Application of Biotechnologies to Wheat Breeding

INIA La Estanzuela, Colonia, Uruguay
November, 19-20, 1998

Man Mohan Kohli - Martha Francis
Editors
Application of Biotechnologies to Wheat Breeding
Application of Biotechnologies to Wheat Breeding

INIA La Estanzuela, Colonia, Uruguay
November, 19-20, 1998

Man Mohan Kohli and Martha Francis
Editors

Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT)
Instituto Nacional de Investigación Agropecuaria (INIA)
CIMMYT® (www.cimmyt.cgiar.org) is an internationally funded, nonprofit scientific research and training organization. Headquartered in Mexico, the Center works with agricultural research institutions worldwide to improve the productivity, profitability, and sustainability of maize and wheat systems for poor farmers in developing countries. It is one of 16 food and environmental centers known as the Future Harvest Centers. The centers, located around the world, conduct research in partnership with farmers, scientists and policymakers to help alleviate poverty and increase food security while protecting natural resources. They are principally funded through the early 60 countries, private foundations, and regional and international organizations that make up the Consultative Group on International Agricultural Research (CGIAR), (www.cgiar.org). Financial support for CIMMYT’s research agenda also comes from many other sources, including foundations, development banks, and public and private agencies.

In 1998, the centers supported by the CGIAR created Future Harvest, a charitable and educational organization that reaches out to media, scholars, and scientists in the world’s premier peace, environment, health, population, and development, research organizations, as well as policymakers and civil society. Future Harvest catalyzes action for a world with less poverty, a healthier human family, well-nourished children, and a better environment. It supports research, promotes partnerships, and sponsors on-the-ground projects that bring the results of research to farmers in Africa, Asia and Latin America (see www.futureharvest.org).

The National Agriculture and Livestock Research Institute (INIA) was created by law 16065 dated October 1989 as a requirement to set up a new research structure for developing appropriate technologies to improve agriculture production and permit the most efficient exploitation of Uruguay’s limited land area. INIA is financed by public funds and contributions from farmer’s organizations, in equal proportion. Delegates from public sector and farmer’s organizations govern INIA through a four-member board. INIA is composed of a network of five regional experimental stations. It has strong relationship with other national and international organizations dedicated to agricultural research and technology transfer.

ä International Maize and Wheat Improvement Center (CIMMYT) and National Agriculture and Livestock Research Institute (INIA) 2000. All rights reserved. The opinions expressed in this publication are sole responsibility of the authors. The designations employed in the presentation of material in this publication do not imply the expressions of any opinion whatsoever on the part of CIMMYT, INIA, or contributory organizations concerning the legal status of any country, territory, city, or area, or of its authorities, or concerning the delimitation of its frontiers or boundaries. CIMMYT and INIA encourage fair use of this material. Proper citation is requested.


ISBN: 9974-7586-1-0

AGRIS descriptors: Wheat; Triticum aestivum; Biotechnology; Genetic transformation; Molecular markers; Plant breeding; Research projects; Argentina; Uruguay; Mexico; USA; South America.

AGRIS category codes: F30

Dewey decimal classification: 631.53 MMKa

Printed in Uruguay
Prologue

Application of Biotechnologies to Wheat Breeding

The application of biotechnology to crop improvement has been the focus of much research around the world. Tissue culture and selection of somaclonal variation were developed many years ago, followed more recently by molecular markers and genetic engineering. This meeting focused on providing updates on the actual successes to date on the application of several areas of biotechnology to wheat improvement. What is clear is that there continues to be optimism regarding the potential for biotechnology to be applied to wheat improvement. It is also noteworthy that as was predicted by many scientists, many approaches are proving more difficult in wheat than in other crops.

Molecular markers, for example, are difficult to apply routinely given the large genome, hexaploid genetics, and lower levels of polymorphisms. New marker systems such as AFLPs and microsatellites are an improvement over previous RFLPs, but still require significant investments to achieve similar results possible in other, more amenable species. What is encouraging are the successes to identify the genetics of complex traits in wheat such as quality, Fusarium and rust resistance. Some of these will surely provide useful applications in wheat improvement and all mapping efforts provide critical genetic information for further studies at the gene level. These will be increasingly important as genomic approaches (gene expression, proteomics, etc.) become available and are applied to wheat. In many ways, these latest genomics approaches should be more ‘genome independent’ and thus readily applicable to large, complex genome species such as wheat. Coupled with the relatively easy ability to produce doubled haploids, wheat may prove to be an extremely important species for genomic studies.

In addition to the molecular tools available, wheat has been amenable to genetic engineering. Transformed lines with altered quality and pathogen resistant are available and may provide useful materials for breeding programs. While there will be many possible applications of genetic engineering to wheat improvement, the availability of the high-throughput transformation methods will be extremely valuable for studying gene expression via genomics. An efficient transformation system is critical to fully study and understand the genetic basis of most characteristics, and wheat appears to be a relatively easy species to transform.

Why hasn’t biotechnology been more incorporated into wheat research and breeding programs? The technology-associated issues above provide some of the reasons. Others included the availability of funds, which even in developed countries, are often directed to other, more seemingly, easier species. In addition, private sector investment
has focused more on commercially important crops such as maize, soybeans and cotton. Technologies developed for these species are now being applied in wheat research. Finally, intellectual property, while not limited to wheat, seems to complicate all scientists' lives and research programs. Clearly, intellectual property will not disappear and may not become less complicated for several years. Thus, all scientists and research institutes, both the in developed and developing countries, must learn how to deal with the various issues involved. Often the actual process of IP management and negotiation is much easier than thought, and the real difficulty is deciding to delve into the area.

What may be most critical for future applications of biotechnology is to seriously consider the environment necessary to ensure that the tools of biotechnology can be used. This will require countries and institutions to adopt appropriate regulatory and biosafety procedures that provide required safety assurance without overly complicating the procedures to follow. In addition, many persons, scientists included, are not fully aware of the technologies biotechnology has to offer nor even the science behind them and, thus, are critical from a point of ignorance. Public awareness and education of all stakeholders are a must if those who need the products of biotechnology are to receive them in time, if at all.

I am confident that biotechnology is critical to ensure the continued yield and quality gains in wheat, and that we will see in the near future practical results of the hard efforts outlined in this workshop. CIMMYT remains committed to using all tools available and to helping our partners around the world, especially in developing countries, to understand the possibilities and where appropriate, to apply these in their programs.

Dave Hoisington
Director, Applied Biotechnology Center and Bioinformatics
CIMMYT, Mexico
## Contents

**Use of Biotechnology in Wheat Breeding in the Southern Cone Region** ............. 1  
*Man Mohan Kohli*

**The Application of Comparative Genetics to Wheat Improvement** ................. 17  
*Mark E. Sorrells*

**An Analysis of the Use of Haploidy in Wheat Improvement** ....................... 33  
*A. Mujeeb-Kazi*

**The Application of Molecular Markers in Wheat Improvement at CIMMYT** ......... 49  
*H. M. William, M. Khairallah, L. Ayala, R. P. Singh, A. Mujeeb-Kazi, D. Hoisington*

**Advances in Molecular Markers for Bread Making Quality** .......................... 57  
*Jorge Dubcovsky, G. Tranquilli, D. Lijavetzky; I. A. Khan, A. R. Schlatter,  
M. M. Manifesto, S. Marcucci-Poltri*

**Marker-Assisted Selection of Disease Resistance Genes in Wheat** .................. 71  
*James A. Anderson*

**Microsatellites in Wheat: An Useful Tool for Variety Identification and Breeding** ........................................... 85  
*M. M. Manifesto, A. R. Schlatter, H. E. Hopp, E. Y. Suárez, J. Dubcovsky*

**Maker Assisted Selection: Is it Practical?** ............................................. 103  
*Mark E. Sorrells*

**Routine Transformation System for Use with CIMMYT Wheat Varieties** .......... 111  
*A. Pellegrineschi, S. Fennell, S. McLean, R. M. Brito, L Velázquez, M. Salgado,  
J. J. Olivares, R. Hernández, D. Hoisington*

**Progress and Prospects for Engineering Wheat Quality Characteristics** ...... 121  
*Olin D. Anderson*

**Intellectual Property and Wheat Breeding for the Southern Cone** ............... 135  
*John H. Barton*

**Opportunities for Utilization of Biotechnology in INIA`s Wheat Breeding Program in Uruguay** ................................ 151  
*Martha Francis, Fabián Capdevielle*

**Closing Remarks** ...................................................................................... 163  
*Pedro Bonino*

**List of Participants** .................................................................................. 165
Use of Biotechnology in Wheat Breeding in the Southern Cone Region

Mohan Kohli*

Abstract

Wheat is an important crop for the winter season in the Southern cone region of South America. Given the spread of its cultivation under different ecological and management environments, it makes a critical contribution to the regional economy. Most wheat breeding programs are conventional and have made significant progress in grain yield and other agronomic characters.

The present study reports the responses to a biotech survey among major wheat breeding programs of the region and CIMMYT. The results show a very high level of interest and knowledge among the wheat breeders regarding different biotechnologies and methodologies available for germplasm improvement, but very few of them have been utilized with varying rates of success. The reasons for the lack of adoption are explored. Most believe that application of some technologies such as double-haploids and molecular markers will probably be widespread in wheat improvement programs over the next decade or so. The use of molecular markers will be primarily sought to screen for industrial quality and biotic stress characteristics.

There is visible concern regarding foreseeable reduction in the free exchange of germplasm internationally and its safe keeping in the gene-banks. Several ideas to keep genetic variability flowing among the programs are proposed.

Introduction and wheat production environment

Wheat is the most important winter season crop forming part of different agricultural systems in the Southern Cone region of South America. Seeded primarily in wheat-soybean-wheat or wheat-soybean-maize rotation, the crop is an economic pillar of the regional agriculture. Approximately eight million hectares is planted to the crop annually to produce around 18 million tons of grain in the region (Table 1). From the standpoint of geographic distribution, the crop area spreads from the mesothermic valleys and tropical lowlands in Bolivia to the temperate pre-Andean mountain region in the southern

* CIMMYT, Andes 1365 of. 314, Montevideo, Uruguay. e-mail: cimmyt@inia.org.uy
part of Chile. Within this vast region, wheat is grown on the fertile Pampas in Argentina, and on the acid soils with aluminum toxicity in Brazil. The majority of the crop is grown under the rainfed conditions except for some irrigated areas in the Central Valley of Chile and in the Cerrado region of Brazil (Table 2).

In spite of several abiotic and biotic stresses affecting the crop over this vast area, the average yield of wheat has increased moderately over the last 20 years and the adoption of semi-dwarf germplasm is widespread (Kohli and Rajaram, 1995). Among the abiotic stresses, early drought between tillering and heading stage, high temperatures (in the tropical region) and frost damage (in the south), hot winds during the grain filling period, shattering and pre-harvest sprouting are important yield and quality limiting factors (Kohli, 1984). Biotic stresses include a wide array of diseases and insect pests varying from one part of the region to another. The most important diseases present in the region are the three rusts, the foliar blights caused by

**Table 1. Wheat Production Statistics of the Southern Cone Region*.**

<table>
<thead>
<tr>
<th>Year</th>
<th>Area (000 ha)</th>
<th>Production (000 mt.)</th>
<th>Yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1979-81</td>
<td>9.15</td>
<td>12.07</td>
<td>1319</td>
</tr>
<tr>
<td>1991</td>
<td>10.40</td>
<td>16.76</td>
<td>1611</td>
</tr>
<tr>
<td>1993</td>
<td>7.36</td>
<td>14.61</td>
<td>1955</td>
</tr>
<tr>
<td>1995</td>
<td>6.48</td>
<td>11.83</td>
<td>1827</td>
</tr>
<tr>
<td>1997</td>
<td>8.15</td>
<td>18.45</td>
<td>2263</td>
</tr>
<tr>
<td>1998</td>
<td>7.35</td>
<td>16.36</td>
<td>2226</td>
</tr>
</tbody>
</table>

* Source: FAO files and other estimates.

**Table 2. Wheat megaenvironments (ME) in the Southern Cone region.**

<table>
<thead>
<tr>
<th>Mega-environment *</th>
<th>Character</th>
<th>Countries involved</th>
<th>Estimated area ** (000 ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME 1</td>
<td>Irrigated</td>
<td>Arg, Brz, Chl</td>
<td>250</td>
</tr>
<tr>
<td>ME 2</td>
<td>High rainfall</td>
<td>Arg, Brz, Chl, Parg, Urug</td>
<td>2750</td>
</tr>
<tr>
<td>ME 3</td>
<td>Acid soil</td>
<td>Brz</td>
<td>1700</td>
</tr>
<tr>
<td>ME 4</td>
<td>Low rainfall</td>
<td>Arg, Bol, Brz, Chl, Parg</td>
<td>5500</td>
</tr>
<tr>
<td>ME 5</td>
<td>High temperature</td>
<td>Arg, Bol, Brz, Parg</td>
<td>1800</td>
</tr>
<tr>
<td>ME 8</td>
<td>Facultative wheats</td>
<td>Arg, Chl, Urug</td>
<td>2450</td>
</tr>
</tbody>
</table>

* Megaenvironments defined by CIMMYT.
** Estimated area may represent overlap among different megaenvironments.
Helminthosporium and Septoria spp, Fusarium head blight, bacterial leaf stripe and barley yellow dwarf virus (BYDV). Given that this vast region does not have any geographical barrier except for the Andean Cordillera that separates Chile from the rest, the microorganisms causing diseases and their different forms or virulences spread very rapidly from one country to another. Among insects, various species of aphids are most serious (as vectors of BYDV) all over the region and Russian wheat aphid has been added to the list recently.

To solve the problems mentioned above, there is a long history of successful wheat breeding programs in all the countries. For example, wheat breeding in Uruguay started with the establishment of this station at La Estanzuela in 1914. Several other programs have also contributed to wheat breeding for over 50 years. Besides producing newer varieties to suit local needs, some of the germplasm developed in the region is heavily used to incorporate desirable characters, especially disease resistance, globally. Brazilian variety, Frontana has been used internationally to incorporate characters such as: adult plant resistance to leaf rust, resistance to Fusarium head blight and to pre-harvest sprouting etc. (Singh and Rajaram, 1992).

Early in the 1950s, Dr. Norman Borlaug at CIMMYT's predecessor Office of the Special Studies (OSS) of the Rockefeller Foundation started collaborating with the national wheat breeding programs of the region. CIMMYT's Regional Network of collaboration with the National Institutes and private-breeding enterprises, first based in Chile, started in 1978. The regional presence of CIMMYT not only enhanced earlier collaboration but could also identify specific germplasm needs of different breeding programs (Kohli and Rajaram, 1995). The availability of a larger set of targeted international germplasm, including recently developed lines based on inter-specific and inter-generic crosses and the synthetic wheats have increased the proportion of material being selected and used by the local programs.

Wheat breeding programs of the region have shown an excellent record of releasing improved varieties regularly that have not only helped stabilize production, but also increase it to a moderate extent. Over one hundred new varieties have been released in the region between 1993 and 1998. In terms of adoption, over 60% of the wheat area in the Southern Cone is covered by the varieties released between 1993 and 1996.

Use of biotechnology in wheat breeding

A survey was conducted among the wheat breeders of the region to assess the use and status of biotechnology in each wheat breeding program of Southern Cone region. The objective of the study was to determine the interest and the knowledge of the participants regarding new breeding methodologies (biotechnologies) becoming available and their utilization. It also intended to identify their preferences among
different methodologies, if any, the targeted characters as well as any results achieved so far. For the programs completely based on the conventional methods, the reasons for the lack of use of biotechnology and possible plans for the future were also explored.

Twenty-three of the 27 wheat breeding programs surveyed from the Southern Cone region and two respondents from CIMMYT, Mexico, provided the data presented here. They represent Argentina (10), Bolivia (2), Brazil (7), Chile (2), Paraguay (1), and Uruguay (1) programs. In addition to the aspects explored in the survey, the programs made some excellent observations regarding use of newer technologies and their promotion in the national breeding programs.

The results show that a very large proportion (84%) of the programs surveyed are completely based on conventional breeding methods. Only four programs used some methodologies falling under the category of biotechnology. Varied reasons ranging from the shortage of funds and lack of training or required technical assistance to the need of some concrete results and intellectual property rights were responsible for keeping biotechnology on hold (Table 3). Lack of international collaboration to promote these technologies among the national programs was considered to be a major reason behind their slow or non-adoption by one fourth of the respondents. However, if such collaboration and other facilities were put in place, over 80% of the participating breeders would use it immediately, while 12% of them would rather wait to see some results before committing their time and resources to it.

The advances in the development and use of newer techniques to assist wheat breeding programs have been rapid since mid 1980s (Inagaki and Tahir, 1990; Laurie

<table>
<thead>
<tr>
<th>Program need</th>
<th>% Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funding</td>
<td>56</td>
</tr>
<tr>
<td>Training</td>
<td>52</td>
</tr>
<tr>
<td>Knowledge base</td>
<td>36</td>
</tr>
<tr>
<td>Technical assistance</td>
<td>32</td>
</tr>
<tr>
<td>Visualization of concrete results</td>
<td>28</td>
</tr>
<tr>
<td>Institutional collaboration national/international</td>
<td>25</td>
</tr>
<tr>
<td>Availability of technology</td>
<td>24</td>
</tr>
<tr>
<td>Intellectual property right issues</td>
<td>12</td>
</tr>
</tbody>
</table>
and Bennett, 1986; Laurie and Reymondie, 1991; Payne et al., 1987, 1988; Snape, 1989). However, being a polyploid specie and with low level of polymorphism, it has remained relatively difficult crop for the biotechnologists around the world (Snape, 1996; Sorrells, 1996). This situation seems to be changing rapidly for the past five years or so and new reports indicate the applicability of biotechnology to wheat as to any other crop (Anderson et al., 1993; Banks et al., 1994; Korzun et al., 1997; Ma et al., 1995). Several major breeding efforts in Australia, Europe and North America have been presenting preliminary successes with the use of specific techniques towards targeted objectives (Barcelo et al., 1998; Chee et al., 1998; Gianibelli et al., 1998).

Use of haploidy

While most the wheat breeders are aware of these developments and agree that their programs need to be adapted to incorporate these technologies, their priorities regarding importance of different techniques vary significantly. Highest number (76 %) of respondents favored immediate use of techniques involved with haploidy in wheat to fix genetic variability in a hybrid population in the early generations (Table 4).

Several programs use the development of doubled haploids as complementary to their conventional breeding efforts. The use of anther culture by National Wheat Center, EMBRAPA, Brazil, produced very positive results leading to the release of BR 43 as a commercial variety. However, many breeders are encouraged with the results achieved through maize mediated crosses (Inagaki and Tahir, 1990; Inagaki and Mujeeb Kazi, 1995; Kisana et al., 1993; Laurie and Bennett, 1986; Laurie and Reymondie, 1991; Snape, 1989) and the use of microspores to develop doubled haploids. Yet, given the limitations mentioned earlier, only a few programs are utilizing this technique or have achieved any significant results (Table 5).

<table>
<thead>
<tr>
<th>Methodology involved</th>
<th>Are Interested</th>
<th>Are Knowledgeable</th>
<th>Have Utilized</th>
<th>Have Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development of Doubled haploids</td>
<td>76</td>
<td>68</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Marker assisted selection</td>
<td>52</td>
<td>44</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Genetic transformation</td>
<td>48</td>
<td>40</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Use of existing genetic data</td>
<td>48</td>
<td>32</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>
Marker assisted selection

Fifty percent of the responding breeders showed interest in the methodologies involved with the marker-assisted selection, MAS (Table 4). While most programs are familiar with the relationship between High Molecular Weight Glutenins (HMWG) and industrial quality characters (Payne et al., 1987, 1988), only a few are generating information on their germplasm or using the available data to combine positive bands through hybridization. Responding programs had little information or experience with the use of the markers associated with Low Molecular Weight Glutenins (LMWG) or Gliadins and their effect on industrial quality (Devos et al., 1995; Metacovsky et al., 1990, and Röder et al., 1995).

Recently there is some effort in Argentina to identify and utilize molecular markers related with the industrial quality characters such as grain hardness and protein content, etc. (Giroux and Morris, 1998; Joppa et al., 1997; Sourdille et al., 1996 and Stephenson et al., 1998). At the same time EMBRAPA, Brazil, in collaboration with University of Manitoba, Canada, is working to identify markers associated with adult plant resistance to leaf rust in wheat. The work associated with resistance to leaf rust, being pursued by Dr. Manilal William and his team at CIMMYT, Mexico, (Khairallah et al., 1998 and William et al., 1997) as well as other activities related with MAS will be presented later at this workshop.

Development of genetic populations with regard to a desired character based on parental identification is one of the pre-requisites to use marker technology in a breeding program. Only 36% of the respondents, including CIMMYT, were adapting their programs for an eventual use of MAS, while the other 60% were not doing so. Of the programs interested in MAS, only 20% were generating segregating populations of their own while another 4% were acquiring these from an international collaborator.

Among different molecular marker technologies, respondents were more interested and knowledgeable about RAPDs, RFLPs, and Micro-satellites than about AFLPs, probably because of their recent development (Table 5). CIMMYT, Mexico and INTA, Castelar, Argentina, were the only institutions to have tried using these techniques and have achieved some success.

In terms of characters most prioritized for screening with markers, disease resistance, better industrial quality and resistance to abiotic factors ranked high (Table 6). Almost half of the respondents were interested to use MAS for improving grain yield, however, most believed that complex (quantitative) characters such as this, under the control of many genes with minor (and probably additive) effects, were not likely to be targeted by this technology for some time to come. Now that most countries in the region have variety protection laws in place, the immediate applicability of the marker technology was seen in the field of varietal identification or passport development (to be presented
**Table 5.** Percent of responding wheat breeders interested and knowledgeable in specific methodologies and those having utilized them with some results.

<table>
<thead>
<tr>
<th>Methodology involved</th>
<th>Are Interested</th>
<th>Knowledgeable</th>
<th>Have Utilized</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Development of Doubled haploids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anther culture</td>
<td>52</td>
<td>52</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Barley mediated</td>
<td>16</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maize mediated</td>
<td>72</td>
<td>68</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Microspores</td>
<td>24</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Marker assisted selection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAPD</td>
<td>56</td>
<td>48</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>RFLP</td>
<td>56</td>
<td>44</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>AFLP</td>
<td>44</td>
<td>36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Microsatellite</td>
<td>56</td>
<td>48</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>Genetic manipulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct transformation</td>
<td>48</td>
<td>40</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Agrobacterium mediated</td>
<td>32</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 6.** Priority of different agronomic characters to be evaluated using molecular marker assisted selection technology and some results achieved.

<table>
<thead>
<tr>
<th>Agronomic characters</th>
<th>Percent wheat breeding programs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>interested</td>
</tr>
<tr>
<td>Grain yield improvement</td>
<td>56</td>
</tr>
<tr>
<td>Improvement in industrial quality</td>
<td>76</td>
</tr>
<tr>
<td>Improvement in nutritional quality</td>
<td>52</td>
</tr>
<tr>
<td>Disease resistance</td>
<td>76</td>
</tr>
<tr>
<td>Insect resistance</td>
<td>52</td>
</tr>
<tr>
<td>Resistance to abiotic stresses</td>
<td>68</td>
</tr>
<tr>
<td>Resistance to mineral deficiency/toxicity</td>
<td>36</td>
</tr>
<tr>
<td>Varietal identification</td>
<td>56</td>
</tr>
</tbody>
</table>
by Marcela Manifiesto at this workshop). Depending on the availability of markers and relative labor and cost involved with their utilization, better disease resistance and industrial and nutritional quality were considered next priorities.

The programs using molecular markers for screening characters mentioned earlier have achieved moderate success in developing populations for improvements in industrial quality and disease resistance. In general, the respondents believed that increasing availability and abundance of microsatellites markers would promote their utilization in wheat to produce quicker results than RAPD, RFLP or AFLP markers.

*Genetic transformation*

The application of genetic manipulation or transformation technology to wheat was found to be of least interest among the participants (Tables 4 and 5). Only two respondents, including CIMMYT, had used genetic transformation in wheat and Dr. Alessandro Pellegrineschi (CIMMYT) will report some initial results at this workshop. In spite of the recent successes reported by the USDA scientists (Olin Anderson's presentation at this workshop) and others in developing transgenic wheats (Alpeter *et al.*, 1996; Barcelo *et al.*, 1998; Becker *et al.*, 1994; Fennel *et al.*, 1996; Hoisington *et al.*, 1998; and Vasil *et al.*, 1992 and 1993), only 12% of the respondents from the region consider doing any genetic engineering in short term. Most favor vigorous international collaboration to spread results generating from genetic transformation of wheat.

Direct gene transfer using particle bombardment (biolistics or gene gun) is considered an easier methodology to adopt in developing transgenic wheats (Table 5). There was little knowledge about *Agrobacterium tumefaciens* mediated gene transfer and none of the participants had utilized it. No other technologies for direct gene transfer such as microinjection or electroporation etc. was mentioned or reported by the respondents.

*Use of molecular maps*

There was significant interest among the participants to utilize genetic maps being developed around the world (Gale *et al.*, 1995; Joppa *et al.*, 1997; Khairallah *et al.*, 1998; Nelson *et al.*, 1995a, b, c; Röder *et al.*, 1998 and Van Deynze, 1995), but very few had knowledge regarding their existence or usefulness. It was also unclear as to how such genetic data could be utilized to help wheat breeders select better varieties.
Utilization of biotechnology in wheat breeding: Concerns and Possibilities

In spite of such overwhelming gap in the knowledge and utilization of newer processes or methodologies available, over two thirds of the participants believe that some biotechnology will be in general use in wheat development programs within the next ten years or so. While almost 30% the respondents believed that wheat breeding will incorporate molecular techniques globally in less than 5 years, another 12% believe that it to take almost 25 years before these techniques will be in general use.

Future restriction on the free exchange of germplasm developed through MAS or transgenics came out to be an issue of major concern to the participants. As discussed earlier, this region has maintained a strong history in international collaboration for wheat germplasm development. Almost 75% of respondents believed that the property rights issues attached with the new techniques will restrict the sharing of such germplasm freely. However, 16% of the respondents believed that germplasm exchange would probably increase due to newer and targeted nature of the modified genetic pools or advanced lines.

In order to maintain a dynamic and equitable sharing of germplasm among everyone, the participants of the survey believed that:

1. Internal capacity of all National Agricultural Research Systems (NARS) to use biotechnology must be increased.
2. Wheat programs of the NARS in the region must be integrated among themselves and with CIMMYT regionally and internationally.
3. Protocols for institutional agreements, especially with Advanced Research Centers, need to be developed.
4. Joint venture programs among public research institutions (INIAs), the private sector and the universities of the region should be enhanced.
5. International associations to exchange biotechnologically modified germplasm should be created.
6. Legal frameworks for the exchange of genetically modified germplasm must be worked out.
7. Public and political awareness campaigns should be started highlighting the value of improved germplasm and the future demands and goals of national and international agriculture.
8. An open policy and forum to discuss the pros and cons of the newer technologies including cost benefit relationships and real or perceived threats generated by genetically modified varieties should be put in place.

Conclusions

To summarize the results of the survey, it is evident that all wheat breeders in the region are highly interested in future development of the new tools and methodologies grouped as biotechnology. There is moderate knowledge among the group regarding what is available for wheat improvement and the progress being made by the Advanced Research Centers (ARCs) and International Centers (IARCs). However, very few participants are actively using or planning to use biotechnology as a tool to assist wheat breeding programs in the region and except for the release of BR 43 mentioned earlier, no other results have been achieved.

These results provide a challenging opportunity for developing a training program for the wheat improvement scientists of the region, as well as tremendous potential for collaboration among NARS, ARCs and IARCs such as CIMMYT. Further, there is an urgent need to streamline the development of protocols to insure a rapid and equitable transfer of these technologies as well as modified germplasm to the national programs in order to promote the adoption of biotechnology in the region.

The results presented at this workshop and the discussions that follow will hopefully bring a new level of awareness regarding the status and availability of successful biotechnologies in wheat. Besides enhancing the possibilities for regional and international collaboration, we believe that the discussions here will serve to identify the research and resource requirements that the region must commit to incorporate the newer technologies in wheat breeding. It is an essential aspect to gain further efficiency in obtaining research results, especially under the reduced funding scenarios.

