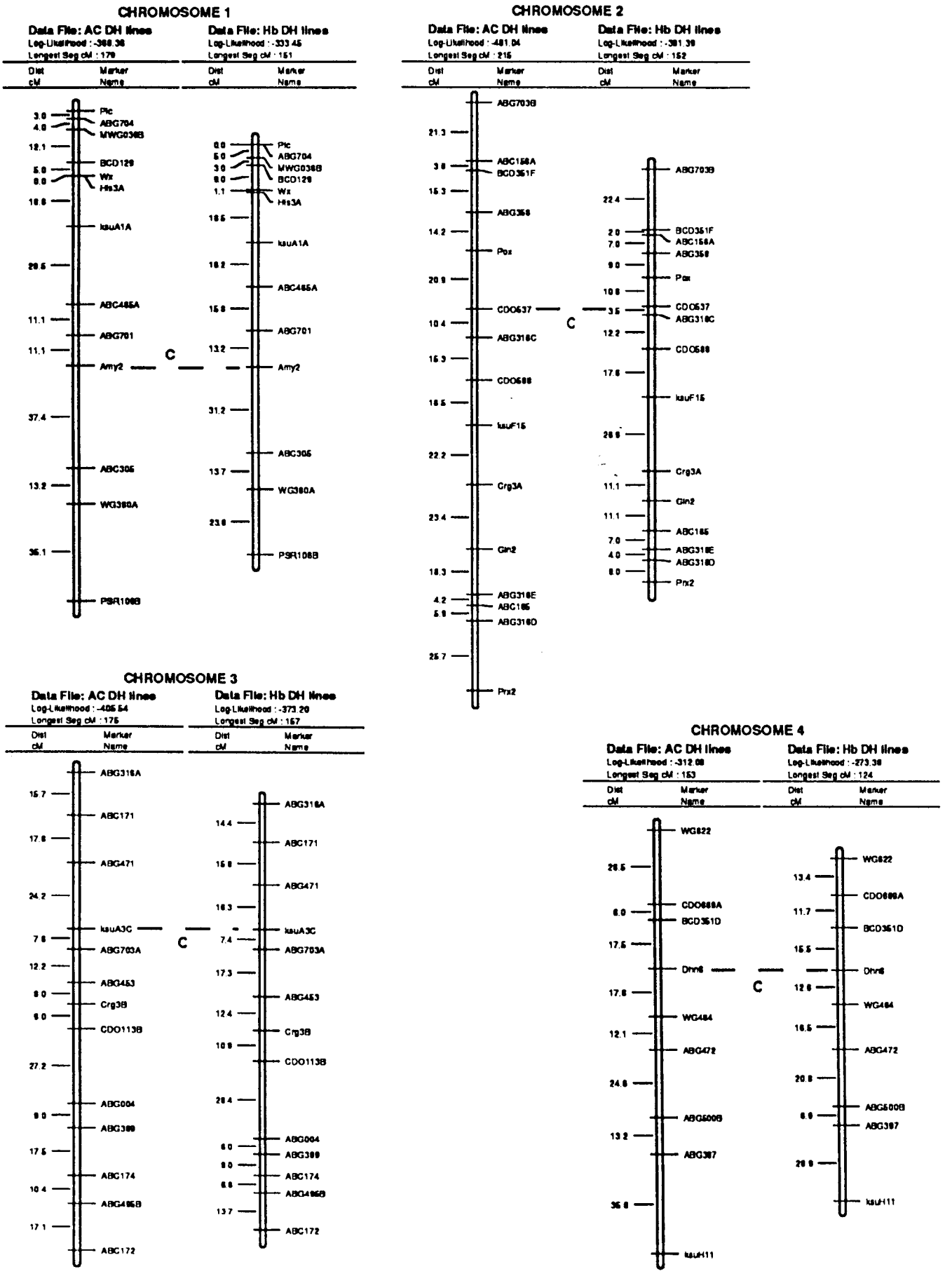
A total of 86 loci were included in the analysis (Table 1). These markers cover 94% of the barley genome (based on the latest Steptoe × Morex map developed using 150 Hb-derived DH lines and > 450 loci; Kleinhofs 1994). Most of the chromosomes are completely covered except for a few cM but a 43 cM segment of the minus (M, long) arm of chromosome 6 from PSR154 to MWG798A, representing 23% of the chromosome, is missing due to lack of intermediate markers.

The overall genome genetic map calculated for the AC-derived DH lines represents 118% of the Hb DH line map (Table 1; Fig. 1). This increase in the genetic map length is observed for every chromosome, with the smallest increase for chromosome 7 (108%) and the largest for chromosome 2 (141%). Comparison of the genetic map distances of individual chromosome arms (calculated from the marker closest to the centromere) yielded similar results, with the exception of chromosome 5 P (85%), chromosome 6 M (94%) and chromosome 7 P (79%). The differences between the maps in chromosome arm lengths were statistically

Table 1 Comparison of genetic distances in barley determined using segregating anther culture (AC)- or *Hordeum bulbosum* (Hb)-derived doubled haploid lines

Chr	Loci	Genetic distance (cM)								
		Total			P arm			M arm		
		AC	Hb	AC/Hb	AC	Hb	AC/Hb	AC	Hb	AC/Hb
1	13	179	151	1.19	93	82	1.13	86	69	1.25
2	15	215	152	1.41	75	51	1.47	140	101	1.39
3	13	175	157	1.11	57	46	1.24	118	111	1.06
4	9	153	124	1.23	50	41	1.22	103	84	1.23
5	12	154	135	1.14	58	68	0.85	96	67	1.43
6	10	115	105	1.10	84	72	1.17	31	33	0.94
7	14	206	191	1.08	42	53	0.79	164	137	1.20
Total	86	1197	1015	1.18	459	413	1.11	738	602	1.23

602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Data File: AC DH lines
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Longest Seg cM : 179
Dist cM
Marker Name
Plc
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MWG036B
BCD129
Wx
His3A
ksuA1A
ABC485A
ABG701
Amy2
ABC305
WG380A
PSR108B
Data File: Hb DH lines
Log-Likelihood : -333.45
Longest Seg cM : 151
C
CHROMOSOME 2
Data File: AC DH lines
Log-Likelihood : -481.04
Longest Seg cM : 215
ABG703B
ABC158A
BCD351F
ABG358
Pox
CDO637
ABG318C
CDO688
ksuF15
Crg3A
Gln2
ABG318E
ABC185
ABG318D
Prx2
ABG703A
Data File: Hb DH lines
Log-Likelihood : -381.39
Longest Seg cM : 152
CHROMOSOME 3
Data File: AC DH lines
Log-Likelihood : -405.54
Longest Seg cM : 175
ABG316A
ABC171
ABG471
ksuA3C
ABG703A
ABG453
Crg3B
CDO113B
ABG004
ABG389
ABC174
ABG466B
ABC172
Data File: Hb DH lines
Log-Likelihood : -373.29
Longest Seg cM : 157
CHROMOSOME 4
Data File: AC DH lines
Log-Likelihood : -312.68
Longest Seg cM : 153
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CDO668A
BCD351D
Dhn6
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Data File: Hb DH lines
Log-Likelihood : -273.38
Longest Seg cM : 124

603

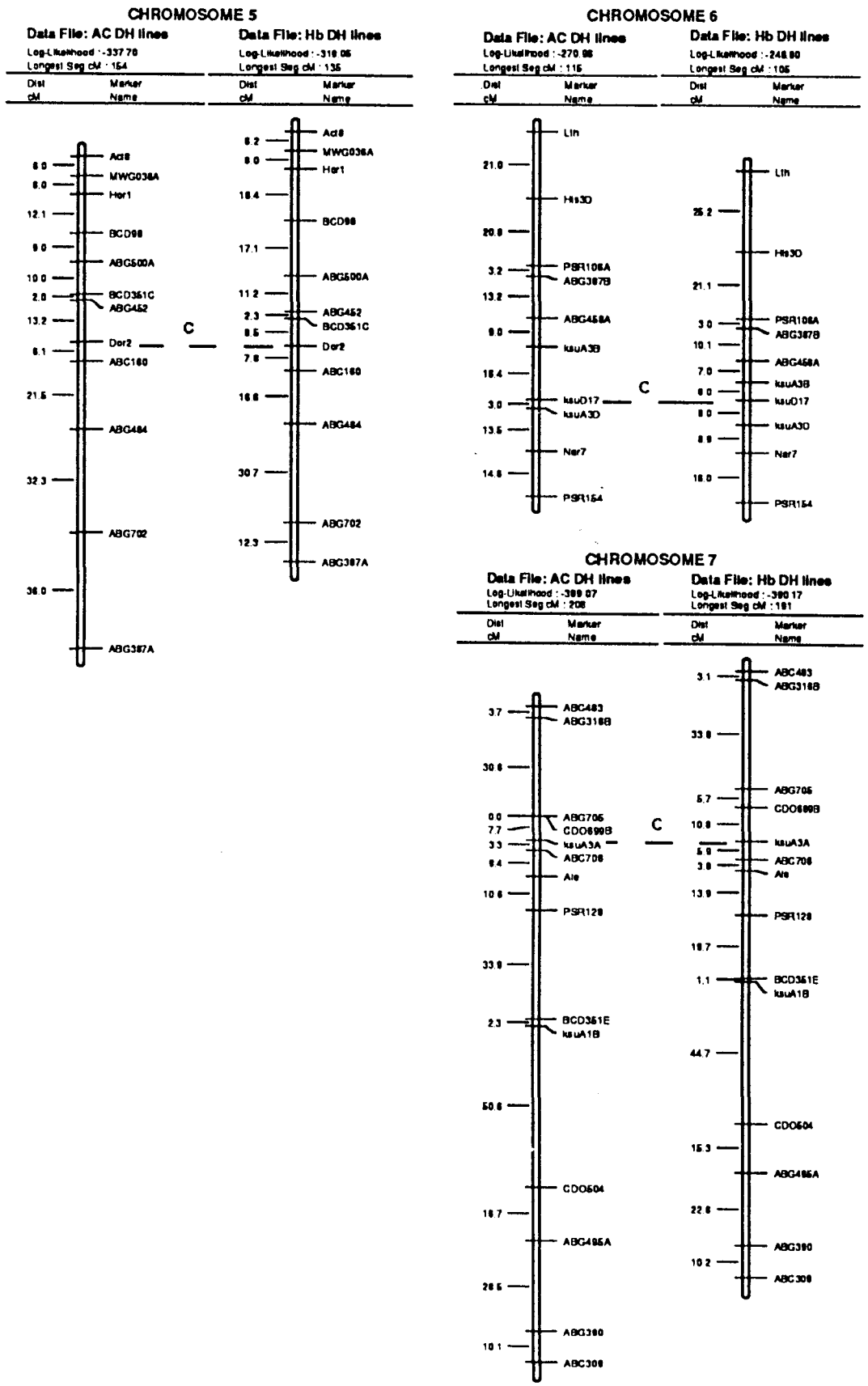

Fig. 1 Barley chromosome genetic maps developed with anther culture (AC)-derived (male recombination) and *Hordeum bulbosum* (Hb)-derived (female recombination) doubled haploid populations. The maps of individual chromosomes are aligned at the locus closest to the centromere. The actual centromere location above or below this marker is indicated by the "C" above or below the line connecting the loci

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Table 2 Comparison of genetic distances between linked loci determined in anther culture (AC)-vs. *Hordeum bulbosum* (Hb)-derived doubled haploid populations. Distances that significantly differ at $P < 0.05$ are shown

Chr	Region	Loci	AC	Hb	χ^2	P
1	P telo	*MWG036B-BCD129*	12.1	3.0	5.65	0.02
2	Centr	*Pox-CDO537*	20.9	10.6	4.79	0.03
	Centr	*CDO537-ABG316C*	10.4	3.5	5.53	0.02
	M telo	*Crg3A-Gln2*	23.4	11.1	4.39	0.03
	M telo	*ABG316D-Prx2*	25.7	8.0	12.25	0.001
4	P telo	*WG622-CDO669A*	26.5	13.4	3.64	0.05
5	M telo	*ABG702-ABG38A*	36.0	12.3	11.28	0.001
6	Centr	*ksuA3B-ksuD17*	16.4	6.0	5.79	0.02
7	Centr	*ABG705-CDO699B*	0	5.7	6.38	0.01

P telo, Plus arm telomeric region, upper 0.5 of the P arm; Centr, centromeric region, includes 0.5 of the P and M arms distal of the centromere marker; M telo, Minus arm telomeric region, lower 0.5 of the M arm

significant as judged by Wilcoxon's signed rank test ($P < 0.05$).

Comparison of the number of recombinations per genome also showed a higher number in the AC-derived DH lines (1.047/chromosome) than in the Hb-derived DH lines (0.912/chromosome).

All pair-wise distances between individual loci were examined using the χ squared test. Nine distances were significantly different at $P < 0.05$ (Table 2). Of these, eight showed higher recombination in the AC population and one in the Hb population. Dividing the chromosome into arbitrary telomeric and centromeric regions failed to show preferential map expansion for either region in AC populations (5 telomeric and 3 centromeric) (Table 2). However, the two most significantly different distances ($P < 0.001$) were located in telomeric regions, in both cases representing a threefold increase in the map distance for the AC population relative to Hb-derived lines. The most telomeric intervals from 13 chromosomal arms (excluding 6M, for which no markers were available in the telomeric half) were 43% longer in the AC compared to the Hb population, while nontelomeric intervals were only 12.5% longer. Wilcoxon's signed rank test confirmed the significance of the difference in telomeric interval length ($P < 0.05$), while the differences in nontelomeric intervals were judged insignificant by the same test.

Segregation distortions

Although the number of single-locus distortions was only slightly higher for the AC than for the Hb population (20 vs. 16), there was a clear difference in the magnitude of the distortions between the two systems (Table 3). Eight markers from chromosome 5, covering 50% of its length, showed significant segregation distortion in favor of the Morex allele in the AC population. The strongest distortion (only 21 Steptoe alleles compared to 80 from Morex) was found for the Dor2 marker located close to the centromere, with progressively reduced levels of distortion towards both telomeres. There are distortions favoring Morex alleles in Hb population that overlap with the distorted region in AC, however the magnitude of the effect is much smaller and the peak of the effect (at ABG500A locus) is ca. 20 cM away from the Dor2 locus. Another region of

Table 3 Single-locus segregation distortions in anther culture (AC)- and *Hordeum bulbosum* (Hb)-derived doubled haploid populations. Loci showing segregation ratios statistically different from 1:1 at $P < 0.05$ are listed

Chr	Locus	Steptoe allele/Morex allele[a]	
		AC population	Hb population
2	ABC156A	–	1.51
	KsuF15	–	1.51
	Crg3A	–	1.53
	ABG316E	–	1.48
3	ABG316A	0.62	–
	ABC171	0.62	–
	ABC174	–	0.64
	ABG495B	–	0.62
4	WG622	–	1.49
	CDO669A	–	1.58
	ABG500B	–	2.12
	ABG397	–	1.58
	KsuH11	1.45	–
5	Horl	0.60	–
	BCD98	0.51	0.55
	ABG500A	0.36	0.54
	BCD351C	0.33	0.67
	ABG452	0.33	0.67
	Dor2	0.26	–
	ABC160	0.39	–
	ABG464	0.60	–
6	ABG458	1.50	–
	PSR154	1.68	–
7	ABG705	1.60	–
	CDO699B	1.70	–
	KsuA3A	1.94	–
	ABC706	2.06	1.54
	Ale	1.86	–
	PSR128	1.53	–
	ABG495A	–	0.60
	ABC309	0.64	–

[a] Segregation ratios not significantly different from 1:1 at $P < 0.05$ are indicated by dashes

strong distortion in AC population, this time in favor of the Steptoe allele, is delineated by ABG705 and PSR128 and covers a ca. 30 cM segment of chromosome 7. The peak of the effect is located near the centromere (at ABC706 locus) and coincides with a single distorted locus in Hb population. Remaining distortions are limited to a single locus or two neighboring loci, without a distinct preference for telomeric or internal locations.

The comparison of distortions over the whole genome does not show any preference for a particular genotype in either system. Eight loci were distorted towards Steptoe and 11 towards Morex in the AC population, while the respective values for Hb were 9 and 7. Segregation distortions did not influence the linkage values strongly. Of 31 distorted loci, only 5 were involved in the 3 (out of 9) intervals that showed significant differences in map distances between the AC and Hb systems. The most distorted loci were not involved in the intervals that differed significantly in map distance.

Discussion

Homozygosity of mapping material

All of the plants regenerated by AC in the present study were homozygous, except one, which was partially heterozygous. Homozygosity of AC-regenerated barley plants based on phenotypic (Foroughi-Wehr et al. 1982) and genetic studies (Franzone et al. 1984, Powell et al. 1986) has been reported previously. The one partially heterozygous plant identified in this study could not have originated from a somatic cell or an unreduced microspore since only about one-half of the tested loci were heterozygous. Thus, the most likely explanation is that this plant originated from fused microspores. Chen et al. (1984) have reported fusion of barley microspores from the 4th to the 10th day of AC.

Male/female recombination frequency

Our results suggest an excess of crossing-over in barley male gametogenesis (anther culture) relative to female gametogenesis (*Hordeum bulbosum*). This increased recombination was observed for all chromosomes and for 11 out of the 14 chromosome arms. Of the nine linkage segments shown to be significantly different at $P < 0.05$, eight were larger in the male recombination-derived population.

These results contrast with those of other studies showing reduced crossing-over during male gametogenesis in tomato (De Vicente and Tanksley 1991) and humans (Donix-Keller et al. 1987). However, careful examination of the data reveals some interesting similarities with the results in other systems. Although 3 out of 8 segments that appear significantly longer in the AC population are non-telomeric, the most significant increases were noted for the telomeres of the long arm of chromosome 2 and 5. In tomato, the most dramatic (5.6-fold) difference in genetic distance was in favor of the male gamete for the most distal interval of chromosome 9. In humans, an overall excess of female recombination of 1.9 has been reported, but several distal regions did not show the sex differences while the terminal region of 20q had a 2-fold higher recombination frequency in male meiosis (Donis-Keller et al. 1987). Significant excess of male recombination at the most distal regions was also found for human chromosomes 5 (p and q), 11 (p and q), 16 and 17 (Weiffenbach et al. 1991; Julier et al. 1990a, b; O'Connell et al. 1987; Nakamura et al. 1988). The most pronounced increase in the recombination frequency in the male relative to female meiosis was observed for the pseudoautosomal region of the human allosome, where male and female genetic lengths are 50 and 3 cM, respectively.