The Southern Cone of South America represents one of the largest under explored regions of the world with possibilities to increase area under crop production. The increase in world population and demand for food production will need to be met by regions such as this. In terms of global food and feed consumption wheat has become the number one commodity during the last few years. The recent data from IFPRI also identify wheat to be the largest component of cereal imports by developing countries in the year 2020, approaching approximately 150 million tons. While such scenario demands an urgent reaction or plan for solutions, the task is being further complicated by the stagnation of yields in some of the critical wheat producing regions of the world. In spite of the utilization of modern crop management techniques, including higher input levels, the yield advances are slower during the last 10 years in many countries including this region. This urgency imposes a higher level of responsibility on all wheat scientists to find efficient methods in order to give a renewed boost to the global wheat production capacity.
Under such circumstances, it is only conceivable that wheat breeders of this region will need to use biotechnology as a tool to gain efficiency in the development of superior varieties both higher in yield potential and better in industrial/nutritional quality. In their fight against globally lowering levels of per capita food production, they only hope that biotechnology is just not another trend or unfulfilled promise.

Acknowledgements

I would like to express my appreciation to all colleagues, wheat breeders of the region and CIMMYT, for their valuable time to fill the survey and share their results and views on the applications of biotechnology to wheat breeding.

References


Estado de Utilización de Biotecnología en el Mejoramiento de Trigo en la Región Cono Sur

El trigo es un cultivo importante durante el ciclo del invierno en la región Cono Sur de Sudamérica. Dada la extensión de su siembra bajo diferentes sistemas de manejo y ambientes ecológicos, ésta hace una contribución crítica a la economía regional. La mayoría de los programas de mejoramiento de trigo se consideran convencionales y han hecho un progreso significativo en la producción de granos y otros caracteres agronómicos.

El presente estudio, analiza las respuestas a un sondeo biotecnológico entre los principales programas de mejoramiento de trigo de la región y el CIMMYT. Los resultados muestran un nivel muy alto de interés y conocimiento entre los mejoradores de trigo en lo que se refiere a diferentes biotecnologías y metodologías disponibles para el mejoramiento de germoplasma, pero muy pocos de ellos han sido utilizados con variados índices de éxito. Se discuten las razones para tal carencia de adopción. La mayoría cree que la aplicación de algunas tecnologías tales como doble-haploides y marcadores moleculares será probablemente extendido en los programas de mejoramiento de trigo en la próxima década o más. Los marcadores moleculares serán principalmente usados para evaluar las características de calidad industrial y de estrés biótico.

Existe una preocupación visible en lo que se refiere a una reducción posible en el intercambio libre de germoplasma internacionalmente y su mantenimiento en los bancos de genes. Algunas propuestas concretas para mantener la fluidez de la variabilidad son analizadas.
The Application of Comparative Genetics to Wheat Improvement

Mark E. Sorrells*

Abstract

Comparative genetics is a broad field of research with the general goal of estimating similarity at some specific level of organization. The evolution of comparative genetics research from the whole plant level to the DNA level has synergistically expanded our knowledge of genome structure and function due to the complementarity of research among scientists working on different species. In this review, I will present a brief overview of comparative genetics with examples from the Gramineae family that illustrate future directions of comparative genetics research. This presentation will focus on the role of genomic research in crop improvement especially for species with large polyploid genomes and minor crops. The mapping of loci controlling economic traits, trait dissection, and candidate gene analysis will be presented along with examples. The long-term goal is to facilitate the identification of superior alleles for genes of economic importance so that they can be assembled in superior crop cultivars.

Introduction

Comparative genetics is a broad field of research with the general goal of estimating similarity at some level of organization. The structure or patterns of relationships discovered can then lead to new knowledge, hypotheses, and predictions about those species. The evolution of comparative genetics research from the whole plant level to the DNA level has synergistically expanded our knowledge of genome structure and function due to the complementarity of research among scientists working on different species. There has been a rapidly growing interest in comparative genomics over the past couple of years for several reasons, three of which are discussed below.

Industrialization of crop improvement

The rapid industrialization of crop improvement that we are witnessing today is based on intellectual and material property rights, the efficiencies gained through consolidation
of high cost operations, and the vertical integration for unique plant products. This is having a dramatic impact on relationships between public and private researchers.

**Changing technologies, methods and goals**

There has been an increased emphasis on the use of model species for genomics research, largely due to the ability to make rapid advances in the discovery of genes and their function. New technologies and methods are being discovered daily and are changing the way we think about crop improvement. New marker systems as well as unique traits now accessible through transformation are becoming available. Finally, the scenario of gene discovery through QTL analysis followed by determination of gene function and assessment of allelic value is approaching reality.

**Integration of information from disparate sources**

Databases are making information more available, especially for germplasm, genes, and maps and they are presenting the information in ways that facilitate interpretation. One of the most exciting prospects is the integration of information about metabolic pathways and phenotypes. Comparative genetics is essential for facilitating the transfer of information across species. Ultimately, linking gene to phenotype is the goal. Figure 1 illustrates the integration of various sources of information that allow identifying the genes controlling a trait of interest and eventually understanding their function. Given a trait of interest, it is necessary to learn how many important genes control the trait and where they are located in the genome. QTL mapping is still the most common approach to acquiring that information. Once the approximate location of the genes is known, it will be important to learn their function. Some knowledge about the metabolic pathway that might be involved will allow one to select a subset of candidate genes that have been previously located to that region of the genome. These may be cloned and characterized genes or expressed sequence tags (ESTs) that have been assigned a putative function. Supporting information about the candidate genes can be gained if the information about gene expression can be obtained regarding tissue or developmental specificity. Finally, once there is ample evidence for the role of a particular gene, the final but most important step is to characterize allelic variation in the gene. Once the superior alleles are identified, they can be used for variety improvement.

**Goals of comparative genetics**

There are three primary goals of comparative genomics. The first is to build on information from model species and simpler organisms. Secondly comparative genetics facilitates the integration of information on gene location and expression across species and disciplines. Finally, the primary beneficiaries of comparative genetics are the
The Application of Comparative Genetics to Wheat Improvement

Fig. 1. Use of genomics in integration of information.

genomically challenged, the economically disadvantaged, and the technologically deficient crop species.

Levels of organization

Comparative scientific analyses can be performed on virtually any characteristic or level of organization. The results and interpretations of a study depend on the character, level, and methods employed and may differ from study to study. Also some entities can be compared in different ways. For example, comparisons of genes can be based on DNA sequence, position in a phylogenetic tree, chromosome location, function, gene product interactions, substrate specificity, or physiological role.

Classification of genes and proteins

The universal system of classification of living organisms introduced by Linnaeus effectively organized the complexity of biological relationships and provided scientists with a valuable framework for the study of plant evolution and genetic relationships.
Variation in a wide range of different traits from obscure plant structures to biochemical pathways have been evaluated and compared across species. This paradigm has been extended to genes and proteins, and their families, subfamilies and superfamilies to create a molecular taxonomy (e.g. Henikoff et al., 1997). Ancestral duplications and rearrangements of genes or parts of genes has lead to a complex evolution of gene family relationships and genome structure. The understanding of how these complex patterns of gene evolution resulted in the diversity of living organisms we see today will be among our most important scientific accomplishments.

Role of evolutionary genetics

Comparative genetics will continue to evolve as new technologies, methods, and information become available. Current and future research will emphasize comparisons of genes and genomes across species and genera using sequence and map-based tools that will utilize evolutionary continuities among organisms at both the structural and functional levels. Genomic research is rapidly evolving from a descriptive science to predictive science where we can predict gene or protein function and either modify or reverse engineer those genes for transfer into elite varieties.

In this review, a brief overview of comparative genetics with examples from the Gramineae family that illustrate future directions of research in this field is presented. The discussion will focus on the role of genomic research in crop improvement especially for species with large polyploid genomes and minor crops. The long-term goal is to facilitate the identification of superior alleles for genes of economic importance so that they can be assembled in superior crop cultivars.

Benefits of comparative mapping

Recent advances in molecular genetics have enabled a more complete understanding of the genomic structures of the Triticeae, rice, and maize. This has encouraged researchers to expand their vision of what might be possible if we examine species further out on the evolutionary tree. Comparative maps allow transfer of information from species with small diploid genomes, such as rice, to species with more complex genomic structures (increased repetitive DNA, polyploidy) and less economic support. Because of the size and complexity of the genomes, it is scientifically inappropriate to sequence the entire genomes of wheat, rye, oat, or barley. However, alternative strategies involving identification of gene-rich regions of the Triticeae genome and comparison of the genome structure and genetic colinearity with rice, maize, sorghum, and other species provide Triticeae researchers with the knowledge and tools necessary for genetic parity with simpler genomes.
Gramineae maps

Crop species of the Poaceae display a remarkable level of genetic similarity despite their evolutionary divergence 65 million years ago (Bennetzen and Freeling, 1993; Paterson et al., 1995). Molecular markers have been used to develop comparative chromosome maps for several members of the Gramineae and these have been used to study genes of agronomic importance across species (for review see Snape and Laurie, 1998). Large segments of the genomes of maize, sorghum, rice, wheat, and barley conserve gene content and order (Ahn and Tanksley, 1993; Ahn et al., 1993; Gale and Devos 1998; Hulbert et al., 1990; Kurata et al., 1994; Van Deynze et al., 1995a,b,c), although the correspondence has been modified by chromosome duplications, inversions, and translocations. For the domesticated grasses, the conserved linkage blocks and their relationships with rice linkage groups provides the insight into the basic organization of the ancestral grass genome (Moore et al., 1995; Wilson et al, 1998).

In a recent paper due to appear in Genetics, a higher resolution Rice/Maize comparative map that details more than 20 arrangements has been described. This was a collaborative project with Mike Lee at Iowa State and Susan McCouch. In comparative mapping studies involving maize, the tetraploid nature of the genome is readily apparent. The rearrangements included telosomic fusions, intrachromosomal inversions, and non-reciprocal translocations. An example of this is the comparison of Rice 4 with Maize 2 and 10. These rearrangements and the homeoelodies with related species lead us to propose that the maize genome progenitor was probably composed of 8 chromosomes rather than the 5 or 10 proposed earlier.

Future genomics research – Where is it leading us?

Comparative genomics research falls into three major categories:

1) Gene sequence and genome structural relationships for integrating genomic information across species.

2) Characterization of the function of all genes that affect agronomic performance and quality.

3) Bioinformatics - Integration, visualization, and analysis of complex data.

The large volumes of information from genome sequencing and gene expression studies now requires far more sophisticated computational tools for display, analysis and integration.
Genome-wide expressed gene sequencing

Although there are undoubtedly fascinating mysteries locked in the ubiquitous repeated DNA sequences of large genome species (LGS), for the near future, the primary focus will be on coding regions in the large genome species. Expressed sequence tag (EST) analysis was first proposed in 1991 by The Institute for Genomic Research (TIGR) for efficiently sampling a genome for information about genes that could be used to search existing databases (Adams et al., 1991). From those searches, one can determine if a specific gene (or gene motif) has been found in the same or other organisms and if its function has been determined. These ESTs can also be useful for further laboratory work in understanding organism function, mapping, and identification of traits, and direct alteration of the organism.

A major portion of both the Human Genome Project and the Arabidopsis Genome Research Project have focused on the isolation, DNA sequence determination, and analysis of ESTs. It is now one of the primary activities with over 25,000 Arabidopsis sequences (Newman et al., 1994; Cooke et al., 1996; Rounsley et al., 1996). They estimated that more than 70% of the estimated 20,000 expressed genes have been identified. However, where large contiguous stretches of the genome have been sequenced, previously isolated ESTs only account for about 35% of the genes identified (M. Bevan, presentation at Cornell, 6/98). This indicates that whole genome sequencing reveals a large number of genes not found among ESTs. Eventually information will be merged with that of a protein database to provide complementary information on patterns of gene expression. Over all sequence databases, there are more than 200,000 sequences from more than 26 different organisms (Cooke et al., 1996), and this number will continue to grow. For the long term, EST information will be a critical resource for crop improvement and will be used extensively for locating genes, understanding changing patterns of gene expression, and biotechnological modifications of traits.

Identifying and mapping all the expressed genes in a species without sequencing the entire genome is a difficult task. This is evidenced by the large cDNA sequencing efforts of the human genome and Arabidopsis genome projects in which commonly expressed genes are sequenced many times but it is difficult to obtain rare sequences. These sequences may be rare or absent either because they were not expressed under the conditions in which the organisms were grown for library construction or because they were not expressed in the tissues sampled at the particular stage of development. Several strategies have been devised to minimize re-sequencing these commonly expressed genes and specific groups of genes can be targeted with differential expression techniques.
Comparative mapping by sequence matching

Southern hybridization using anchor probes has been the method of choice for evaluation of relationships among species and genera especially for comparative mapping (Van Deynze et al., 1998). This is because other molecular methods such as PCR-based fragment amplification may be an all or none reaction (dominant) and may amplify non-orthologous loci or inadequately sample sequence variation. Also, fidelity of PCR replication can pose problems. New methods of enhancing comparative map information are needed that take advantage of existing information in the literature and genome databases. One alternative is to sequence previously mapped probes or generate new ESTs for the LGS, map them in the LGS, and then cross-reference them by sequence matching using BLAST searches against the model species. The information generated can be used to develop high-density comparative maps for genetic linkage and physical distance between genes as well as evolutionary information about the genes and their phenotypes in the two species. The utility of this approach is assessed below.

Automated sequence comparisons based on sequence alignments are among the most popular queries in biological research. The BLAST Internet servers currently execute more 8000 sequence queries per day. Another approach that utilizes anonymous or partial gene sequences known as expressed sequence tags (ESTs) is cross-species sequence matching. Recently, Lazo et al. (1998) reported on DNA sequence analyses and graphical displays of oat and barley cDNA clones some of which were mapped in rice, maize, oat, sugarcane, and Triticeae species. In their BLAST searches a large number of oat and barley cDNA sequences matched rice EST sequences which had been mapped by the Rice Genome Program in Japan. Recently, an effort was made to compare the rice chromosome locations of the oat and barley cDNAs that matched the mapped rice ESTs according to BLAST searches (Table 1).

Nine of the oat or barley probes were both mapped in rice by Southern analysis and matched a rice EST that was mapped by Southern analysis. Out of those nine matches, five of the rice chromosome locations were similar or identical for the cDNA and the EST while the other four mapped to different chromosomes. Then we used our comparative maps for maize, barley and wheat to predict the map location of those clones as well as other oat and barley cDNA-probed loci in rice. Six matching rice ESTs were available for maize and 18 were available for barley and wheat. In maize, four out of six matched the predicted location and for wheat/barley, 12 out of 18 matched the predicted location. These results suggest that it is possible to identify homologous sequences and their map location by comparing anonymous cDNA sequences. Furthermore, it may be possible to resolve gene order relationships in gene rich regions. Most importantly, sequence matching can supplement the costly data provided by Southern analysis and greatly enhance the resolution of comparative maps required for information transfer between species.
Two primary limitations to using sequence matching are that 1) different genes are known to evolve at different rates and 2) the accuracy and sensitivity of the sequence comparisons decline in more distantly related species. However, it may be possible to estimate the rate of evolution of most any gene by comparing divergence with taxonomic relationship, especially for tribes with well-defined taxonomic relationships. Intragenomic duplications may be resolvable by comparing adjacent markers. Ultimately, it is anticipated that classification of all plant genes into gene families based on conserved sequences or motifs will provide a common language for predicting gene function(s) (Bork and Koonin, 1996).

**Microcolinearity**

In recent years, comparative mapping at the genome level has a been a major focus, however, current and future efforts will become increasingly directed at the megabase level for the purpose of evaluating micro-colinearity and interspecies gene cloning.
Interspecies gene cloning for a LGS utilizes the predicted colinearity between genomes and the low ratio of physical to genetic distance between genes in a model species such as rice to facilitate the isolation of the gene of interest. Once the gene is isolated in rice it can then be used as a probe to isolate the gene in a cDNA library from the LGS. Even if a homologue of the gene is not identified in rice, additional genes that may be closer to the target gene can be used for fine mapping in the LGS.

In the grasses, micro-colinearity of coding regions has been shown to be largely conserved over short regions for rice, maize and sorghum (Chen et al., 1997) and for rice and barley (Killian, et al., 1995; Dunford et al., 1995). The primary limitation for several species is proving the identity of the gene. Correlation of phenotypic variation with variation in the gene is regarded as circumstantial evidence due to the possibility of tight genetic linkage. Transformation of genotypes that will display the effect of the isolated gene is apparently the only method for proving that a functional entity of the target gene has been cloned.

Species that have large portions of the genome sequenced will provide a useful template for sequence matching genes between related species. Mapped clones and ESTs can be used to cross-reference large insert libraries in the respective species. This can be very useful for locating genes and gene-rich regions in the LGS, for generating additional probes for fine mapping, and for reducing the ambiguity in the alignment of large insert clones for physical mapping in the LGS.

**Functional genomics**

Functional genomics (the genome-wide analysis of gene function) is now receiving greater emphasis because of the realization that we do not know the function of the majority of the genes being isolated, and their function is not trivial to discern. Furthermore, for those genes with a known or hypothesized function, there is often knowledge of the natural allelic variation for the gene product, its regulation or its interaction with other genes. For example, it is important to know when functional variation in a particular enzyme has a higher order effect on a plant phenotype such as height or grain yield.

There are a wide variety of experimental approaches addressing the assignment of functions to genes such as transposon mutagenesis, differential gene expression, mutant phenotypes, quantitative trait locus mapping, transformation, candidate gene analysis, and modeling of protein structure and function. Most of these techniques are most efficient or can only be used with model organisms that have small, diploid genomes. In the plant world, Arabidopsis, rice, tomato, brassica and maize have received the most attention.
Gene expression patterns

Microarrays or “DNA-chips” are new technologies that are revolutionizing many areas of plant biology. DNA is fixed to a solid surface (glass or a membrane) and hybridized with a fluorochrome-labeled nucleic acid probe. The degree of hybridization to each DNA sequence is a measure of the amount of probe complementary to the immobilized DNA. Some potential uses include the ability to search for clones directly or indirectly that are related to major-gene differences, mutations, QTLs, and for genes showing changes in gene expression during a developmental time-course. In grain crops, time-course studies of seed development, gene expression during meiosis, and responses to specific environmental stimuli will identify expressed genes. This will assign function to ESTs that will serve as potential candidates for mapped qualitative or quantitative loci affecting important traits. Characterization of the expression patterns of genes involved in genotype x environment interaction may eventually help unravel the complexities of this phenomenon.

Cataloging allelic variation

Sometimes gene functions can be deduced by comparing the phenotypes of individuals with different alleles. Characterization of allelic variation provides the needed information for determining relative value of alleles, the fundamental basis for crop improvement. A catalog of common and rare allelic variants for a few of the economically important genes would provide plant breeders with an invaluable resource for evaluating and pyramiding useful alleles into their elite varieties and lines. Such a project would require only modest resources for the genes that are already cloned.

Contributions from large genome species

Comparative genetics information is critical to those crop species that might otherwise be orphaned in the genomics revolution because of large, complex genomes or less economic importance. Although it is unlikely that the genomes of polyploid crop species such as wheat, oat, cotton, and others will be sequenced in their entirety, strategies are emerging that will allow the identification of virtually all expressed genes in these species. Polyploid crop species are likely to be the primary beneficiaries of comparative genetics research; however, this is not to imply that polyploids cannot contribute to the general knowledge of gene function. A wealth of genetic stocks, the ability to produce true-breeding mapping populations via haploidy and aneuploidy, large-scale development of recombinant inbred populations facilitated by naturally high levels of self-pollination in some species, the potential for development of an endless variety of homozygous sub-chromosome deletion stocks and the ability to dissect alien genomes are a few examples of many assets of polyploid species for gene discovery and characterization.
The Application of Comparative Genetics to Wheat Improvement

Quantitative trait locus mapping

Even with detailed comparative maps, the potential application of new molecular technologies, especially for LGSs will not be realized unless there is adequate species-specific data that identifies the genes controlling quantitative traits.

A quantitative trait locus (QTL) is the location of a gene that affects a trait that is measured on a quantitative scale. Examples of quantitative traits include plant height, grain yield and grain quality. These traits are typically affected by more than one gene, and also by the environment. Thus, mapping QTL is not as simple as mapping a single gene that affects a qualitative trait (such as flower color). Molecular marker technologies have allowed the development of saturated linkage maps to initiate the dissection of quantitative traits. A major objective for QTL detection is to manipulate the underlying determinants in an applied breeding program. Paterson et al. (1991) and Dudley (1993) provided excellent overviews of the potential applications of these techniques to breeding.

The QTL for a range of traits - yield, malting quality, winter hardiness, disease resistance - have been located in a number of barley germplasm sources (reviewed by Hayes et al., 1996). The genetic bases of these QTL, are of great theoretical interest and practical importance. Robertson (1989) proposed that QTL are actually the result of loci where the alleles in question have a smaller differential effect on the phenotype than when mapped as a qualitative trait. Relatively few QTL are often detected for complex phenotypes, such as yield, components of malting quality, and quantitative resistance to biotic and abiotic stresses (reviewed by Hayes et al., 1996). For some traits, candidate genes can be proposed as QTL determinants; e.g., shattering resistance as a determinant of yield (Hayes et al., 1993).

Comparative QTL maps

Comparative QTL analysis has shown that convergent domestication of the grasses has resulted from selection for a few important genes for traits such as non-shattering, short plant height, optimum flowering time and others. Studies that combine QTL results from different populations grown in different environments can be used to construct consensus QTL maps. Such maps can then be related across species using anchor loci that have been previously mapped. This approach combined with trait dissection can be utilized in candidate gene analysis.

Candidate gene analysis

As the genomic maps become more densely populated with gene sequences of known function and expression, candidate gene analysis becomes more efficient and may eventually replace other methods of gene discovery but it does not eliminate the need for phenotypic data. A candidate gene is a gene that is associated with variation in a trait and believed to be involved with the development or physiology of the trait.
Candidate gene analysis has the goal of finding genes responsible for genetic variation in traits of interest. Candidate genes are often sequenced genes of known or suspected function and may belong to biochemical or regulatory pathway (Rothchild and Soller, 1997).

Genetic dissection of complex traits refers to QTL analysis of components of a trait and can lead to candidate gene hypotheses. Breaking down a complex trait into its components (trait dissection) can be very helpful in understanding the effects of genes on a trait and their interactions. Often the components will have a higher heritability and be less affected by the environment. Candidate gene analysis is complementary to QTL analysis as it provides different kinds of genetic information, often over a broader range of germplasm. Because there are large numbers of genes located in the region of a QTL, the odds of identifying the gene that actually controls the trait appear to be quite low; however, a number of factors can increase the odds of success, especially as the number of genes sequenced increases.

The methodology begins with choosing candidate genes based on biological system or QTL mapping information. Next a technique is chosen for amplifying part or the entire gene using consensus or degenerate primers. Once the gene has been amplified and sequenced for a few genotypes, polymorphisms are identified in the candidate genes' sequences or restriction sites. It is not critical at this point that these polymorphisms be responsible for the phenotypic variation because only the association is tested. Eventually, an understanding of the relationship between variation in the gene product and phenotype will be necessary for proving gene identity and especially important for predicting and constructing uniquely useful genes. A method is then chosen for scaling up genotyping using allele-specific primers or PCR-RFLP. A suitable population is then identified and the primers are used to genotype the population. The population does not require a particular structure as conventionally used for segregation analysis. The final step is to analyze the association between allele and phenotype. Validation using another population is important as it is in other kinds of genetic analysis.

Advantages to candidate gene analysis include the ability to scale up the screening, statistical power comparable to an $F_2$ population, flexibility in population structure, modest cost, building an information base, and it is adaptable to breeding programs. Some disadvantages inherent to this approach include a need for a priori knowledge of genes and a putative function. Also, non-trait genes may influence target trait making it difficult to narrow down the list of candidate genes. Variation in closely linked genes may complicate proof of gene identity. In spite, of these difficulties candidate gene analysis is another useful tool in our long term goal of rapid, large-scale gene discovery and characterization. Characterized alleles can then be ranked for their usefulness or desirability and pyramided into superior cultivars.
Conclusions

Genomic research has emphasized structural aspects in recent years, however, the focus will gradually shift to determining the functional role of genes and the mechanisms of evolutionary change that have resulted in the diversity of living organisms seen today. Methods for genome-wide gene expression studies are developing rapidly and will lead to enhanced understanding of protein structure-function relationships that are necessary for predicting gene function and for rationally engineering genes. Bioinformatics will play an increasingly important role in the integration of information from different species and sources through the use of novel approaches to analysis and visualization of complex data.

Structural genomic research linking genes and genomes across species benefits all species but is especially important for LGS. We must not lose sight of our long-term goal, which is crop improvement. Breeding progress depends on i) discovery and generation of genetic variation for agronomic traits and ii) accurate selection of rare genotypes that possess new or improved attributes due to superior combinations of alleles at multiple loci. Consequently, efficient methods are needed for identifying and evaluating allelic effects on a large scale so that desirable alleles can be assembled in superior varieties. This can be facilitated by integration of genetic information across species, identification of superior alleles, and by focusing on the most important genes and traits for the species of interest.

Acknowledgements

I wish to thank Olin Anderson, Gerry Lazo, and David Matthews for their contributions to the sequence matching experiments. Finally, I am grateful to Dr. Mohan Kohli and his staff for their hospitality and for making it possible for me to participate in the International Workshop on the Application of Biotechnologies to wheat breeding.

References


The Application of Comparative Genetics to Wheat Improvement


Resumen

Aplicación de Genética Comparativa al Mejoramiento de Trigo

La genética comparativa es un amplio campo de investigación con el objetivo general de estimar la similaridad en diferentes niveles específicos de organización. La evolución de la investigación en genética comparativa, desde el nivel de una planta hasta el nivel del ADN ha expandido sinérgicamente nuestro conocimiento de la estructura y función del genoma, debido a la complementariedad entre las investigaciones de científicos que trabajan en diferentes especies. En esta revisión se presentará un breve panorama de la genética comparativa con ejemplos provenientes de la familia Gramineae que ilustrarán las futuras orientaciones en esta área. Esta presentación se enfoca en el rol de la investigación genómica en el mejoramiento de cultivares, especialmente para especies con grandes genomas poliploides y en cultivares menores. El mapeo de loci que controlan caracteres de importancia económica, la disección de caracteres complejos, y el análisis de genes candidatos serán presentados conjuntamente con ejemplos. El objetivo de largo plazo es facilitar la identificación de alelos superiores para genes de importancia económica que puedan ser reunidos en cultivares superiores.
An Analysis of the Use of Haploidy in Wheat Improvement

A. Mujeeb-Kazi*

Abstract

Polyhaploid production in wheat has relied heavily on anther culture and sexual crosses with Hordeum bulbosum. The occurrence of somaclonal variation, aneuploidy and genotypic specificity are major limitations of anther culture. The homoeologous group 5 crossability loci (K5) influence the sexual crossings of wheat with H. bulbosum. Producing wheat haploids by sexual crosses of bread wheat x maize, pearl millet or Tripsacum has become a significant procedure since the production constraints of anther culture and H. bulbosum crosses are not present. Currently this procedure is being routinely used in wheat cytogenetics, wide crosses, wheat breeding, with extensions of the application into genetic engineering and molecular mapping. Recent technique advances in enhancing efficiency of haploid production utilize detached tillers from selected plants, their culture in a nutrient solution including sulfurous acid to avoid contamination, and hot water (43°C) immersion of the spikes for three minutes to effect emasculation. Hormonal treatment (2,4-dichlorophenoxy acetic acid; 100 ppm) is essential, as is embryo rescue 14 days after pollination. The protocol is almost 100% effective for all bread wheat-cultivars. It has genotypic specificity for durums and TriticoSecale. The contribution of D genome chromosomes is a definitive factor in cross success. Mean frequencies of embryo excision are 25%, plantlet differentiation 80%, with a colchicine induced doubling range from 80 to 95%. Results of the techniques application in wide crosses, genetics, cytogenetics, breeding, and genetic transformation will be presented. Use of stored millet pollen to facilitate haploid production, due to unavailability of fresh pollen when wheat is grown, will be elucidated.

Introduction

Polyhaploid plants are important in efforts to reduce the number of generations it takes to fix the homozygosity of wheat and other cereal plants. A homozygous plant is obtained when a polyhaploid’s chromosomes are doubled. Until recently, polyhaploid production in the Triticeae had relied mostly on anther culture and sexual crossings with the perennial barley relative Hordeum bulbosum L. The occurrence of somaclonal

*CIMMYT, Aptdo. Postal 6-641, CP 06600 Mexico D.F., Mexico. email:m.kazi@cgiar.org
variation, aneuploidy, and genotypic specificity (Picard 1989) are major limitations of anther culture in polyploid production. The homoeologous group 5 crossability loci (Kr1, Kr2, Kr3) are the limiting factors in *H. bulbosum* sexual crossings (Snape et al., 1979; Falk and Kasha 1981, 1983; Sitch and Snape 1986, 1987; Mujeeb-Kazi and Asiedu 1990). In order to avoid tissue culture-associated somaclonal variation, the sexual route to polyploid production appears as more desirable; however, a substitute for the *H. bulbosum* technique was needed in order to overcome genotypic specificity. *Zea mays* L. (Laurie and Bennett 1986, 1988a,c; O’Donoughue and Bennett 1988; Laurie et al., 1990; Zenkteler and Nitzsche 1984) and *Tripsacum dactyloides* (Riera-Lizarazu and Mujeeb-Kazi 1993) have emerged as alternative sexual routes for polyploid production in the Triticeae, and led to documented production of polyploid plants. There have also been successful crosses between *Z. mays* and *T. turgidum* L. as well as other *Triticum* and *Aegilops* spp. (O’Donoughue and Bennett 1988). Successful fertilizations have also been accomplished in crosses between wheat and *Sorghum bicolor* L. Moench, sorghum (Laurie and Bennett 1988a,b); *Pennisetum glaucum* R. Br., pearl millet (Laurie 1989); *Z. mays* ssp. *mexicana*, teosinte (Ushiyama et al., 1991); *Hordeum vulgare* L., barley (Laurie and Bennett 1988c); and *Secale cereale* L., rye (Laurie et al., 1990).

After fertilization occurs in any of the above crosses, chromosomes of the male parent are eliminated very early (Laurie and Bennett 1988a) thus producing a polyploid embryo that retains only the chromosomes of the female parent. Normally, the embryo soon aborts; however, exogenous treatment with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) promotes seed and embryo development until the embryo can be excised and plated onto a synthetic medium for continued growth and plantlet regeneration (Laurie et al., 1990).

Using this methodology, polyploid cereal plants have been recovered from crosses of bread wheat (*T. aestivum*) x maize (Comeau et al., 1988; Laurie and Bennett 1988c, Suenaga and Nakajima 1989; Inagaki and Tahir 1990; Rines et al., 1990; Riera-Lizarazu and Mujeeb-Kazi 1990; Laurie and Reymondie 1991); durum wheat x maize (Riera-Lizarazu and Mujeeb-Kazi 1993); wheat x pearl millet (Ahmad and Comeau 1990); bread wheat x sorghum (Ohkawa et al., 1992); bread wheat x *teosinte* (Ushiyama et al., 1991); barley x maize (Furusho et al., 1991); and *T. aestivum* x *Tripsacum* (Riera-Lizarazu and Mujeeb-Kazi 1993).

This paper presents some diverse applications of the doubled haploid (DH) procedure with special emphasis on the effects of pollen storage and detached-tiller culture on wheat polyploid production frequencies in maize and pearl millet crosses. Protocol modifications and some output constraints shall also be elucidated.
An Analysis of the Use of Haploidy in Wheat Improvement

Haploid production

Via anther culture

Anther culture, a method for haploid induction, is widely used in wheat and many other crops. Significant genotypic differences are present for anther culture response in wheat (Andersen et al., 1987). Few responding genotypes, low haploid recovery and aneuploidy have been some major limitations with the anther culture method. Advances in its application are still being pursued (Kisana et al., 1993).

Via crosses with Hordeum bulbosum

Wide crosses of wheat with a bulbous wild barley (Hordeum bulbosum L.) results in the production of immature haploid embryos of wheat after preferential elimination of H. bulbosum chromosomes from hybrid zygotes (Barclay 1975). However, the crossability of wheat with H. bulbosum is genetically controlled by the genes Kr1 and Kr2 located on chromosomes 5B and 5A, respectively (Falk and Kasha 1983; Snape et al., 1979). According to the pedigrees of wheat varieties, crossable genotypes can be traced to the variety Chinese Spring or to materials of Asian origin (Falk and Kasha 1981). Both Japanese and Chinese wheat varieties, and in particular, local varieties, are highly crossable with H. bulbosum (Inagaki and Snape 1982; Inagaki 1986; Li and Hu 1986). Wheat genotypes carrying the dominant Kr gene(s) are not crossable with H. bulbosum and cannot produce haploid embryos. The lack of hybridization of wheat genotypes with H. bulbosum is due to the failure of the pollen tube to penetrate the embryo sac (Snape et al., 1980). Application of plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) promotes seed setting and embryo formation when crossable genotypes are used for crosses, but is unable to break the barrier of cross-incompatibility (Inagaki 1986). Since the efficiency of wheat haploid production is greatly influenced by the crossability of H. bulbosum onto wheat, the method using H. bulbosum crosses is restricted only to crossable wheat genotypes.

Via maize and other sources

Ultra-wide crosses of wheat with members of the Panicoides subfamily have been attempted in alien genetic transfer. Maize (Zea mays L.) pollen can successfully hybridize wheat egg cells and produce hybrid zygotes (Laurie and Bennett 1986), irrespective of the presence of Kr gene(s). Maize chromosomes are rapidly eliminated from the hybrid zygotes, requiring artificial rescue of proembryos at an early developmental stage (Comeau et al., 1992; Laurie and Bennett 1987, 1988c). A post-pollination 2,4-D treatment is critical to enhance embryo development in wheat x maize crosses (Suenaga and Nakajima 1989). Maize pollination and subsequent 2,4-D treatment results in the production of immature wheat embryos capable of regenerating haploid plants, even
for wheat varieties that are cross-incompatible with *H. bulbosum*. Wheat haploid production through maize crosses has been achieved using diverse wheat varieties (Inagaki and Tahir 1990; Laurie and Reymondie 1991). Some species related to maize, such as teosinte (*Zea mays* L. spp. *Mexicana*) (Ushiyama *et al.*, 1991) and eastern gamagrass (*Tripsacum dactyloides* (L.) L.) (Riera-Lizarazu and Mujeeb-Kazi 1993), are efficient alternative pollen donors for wheat haploid production.

Cytological evidence indicates successful fertilization and elimination of paternal chromosomes from hybrid zygotes in sorghum (*Sorghum bicolor* (L.) Moench) and pearl millet (*Pennisetum glaucum* (L.) R. Br.) crosses, which suggests that sorghum and pearl millet are potential pollen sources for wheat haploid production (Ahmad and Comeau 1990; Comeau *et al.*, 1992; Laurie and Bennett 1988b; Laurie 1989). Wheat haploids were obtained at high frequencies from sorghum (Ohkawa *et al.*, 1992) and pearl millet (Inagaki and Bohorova 1995) crosses followed by 2,4-D treatment after pollination. However, sorghum crosses expressed a strong genotypic barrier of wheat to embryo formation (Inagaki and Mujeeb-Kazi 1995). Therefore, haploid production through crosses with maize and pearl millet appears more stable than other methods because of its less pronounced genotypic effect on haploid embryo formation.

**The protocol**

**Germplasm**

Sowing times of seed materials need to be determined in order to synchronize flowering times of wheat and pollen donors. A continuous supply of pollen from pollen parents sown at one or two-week intervals should be ensured.

**For intact plants**

At the time of ear emergence, wheat spikes on plants were conventionally emasculated and pollinated with maize or pearl millet one day before the estimated conventional wheat anthesis. On two consecutive days after pollination, the uppermost internodes of the wheat culms with pollinated spikes get needle-injected with a 100 mg/l 2,4-D solution according to the method of Inagaki and Tahir (1990).

**For detached tillers**

Wheat tillers with spikes are cut at the base of each tiller from growing plants and cultured in a flask with tap water. These spikes are then emasculated and pollinated as described above. After pollination, the detached wheat tillers are cultured in a solution containing 40 g/l sucrose, 8 ml/l sulfurous acid (6% SO₂) and 100 mg/l 2,4-D and cultured until embryo rescue. Culture conditions are 22.5°C, 12-hr daylength and 60 - 70% relative humidity in a growth chamber.
Regeneration and rescue

At 14 days after pollination, immature embryos get aseptically excised from wheat seeds, and transferred onto a half strength Murashige and Skoog (1962) culture medium supplemented with 20 g/l sucrose and 6 g/l agarose. The embryos are incubated at 25°C, 12-hr daylength and approximately 5000 lux light intensity. Plants regenerated from embryos are cytologically examined in root-tip mitotic preparations stained with aceto-orcein, according to the method of Mujeeb-Kazi and Miranda (1985).

Modifications

Pollen storage

Fresh pollen collected between 9:30 and 10:00 a.m. is screened through a 0.5 mm aperture sieve to remove anthers. Ten grams of pollen get spread on a paper tray and dried with gentle ventilation at 35°C and 35 - 40% relative humidity. Pollen water content is reduced to 11.8% in maize and 5.3% in pearl millet in approximately two hours of drying. Pollen water content is determined from a 0.5 g pollen sample dried at 95°C for five hours. The dried pollen is then distributed among cryopreservation tubes (1.5 ml volume). These sealed tubes are immediately immersed in liquid nitrogen (-196°C). Maize and pearl millet pollen was stored for 8 months and 10 months, respectively. After thawing the tubes containing pollen in a water-bath at 38°C for five minutes, pollen can be used for crossing. Pearl millet pollen is better suited for storage and delayed use since the induction frequency of haploids remains unaltered as happens with maize (Inagaki and Mujeeb-Kazi 1994).

Emasculation

When numbers of spikes to be emasculated is excessive, these spikes are immersed in hot water maintained at 43°C for three minutes one day before pollen shed. The treatment renders anthers to be sterile. Spikes are then pollinated with maize or millet without removing the wheat anthers.

Applications

Some applications of the haploid methodology are elucidated. These span over areas like cytogenetics, breeding, molecular diagnostics, transformation, and genetic analyses.

Disomic additions

In cyogenetic studies when alien chromosomes are to be added to wheat to yield addition lines, a constraint identified is in the paternal transmission of the alien
chromosome. Hence, 44 chromosome derivatives do not result. Often multiple disomics may be a requirement and their stability a factor in the stocks maintenance. Haploid induction on 43 chromosome plants (42 wheat plus 1 alien) or for example 45 chromosomes (42 wheat plus 3 different aliens) yield haploids ranging from 21, 21 + 1, or 21 and different, 21+1, 21+2 or 21+3 combinations. These upon doubling result in disomic or multiple disomic additions (see schematic 1 for the disomic product). It is plausible that such homozygous additions may be more stable than those conventionally produced is a concept that needs testing.

**Schematic 1. Development of a disomic alien addition line from the monosomic addition parent via the maize haploid induction protocol.**

```
Monosomic addition (2n=6x=42 + 1J) x Maize
Polyhaploids
- n=3x=21
- n=3x=21 + 1J
Chromosome doubling
2n=6x=42 + 1J1J
(Disomic addition)
```

* Ph manipulation*

The doubled haploid wheat/maize based manipulation protocol is applicable to *Ph* F1 wheat/alien F1 hybrids. This is also applicable to amphiploids and fertile BCI combinations where the *Ph* locus is homozygous dominant.

The DH role in salvaging *Ph* based F1 hybrids has become an option to enable *ph* mediated alien introgression/s without having to remake complex F1 hybrids using the *ph* genetic stock (Sears, 1977) as the maternal parent. Because of our living F1 herbarium involving wheat and several alien species (*Ph* locus present), BCI derivatives
can be produced by pollinating these $Ph F_1$ wheat/alien hybrids with the Chinese Spring $ph ph$ wheat genetic stock. The BCI progenies ($Ph ph$) are crossed with maize to yield polyhaploids that possess the $Ph$ or $ph$ locus. The entire wheat and alien chromosomal complement is represented. The $ph$ based haploids derived from the $Ph ph$ BCI derivatives identified at the seedling stage by a PCR based diagnostic analysis (Gill and Gill 1996; Qu, et al., 1998), can enhance program efficiency (Schematic 2), and allow a crop improvement program to use the integration of the breeding methodologies. The BCI derivatives can also be selfed and the $Ph Ph$, $Ph ph$ or $ph ph$ progeny similarly identified.

**Schematic 2.** *Use of a PCR probe to detect* $Ph$ *and* $ph$ *plants in intergeneric derivatives obtained from backcross I* ($Ph ph$) *x Maize crosses.*

---

$T. aesitivum$  
(2n=6x=42, AABBDD)  
($Ph$)

$Th. bessarabicum$  
(2n=2x=14, JJ)

$F_1$ hybrid  
(2n=4x=28, ABDJ)  
($Ph$)

$T. aesitivum$  
(2n=6x=42; $ph$)

BCI  
(2n=7x=49, AABBDDJ)  
($Ph ph$)

Maize

Haploids

Identify by **PCR probe** at seedling stage (Gill & Gill 1996; Qu, et al., 1998.)

* Identify by PCR probe at seedling stage (Gill & Gill 1996; Qu, et al., 1998).  
* Do meiosis on plants NULL for band.  
These are $ph$ and will show high pairing.
**Breeding**

In self-pollinating crops, such as bread wheat (*Triticum aestivum* L.), breeding programs include a three-step process for developing germplasm: 1) genetic recombination for enlarging variation, 2) identification and selection of recombinant genotypes according to their agronomic types, and 3) fixation of genes in homozygous genotypes. Pedigree selection is the conventional method of accumulating genetic recombination in each generation. The heterozygosity in early generations makes the efficient identification and selection of recombinant genotypes more difficult. Repeated pedigree selection increases homozygosity, but requires many generation cycles to attain homozygosity for loci associated with agronomic traits. The single-seed descent method is used to obtain homozygous inbreds by accelerating generation cycles. Its application however, is dependent on the growth habit of the plant materials used.

Artificial production of haploid plants followed by chromosome doubling is a quick method for obtaining homozygous recombinant genotypes from heterozygous parental genotypes in a single generation (Nei 1963). This DH method has the advantage to commence yield evaluation earlier and speed up varietal development. This method also has the great advantage of increasing the efficiency of selection because doubled haploid lines do not express dominance variation and segregation within lines (Snape 1989).

Recently the yield performance of each group of 10 bread wheat lines selected by doubled haploid (DH), single-seed descent (SSD) and pedigree selection (PS) methods from three F1 crosses was compared with the aim of evaluating the DH method in breeding programs (Inagaki et al., 1998). Their results confirmed that the DH method saves time in obtaining recombinant inbred lines ready for yield evaluation. However, a larger DH population is required to achieve the same level of genetic advance with the PS method in crosses containing greater genetic variation.

Since the DH procedure is accurate and genotype independent in bread wheat, its application can fit almost any filial generation. The techniques perfection allows one to work on breeder selected F2 or F3 plants in diverse populations for which the detached spike modification is an asset if larger numbers are involved. Fixing lines for achieving homozygosity is also seen as a boon to assist global testing of elite lines, and economize country wheat improvement programs.

**Mapping populations**

- Based on F1 or backcross I (BC1)

The development of molecular mapping populations based upon conventional or distant species involvement has been approached by the DH route. Desired parents are crossed to produce the F1 seed, which are then crossed with maize or an alternate
source to generate haploids. CIMMYT has produced a DH conventional bread wheat population for KB using the susceptible WL711 and resistant HD29 cultivars as parents and generated 290 DH for the direct, and also for the reciprocal crosses. A similar DH population has been produced for scab using Norin (Susceptible) crossed with the resistant Sumai 3 and Frontana bread wheat cultivars. Ae. tauschii has been used to produce synthetic hexaploid wheats (SH's). Such a synthetic has been used to develop populations with three bread wheat cultivars that demonstrate a high polymorphism. Such mapping populations with Ae. tauschii as the alien source are currently being developed for resistance to drought, spot blotch and Fusarium head blight. The DH route offers stability, homozygosity, and these stocks can be utilized very broadly by researchers. These mapping populations can also be produced on BCI germplasm.

*Transformation studies*

The stability of the DH products has also been applied in conferring this attribute to wheat transformants. It is recognized that transformants may tend to be unstable in the sense that the gene expression in the transformants selfed progenies may not remain consistent. Could it be due to segregation as a consequence of incipient heterozygosity, apart from other reasons like methylation. Having a homozygous DH transformant initially positive for the transgene expression, will hopefully address this question of expressive gene stability over each DH selfed analysis, and allow the germplasms comparison with normal selfed derivatives (Schematic 3).

**Schematic 3.** Production of DH derivatives from wheat transformants to evaluate gene expression stability.
**Monosomic analysis**

The DH approach is being effectively used to conduct genetic analyses for identifying physical gene locations through complete or partial monosomic analysis. The partial analysis is conducted when resistance is associated with the D genome of synthetic hexaploid (SH) wheats or their resistant SH/susceptible bread wheat derivatives that are resistant. This resistance in the derivatives is attributed to the D genome chromosomes of *Ae. tauschii* (Schematic 4). The F$_1$ monosomics of 1D to 7D chromosomes (2n=6x=40 +1D to 40+7D) when crossed with maize yield 21 chromosome polyhaploids with the 1D to 7D contributions coming from resistant SH x BW derivatives. Doubling these n=3x=21 polyhaploid plants with colchicine, results in stable 42 chromosome double haploids. Each DH now possesses the homozygous 1D to 7D chromosomes of the resistant SH parent being analyzed for the chromosomal location/s of the resistant gene/s. Upon screening, the non-segregating resistant DH's are attributed with having the gene/s in them. The stable monosomic derived DH germplasm apart from simplifying the conventional monosomic analysis also facilitates global distribution of the developed germplasm. The germplasm enables experimental repetition without having to re-build the analytical germplasm, as is necessary when the conventional monosomic analytical procedure is followed.

**Constraints**

Bread wheat germplasm of diverse growth habit (spring, winter, facultative) all respond to the haploid induction procedure using maize or other species as pollen sources. This makes its application attractive and routine in bread wheat improvement. Genotypic specificity is non-existent, and production frequencies render the utilization of these techniques quite simple for crop improvement. A similar trend, however, is not seen so far for durums or *TriticoSecale* (Almouslem et al., 1998; Inagaki et al., 1998). Manipulations to enhance frequencies of haploid production have been positive (Inagaki et al. 1998) but breaking barriers to render the incompatible types compatible has not been achieved. The D genome and some of its chromosomes decidedly have an influence as observed from inferences made by Almouslem et al. (1998), Inagaki et al., (1998) for durums and Inagaki et al. (1997) for *TriticoSecale*. The positive contributions of D genome to haploid production are also apparent from durum and their derived SH (Durum x *Ae. tauschii*) crosses with maize. The haploid production is significantly higher in the SH's as compared with their durum parents that lack the D genome (Unpublished Data).
Schematic 4. Steps involved in conducting a partial D genome based monosomic analysis utilizing doubled haploidy.

Glennson mono 1D to 7D (40 + 1D) x SH x Bread wheats (40 + 1D + 1D)

\[ F_1 \]

20+20+1D+ 1D =42

20+20+ 1D =41

Identify \( F_1 \) x Maize (20+20+ 1D =41)

Polyhaploids: 1) 20
2) 20 + 1D

40 + 1D + 1D

Increase seed

7 such DH's result;
Non-segregating resistant 1D to 7D doubled haploid possesses the resistant genetic control

Conclusions

Crosses between wheat (Triticum aestivum) and maize are an effective means of producing wheat polyhaploids. Haploid generation is independent of the source of maize pollen and of the recipient wheat cultivar, though some pollen source preference does exist. Application of the haploid procedure is useful in breeding programs, genetic analysis, developing mapping populations, producing cytogenetic stocks, and as an off-shoot in assisting wide crossing programs. Techniques that complement satisfactory hybridization product frequencies strongly favor the use of detached spikes, sucrose
as a nutrient source and sulfurous acid for overcoming contamination. Post pollination 2,4-dichlorophenoxy acetic acid treatment remains crucial. Use of stored maize pollen gives positive results with a low frequency. Improved frequencies result by using stored pearl millet pollen. The technique gives a variable response with durum wheats and X TriticoSecale.

References


Resumen

Un Análisis del Uso de Haploides en el Mejoramiento de Trigo

La producción de haploides en trigo se ha basado fuertemente en el cultivo de anteras y cruzamientos sexuales con Hordeum bulbosum. La ocurrencia de variación somaclonal, aneuploidía y la especificidad genotípica son las limitantes mayores del cultivo de anteras. El locus de cruzabilidad (Kr) del grupo homeólogo 5 influye en los cruzamientos sexuales de trigo con H. bulbosum. La producción de trigos haploides por cruzamientos sexuales de trigo pan x maíz, mijo, Tripsacum se ha vuelto un procedimiento significativo debido a que no presentan las limitantes del cultivo de anteras y los cruzamientos con H. bulbosum. Corrientemente este procedimiento es usado rutinariamente por nosotros en citogenética, cruzamientos amplios, mejoramiento de trigo con extensiones de su aplicación en ingeniería genética y mapeo molecular. Recientemente la técnica ha avanzado en el aumento de la eficiencia de la producción de haploides utilizando macollos colectados de plantas seleccionadas, cultivándolas en una solución nutritiva que incluye ácido sulfúrico para evitar las contaminaciones e inmersión en agua caliente (43°C) de las espiguillas por tres minutos para efectuar la emasculación. El tratamiento hormonal, (ácido 2,4 diclorofenoxiacético; 100 ppm) es esencial, así como realizar el rescate de embriones 14 días después de la polinización. El protocolo es prácticamente 100 % efectivo para todos los cultivares de trigo pan. Tiene especificidad genotípica para los trigos durum y triticales. La contribución de los cromosomas del genómico D es un factor definitivo en el éxito de los cruzamientos. La frecuencia promedio de excisión de embriones es del 25%, de diferenciación de plantas es de 80% y con colchicina se induce la duplicación cromósómica en un rango de 80 a 95%. Se presentarán los resultados de la aplicación en cruzamientos amplios, genética, citogenética, mejoramiento y transformación genética. El uso de polen almacenado de mijo para la producción de haploides, debido a la no disponibilidad de polen fresco cuando el trigo está cultivado será considerado.
Abstract

Molecular markers have many applications to crop improvement. When tightly linked to genes of interest, they can be used to indirectly select for the desirable alleles. In addition, molecular markers can be used for dissecting polygenic traits into their Mendelian components or quantitative trait loci (QTL), thus increasing our understanding of the inheritance of such traits. Scientists in the Applied Biotechnology Center at CIMMYT have been collaborating with the wheat program in the development and use of molecular markers for wheat improvement. Development of cultivars with durable leaf rust and yellow rust resistance is an important breeding objective of CIMMYT. Resistance to leaf and yellow rust is controlled by a number of minor genes and is referred to as adult plant resistance (APR). Linkage mapping and bulked segregant analysis are being used with several recombinant inbred line populations segregating for leaf rust and yellow rust resistance in order to find molecular markers associated with APR genes. Three markers with significant association with durable leaf rust resistance have been found in one population. In a second population, five and two quantitative trait loci (QTL) were detected for resistance to leaf and yellow rust, respectively. In addition, molecular markers are being used to transfer Thinopyrum intermedium derived resistance to barley yellow dwarf virus into different bread wheats. Markers are also being used to detect wide cross derivatives with enhanced meiotic pairing that would facilitate the detection of introgression of chromosomal segments from wild relatives of wheat that carry important biotic and abiotic attributes into cultivated wheat.

Introduction

Molecular markers (DNA markers), reveal sites of variation at the DNA level. These markers have the advantage of being numerous in nature and not affected by the environment as in the case of morphological markers. Molecular markers reveal neutral sites of variation and therefore, unlike morphological markers, do not show phenotypic effects. The expression of most genes is quantitative in segregating populations and is confounded by the environment.
Progress of identifying molecular markers in wheat has been comparatively slow due to its large genome size and low levels of polymorphism at the molecular level. However, there have been considerable successes in developing molecular markers to tag leaf rust resistance and other genes derived from the wild species (reviewed in Hoisington et al., 1998). This is often possible due to the presence of large segments of introgressed chromatin from the wild species in wheat background and high frequency of polymorphism between the introgressed segments and the corresponding counterparts of the wheat genome. Further, once molecular polymorphisms are identified and genes derived from wild species are tagged, these markers often become stable enough to be used in practical breeding programs due to lack of pairing between the introgressed chromosomal segment and the wheat homoeologs. The presence or absence of genes and the number of genes for traits such as adult plant resistance (APR) to leaf rust and yellow rust can be suggested or hypothesized in different breeding populations. However, identification of these genes and combining them together in cultivars can be facilitated when markers are available for the genes of interest.

In the Applied Biotechnology Centre at CIMMYT, efforts are underway to develop molecular markers associated with various biotic parameters in wheat such as durable leaf and yellow rust resistance, barley yellow dwarf virus (BYDV) resistance/tolerance and resistance to head scab. Considerable success has also been made in identifying molecular markers with tight linkage to genes controlling aluminum tolerance in rye (Secale cereale) as well as studying genetic diversity in a set of historically important wheat cultivars. Efforts are also being made to apply molecular markers developed in other laboratories in facilitating breeding efforts. The work underway in the identification of markers associated with APR, BYDV and the utilization of markers for the detection of wide hybrid derivatives of wheat with enhanced meiotic pairing will be presented in detail.

Mapping durable leaf rust resistance

Rusts are the most common diseases of cultivated wheat and the use of resistant cultivars offers the most effective form of control of the disease. Over 40 mainly race-specific leaf rust genes identified from the wheat gene pool or derived from the wild relatives of cultivated wheat are known (Knott, 1989; Roelfs et al., 1992). Most of these genes have been used either singly or in combinations to develop cultivars with rust resistance. The slow rusting, durable type of resistance, is effective in adult plants and is known to be complex in inheritance (Knott and Yadav, 1993). Gavinlertvatana and Wilcoxson (1978) reported that between 3 to 21 genes could be involved in slow rusting type of resistance. The gene Lr34 has been reported to increase the latent period, decrease the infection frequency and urdial size (Drijepondt and Pretorius, 1989). Although Lr34 itself may not confer adequate protection against high disease pressure (Singh and Gupta, 1992), the Lr34 complex, defined as the result of additive interaction of Lr34 with several other slow rusting genes, would confer adequate adult
plant resistance (Singh and Rajaram, 1992; Roelfs, 1988). \textit{Lr34} is known to be either pleotrophic or closely linked to the phenological trait leaf tip necrosis (LTN) of adult plants (Singh, 1992).

Several recombinant inbred line (RILs) populations are being used at CIMMYT with the objective of developing molecular markers associated with \textit{Lr34} and other durable leaf rust/yellow rust resistance genes. The availability of such markers would facilitate the breeding efforts where considerable efforts are being made to combine several slow rusting genes in CIMMYT wheat germplasm for obtaining cultivars with durable resistance under high disease pressure. One such population is composed of 77 RILs derived from the cross ‘Parula’ (resistant) X ‘Siete Cerros’ (partially susceptible). Leaf rust data were available for two years from replicated field trials conducted at Ciudad Obregón (Sonora, Mexico). Initial studies with the population were conducted with restriction fragment length polymorphism (RFLP) analysis with parental screening and mapping with approximately 250 RFLP probes. Subsequently, bulked segregant analysis was conducted with 500 Operon decamer primers (Operon Technologies, Alameda, Calif.). Two bulks made from the 10 most resistant entries and 10 most susceptible entries were analyzed. However, no significant association between molecular markers and leaf rust resistance could be observed in these studies further emphasizing the difficulty associated with wheat at the molecular marker level.

A subsequent study was conducted with genomic DNA enriched for low copy sequences (William et al., 1997). Screening the two bulks with 400 Operon decamer primers identified three polymorphisms between the two bulks. The three amplification products were cloned and were used as probes in Southern analysis with the 77 RIL populations. The three clones derived from Operon decamer primers OPG-05, OPI-16 and OPR-03 were designated CMTG05-500, CMT116-1500 and CMTRO3-500 respectively. Linkage analysis using MAPMAKER indicated that the two loci \textit{Xcmtg05-500} and \textit{Xcmti16-1500} were tightly linked with 2% recombination (LOD score of 17.1). There was no significant linkage between these two loci and the third locus \textit{Xcmtr03-500}. There was also no linkage between the three molecular markers and leaf tip necrosis (\textit{Ltn}).