Recent comparisons of sex-dependent differences in recombination frequency from two plant species show trends similar to our data. Enhanced male recombination frequencies were observed at proterminal regions of several linkage groups in *Brassica nigra* (Lagercrantz and Lydiate 1995). Male recombination was only slightly increased (14%) in *Pennisetum glaucum*. but 2 of 3 significantly different intervals were the most terminal (Busso et al. 1995). Additional evidence for increased recombination frequency at the telomeres in male meiosis comes from extensive mapping of the *Rpg1* region in barley (Kilian and Kleinhofs, unpublished). A small (1.5 cM) segment just 1 cM proximal of the telomeric marker on barley chromosome 1P was mapped using 230 AC-derived (male) gametes and 860 Hb-derived (female) gametes. Of the 16 confirmed recombinants within the target region, 7 were from the AC and 9 from the Hb-derived populations. This represents a statistically significant, 3-fold greater recombination frequency in the male compared to the female gametes.

Rouyer et al. (1990) have proposed that the excess of male recombination observed in the terminal regions of human chromosomes could be a consequence of the pairing process of homologs at meiosis. In spermatocytes, chromosomal pairing starts from the chromosome telomeres and recombination nodules are observed first at the terminal regions of chromosomes. In oocytes, pairing of homologs also starts at the termini but interstitial pairing is much more frequent than in male meiosis (Bojko 1983). A similar pattern of chiasma distribution was reported also for the newt *Triturus helveticus* (Watson and Callan 1963). Based on these observations, Rouyer et al. (1990) hypothesized that more random initiation of pairing could explain the lesser expansion of the female genetic map in the distal regions of chromosomes. In order to critically

evaluate this hypothesis in any species a complete genetic map is needed. In barley, most of the chromosome termini have been tagged with telomeric markers that were mapped in the Steptoe × Morex Hb population (Kilian and Kleinhofs, in preparation). Mapping these markers in the AC population will allow us to test whether an increased frequency of recombination at the telomeric regions is a general phenomenon in barley or is limited to particular chromosomal arms.

From the discussion presented above it is apparent that recombination frequencies differ in favor of either sex, with the extremes characterized by lack of recombination in *Drosophila* males (Morgan 1914) and silkworm females (Maeda 1939). With the more complete genetic maps now available for between-sex comparisons, both in plants and animals, the tendency toward increased recombination frequency at the telomeres in male meiosis seems to be emerging as a general phenomenon. Since the differences in chiasma distribution show a similar trend, increased recombination frequency at the telomeres may be a biologically significant phenomenon. There is some evidence that subtelomeric regions of human (Saccone et al. 1992) and cereal (Moore et al. 1993) genomes are much more "gene-rich" than proximal regions. If this proves to be true for other species, one could speculate that increased male recombination at the telomeric regions is selectively advantageous and therefore evolutionarily conserved, since it results in an increased number of gene assortments in the more abundant male gametes. Male gametes are not only more abundant, but also energetically less costly and subject to selection at the pollination and/or fertilization stages.

Genetic factors are known to influence the frequency of recombination (Reeves et al. 1990; Sall et al. 1990; Nilsson and Pelger 1991). Exactly the same parents were used in our study to generate the male gamete-derived doubled haploids (anther culture) and the female gamete-derived doubled haploids (*Hordeum bulbosum*). However, the two techniques are quite different and involve the use of different tissue culture conditions. Perhaps the most important difference, besides sex, is the temperature used for growing donor plants. The Hb donor plants were grown at ca. 18° C, while the AC donor plants were grown at 12° C. Increasing temperature has been reported to result in increased recombination frequency (Jensen 1981). This implies that the difference between male and female recombination in barley may be even larger than observed in our study.

Segregation distortions

Different tissue culture conditions used during doubled haploid production impose different selective forces on AC and Hb populations, as manifested by various levels of single-locus segregation distortions. Distorted segregation ratios for AC-derived DH populations were noted previously (Graner et al. 1991; Heun et al. 1991; Thompson et al. 1991; Zivy et al. 1992). In the present study, the AC-derived DH population showed a strong segregation distortion in the centromeric regions of chromosomes 5 and 7. Devaux and Zivy (1994) demonstrated that some markers showing distorted segregation were linked to genes involved in anther culture response. Since neither of the parents involved in this cross is particularly anther culture-responsive, it seems more likely that the distorted regions include genes important in overcoming stress imposed by the tissue culture conditions. The fact that distortions favored both Steptoe and Morex alleles agrees with the similar (low) levels of responsiveness of these genotypes. In the context of the present paper, it is important to emphasize that single-locus distortions did not influence recombination values very significantly.

The number of recombinants observed in this study is very close to one per chromosome. This is very close to the number of chiasmata per chromosome observed in barley (Nilsson and Pelger 1991). The higher number of recombinants per chromosome previously reported was based on a denser, but less uniformly distributed marker map (Nilsson et al. 1993). Maps reported here measure linkage in about 15 cM increments. Double cross-overs would be expected to be rare in these intervals especially considering the overall low rate of recombination in barley. Some of the apparent double cross-overs in denser maps are, no doubt, due to errors. However, not all of the differences in map distances between dense and skeletal maps (compare maps reported here with the dense Steptoe × Morex map) can be explained by assuming errors and it is possible that some of the apparent double cross-overs in small chromosome regions are due to gene conversions or other mechanisms.

Conclusions

Our results suggest that there is a general excess of recombination in the AC-derived population relative to the Hb population. Even though there appear to be real differences in recombination frequencies in some chromosome segments between anther culture and *Hordeum bulbosum*-derived doubled haploid populations, it should be possible to use populations derived by the different methods for the construction of a single barley genome map. Such merged maps are likely to have some errors at the fine structure level, but should be accurate enough to allow investigators to choose markers in the region of interest with sufficient confidence to initiate the fine-structure mapping needed for positional cloning of barley genes.

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References

Bojko M (1983) Human meiosis. VIII. Chromosome pairing and formation of the synaptonemal complex in oocytes. Carlsberg Res Commun 48:457–483

Busso CS, Liu CJ, Hash CT, Witcombe JR, Devos KM, deWet JMJ, Gale MD (1995) Analysis of recombination rate in female and male gametogenesis in pearl millet (*Pennisetum glaucum*) using RFLP markers. Theor Appl Genet 90:242–246

Chen CC, Kasha KJ, Marsolais A (1984) Segmentation patterns and mechanisms of genome multiplication in cultured microspores of barley. Can J Genet Cytol 26:475–483

Chen F, Hayes PM (1989) A comparison of *Hordeum bulbosum*-mediated haploid production efficiency in barley using in vitro floret and tiller culture. Theor Appl Genet 77:701–704

Devaux P (1992) Haploidy in barley and wheat improvement. In: Datto Y, Dumas C, Gallais A (eds) *Reproductive biology and plant breeding*. Springer-Verlag, Berlin, pp 139–151

Devaux P, Zivy M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701–706

Devaux P, Hou L, Ullrich SE, Huang Z, Kleinhofs A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32–36

De Vicente MC, Tanksley SD (1991) Genome-wide reduction in recombination of backcross progeny derived from male versus female gametes in an interspecific cross of tomato. Theor Appl Genet 83:173–178

Donis-Keller H, Green P, Helms C, Cartinhour S, Weiffenbach B, Stephens K, Keith TP, Bowden DW, Smith DR, Lander E, Botstein D, Akots G, Rediker KS, Gravius T, Brown VA, Rising MB, Parker C, Powers JA, Watt DE, Kauffman ER, Bricker A, Phipps P, Muller Kahle H, Fulton TR, Ng S, Schumm JW, Braman JC, Knowlton RG, Barker DF, Crooks SM, Lincoln SE, Daly MJ, Abrahamson J (1987) A genetic linkage map of the human genome. Cell 51:319–337

Foroughi-Wehr B, Friedt W, Wenzel G (1982) Field experiments with anther-derived lines of barley (*Hordeum vulgare*) and rye (*Secale cereale*). In: Giles KL, Sen SK (eds) Plant cell culture in crop improvement. Plenum Publishing, New York

Franzone PM, Fishbeck G, Foroughi-Wehr B, Friedt W (1984) Analysis of origin of androgenetic plants of *Hordeum vulgare* L. by the use of balanced lethal systems. Z. Pflanzenzüchtg 93:320–330

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

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map of barley (*Hordeum vulgare*). Genome 34: 437–447

Jensen J (1981) Effect of temperature on genetic recombination in barley. Hereditas 94:215–218

Julier C, Nakamura Y, Lathrop M, O'Connell P, Leppert M, Litt M, Mohandas T, Lalouel J-M, White R (1990a) A detailed genetic map of the long arm of chromosome 11. Genomics 7:335–345

Julier C, Nakamura Y, Lathrop M, O'Connell P, Leppert M, Mohandas T, Lalouel J-M, White R (1990b) A primary map of 24 loci on human chromosome 16. Genomics 6:419–427

Kasha KJ, Reinbergs E (1982) Recent developments in the production and utilization of haploids in barley. In: Asher MJC, Ellis RP, Whitehouse RNH (eds) Proceedings 4th International Barley Genetics Symposium, July 22–29, 1981. Edinburgh University Press, Edinburgh, pp 655–665

Kleinhofs A (1994) Barley Steptoe × Morex map. File available via Internet Gopher, host greengenes.cit.cornell.edu, port 70, menu "Grains files to browse"/"Barley Steptoe × Morex map"

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

Lagercrantz U, Lydiate D (1995) RFLP mapping in *Brassica nigra* indicates different recombination rates in male and female meioses. Genome 38:255–264

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Liu BH, Knapp SJ (1990) GMENDEL: a program for Mendelian segregation and linkage analysis of individual or multiple progeny populations using log-likelihood ratios. J Hered 81:407

Maeda T (1939) Chiasma studies in the silk worm, *Bombyx mori* L. Jpn J Genet 15:118–127

Moore G, Abbo S, Cheung W, Foote T, Gale MD, Koebner R, Leitch A, Leitch I, Money T, Stancombe P, Yano M, Flavell R (1993) Key features of cereal genome organization as revealed by the use of cytosine-methylation sensitive restriction endonucleases. Genomics 15:472–482

Morgan TH (1914) No crossing over in the male of *Drosophila* of genes in the second and third pair of chromosomes. Biol Bull (Woods Hole)26:195–204

Nakamura Y, Lathrop M, O'Connell P, Leppert M, Barker D, Wright E, Skolnick M, Kandoleon S, Litt M, Lalouel J-M, White R (1989) A mapped set of DNA markers for human chromosome 17. Genomics 2:302–309

Nilsson N-O, Pelger S (1991) The relationship between natural variation in chiasma frequencies and recombination frequencies in barley. Hereditas 115:121–126

Nilsson N-O, Sall T, Bengtsson BO (1993) Chiasma and recombination data in plants: are they compatible? Trends Gene 9:344–348

O'Connell P, Lathrop M, Law M, Leppert M, Nakamura Y, Hoff M, Kumlin E, Thomas W, Elsner T, Ballard L, Goodman P, Azen E, Sadler J, Cai G, Laloul J-M, White R (1987) A primary genetic linkage map for human chromosome 12. Genomics 1:89–102

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

Powell JB, Nilan RA (1963) Influence of temperature on crossing over in an inversion heterozygote in barley. Crop Sci 3:11–13

Powell W, Borrino EM, Allison MJ, Griffiths DW, Asher MJC, Dunwell JM (1986) Genetical analysis of microspore-derived plants of barley (*Hordeum vulgare*). Theor Appl Genet 72: 619–626

Reeves RH, Crowley MR, O'hara BF, Gearhart JD (1990) Sex, strain, and species differences affect recombination across an evolutionarily conserved segment of mouse chromosome 16. Genomics 8:141–148

Robertson DS (1984) Different frequency in the recovery of crossover products from male and female gametes of plants hypoploid for B-A translocations in maize. Genetics 107:117–134

Rouyer F, De La Chapelle A, Andersson M, Weissenbach J (1990) An interspersed repeated sequence specific for human subtelomeric regions. EMBO J 9:505–514

Saccone S, De Sarino A, Della Valle G, Bernardi G (1992) The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. Proc Natl Acad Sci USA 89:4913–4917

Sall T, Flink J, Bengtsson BO (1990) Genetic control of recombination in barley. Hereditas 112:157–170

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Shiroishi T, Sagai T, Hanzawa N, Gotoh H, Moriwaki K (1991) Genetic control of sex-dependent meiotic recombination in the major histocompatibility complex of the mouse. EMBO J 10: 681–686

Sinha RP, Helgason SB (1969) The action of actinomycin D and diepoxybutane on recombination of two closely linked loci in Hordeum. Can J Genet Cytol 11:745–751

Sokoloff A (1964) Sex and crossing over in *Tribolium castaneum.* Genetics 50:491–496

Sved JA, Blackman LM, Gilchrist AS, Engels WR (1991) High levels of recombination induced by homologous P elements in *Drosophila melanogaster.* Mol Gen Genet 225:443–447

Thompson DM, Chalmers K, Waugh R, Foster BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L. Theor Appl Genet 81:487–492

Watson ID, Callan HG (1963) The form of bivalent chromosomes in newt oocytes at first metaphase of meiosis. Quart J Microsc Sci 104:281–295

Weiffenbach B, Falls K, Bricker A, Hall L, McMahon J, Wasmuth J, Funanage V, Donis-Keller H (1991) A genetic linkage map of human chromosome 5 with 60 RFLP loci. Genomics 10: 173–185

Weitkamp LR, Guttormsen SA, Costello Leary P (1982) Equine gene mapping: a sex difference in recombination frequency for linkage group II. Animal Blood Groups Biochem Genet 13: 305–306

Zetka MC, Rose AM (1990) Sex-related differences in crossing over in *Caenorhabditis elegans.* Genetics 126:355–363

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

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Segregation distortion and linkage studies in microspore-derived double haploid lines of *Hordeum vulgare* L.

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Summary. A total of 62 doubled haploid (DH) lines was derived from a cross between tow lines of barley by anther culture. By two-dimensional electrophoresis of seedling proteins, the segregation of 28 loci in the population of DH lines was studied and a linkage map was constructed. The linkage map covered a large part of the length of the genome. A deviation to the 1:1 segregation expected in the absence of selection was observed for at least one chromosome segment. This might be linked to a gene or group of genes selected because of their involvment in the process of haploid production.

Key words: *Hordeum vulgare* – Doubled haploid lines – Segregation distortion – Linkage map – Two-dimensional electrophoresis

Introduction

One question which has arisen with the development of doubled haploid (DH) production techniques in plant breeding schemes is whether or not *in vitro* procedures, i.e., mainly male or female gametophyte culture or embryo rescue after interspecific hybridization, induce selection pressures leading to significant segregation distortions. Several experiments have been reported on these topics, e.g., in tobacco (Kumashiro and Oinuma 1985), rape (Hoffmann et al. 1982), wheat (de Buyser et al. 1985; Winzeler et al. 1987), triticale (Charmet and Branlard 1985), and maize (Lashermes et al. 1988).

In cultivated barley (*Hordeum vulgare* L.), haploid plants can be obtained through the *in vitro* culture of male gametophytes by anther culture or more recently by microspore culture, and by the technique of chromosome elimination following interspecific hybridization with *H. bulbosum* (for review, see Pickering and Devaux 1991).

Most studies using monogenetic phenotypic traits (Powell et al. 1984; Doll et al. 1989; Kjær et al. 1990) or protein encoding genes (Powell et al. 1990) as genetic markers support the hypothesis that no selection is induced by the *H. bulbosum* technique. Moreover, none of the 23 genetic markers (morphological traits, enzyme encoding genes, RFLP, and PCR loci) used by Schön et al. (1990) showed significative deviation from the 1:1 segregation in 42 DH lines derived from F_1 plants. However, Furusho et al. (1990) observed a deviation from the 1:1 ratio for resistance to powdery mildew in DH lines derived from F_1 plants, although the F_2 segregation did not deviate from 3:1. Interestingly, this deviation depended on the genotype of the parental lines.

Concerning the androgenetic method, Foroughi-Wehr and Friedt (1984) and Kao (1988) observed a deviation from the 1:1 ratio for the number of rows per spike; Powell et al. (1986) observed a deviation for three of the five genes tested, but only 14 DH lines were studied.

In this study, we have tried to answer the question of whether or not there are selection pressures during the process of DH production by anther culture. The occurrence of selection was tested for by scoring segregation distortions among protein markers separated by two-dimensional (2D) electrophoresis in a population of 62 DH lines derived from a single F_1 hybrid. As a high level of polymorphism was found for heat shock proteins (HSP) in wheat (Zivy 1987), plants were submitted to heat shock before protein extraction, to increase the number of markers.

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Materials and methods

Plant material

The two inbred parental lines "Kaskade" and "DH8293" were crossed, and haploid and spontaneously DH plants were regenerated from the F_1 through anther culture according to Devaux (1987). The haploid plants were treated with colchicine according to Pickering (1980). At maturity, grains were collected from spontaneous DH plants and fertile colchicine-treated haploid plants. Eleven DH plants were obtained in a first series, and 51 were obtained in a subsequent series, using the same genotype and methods.

Several grains of the two parental lines and of their 62 DH progenies were sown on moist filter paper in petri dishes and allowed to germinate in the dark at a constant 20 °C. On the 7th day seedlings were submitted or not (for controls) to heat shock (3 h at 40 °C), then their aerial parts were cut off and immediately plunged into liquid nitrogen, where they were stored until extraction. Controls (no heat shock) were done only on seedlings of the parental lines, to identify HSP.

2D Electrophoresis

Proteins were extracted according to the procedure of Zivy (Damerval et al. 1986) and 2D electrophoresis was performed as described in Bahrman and Thiellement (1987). The 240 × 200 × 1 mm 2D gels bound to Gelbond were silver stained according to Heukeshoven and Dernick (1985) modified by Damerval et al. (1987) in the apparatus described by Granier and de Vienne (1986).

Gel comparisons

The two parental lines were first compared (at least four gels from different extractions), to identify spot variations reproducible enough to be scored without ambiguity with onyl one gel per genotype: only one quantitative variation was retained; all others were presence/absence variations. The segregation of these spots was then scored in the DH progeny. When technical problems prevented reliable notations on a gel, 2D electrophoresis was repeated. To take in account variations between batches of 2D gels, samples from parental lines or 1:1 mixtures of parental samples were run in all batches. Identification of HSP was done by comparison of at least four gels of each parental line with at least four gels of their respective controls.

Spot identification

Only spots found variable between the two parental lines were numbered. The identification number was suffixed with a "k" or a "d," when the spot was found specific, respectively, to Kaskade or DH8293, and not suffixed when the spot was present in both parents (quantitative variation). It was prefixed with a "h" when the spot corresponded to a HSP. When allelic relationships were found between two spots, they took the same number and different suffixes.

Linkage map

The linkage map was built with the aid of the "Mapmaker" software (Lander et al. 1987).

Results

Comparison between the two parental lines allowed the identification of 50 variable spots (Fig. 1). Five of them corresponded to heat shock proteins (h23k, h23d, h28k, h28d, h27k). All of them showed a presence/absence variation, except for spot 26, which was clearly more intense in DH8293 than in Kaskade. The segregation of these spots was then studied in the 62 DH lines. Of the 3,100 possible observations, 22 were not scored because of ambiguities or bad resolution in regions of some gels.

Allelic relationships

Identification of pairs of spots corresponding to different allelic products of the same structural gene was done according to the following criteria: (i) two allelic products must originate from different parental lines, and they cannot be both present in the same DH line; (ii) they must be relatively close to each other on 2D patterns, i.e., they have similar pI and apparent molecular masses. Accordingly, 44 spots were found to correspond to 22 pairs of allelic products (see Fig. 1). Different reasons can explain the absence of alternative allelic product for the 6 remaining spots 15d, 10k, 25k, h27k, 26, and 16k (see Discussion). The simplest hypothesis will be assumed in the following section, i.e., control by biallelic loci, one allelic form corresponding to the presence of the spot (or presence of a large spot for spot 26), and the alternative one corresponding to the absence of the spot (or presence of a small spot for spot 26). Thus, 28 loci were considered.

Linkage map

For haploid segregations, the maximum-likehood estimate of the recombination rate between two loci is equal to the observed recombination rate. This rate remains unchanged when an allelic form at one locus affects viability. The software "Mapmaker" (Lander et al. 1987) was used to build the map shown in Fig. 2. The linkage groups were defined using a lod score threshold of 3.0 (likehood for linkage between two loci 1,000 times greater than likehood for independence). The highest recombination rate taken into account was 0.283 (between loci 15 and 17). Three-point and multiple-point tests were performed in groups comprising more than two loci, to determine their order. As no significative interference was observed, the recombination rates were converted into centiMorgans using Haldane's formula: $x(r) = -1/2ln(1-2r)$ (Bailey 1961), where x is the estimated distance in Morgans and r is the recombination rate.

The 28 loci were found to fall into 14 independent loci or linkage groups. In two cases, no recombination was observed between pairs of loci: between 20 and 21, and between 7 and 14. The latter observation could be explained in a different way: there could be actually only one locus encoding polypeptides 20 and 21, and one locus encoding polypeptides 7 and 14; the difference between

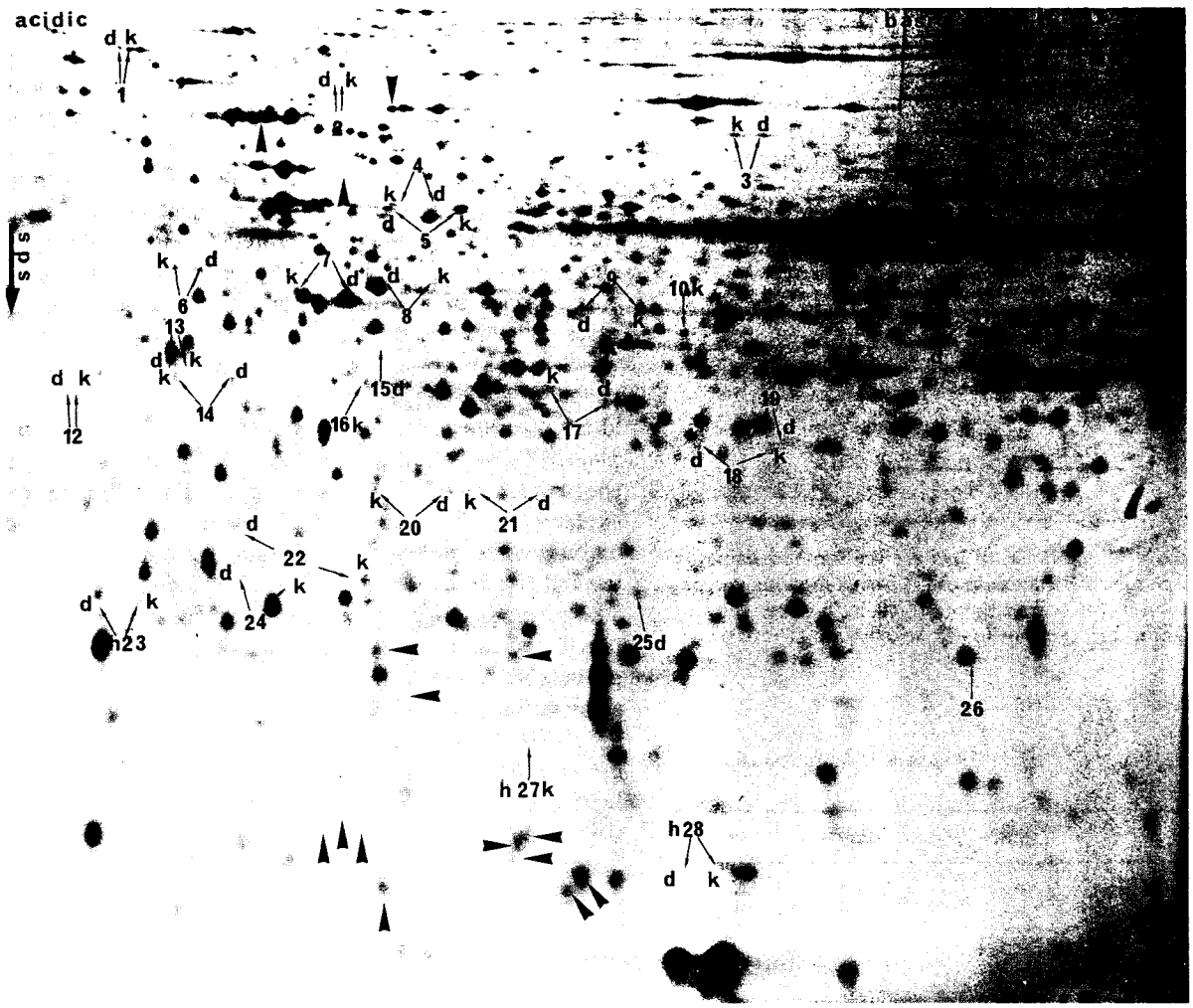

Fig. 1. 2D electrophoresis of the 1:1 mixture of proteins from the two parental lines DH8293 and Kaskade. Only variable spots are numbered. *Linked arrows* show pairs of allelic form. For identification code, see 'Material and methods'. *Large arrowheads* show non-variable HSPs. Although some faint spots are not visible on this photography, their positions is shown

the two products of the same gene could be caused by post-translational modifications. This hypothesis is unlikely for polypeptides 7 and 14, since the two polypeptides have very different physical properties, but it is possible for polypeptides 20 and 21 (see Fig. 1). The three loci encoding HSPs or controlling their synthesis were found to be unlinked. However, the HSP encoding locus h23 was found to be linked to locus 25 (9.5 cM).

Segregation distortions

Table 1 shows the frequency (f) of the DH8293 allelic form at each locus in the population of DH lines. For seven loci, a significant difference from 0.5 was found: loci 16 ($f=0.300$, $p=0.004$), 17 ($f=0.689$, $p=0.006$), 1 ($f=0.661$, $p=0.012$), 4 ($f=0.645$, $p=0.024$), 5 ($f=0.645$, $p=0.024$), 7 ($f=0.645$, $p=0.024$), and 14 ($f=0.645$, $p=0.024$). However, three of them (17, 1, and 4) were closely linked and two others (7 and 14) did not recombine at all (see Fig. 2). Thus, the seven loci actually mark the four following chromosome segments: 17-1-4, 7-14, 16, and 5. For all of them except one (locus 16), the selected form originated from DH8293.

The significance level that should be taken into account for several independent comparisons should be divided by the number of comparisons. However, the 28 comparisons were not independent, since some loci were linked. Taking into account the 14 independent linkage groups or loci, the significance level for each individual comparison is 0.4%. Two loci showed a deviation from the 1:1 segregation with a risk of about this magnitude: 16 and 17 (respectively, 0.4 and 0.6%).

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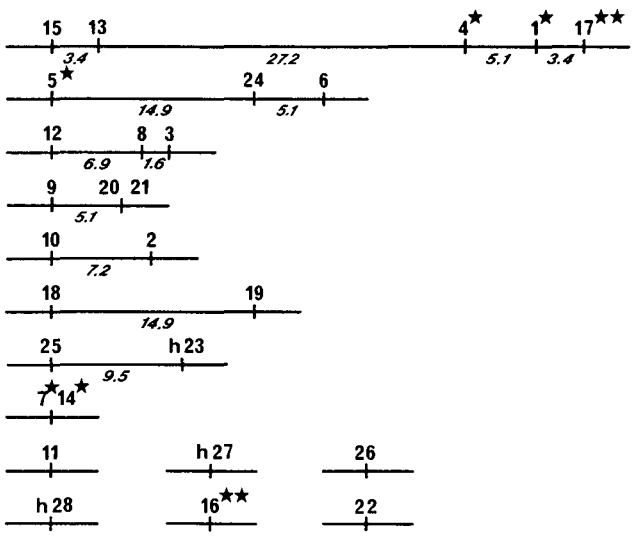

Fig. 2. Linkage map. Names of loci are the same as names of spots. Italic numbers: distances in cM. * Locus showing a significant deviation to 1:1 segregation ($P<0.05$); ** Locus showing a highly significant deviation to 1:1 segregation ($P<0.01$)

Table 1. Frequency of the DH8293 allelic form at the 28 loci in the 62 DH lines

Locus	p	nobs	freq	χ^2
1	41	62	0.661	6.45*
2	29	62	0.468	0.26
3	37	62	0.597	2.32
4	40	62	0.645	5.23*
5	40	62	0.645	5.23*
6	33	62	0.532	0.26
7	40	62	0.645	5.23*
8	38	62	0.613	3.16
9	26	62	0.419	1.61
10	27	60	0.450	0.60
11	35	62	0.565	1.03
12	36	62	0.581	1.61
13	26	61	0.426	1.33
14	40	62	0.645	5.23*
15	27	61	0.443	0.80
16	18	60	0.300	9.60**
17	42	61	0.689	8.67**
18	32	62	0.516	0.06
19	36	62	0.581	1.61
20	27	62	0.435	1.03
21	27	62	0.435	1.03
22	36	62	0.581	1.61
h23	32	58	0.552	0.62
24	32	61	0.525	0.15
25	34	62	0.548	0.58
26	36	62	0.581	1.61
h27	30	61	0.492	0.02
h28	32	61	0.525	0.15

p: number of scored presences; nobs: number of observations; χ^2: conformity to 1:1 segregation (1 *df*); Freq: frequency
* Frequency significantly different form 0.5 ($P<0.05$)
** Frequency highly significantly different from 0.5 ($P<0.01$)

Discussion

In this study, the occurrence of selection pressures was tested by looking for segregation distortions. The expected segregation for two allelic forms of one gene in the absence of selection is 1:1. Our results are in the form of a presence/absence of polypeptides (except for one quantitative variation), and our interpretation is that the presence/absence of one polypeptide corresponds to the presence/absence of an allelic form of one gene. In most cases, pairs of allelic products were observed (22 pairs), which shows that the variation was in the structural gene encoding the polypeptide. However, in six instances no alternative allelic form was found. This can still be explained by variation for the structural gene (alternative spot hidden by others or a silent gene), but it can also be explained by the variation of a regulator controlling the amount of the polypeptide. The segregation of a regulator will also be expected to be in a 1:1 ratio. However, as a polypeptide can be under the control of several regulators (Colas des Francs and Thiellement 1985), there is the probability that two or more regulators controlling the same polypeptide are different in the two parental lines; in this case, a 1:1 segregation is not expected. Thus, the use of a polypeptide spot without an alternative allelic form (and of polypeptides showing quantitative variation) is less safe for the determination of segregation distortions. For the same reasons, their localization in linkage groups is also less secure, and it cannot be determined whether the encoding gene or a regulator was localized.

Construction of a linkage map

The study of recombination frequencies allowed the construction of a linkage map showing 14 independent loci or linkage groups (6 independent loci and 8 linkage groups comprising two to five loci: see Fig. 2). The three variable loci encoding HSP were found to be independent of each other, but one of them was found linked to a locus encoding a non-HSP protein. Among 20 HSP spots observed in the parental lines, 5 were variable: as expected, the level of polymorphism in HSP was found to be higher than in non-HSP (Zivy 1987).

The localization of spots with no alternative allelic form was performed according to the simplest hypothesis, i.e., segregation of one gene encoding the protein or a regulator of its synthesis. For five of them, no difference to the 1:1 segregation was observed, indicating that this hypothesis was probably true. For the last one (spot 16), this could not be verified therefore the localization of the locus might be erroneous.

In this study, the linkage between two loci was significant if the recombination frequency was lower or equal to 0.283, which corresponds to 41.7 cM. Accordingly,

each identified linkage group or independent locus marked a chromosome segment of 41.7 cM at both ends of its position, plus the length of the linkage group itself. Thus, the total length of marked chromosomes was between 1,272 cM (if the 14 marked chromosome segments did not overlap each other) and 730 cM (maximum overlap). To our knowledge the total length of the genome of barley has not been estimated. However, those of rice and maize were estimated to be between 1,400 and 1,500 cM (McCough et al. 1988; Murray et al. 1988). Thus, a relatively large part of the genome of barley was marked with the 28 markers, and is is likely that the study of a few other crosses with the same methodology could allow the production of a complete linkage map.

Selective effects

Two chromosome segments showed a significant distortion of segregation. As one of them was represented by a spot without an alternate allelic form (spot 16k), the non-conformity to the 1:1 segregation could be interpreted in another way, as already discussed. The other one is marked by three closely linked loci (17-1-4). As there was a positive gradient of deviation to the 1:1 ratio from locus 4 to locus 17 (the frequency of the DH allelic form in loci 4, 1, and 17 being, respectively, 0.645, 0.661, and 0.689), the chromosome part that is actually submitted to selection should lie on the other side of (or at) locus 17. Two other chromosome segments showed a segregation distortion, but with a significance level insufficient for this study ($p < 0.05$): other genotypes should be studied to confirm the distortion.

As the studied progeny were not compared to a F_2 progeny, it is not known whether selection took place at a stage common to both modes of reproduction (microspore production and maturation in the anther) or during the different steps specific to DH line production (induction of embryo formation from microspores and green plant development). It should noted that except for locus 16, all the possibly selected allelic forms originated from parental line DH8293, which itself is a DH line obtained by the *Hordeum bulbosum* method. This observation favors the hypothesis that the character submitted to selection is related to DH production, but is not specific to the method of haploid obtainment: e.g., ability to produce barley haploid embryos and, subsequently, plants. Nevertheless, whether the observed selection occurs only during DH line production or also during natural reproduction, the presence of the selected chromosome segment should be advantageous for DH production, at least with the two parental lines studied here; this is currently under investigation. If it is confirmed that the protein markers found in this study actually mark a chromosome segment that is actively selected during haploid production, they could become valuable tools for the breeders to select for improved doubled haploid production.

References

Bahrman N, Thiellement H (1987) Parental genome expression in synthetic wheats (*Triticum turgidum* sp. × *T. tauschii* sp.) revealed by two-dimensional electrophoresis of seedling proteins. Theor Appl Genet 74:218–223

Bailey NTJ (1961) Introduction to the mathematical theory of genetic linkage. Clarendon Press, Oxford, 140 pp

Buyser J de, Henry Y, Taleb G (1985) Wheat androgenesis: cytogenetical analysis and agronomic performance of doubled haploids. Z Pflanzenzuecht 95:23–34

Charmet G, Branlard G (1985) A comparison of androgenetic doubled-haploid and single-seed descent lines in *Triticale*. Theor Appl Genet 71:193–200

Colas des Francs C, Thiellement H (1985) Chromosomal localization of structural genes and regulators in wheat by 2D electrophoresis of ditelosomic lines. Theor Appl Genet 71:31–38

Damerval C, Vienne D de, Zivy, M, Thiellement H (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. Electrophoresis 7:52–54

Damerval C, Le Guilloux M, Blaisonneau J, Vienne D de (1987) Simplification of the Heukeshoven and Dernick's silver staining of proteins. Electrophoresis 8:158–159

Devaux P (1987) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploid in winter barley. I. Production of green plants. Plant Breeding 98:215–219

Doll H, Haahr V, Søgaard B (1989) Relationship between vernalization requirement and winter hardiness in doubled haploids of barley. Euphytica 42:209–213

Foroughi-Wehr B, Friedt W (1984) Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture. Theor Appl Genet 67:377–382

Furusho M, Hamachi Y, Yoshida T (1990) Varietal difference in crossability between Japanese barley and *Hordeum bulbosum* L. Jpn J Breed 40:411–417

Granier F, Vienne D de (1986) Silver staining of proteins: standardized procedure for two-dimensional gels bound to polyester sheets. Anal Biochem 155:45–50

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103–112

Hoffman F, Thomas E, Wenzel G (1982) Anther culture as a breeding tool in rape. 2 Progeny analyses of androgenetic lines and induced mutants from haploid cultures. Theor Appl Genet 61:225–232

Kao KN (1988) *In vivo* culture in barley breeding. In: Semidwarf cereal mutants and their use in cross-breeding III. In: Proc Final Res Coordination Meeting, Joint FAO/IAEA Divisions, Rome 1985, pp 183–191

Kjær B, Jensen HP, Jensen J, Jørgensen JH (1990) Associations between three ml-o powdery mildew resistant genes and agronomic traits in barley. Euphytica 46:185–193

Kumashiro T, Oinuma T (1985) Comparison of genetic variability among anther-derived and ovule-derived doubled haploid lines of tobacco. Jpn J Breed 35:301–310

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

924

Lashermes P, Gaillard A, Beckert M (1988) Gynogenetic haploid plants analysis for agronomic and enzymatic markers in maize (*Zea mays* L.). Theor Appl Genet 76:570–572

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829

Murray M, Cramer J, Ma Y, West D, Romero-Severson J, Pitas J, DeMars S, Vilbrandt L, Kirshman J, McLeester R, Schilz J, Lotzer J (1988) Agrigenetics maize linkage map. Maize Genet Coop Newsl 62:89–91

Pickering RA (1980) Use of the doubled haploid technique in barley breeding at the Welsh Plant Breeding Station. Rep Welsh Plant Breed for 1979, pp 208–226

Pickering RA, Devaux P (1991) Haploid production: approaches and use in plant breeding. In: Shewry PR (ed) Barley: genetics, molecular biology and biotechenolgy. CAB, Wallingford, UK, pp 511–539

Powell W, Asher MJC, Wood W, Hayter AM (1984) The manipulation of mildew resistance genes in a barley breeding programme by the use of doubled haploids. Z Pflanzenzuecht 93:43–48

Powell W, Borrino EM, Allison MJ, Griffith DW, Asher MJC, Dunwell JM (1986) Genetic analysis of microspore derived plants of barley (*Hordeum vulgare*) . Theor Appl Genet 72:619–626

Powell W, Ellis RP, Macauley M, McNicol J, Forster BP (1990) The effect of selection for protein and isozyme loci on quantitative traits in a doubled haploid population of barley. Heredity 65:115–122

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

Winzeler H, Schmidt J, Fried PM (1987) Field performance of androgenetic doubled haploid spring wheat lines in comparison with lines selected by the Pedigree system. Plant Breed 99:41–48

Zivy M (1987) Genetic variability for heat shock proteins in common wheat. Theor Appl Genet 74:209–213

Note added in proof

A restriction fragment length polymorphism map of barley developped on the basis of anther culture derived DH lines was recently published (Heun et al. 1991, Genome 34:437–447). Several loci showed also disturbed segregation.