One way analysis of variance showed significant association of the three molecular markers as well as the phenological marker, LTN, with factors controlling leaf rust resistance. Leaf tip necrosis, confirming its tight linkage with \textit{Lr34} explained 20–30\% of the total phenotypic variation. The two tightly linked loci \textit{Xcmtg05-500} and \textit{Xcmti16-1500}, also explained a similar proportion of the total phenotypic variation (22 – 30\%). The alleles detected by \textit{Xcmtg05-500} and \textit{Xcmti16-1500} were present in the susceptible parent ‘Siete Cerros’ and were associated with susceptibility. Using these two markers, homozygous resistant parents could be detected (absence of band). The third locus \textit{Xcmtr03-500}, the allele identified in the resistant parent ‘Parula’ explained approximately 10\% of the total phenotypic variation. Chromosomal locations
for the three clones were determined using cytogenetic stocks of ‘Chinese Spring’. The two loci Xcmtg05-50 and Xcmti16-1500 could be located on chromosome 7BL. Clone CMTR03–500 revealed two loci, one of that could be located on chromosome 1BS. This study has revealed two loci associated with leaf rust resistance on chromosome 7BL that seem to be as effective as Lr34 (Ltn) in conferring resistance as well as another component of slow rusting resistance located on chromosome 1B (William et al., 1997).

Another population being used for mapping APR genes for leaf rust and yellow rust is a recombinant inbred line population (220 RILs) derived from ‘Frontana’ X ‘INIA66’. A full molecular map is being developed using this population. Currently, the map consists of 451 markers on 33 linkage groups. The markers were derived from 118 RFLPs, 19 microsatellites (SSRs), 312 amplified fragment length polymorphisms (AFLPs) and two morphological markers (LTN, and pseudo black chaff, PBC). Phenotypic data have been collected for leaf rust for 1991/1992, 1994/1995 (Ciudad Obregón, Sonora, Mexico) and yellow rust (data collected in Toluca, Mexico, 1993). Quantitative trait loci (QTL) have been identified for both leaf rust and yellow rust resistance. Five QTLs were associated with leaf rust and two with yellow rust resistance. As in the previous study, Ltn explained 51% and 37% of the phenotypic variation for leaf rust and yellow rust, respectively, further indicating its strong linkage with Lr34 and Yr18 (Table 1). The second QTL for yellow rust resistance coincided with the gene Sr2, identified by its association with the morphological marker pseudo black chaff (PBC), where a QTL with minor effects was identified for leaf rust as well. Genotyping the population with new microsatellite markers and sequence tagged sites (STS) is currently underway (Khairallah et al., 1998).

Other populations that are segregating for leaf rust and yellow rust and are currently being utilized in the molecular marker work include, ‘Avocet’ X ‘Parula’, ‘Avocet’ X ‘Tonichi’ and ‘Avocet’ X ‘Pavon’. Each population has approximately 150 F₅ lines, which are being used in bulked segregant analysis and development of molecular maps.

Markers for barley yellow dwarf resistance

Barley yellow dwarf is caused by a group of phloem limited luteoviruses collectively known as the barley yellow dwarf viruses (BYDVs), and is transmitted by aphids. Significant economic losses have been reported in most cultivated cereals including wheat (Pike, 1990). Although some tolerance to BYDV has been identified in wheat, it is known to be only partially effective and is affected by environmental influence (Zhou et al., 1990). Resistance to BYDV has been incorporated into cultivated wheat by tissue culture derived chromosomal translocation lines from Thinopyrum (Agropyrum) intermedium (Banks et al., 1995; Larkin et al., 1995).
Table 1. Quantitative trait loci detected for leaf rust and yellow rust resistance in 'Frontana' x 'Inia-66' population.

<table>
<thead>
<tr>
<th>Linkage Group</th>
<th>Position (cM)</th>
<th>Likelihood ratio</th>
<th>Phenotypic Variation (R²%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf rust</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>76</td>
<td>15.7</td>
<td>5.0</td>
</tr>
<tr>
<td>4B</td>
<td>29</td>
<td>14.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Group 6-2</td>
<td>12</td>
<td>14.4</td>
<td>2.0</td>
</tr>
<tr>
<td>7D</td>
<td>54</td>
<td>88.4</td>
<td>51.1</td>
</tr>
<tr>
<td>Group 7-2</td>
<td>159</td>
<td>15.4</td>
<td>12.2</td>
</tr>
<tr>
<td><strong>Yellow rust</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>77</td>
<td>10.4</td>
<td>18.6</td>
</tr>
<tr>
<td>7D</td>
<td>54</td>
<td>44.9</td>
<td>36.6</td>
</tr>
</tbody>
</table>

*Likelihood ratio = 11.5 is equivalent to LOD score of 2.5.

Efforts are underway to develop molecular markers and utilize them to transfer the introgressed chromosomal segment of *Th. intermedium* into different wheat backgrounds and to study its effectiveness and inheritance against the background of the tolerant gene *Bdv1* identified in CIMMYT wheats (Singh et al., 1993). Currently, four RFLP markers and two microsatellite markers have been identified and a microsatellite marker is routinely used to identify the presence of the translocated chromosomal segment on 7DL in different bread wheat backgrounds (Ayala et al., unpublished). Efforts are also underway to convert the RFLP markers into more convenient STS markers. In addition, phenotypic data have been collected on BYDV tolerance for the RIL populations of 'Frontana' X 'Inia 66' and 'Opata' X 'Synthetic'. Molecular marker analysis is underway to identify the loci controlling tolerance to BYDV in these populations.

Application of molecular markers in wide hybrids in wheat

Intergeneric hybridization of wheat with its wild relatives is an attractive method of transferring certain desirable traits from wild relatives into cultivated wheat. However, the limitations associated with the procedure include lack of interchromosomal associations even when the hybrids are successfully made, mainly due to the presence of several genes controlling pairing (*Ph* genes) between homoeologous chromosomes. The strongest effect has been observed for the *Ph1* locus (located on 5BL) which effectively suppresses pairing between homoeologous chromosomes (Sears and Okomoto, 1958; Riley and Chapman, 1958). Wheat lines carrying deletions of the
Ph1 locus (ph1b) have been identified (Sears, 1977). Molecular markers based on the polymerase chain reaction (PCR) that facilitate identification of the ph1b deletion stocks have been developed (Qu et al., 1998; Gill and Gill, 1996).

At CIMMYT, we have successfully applied one of the markers (Qu et al., 1998) to facilitate the identification of wide hybrid derivatives, that carry alien chromosomes from species such as Thinopyrum bessarabicum in the selfed BC₁ progenies of the F₁ intergeneric hybrids with 'Chinese Spring' ph1b deletion stocks. Over 300 lines were analyzed with the PCR marker and lines that carry the ph1b deletion identified and later confirmed by meiotic analysis to have multivalent pairing involving wheat and Th. bessarabicum chromosomes. This PCR marker is being used in the identification of wide hybrid derivatives involving several other wild relatives of wheat that carry important biotic and abiotic attributes.

Acknowledgement

This research is part of and partially funded by the Australian-funded Cooperative Research Center for Molecular Plant Breeding, of which, CIMMYT is a core participant.

References


Resumen

La Aplicación de Marcadores Moleculares en el Mejoramiento de Trigo en CIMMYT

Los marcadores moleculares tienen varias aplicaciones en los programas de mejoramiento de cultivos. Estos, cuando están estrechamente vinculados a genes de interés, pueden ser utilizados indirectamente para seleccionar los alelos deseados. Además, los marcadores moleculares pueden ser usados para disecionar características poligénicas en sus componentes Mendelianos o loci para caracteres cuantitativos (QTL), aumentando así nuestro conocimiento sobre la herencia de estos caracteres. Los científicos en el Centro de Biotecnología Aplicada del CIMMYT están colaborando con el Programa de Trigo en el desarrollo y uso de marcadores moleculares para el mejoramiento de trigo. El desarrollo de cultivares con resistencia duradera a la roya de la hoja y roya estriada es un objetivo importante para el CIMMYT. La resistencia a estas royas está controlada por un gran número de genes menores y es referida como resistencia en estado de planta adulta (RPA). El mapeo de ligamientos y el análisis de masales segregantes están siendo utilizado con varias poblaciones de líneas recombinantes endocriadas que están segregando para genes de resistencia a la roya de la hoja y roya estriada, con el propósito de encontrar marcadores moleculares asociados con los genes para RPA. Tres marcadores que muestran asociación significativa con la resistencia duradera a la roya de la hoja han sido encontrados en una población. En una segunda población, 5 y 2 QTLs fueron detectados para resistencia a la roya de la hoja y roya estriada respectivamente. Además, los marcadores moleculares están siendo utilizados para transferir resistencia al virus del enanismo amarillo de la cebada derivado de Thinopyrum intermedium en diferentes trigos harineros. Los marcadores también están siendo utilizados para detectar los derivados de las cruces amplias con una mayor frecuencia de apareamiento meiótico que facilitarán la detección de la introgresión de los segmentos cromosomales de las especies aliadas del trigo que poseen características bióticas y abióticas importantes para su transferencia al trigo cultivado.
Advances in Molecular Markers for Bread Making Quality

Jorge Dubcovsky *, G. Tranquilli¹, D. Lijavetzky¹, I. A. Khan¹, A. R. Schlatter², M.M. Manifesto² and S. Marcucci-Poltri²

Abstract

Composition of the grain storage proteins, grain protein content (GPC) and hardness are the major factors determining bread making quality. Though the effect of specific high molecular weight glutenin subunits on bread making performance is well characterized, they usually account for less than 50% of the inter-cultivar variation in gluten strength. A specific Gli-B1/Glu-B3 allele from Klein 32 that accounts for a significant proportion of the variation in gluten strength has been recently identified using a microsatellite marker. Introgression of this allele in combination with appropriate high molecular weight glutenin subunits may allow a more efficient manipulation of gluten strength. Besides gluten strength, GPC is probably the major factor affecting bread making and pasta quality. In spite of the importance of this character, progress in breeding for high GPC has been slow and difficult because of the large effect of the environment and the strong negative relationship between grain protein percentage and grain yield. Molecular markers linked to a gene for high GPC have been recently identified in the short arm of Triticum dicoccoides chromosome 6B. A large segment of this chromosome was detected in the high GPC hexaploid variety Glupro. Various molecular markers can be used to introgress the high GPC gene into both tetraploid and hexaploid wheat varieties. Manipulation of GPC and protein quality with molecular markers can be now complemented with the manipulation of grain hardness using similar techniques. Tight linkage between the grain softness related protein and the puroindoline-a loci (both associated with hardness) allow the combination of the molecular markers for both loci. A strategy combining PCR and restriction enzymes was developed to facilitate the introgression of different hardness alleles in marker assisted selection programs.
Application of molecular markers to wheat breeding

Progress in molecular markers in wheat has been slower than in other crops because of the hexaploid nature of bread wheat, the low level of polymorphism present in cultivated varieties, and the huge size of the wheat genome (almost forty times larger than the rice genome (Arumuganathan and Earle 1991)). In spite of these difficulties, important progress has been made during the last four years in the construction of complete restriction fragment length polymorphism (RFLP) maps of the wheat genome. These maps include approximately 2000 RFLP markers (Dubcovsky et al., 1995a; Dubcovsky et al., 1995b; Gale et al., 1995; Nelson et al., 1995a; Nelson et al., 1995b; Nelson et al., 1995c; Van Deynze et al., 1995; Dubcovsky et al., 1996a; Dubcovsky et al., 1996b; Marino et al., 1996) and more than 300 microsatellite markers (Devos et al., 1995; Ma et al., 1995; Korzun et al., 1997; Röder et al., 1998; Stephenson et al., 1998).

Many of these RFLP and microsatellite markers are linked to important agronomic traits and can be used to facilitate the incorporation of the selected genes or chromosome segments into adapted materials using backcross (BC) and marker assisted selection. Theoretically, the recovery of the recurrent parent is expected to be ninety-nine percent after six backcrosses (Hospital et al. 1992) and the analysis of five BC plants allow the recovery of a desired genotype, with a probability higher than 0.95, if only one gene is tagged. At least 11 BC plants are required if two segments are tagged simultaneously. It is preferable to incorporate each gene separately and then combine them through hybridization, self-pollination and selection of homozygous plants using molecular markers. The complete backcross process can be done in three years growing three generations per year in greenhouses. However, the process can be accelerated by the use of rapid generation advancement protocols (US patent 5682708).

Many molecular markers have been recently linked to different genes for protein quantity, gluten strength or grain hardness that affect bread making quality (BMQ). In spite of this potential, the use of molecular markers for BMQ has been limited to few breeding programs and mainly to the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for high molecular weight glutenins. The limited use of marker assisted selection (MAS) for BMQ can be due to the very recent development of many of these markers. Other reasons such as lack of information and technical expertise to use molecular markers in some breeding programs and further lack of examples of successful varieties released with the aid of MAS are also important. A gap in public funding between the development of the markers and their use in the development of wheat varieties is one reason for the delay in these releases. However, it is unlikely that the research and development granting agencies will support proposals aimed at the transfer of genes into adapted materials because of the relatively low scientific impact of this kind of projects. On the other hand, the grower associations...
that support public-breeding programs usually can not direct their limited funding to
test the usefulness of these new biotechnological tools. That the small breeding
programs and most grower associations and end-users do not have adequate
information on the recent advances in this area presents an additional barrier. This
workshop on “Application of Biotechnologies to Wheat Breeding” is a useful forum to
bridge this gap of information between traditional breeders, science administrators,
and wheat biotechnology researchers. This paper will try to summarize the information
available on molecular markers for BMQ in three critical areas: protein content, protein
quality and grain hardness.

Molecular markers for grain protein content in bread and pasta wheat

Grain protein content (GPC) is one of the major factors affecting bread making and
pasta quality. As protein content increases within a bread wheat variety, its water
absorption capacity and loaf volume also increase (Finney et al., 1987). In durum wheat,
the increase in protein quantity causes increase in the dough strength, cooked spaghetti
firmness, and cooked weight and decreases in the loss of solids to the cooking water
(Dick and Youngs 1988). In spite of the importance of this character, the progress in
breeding for high protein has been slow and difficult. The first limitation is that genetic
variation for protein content is much less than that caused by differences in the growing
environments. At least 70% of the variation in protein content is determined by the
availability of nitrogen. The second limitation is that there is a very strong negative
relationship between grain protein percentage and yield; so that varieties with high
protein content tend to be low yielders.

However, there are exceptional varieties that combine excellent yield potential and
high protein content, probably by a more efficient relocation of nitrogen from senescing
tissues to grain, or by a more efficient uptake of nitrate and ammonia from the soil
(Blackman and Payne 1987). Examples of these varieties are Atlas 66 (from USA) and
Avalon from UK (Bingham et al., 1991). Avalon has between 0.5% and 1% more protein
than would be expected from its yield. In both cases the use of chromosome substitution
lines showed that chromosome 5D was responsible for a large proportion of the higher
seed protein content observed in these two varieties (Morris et al., 1973; Snape et al.,
1996). A recent study using recombinant lines of Avalon chromosome 5D demonstrated
that a large proportion of the high protein of this variety is due to a major gene located
on the 5D short arm and closely linked to the locus Ha determining grain hardness
(Snape et al., 1996). Molecular markers are available for this distal region of
chromosome arm 5DS (Dubcovsky and Dvorak 1995, Giroux et al., 1997) that allow
the manipulation of this chromosome segment in marker assisted selection programs.

An additional source of high GPC was detected in a survey of wild tetraploid Triticum
turgidum ssp. dicoccoides (hereafter referred as dicoccoides) (Avivi 1978). Substitution
lines of the chromosomes of one high grain protein accession in the cultivated durum variety Langdon showed gene(s) for high protein content to be present on chromosome 6B (Joppa and Cantrell 1990). Quantitative trait loci analysis (QTL) of the recombinant substitution lines of chromosome 6B from dicoccoides and chromosome 6B from Langdon was used to determine the location of the gene(s) responsible for the high GPC. RFLP markers flanking the dicoccoides segment were located in the centromeric region of the short arm of chromosome 6B (Joppa et al. 1997). This segment accounted for 66% of the variation in GPC observed in this cross.

R. Frohberg crossed the same dicoccoides accession with hexaploid wheat to develop a high GPC variety Glupro. Molecular characterization of Glupro done recently show that Glupro has the complete long arm of chromosome 6B from dicoccoides and a proximal segment of the short arm that includes the segment where the QTL for high GPC is located (Khan, I.A., Tranquilli, G. and Dubcovsky, J. unpublished). Currently a ribosomal gene is being used as a molecular marker for the GPC gene in the MAS program.

The ribosomal genes completely linked to the RFLP locus Xmwg79 were mapped. These showed the strongest association with the QTL for high GPC in the tetraploid mapping population used by Joppa et al., 1997, (Khan, I.A., Tranquilli, G. and Dubcovsky, J. unpublished). The same molecular markers can be used to manipulate this segment in tetraploid and hexaploid wheat. In both cases DNAs are digested with the restriction enzyme Taq I, transferred to nylon membranes, and hybridized with the clone pTa250.15 (Appels and Dvorak 1982) that encompasses the intergenic spacer of the ribosomal gene (Fig. 1). Since ribosomal genes are repeated thousands of times in the NOR loci, an autoradiography from the hybridization with pTa250.15 can be developed in less than an hour, and the probe can be reused many times for up to one month. Alternatively microsatellite markers Xwms508 and Xwms192 flanking the QTL can be also used to manipulate the trait in a MAS program using a PCR based technique (A. R. Schlatter and J. Dubcovsky unpublished).

Molecular markers are particularly well suited to manipulate traits with a large environmental variance like GPC. Moreover, the use of molecular markers for this trait is helpful to avoid the undesired negative selection for yield that would result from a direct selection for high grain protein content.

**Molecular markers for gluten strength**

*Bread making* quality depends not only on the quantity but also on the composition of the grain storage proteins. Since the composition of the grain storage proteins is less affected by the environment than the protein content, it is easier to manipulate in wheat breeding programs. The composition of gliadins and glutenins is particularly important for BMQ because they impart the viscoelasticity to dough.
Gliadins are monomeric proteins with intramolecular disulfide bonds and have a relatively lower effect on quality than glutenins. The reported association between grain quality and the presence of some gliadin alleles was generally explained by the tight genetic linkage between the \textit{Gli-1} locus encoding for gliadins and the \textit{Glu-3} locus encoding for LMr-GS (Metakovsky \textit{et al.}, 1990; Pogna \textit{et al.}, 1990). However, it is difficult to rule out the possibility of a direct effect of gliadins on BMQ because mutant \(\alpha\)-type and \(\gamma\)-type gliadin subunits can form intermolecular disulfide bonds. These mutant gliadins can function as chain terminators and modify the average length of the gluten polymers (Lew \textit{et al.}, 1992).

Glutenins, on the other hand have a polymeric structure, determined by the presence of intermolecular disulfide bonds that allow retention of \(\text{CO}_2\) and have a major effect on BMQ. This protein fraction has been categorized according to the relative mobility \((M)\) of the subunits following their reduction in SDS-PAGE into high \(M\), glutenin subunits (HMr-GS) and low \(M\), glutenin subunits (LMr-GS). Correlations between specific HMr, glutenin subunits and the \textit{bread making} quality of the wheat flour have been extensively studied. These studies have shown that variation in HMr-GS composition can account for up to 50\% of the variation in BMQ (Payne \textit{et al.}, 1987; Payne \textit{et al.}, 1988; Rogers \textit{et al.}, 1989). The genetic origin of the variation not explained by the HMr-GS is an area of active research in our laboratory.

Although LMr-GS are present in larger proportions than the HMr-GS, correlations between these polypeptides and BMQ have been studied only recently. Studies using SDS-PAGE and sequential extraction procedures for separating LMr-GS from gliadins.
(Gupta and MacRitchie 1991; Singh et al., 1991) showed that allelic variation at loci encoding LMr-GS can have effects on quality approaching those of the HMr-GS (Gupta et al., 1991; Khelifi and Branlard 1992; Gupta and MacRitchie 1994; Rousset et al., 1996; Manifesto et al., 1998).

The association between molecular markers and bread making quality (BMQ) in a cross between Chinese Spring and Klein 32, with same high Mr-glutenin SDS gel electrophoresis pattern, has been studied recently. Highly significant (p < 0.01) differences in SDS sedimentation tests and mixograms were detected for XGli-B1 / XGlu-B3 loci on chromosome arm 1BS. The increase in the number of Klein 32 alleles at these loci determined a linear increase in sedimentation and mixogram values. It was not possible to differentiate the effect of XGli-B1 from that of XGlu-B3 because of the close linkage between these two loci. These two loci considered together explained from 11% to 15% of the variation in BMQ observed in this cross. The inclusion of the protein content of each sample as a covariable in the model increased the proportion of variation in SDS sedimentation explained by the analysis up to 46%. Similar results were obtained in a different study using recombinant substitution lines of Cheyenne and Chinese Spring. Chinese Spring alleles at the XGli-B1/XGlu-B3 loci also showed a detrimental effect on BMQ parameters compared with the positive effect of Cheyenne alleles (Rousset et al., 1996).

At present XGli-B1/XGlu-B3 alleles from Klein 32 (Manifesto et al., 1998) and Cheyenne (Rousset et al., 1996) are being introgressed into adapted cultivars to evaluate their relative value to other alleles. A microsatellite marker located within the gliadin gene in chromosome 1B (Devos et al., 1995) is being used to facilitate this introgression. This marker produces a 252 bp marker in Klein 32 and a 219 bp band in Cheyenne. The strategy is to simultaneously introgress the variation in HMr-GS and LMr-GS in order to manipulate a larger proportion of the variation in gluten strength than with the HMr-GS alone.

With the incorporation of different combinations of transgenic HMr-GS, an alternative strategy to manipulate gluten strength is now available to wheat breeders. These new loci have been stable for many generations and can be readily transferred to adapted germplasm by backcrossing. Dr. O. Anderson presents a detailed description of the current status of transgenic HMr-GS in wheat in another paper in these Proceedings.

**Molecular markers for hardness**

Wheat grain end-use properties are affected markedly by endosperm texture, i.e. the hardness or softness of the grain, making this an important quality criterion. Hard wheats require more grinding energy to reduce endosperm into flour and during this milling process a considerable number of starch granules become physically damaged. Soft wheats, by contrast, produce flours with lower levels of damaged starch. Since
damaged starch granules absorb more water than intact undamaged granules, water absorption is generally higher for hard wheat flours than for soft wheat flours at an equivalent level of protein. Water absorption is an important quality character for baking because it is related directly to the amount of bread that can be produced from a given weight of flour. It also influences the crumb softness and bread-keeping characteristics strongly. Flours from hard wheats are preferred for bread making while flours from soft wheats are preferred to manufacture cookies and cakes.

During the last five years several studies have been directed to define the biochemical basis for the regulation of endosperm texture. Micropenetrometer tests on starch granules and the surrounding protein matrix showed no differences in hardness between hard and soft wheats for these components. This indicates that differences in endosperm texture are due to the differences in the strength of the adhesion between the starch granule surface and the surrounding matrix. Protein examination from well-washed starch preparations by SDS-PAGE has shown that the amount of a small, Mₘ 15k starchy surface-associated protein is present at relatively low levels on hard wheat starches and at relatively high levels on soft wheat starches (Schofield 1994). This association provides an indication, although not proof, of a causal role for this protein named ‘friabilin’, in controlling endosperm texture. Though the main locus, in the distal part of chromosome arm 5D, controlling endosperm texture is referred as the ‘hardness’ (Ha) locus, softness is in fact the dominant trait.

Friabilin is not a single polypeptide but a composite of related proteins that include puroindoline a (pinA), puroindoline b (pinB), and grain softness protein (GSP). GSP, pinB and pinA have been mapped on the distal part of chromosome 5DS completely linked to the Ha locus (Jolly et al., 1996; Sourdille et al., 1996; Giroux and Morris 1997). Deletion of the pinA gene or a glycine-to-serine mutation in the pinB gene have been associated with hard textured endosperm (Giroux and Morris, 1997; Giroux and Morris, 1998). These results suggest that both pinA and pinB proteins work in concert for the expression of grain softness and reinforce the conclusion that the presence of high levels of friabilin on starch granules and endosperm softness may be more than causally related. Friabilin appears to be a starch granule surface protein and is therefore appropriately located to influence the adhesion between the granule surface and the surrounding protein matrix.

Preliminary results suggest that bread wheat varieties with the null pinA deletion be harder than the varieties with glycine-to-serine mutation in the pinB gene (Giroux et al., 1998; Morris et al., 1998). This indicates that it may be possible to make smaller adjustments in the hardness level of a hard variety by replacing the glycine-to-serine mutation in pinB by the deletion of pinA.

Recent results have shown that GSP and pinA are very tightly linked. No recombinants between these two markers were found in a population of 550 F₂ plants of diploid wheat
(Tranquilli, G. Lijavetzky, D. and J. Dubcovsky, unpublished), suggesting a maximum distance of 0.03 cM with a probability of 95% (Hanson, 1959). Since no recombination has been observed between Ha and pinA, pinB and GSP, it is possible to conclude that these three genes are tightly linked. Consequently, polymorphism at any of these genes can be used as molecular marker for grain hardness.

The RFLP clone MTA9 for the pin A gene (Gautier et al., 1994) can be used in MAS to introgress the active pinA gene from a soft variety into pinA null hard variety. However, this marker can not be used to introgress a pinA null mutation by backcrossing into a soft variety because the heterozygous BC plants can not be differentiated. A better alternative is to use a PCR marker for the single base pair mutation in the pinB gene. Giroux and Morris (1997, 1998) designed PCR primers based on this mutation that discriminate between hard and soft textured varieties. However, since the discrimination between these two alleles is based on a single base pair difference at the 3' end of the primers, occasional false positives are observed if the PCR conditions are not perfectly optimized. An alternative strategy to avoid this problem has been developed recently. The pinB gene is amplified using the external primers designed by Gautier et al. (1994) and then the PCR product is digested with the restriction enzyme Bsr BI. This restriction enzyme cuts the sequence present in the allele of the hard textured varieties but not the allele present in the soft textured varieties. In this way, a codominant PCR marker is available for the Ha locus.

This PCR marker can be used not only to trace the glycine-to-serine mutation in the pinB protein but also to follow the closely linked pinA mutation. Since varieties showing the pinA null mutations have always the pinB-glycine allele (also present in soft varieties), the PCR marker can be used to increase slightly the grain hardness of a hard variety by replacing the glycine-to-serine mutation in the pinB gene by the null mutant in pinA.