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Protein markers for anther culturability in barley

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Abstract Two-dimensional electrophoresis of proteins from a recombinant population of anther culture-derived doubled haploid lines identified 4 loci or linkage groups showing a deviation from an expected 1:1 segregation. It was hypothesized that these markers are linked to genes involved in the process of haploid plant production and that the deviation was due to a selection for alleles conferring higher anther culture response. To check this hypothesis, the anther culturability of 50 of the doubled haploid lines and their two inbred parents was assessed. It was found that 2 of the loci which had a distortion of segregation showed a significant effect on anther culture response, the most efficient allele being the most frequent in both loci. In addition, 2 more markers associated with anther culturability were found. One of the first mentioned 2 loci and one of the latter 2 were found to be linked to genes involved in both embryoid production and subsequent green plant regeneration. The remaining two were linked to genes involved only in green plant regeneration. Of the 4 favorable alleles 3 were inherited from one parent.

Key words *Hordeum vulgare* · Anther culture · Linkage · Two-dimensional electrophoresis markers

Introduction

Anther culture is a technique used for doubled haploid (DH) production in barley. Both environmental and genetic factors may influence DH production, and a great deal of effort has been devoted to increasing the efficiency of producing them (reviewed by Pickering and Devaux 1992). Many investigators have studied the inheritance of this character in barley. Foroughi-Wehr et al. (1982) showed that the technique involves two main mechanisms separately inherited, the induction of embryoids and their subsequent regeneration into plantlets. From a complete diallel cross experiment among seven barley cultivars, Powell (1988) detected significant additive and dominance effects for percentage of responding anthers and for green or albino plants produced per 100 anthers cultured. Investigating 22 reciprocal and 1 single hybrid, Larsen et al. (1991) found that genetic variation for embryo and green plant regeneration could be explained by general combining ability. The value of a parental barley line appears to be its suitability for predicting the level of anther culture response expressed in a cross. Most of the barley yellow mosaic virus-resistant DH lines produced by Foroughi-Wehr and Friedt (1984) were derived from F_1 hybrids having an anther culture-responsive parent.

The segregation of anther culturability characters among plants obtained from single crosses has been studied in different species. Dunwell et al. (1987) found that the percentage of responding anthers was significantly different between F_2 plants in barley. The quantitative inheritance of different components of anther culturability, including percentage of responding anthers, embryoid production, and plant regeneration, has been reported in wheat (Lazar et al. 1984, Deaton et al. 1987), triticale (Charmet and Bernard 1984), maize (Petolino and Thompson 1987, Petolino et al. 1988, Afele and Kannenberg 1990), and rice (Quimio and Zapata 1990). In addition, transgressive segregation for green plant regeneration has been reported by Hou (1992) in a F_2 population of barley, and Agache et al. (1988) identified transgressive DH lines combining high embryo production and regeneration ability through anther culture in wheat.

Thus, it is possible to transfer anther culturability by selective crosses. However, the assessment of anther culturability is very labor intensive: at least 2 months of tissue culture are required to obtain valid results, and the procedure is environmentally sensitive. An alternative method would be to identify markers linked to genes involved in

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the anther culture response. In maize, Cowen et al. (1992) identified four RFLP loci tagging embryoid production.

Distortions of segregation for genes encoding polypeptides were found in a barley DH population derived from a cross between the two inbred lines 'Kaskade' and 'DH8293' (Zivy et al. 1992). It was hypothesized that these markers are genetically linked to factors involved in the process of haploid production that have been submitted to selection during this process. They could therefore be used as markers of anther culturability. The aim of the study presented here was to test this hypothesis by culturing the anthers of DH lines that differed in the presence or absence of the selected alleles.

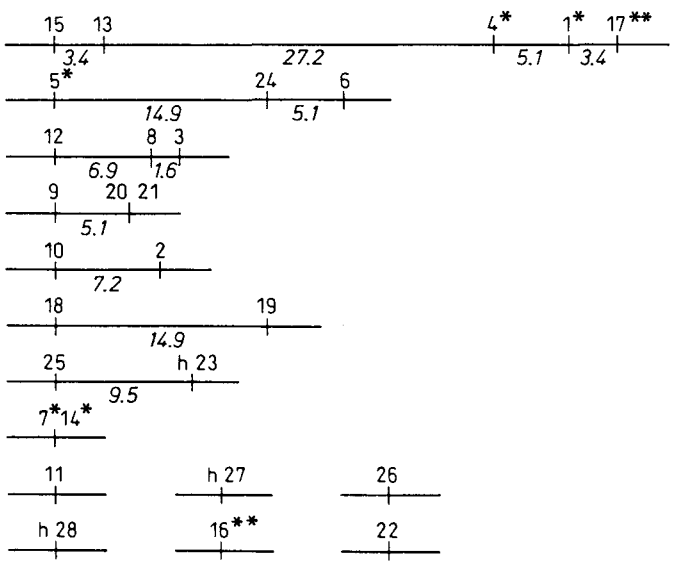
Materials and methods

Fifty DH lines derived from the F_1 hybrid between 'Kaskade' and 'DH8293' were selected from a population of 62 DH lines investigated by two-dimensional electrophoresis (Zivy et al. 1992). In this previous study, 28 protein markers were arranged in a genetic map (Fig. 1). Taking into account approximately 40 cM at both ends of the 14 independant loci and linkage groups, they estimated the total length of the marked genome to be between 730 and 1270 cM. According to the length of published high-resolution genetic maps (Graner et al. 1991; Heun et al. 1991; Kleinhofs et al. 1993), the length of the barley genome is between 1250 and 1450 cM. Although our map did not mark the genome with high resolution, it enabled the identification of four chromosome regions showing a significantly distorted segregation: 17-1–4, 5, 7–14, and 16 (see Fig. 1). The 50 DH lines selected for the present study were those that did not show recombination within the linkage group 17-1–4. They were divided into 13 groups on the basis of their genotype at 17-1–4, 5, 7–14, and 16. The number of DH lines within a group ranged from 1 to 7.

After vernalization, 2–4 plants of 'Kaskade', 'DH8293' and of each F_1-derived DH line were raised in a greenhouse. Plant growth conditions and anther culture were the same as for the production of the F_1-derived DH lines (Zivy et al. 1992). Spikes were sampled from each DH line within a group with a total of 14–36 spikes per group being used. Numbers of anthers, embryoids, and green plants were recorded.

Statistical analyses were conducted using the generalized linear model (GLM) procedure in the statistical analysis system (SAS 1988). Analyses of variance were performed on the 52 genotypes (DH lines and two parents), except for loci 2, 13, and 16, which had one missing value, and h23, which had two missing values.

Fig. 1 Linkage map. (*numbers in italics*, distances in cM, * locus showing a significant deviation to 1:1 segregation ($P< 0.05$), ** locus showing a highly significant deviation to 1:1 segregation ($P< 0.01$) (from Zivy et al. 1992))



Results

Anther culturability of 'Kaskade', 'DH8293', and their F_1-derived DH progeny

Anther culturability was defined as the number of green plants per 100 anthers (%GPL/ANTH). Two intermediate steps were also computed: number of embryoids per 100 anthers (%EMB/ANTH) and number of green plants per 100 embryoids (%GPL/EMB).

A large amount of variation was found among the 52 genotypes for the three variables. The distribution was relatively normal for %EMB/ANTH, but not for %GPL/EMB and %GPL/ANTH, which were skewed. Few genotypes produced large quantities of green plants, and many produced only small numbers. Of the 52 genotypes 13 did not produce any green plants at all. 'Kaskade' and 'DH8293' produced relatively small numbers of embryoids, but 'DH8293' produced more green plants than 'Kaskade'.

There was no correlation between %EMB/ANTH and %GPL/EMB, but both characters were positively correlated to %GPL/ANTH with r=0.547 and 0.826, respectively, thereby being significant at the 1% level. The skewness of %GPL/EMB and %GPL/ANTH led us to use the transformed variables Log(%GPL/EMB+1) and Log(%GPL/ ANTH+1) for variance and regression analyses (Steel and Torrie 1980).

Marker associations with anther culturability

The means for %GPL/ANTH for the different lines, grouped according to their genotype at the four loci that showed a deviation to the 1:1 segregation, are indicated in Table 1. One-way analyses of variance were performed separately for each locus. Loci 17 and 5 showed highly significant effects (P=0.0004 and P=0.0033, respectively, Table 2). In both cases, the selected allele from 'DH8293' resulted in a higher yield of green plants, as illustrated in Fig. 2A and B. Loci 7 and 16 did not show any significant effect.

Lines having the 4 selected alleles were the most efficient (see Table 1). Of the 5 top performing lines, 4, including the best one, have these alleles. The number of genotypes studied was too small to study the interaction between the 4 loci. However, in the one-way analysis of variance among the 13 groups defined according to genotype at the 4 loci, the contrast between genotypes having

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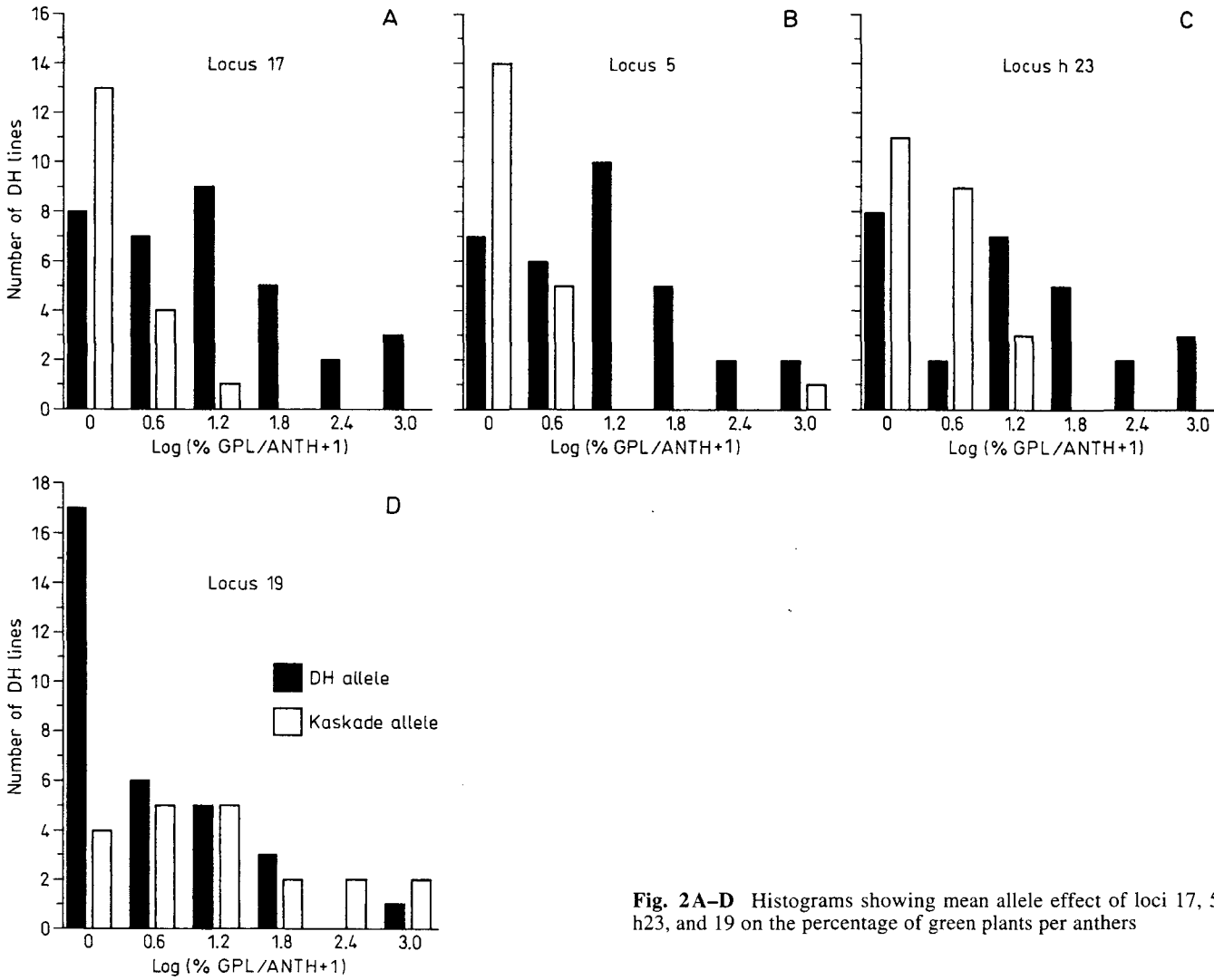

Fig. 2A–D Histograms showing mean allele effect of loci 17, 5, h23, and 19 on the percentage of green plants per anthers

Table 1 Means for anther culturability characters according to the genotype at loci showing distorted segregation. One DH line had data missing at locus 16 and was excluded from this table

Group	Locus[a] 17 7 5 16	Number of selected alleles	Number of DH lines	% EMB/ANTH	% GPL/EMB	% GPL/ANTH
1[b]	0 0 0 1	1	3	158.5	0.155	0.137
2	0 0 1 0	1	1	48.0	0.234	0.110
3	0 1 0 0	1	4	143.0	0.049	0.095
4	1 1 0 0	2	1	112.9	0.353	0.400
5	1 0 1 0	2	5	366.1	0.935	3.288
6	1 0 0 1	2	2	117.3	0.911	0.765
7	0 0 1 1	2	3	257.6	0.241	0.597
8	0 1 0 1	2	3	114.6	0.151	0.113
9[c]	1 1 1 0	3	6	150.7	1.547	2.260
10	1 1 0 1	3	7	332.0	0.606	4.477
11	0 1 1 1	3	4	147.0	0.510	0.968
12	1 0 1 1	3	5	260.1	0.935	2.028
13	1 1 1 1	4	7	296.8	3.859	11.251

[a] 1, Presence of selected allele; 0, absence of selected allele: 17, 7, and 5 in 'DH8293', and 16 in 'Kaskade'
[b] 'Kaskade' is in group 1 [c] 'DH8293' is in group 9

Table 2 Effect of the most significant marker of different linkage groups on the percentage of green plants per anther. Analyses of variance were done on the log-transformed variable, but means shown here are on the untransformed variable (*P* Probability of Fisher's test, *R-square* ratio of the sum of squares explained by the locus to the total sum of squares)

Locus	*P*	R-square	Mean for DH8293 allele	Mean for Kaskade allele
17	0.0004	0.221	4.48	0.38
5	0.0033	0.160	3.90	1.72
h23	0.0009	0.207	5.32	0.66
19	0.0102	0.125	1.55	5.47

the selected allele at the 4 loci and those having the selected allele only at loci 17 and 5 was not significant (P=0.09): selected alleles at loci 7 and 16 did not significantly modify %GPL/ANTH in the presence of selected alleles at loci 17 and 5.

Of the other loci revealing polymorphism between 'Kaskade' and 'DH8293' (Zivy et al. 1992) but not different from a 1:1 ratio in the F_1-derived DH progeny, 6 (15,

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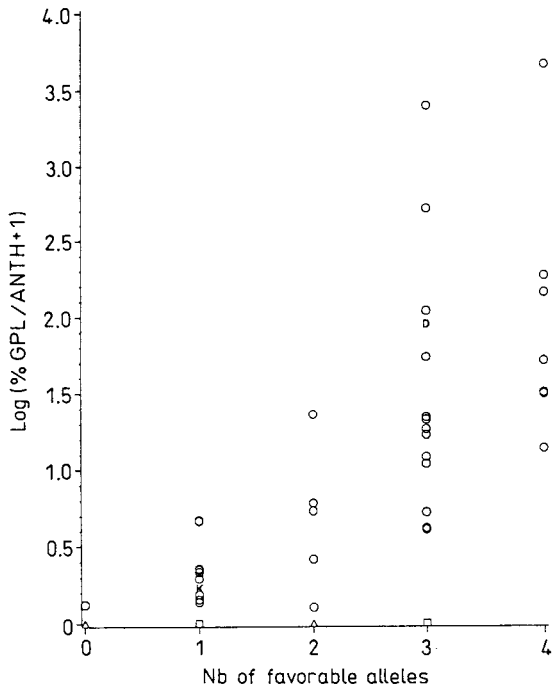

Fig. 3 Percentage of green plants per anther as a function of the number of favorable alleles at the 4 loci showing a significant effect (*K* 'Kaskade', *D* 'DH8293', ○ 1 DH line, △ 2 DH lines, □ 4 DH lines)

Table 3 Mean effects of alleles of different linkage groups on the percentages of embryos per anther and of green plants per embryoid. For the percentage of green plants per embryoid, analyses of variance were done on the log-transformed variable, but means shown here are on the untransformed variable.