**Conclusion**

In case these new molecular markers are proven to be adequate to manipulate a large proportion of the variation in BMQ, they can have a significant impact on the strategies of plant breeding. Currently, the SDS-sedimentation test is used extensively as an early-generation screen for breeding bread-quality in many wheat breeding programs around the world (Blackman and Payne, 1987). This screen usually results in the elimination of a large portion of the breeding lines before yield testing resulting in a lower probability of recovering higher yielding genotypes. If an appropriate BMQ can be recovered from poor BMQ lines by using marker assisted selection (MAS) for gluten strength, protein content, and grain hardness, selection for SDS sedimentation may be delayed in the breeding program. Breeding lines with low quality but outstanding yield potential can be corrected later on by marker assisted selection.
References


Avances en Marcadores Moleculares Para Calidad de Panificación

La composición de las proteínas de reserva en el grano, el contenido de proteínas en el grano y la dureza del grano son los factores principales que determinan la calidad de panificación. A pesar de que el efecto de las subunidades específicas de gluteninas específicas de alto peso molecular en la calidad de panificación está bien caracterizado, usualmente son responsables de menos del 50% de la variación entre cultivares de la fuerza del gluten. Un alelo específico Gli-B1/Glu-B3 de Klein 32 que es responsable de una proporción significativa de la variación en la fuerza del gluten ha sido identificado recientemente usando un marcador microsatélite. La introgresión de este alelo en combinación con subunidades apropiadas de glutenina de alto peso molecular pueden permitir una manipulación más eficiente de la fuerza del gluten. Además de la fuerza del gluten, el contenido de proteínas del grano (CPG) es probablemente el factor principal que afecta la calidad de panificación y para pastas. A pesar de la importancia de esta característica, los progresos en mejoramiento para alto CPG han sido lentos y difíciles debido al gran efecto del ambiente y a la fuerte correlación negativa entre el porcentaje de proteínas del grano y el rendimiento. Los marcadores moleculares ligados a un gen de alto CPG han sido recientemente identificados en el brazo corto del cromosoma 6B de Triticum dicoccoides. Un gran segmento de este cromosoma ha sido detectado en la variedad hexaploide Glupro de alto CPG. Varios marcadores moleculares pueden ser usados para introducir el gen de alto CPG tanto en variedades de trigos tetraploides como hexaploides. La manipulación del CPG y de la calidad de proteínas con marcadores moleculares puede ser ahora complementada con la manipulación de la dureza del grano usando técnicas similares. Un estrecho ligamiento entre la proteína relacionada con el tipo de grano blando y el locus de la purindoline-a (ambos asociados con la dureza) permite la combinación de los marcadores moleculares para ambos loci. Una estrategia que combina PCR y enzimas de restricción fue desarrollada para facilitar la introgresión de diferentes alelos de dureza en los programas de selección asistidos por marcadores.
Marker-Assisted Selection of Disease Resistance Genes in Wheat

James A. Anderson*

Abstract

Markers can increase selection efficiencies when breeding for disease resistance. The number of resistance genes for simply inherited wheat diseases mapped with markers is increasing on a monthly basis. However, their adoption in breeding programs is often slowed by the cost of this technology in terms of labor, equipment, and supplies. One widely used disease resistance marker is the endopeptidase isozyme that is linked with eyespot resistance derived from Aegilops ventricosum. This marker is widely used to introgress this chromosome segment into winter wheat in the Pacific Northwest of the U.S. Another use of markers in disease resistance is expected to arise from those diseases that are quantitatively inherited, but are difficult to screen for using conventional methods [e.g. Fusarium head blight (FHB) and tan spot]. FHB resistance genes have been mapped with the intent of using the markers in selection. Two populations were analyzed, resulting in the identification of six quantitative trait loci (QTL) associated with resistance. One QTL on chromosome 3BS explained more than 15% of the variation for FHB resistance in both populations, and two other QTL also were identified in both populations. PCR markers are being developed for two of these QTL to facilitate their introgression in adapted germplasm. Two major genes are largely responsible for tan spot resistance. One gene on 5BL conditions resistance to tan necrosis, and a QTL on 1AS in combination with other minor genes conditions resistance to chlorosis. Selection based on these two genes alone provides a high level of resistance to this disease.

Introduction

Genetic markers for disease resistance genes can increase efficiency of selection and will most likely be useful in the following cases:

i) Low heritability diseases (e.g. Fusarium head blight).
ii) Pyramiding genes (e.g. rusts, powdery mildew).

* Department of Agronomy and Plant Genetics, 411 Borlaug Hall, University of Minnesota, St. Paul, MN 55108, USA
Phone: 612-625-9763, FAX: 612-625-1268, email: ander319@tc.umn.edu.
iii) Lack of affordable or effective screening methods.

iv) Absence of pathogen (e.g. karnal bunt).

v) Accelerated backcrossing to recover the recurrent parent, especially in cases where non-adapted germplasm is used as the gene donor.

The identification of markers for qualitatively inherited genes is straightforward, typically based on $F_2$, $F_3$, doubled haploid, or recombinant inbred progeny from single crosses. Quantitative traits, on the other hand, require inbred lines or doubled haploids that can be replicated to allow for phenotypic evaluation in multiple environments. Quantitative trait loci (QTL) have been identified in wheat for resistance to leaf rust (Nelson et al., 1997; Williams et al., 1997), karnal bunt (Nelson et al., 1998), tan spot (Faris et al., 1997), and Fusarium head blight (Anderson et al., 1998a, b; Waldron et al., 1999).

More than 40 wheat disease resistance genes have been tagged with molecular markers (Feuillet and Keller, 1998; Langridge and Chalmers, 1998) (Table 1). The majority of these markers have been located using bulked segregant analysis (Michelmore et al., 1991) with RAPDs, or targeting specific chromosomes known to contain the gene(s) with RFLPs that were previously mapped. These approaches are used, when possible, because of the time and resource requirements necessary to

<table>
<thead>
<tr>
<th>Disease</th>
<th>Nº genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf rust$^2$</td>
<td>14+</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>8</td>
</tr>
<tr>
<td>Stem rust</td>
<td>5</td>
</tr>
<tr>
<td>Cereal cyst nematode</td>
<td>3</td>
</tr>
<tr>
<td><em>Fusarium</em> head blight (scab)$^2$</td>
<td>2+</td>
</tr>
<tr>
<td>Tan spot$^2$</td>
<td>2+</td>
</tr>
<tr>
<td>Karnal bunt$^2$</td>
<td>2+</td>
</tr>
<tr>
<td>Eyespot</td>
<td>2</td>
</tr>
<tr>
<td>Loose smut</td>
<td>2</td>
</tr>
<tr>
<td>Common Bunt $^1$(BT10)</td>
<td>1</td>
</tr>
<tr>
<td>Stripe rust $^1$(Yr15)</td>
<td>1</td>
</tr>
<tr>
<td>Wheat streak mosaic virus $^1$</td>
<td>1 (Wsm1)</td>
</tr>
</tbody>
</table>


$^2$ Includes quantitative trait loci (QTL).
create complete linkage maps of wheat (Marino et al., 1996; Nelson et al., 1995a, b, c; Van Deynze et al., 1995) for the purpose of finding markers for genes of unknown chromosomal location.

Although the number of DNA markers for disease resistance genes in wheat increases on a monthly basis, few examples of their use in breeding have emerged. Known examples, most of which have not been documented in the literature with regard to their specific use in breeding, include cereal cyst nematode (Cre1, Cre2, Cre3), eyespot (Pch1), leaf rust (Lr37), powdery mildew (Pm1), stem rust (Sr6, Sr22, and Sr38), stripe rust (Yr17), and wheat streak mosaic virus (Wsm1). These examples have emerged, despite considerable barriers to the use of markers in wheat breeding. These include:

i) Cost in terms of time, labor, equipment, and supplies.
ii) Technical expertise required.
iii) Low throughput compared to many conventional disease screening methods.
iv) Difficulty in recovering informative polymorphisms in breeding germplasm.
v) Changing magnitude of effects of genes (especially QTLs) across environments and in different genetic backgrounds.

Most wheat breeding programs are not equipped for the use of marker systems requiring PCR and/or gel electrophoresis. Instead, they must cooperate with molecular geneticists for this equipment and expertise required to utilize marker technology. A frequent statement at the end of abstracts of papers reporting associations of DNA markers with disease resistance genes in wheat is that the markers “...should be useful in MAS...”. Although the promise of markers for disease resistance is evident, the follow-through necessary to develop allele-specific primers and implement them in marker-assisted selection has been lacking in all but a few instances. Several examples follow that illustrate different aspects of the identification and implementation of markers for qualitatively and quantitatively inherited diseases of wheat.

Use of markers for screening disease resistance

Eyespot (Pch1)

One of the best examples of marker-assisted selection in all of plant breeding is the case of eyespot resistance in wheat. Eyespot (syn. strawbreaker foot rot) is caused by Pseudocercosporella herpotrichoides and results in weakening of stem bases and lodging. Disease severity of lines is assessed in a quantitative manner following field-based inoculations or growth chamber evaluation after inoculation with a GUS-transformed strain (de la Peña and Murray, 1994). One potent eyespot resistance gene (Pch1) is derived from Aegilops ventricosa (Maia, 1967). This gene was introgressed to a segment on chromosome 7D and is associated with the isozyme marker for endopeptidase (Ep-D1b).
James A. Anderson

(McMillin et al., 1986; Worland et al., 1988). This marker has been widely used by wheat breeding programs in the Pacific Northwest, USA, and Europe.

In the USDA-ARS wheat breeding program at Pullman, Washington, lines were screened for presence of the diagnostic A. ventricosa derived isozyme prior to preliminary yield trials. Prior selection in headrows was applied for such characters as heading date, height, agronomic type, rust resistance, and grain quality to reduce the number of lines for isozyme testing. All lines that survived the screening at the headrow generation and had a parent containing Pch1 were screened for presence of the marker. A single person could process about 60 samples in a day and a total of 200-400 lines were tested for the isozyme marker on a yearly basis. Due to the presence of a third isozyme phenotype that was not associated with resistance or susceptibility, all lines believed to possess Pch1 were tested under field conditions to confirm their eyespot resistance for at least two years prior to cultivar release. The field screening also allowed identification of germplasm containing genes that provided greater levels of resistance than Pch1 alone.

**Wheat streak mosaic virus (Wsm1)**

Wheat streak mosaic virus is transmitted by the wheat curl mite. Screening for resistance is accomplished by infecting with the virus, followed by visual observations of disease symptoms and/or ELISA. The resistance gene Wsm1 is on an Agropyron intermedium chromosome segment that has been translocated to chromosome 4D (Friebe et al., 1991). Talbert et al. (1996) developed a STS marker derived from a RAPD that is diagnostic for the presence of the resistance gene at an incidence of 98-99%. This marker is being used in spring and winter wheat breeding programs at Montana State University (L.E. Talbert, personal communication). Undergraduate labor is used during the summer months to screen approximately 500 lines per year for the presence of the marker. The fact that this gene is derived from A. intermedium means that little or no recombination will be expected between the gene and marker. The 1-2% recombination observed has not been explained.

**Leaf rust (Lr10)**

The leaf rust resistance gene Lr10 is derived from CIMMYT germplasm and is a component of multigene resistance of many North American wheat cultivars (McIntosh et al., 1995; Pretorius and Roelfs, 1996). A putative resistance gene at this locus (Lrk10) has been cloned (Feuillet et al., 1998). By sequencing this gene in different germplasm, primers that amplify a fragment unique to germplasm carrying Lr10 have been produced (Schachermayr et al., 1997). This represents perhaps the best example to date of the development of allele-specific primers for a disease resistance gene in wheat. Despite the availability of diagnostic PCR markers for this gene, they remain largely unused in breeding programs (M. Winzeler, personal communication).
**Leaf rust (quantitative)**

The leaf rust resistance gene *Lr34* in combination with two to three other genes has provided durable resistance to this pathogen (Singh and Rajaram, 1992). *Lr13* also has been effective in combination with *Lr34* and other genes (Roelfs, 1988; Kolmer, 1992). *Lr34* is associated with leaf tip necrosis (Singh, 1992), but this is difficult to score in some environments. Markers for adult-plant, slow rusting genes are desired to keep these effective complexes intact, especially in the presence of seedling resistance genes. To date, nine wheat genomic regions have been identified that either enhance or suppress adult-plant leaf rust resistance, six of which were hypothesized to be known *Lr* genes or suppressors (Nelson *et al.*, 1997; William *et al.*, 1997). The QTLs individually accounted for 9 - 45% of the phenotypic variation in leaf rust resistance in recombinant inbred populations. The only two QTL with consistent effects were the *Lr34* region on 7DS and a region on 7BL. The *Lr34* region was significantly associated with reduction in leaf rust disease in all 12 environments examined and accounted for 16 - 45% of the phenotypic variation. Because of the variable effects of the QTLs detected in different environments (Nelson *et al.*, 1997), despite being in the same genetic background, the power of these genes will be in their specific combinations that are more likely to be effective across environments and in different germplasm.

**Tan spot**

Tan spot, caused by *Pyrenophora tritici-repentis*, behaves in many breeding populations as a quantitatively inherited disease (Nagle *et al.*, 1982; Elias *et al.*, 1989). Lamari and Bernier (1991) reported two gene inheritance of this disease with a dominant gene responsible for a chlorosis factor and a recessive gene responsible for a necrosis factor. In the genetic dissection of the chlorosis component, a major QTL (*QTsc.ndsu-1A*) was identified on chromosome 1AS, a minor QTL near the centromere of 4A, and an epistatic interaction between *QTsc.ndsu-1A* and a locus near the centromere of 2DL (Faris *et al.*, 1997). Together, these QTLs explained 49.0% of the variation in tan spot reaction. A qualitative gene (*tsn1*) for insensitivity to a tan spot necrosis toxin was mapped on chromosome 5BL (Faris *et al.*, 1996). These markers have not been used in wheat breeding because of the comparative ease of screening by infusing young leaves with either chlorosis or necrosis toxin and observing the symptoms that develop. Nevertheless, these experiments illustrate the power of genetic markers to dissect a relatively complex inheritance into distinct components controlled by different genetic systems.

**Fusarium head blight**

Recent epidemics of *Fusarium* head blight (FHB) in the USA and Canada have caused severe yield and end-use quality losses in common (*Triticum aestivum* L.)
and durum (*T. turgidum* L.) wheat (McMullen *et al.*, 1997). *Fusarium* head blight resistance is inherited as a quantitative trait and numerous chromosomal regions have been reported to influence resistance (reviewed by Bai and Shaner, 1994). Because of the difficulties in breeding wheat for resistance to this disease using conventional methods, the identification of DNA markers associated with resistance would be useful for wheat breeders and geneticists.

Two recombinant inbred populations (Sumai 3/Stoa and ND2603(Sumai 3/Wheaton)/Butte 86) were grown in the greenhouse and evaluated for reaction to inoculation with conidia from *Fusarium graminearum* in two experiments each (Anderson *et al.*, 1998a, b; Mitchell Fetch *et al.*, 1998; Waldron *et al.*, 1999). The procedure used, placing a conidial suspension directly into a single spikelet near the center of the spike, bypasses primary infection and targets Type II resistance (Schroeder and Christensen, 1963; Mesterhazy, 1995).

Both populations displayed a near normal distribution, transgressive segregants, and significant variation among RILs for FHB severity. A map of 511 RFLP and AFLP markers was utilized in the Sumai 3/Stoa population to detect QTL. Those markers associated with FHB resistance in this population were subsequently mapped in the ND2603/Butte 86 population. Six genomic regions containing putative quantitative trait loci (QTL) were associated with FHB resistance in the Sumai 3/Stoa population from the combined analysis of two experiments (Table 2).

**Table 2.** Coefficients of determination and *P* values for DNA markers associated with *Fusarium* head blight resistance in the Sumai 3/Stoa and ND2603/Butte 86 recombinant inbred populations.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome</th>
<th>Resistant Source</th>
<th>Sumai 3/Stoa <em>R</em>² x 100</th>
<th><em>P</em></th>
<th>ND2603/Butte 86 <em>R</em>² x 100</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>X*EagcMeta.1</strong></td>
<td>3BS</td>
<td>Sumai 3</td>
<td>17.6</td>
<td>&lt;0.001</td>
<td>15.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>X*ksuH16</strong></td>
<td>2AL</td>
<td>Stoa</td>
<td>14.3</td>
<td>&lt;0.001</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>X*PaccMcga.1</strong></td>
<td></td>
<td>Sumai 3/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>X*ffbb82</strong></td>
<td>6BL1</td>
<td>ND2603</td>
<td>8.9</td>
<td>0.004</td>
<td>6.3</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>X*wgw909</strong></td>
<td>4BL</td>
<td>Stoa</td>
<td>7.2</td>
<td>0.007</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>X*bcd331</strong></td>
<td>6BS</td>
<td>Sumai 3</td>
<td>6.0</td>
<td>0.010</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>X*cd0524</strong></td>
<td>6BS</td>
<td>Sumai 3</td>
<td>3.9</td>
<td>0.049</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>X*bcd941</strong></td>
<td>3AL</td>
<td>ND2603</td>
<td>1.9</td>
<td>0.27</td>
<td>9.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1 Both **X*PaccMcga.1** and **X*ffbb82** were mapped in the Sumai 3/Stoa population and are less than 10 cM apart on chromosome 6BL, thus likely representing the same QTL. Only **X*ffbb82** was mapped in the ND2603/Butte 86 population.
Encouragingly, the two markers associated with FHB resistance from Sumai 3 in the Sumai 3/Stoa population were also associated with resistance in the ND2603/Butte 86 population (Table 2). This is one important step in verifying the effectiveness of these markers in other genetic backgrounds. In addition, another RFLP marker associated with resistance from ND2603 was serendipitously discovered in this population. Loci associated with FHB resistance from Stoa have not been mapped in ND2603/Butte 86 due to lack of polymorphism.

A large proportion of the lines carrying the Sumai 3 allele of the AFLP locus XEagcMcta.1 were relatively resistant (Fig. 1), including 12 of the 14 most resistant lines. Lines carrying the Stoa allele were uniformly distributed across FHB severity scores. These results indicate that the effect of the resistance gene linked to XEagcMcta.1 was partially masked by the Stoa allele at this locus or other QTL; therefore, this QTL may have a greater effect on conditioning FHB resistance in other genetic backgrounds. The marker XksuH16 showed the opposite phenotypic pattern in that a high proportion of the RI lines having the Stoa allele were resistant, whereas those having the Sumai 3 allele showed a uniform distribution across FHB severity scores.

![Histograms](image)

**Fig. 1.** Histograms for the Sumai 3 and Stoa alleles in their recombinant inbred population at the two most important QTLs for Fusarium head blight resistance.
A major concern in the implementation of MAS for QTL is the consistency of the gene effects across environments. Both of these populations were evaluated twice. The four Sumai 3/Stoa markers with the greatest effects were stable in the two experiments (Fig. 2), indicating that they may be useful in selection. Results from the ND2603/Butte 86 population were more disappointing as none of the three QTL were significantly associated with FHB resistance at the 0.001 probability level in both experiments.

Fig. 2. Percent of phenotypic variation explained of DNA markers associated with Fusarium head blight resistance in a recombinant inbred population of Sumai 3/Stoa. The symbols *, **, *** indicate significance at the 0.05, 0.01 and 0.001 levels of probability, respectively.

The most significant genomic region associated with FHB resistance in these populations is located on the short arm of chromosome 3B. Interval analysis revealed a peak LOD score of 6.3 for this region in the Sumai 3/Stoa population. The leaf rust resistance gene Lr27 also has been located to this region in a different population (Nelson et al., 1997). These markers may have utility in indirectly selecting for FHB resistance, thereby accelerating the development of resistant varieties. Future research will focus on i) mapping additional markers on 3BS and obtaining PCRable markers for this region; and ii) verifying these and other new markers in the ND2603/Butte 86 population and others (Fig. 3).
Fig. 3. Percent of phenotypic variation explained of DNA markers associated with Fusarium head blight resistance in a recombinant inbred population of ND2603/Butte 86. The symbols *, **, *** indicate significance at the 0.05, 0.01 and 0.001 levels of probability, respectively.

Conclusions

To date, marker-assisted selection has been most often employed for disease resistance genes in wheat when the objective is to pyramid resistance genes or avoid difficult phenotypic screens. Also, it is probably not a coincidence that of the few examples of MAS for disease resistance in wheat, several are for genes not from the primary gene pool of Triticum (e.g. Cre2, Lr37, Pch1, Sr38, Wsm1, Yr17), thus increasing the likelihood of generating allele-specific primers and decreasing the likelihood of recombination between the gene and marker.

Remedies to several of the existing barriers to the implementation of MAS for disease resistance in wheat are emerging. There have been incremental improvements on a yearly basis to reduce cost, improve throughput and reduce the technical expertise required. The recent availability of SSR markers (Röder et al., 1998) mean that wheat workers may finally have a set of highly polymorphic PCR markers for gene mapping
and selection. If DNA chips (Lemieux et al., 1998) can be affordably produced, they may remedy these problems and provide a high throughput means of diagnosing the presence of disease resistance genes in wheat. The problem of varying magnitude of gene effects over germplasm and environments can be addressed by i) selecting those markers (genes) with consistent, non-epistatic effects; ii) concentrating on major genes or specific resistance components; or iii) transferring groups of genes. The chances of recovering diagnostic polymorphisms can be enhanced by obtaining marker sequence from germplasm with and without the gene. As more disease resistance genes are cloned, direct allele selection (Sorrells and Wilson, 1997) can be employed to search for germplasm with superior resistance alleles.

References


Selección de Genes para Resistencia a Enfermedades en Trigo Asistida por Marcadores

Los marcadores pueden aumentar la eficiencia de selección en mejoramiento para resistencia a enfermedades. El número de genes con herencia simple para resistencia a las enfermedades de trigo, mapeados con marcadores aumenta mensualmente. Sin embargo su adopción en los programas de mejoramiento a menudo es lenta debido al costo de esta tecnología en términos de labor, equipamiento y suministros. Uno de los marcadores ampliamente utilizado para resistencia a enfermedades es la isoenzima endopeptidasa que está ligada a la resistencia a mancha ocular derivada de Aegilops ventricosum. Este marcador está ampliamente utilizado para introducir este segmento cromosómico en trigos invernales en la costa Noroeste del Pacífico en los EEUU. Otro uso de marcadores en la resistencia a enfermedades se prevé que provenga de aquellas enfermedades que se heredan en forma cuantitativa, pero que son difíciles de seleccionar usando métodos convencionales. (por Ejem. Fusariosis de la espiga y mancha amarilla o parda). Los genes de resistencia para fusariosis han sido mapeados con el propósito de utilizar los marcadores en selección. Dos poblaciones fueron analizadas, resultando en la identificación de 6 loci para caracteres cuantitativos (QTL’s) asociados a la resistencia. Un QTL en el cromosoma 3BS explica más del 15% de la variación para la resistencia a Fusarium en ambas poblaciones y otros dos QTL’s también fueron identificados en ambas poblaciones. Marcadores para PCR están siendo desarrollados para dos de estos QTL’s, para facilitar su introgresión en germoplasma adaptado. Dos genes mayores son los responsables principalmente de la resistencia a mancha amarilla. Un gen en el cromosoma 5BL condiciona la resistencia a la necrosis y un QTL en el cromosoma 1AS en combinación con otros genes menores condiciona la resistencia a la clorosis. La selección, basada en sólo estos dos genes, proporciona un alto nivel de resistencia a esta enfermedad.
Microsatellites in Wheat: An Useful Tool for Variety Identification and Breeding

M.M. Manifesto*1,2, A.R. Schlatter1, H.E. Hopp2, E.Y.Suárez1 and J. Dubcovsky3

Abstract

Thousands of hypervariable regions called microsatellites or simple sequence repeats (SSR), present in plant and animal genomes, are characterized by two to four pairs of bases in tandem repeats. These loci can be amplified through Polymerase Chain Reaction (PCR) using specific primers (18-20 pb long), which recognize unique flanking sequences of the hypervariable regions. Abundant variability present within these loci together with loci specificity and its codominant nature makes this kind of marker a powerful tool for wheat research. In this study the microsatellite markers were evaluated as a potential tool for finger printing of the wheat cultivars and for the studies on genetic variability.

Microsatellites, located at different chromosomes, were used to analyze a set of 105 bread wheat varieties from Argentina. Variability for each microsatellite locus was measured using the Polymorphism Information Content (PIC). PIC values for the different loci varied between 0.40 and 0.84. These values were compared with those obtained through RFLP in the same set of varieties, that reached an average value of 0.46 for the HMW-GS loci. These results suggest that microsatellite markers were an appropriate tool to discriminate this set of varieties.

Closely related varieties, that present high similarity coefficients, can be discriminated using selected microsatellites. Finally, a subset of microsatellites was used to construct an Identity Matrix for the registered bread wheat cultivars that will allow an unequivocal identification of each variety. The chromosome location, range in size, number of alleles and PIC of each microsatellite loci were parameters used to build the Identity Matrix mentioned above.

1 Laboratorio de Marcadores Moleculares, Instituto de Recursos Biológicos CIRN-INTA Castelar.
2 Instituto de Biotecnología CNIA, INTA Castelar. Cabañas y Reseros s/n, 1712 Castelar, Buenos Aires, Argentina.
3 Department of Agronomy and Range Science, University of California, Davis, 95616 CA, USA.
Corresponding author email: mmanifes@cicv.inta.gov.ar
Introduction

Characterization of wheat germplasm using DNA fingerprinting can provide the information required for a rational utilization of genetic diversity both in basic and practical research projects. Recently, one of the most common uses of fingerprinting has been to provide legal protection for the plant breeders to register new varieties. Although it has become an important feature of the fingerprinting, the technique can be used in the following areas:

* Discrimination of a large number of varieties to speed up their official registration and provide better legal protection to breeders of new varieties. Such a protection is essential to encourage private investment in plant breeding endeavors and develop novel and better cultivars.

* Pedigree and genetic relationship verification between varieties to facilitate an accurate selection of germplasm to be used in the breeding programs.

* Uniformity and stability verification of advanced lines through consecutive backcross cycles. Availability of uniform and properly identified lines is essential for breeding programs and for an efficient handling of different varieties.

* Genotypic fingerprinting of germplasm from Gene Banks to improve conservation criteria. A proper identification and genetic characterization allows detection of duplicates and defines a "core collection" to maximize genetic diversity.

* Molecular pedigrees can be used to determine the allelic contribution of each parent. Codominant markers such as microsatellites are very useful for this purpose.

* Analysis of variation in the gene pool of crops. Microsatellite markers can be used to quantify the genetic diversity in modern cultivars in relation to progenitor land races.

* Understanding the process of domestication involved in the evolution of crop plants. The data generated with this analysis can also provide useful criteria for enriching the gene pool of crop plants, and determine the efficiency of plant breeders in accessing all existing variation.

* To study genetic variation in natural populations or old germplasm as source of characters of interest.

* Screening of agronomic traits linked to some molecular markers.

* Fast and accurate identification of varieties will increase breeders' efficiency and reduce the period for registration of new varieties.
Criteria for development of an identification matrix

Identification and registration of bread wheat varieties (*Triticum aestivum*) is currently based on morphologic and physiologic characteristics. Even though these descriptors are useful, they are limited in number and may be affected by environmental factors. Being neutral to environmental effects, the molecular markers allow cultivar identification in early stages of plant development. Smith (1992) cited some prerequisites necessary for providing a reference system. To generate a practical, trust worthy and legally valuable system for variety identification, selected marker techniques must satisfy several criteria:

1) Descriptors must demonstrate high resolution for discrimination.

2) They should exhibit none or minimum interaction with environment.

3) Objective criteria must be developed for the accurate and repeatable recording of variety profiles and for translating them into an information database and also making valid comparisons among profiles.

4) The same class of descriptors should be capable to generate equivalent results amongst different laboratories.

5) They must allow the estimation of distances between genotypes consistent to other methods such as in pedigree. This is important because distance between cultivars is an integral component of evaluating novelty and of the degree of effort that has been necessary to produce a new genotype.

6) Known chromosomal location. When chromosome location is known, molecular markers can be selected to provide a better coverage of the genome.

7) Methodologies to generate band profiles into discrete identifiers must be publicly available, not only for research use but also for routine and widespread usage.

8) Selected methodologies can be automated.

9) They must have adequate cost/benefit relationship.

Molecular markers

The advent of DNA-based profiling methodology represents an increase in the power of discrimination among inbred lines and varieties. However, different molecular techniques have different limitations. Restriction Fragment Length Polymorphism technique, RFLP (Botstein *et al.*, 1980) is used widely to study germplasm associations according to the pedigree data. However, this technique has been of limited use in wheat (Dubcovsky *et al.*, 1995) and in other autogamous species like soybean (Skrupska *et al.*, 1993), due to narrow genetic base that determines
insufficient level of polymorphism to identify commercial varieties. It is also very laborious and requires DNA hybridization process that is costly and time consuming.

A more recent generation of molecular markers are based on the Polymerase Chain Reaction (PCR) technique. The first markers to be developed based on this technique were Random Amplified Polymorphism Detection, RAPD Markers (Williams et al., 1990). The advantage of this technique is that the primers can be made without previous knowledge of specific target DNA sequence. These primers are used single rather than in pairs, and provide amplification profiles that also reveal polymorphism across cultivars. Other advantages of this technique are that a) it is amenable to automation for breeding applications, b) small amounts of DNA are required, c) the assay is non-radioactive and can be performed in few hours, and d) no expensive laboratory equipment is needed. However, lack of reproducibility severely limits the application of RAPDs for varietal identification. This technique is extremely sensitive to the technical variations such as difference of thermocyclers, enzyme (Taq polymerase) or even the thickness of the tube for reaction. These characteristics limit the standardization of the technique and its value for legal purposes. Another disadvantage is that the chromosome location of these markers is generally unknown. Devos and Gale (1992) analyzed the use of random amplified polymorphic DNA markers and their limitations in wheat.

The Amplification Fragment Length Polymorphism (AFLP) technique combines digestion of DNA with restriction enzymes and PCR amplification (Zabeau 1993, Vos, et al., 1995). The method involves selective amplification of an arbitrary subset of restriction fragments, generated by enzymatic digestion of genomic DNA. Prior to the amplification, the end of every fragment in the digestion is modified by the addition of enzyme specific double strand adapters. The amplification is selective because each pair of primers used span the adapter as well as the nucleotides of the restriction fragment itself. Two to three random nucleotides are added to the 3' end of each primer to discriminate a subset of the restriction fragments. Only the restriction fragments that have ends homologous to the primers arbitrary 3'-sequences will be amplified out of the entire population. These additional bases can be modified to reveal different sets of bands.

This method is potentially very informative for the genome analysis and will probably be used widely. However, the markers developed by this technique are dominant, and it involves multiple laborious steps that require considerable technical skill. The large amount of genomic DNA used for AFLP assays must be of the highest quality and free of inhibitors or contaminants. Its application for fingerprinting is recommended only in those cases when no other marker system is available.

Microsatellites are an alternative PCR technique. Thousands of the hypervariable regions called microsatellites or simple sequence repeats (SSR), characterized by tandem repeats of 2 to 4 base pairs flanked by conserved regions are present in plant and animal genomes. These loci can be amplified through Polymerase Chain Reaction
(PCR) using specific primers (18-20 pb long) which recognize unique flanking sequences of the hypervariable regions.

The existence of these regions was first reported over 15 years ago by Hamada et al. (1982). Later Tauz and Renzhave (1984), confirmed abundance and ubiquity of microsatellites throughout the eukaryotic genomes. Though microsatellites were first discovered and used in human and mammal biology, their use for plant research can be significant. At present, large effort is being made to develop microsatellites in several crop plants such as tomato, barley, rice, sugar beet, maize, soybean, sunflower, etc. Different molecular markers systems are compared in Table 1.

**Table 1. Characteristics of different molecular markers systems.**

<table>
<thead>
<tr>
<th></th>
<th>RFLP</th>
<th>RAPD</th>
<th>AFLP</th>
<th>SSR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Principle</strong></td>
<td>Endonuclease restriction, Southern blotting, Hybridization</td>
<td>Amplification with random primers (10 pb long)</td>
<td>Endonuclease restriction, adapter linked, amplification with known sequence primers</td>
<td>Amplification with sequence specific primers (20 pb long)</td>
</tr>
<tr>
<td><strong>Type of polymorphism</strong></td>
<td>Single base changes, insertions, deletions</td>
<td>Single base changes, insertions, deletions</td>
<td>Single base changes, insertions, deletions</td>
<td>Number of repeats</td>
</tr>
<tr>
<td><strong>Level of polymorphism</strong></td>
<td>Medium</td>
<td>Medium</td>
<td>Medium / High</td>
<td>High</td>
</tr>
<tr>
<td><strong>Dominance</strong></td>
<td>Co-dominant</td>
<td>Dominant</td>
<td>Dominant</td>
<td>Co-dominant</td>
</tr>
<tr>
<td><strong>Amount of DNA required</strong></td>
<td>10 ug/ lane</td>
<td>10-25 ng/reaction</td>
<td>1 ug/ initial amplification</td>
<td>25-35 ng/ reaction</td>
</tr>
<tr>
<td><strong>Quality of DNA</strong></td>
<td>High</td>
<td>Medium</td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td><strong>Sequence information</strong></td>
<td>Yes/no</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Radioactive detection required</strong></td>
<td>Yes/no</td>
<td>No</td>
<td>Yes/no</td>
<td>Yes/no</td>
</tr>
<tr>
<td><strong>Development</strong></td>
<td>Complex</td>
<td>Easy</td>
<td>Easy</td>
<td>Complex</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>Medium/high</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td><strong>Complexity</strong></td>
<td>Medium/high</td>
<td>Low</td>
<td>Low</td>
<td>Medium/high</td>
</tr>
<tr>
<td><strong>Initial costs</strong></td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
<td>Medium/high</td>
</tr>
<tr>
<td><strong>Application time</strong></td>
<td>Time consuming</td>
<td>Fast</td>
<td>Time consuming</td>
<td>Fast</td>
</tr>
<tr>
<td><strong>Reproducibility</strong></td>
<td>Medium/high</td>
<td>Low extremely influenced by experimental conditions</td>
<td>Medium/High</td>
<td>High</td>
</tr>
</tbody>
</table>
Microsatellites

Microsatellites have been the markers of choice for identification analysis. Though microsatellites are based on a PCR technique like RAPDs, they differ from the latter in the amplification of known sequences from specific loci, and in the utilization of longer primers (18 to 24 bp) that result in more stable and reproducible results. Recently, numerous microsatellites have been developed in wheat (Stephenson et al., 1998, Röder et al., 1998) and have some particular characteristics that make them useful molecular tools:

* Multiallelic nature. They present high PIC values and demonstrate a greater discrimination power than RFLP (Röder et al., 1995), especially in self-pollinated crops and low genetic variability situations.
* High reproducibility based on PCR amplification technique.
* Co-dominant nature (visualization).
* Allow analysis of a single locus among the homoeologous groups of polyploid species.
* Easy detection by PCR.
* Relative abundance.
* Extensive genome coverage.
* Small amount of starting DNA.
* Mendelian heredity.
* Somatic stability.
* Exchangeable information consists on DNA sequences (no clones).

Microsatellites in wheat have been researched with respect to their abundance, chromosomal location, usefulness in related species and their polymorphic content compared with RFLP (Röder et al., 1995, Plaschke et al., 1996). They provide a good alternative marker system that can detect higher level of intraspecific polymorphism in wheat. Sequence characteristics of microsatellites in wheat genome was investigated and potential relationship between the usefulness of each microsatellite locus and their sequence features was discussed (Ma et al., 1996).

It has been estimated that on an average a repeat longer than 20 pb in length occurs every 33 Kb in plant nuclear genomes. Wheat genome contains on an average one (GT)n block every 704 Kb and one (GA)n block every 440 Kb. The average distance between any of the two-dinucleotide blocks is approximately 271 Kb. With a genome size of $16 \times 10^9$ bp per haploid genome of wheat (Bennett and Smith, 1976), the total number of microsatellite loci available is $3.6 \times 10^4$ (GA)n and $2.3 \times 10^4$ (GT)n (Röder et al., 1995).
Poly (A-C) and poly (A-G) were detected at a frequency of $5 \times 10^3$ and $3 \times 10^5$ respectively. The major disadvantage of these markers is their initial high cost to develop primers. However, this original investment is usually paid off by the repeated use of the same markers in multiple studies.

### Availability of microsatellites

There are two methods for microsatellite isolation, a) sequence analysis from databases and b) screening from a library. Standard method for the isolation of SSRs from a library involves creation of a small insert genomic library, its screening by hybridization, DNA sequencing of positive clones, primer design and locus-specific PCR analysis and identification of polymorphisms.

Currently there are more than four hundred microsatellite primers available publicly (Plaschke et al., 1995, Devos et al., 1995; Röder et al. 1995,1998; and Sorrells, 1995). Röder et al. (1998) reported the development of 230 polymorphic primer sets and a genomic map of wheat containing 279 microsatellite covering the seven homoeologous chromosome groups. At present, a set of microsatellites developed by Röder and Korzum, Germany, are being used in our laboratory to map *Triticum monococcum*. INTA, Castelar, also forms a part of the Wheat Microsatellite Consortium that includes many laboratories from different countries. These efforts have led to the sequencing of 48 clones and designing of 16 pairs of primers. Seven of them were mapped in the Synthetic Xopata population. Four of the remnant were monomorphic while the other five, though assayed under different amplification conditions (more than ten temperatures, several Mg concentrations), showed smears or no amplification at all. Currently more than 100 microsatellites from the Consortium are being mapped.

Two interesting microsatellite loci located within the coding region of seed storage proteins of low molecular weight LMW (XGlu-A3) and gamma-gli (XGli-B1) were isolated and characterized (Röder et al., 1995). These microsatellites have facilitated the characterization of allelic variation of these storage protein loci, and are currently being used in INTA wheat breeding program to select allelic variants with increased gluten strength. These SSR markers were used to characterize eight European cultivars (Devos et al., 1995) and eleven Canadian cultivars (Lee et al., 1995) of wheat bread. Same microsatellites have been used to characterize 105 Argentine bread wheats and another 100 bread wheat varieties from California (unpublished).

Other microsatellites have been used to provide markers for agronomically important genes and quantitative inherited traits and to facilitate their handling in the segregating progenies of the populations. The use of microsatellites linked to genes that affect breadmaking quality (Manifesto et al., 1998), or to the dwarfing gene Rht8 (Korzun et al., 1998 and Worland et al., 1998) has been successful. Wheat microsatellite WMS261, whose 192 pb allele was to be diagnostic tool for dwarfing gene Rht8 in the commercially
Use of microsatellites in fingerprinting wheat varieties

Potential of microsatellites in cultivar identification has been recognized by the Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular (UPOV, 1995), but it has still not been accepted as a single criteria for identity determination. In order to confirm their usefulness as a powerful tool for wheat cultivar fingerprinting and for studies of genetic variability, a set of 105 bread wheat varieties from Argentina was analyzed using microsatellites, located at different chromosomes.

Before going into fingerprinting, a screening was conducted to select primers showing high variability and clear band patterns. Amplification reactions were carried out according to the protocol recommendations and in a few cases with adjustments of annealing temperatures and Mg++ concentrations. The products of amplification were separated on (6%) polyacrilamide denaturing gels and were detected by silver staining. Size of each band was measured with a molecular weight standard and the aid of a sequence reaction. The results were scored manually. Differences in the size of the amplified fragments represent a different allele. The patterns of bands of different microsatellites used are shown in Figures 1, 2 and 3.

Variability for each microsatellite locus was measured using the Polymorphism Information Content (PIC). The PIC were calculated using the following formula (Anderson et al., 1993):

\[ \text{PIC}_i = 1 - \Sigma (n_j p_{ij}^2) \]

where \( p_{ij}^2 \) is the frequency of the \( i^{th} \) allele

PIC values provide an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values range from 0 (monomorphic) to 1 (very high discriminative, with many alleles in equal frequencies).

PIC values for the different loci analyzed in this study ranged from 0.40 to 0.84 with an average value of 0.62. These values were compared with those obtained with RFLP for HMW-GS loci, in the same set of varieties. The average value of PIC in the study using RFLP was 0.46. These results are similar to those reported by Röder et al. (1995) and suggest that the selected set of microsatellite markers was successful to characterize these varieties.

Later, a subset of 10 microsatellites was selected to build an Identity Matrix of the registered bread wheat cultivars in Argentina, in order to provide an unequivocal identification of each variety. Characteristics of the microsatellite loci used to build the
Figure 1. Microsatellites XGlu3-A (1AS) and Xgli-B1 (1BS). Each allele is represented by a major band and additional bands, which characterize each microsatellite. Reference band of each allele is pointed and its size in pair of bases is mentioned at the bottom of each gel.
Figure 2. Microsatellites Xcn15 (1AL) and Xwms-46 (7BS). Each allele is represented by a major band and additional bands, which characterize each microsatellite. Reference band of each allele is pointed, its size in pair of bases is mentioned at the bottom of each gel.
Figure 3. Microsatellites Xwms-44 (7DS) and Xwms-5 (3AS). Each allele is represented by a major band and additional bands, which characterised each microsatellite. Reference band of each allele is pointed, its size in pair of bases is mentioned at the bottom of each gel.
Identity Matrix and results obtained for each primer, including number of alleles and PIC value are shown in Table 2.

Microsatellites in XGlu-A3 and XGlsl-B1 are located within the seed storage protein loci (Devos et al., 1995). The null allele in XGlu-A3 correspond to the rye translocation 1AL/1RS (confirmed by gliadins) and the null allele in XGlsl-B1 corresponds to rye translocation 1BL/1RS (confirmed by cytology). This indicates that these primers do not anneal to the homoeologous rye sequence. All data obtained was used to construct a similarity matrix following the similarity coefficient of Jaccard. Cluster analysis was done using UPGMA and NTSys pc v. 2.0 computational programs. A dendrogram representing 105 bread wheat varieties is presented in Fig. 4.

The selected set of microsatellites was able to separate even closely related varieties representing high coefficient of similarity. In most of cases, the clustering of the varieties is in agreement with the data shown by the pedigree. Only two varieties presented a similarity coefficient of 1 (Prointa Don Alberto y Precoz Parana INTA) that can be differentiated by alleles at HMW-GS loci.

The relationship between different microsatellite alleles was also investigated by clustering analysis to evaluate character independence. A dendrogram of loci used to build the identification matrix is shown in Fig. 5. A low similarity coefficient value points out that the characters used were independent and non-redundant. Finally, the selected subset of the microsatellites was used to build an Identity Matrix of the cultivars to provide an unequivocal identification of each variety.

Table 2. Microsatellite loci, chromosome location, range in size, number of alleles and polymorphism information content (PIC) of each microsatellite used to build the Identity Matrix in 105 bread wheat varieties from Argentina.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome location</th>
<th>Range in size of base pairs</th>
<th>N° of alleles</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>XGlu-A3</td>
<td>1AS</td>
<td>133 a 157</td>
<td>10 + 1 null</td>
<td>0.84</td>
</tr>
<tr>
<td>XGlsl-B1</td>
<td>1BS</td>
<td>213 a 285</td>
<td>12 + 1 null</td>
<td>0.84</td>
</tr>
<tr>
<td>Xcnl5</td>
<td>1AL</td>
<td>115 a 129</td>
<td>5</td>
<td>0.40</td>
</tr>
<tr>
<td>Xcnl3</td>
<td>6BS</td>
<td>117 a 151</td>
<td>7</td>
<td>0.63</td>
</tr>
<tr>
<td>Xgwm46</td>
<td>7BS</td>
<td>159 a 187</td>
<td>11</td>
<td>0.76</td>
</tr>
<tr>
<td>Xgwm44</td>
<td>7DS</td>
<td>160 a 180</td>
<td>9</td>
<td>0.85</td>
</tr>
<tr>
<td>Xgwm2</td>
<td>2AS</td>
<td>140 a 164</td>
<td>7</td>
<td>0.73</td>
</tr>
<tr>
<td>Xgwm18</td>
<td>4BS</td>
<td>184 a 196</td>
<td>5</td>
<td>0.67</td>
</tr>
<tr>
<td>Xgwm33</td>
<td>1A, 1B, 1D</td>
<td>138 a 206</td>
<td>11</td>
<td>0.81</td>
</tr>
<tr>
<td>Xgwm5</td>
<td>3AS</td>
<td>167 a 173</td>
<td>8</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Fig. 4. Dendrogram showing family tree and similarity coefficients among 105 bread wheat varieties from Argentina.
Fig. 5. Dendrogram showing relationship among loci used to build Identification Matrix of 105 bread wheat varieties from Argentina.
Conclusions

High level of polymorphism shown by the microsatellite or SSR markers analyzed indicates that these molecular markers will be very useful in genetic studies in bread wheat which demonstrates relatively low levels of polymorphism. These generated polymorphism information content values are higher than those observed with RFLP. It was possible to estimate genetic variability present in this set of bread wheat varieties. The selected subset of microsatellites allowed the construction of an Identity Matrix that can be used to discriminate all the varieties analyzed in this work including those that are closely related.

References


Dubcovsky, J, M.C. Luo and J. Dvorak. 1995. Differentiation between homoeologous chromosome 1A of wheat and 1A\textsuperscript{th} of Triticum monococcum and its recognition by the wheat Ph1 locus. PNAS. In press.

Hamada et al. (1982). A novel repeated element with Z-DNA-forming potential is widely found in evolutionary diverse eukaryotic genomes. Nucleic Acid Research 10, 4127-4130


Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular. International Union for the Protection of New Varieties of


Resumen

**Microsatélites en Trigo: Una Herramienta Util para la Identificación Varietal y el Mejoramiento**

En los genomas de plantas y animales existen miles de regiones hipervariables denominadas microsatélites o secuencias de repetición simple (SSR), las cuales se caracterizan por 2 a 4 pares de bases repetidas en tandem. Estos loci pueden ser amplificados a través de la reacción de polimerización en cadena (PCR) del ADN, utilizando "primers" específicos (18-20 pares de bases de longitud) los cuales reconocen secuencias únicas en los flancos de las regiones hipervariables. La abundante variabilidad existente dentro de estos loci, así como su especificidad y naturaleza codominante hacen de este tipo de marcador una herramienta poderosa en trigo.

Se evaluaron microsatélites como marcadores con potencial de utilización para la identificación de cultivares de trigo y para estudios de variabilidad genética. Con este objetivo se analizó un conjunto de 105 variedades de trigo panadero de Argentina utilizando microsatélites localizados en diferentes cromosomas. La variabilidad para cada locus correspondiente a un microsatélite fue medida utilizando el índice de contenido polimórfico (PIC). Los valores de PIC para los diferentes loci variaron entre 0.40 y 0.84. Estos valores fueron comparados con aquellos obtenidos utilizando RFLP en el mismo conjunto de variedades, donde el valor promedio de PIC para los loci HMW-GS fue 0.46. Esto sugiere que los marcadores microsatélites son una herramienta apropiada para discriminar este conjunto de variedades.

Variedades cercanamente relacionadas, las cuales presentan altos coeficientes de similaridad podrían ser discriminadas utilizando microsatélites seleccionados. Finalmente, un subconjunto de microsatélites fue utilizado para construir una matriz de identidad de los cultivares de trigo panadero registrados, lo cual permitirá una identificación inequívoca de cada variedad. La localización cromosómica, el rango de tamaño, el número de alelos y los índices de contenido polimórfico de cada loci de microsatélite fueron utilizados para construir la matriz de identidad mencionada previamente.
Marker Assisted Selection: Is it Practical?

Mark E. Sorrells*

Abstract

Molecular markers have been proposed for use in fingerprinting, parental selection, monitoring homozygosity, outcrossing, or genetic stocks, for selection of loci controlling traits that are difficult or costly to evaluate, or have low heritability, and for selection against donor genome in backcrosses. The use of markers for these activities to date has been minimal, especially for crops such as wheat where the genetic system is complex. There are many potential problems. Some of the limitations include low polymorphism, complex inheritance, low precision in mapping, errors in assigning relative weights or in choosing loci, cost of developing and using molecular markers, undetected recombination between markers and trait loci, unknown linkage relationships between marker and trait alleles in genotypes other than parents of mapping populations, locus x year interaction, and negative pleiotropic effects. Because conventional breeding methods have been effective for improving quantitative traits, there is resistance to implementation of marker assisted selection due to labor and cost. Initially, marker assisted selection (MAS) in backcross populations using elite recurrent parents is the most efficient method for testing the efficacy of MAS. For species with low polymorphism, the use of hypervariable markers such as microsatellites is critical.

Applications of molecular markers

Molecular markers can be applied to crop improvement in several ways: 1) Markers can be used to identify and combine desirable genes from different varieties 2) Selection against undesirable linked genes and gene interactions can be practiced 3) Molecular markers expedite the introgression of novel genes from related species 4) Genetic diversity in the primary gene pool can be accurately assessed 5) Cultivars and germplasm can be positively identified by fingerprinting and 6) Genetic stocks can be monitored for homozygosity or outcrossing. Large numbers of molecular markers are now available for all major crop species and generally markers can be found that are closely linked to genes of interest.

* Department of Plant Breeding, Cornell University, 252 Emerson Hall, Ithaca, NY 14853-1902 USA 1902 USA. email:mes12@cornell.edu
The use of molecular markers has the potential to increase the efficiency of backcross breeding and overcome some limitations of the method. First, the segregating population can be screened at the seedling stage for RFLP markers and desirable individuals can be identified prior to hybridization with the recurrent parent. Segments from the non-recurrent parent can be monitored and individuals containing the highest proportion of the recurrent parent genome can be used in the next backcross. Individuals with desirable recombination events close to the genes of interest can be selected with accuracy. Finally, markers can be used to transfer genes controlling traits that are difficult to evaluate because of cost, complexity or low heritability. In spite of the many advantages, molecular markers have not received wide usage for wheat or barley for a number of reasons.

Potential limitations of marker assisted selection (MAS)

Low polymorphism

Low polymorphism is probably the most universal problem for breeders because the most frequently used germplasm is often closely related. Even if two genotypes differ for the trait and the genes that control it, linked molecular markers are frequently monomorphic making them impossible to use. Microsatellite markers are generally more polymorphic and should be used whenever possible. Recently, Röder et al. (1998) have published the chromosome locations of a large number of microsatellites for wheat; however, genome coverage using microsatellites is still inadequate for wheat.

Polygenic traits

Another common problem for breeders is that most traits of economic importance are quantitatively inherited and controlled by several genes with relatively small effects making them difficult to map precisely (Paterson et al., 1991). If the loci are not mapped precisely, recombination between marker and trait loci can go undetected. The alternative is to select very large portions of the chromosome. This increases the risk of including undesirable genes in the derived lines. In addition, estimates of locus effects are subject to sampling error causing errors when deciding which loci to select or when assigning relative weights. Because of the cost and logistics of following multiple loci in segregating populations, manipulating several loci for multiple traits becomes prohibitive. Lande and Thompson (1990) have elaborated theoretical aspects of marker-assisted selection for improvement of quantitative traits. They concluded that the efficiency of conventional methods of phenotypic selection could be substantially enhanced by combining them with marker-assisted selection for traits of moderate to high heritability. Currently, microsatellite markers are the least expensive kind of marker to use for MAS in wheat.
Unknown genotypes

In conventional breeding programs it is routine to introduce new or exotic germplasm into the crossing program; however, when using MAS if the linkage relationships between marker and trait alleles is different from the parents of mapping populations, it is impossible to know if the same markers can be used. Even more serious is the fact that the allele value of the quantitative trait loci (QTL) being selected is unknown because it has not been evaluated in a mapping population. Also, QTL may be expressed differently in different genetic backgrounds. If the size of the fragment in the new genotype is the same as in the original source genotype of the allele and the original source genotype is a recent parent in the pedigree, there is a good chance that the linkage relationship has been maintained. For other situations, unless a mapping population is constructed and trait and marker data analyzed for associations, there is no basis for assuming that a particular fragment is linked to a locus controlling the trait of interest.

Genotype x environment interactions

Paterson et al. (1991) and Dudley (1993) provided excellent overviews of the potential applications of QTL analysis to breeding. QTL for a range of traits - yield, malting quality, winterhardiness, disease resistance - have been located in a number of barley germplasm sources (reviewed by Hayes et al., 1996). The genetic bases of these QTL, are of great theoretical interest and practical importance. Robertson (1989) proposed that QTL are actually the result of loci where the alleles in question have a smaller differential effect on the phenotype than when mapped as a qualitative trait. For some traits, candidate genes can be proposed as QTL determinants; e.g., shattering resistance as a determinant of yield (Hayes et al., 1993). Several reports of significant QTL x environment interaction effects suggest that this is a common occurrence. In particular, QTL x year effects are difficult to assess and manage because year effects may not repeat in subsequent years. Genes that are not consistently expressed over multiple environments are not good candidates for MAS.

Undesirable pleiotropic effects

Negative pleiotropic effects where desirable effects on a trait are associated with undesirable effects on another trait are common and breeders have to deal with them constantly in conventional selection. Common negative associations include late maturity or taller plants and higher yield (Siripoonwiwat et al., 1996). Loci controlling these traits are not useful for MAS if later and taller genotypes are undesirable for commercial use. Quantitative trait mapping studies must incorporate evaluation for as many traits as possible to avoid this pitfall. There are numerous causes for negative associations and frequently they are caused by the same genes and cannot be separated by recombination. These genes that cause negative pleiotropic effects are not useful for MAS and if the undesirable traits are ignored in
QTL studies the result will be derived lines with little or no commercial value. One approach that has not been tested is to identify independent genes that do not exhibit negative pleiotropy and select desirable alleles at those loci to counteract the loci with the negative pleiotropic effects.

Conventional breeding methods have been effective for improving quantitative traits for many decades and until MAS techniques become routine and cost effective, there is little justification for implementation.

Considerations for implementing MAS

QTL mapping studies

When initiating a MAS selection project for a trait of interest there are several considerations. Before we can begin, a comprehensive QTL study must be conducted using a suitable mapping population that is segregating for traits of interest. The evaluation of traits of interest as well as traits that may be negatively associated must be conducted over years and locations that are representative of the commercial production region (Anderson et al., 1993). Then the phenotypic datasets for all environments are assembled and analyzed. The association of each marker with each trait in each trial is determined by ANOVA or simple regression. From these analyses, we can identify QTL that are associated with variation for a trait in one or more environments. Initially, the evaluation in the target region is critical, however, later it may be possible to combine QTL studies that have been conducted in different environments to enhance our understanding of the trait.

Selection of QTL and markers

Once we have completed a QTL study, we first identify the significant chromosome regions and select the markers. This must take into consideration the size of the locus effects, the number of significant loci, the proportion of the trait variance it explains and any negative pleiotropic effects. Selection of markers can also be difficult if the location of the QTL is not well resolved or if the location shifts slightly from one environment to the next. In addition, those QTL that are expressed in most environments should be given first priority. The selected genomic regions and the allele and trait of interest in each are then used to screen potential parents for marker allele polymorphism in each of these regions. Only those loci that are polymorphic between the source and recurrent parent for markers linked to a QTL can be used and selected in segregating populations.

Selection of donor parents

Parental selection may be complicated for MAS because different recurrent parents differ in their strengths and weaknesses. When there are multiple loci, we first need
a genotype that includes the desirable allele at each of the loci to be selected. If we are backcrossing to one of the same parents used to map the loci in a population, then only the alleles from the other parent need to be selected. If there were desirable alleles at different loci from each parent of the mapping population and we are backcrossing to a different genotype, then one of the transgressive segregant lines from the population would be a superior source parent. Recurrent parents of unknown genotype must be surveyed using the markers to determine if they are polymorphic with the source parent. Any QTL linked to monomorphic markers obviously cannot be selected.

Lines from the mapping population can be selected for use as source parents using an index. This allows one to chose lines that may be superior for traits other than those to be used in MAS. Typically we begin by calculating a mean rank of all lines across all trials for each trait. Then we weigh the breeding value of the line according to presence of desired alleles in the genomic regions chosen. Optional weighting factors could include estimates of the locus effect or of "economic value" of the trait. The total breeding value of each line is thus the product of the above weights taken at each of the regions and summed across loci. Finally we sort the lines on their breeding values and take the top few lines as donor parents. Further selection can be based on the haplotypes of the best individuals to identify those individuals with desirable crossovers and less non-recurrent parent genome.

Population size and generation of selection

After the parents, the QTL, and their markers have been selected, the next step is to determine how large the population must be to have a reasonable chance of recovering the selected alleles in one genotype. The volume of material that can be handled will depend on many factors, however, with careful planning, it is possible to harvest and store plant material or DNA from populations and spread out the screening operation. If there are doubts about the expression of the genes or linkage relationships, it may be wise to evaluate traits in intermediate generations using appropriate field plot technique.

The relative cost of marker-assisted selection at various stages of inbreeding or testing depends on the number of individuals evaluated and the number of markers used. In general, traits conditioned by one or very few loci will favor marker-assisted selection at advanced stages in the breeding program because a large proportion of the progeny will carry the desired allele(s) and the population can be screened for less costly traits first. However, in most situations (especially for wheat) marker-assisted selection will be most efficient in the $F_2$ or first backcross because these are the generations of maximum segregation. This will result in a population either homozygous or highly enriched for the desired alleles, depending on whether homozygotes or heterozygotes are selected. Cost of developing and using molecular
markers is still relatively high compared to conventional selection methods; however, the cost of the marker technologies and the infrastructure necessary for the research are gradually declining. Molecular markers are generally cost effective for only those traits that are difficult or costly to evaluate and are controlled by a few genes.

**Approaches to implementation**

There are several possible methods for implementation of MAS in a conventional breeding program and in general, the same principles apply that are used for other selection techniques. Backcrossing desirable alleles into elite genotypes is generally regarded as the most efficient approach and can be modified to suit the specific breeding goals and resources available. Integration of conventional and MAS using index and/or culling selection (multiple stage selection) may be useful for specific traits and germplasm provided population sizes can be kept small.