Locus	*P*	Mean for DH8293 allele	Mean for Kaskade allele
%EMB/ANTH			
17	0.0232	263.3	155.6
5	0.4339	240.3	203.2
h23	0.4967	242.5	209.8
19	0.0354	188.3	286.3
%GPL/EMB			
17	0.0017	1.53	0.23
5	0.0061	1.52	0.38
h23	0.0010	1.80	0.32
19	0.0506	0.77	1.58

13, 25, h23, 19 and 18) also had a significant effect on anther culturability. Loci 15 and 13 were tightly linked and located 27.2 cM from group 17-1–4 (Fig. 1). Thus, the significant effect of loci 15 and 13 can be explained by linkage with locus 17, which showed a highly significant effect (see above). This hypothesis is strengthened by the fact that there was no significant difference between genotypes having the favorable allele at locus 17 and at loci 15 and 13, and those having the favorable allele only at locus 17.

Loci h23-25 constitute an independant linkage group on the derived map (Fig. 1), although linkage with loci 25 and h23 was apparent but not significant when using the LOD score of 3 to build the map. Thus, the significant effect found for loci 5, 25, and h23 could be due to the fact that they actually mark the same chromosomal segment. There was no significant difference between genotypes having the favorable allele at loci 5 and h23-25 and those having the favorable allele at locus 5 only. On the other hand, h23, which would be further than 25 from 5, had a higher effect on anther culturability (P=0.002 vs 0.033). Therefore, the hypothesis that the linkage group h23-25 and locus 5 tag different genes involved in %GPL/ANTH cannot be excluded. The effect of h23 is illustrated in Fig. 2C.

The 2 linked loci 19 and 18 did not show linkage with any other marker (Fig. 1). In contrast with the other markers showing a significant effect, the favorable allele originated from 'Kaskade'. They mainly affected %GPL/ANTH in the percentage of anthers producing no green plants. This was confirmed by a chi-square test of independence. The effect of locus 19, the most significant one, is illustrated in Fig. 2D.

In summary, four linkage groups containing markers for anther culturability were identified. In order of decreasing significance they are: 17, h23, 5, and 19 (Table 2). A regression of %GPL/ANTH against the number of favorable alleles (0–4) was computed, and the regression was highly significant (P=0.0001). A graph of %GPL/ANTH against the number of favorable alleles is shown in Fig. 3.

The results of analyses of variance on %EMB/ANTH and %GPL/EMB for the 4 loci that showed a significant effect on %GPL/ANTH are shown in Table 3. Loci 17 and 19 showed a significant effect on both characters, while loci 5 and h23 showed a significant effect only on %GPL/EMB. As expected from the linkage between 5 and h23, these 2 loci acted in the same direction on the same character.

Discussion

In an earlier study of DH lines derived from a cross between 'DH8293' and 'Kaskade' Zivy et al. (1992) observed a significant deviation from the 1:1 segregation for markers or linkage groups 17-1–4, 5, 7–14, and 16. Alleles from 'DH8293' were found more frequently at 17-1–4, 7–14, and 5, while the 'Kaskade' form was more often found for marker 16. Two groups (17-1–4 and 5) had a significant effect on anther culturability. Linkage group 17-1–4 had a greater effect than locus 5 and also showed the most deviant segregation. For both loci, the allele originating from 'DH8293' was the most efficient. This is in accordance with the hypothesis tested in this study. The mean value for anther culturability for DH lines having the 4 selected alleles was higher than that for DH lines having other allelic combinations, although not significantly. An epistatic interaction between them cannot definitely be ruled out. However, it was not expected that all markers showing a deviation to the 1:1 segregation would have an effect on anther culturability, since some deviations could have occurred by random chance. In addition, the monogenic de-

terminism of marker 16 was uncertain. The marker that was most likely to show an effect on anther culturability was marker 17 (Zivy et al. 1992).

In addition to 17-1–4 and 5, two other linkage groups showed a significant effect on anther culturability: 18–19 and h23-25. Weak linkage was found between h23-25 and 5, and these two groups could actually mark a single chromosome segment containing a gene involved in anther culturability. More DH lines should be studied to confirm this. Several hypotheses can explain the fact that an unselected locus shows an effect on anther culturability: (1) the locus is weakly linked to a selected chromosome region, as hypothesized above for h23-25 and 5. For 18–19, the selected region would not be located on our map. (2) Epistatic interaction: the locus affects anther culturability only in the presence of another unmarked locus subjected to selection. (3) Anther effect: since the first generation of DH lines was derived from a F_1 hybrid, anther tissues were heterozygous at all of the polymorphic loci. On the contrary, the second generation of regenerants was derived from DH lines where anther tissues were homozygous. Since tapetal cells are known to play a role in microspore development (Pelletier and Ilami 1972, Sunderland and Huang 1985), it is possible that different interactions occur in the different tissues and that some genes have an effect in one tissue but not in the other one.

Two types of genes involved in anther culturability were tagged: linkage groups 18–19 and 17–1–4 were subsequently seen to have significant effects at both steps of DH production, the percentage of embryoids produced per anther and the percentage of green plants produced per embryoid, while 5 and h23-25 showed a significant effect only on the latter character.

As with 17-1–4 and 5 the favorable alleles for h23-25 originated from 'DH8293' although they originated from 'Kaskade' for 18–19. Thus 'DH8293' and 'Kaskade' can be hypothesized to contain 3 and 1 favorable alleles, respectively. This could explain the observation that transgressive DH lines were obtained in both directions for anther culturability. The difference between the means of lines having the 4 favorable alleles (%GPL/ANTH=9.73) and those having none of them (%GPL/ANTH=0.043) was very large, although the amount of variability explained by the 4 markers was 44%. The remaining unexplained portion could be due to environmental variation, which is known to have an important role in anther culture experiments (Dunwell et al. 1987; Henry and De Buyser 1990) and possibly to the fact that the markers are only partially linked to the genes responsible for the variation. In addition, one cannot exclude the occurrence of other genes, as yet unmarked, controlling this trait. It is not known whether the genetic effects evidenced in the present study are specific to the cross or not. Other crosses should be studied and/or other types of markers should be added to the present map.

Distorted segregation for genetic markers has already been reported in anther culture-derived DH progenies of maize (Bentolila et al. 1992; Wan et al. 1992), wheat (Agache et al. 1989; Müller et al. 1989; Devaux et al. 1990), and barley (Graner et al. 1991; Heun et al. 1991; Thompson et al. 1991). The same phenomenon was observed among barley DH lines derived by the *Hordeum bulbosum* method (Kleinhofs et al. 1993). In most of these studies, the loci have been proposed as potential markers for haploid production capability. The results of the present study confirm this hypothesis.

References

Afele JC, Kannenberg LW (1990) Genetic studies of corn (*Zea mays* L.) anther culture response. Theor Appl Genet 80: 459–464

Agache S, De Buyser J, Henry Y, Snape JW (1988) Studies on the genetic relationship between anther culture and somatic tissue culture abilities in wheat. Plant Breed 100:26–33

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

Bentolila S, Hardy T, Guitton C, Freyssinet G (1992) Comparative genetic analyses of F_2 plants and anther culture derived plants of maize. Genome 35: 575–582

Charmet G, Bernard S (1984) Diallel analysis of androgenetic plant production in hexaploid triticale (× *Triticosecale* Wittmack). Theor Appl Genet 69: 55–61

Cowen NM, Johnson CD, Armstrong K, Miller M, Woosley A, Pescitelli S, Skokut M, Belmar S, Petolino JF (1992) Mapping genes conditioning in vitro androgenesis in maize using RFLP analysis. Theor Appl Genet 84: 720–724

Deaton WR, Metz SG, Armstrong TA, Mascia PN (1987) Genetic analysis of the anther-culture response of three spring wheat crosses. Theor Appl Genet 74: 334–338

Devaux P, Lonnet P, Jean R (1990) Significant higher proportions of 1BL-1RS wheat-rye translocation lines among doubled haploid progenies derived from F_1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp HJJ, Van der Plas LHW, Van Aartrijk J (eds) Progress in plant cellular and molecular biology. Proc 7th Int Cong Plant Tissue Cell Cult. Kluwer Amsterdam, pp 422–426

Dunwell JM, Francis RJ, Powell W (1987) Anther culture of *Hordeum vulgare* L.: a genetic study of microspore callus production and differentiation. Theor Appl Genet 74: 60–64

Foroughi-Wehr B, Friedt W (1984) Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture. Theor Appl Genet 67: 377–382

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

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

Henry Y, De Buyser J (1990) Wheat anther culture: agronomic performance of doubled haploid lines and the release of a new variety 'Florin'. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 13. Springer, Berlin Heidelberg New York, pp 285–352

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34: 437–447

Hou L (1992) Evaluation of barley (*Hordeum vulgare* L.) anther culture for breeding applications. PhD thesis, Washington State University, Pullman, Wash., USA

Kleinhofs A, Kilian A, Kudrna D, Bollinger J, Hayes P, Chen FQ, Saghai Maroof MA, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ, Knapp SJ

(1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86: 705–712

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

Lazar MD, Baenziger PS, Schaeffer GW (1984) Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor Appl Genet 68: 131–134

Müller G, Borschel H, Vahl U, Wiberg A, Hartel H, Damisch W (1989) Die nutzung der antherenkulturmethode im zuchtprozess von winterweizen. I. Zur androgenesefahigkeit von 1B-1R weizen-roggen-translokationsformen. Plant Breed 102: 196–207

Pelletier G, Ilami M (1972) Les facteurs de l'androgenèse in vitro chez *Nicotiana tabacum*. Z Pflanzenphysiol 68: 97–114

Petolino JF, Thompson SA (1987) Genetic analysis of anther culture response in maize. Theor Appl Genet 74: 284–286

Petolino JF, Jones AM, Thompson SA (1988) Selection for increased anther culture response in maize. Theor Appl Genet 76: 157–159

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

Powell W (1988) Diallel analysis of barley anther culture response. Genome 30: 152–157

Quimio CA, Zapata FJ (1990) Diallel analysis of callus induction and green plant regeneration in rice anther culture. Crop Sci 30: 188–192

SAS (1988) SAS/STAT user's guide, release 6.03 edn. SAS Institute Inc, Cary, N.C., USA

Steel RGD, Torrie JH (1980) Principles and procedures of statistics: a biometrical approach, 2nd edn. McGraw-Hill, New York

Sunderland N, Huang B (1985) Barley anther culture – the switch of programme and albinism. Hereditas [Suppl] 3: 27–40

Thompson DM, Chalmers K, Waugh R, Forster BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L. Theor Appl Genet 81: 487–492

Wan Y, Rocheford TR, Widholm (1992) RFLP analysis to identify putative chromosomal regions involved in the anther culture response and callus formation of maize. Theor Appl Genet 85: 360–365

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

SIGNIFICANT HIGHER PROPORTIONS OF 1BL-1RS WHEAT-RYE TRANSLOCATION LINES AMONG DOUBLED HAPLOID PROGENIES DERIVED FROM F1 HYBRIDS BETWEEN TRANSLOCATED AND NON-TRANSLOCATED WHEAT CULTIVARS.

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1. INTRODUCTION

Many plant breeding programmes have been using doubled haploid plants (DHs) for increasing their efficiency and/or for development and release of new cultivars. In common wheat, Triticum aestivum L., anther culture is the most efficient technique for obtaining haploids and DHs, and many efforts have been devoted to its improvement (for recent review, see 1). Genetic analyses have shown that male gametophyte-derived plant production is under the control of at least three independently inherited characters i.e. embryo induction rate, regeneration ability and the ratio of green to albino plants (2).

In the cytotype of wheat which carries the 1BL-1RS wheat-rye translocated chromosome, higher regeneration rate has been observed leading to the deduction that gene(s) involved in regeneration ability are located on the 1RS chromosome arm (2).

From a breeding point of view, the 1RS chromosome arm is known to carry many important genes for disease resistance (3) and has been therefore incorporated into several wheat cultivars (4, 5). Unfortunately, the 1BL-1RS transfer causes a decrease in bread-making quality (6).

Our wheat breeding programme involves some crosses between 1BL-1RS translocated cultivars and those having a normal 1B chromosome. Such F1 hybrids i.e. 1BL-1RS/ 1BL-1BS structural heterozygotes have been used for DH production and for assessing in vitro selection within male gametophyte-derived embryos in their ability to regenerate plants.

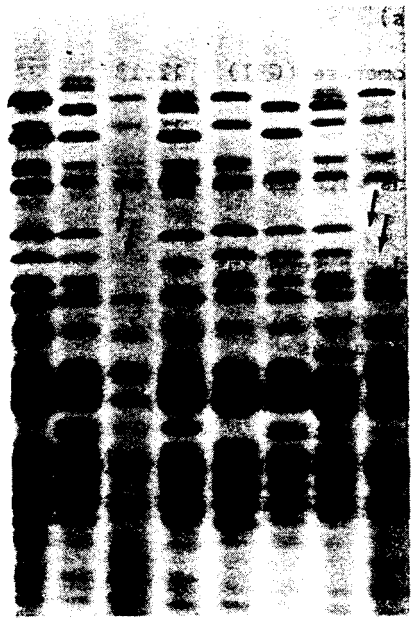
2. MATERIALS AND METHODS

The plant material for DH production consisted of seven F1 hybrids between cultivars having a normal 1B (1BL-1BS) chromosome namely Camp Remy (CR), Dartagnan (Da), Donau (Do), Fresco (F), Pernel (P), Soissons (S) and one cultivar, Apollo (A), or advanced line, FD1692 (FD), both with a homozygous 1BL-1RS translocated chromosome. All of them are winter wheats.

One hundred and forty seven fertile DHs have been developped by classical anther culture techniques (7) and subsequently analyzed for

presence or absence of the 1BL-1RS translocated chromosome. Among the main systems available for characterization of 1BL-1RS translocation lines and listed in Table 1, sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) of the storage proteins has been used in the present experiment and performed on parents of the 7 F1 hybrids as well as on the 147 derived DHs. The technique was the same as described by Branlard and Le Blanc (18). Lines with the 1BL-1RS translocated chromosome can therefore be identified by the absence of 1BS-encoded ω-gliadins on such gels (Figure 1).

Figure 1. Analysis by SDS-PAGE of the storage proteins of wheat lines. Translocated 1BL-1RS lines have been identified by the absence of 1BS-encoded ω-gliadins as indicated by the arrows.



3. RESULTS AND DISCUSSION.

The frequencies of 1BL-1RS wheat-rye translocation DH lines and those having a normal 1BL-1BS chromosome are indicated in Table 2. 103 (70.1%) of the 147 DHs analyzed have been found to possess a 1BL-1RS translocated chromosome while 44 (29.9%) have a 1BL-1BS chromosome. A χ^2 test applied to the data indicates that the overall ratio translocated:non-translocated DHs is significantly different from the 1:1 expected one at the 0.001 level. Percentages of 1BL-1RS cytotypes per F1 hybrid, from which DHs have been derived, vary from 59.3% to 100%.

Our results agree closely with those reported recently (10, 14) in which 67.3% and 75% of microspore-derived plants respectively had a 1BL-1RS translocated chromosome and confirm that there is a

Table 1. Main systems for characterization of 1BL-1RS wheat-rye translocation lines.

Markers	Techniques	References
Cytological	Mitosis:	
	Nucleolus organiser competition (Feulgen, Ag-NOR banding)	8.2.4.9.10.11
	C-banding	3.11
	N-banding	9
	Meiosis in PMCs (for heterozygous 1BL-1BS/1BL-1RS lines)	3
Isozyme	Glucose phosphate isomerase (GPI)	12.13
	Peroxidase	14
Storage protein	SDS-PAGE of ω-gliadins with HMW glutenin subunits	15.16
	APAGE of gliadins and rye secalins	2
	Two-dimensional electrophoresis of gliadins and rye secalins	16.5
	HPLC of gliadins and rye secalins	(*)
RFLP	Use of pTA71, PSR161 probes	17. (**)

(*) J.A. Bietz, R.A. Graybosch, B. Lookhart, personal communication.
(**) M.D. Gale, personal communication.

preferential developpement of microspore-derived embryos for those with the 1RS chromosome arm. This difference in frequency is maintained up to the obtaining of fertile DHs, since grains collected on them have been analyzed in our experiment by SDS-PAGE. Thus, the ability for chromosome doubling, either spontaneously or after colchicine treatment, seems not to be linked to the presence or absence of the 1BL-1RS chromosome.

In a more recent study, Due et al. (13) have found distorted segregation of 1BL-1RS (74%) and 1BL-1BS (26%) among the green regenerants of microspore-derived embryos while a 1:1 ratio among the albino regenerants . They have made the hypothesis that the 1BS chromosome arm is acting as a semi-lethal in structures determined to become green.

From a breeding point of view, our results can be exploited as follows. From heterozygous 1BL-1BS/1BL-1RS hybrids, two kinds of DHs

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Table 2. Numbers of 1BL-1RS wheat-rye translocation and normal 1BL-1BS lines among DH progenies derived from 7 F1 hybrids heterozygous 1BL-1RS/1BL-1BS.

F1 hybrid	No. of DHs with:		% 1BL-1RS	Total
	1BL-1RS	1BL-1BS		
A x CR	16	9	64.0	25
A x Da	15	1	93.8	16
A x Do	8	3	72.7	11
F x A	22	15	59.5	37
A x P	14	0	100.0	14
A x S	16	11	59.3	27
S x FD	12	5	70.6	17
Total	103	44	70.1	147

$\chi^2 = 23.68$ with 1 d.f. ($\chi^2_{.001} \geq 10.83$)

are produced by anther culture : one carrying 1BL-1RS, the second with a normal 1B chromosome. The former can be used in feed-wheat breeding, since the 1RS chromosome arm induces a detrimental effect on bread-making quality. The latter, although in fewer proportions, can be evaluated further for quality which has become an important trait for modern cultivars.

REFERENCES

1 Picard, E. (1989) Genome 31, 1005-1013
2 Henry, Y. and De Buyser, J. (1985) Plant Cell Rep. 4, 307-310
3 Friebe, B., Zeller, F.J. and Kunzmann, R. (1987) Theor. Appl. Genet. 74, 423-425
4 Lange, W. (1988) Euphytica S, 7-25
5 Rogers, W.J., Payne, P.I. and Harinder, K. (1989) Plant Breeding 103, 89-100
6 Zeller, F.J., Günzel, G., Fischbeck, G., Gerstenkorn P. and Weipert, D. (1982) Getr. Mehl. Brot. 36, 141-143
7 Picard, E. (1984) Thèse de Doctorat d'Etat, Université Paris XI, Orsay, France
8 Miller, T.E. (1984) Can. J. Genet. Cytol. 26, 578-589
9 Rayburn, A.L. and Carver, B.F. (1988) Euphytica 38, 237-240
10 Agache, S., Bachelier, B., De Buyser, J., Henry, Y. and Snape, J.W. (1989) Theor. Appl. Genet. 77, 7-11
11 Friebe, B., Heun, M. and Bushuk, W. (1989) Theor. Appl. Genet. 78, 425-432
12 Ainsworth, C.C. and Gale, M.D. (1987) in Enzymes And Their Role In

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Cereal Technology (Kruger, J.E., Lineback, D.R. and Stauffer, C.E., ed.), pp.53-82, American Association of Cereal Chemists, St Paul
13 Due, I.K., Pedersen, S., Olesen, A. and Andersen, S.B. (1990) Plant Cell Rep. (submitted)
14 Müller, G., Borschel, H., Vahl, U., Wiberg, A., Härtel, H. and Damisch W. (1989) Plant Breeding 102, 196-207
15 Payne, P.I., Holt, L.M., Hutchinson, J. and Bennett M.D. (1984) Theor. Appl. Genet. 68, 327-334
16 Payne, P.I., Nightingale, M.A., Krattiger, A.F. and Holt, L.M. (1987) J. Sci. Food Agric. 40, 51-65
17 Sharp, P.J., Chao, S., Desai, S. and Gale, M.D.(1989) Theor. Appl. Genet. 78, 342-348
18 Branlard, G. and Le Blanc, A. (1985) Agronomie 5, 467-477

CONCLUSION GÉNÉRALE

Nous venons d'esquisser l'histoire de la production d'haploïdes doublés d'orge sur une période de 25 années. Les techniques de culture d'anthère et de l'hybridation interspécifique ont été utilisées de manière extensive pour la production de cultivars. Le point fort de notre recherche réside dans le nombre extraordinaire et inhabituel de génotypes que nous avons manipulés. Il est évident que les génotypes récalcitrants à l'une ou l'autre technique existent dans le réservoir génétique de l'orge, et que, pour contourner cet écueil et rendre la méthode de production des haploïdes doublés performante, nous recommandons l'utilisation en parallèle des méthodes *Hordeum bulbosum* et culture d'anthères.

Dans les programmes d'amélioration de blé, le croisement blé x maïs a fait suffisamment ses preuves de fiabilité pour devenir l'unique système de production d'haploïdes doublés. Bien que des différences génotypiques aient été mises en évidence sur les plans de l'efficacité de la production et de l'aptitude au doublement chromosomique, aucun génotype récalcitrant n'a été identifié à ce jour.

À la vue du potentiel élevé pour la production d'haploïdes de la méthode microspores isolées, notre recherche principale s'est tournée récemment vers cette méthode aussi bien pour l'orge que pour le blé. Des percées récentes dans la technique vont probablement fortifier notre choix dans le futur.

Nos données indiquent que les lignées haploïdes doublées, obtenues par la culture d'anthère ou par *Hordeum bulbosum,* présentent un niveau très bas de modifications induites de l'ADN chez l'orge. Le niveau de méthylation de l'ADN est altéré dans les lignées d'haploïdes doublés obtenues par culture d'anthères, et, à un moindre degré, dans celles issues de la méthode *Hordeum bulbosum.* Ces modifications de méthylation de l'ADN sont probablement induites durant la culture *in vitro* des microspores à l'intérieur de l'anthère. Des recherches ultérieures devront être menées pour vérifier le niveau initial de la méthylation de l'ADN dans les embryons zygotiques immatures comparé à celui des microspores et d'étudier cette possible hérédité d'altérations de la méthylation de l'ADN induites par le milieu.

Disposant de populations d'haploïdes doublés obtenues par voies mâle et femelle, nous avons montré qu'il y a régulièrement un excès de recombinaison dans la méiose mâle par rapport à la méiose femelle. Nous avons sélectionné des sondes pour détecter des loci couvrant l'essentiel du génome de l'orge, et ceci nous a permis de localiser la majorité des distances significativement différentes entre les deux cartes dans les régions télomériques des chromosomes. Ces découvertes sont fondamentales en génétique et en amélioration, car il y a

une certitude croissante que les régions subtélomériques sont plus riches en gènes que les régions proximales. C'est pourquoi, en absence de sélection, on doit s'attendre à un assortiment de gènes plus important dans les populations d'haploïdes doublés obtenues par la voie mâle (culture d'anthères) que dans celles obtenues par la voie femelle (*Hordeum bulbosum).* Comme les études cartographiques progressent, il sera possible de vérifier cela quand davantage de gènes associés à des changements phénotypiques auront été cartographiés. En outre, si la génétique des plantes haploïdes démontre que le site de déroulement de la méiose (sac pollinique pour la méiose mâle, sac embryonnaire pour la méiose femelle) a un effet sur la recombinaison, que nous avons appelé effet sexe, elle peut aussi servir de test du facteur température. Car l'effet des différences de température pourrait être expérimenté sur des haploïdes doublés obtenus par culture d'anthère à partir d'hybrides F1 donneurs de pollen qui seront soumis à des températures différentes durant leur croissance. De telles recherches seront également très utiles aux généticiens et aux sélectionneurs.

Il a été démontré que les phases de culture *in vitro,* qui revêtent des modalités différentes dans les techniques des cultures d'anthères et d'*Hordeum bulbosum,* agissent sur la distribution des génotypes de gamétophytes (sous la forme de plantes haploïdes) à la suite de la sélection gamétophytique et/ou sporophytique. Les lignées haploïdes doublées obtenues par culture d'anthères présentent davantage de ségrégations déviées que celles obtenues par la méthode *Hordeum bulbosum.* Dans le contexte de l'amélioration, ce phénomène nous apparaît, dans la majorité des cas, comme indésirable, mais il peut nous aider à identifier des gènes intervenant dans l'aptitude à la culture d'anthères chez l'orge. Des analyses de ségrégation devraient être menées sur des populations d'haploïdes doublés obtenues par culture d'anthères à partir d'hybrides F_1 issus de deux parents ayant une bonne aptitude à la culture d'anthères. Des analyses de même type seraient nécéssaires si de nouvelles techniques de production d'haploïdes doublés indépendantes du génotype étaient découvertes. Elles nous diraient si d'autres facteurs que ceux de la culture *in vitro* pourraient générer des ségrégations déviées dans les descendances haploïdes doublées de l'orge.

GENERAL CONCLUSION

We have outlined the history of doubled haploid barley production over a 25-year period. The techniques of anther culture and interspecific hybridization have been used extensively for cultivar production. The strength of our researches relies on the extraordinary and unusual large number of genotypes that have been manipulated. It is evident that recalcitrant genotypes to one or the other technique exist in the barley pool and that to overcome this problem and make the doubled haploid method more efficient for breeding, we recommand using the *Hordeum bulbosum* and anther culture methods in parallel. The wheat x maize cross by itself has proven to be reliable enough to be a unique system for doubled haploid wheat production. Although genotypic differences have been found for haploid production efficiency and chromosome doubling ability, no recalcitrant genotype has been identified so far. Considering the high potential of the isolated microspore system for haploid production, our more basic researches have recently been focusing on this topic both in barley and wheat. Recent breakthroughs in this technique are likely to make it the choice in the future.

Our data indicates that anther culture- and *Hordeum bulbosum*-derived doubled haploid lines have very low levels of induced DNA changes in barley. The level of DNA methylation was altered in the anther culture- and to a much smaller extend, in the *Hordeum bulbosum*-derived doubled haploid lines.These changes in the DNA methylation pattern were probably induced during the *in vitro* culture of microspores. Further research should be performed to check the initial levels of DNA methylation in the immature zygotic embryos *versus* microspores and to study the possible inheritance of these environmentally-induced DNA methylation alterations.

Using male and female derived, doubled haploid populations we have shown that there is a general excess of recombination in barley male gametogenesis relative to female gametogenesis. A selection of probes was undertaken to detect loci covering most of the barley genome and this enabled the most significant different distances between the two maps to be localized in the telomeric regions of the chromosomes. These findings are of importance for genetics and breeding since there is increasing evidence that subtelomeric regions are more gene-rich than proximal regions. Therefore in absence of selection, increased number of gene assortments are likely to be expected in male (anther culture) than in female (*Hordeum bulbosum*) derived, doubled haploid populations. As mapping studies progress, it will become possible to check when more gene associated with phenotypic changes would have been mapped. Perhaps the most important difference, besides sex, is temperature at the time meiotic recombination occurs. Temperature-related differences could be assessed from anther

culture doubled haploid populations derived from an F_1 hybrid for which donor plants would have been raised at different temperatures. Such research would also be very useful to genetists and breeders.

It has been shown that the *in vitro* culture phases which are distinct from anther culture and *Hordeum bulbosum* techniques have influenced the representation of recovered gametes through gametophytic and/or sporophytic selection. The anther culture-derived doubled haploid lines show more segregation distortion than is observed with the *Hordeum bulbosum*-derived lines. This is probably an undesirable characteristic in most case, but may help to identify genes important for anther culturability in barley. Segregation analyses should be performed in a doubled haploid population derived from a F_1 hybrid between two highly anther culture responders and or if a genotype-independent doubled haploid production technique becomes available. This would tell us whether other factors such as *in vitro* culture could generate distortions of segregation in barley doubled haploid progenies.

Références bibliographiques
References

Nous n'avons pas inclus dans cette liste des références de l'article Pickering RA, Devaux P (1992) 'Haploid production: approaches and use in plant breeding', p 59, parce qu'il porte sur les haploïdes doublés des plantes cultivées en général.
The references of the paper Pickering RA, Devaux P (1992) *'Haploid production: approaches and use in plant breeding', p 59, have not been included in this list because this paper comprises data on doubled haploid plants of several crops.*

1. Adams RLP (1990) DNA methylation. The effect of minor bases on DNA-protein interactions. Biochem J 265:309-320
2. Adams RLP, Burdon RH (1985) Molecular biology of DNA methylation. Springer, New York
3. Adamski T, Kaczmarek Z, Surma M, Madejewski R, Madejewska M (1995) Efektywnosc hodowli jeczmienia metoda rodowodowa i metoda wykorzystujaca lilie podwojonych haploidow. Hod Rosl 2:16-19
4. Afele JC, Kannenberg LW (1990) Genetic studies of corn (*Zea mays* L.) anther culture response. Theor Appl Genet 80:459-464
5. 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
6. Agache S, Buyser J de, Henry Y, Snape JW (1988) Studies on the genetic relationship between anther culture and somatic tissue culture abilities in wheat. Plant Breed 100:26-33
7. Ahokas H (1978) Cytoplasmic male sterility in barley. I. Anther and pollen characteristics of *msm1,* restored and partially restored *msm1* genotypes. Z Pflanzenzüchtg 81:327-332
8. Ainsworth C, Gale MD (1987) Enzyme structural genes and their exploitation in wheat genetics and breeding. In: Enzymes and their role in cereal technology. Kruger JE, Staufer CE, Lineback DR (eds) Amer Assoc Cereal Chem, St Paul pp53-57
9. Amasino RM, John MC, Klaas M, Crowell DN (1990) Role of DNA methylation in the regulation of gene expression in plants. In: Clawson GA, Willis DB, Weissbach A, Jones PA (eds) Nucleic acid methylation. Wiley-Liss, New York, pp187-198
10. Anderson MK, Reinbergs E (1985) Barley breeding. In: Rasmusson DC (ed) Barley. Amer Soc Agron, Madison WI, pp231-268
11. Baenziger PS, Wesenberg DM, Schaeffer GW, Galun E, Feldman M (1983) Variation among anther culture derived doubled haploids of 'Kitt' wheat. In: Sakamoto S (ed) Proc 6th Int Wheat Genetics Symp, Kyoto University, Japan, pp 575-582
12. Bahrman N, Thiellement H (1987) Parental genome expression in synthetic wheats (*Triticum turgidum* sp. x T. *tauschii* sp.) revealed by two-dimensional electrophoresis of seedling proteins. Theor Appl Genet 74:218-223
13. Bailey NTJ (1961) Introduction to the mathematical theory of genetic linkage. Clarendon Press, Oxford, 140p

14. Baillie AMR, Rossnagel BG, Kartha KK (1992) Field evaluation of barley (*Hordeum vulgare* L.) genotypes derived from tissue culture. Can J Plant Sci 72:725-733
15. Benito C, Romero MP, Henriques-Gil N, Llorente F, Figueiras AM (1996) Sex influence on recombination frequency in *Secale cereale.* Theor Appl Genet 93:926-931
16. Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Phil Trans R Soc London (Biol) 274:227-274
17. Bentolila S, Hardy T, Guitton C, Freyssinet G (1992) Comparative genetic analyses of F_2 plants and anther culture derived plants of maize. Genome 35:575-582
18. Bergounioux C, Perennes C, Miège C, Gadal P (1986) Male sterility effect upon protoplast division in *Petunia hybrida.* Protoplasma 130:138-144
19. Biom (1989) A package of statistical programs. Applied Biostatistics, Setauket, NY
20. Bjørnstad Å (1986) Partial incompatibility between Scandinavian six-rowed barleys (*Hordeum vulgare* L.) and *Hordeum bulbosum* L., and its genetical basis. Hereditas 104:171-191
21. Bojko M (1983) Human meiosis VIII. Chromosome pairing and formation of the synaptonemal complex in oocytes. Carlsberg Res Comm 48:457-483
22. Branlard G, Le Blanc A (1985) Les sous-unités gluténines de haut poids moléculaire des blés tendres et des blé durs cultivés en France. Agronomie 5:467-477
23. Breiman A, Rotem-Abarbanell D, Karp A, Shaskin H (1987) Heritable somaclonal variation in wild barley *(Hordeum spontaneum).* Theor Appl Genet 74:104-112
24. Brettell RIS, Dennis ES (1991) Reactivation of a silent *Ac* following tissue culture is associated with heritable alterations in its methylation pattern. Mol Gen Genet 229:365-372
25. Bridges WC, Knapp SJ (1990) Using molecular markers to estimate quantitative trait locus parameters: Quantitative genetic theory for replicated progeny. In: Agronomy abstracts, ASA, Madison,WI, p194
26. Brophy M (1996) Global production and markers for barley in the 21st century. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp. University Extension Press, Saskatoon, Sask, pp37-43
27. Brown PTH (1989) DNA methylation in plants and its role in tissue culture. Genome 31:717-729
28. Brown PTH, Kyozuka J, Sukekiyo, Kimura Y, Shimamoto K, Lörz H (1990) Molecular changes in protoplast-derived rice plants. Mol Gen Genet 223:324-328
29. Brown S, Devaux P, Bergounioux C, Petit PX (1991) Analyse de la ploidie par cytométrie. Biofutur, Le Technoscope 105:1-16
30. Busso CS, Liu CJ, Hash CT, Witcombe JR, Devos KM, de Wet JMJ, Gale MD (1995) Analysis of recombination rate in female and male gametogenesis in pearl millet (*Pennisetum glaucum*) using RFLP markers. Theor Appl Genet 90:242-246
31. Buyser J de, Henry Y, Taleb G (1985) Wheat androgenesis: cytological analysis and agronomic performance of doubled haploids. Z Pflanzenzüchtg 95:23-34
32. Charmet G, Bernard S (1984) Diallel analysis of androgenetic plant production in hexaploid triticale (x *Triticosecale* Wittmack). Theor Appl Genet 69:55-61

33. Charmet G, Bernard S, Bernard M (1986) Origin of aneuploid plants obtained by anther culture in triticale. Can J Genet Cytol 28:444-452
34. Charmet G, Branlard G (1985) A comparison of androgenetic doubled-haploid and single-seed descent lines in *Triticale.* Theor Appl Genet 71:193-200
35. Campbell KW, Brawn RI, Ho KM (1984) Rodeo barley. Can J Plant Sci 64:203-205
36. Chen CC, Kasha KJ, Marsolais A (1984) Segmentation patterns and mechanisms of genome multiplication in cultured microspores of barley. Can J Genet Cytol 26:475-483
37. Chen F, Hayes PM (1989) A comparison of *Hordeum bulbosum*-mediated haploid production efficiency in barley using *in vitro* floret and tiller culture. Theor Appl Genet 77:701-704
38. Choo TM, Reinbergs E, Kasha KJ (1985) Use of haploids in breeding barley. Plant Breed Rev 3:219-251
39. Choo TM, Shugar L, Bastien D (1995) *Bulbosum* method in Eastern Canada: an observation. Barley Newsl 39:111-112
40. Cistué L, Ziauddin A, Simion E, Kasha KJ (1995) Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss Org Cult 42:163-169
41. Clapham D (1973) Haploid *Hordeum* plants from anthers *in vitro.* Z Pflanzenzüchtg 69:142-155
42. Colas des Francs C, Thiellement H (1985) Chromosomal localization of structural genes and regulators in wheat by 2D electrophoresis of ditelosomic lines. Theor Appl Genet 71:31-38
43. Coles GD (1986) Gwylan barley (*Hordeum vulgare* L.). New Zealand J Exp Agri 14:101-103
44. Comeau A, Nadeau P, Plourde A, Simard R, Maës O, Kelly S, Harper L, Lettre J, Landry B, St-Pierre CA (1992) Media for the *in ovulo* culture of proembryos of wheat and wheat-derived interspecifc hybrids or haploids. Plant Sci 81:117-125
45. Cowen NM, Johnson CD, Armstrong K, Miller M, Woosley A, Pescitelli S, Skokut M, Belmar S, Petolino JF (1992) Mapping genes conditioning in vitro androgenesis in maize using RFLP analysis. Theor Appl Genet 84:720-724
46. D'Amato F (1990) Somatic nuclear mutations *in vivo* and *in vitro* in higher plants. Caryologia 43:191-204
47. Damerval C, Vienne D de, Zivy M, Thiellement H (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. Electrophoresis 7:52-54
48. Damerval C, Le Guilloux M, Blaisonneau J, Vienne D de (1987) Simplification of the Heukeshoven and Dernick's silver staining of proteins. Electrophoresis 8:158-159
49. Davies PA, Morton S (1995) Barley doubled haploids from isolated microspore cultures. In: Proc 7th Aus Barley Tech Symp. Perth WA, pp195-199
50. Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9:671-678
51. Deaton WR, Metz SG, Armstrong TA, Mascia PN (1987) Genetic analysis of the anther-culture response of three spring wheat crosses. Theor Appl Genet 74:334-338