Other strategies that might facilitate MAS include the development and maintenance of a core set of germplasm accessions that has been evaluated for marker and trait phenotypes. Enhanced germplasm databases that combine information from various sources would enable the breeder to make more informed parental choices for the crossing block. Comparative maps for locating trait loci and markers can be quite useful (Van Deynze *et al.*, 1995). Comparative maps allow breeders to use genetic, physiologic, and other kinds of information that have been researched in other crop species to the benefit of wheat.

Markers can also be used as diagnostic for a particular allele provided the marker is the actual gene and the source of the allele is a genotype in the pedigree (Sorrells and Wilson, 1997). This has been referred to as “direct allele selection”. Identification of the genes controlling a trait and knowledge of their DNA sequence would facilitate classification of variation in the germplasm pool based on gene fingerprinting or characterization of variation in key DNA sequences. Classification of sequence variants at a targeted locus would substantially reduce the amount of work required to determine their relative breeding value and lead to the identification of superior alleles. Combining direct allele selection (DAS) with conventional selection, would allow more rapid and precise improvement of populations and breeding lines. Limitations of current technology can be minimized by transfer of genetic information across species, identification of highly variable genes, and focusing on the most important genes and traits for the species of interest.

Marker assisted selection is gradually being incorporated into conventional wheat breeding programs; however, because of cost MAS is currently only used to select loci of traits that are otherwise difficult or costly to evaluate.
Acknowledgements

I wish to thank Dr. Mohan Kohli and his staff for their hospitality and for making it possible for me to participate in the International Workshop on the Application of Biotechnologies to wheat breeding.

References


Resumen

La Selección Asistida por Marcadores: ¿Es Práctica?

Los marcadores moleculares han sido propuestos para utilización en identificación genética, selección de progenitores, monitoreo de homocigosis y alogamia, stocks genéticos, para selección de loci que controlan caracteres de evaluación difícil, costosa o que tienen baja heredabilidad, y para selección contra un genoma donador en retrocruzamientos. El uso de marcadores para estas actividades ha sido mínimo hasta ahora, especialmente para cultivos como trigo donde el sistema genético es complejo. Existen numerosos problemas potenciales incluyendo bajos niveles de polimorfismo, herencia compleja, baja precisión en el mapeo, errores en la asignación de las contribuciones relativas o la elección de loci de interés, costos para desarrollar y utilizar marcadores moleculares, recombinación no detectada entre los marcadores y el/los loci para los caracteres, relaciones de ligamiento desconocidas entre los alelos del marcador y del carácter en genotipos diferentes a los progenitores de las poblaciones de mapeo, interacción locus x año, y efectos pleiotrópicos negativos. Debido a que los métodos de mejoramiento convencionales han sido efectivos para mejorar caracteres cuantitativos, existe cierta resistencia a la implementación de la selección asistida por marcadores por sus requerimientos de trabajo y costos. Inicialmente, la selección asistida por marcadores (MAS) en poblaciones de retrocruzamiento que utilizan progenitores recurrentes superiores es el método más eficiente. Para especies con bajo polimorfismo, el uso de marcadores hipervariables como los microsatélites es crítico.
Routine Transformation System for Use with CIMMYT Wheat Varieties


Abstract

The inability to reach and regenerate the proper cells in the target tissue and problems related to gene expression have been major limiting factors affecting wheat transformations. This paper describes a routine biolistic bombardment-based transformation technology that overcomes these barriers and allows DNA transfer in wheat. The method involves subjecting the plant tissue to microprojectile bombardment with purified DNA and reduced levels of tissue culture manipulation. In vitro culture response of bombarded immature embryos and the production of transgenic plants were investigated in 3 wheat cultivars: Bobwhite, Baviacora, and Attila. In the three genotypes, at least 700 immature embryos were bombarded with a plasmid containing a PPT-resistant gene (Bar) under the control of the ubiquitin promoter that included the reporter gene Gus (β-glucuronidase). Although the transient expression of the reporter gene β-glucuronidase fused with the same promoter was similar in the three cultivars tested, the frequency of stable transformations varied with the genotype. The transformation frequency was highest in Bobwhite in which 0.24 to 0.44% of bombarded embryos produced plants. The other two varieties, Baviacora and Attila, also expressed Basta resistance but at a lower frequency than Bobwhite. Preliminary data suggests that the variation in the rate of transformation is more influenced by the differences among the genotypes than by the efficiency of the particle bombardment procedure.

Abbreviations: Phosphoeritromicin (PPT); Dichlorophenoxyacetic Acid (2,4-D); β-glucuronidase (Gus); Phosphinothricin acetyltransferase (Bar); inches cm square (ics).

Introduction

Microprojectile bombardment is currently the most widely applied technique for transferring genes to some Agrobacterium transformation recalcitrant species (Christou 1996). Transgenic cereals are commonly generated using this technology. Among the particle bombardments methods, the most successful one is based on Helium driven gun (Barcelo and Lazzeri 1995), that introduces gold coated particles of DNA into the target cells which proliferate in response to the somatic embryo induction medium.

* CIMMYT, Apdo. Postal 6-641, CP06600 Mexico D.F., Mexico. Corresponding author email: a.pellegrineschi@cgiar.org
The transformation of wheat through particle bombardment was first reported by Vasil et al. (1992) and since then the reports have followed from several others laboratories (Becker et al., 1994; Altpeter et al., 1996). Transformation efficiencies have varied in different studies between 0.15 to 0.5% (Weeks et al., 1993; Altpeter et al., 1996a, b) and 0.5% to 1.5% (Vasil et al., 1993, Becker et al., 1994; Nehera et al., 1994). While initial successes were achieved using established long-term embryogenic cultures (Vasil et al., 1992), recent studies have demonstrated consistent results using freshly initiated cultures from immature embryos (Nehera et al., 1994). However, all reported transformation frequencies are based on a single genotype (Bobwhite). Most wheat transformation groups still use one or two specific wheat varieties that have demonstrated favorable response in the tissue culture (Barcelo and Lazzeri, 1995). This genotype dependence is also evident in the development of alternative methods for wheat transformation, such as direct gene transfer to protoplasts (He et al., 1994), which attempt to overcome the genotype effects.

These different efforts show the level of difficulty in developing transgenic wheat plants. The variations in available protocols are due to our poor understanding of the initial factors that influence stable transformations; such as the lack of knowledge of the processes involved with the integration of DNA and how the cells may be treated in order to improve it.

In an effort to overcome these problems, the effects of the standardization on the plant transformation parameters (such as mother plant health, embryo size and developmental stage, culture medium, and bombardment parameters) were analyzed. As a result, a routine transformation system has been developed which allows a steady production of transgenic plants.

**Materials and methods**

**Plant material**

Three wheat cultivars (Bobwhite, Baviacora, and Attila) were used for the transformation experiments based on their regeneration ability (Fennell et al., 1996). The plants were grown in a screen-house under controlled conditions (day temperature of 20-22 °C), with supplementary lightening provided by sodium lamps. Fifteen days after heading, immature seeds were harvested, sterilized, and transformation protocols were initiated.

**DNA plasmid vector and preparation of the microcarrier**

The plasmid used in this experiment was pACH25, containing the Gus gene encoding for the β-glucuronidase enzyme, and the selectable Bar gene that confers resistance to the herbicide Basta. Both genes were under the control of maize ubiquitin
promoter (Christensen et al., 1992). Plasmid DNA was precipitated on gold particles following the protocol modified from the original Bio-Rad procedure. The precipitation mixture comprised 2 mg of gold particles (0.6 µm and 1-3 µm diameter), 50 µl of 2.5 M CaCl₂, 20 µl of 100 mM spermidine free base, and 50 µl DNA (14 µg total). After microprojectile-DNA precipitation the supernatant was discarded. Particles were washed in 250 µl absolute ethanol and resuspended in 250 µl absolute ethanol. For each bombardment, 10 µl of microparticles-DNA were placed on to the macrocarrier. Bombardments were conducted at a distance of 5 cm from the stopping plate using a PDS 1000/He microprojectile gun (Bio-Rad) with an array of different rapture discs (650, 900, 1100, 1350, and 1550 ics [inches cm square]).

**Culture conditions and recovery of transformed plants**

Freshly isolated embryos were directly transferred to the osmotic medium (MS plus 15% maltose) for dehydration. After bombardment, the embryos (1 to 2 mm long) were placed on MS (Murashige and Skoog 1962) medium containing 2.5 mg l⁻¹ 2,4-Dichlorophenoxyacetic Acid (2,4-D), 30 g l⁻¹ sucrose, and 8 g Bacto-Agar for somatic embryo induction. Twenty days later the treated embryos were transferred to the MS medium with 5 mg l⁻¹ PPT (Phosphoeritromicin). Green shoots surviving the treatment were transferred again to the same medium for rooting and 20 days later, rooted shoots were transferred to soil.

**Gus enzyme assays**

Expression of *Gus* A was visualized by X-Gluc staining (Jefferson et al., 1987). Bombarded embryos, regenerating shoots and young leaves were transferred to *Gus* substrate and incubated for 6 hrs, as described by Jefferson et al. (1987).

**Molecular analyses**

Total genomic DNA was isolated from the leaf tissue of the primary transformants and their progeny using the Edwards method (Edwards et al., 1991). PCR analyses were carried out using 1 µl of the extracted DNA in 20 µl total reaction volume containing 50 mM KCl, 1.0 mM tris-HCl (pH 8.8), 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µM of each dNTP, 0.3 µM of each primer, and 0.5 units of Taq DNA polymerase. The PCR profile used for all amplifications was as follows: 94°C for 1 minute, 62°C for 2 minutes, and 72°C for 2 minutes. The primers used for *Gus* gene amplification were 5'-AGTGTACGTATATCACGGTTTGTGAAC-3', and 5'-ATCGCCGCTTTGACGATACTCCGTA-3'. For the *Bar* gene, the primers were 5'-GTCTGCACCATCGTCAACC-3' and 5'-GAAGTCCAGCTGCCAGAAAC-3'. The 62°C hybridization temperature was used for *Gus*. PTC-225 Tetrad DNA engine thermocycler (MJ Research) was used for PCR amplifications.
Southern blot analyses were carried out according to Hoisington et al. (1997), using genomic DNA digested with Hind III isolated from PPT resistant plants transformed with the pACH25 plasmid. The membrane was hybridized with the digoxigenin labeled pACH25 plasmid. Probe labelling and Southern hybridization conditions were conducted according to the protocols described by Hoisington et al. (1997).

**Phenotypical analyses**

Herbicide resistance of the putative transgenic wheat plants was determined by spraying localized applications of Basta on to the leaves when the survived plants were at the fifth leaf stage. The central part of the leaves was painted with 0.3% Basta solution. Plants were scored as susceptible, partially resistant or resistant, according to the degree of leaf desiccation after 7 days.

**Results**

**Comparison of bombardment and selection parameters**

Cultured embryos of Attila, Baviacora, and Bobwhite were bombarded as described above and transferred directly to the induction medium (with and without selection) or for Gus staining. In the initial experiments, the expression vector pACH25 was used to monitor gene transfer into wheat embryos by bombardment under different pressures (between 650 and 1550 ics). Adequate transient expression was obtained with lower levels of bombardment pressure (Table 1). The spots were well defined and isolated up to 1100 ics. With higher-pressure, large spots or diffusion of the Gus staining was observed. The transient expression was higher with frequent formation of large spots when 1-3 μ diameter gold particles were used (Table 1). On the other hand, with 0.6 μ diameter gold particles, the spots were well defined and transient expression was obtained only under higher pressure. The number of well-defined spots in bombarded zygotic embryos in relation to bombardment pressure and gold quantity is illustrated in Table 1. When less gold particles were used, the frequency of transformation was slightly higher than with the reported quantity (Table 2). It appears that a lower gold quantity produces less damage to the embryo surface, which favors higher production of somatic embryos. The highest number of PCR positive and Basta resistant plants were obtained with 1,100 ics. Five plants tested with Southern blot were derived from the 20 obtained with 1100 ics and 10 μg gold particles, Table 2.

**Recovery of transgenic plants from cultures selected with PPT**

Green, vigorously growing plants (2-3 cm tall) were obtained from the selective regeneration medium (Fig. 1 no.3) and transferred again to the same medium for rooting. For comparison, non-transformed shoots utilized as controls were also
Table 1. Relationship between bombardment pressure and quantity of gold particles to produce transient expression.

<table>
<thead>
<tr>
<th>Rupture disk pressure</th>
<th>Transient Gus effects with 5 µg gold</th>
<th>Transient Gus effects with 10 µg gold</th>
</tr>
</thead>
<tbody>
<tr>
<td>650 ics</td>
<td>Spots well-defined (50-80 per embryo)</td>
<td>Spots well-defined (70-100 per embryo)</td>
</tr>
<tr>
<td>900 ics</td>
<td>Spots well-defined (70-110 per embryo)</td>
<td>Spots well-defined (80-150 per embryo)</td>
</tr>
<tr>
<td>1,100 ics</td>
<td>Spots well-defined (90-150 per embryo). Some big spots occasionally</td>
<td>Spots well-defined (100-180 per embryo). Some big spots.</td>
</tr>
<tr>
<td>1,350 ics</td>
<td>Spots well-defined (100-180 per embryo). Some big spots.</td>
<td>Spots well-defined (150- over 250 per embryo). Frequent big spots.</td>
</tr>
<tr>
<td>1,550 ics</td>
<td>Spots-defined (120 – over 250 per embryo). Some big spots and transient expression in the solution.</td>
<td>Spots-defined (150–over 250 per embryo). Some big spots and lot of transient expression in the solution.</td>
</tr>
</tbody>
</table>

Table 2. Number of transgenic plants obtained using different bombardment pressures and DNA-particle suspensions.

<table>
<thead>
<tr>
<th>Rupture disk pressure</th>
<th>5 µl DNA-particle suspension</th>
<th>10 µl DNA-particle suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>650 ics</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>900 ics</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>1,100 ics</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>1,350 ics</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>1,550 ics</td>
<td>1</td>
<td>None</td>
</tr>
</tbody>
</table>

Transferred to the same medium. Inhibition effects in the control plants included stunting of growth and leaf necrosis. At 5 mg l⁻¹ PPT, root growth was inhibited in all the evaluated non-transformed shoots. A portion of the putative transformants showed no similar effect when compared to control plants. Putative transformed plantlets showed vigorous root growth at 5 mg l⁻¹ PPT.
Recognizing that this selection system may not be stringent and could permit false positive plants, a second selection was done by spraying the plantlets with 0.1% Basta solution. After 15 days of spraying, the surviving plants were assayed again for Basta resistance by painting leaves with 0.3% Basta solution. The plants that survived through this last selection were phenotypically normal.

**Molecular analyses**

The $T_o$ plants showed a good correlation between PCR results and resistance to Basta. The tested plants fell into positive and negative groups from their PCR results, and for resistance or susceptibility to Basta (Fig. 1, no.7).

An essential proof of stable genetic transformation requires a test of integration of foreign DNA into the chromosome of the recipient plants. Southern analyses was conducted on the plants that gave evidence of being transgenic based on the Gus staining, phenotypical (Basta resistance) and PCR analyses (Fig. 1, no. 3, 4, and 7). Genomic DNA from control and five randomly picked putative transgenic plants (each recovered independently) were isolated and analyzed for the presence of Bar and Gus genes (Fig. 1, no. 8). The hybridizing bands were of the same apparent molecular weight in each of the putative plants studied.

**Discussion**

Transgenic plants from CIMMYT genotypes (Attila, Baviacora, and Bobwhite) have been produced by microprojectile bombardment into cultured immature embryos, followed by selection for resistance to PPT. The results demonstrated transformation frequencies between 0.24-0.44% of bombarded embryos with Bar and Gus genes. Similar wheat transformation frequencies have been reported (Bommineni and Jauhar, 1997; Weeks et al., 1993; Altpeter et al., 1996a, b) in the Bar gene, in combination with bialaphos and a chimeric aphA-uidA fusion, where geneticin was used to select for transgenic tissues (Weeks et al., 1993). Considering that the proportion of non-transformed escapes regenerated was not much higher when strong selection pressure was used, it was decided to reduce the stringency of the selection pressure and spray the plants directly in the biosafety greenhouse. High escape frequencies have also been reported in other wheat transformation studies (Altpeter et al., 1996a,b).

Another interesting aspect relates to the effect of the quantity and size of gold used for bombardment in the production of transgenic plants. The results show that a lower quantity and particle size of gold may give a higher rate of transformation with a larger window of pressures (Table1).

Molecular evidence to verify that plants selected after bombardment were genetically transformed was undertaken. This was accomplished by genomic DNA characterization
Figure 1. 1) Development of the transgenic tissue (Gus staining) 2, 5, 10, and 15 days after bombardment. 2) Gus staining after 20 days in an embryogenic scutellum. 3) Regenerating shoots after Gus staining Transgenic (up) and control (down). 4) Basta resistant leaves (first 3 leaves from the top) and control (last two). 5) Plantlets resistant after spraying (right) and controls (left). 6) Transgenic plants tested for PCR and Basta resistance. 7) PCR results (control plasmid: fourth from the right). 8) Southern blot analyses.
of the regenerates. PCR assays gave first indication that *Gus* and *Bar* genes were integrated in the genome of the T₀ plants. Southern analyses of the genomic DNA of controls and PCR positive plants showed banding patterns in digested samples that varied among the transformants, which is consistent with random chromosomal integration (data not shown).

The ability of the particle gun to consistently transform wheat was previously reported (Vasil *et al.*, 1992; Vasil *et al.*, 1993). However, it has been noted that cereal transformation is still difficult because of a number of parameters related to the technique. Standardizing these parameters may reduce the difficulties, but there are still insufficient data to predict which parameters will be optimal for the efficient production of transgenic wheat.

In conclusion, a method for large-scale production of transgenic wheat has been developed at CIMMYT. Based on the average of the transformation experiments and the different cultivars, the transformation efficiency has ranged between 0.24 and 0.44%. The transformed plants show a normal phenotype and are fertile. The inheritance of these genes in the future generations will be demonstrated.

Acknowledgments

The authors wish to thank the Australian Cereal Research Council (CRC) for its support of this research.

References


Resumen

Sistemas Rutinarios de Transformación Utilizados con las Variedades de Trigo del CIMMYT

La transformación genética de trigo ha estado limitada por la incapacidad para obtener y regenerar las células apropiadas en los tejidos objeto de transformación y por problemas relacionados con el silenciamiento de genes. En este artículo se describirá una tecnología de uso rutinario basada en el método biolítico de bombardeo para superar dichas barreras y potenciar la transferencia de ADN en trigo. Este método involucra la exposición de los tejidos vegetales al bombardeo con ADN altamente purificado y con niveles reducidos de manipulación por cultivos de tejidos. La respuesta al cultivo in-vitro de los embrones inmaduros bombardeados y la producción de plantas transgénicas fue investigada en tres cultivares de trigo, Bobwhite, Baviacora y Attila. En todos los genotipos se bombardeó un mínimo de 700 embriones inmaduros con un plásmido conteniendo un gen de resistencia a Bialaphos (bar) bajo el control de un promotor de ubiquitina y el gen de la beta-glucuronidasa (GUS). A pesar que la expresión transiente del gen reportero GUS unido con el mismo promotor fue similar en los tres cultivares evaluados, la frecuencia de transformación estable varió con los genotipos. La frecuencia de transformación mayor correspondió a Bobwhite, produciendo plantas a partir del 0,24-0,44 % de los embrones bombardeados. En las otras dos variedades se obtuvieron en menor proporción plantas transgénicas expresando resistencia a Basta. Los datos preliminares sugieren que la variación en transformación fue causada por diferencias en los genotipos y no por la eficiencia del procedimiento de bombardeo por partículas.
Progress and Prospects for Engineering Wheat Quality Characteristics

Olin D. Anderson*

---

Abstract

The contribution of the wheat HMW-glutenin subunits to wheat quality is reviewed along with some of the results related to using the HMW-glutenin genes to engineer altered wheat quality. The successful use of bioengineering of wheat quality traits through wheat transformation has already been achieved in several laboratories. This opens the door to controlling wheat quality to a degree not otherwise possible. The technology of genetic engineering is the long-term future of wheat improvement. However, this technology is also associated with scientific, societal, and economic concerns different from current wheat breeding practices. Southern Cone countries must carefully balance this combination of opportunity and problems in planning the future of wheat improvement.

Introduction

Since the beginning of the modern molecular biology era one of the dreams has been to engineer improved crops to feed the ever-increasing human population. It has been perhaps 20 years since the first overly optimistic projections that engineered crops would soon be in the fields. However, it is now fair to say that finally the projections are coming true. Estimates are that upwards of 32 million hectares of planted acreage in the United States alone is occupied by genetically engineered crops. These first transgenics involve simple single dominant gene traits for crops like maize, soybeans, and cotton; i.e., mainly insect and herbicide resistances. It now seems inevitable that other crops and more complex traits will soon follow. Among these additional crops will be wheat and some of the traits that make wheat unique among crops.

Wheat is unquestionably one of the basic pillars of the human diet along with rice and maize. In some respects wheat can be considered most important, particularly

* U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, Albany, CA 94710, USA. email: oandersn@pw.usda.gov
when including barley - as they are closely related and along with the rest of the Triticeae tribe (which also includes rye and triticale) often considered a single genetic system. Rice is critical in tropical areas, but is basically a single-use crop - boiled and consumed by humans. Maize is widely cultivated, but its main use is in animal feed industry, and thus only indirectly for human consumption. Wheat, on the other hand, is the most widely adapted and grown crop in the world, is the major source of human dietary protein, and is almost entirely consumed by humans in a wide variety of products (raised and flat breads, cakes and cookies, pastas, and numerous varieties of noodles, just to mention the major product classes).

The reason wheat is so versatile in product creation is due mainly to the unique wheat seed proteins which confer physical/chemical properties, such as visco-elasticity, to wheat flour-water mixtures (doughs). The set of dough characteristics is one basis of what is referred to as wheat “quality”, or the ability of a specific wheat cultivar to provide favorable characteristics for a specific use. Thus, for bread making quality wheat requires dough strength to trap gas bubbles during leavening and baking, while quality for a noodle process may be white color and lack of strength. A good quality wheat thus must be defined not only by the physical/chemical properties of the doughs it produces but also the specific market or product target.

In the past, the options for breeding for improved quality were restricted to the genes available in existing wheat germplasm, and if necessary, in wild grasses whose genes could be moved into wheat. The latter can involve considerable technical challenges and take many years to accomplish. We now have the ability to modify genes in the laboratory and to transform wheat with natural and modified genes. These abilities are in the process of having a revolutionary effect on both our understanding of the factors effecting wheat quality, and our capability to engineer improved wheat lines.

**Engineering wheat quality**

*Targets for engineering wheat quality*

While wheat is also subject to many of the same genetic engineering efforts as other crops, such as insect and herbicide resistances, we shall here concentrate on those traits unique to wheat that determine the utilization of the crop in food products, particularly the visco-elastic properties of wheat doughs. These properties are conferred by a complex interaction of all of the seed components, but particularly starch (not to be addressed here) and the seed storage proteins. Of the latter, the class referred to as the prolamines (high in proline and glutamine) are the most important, and particularly the high-molecular-weight (HMW) and low-molecular-
weight (LMW) glutenin subunits. Of these two, the HMW-glutenins have shown the highest correlation to wheat quality. There are two key characteristic structural features of the HMW-glutenin polypeptides: a central, repetitive domain theorized to relate to elasticity, possibly through secondary conformations similar to the vertebrate connective tissue protein elastin; and placement of the cysteine residues at the two, non-repetitive, terminal domains of the polypeptide. The glutenins disulfide crosslink into enormously long polymers, the glutenin macropolymer. Fig. 1 models the glutenin subunit and structure. By mechanism not yet completely understood, these polymers confer strength and elasticity to doughs.

![Glutenin Polymers](https://via.placeholder.com/150)

**Fig. 1.** Glutenin polymers. A simplified model of the glutenin macro-polymer shows the polymerization of both HMW- and LMW-glutenin subunits. The LMW-glutenin subunits can form monomers, while the HMW-glutenins join in a mixed polymer with the LMW-glutenin subunits. Standard models of polymer formation envision the HMW-glutenins forming intermolecular disulfide bonds from free cysteine residues at the two terminal domains.

Once transformation of wheats became a technical reality, it became practical to consider what genes and traits were to be attempted to engineer. The easiest first candidates were single gene systems that have a significant impact on desired traits. One obvious candidate was the HMW-glutenin genes and their contribution to dough properties. It is anticipated from past work, in many laboratories, that increasing the relative amounts of the HMW-glutenin polypeptides will likely result in stronger doughs.
Two of these works are summarized in Fig. 2, where for near isogenic lines whose main difference is in the number of active HMW-glutenin genes (from 0 to 5), two important parameters (loaf volume and mixing time) are correlated positively with increasing numbers of active HMW-glutenin genes. It is also known that even within the HMW-glutenin gene family there are genes and alleles with differing effects on quality. The best known example is the two HMW-glutenin subunits encoded in the D-genome of hexaploid wheat. The allelic pair encoding the 2 and 12 subunits is consistently associated with poorer quality wheat, while the 5 plus 10 pair is associated with good quality and strong doughs; e.g. Fig. 3 shows results reported by Mansur et al. (1990). When the chromosome carrying the 5 plus 10 pair from the high quality bread wheat cultivar Cheyenne is substituted into the poor quality cultivar Chinese Spring, a dramatic increase in bread loaf volume results. The increase is due to the dough being stronger and able to retain gas bubbles longer during baking. The better dough mixing characteristics associated with the chromosome substitution includes longer peak mixing times and a more favorable wide mixing trace over longer mixing intervals. Thus, from such results the simple prediction is that the addition of even a single copy of a HMW-glutenin gene through genetic engineering will result in stronger doughs.

**Fig. 2.** Effects of increasing numbers of HMW-glutenin genes. Isogenic wheat lines with different numbers of active HMW-glutenin genes show clear relationships to dough quality characteristics. A) Loaf volume (liters) increases with more HMW-glutenin genes (Payne et al., 1987). B) Peak mixing time (shown as percent of control) increases with more HMW-glutenin genes (Lawrence et al., 1988).
Several examples indicate how this might be useful. If a specific wheat cultivar has otherwise good agronomic traits, but is unsuitable because of weak doughs, adding more HMW-glutenin genes could strengthen the doughs. Also, as humans tend toward more whole-grain products, stronger doughs will be needed to counter the tendency of seed coat fragments to weaken doughs. This last example also relates to the yield of flour in wheat milling. Currently, a large fraction of the endosperm is lost in milling, since in many countries mills must avoid including too much bran in flour in order not to weaken the resulting doughs. Stronger doughs could counter the weakening action of bran and allow milling closer to the bran and result in a larger flour yield from a given quantity of grain. In addition to practical applications, the ability to transform wheat with genes for natural and modified glutenin polypeptides allows approaches to dissecting the molecular basis of dough properties by strategies not otherwise available.
In vitro systems for studying wheat quality parameters. A bacterial expression system is used to synthesize HMW-glutenin subunits. The purified protein is then used in dough micromixing experiments (Mixing Time) to test the effects on dough of native and modified HMW-glutenin subunits. Results show the effects of adding a control subunit preparation (sample 1) and a series of modified HMW-glutenin subunits (samples 2-11). Thus, it is possible to systematically alter dough properties with engineered genes.

In Western Regional Research Center (WRRC), it has been possible to combine wheat transformation experiments with in vitro studies of HMW-glutenin structure and function utilizing microbial expression systems. Fig. 4 summarizes the results of a series of dough micromixing experiments (performed by F. Bekes, CSIRO, Sydney) incorporating HMW-glutenins synthesized by bacteria using HMW-glutenin genes modified in the laboratory. A large number of modifications of gene structure are being tested. Fig. 4 shows simply the different effects on peak mixing time from 10 different constructs. Such experiments are anticipated to give us detailed information on how the various domains of the HMW-glutenin polypeptide structure relate to functional properties of doughs. An entirely synthetic HMW-glutenin genes has also been constructed (Anderson et al., 1996, and unpublished). Such synthetic genes allow one to design genes for maximum ease of future modifications, and allow easier separation of the different polypeptide domains in analyzing protein structure/function relationships. In addition, WRRC has also constructed a shuttle-vector system so that HMW-glutenin
gene constructs, first studied in bacterial expression systems, can then be easily moved into a wheat promoter DNA context for wheat transformation (not shown).