52. De Paepe R, Prat D, Huguet T (1983) Heritable nuclear DNA changes in doubled haploid plants obtained by pollen culture of *Nicotiana sylvestris*. Plant Sci Lett 28:11-28
53. Devaux P (1983) Haploïdes doublés, issus d'orges d'hiver, par la méthode *bulbosum*. Analyse cytogénétique et recherche de meilleures souches d'*Hordeum bulbosum*. Thèse de 3ème cycle, Université des Sciences et Techniques de Lille, 125p
54. Devaux P (1986) Yield of haploid production through the *bulbosum* method in a winter barley programme. Cereal Res Comm 14:273-279
55. Devaux P (1987) Comparison of anther culture and *Hordeum bulbosum* method for the production of doubled haploids in winter barley. I. Production of green plants. Plant Breed 98:215-219
56. Devaux P (1992) Haploidy in barley and wheat improvement. In: Dattée Y, Dumas C, Gallais A (eds) Reproductive Biology and Plant Breeding, Proc 8th Eucarpia Cong. Springer-Verlag, Berlin, pp139-151
57. Devaux P, Adamski T, Surma M (1990) Studies on low crossabilities encountered with the *Hordeum bulbosum* method for haploid production of barley, *Hordeum vulgare* L. Plant Breed 104:305-311
58. Devaux P, Adamski T, Surma M (1992) Inheritance of seed set in crosses of spring barley and *Hordeum bulbosum* L. Crop Sci 32:269-271
59. Devaux P, Hou L, Ullrich SE, Huang ZX, Kleinhofs A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32-36
60. Devaux P, Kilian A, Kleinhofs A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids: mutations and methylation. Mol Gen Genet 241:674-679
61. 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
62. Devaux P, Lonnet P, Jean R (1990) Significant higher proportions of *1BL-1RS* wheat-rye translocation lines among doubled haploid progenies derived from F_1 hybrids between translocated and non-translocated wheat cultivars. In: Nijkamp HJJ, Van der Plas LHW, Van Aartrijk J (eds) Progress in plant cellular and molecular biology. Proc. 7th Int Cong Plant Tissue and Cell Cult. Kluwer, Amsterdam, pp422-426
63. Devaux P, Zivy M (1994) Protein markers for anther culturability in barley. Theor Appl Genet 88:701-706
64. Devaux P, Zivy M, Kilian A, Kleinhofs A (1996) Doubled haploids in barley. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp, University Extension Press, Saskatoon, Sask pp213-222
65. De Vicente MC, Tanksley SD (1991) Genome-wide reduction in recombination of backcross progeny derived from male *versus* female gametes in an interspecific cross of tomato. Theor Appl Genet 83:173-178
66. De Vries SC, Booij H, Janssens R, Vogels R, Saris L, Lo Schiaro F, Terzi M, Van Kammen A (1988) Carrot somatic embryogenesis depends on the phytohormone-controlled presence of correctly glycosylated extracellular proteins. Genes Dev 2:462-476
67. Doll H, Haahr V, Søgaard B (1989) Relationship between vernalization requirement and winter hardiness in doubled haploids of barley. Euphytica 42:209-213

68. Donis-Keller H, Green P, Helms C, Cartinhour S, Weiffenbach B, Stephens K, Keith TP, Bowden DW, Smith DR, Lander E, Botstein D, Akots G, Rediker KS, Gravius T, Brown VA, Rising MB, Parker C, Powers JA, Watt DE, Kauffman ER, Bricker A, Phipps P, Muller Kahle H, Fulton TR, Ng S, Schumm JW, Braman JC, Knowlton TG, Barker DF, Crooks SM, Lincoln SE, Daly MJ, Abrahamson J (1987) A genetic linkage map of the human genome. Cell 51:319-337
69. Dpac (1994) Data pool application software for CA-II, version 2.1. Partec, Münster, Germany
70. Dunnet CW (1955) A multiple procedure for comparing several treatments with the control. J Am Stat Assoc 50:1096
71. Dunwell JM, Francis RJ, Powell W (1987) Anther culture of *Hordeum vulgare* L.: a genetic study of microspore callus production and differentiation. Theor Appl Genet 74:60-64
72. Evans DA, Sharp WR (1983) Single gene mutations in tomato plants regenerated from tissue culture. Science 221:949-951
73. Fadel F, Wenzel G. (1990) Medium-genotype-interaction on androgenetic haploid production in wheat. Plant Breed 105:278-282
74. Falk DE, Kasha KJ, Reinbegs E (1981) Presowing selection on genetic male sterile plants to facilitate hybridization in barley. In: Asher MJC, Ellis RP, Hayter AM, Whitehouse RNH (eds) Proc 4th Int Barley Genet Symp. Edinburgh University Press, Edinburgh, pp778-785
75. Finnie SJ, Forster BP, Chalmers KJ, Dyer AF, Waugh R, Powell W (1991) Genetic stability of microspore-derived doubled haploids of barley: a cytological, biochemical, and molecular study. Genome 34:923-928
76. 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
77. Flehinghaus T, Deimling S, Geiger HH (1991) Methodical improvements in rye anther culture. Plant Cell Rep 10:397-400
78. Foroughi-Wehr B, Friedt W (1984) Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture. Theor Appl Genet 67:377-382
79. Foroughi-Wehr B, Friedt W, Wenzel G (1982) On genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor Appl Genet 62:233-239
80. Foroughi-Wehr B, Friedt W, Wenzel G (1982) Field experiments with anther-derived lines of barley (*Hordeum vulgare*) and rye (*Secale cereale*). In: Giles KL, Sen SK (eds) Plant cell culture in crop improvement. Plenum Publishing, New York
81. Foroughi-Wehr B, Mix G, Gaul H, Wilson HM (1976) Plant production from cultured anthers of *Hordeum vulgare* L. Z Pflanzenzüchtg 77:198-204
82. Foroughi-Wehr B, Mix G (1979) *In vitro* response of *Hordeum vulgare* L. anthers cultured from plants grown under different environments. Environ Exp Bot 19:303-309
83. Foroughi-Wehr B, Pickering RA, Friedt W (1981) Related reponses of barley cultivars to the *'bulbosum'* and anther-culture technique of haploid production. Barley Genet Newsl 11:54-59

84. Foroughi-Wehr B, Zeller FJ (1990) *In vitro* microspore reaction of different German wheat cultivars. Theor Appl Genet 79:77-80
85. Franzone PM, Fishbeck G, Foroughi-Wehr B, Friedt W (1984) Analysis of origin of androgenetic plants of *Hordeum vulgare* L. by the use of balanced lethal systems. Z Pflanzenzüchtg 93:320-330
86. Friebe B, Heun M, Bushuk W (1989) Cytological characterization, powdery mildew resistance and storage protein composition of tetraploid and hexaploid *1BL/1RS* wheat-rye translocation lines. Theor Appl Genet 78:425-432
87. Friebe B, Zeller FJ, Kunzmann R (1987) Transfer of the *1BL/1RS* wheat-rye-translocation from hexaploid bread wheat to tetraploid durum wheat. Theor Appl Genet 74:423-425
88. Friedt W, Foroughi-Wehr B, Snape JW (1987) The significance of biotechnology for the evaluation of barley breeding methods. In: Yasuda S, Konishi T (eds) Barley Genetics V, Proc 5th Int Barley Genet Symp. Mazuren, Okayama, pp903-913
89. Furusho M, Hamachi Y, Yoshida T (1990) Varietal difference in crossability between Japanese barley and *Hordeum bulbosum* L. Jpn J Breed 40:411-417
90. Gamborg OL, Miller RA, Olima K (1968) Nutrient requirements of suspension cultures of soybean root cell. Exp Cell Res 50:151-158
91. Gill KS, Gill BS, Endo TR (1993) A chromosome region-specific mapping strategy reveals gene-rich telomeric ends in wheat. Chromosoma 102:374-381
92. Gill KS, Gill BS, Endo TR, Boyko EV (1996) Identification and high-density mapping of gene-rich regions in chromosome group 5 of wheat. Genetics 143:1001-1012
93. Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869-1885
94. 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
95. Granier F, Vienne D de (1986) Silver staining of proteins: standardized procedure for two-dimensional gels bound to polyester sheets. Anal Biochem 155:45-50
96. Groover AT, Williams CG, Devey ME, Lee JM, Neale DB (1995) Sex related differences in meiotic recombination frequency in *Pinus taeda.* J Hered 86:157-158
97. Gruenbaum Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860
98. Hayes PM, Chen FQ (1989) Genotypic variation for *Hordeum bulbosum* L.-mediated haploid production in winter and facultative barley. Crop Sci 29:1184-1188
99. Henry Y, Bernard S, Bernard M, Gay G, Marcotte JL, De Buyser J (1993) Nuclear gametophytic genes from chromosome arm 1RS improve regeneration of wheat microspore-derived embryos. Genome 36:808-814
100. Henry Y, De Buyser J (1981) Float culture of wheat anthers. Theor Appl Genet 60:77-79
101. Henry Y, De Buyser J (1985) Effect of the *1B/1R* translocation on anther culture ability in wheat (*Triticum aestivum* L.). Plant Cell Rep 4:307-310

102. Henry Y, De Buyser J (1990) Wheat anther culture: agronomic performance of doubled haploid lines and the release of a new variety 'Florin'. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 13. Springer, Berlin, Heidelberg, New-York, pp285-352
103. Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103-112
104. Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*) Genome 34:437-447
105. Ho KM, Jones GE (1980) Mingo barley. Can J Plant Sci 60:279-280
106. 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
107. 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
108. Hoffman F, Thomas E, Wenzel G (1982) Anther culture as a breeding tool in rape. 2. Progeny analyses of androgenetic lines and induced mutants from haploid cultures. Theor Appl Genet 61:225-232
109. Hou L (1992) Evaluation of barley (*Hordeum vulgare* L.) anther culture for breeding applications. PhD Thesis, Washington State Univ, Pullman
110. Hou L, Ullrich SE, Kleinhofs A, Stiff CM (1993) Improvement of anther culture methods for doubled haploid production in barley breeding. Plant Cell Rep 12:334-338
111. Hougas RW, Peloquin SJ, Gabert AC (1964) Effect of seed-parent and pollinator on frequency of haploids in *Solanum tuberosum.* Crop Sci 4:593-595
112. Huang B, Dunwell JM, Powell W, Hayter AM, Wood W (1984) The relative efficiency of microspore culture and chromosome elimination as methods of haploid production in *Hordeum vulgare* L. Z Pflanzenzüchtg 92:22-29
113. Huang B, Sunderland N (1982) Temperature-stress pretreatment in barley anther culture. Ann Bot 49:77-88
114. Hunter CP (1988) Plant regeneration from microspores of barley, *Hordeum vulgare* L. PhD Thesis, Wye College, Univ of London
115. Inagaki M, Tagir M (1990) Comparison of haploid production frequencies in wheat varieties crossed with *Hordeum bulbosum* L. and maize. Jpn J Breed 40:209-216
116. Iyamabo OE, Hayes PM (1995) Effects of selection and opportunities for recombination in doubled haploid populations of barley (*Hordeum vulgare* L.). Plant Breed 114:131-136
117. Jensen J (1981) Effect of temperature on genetic recombination in barley. Hereditas 94:215-218
118. Jeter JM (1987) Molecular analysis of the *Nar1* locus in barley. MS Thesis, Washington State Univ, Pullman
119. Jiang J, Friebe B, Gill BS (1994) Recent advances in alien gene transfer in wheat. Euphytica 73:199-212

120. Jones DM, Clothier RB, Griffiths TER (1986) Cereal breeding, barley. In: Annual Rep WPBS, pp74-75
121. Jones DL, Jones DM, Clifford BC, Clothier RB, Cook R, Griffiths TER, Jones EWC, Jones IT (1985) Cereal breeding, barley. In: Annual Rep WPBS, pp65-66
122. Julier C, Nakamura Y, Lathrop M, O'Connell M, Leppert M, Litt M, Mohandas T, Lalouel J-M, White R (1990a) A detailed genetic map of the long arm of chromosome 11. Genomics 7:335-345
123. Julier C, Nakamura Y, Lathrop M, O'Connell M, Leppert M, Mohandas T, Lalouel J-M, White R (1990b) A primary map of 24 loci on human chromosome 16. Genomics 6:419-427
124. Kao KN (1981) Plant formation from barley anther cultures with Ficoll media. Z Pflanzenphysiol 103:437-443
125. Kao KN (1988) *In vivo* culture in barley breeding. In: Semi-dwarf cereal mutants and their use in cross-breeding III. In: Proc Final Res Coordination Meeting, Joint FAO/IAEA Divisions, Rome 1985, pp183-191
126. Karp A (1991) On the current understanding of somaclonal variation. Oxf Surv Plant Mol Cell Biol 7:1-58
127. Karp A, Owen PG, Steele SH, Bebeli PJ, Kaltsiges PJ (1992) Variation in telomeric heterochromatin in somaclones of rye. Genome 35:590-593
128. Kasha KJ (1989) Production of haploids in cereals. In: Maluszynski M (ed) Current Options for Cereal Improvement: Doubled Haploids, Mutants and Heterosis. Kluwer, Dordrecht pp71-80
129. Kasha KJ (1996) Biotechnology and cereal improvement. In: Scoles G, Rossnagel B (eds) Proc 5th Int Oat Conf & 7th Int Barley Genet Symp. University Extension Press, Saskatoon, Sask, pp133-140
130. Kasha KJ, Kao KN (1970) High frequency haploid production in barley *(Hordeum vulgare* L.). Nature 225:874-876
131. Kasha KJ, Procunier JD (1990) RFLP studies in barley. Univ of Guelph Dep of Crop Sci Rep for 1989-90. Ontario Agric Coll, Guelph, pp80
132. Kasha KJ, Reinbergs E (1975) Utilization of haploid in barley. In: Gaul H (ed) Barley Genetics III, Proc 3rd Int Barley Genet Symp, Garching, pp307-315
133. Kasha KJ, Reinbergs E (1982) Recent developments in the production and utilization of haploids in barley. In: Asher MJC, Ellis RP, Hayter AM, Whitehouse RNH (eds) Proc 4th Int Genet Symp. Edinburgh Univ Press, pp655-665
134. Kintzios S, Islam RM, Fischbeck G (1994) Distorted segregation for mildew resistance in doubled haploid lines of spring barley. Plant Breed 112:248-251
135. Kisana NS, Nkongolo KK, Quick JS, Johnson DL (1993) Production of doubled haploids by anther culture and wheat x maize method in a wheat breeding programme. Plant Breed 110:96-102
136. Kilian A, Gale MD (1991) Induction of RFLPs by mutagenesis. Cereal Res Commun 19:119-127
137. Kilian A, Kleinhofs A (1992) Cloning and mapping of telomere-associated sequences from *Hordeum vulgare* L. Mol Gen Genet 235:153-156
138. Kjær B, Jensen HP, Jensen J, Jørgensen JH (1990) Associations between three *ml-o* powdery mildew resistance genes and agronomic traits in barley. Euphytica 46:185-193

139. Kleinhofs A (1994) Barley Steptoe x Morex map. File available *via* Internet Gopher. host greengenes.cit.cornell.edu. port 70, menu "Grains files to browse" "Barley Steptoe x Morex map"
140. Kleinhofs A, Kilian A, Kudrna D, Bollinger J, Hayes P, Chen FQ, Saghai Maroof MA, Lapitan N, Fenwick A, Blake TM, Kanazin V, Ananiev E, Dahleen L, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ, Knapp SJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705-712
141. Knapp SJ, Bridges WC, Birkes Jr, D (1990) Mapping quantitative trait loci using molecular marker linkage maps. Theor Appl Genet 79:583-592
142. Knudsen S, Due IK, Andersen SB (1989) Components of response in barley anther culture. Plant Breed 103:241-246
143. Kotch GP, Peloquin SJ (1987) A new source of haploid germplasm for genetic and breeding research. Am Potato J 64:137-141
144. Kumashiro T, Oinuma T, (1985) Comparison of genetic variability among anther-derived and ovule-derived doubled haploid lines of tobacco. Jpn J Breed 35:301-310
145. Lagercrantz U, Lydiate D (1995) RFLP mapping in *Brassica nigra* indicates different recombination rates in male and female meioses. Genome 38:255-264
146. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181
147. Lange W (1988) Cereal cytogenetics in retrospect. What came true of some cereal cytogeneticists' pipe dreams? Euphytica S:7-25
148. Larkin PJ, Banks PM, Bhati R, Brettell RIS, Davies PA, Ryan SA, Scowcroft WR, Spindler LH, Tanner GJ (1989) From somatic variation to variant plants: mechanisms and applications. Genome 31:705-711
149. Larkin PJ, Ryan SA, Brettell RIS, Scowcroft WR (1984) Heritable somaclonal variation in wheat. Thoer Appl Genet 67:433-455
150. Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197-214
151. 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
152. Lashermes P, Gaillard A, Beckert M (1988) Gynogenetic haploid plants analysis for agronomic and enzymatic markers in maize. (*Zea mays* L.) Theor Appl Genet 76:570-572
153. Laurie DA, Bennett MD (1986) Wheat x maize hybridization. Can J Gent Cytol 28:313-316
154. Laurie DA, Bennett MD (1988) The production of haploid wheat plants from wheat x maize crosses. Theor Appl Genet 76:393-397
155. Laurie DA, O'Donoughue L, Bennett MD (1990) Wheat x maize and other wide sexual hybrids: their potential for genetic manipulation and crop improvement. In: Gustafson JP (ed) Gene manipulation in plant improvement II. Plenum, New York, pp95-126

156. Laurie DA, Reymondie S (1991) High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. Plant Breed 106:182-189
157. Lazar MD, Baenziger PS, Schaeffer GW (1984) Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor Appl Genet 68:131-134
158. Lazar MD, Schaeffer GW, Baenziger PS (1984) Cultivar and cultivar x environment effects on the development of callus and polyhaploid plants from anther cultures of wheat. Theor Appl Genet 67:273-277
159. Lee M, Phillips RL (1988) The chromosomal basis of somaclonal variation. Annu Rev Plant Physiol Plant Mol Bio 39:413-437
160. 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
161. Lindsey K, Topping JF (1993) Embryogenesis: a question of pattern. J Exp Bot 44:359-374
162. Liu BH, Knapp SJ (1990) GMENDEL: a program for Mendelian segregation and linkage analysis of individual or multiple progeny populations using log-likelihood ratios. J Hered 81:407
163. Logue SJ, Oti-Boateng C, Karakousis A, Kretschmer M, Manning S, Lance RCM, Langridge PJ (1995) Segregation analysis of DNA markers in anther culture-derived populations of barley (*Hordeum vulgare* L.). In: Proc 7th Aus Barley Tech Symp. Perth WA, pp210-217
164. Luckett DJ, Darvey NL (1992) Utilization of microspres culture in wheat and barley improvement. Aus J Bot 40:807-828
165. Luckett DJ, Smithard RA (1992) Doubled haploid production by anther culture for australian barley breeding. Aus J Agric Res 43:67-78
166. Lyne RL, Bennett RI, Hunter CP (1986) Embryoid and plant production from cultured barley anthers. In: Alderson PG, Withers LA (eds) Plant tissue culture and its agricultural applications. Univ Nottingham, Butterworth Scientific Ltd, Guildford, pp405-411
167. Maeda T (1939) Chiasma studies in the silk worm, *Bombyx mori* L. Jpn J Genet 15:118-127
168. Marburger JE, Jauhar PP (1989) Agronomic, isozyme, and cytogenetic characteristics of 'Chris' wheat doubled haploids. Plant Breed 103:73-80
169. Marsolais AA, Séguin-Swartz G, Kasha KJ (1984) The influence of anther cold pretreatments and donor plant genotypes on *in vitro* androgenesis in wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ Cult 3:69-79
170. Mayer U, Torres Ruiz RA, Berleth T, Misera S, Jurgens G (1991) Mutations affecting body organization in the *Arabidopsis* embryo. Nature 33:402-407
171. McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815-829
172. Meinke DW (1985) Embryo-lethal mutants of *Arabidopsis thaliana:* analysis of mutants with a wide range of lethal phases. Theor Appl Genet 69:543-552

173. Messeguer R, Ganal MW, Steffens JC, Tanksley SD (1991) Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. Plant Mol Biol 16:753-770
174. Miller TE (1984) The homoeologous relationship between the chromosomes of rye and wheat. Can J Genet Cytol 26:578-589
175. Mix G, Wilson HM, Foroughi-Wehr B (1978) The cytological status of plants of *Hordeum vulgare* L. regenerated from microspore callus. Z Pflanzenzüchtg 80:89-99
176. Moore G, Abbo S, Cheung W, Foote T, Gale MD, Koebner R, Leitch A, Leitch I, Money T, Stancombe P, Yano M, Flavell R (1993) Key features of cereal genome organization as revealed by the use of cytosine-methylation sensitive restriction endonucleases. Genomics 15:472-482
177. Morgan TH (1914) No crossing over in the male of *Drosophila* of genes in the second and third pair of chromosomes. Biol Bull (Woods Hole) 26:195-204
178. Müller E, Brown PTH, Hartke S, Lörz H (1990) DNA variation in tissue-culture-derived rice plants. Theor Appl Genet 80:673-679
179. Müller G, Borschel H, Vahl U, Wiberg A, Hartel H, Damisch W (1989) Die Nutzung der Antherenkulturmethode im Zuchtprozess von Winterweizen. I. Zur Androgenesefähigkeit von *1B-1R* Weizen-Roggen-Tranlokationsformen. Plant Breed 102:196-207
180. Murray M, Cramer J, Ma Y, West D, Romero-Severson J, Pitas J, DeMars S, Vilbrandt L, Kirshman J, McLeester R, Schilz J, Lotzer J (1988) Agrigenetics maize linkage map. Maize Genet Coop Newsl 62:89-91
181. Nakamura Y, Lathrop M, O'Connell P, Leppert M, Barker D, Wright E, Skolnick M, Kandoleon S, Litt M, Lalouel J-M, White R (1989) A mapped set of DNA markers for human chromosome 17. Genomics 2:302-309
182. NIAB (1988) Recommended varieties of cereals, farmers leaflet number 8. National Institute of Agricultural Botany, Cambridge, UK
183. Nilsson N-O, Pelger S (1991) The relationship between natural variation in chiasma frequencies and recombination frequencies in barley. Hereditas 115:121-126
184. Nilsson N-O, Sall T, Bengtsson BO (1993) Chiasma and recombination data in plants: are they compatible? Trends Gene 9:344-348
185. O'Connell P, Lathrop M, Law M, Leppert M, Nakamura Y, Hoff M, Kumlin E, Thomas W, Elsner T, Ballard L, Goodman P, Azen E, Sadler J, Cai G, Lalouel J-M, White R (1987) A primary genetic linkage map for human chromosome 12. Genomics 1:89-102
186. O'Donoughue LS, Bennett MD (1994) Comparative responses of tetraploid wheats pollinated with *Zea mays* L. and *Hordeum bulbosum* L. Theor Appl Genet 87:673-680
187. Olsen FL (1987) Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carlsberg Res Comm 52:393-404
188. Orlov PA, Mavrishcheva EB, Palilova AN (1993) Estimation of the response to anther culturing in 60 genotypes of different wheat species. Plant Breed 111:339-342

189. Oury FX, Pichon M, Rousset M (1993) Une comparaison entre 2 méthodes d'haplodiploïdisation chez le blé tendre: l'androgenèse *in vitro* et le croisement interspécifique avec le maïs. Agronomie 13:95-103
190. Palmgren G, Mattsson O, Okkels FT (1991) Specific levels of DNA methylation in various tissues, cell lines, and cell types of *Daucus carota.* Plant Physiol 95:174-178
191. Payne PI, Holt LM, Hutchinson J, Bennett MD (1984) Development and characterisation of a line of bread wheat, *Triticum aestivum*, which lacks the short-arm satellite of chromosome 1B and the *Gli-B1* locus. Theor Appl Genet 68:327-334
192. Payne PI, Nightingale MA, Krattiger AF, Holt LM (1987) The relationship between HMW glutenin subunit composition and the bread-making quality of british-grown wheat varieties. J Sci Food Agric 40:51-65
193. Pelletier G, Ilami M (1972) Les facteurs de l'androgenèse in vitro chez *Nicotiana tabacum.* Z Pflanzenphysiol 68:97-114
194. Petolino JF, Thompson SA (1987) Genetic analysis of anther culture response in maize. Theor Appl Genet 74:284-286
195. Petolino JF, Jones AM, Thompson SA (1988) Selection for increased anther culture response in maize. Theor Appl Genet 76:157-159
196. Picard E (1984) Contribution à l'étude de l'hérédité et de l'utilisation en sélection de l'haplodiploïdisation par androgenèse *in vitro* chez une céréale autogame: *Triticum aestivum* L. Thèse d'Etat, Université Paris XI, 269p
197 Picard E (1989) The male gamete as a tool in the genetic improvement of cereals. Genome 31:1005-1013
198. Pickering RA (1980a)[1] Use of the doubled haploid technique in barley breeding at the Welsh Plant Breeding Station. Rep Welsh Plant Breed Stn (Aberystwyth, Wales) [1979] pp208-226
199. Pickering RA (1980b)[2] Attempts to overcome partial incompatibility between *Hordeum vulgare* L. and *Hordeum bulbosum* L. Euphytica 29:369-377
200. Pickering RA (1983a) The influence of genotype on doubled haploid barley production. Euphytica 32:863-876
201. Pickering RA (1983b) The location of a gene for incompatibility in crosses between *Hordeum vulgare* L. and *Hordeum bulbosum* L. Heredity 51:455-459
202. Pickering RA (1984) Crossability relationships between certain species in the *Hordeae*. Barley Genet Newsl 14:14-17
203. Pickering RA (1985) Partial control of chromosome eliminaton by temperature in immature embryos of *Hordeum vulgare* L. x *Hordeum bulbosum* L. Euphytica 34:869-874
204. Pickering RA (1989) Plant regeneration and variants from calli derived from immature embryos of diploid barley (*Hordeum vulgare* L.) and *H. vulgare* L. x *H. bulbosum* L. crosses. Theor Appl Genet 78:105-112

[1]références de Devaux, Adamski, Surma (1992), de Lefebvre et Devaux (1996), Devaux, Kilian, Kleinhofs (1993), Zivy, Devaux, Blaisonneau, Jean, Thiellement (1992).

[2]référence de Devaux, Adamski, Surma (1992).

205. Pickering RA, Devaux P (1992) Haploid production: approaches and use in plant breeding. In: P.R. Shewry (ed) Barley: Genetics, Molecular Biology and Biotechnology. CAB Int Publ, Wallingford, UK, pp511-539
206. Pickering RA, Hayes JD (1976) Partial incompatibility in crosses between *Hordeum vulgare* L. and *Hordeum bulbosum* L. Euphytica 25:671-678
207. Pickering RA , Rennie WF (1990) The evaluation of superior *Hordeum bulbosum* L. genotypes for the use in a doubled haploid barley breeding programme. Euphytica 45:251-255
208. Powell W (1988) Diallel analysis of barley anther culture response. Genome 30:152-157
209. Powell W, Asher MJC, Wood W, Hayter AM (1984) The manipulation of mildew resistance genes in a barley breeding programme by the use of doubled haploids. Z Pflanzenzüchtg 93:43-48
210. Powell W, Borrino EM, Allison MJ, Griffiths DW, Asher MJC, Dunwell JM (1986) Genetical analysis of microspore-derived plants of barley (*Hordeum vulgare*). Theor Appl Genet 72:619-626
211. Powell W, Ellis RP, Macauley M, McNicol J, Forster BP (1990) The effect of selection for protein and isozyme loci on quantitative traits in a doubled haploid population of barley. Heredity 65:115-122
212. Powell W, Hayter AM, Wood W, Dunwell JM, Huang B (1984) Variation in the agronomic characters of microspore-derived plants of *Hordeum vulgare* cv. Sabarlis. Heredity 52:19-23
213. Powell JB, Nilan RA (1963) Influence of temperature on crossing over in an inversion heterozygote in barley. Crop Sci 3:11-13
214. Quimio CA, Zapata FJ (1990) Diallel analysis of callus induction and green plant regeneration in rice anther culture. Crop Sci 30:188-192
215. Rayburn AL, Carver BF (1988) Cytological identification of the *1B/1R* wheat-rye translocations in winter wheat breeding lines. Euphytica 38:237-240
216. Reeves RH, Crowley MR, O'hara BF, Gearhart JD (1990) Sex, strain, and species differences affect recombination across an evolutionarily conserved segment of mouse chromosome 16. Genomics 8:141-148
217. 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
218. Robertson DS (1984) Different frequency in the recovery of cross-over products from male and female gametes of plants hypoploid for B-A translocations in maize. Genetics 107:117-134
219. Rode A, Hartmann C, Benslimane A, Picard E, Quetier F (1987) Gametoclonal variation detected in the nuclear ribosomal DNA from double haploid lines of spring wheat *(Triticum aestivum L.,* cv 'Cesar'). Theor Appl Genet 74:31-37
220. Rogers WJ, Payne PI, Harinder K (1989) The HMW glutenin subunit and gliadin compositions of german-grown wheat varieties and their relationship with bread-making quality. Plant Breed 103:89-100
221. Rouyer F, de la Chapelle A, Andersson M, Weissenbach J (1990) An interspersed repeated sequence specific for human subtelomeric regions. EMBO J 9:505-514

222. Saccone S, De Sario A, Della Valle T, Bernardi G (1992) The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. Proc Natl Acad Sci USA 89:4913-4917
223. Sall T, Flink J, Bengtsson BO (1990) Genetic control of recombination in barley. Hereditas 112:157-170
224. Sarrafi A, Amrani N, Alibert G (1994) Haploid regeneration from tetraploid wheat using a maize pollen. Genome 37:176-178
225. SAS (1988) SAS/STAT user's guide, release 6.03 edn. SAS Institute Inc, Cary, NC, USA.
226. 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
227. Scooler AB (1967) A form of male sterility in barley hybrids. J Hered 58:206-211
228. Sharp EL (1985) Breeding for pest resistance. In: Rasmusson DC (ed) Barley. Amer Soc Agron, Madison WI, pp313-333
229. Sharp PJ, Chao S, Desai S, Gale MD (1989) The isolation, characterization and application in the *Triticeae* of a set of wheat RFLP probes identifying each homoeologous chromosome arm. Theor Appl Genet 8:342-348
230. Sheridan WF, Clark JK (1993) Mutational analysis of morphogenesis of the maize embryo. Plant J 3:347-358
231. Shiroishi T, Sagai T, Hanzawa N, Gotoh H, Moriwaki K (1991) Genetic control of sex-dependent meiotic recombination in the major histocompatibiliy complex of the mouse. EMBO J 10:681-686
232. Shugar L, Etienne M (1994) Doubled haploid barley progress report. Barley Newsl 38:105-106
233. Sinha RP, Helgason SB (1969) The action of actinomycin D and diepoxybutane on recombination of two closely linked loci in *Hordeum*. Can J Genet Cytol 11:745-751
234. Simpson E, Snape JW, Finch RA (1980) Variation between *Hordeum bulbosum* L genotypes in their ability to produce haploids of barley, *Hordeum vulgare* L. Z Pflanzenzücht 85:205-211
235. Smulders MJM, Rus-Kortekaas W, Vosman B (1995) Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. Theor Appl Genet 91:1257-1264
236. Snape JW (1981) The use of doubled haploids in plant breeding. In: Broertjes C (ed) Induced variability in plant breeding. Pudoc, Wageningen pp52-58
237. 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
238. Sokal RR, Rolf FJ (1981) Biometry, 2nd edn. WH Freeman and Co. New York
239. Sokoloff A (1964) Sex and crossing over in *Tribolium castaneum*. Genetics 50:491-496
240. Sorvari S (1986) The effect of starch gelatinized nutrient media in barley anther cultures. Ann Agric Fenn 25:249-254
241. Statistix (1992) Analytical software version 4.0. Statistix, St. Paul, Minn.
242. Steel RGD, Torrie JH (1980) Principles and procedures of statistics: a biometrical approach. 2nd edn. McGraw-Hill, New York

243. Steffenson BJ, Jin Y, Rossnagel BG, Rasmussen JB, Kao K (1995) Genetics of multiple disease resistance in a doubled-haploid population of barley. Plant Breed 114:50-54
244. Suenaga K, Nakajima K (1989) Efficient production of haploid wheat (*Triticum aestivum*) through crosses between Japanese wheat and maize (*Zea mays*). Plant Cell Rep 8:263-266
245. Suenaga K, Tamaki M, Nakajima K (1991) Influence of wheat (*Triticum aestivum*) and maize (*Zea mays*) genotypes on haploid wheat production in crosses between wheat and maize. Bull Natl Inst Agrobiol Resour 6:131-142
246. Sunderland N, Huang B (1985) Barley anther culture - the switch of programme and albinism. Hereditas [Suppl] 3:27-40
247. Sved JA, Blackman LM, Gilchrist AS, Engels WR (1991) High levels of recombination induced by homologous P elements in *Drosophila melanogaster.* Mol Gen Genet 225:443-447
248. Thompson DM, Chalmers K, Waugh R, Foster BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants of barley *Hordeum vulgare* L. Theor Appl Genet 81:487-492
249. Thörn E, Jensen CJ (1985) Haploid barley induction and the influence of embryo size and development on plant production. Hereditas 3:152-153
250. 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
251. Turkula TE, Jalal SM (1985) Increased rates of sister chromatid exchanges induced by the herbicide 2,4 D. J Hered 76:213-214
252. Ullrich SE, Edmiston HM, Kleinhofs A, Kudrna DA, Maatougui MEH (1991) Evaluation of somaclonal variation in barley. Cereal Res Comm 19:245-260
253. 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
254. Van Engelen FA, De Vries SC (1992) Extracellular proteins in plant embryogenesis. Trends Genet 8:66-70
255. Vasil V, Redway F, Vasil IK (1990) Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Bio/Technology 8:429-434
256. Watson ID, Callan HG (1963) The form of bivalent chromosomes in newt oocytes at first metaphase of meiosis. Quart J Microsc Sci 104:281-295
257. Wan Y, Rocheford TR, Widholm JM (1992) RFLP analysis to identify putative chromosomal regions involved in the anther culture response and callus formation of maize. Theor Appl Genet 85:360-365
258. Weiffenbach B, Falls K, Bricker A, Hall L, McMahon J, Wasmuth J, Funanage V, Donis-Keller H (1991) A genetic linkage map of human chromosome 5 with 60 RFLP loci. Genomics 10:173-185
259. Weitkamp LR, Guttormsen SA, Costello Leary P (1982) Equine gene mapping: a sex difference in recombination frequency for linkage group II. Animal Blood Groups Biochem Genet 13:305-306

260. Wendel JF, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Dioscorides Press. Portland. Ore pp5-45
261. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531-6535
262. Winzeler H, Schmidt J, Fried PM (1987) Field performance of androgenetic doubled haploid spring wheat lines in comparison with lines selected by the Pedigree system. Plant Breed 99:41-48
263. Yu KF, Deynze AV, Pauls KP (1993) Random amplified polymorphic DNA (RAPD) analysis. In: Glick B, Thompson JE (eds) Methods in plant molecular biology and biotechnology. CRC Press, Boca Raton
264. Zeller FJ, Gunzel G, Fischbeck G, Gerstenkorn P, Weipert D (1982) Veränderungen der Backeigenschaften des Weizens durch die Weizen-Roggen-Chromosomen Tanslokation *1B/1R*. Getr Mehl Brot 36:141-143
265. Zetka MC, Rose AM (1990) Sex-related differences in crossing over in *Caenorhabditis elegans*. Genetics 126:355-363
266. Zhou H (1990) Influence of the physical condition of induction media on the culture reponse of wheat (*Triticum aestivum* L.) anthers. MS Thesis, Washington State Univ, Pullman
267. Ziegler G, Dressler K, Hess D (1990) Investigations on the anther culturability of four German spring wheat cultivars and the influence of light on regeneration of green vs. albino plants. Plant Breed 105:40-46
268. Zivy M (1987) Genetic variability for heat shock proteins in common wheat. Theor Appl Genet 74:209-213
269. Zivy M, Devaux P, Blaisonneau J, Jean R, Thiellement H (1992) Segregation distortion and linkage studies in microspore-derived double haploid lines of *Hordeum vulgare* L. Theor Appl Genet 83:919-924