The ability to transform wheat has already begun to allow approaches to wheat research not previously available. Even the first few HMW-glutenin transgenic wheat lines have yielded important new information about glutenin structure and function. The hybrid HMW-glutenin construct transformed by Blechl and Anderson (1996) yielded the surprising result that the two terminal domains of a HMW-glutenin subunit can form an intramolecular crosslink (Shimoni et al., 1997), something not previously believed to occur. Further work with this one line has led to both the determination of the exact cysteine residues involved in this intramolecular crosslink and the previously unknown determination of the complete pattern of intramolecular disulfide bonds within a single HMW-glutenin polypeptide (Kasarda, 1999).

**Progress in engineering wheat quality**

Several laboratories, including WRRC, are producing an increasing number of transgenic wheat lines with altered numbers of naturally occurring HMW-glutenin genes plus modified HMW-glutenins not found in nature. The goals are both to understand the molecular basis of wheat quality traits, and use this understanding in engineering specific enhanced or novel quality traits. While details of these experiments are not discussed here, the early results have proven that there is no longer any question that dough physical/chemical properties can be dramatically altered via molecular biology and transformation. As an example, it has been shown by Barro et al. (1997) that increasing the number of active HMW-glutenin genes by transformation can increase both mixing time and the peak mix resistance (diagramed in Fig. 5). He et al. (1999) has shown that durum wheat transformed with HMW-glutenins leads to doughs so strong as to not be directly comparable to untransformed doughs. In WRRC, large numbers of transgenic wheat lines with varying numbers of natural and modified HMW-glutenins have been generated. This has allowed obtaining mixing curves of probably a greater variety than seen in all available wheat germplasm - all coming from the same cultivar that has been used for all transformation experiments (unpublished). Among the results and new insights into HMW-glutenin structure and function include the following: 1) the HMW-glutenin repetitive amino acid domain is directly related to dough properties; 2) the HMW-glutenin polypeptide is not a rigid structure as previously theorized; 3) the N-terminal and C-terminal polypeptide domains have specific roles and affinities for disulfide bond formation; 4) inclusion of additional natural HMW-glutenin genes through transformation can increase dough strength; 5) some combinations of HMW-glutenin transgenes can lead to greater dough mixing tolerance; 6) there are indications that the mixing time is separable from other parameters such as peak dough strength and resistance to breakdown; 7) some transformed wheat lines show different levels of suppression of the transgenic and endogenous HMW-glutenin genes.
The basic question is “can wheat quality traits be engineered via molecular biology and wheat transformation?” The answer is an unqualified “yes”.

**Future prospects in engineering wheat quality**

One aspect of engineering quality traits in wheat that is only just starting to be appreciated is the uncoupling of existing associations. For example, some wheat markets desire low protein as a reliable measure of lower dough strength (particularly important in some noodle manufacturing), typically 9% protein or less. However, WRRC has transgenic wheats with 12-13% protein and low strength conferred by a single dominant transgene locus. In this example there may be nutritional advantages to use higher protein wheats as long as the noodle-making properties are appropriate.

A common target of wheat research is the search for new variability in glutenin proteins to screen for effects on quality characteristics. One of the strengths of the transgenic
approach versus searching through existing cultivars or wild wheats is the unlimited and specific variability available using molecular biology and transformation. If a specific polypeptide domain is shown to relate to a quality characteristic, it is easy (in principle, though not always in practice) to modify this domain through standard molecular biology techniques, and then transform the modified gene into wheat to express a new glutenin of exactly known structure. Further options for modifications, if desired, can be deduced from the effects of this modification. What will eventually follow is the ability to make a specific modification with high expectations of predicable effects on specific quality characteristics. The potential of these new technologies is suggested in Fig. 6, with many of the current constraints to wheat improvement no longer absolute barriers to improvement, and with as yet undefined new options available.

**Fig. 6.** Wheat research and development future capabilities. Past capabilities in wheat research and utilization are represented by the smaller, dark circle. Modern biotechnological tools enable much greater potentials as represented by the larger, white circle.
In spite of the tremendous promise, there are still important problems: transgenic wheat still needs some years of multi-site field trials to finally confirm transgene stability; consumer confidence is critical in the short-term, but long term engineered wheats seem inevitable; variety identification; and adjustments of industries to both handling transgenic wheats and the exploration of new opportunities. While still early, these results indicate that it will eventually be possible to specifically tailor a wheat to any specific characteristics desired for end-use. It can also be speculated that eventually it may show that transgenic wheats can mitigate at least some of the variations in quality related to environmental effects.

Public acceptance of genetically modified organisms

In spite of the enormous potential for genetic engineering of wheat, the technology must be accepted by the consumer. The current controversy over genetically modified organisms (GMOs) could severely hamper implementation of this technology. There is as yet no confirmed case of GMOs causing harm to humans, animals, or the environment, but scientists must take seriously concerns over GMOs. While it is clear that many of the arguments against GMOs are spurious, and based on insufficient knowledge, ignorance, or deliberate misinformation, this does not mean that there are no aspects of the technology that must be carefully considered and tested. It is also important to distinguish each case on its own merits.

The example of engineering wheat quality by means of the HMW-glutenin genes may prove an interesting test case for GMOs. It will be possible to alter wheat quality using already existing natural HMW-glutenin genes, and modified versions of those genes. This avoids some of the more lurid examples in the press of “fish genes” in tomatoes, etc. The trait of quality (dough visco-elasticity) is already an important wheat trait, so it is not transferring some “unnatural” trait into wheat. The introduction of new allergens is not a factor, again since the proteins already exist in wheat. So, what is left that could be controversial? A common argument is against the use of microbial marker genes such as the bar herbicide resistance gene and antibiotic resistance genes. Once again, there is no evidence that such genes cause harm, but for both easing concerns and for some research purposes it would be useful to be able to answer both of these concerns. The elimination of the antibiotic genes used in DNA clone propagation is simple to accomplish; i.e., physically isolate the genes to be transformed from the antibiotic resistance genes used to maintain the clones in bacteria. This standard laboratory procedure adds only a modest amount of additional work to the transformation effort. Eliminating the marker genes is more difficult since they must integrate into the wheat genome along with the gene(s) of interest in order to select and recover transformed plants. Several approaches to this problem have used recombination systems to later excise the marker gene. One such example uses the Cre recombinase/lox DNA element recombination system
from P1 bacteriophage. This system has been successfully used in the laboratory of David Ow (USDA, Plant Gene Expression Center) to remove the bar marker gene from transgenic wheat (Srivastava et al., 1999). The basic marker gene construct used in these wheat experiments is diagramed in Fig. 7. The marker gene is flanked by loxP sites. Once integrated into the wheat genome, any number of bar construct integrated copies can be excised by the Cre recombinase. The Cre gene is transformed into a separate wheat line which is crossed to the transformed wheat line carrying the transgene of interest. Once the Cre recombinase excises the bar genes, the Cre gene can be segregated away from the useful transgene. Thus, if necessary, it is possible to eliminate essentially all the arguments against GMO wheats engineered using the HMW-glutenin genes (except those arguments based on social agendas rather than science).

**Fig. 7.** Removal of marker genes from transgenic wheats. If the bar marker gene is flanked by inverted loxP DNA elements, then in the presence of the Cre recombinase the DNA sequence between the loxP elements is spliced out the genome and is lost.

**Role of bioengineering quality in the Southern Cone countries**

One goal of this meeting is to review the current status of wheat biotechnology and to assess if and when the Southern Cone countries of South America should utilize these technologies to address problems of wheat quality. The specific focus of this paper is genetic engineering of quality through molecular biology and wheat transformation. The question and potential answers will by necessity be complex, with elements of the current status of scientific infrastructure, likely future resource availability, specific needs of each Southern Cone country, availability of technology developed elsewhere in the world, and all of the societal and political considerations of genetically modified organisms. While all of these are important, a simple first consideration is the question “will GMOs developed through modern molecular biology and plant transformation have a significant future in crop development, particularly
wheat?" There is simply no compelling argument that the answer to this question is anything but "yes". The potential of these new technologies is undoubtedly greater than even the most ardent proponent can imagine; although their implementation timetable is uncertain and currently subject to some controversy. A different question, and one obviously critical to planning the agricultural infrastructure of any country is "how and when to introduce these technologies into existing agricultural systems that have, and will continue, to show progress".

One drawback to these technologies is their relative expense compared to present crop improvement strategies. This is unavoidable, and is an issue that must be addressed by both the scientific and political communities planning future agriculture. However, it should also be kept in mind that the technologies will continually become more efficient, so that what requires major resource development now is likely at some point to become routine.

An aspect of these new technologies that has not been a major factor, at least for wheat production in the past, is the emergence of major multi-national agricultural biotechnology companies and their potential to dominate all sectors of agriculture, especially if public agencies worldwide do not maintain a significant presence in agricultural biotechnology. A discussion of the impact of world food production controlled mainly by a few companies is beyond the scope of the present format. However, even company representatives (at least when speaking in private) believe the health of agricultural research and food production will, in the future, be best served by combinations of private and public efforts.

What advice can be given to the Southern Cone Countries? Certainly internal cooperation will be a key. The advantages to coordinated efforts will be critical to the ability of the Southern Cone countries to accelerate and implement agricultural biotechnology into crop production. In addition, the planning of such cooperation cannot begin too soon. The longer the delay in planning cooperation, the harder it will be to integrate ongoing programs into a coordinated effort. In addition to regional cooperation, the Southern Cone should be looking to developing regular interactions with agricultural biotechnology sectors elsewhere in the world, both private and public. Where feasible, participation in collaborative projects with other regions will accelerate the development of Southern Cone scientific infrastructure, and would assure access to the widest range of technologies. Finally, early communication and understandings of expectations will greatly assist the beneficial inclusion of capabilities of the large agricultural biotechnology companies into specific agricultural systems.

Patience combined with foresight will require skilled planning. In no way should there be a reduction of wheat breeding infrastructure as it now exists. These more traditional approaches and technologies will still be critical into the foreseeable future. What will change is the addition of genetic variability provided by modern biotechnology.
There may actually be an increased need for wheat breeders to take full advantage of these new potentials. In addition, basic germplasm development will remain important, possibly even more so if there is a tendency to concentrate planted wheat into a narrower range of germplasm. The role of the public sector may be crucial to maintain enough overall genetic variability to prevent crop production collapse through increasing monoculture, a trend that agriculture biotechnology companies may accelerate as they focus efforts on a limited number of value-added genes in a narrower germplasm base. This is a conundrum for the future; vastly increased genetic variability for specific genes and traits, but a decrease in the overall wheat genome variability within cultivars available for planting. The ultimate challenge will be to make full use of modern biotechnology for wheat improvement both for specific traits and to improve the general vigor and diversity of the cultivated wheat gene pool.

References


---

Resumen

**El Progreso y los Prospectos de Ingeniar Trigo para Características de Calidad**

La contribución de subunidades de gluteninas de alto peso molecular (G-APM) en trigo está revisada junto con algunos resultados sobre el uso de genes de G-APM para alterar la calidad de trigo. El uso exitoso de bioingeniería de las características de calidad de trigo a través de transformaciones ha sido posible en varios laboratorios. Este hecho abre las puertas para controlar la calidad de trigo que no hubiera sido posible de otra manera. La tecnología de ingeniería genética es un futuro de largo plazo para el mejoramiento de trigo. Sin embargo, esta tecnología también está asociada con preocupaciones científicas, sociales y económicas muy distintas a las actuales de las prácticas de mejoramiento de trigo. Esta combinación de oportunidades y problemas deben ser cuidadosamente balanceadas por los países del Cono Sur en planear el futuro de sus programas de mejoramiento de trigo.
Intellectual Property and Wheat Breeding for the Southern Cone

John H. Barton*

Abstract

This talk will explore the implications of current trends in intellectual property rights for wheat breeding for the Southern Cone, and suggest appropriate responses for public sector institutions. It will first review the applicable forms of intellectual property, including both plant variety protection and patent law, and briefly describe some of the relevant trends in U.S. and European patent law. This will include a series of examples of the different kinds of patents that are currently being issued whose claims cover wheat seeds or breeding or relevant production and research techniques. Later it will explore the relevance of these patents to wheat breeding and production in the Southern Cone, including consideration of the factors shaping impacts on the seed production industry within the Southern Cone, and, in addition, of impacts on wheat export to markets in which such patents are in force. Finally, it will explore appropriate responses for public and private sector breeders in the Southern Cone. The alternatives include the design of Southern Cone breeding programs to avoiding infringement, obtaining licenses from or entering in cooperative arrangements with the firms holding leading positions, and obtaining a patent portfolio for negotiating purposes.

Introduction

The new biotechnologies are transforming the scientific substance and the legal and commercial context of agricultural research. This transition is especially significant for the international public research sector that has been the source of much innovation of great importance for the developing nation farmer (1). This paper examines the implications of the transition for wheat research in the Southern Cone region. It attempts to clarify what is likely to happen in the domestic and international seed industries of these nations, to note implications for the farmers, and to explore implications for the research community.

* Stanford Law School, Stanford, CA 94305, USA. email: jbarton@leland.stanford.edu
The existing system and its trends

Wheat is one of the most important products of the Southern Cone, as demonstrated by the summary statistics presented in Table 1. It is typically grown as an open-pollinated crop; thus farmers can save seed from year to year. Thus, there has been relatively little private research in spite of the existence of a substantial wheat seed industry. And there has been substantial research conducted by national agricultural research institutions within the region, as well as by CIMMYT.

Table 1. Wheat producers of the Southern Cone region.

<table>
<thead>
<tr>
<th></th>
<th>Argentina</th>
<th>Brazil</th>
<th>Chile</th>
<th>Uruguay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area in wheat (m ha)*</td>
<td>5.6</td>
<td>1.5</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Production (m mt)*</td>
<td>14.5</td>
<td>2.5</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Portion exported**</td>
<td>45 %</td>
<td></td>
<td>&lt; 1 %</td>
<td></td>
</tr>
<tr>
<td>Plan variety protection</td>
<td>1994</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Medium term prospects for agricultural commoditios, 1994. FAO, calculated by author.

This pattern may well change in the next several years, as a result of agricultural biotechnology and of the rise of intellectual property rights in agricultural biotechnology processes and products. These are likely to lead to the transgenic insertion of new capabilities into wheat, including, for example, likely herbicide resistance, pest resistance, and careful control and modification of the amino acid composition of the grain for various special markets. These capabilities are already being considered for a wide variety of crops, and have been commercialized in a number of cases. Although it is impossible to be confident of the precise timing and pattern of these transitions, it seems likely that the new technologies will be commercialized in the form of seeds for hybrid varieties. The use of hybrids implies that the farmer must return to the seed producer year after year and cannot reuse harvested grain as a new seed. The possibility of creating hybrids has been a strong stimulant to private sector research investment for maize, and may prove so as well for wheat. Wheat hybrids are already on the market in Western Europe.

This creates a new situation for public sector institutions, for they must define an appropriate way to coordinate their taxpayer-funded activities with the private firms' market funded activities. This new relationship may involve a change of focus. The public sector institutions may be able to continue to produce new varieties, and these varieties could be used as breeding material by a growing private sector industry. This, in essence, is what happened with maize in the United States and Europe prior to the development of advanced agricultural biotechnology patenting. On the other hand, the public sector may find it wiser
to concentrate on the concerns of marginal farmers (probably a lesser need in the case of Southern Cone wheat than of many other crops and regions) or on special issues such as improving environmental sustainability or dealing with regional diseases or stresses, issues that may be low on the multinational horizon. This may, in turn, involve cooperation between the public and the private sector.

And it will involve intellectual property issues, for the research institutions will wish to avoid infringement, to cooperate effectively with the private sector, and to maintain their own freedom to maneuver. Since wheat is traded internationally, this may require consideration of intellectual property rights in market nations as well as in producing nations. Should a specific grain be patented within a market nation, for example, it may be illegal to export that grain to that nation without a license from the holder of the patent. Moreover, should the grain be produced by a process that is patented within the market nation, export of the direct product of that process into the nation may also raise infringement issues. It must be anticipated that in today’s highly competitive international agricultural environment, firms will use these patent rights in market nations as part of their competitive weaponry. The possibility of this form of competition is enhanced for specific wheat varieties designed for specific applications or if wheat becomes subject to special labeling requirements designed to track whether the particular material is transgenic. This is an issue that may only develop in the future, however, for most of the current international markets for wheat are within developing nations, who are unlikely at this time to have strong intellectual property systems.

**Intellectual property**

Over the last two decades, patent law has extended into biotechnology, exemplified in the United States by *Diamond vs. Chakrabarty*, 447 U.S. 303 (1980), and in Europe by Directive 98/.../EC of the European Parliament and of the Council on the legal protection of biotechnological inventions (2), issued in 1998 after years of debate. A variety of relevant patents have been issued in these nations; they have clear impact on the ability of public sector entities to conduct research. As will be seen below, they have led to substantial litigation, and perhaps contributed to consolidation, among the leading agricultural biotechnology firms. It is important to note that these patents have legal force only in the nation in which they are issued, they do not affect activities in other nations, save to the extent that these activities are directed toward export to the nations in which the patents are issued.

Before the regular patent system became used in plant biotechnology, plant lines were often protected by Plant Breeder’s Rights (PBR), often also known as Plant Variety Protection (PVP). This is a specialized intellectual property system, covered internationally by the UPOV convention (3). Such a system is in place in Argentina, Chile, and Uruguay and will be in place in Brazil by next year. It is designed specifically for plant breeding, and legally protects a breeder against another’s unauthorized use of the relevant variety for growing
purposes. Under this legislation, which is also in force in most other major wheat exporting areas, and is being implemented on a European Union basis in Europe, a farmer is generally, legally free to reuse harvested material for breeding purposes. And, of extreme importance, a breeder is free to use another’s protected materials for the purpose of developing new varieties (4).

There are now many important patents issued under regular patent laws, however, supplementing protection under this PBR system. A sampling is presented in Chart I, which shows the scope and force of the mesh of rights. Several specific categories deserve

<table>
<thead>
<tr>
<th>Chart I. Some representative patents affecting wheat and wheat research*.</th>
</tr>
</thead>
</table>
| **Varieties**
G123 hard white winter wheat, U.S. Patent 5,498,829, Goertzen et al., Cargill, March 12, 1996. (Specific variety identified by an American Type Culture Collection accession number.)

**Breeding systems**
Rapid generation advancement in winter wheat, U.S. Pat. 5,682,708, Frederick, Purdue, Nov. 4, 1997.

**Transformation methods and related innovations**
Method for transporting substances into living cells and tissues and apparatus therefor, U.S. Pat. 4,945,050, Sanford et al., Jul 31, 1990, Cornell (Gene gun).
Chimeric genes for transforming plant cells using viral promoters, U.S. Pat. 5,352,605, Fraley et al., Oct 4, 1994, Monsanto (35S Promoter).

**Other fundamental patents**
Antisense regulation of gene expression in plant cells, U.S. Pat. 5,107,065, Shewmaker et al., Apr. 21, 1992, Calgene (antisense technology).
Synthetic plant gene, U.S. Pat. 5,500,365, Fischhoff et al, Mar. 19, 1996, Monsanto (Coding sequence modification for better expression).

**Specific genes and gene technologies**
Virus resistant plants, U.S. Pat. 5,185,253, Turner et al., Feb. 9, 1993, Monsanto.
*Bacillus thuringiensis* isolate active against lepidopteran pests, and genes encoding novel lepidopteran-active toxins, U.S. Pat. 5,126,133, Payne, Jun 30, 1992, Mycogen.

*(Note that these are only examples; in most cases, there are many other patents also affecting the relevant area of technology.)*
One is the possibility of patents on particular lines, exemplified in the chart by a recent patent on a specific line of wheat, with the line identified through an American Type Culture Collection number. Such varieties are unpatentable in many nations, including Chile, because of legal provisions designed to permit varieties to be covered only by a PBR system. The claims of these patents (it is the claims which define the technical legal scope of the patents) typically extend to the progeny of the plant itself and its seeds. The patents are thus designed to avoid the limitations of the PBR system, and to keep customers from reusing their harvested product or using the purchased seeds for breeding research. Frequently also, firms in the United States seek to obtain the same result through a seed legend prohibiting buyers from using the seed for breeding purposes or reusing the harvested seed for growing a crop; it is not clear whether these contracts are enforceable in the United States, or that they would be in other nations (5). There are also patents on groups of varieties. There is not known, however, to be a broad patent covering all transgenic wheat, analogous to the Agracetus' patents on transgenic cotton and soybeans (6).

Another category of patents covers breeding systems; these are likely to be available in many legal systems, probably including Chile and Uruguay. These patents affect the ability of a firm to protect its market. As noted above, firms have relatively little interest in investing in research for open-pollinated varieties, for the farmer's ability to reuse the seed creates competition for the variety and lowers the possible price. Hybrids provide a potentially greater return for the breeder, since the farmer is unable to reuse the seeds (and, of course, hybrids also provide the benefits of heterosis). An alternative is the new so-called "terminator technology" — on which a patent has just issued in the United States. This patent claims a broad group of mechanisms of inserting several genes, some of which are activated at specific times in the plant's life-cycle. The process allows to grow a normal first generation crop from the purchased seed, while a gene is activated to prevent seed viability for the second cycle. There are also now patented technologies for apomixis (7). For some species, these can be used to breed new generations of heterozygotic seeds from heterozygotic seeds — and thus enable seed production of "true-to-type" hybrids.

Some transformation methods — and a variety of the research tools used in biotechnology — are patented. Thus, the "gene gun," which has become one of the most common methods of transforming crop plants, is patented in the United States. Similarly, Monsanto holds a patent on the widely used 35S promoter. There are a number of other important fundamental patents, exemplified by patents on the use of antisense technology to control gene expression and on the recoding of DNA sequences for proteins taken from other organisms to permit better expression with a plant's transcription machinery. In several of these cases, there have been significant intellectual property struggles among different firms with conflicting and overlapping rights — the patents listed in the chart are representative of a much broader group of patents held by different firms. The patent network is strong enough that, if the patents are valid,
they can be used to control essentially all research on transgenic crops in the United States and perhaps Europe.

Finally, genes themselves are now routinely patented, typically with claims that cover the isolated gene, various constructs including the gene, plants transformed with those constructs, and the seed and progeny of those plants. Those plants that contain the gene naturally are not novel within the meaning of the patent laws — these patents therefore do not affect those plants nor do they affect breeding with them. Among the important examples for wheat are the mitochondrial sequences for Karnal bunt, and plants transformed to alter their glutenin content. Among the other genes and gene technologies that have been patented are those for the viral coat protein used in providing resistance to a virus, a technology in which Monsanto has a very strong position, and those for the *Bacillus thuringiensis* toxin. This is a technology for which there are many broad and overlapping patent rights held by a number of firms and subject to substantial litigation (8). In a pattern paralleled in several other technologies, three different firms had claims to Bt-transformed maize — one based on initial cloning of the gene for the Bt toxin, one based on transformation of a model organisms with the toxin, and one based on transformation of the actual crop plant with the toxin. Recent litigation suggests that certain of these patents may be invalid (9).

In addition to these patent rights, there may be proprietary rights in genomic information. This information is typically protected through contracts based on trade secrecy principles. Thus, a firm may create a substantial database or map of a genome and then provide access only under agreed terms that may include creation of a mechanism for compensation. For example, Monsanto has recently entered into a major cooperative effort with Millenium, a genomics firm (10). It should also be noted that there are efforts underway to create a publicly funded map for wheat (11).

The international industry

At the same time that these broad intellectual property rights are being created, the U.S. and European seed industries are rapidly consolidating. Chart II shows the status of this integration, and also lists some of the firms known to be currently pursuing research on wheat. Several factors are shaping this consolidation. One, quite simply, is the strategy of the larger firms to “promote the closer working relationships and the greater sharing of technologies that are only possible with full ownership,” (12) — and each of the major firms almost certainly views itself as needing to grow to compete with the others. Another is a pattern in which small firms funded by venture capital explore a scientific area and, if they are successful, offer themselves for acquisition by larger firms. The proceeds of the acquisition provide a financial return for the venture capitalists. Of especial importance to wheat, there is also a pattern of vertical acquisition of seed distributors, including Latin American distributors.
The expanding number of broad patents in the industry encourages litigation aimed at completely excluding competitors from the market for any genetically modified seeds. There has been a great deal of such litigation during the last several years; a May 1997 summary showed 21 such suits, mostly involving the companies listed in Chart II (13). Many of these suits involve quite broad patents, and amount to efforts to expel the defendant from the agricultural biotechnology business. In some cases, a merger is the easiest form of settlement. And — and the point is extremely important for the future of the area — it is typical in such a situation of overlapping patents for litigation to be settled or avoided through a network of cross-licenses among the firm leaders. Some of the cross-licenses may be tacit, based on a fear of counter-suit if one brings a suit. The firms may, of course, continue to use patents to slow entry into the industry oligopoly.

Any estimate at the future of the industry is necessarily a guess. But what seems most likely is that, at least for the lucrative portions of the global grain market, e.g., transgenic maize, wheat, and soybeans, there will be at most five major firms, and that each will have a patent portfolio strong enough to bar any new entrant to the group. The

Chart II. Major agricultural research firms.

<table>
<thead>
<tr>
<th>Name</th>
<th>Wheat activity</th>
<th>Recent acquisitions and alliances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monsanto</td>
<td>PBIC’s research</td>
<td>Cargill international activities (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DeKalb (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agracetus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asgrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Holdens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calgene (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Holdings in Brazil</td>
</tr>
<tr>
<td>Novartis</td>
<td></td>
<td>(Combination of Sandoz and Ciba-Geigy)</td>
</tr>
<tr>
<td>Agrevo</td>
<td></td>
<td>(Joint venture of Schering and Hoechst)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plant Genetic Systems (1996)</td>
</tr>
<tr>
<td>DuPont</td>
<td>John Innes Consortium</td>
<td>Part owner of Pioneer Hi-Bred</td>
</tr>
<tr>
<td>DowElanco</td>
<td></td>
<td>Mycogen (which has recently purchased Brazilian seed companies)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dinamilho (Brazil) (1998)</td>
</tr>
<tr>
<td>Zeneca</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
number of firms may prove to be much smaller than five mentioned above. Moreover, because many of the patents cover technologies relevant to several crops, the patent portfolios will be strong enough to control any research in or entry into the North American or European markets of unlicensed transgenic crops.

The Southern Cone

It is clear that this emerging multinational industry will be interested in the Southern Cone wheat seed industry. Southern Cone wheat offers a substantial global market, and the intellectual property position for wheat seed in the region is reasonably favorable to the industry. Globally, wheat seed is becoming an area of interest. Thus Monsanto has a French subsidiary producing hybrid wheat (14) and has also recently purchased Plant Breeding International, Cambridge, which has a substantial hybrid wheat program (15). DuPont, on the other hand, has formed a strategic alliance with the John Innes Center and a number of other groups to develop new wheat varieties (16), and has also bought a French hybrid wheat seed firm (17). Moreover, herbicide resistance may offer a subsidiary market of interest to the multinationals — there are stories of seed industry majors offering freely to transfer into national lines the genes necessary for herbicide resistance, in order later to sell the herbicide.

National intellectual property systems

For these multinationals, the national intellectual property systems will be of great importance. As noted above, all the four nations examined here, save for Brazil, have recently adopted national PBR legislation and that nation may move to do so reasonably soon. But, for the multinationals, it is coming to be the regular patent legislation (as exemplified to support the kinds of patents shown in Chart I) that is becoming of greatest significance.

Economically, Argentina is the most important of the Southern Cone wheat seed markets. It already has a significant seed industry, which has already been purchased in part by the multinationals. Argentina has a new patent law, enacted in 1996. Article 7 (b) of this law clearly prohibits the patenting of genes and of genetic processes (18); few if any of the U.S. patents discussed earlier could be granted analogues in Argentina. Unless this law is changed, it is therefore unlikely that firms will develop advanced transgenic materials specifically for the Argentine market, and it is possible that they will resist the transfer of some kinds of material to Argentina. At the same time, it is clear that research institutions based in Argentina need not be concerned about domestic infringement of such patents. But there will be significant pressure on Argentina to modify this provision of its law, on the argument that it is inconsistent with the TRIPS agreement, which requires that patents be available for inventions "in all fields of technology," and outlines specific, more narrow exceptions for biotechnology and living organisms.
Brazil's exception for living organisms is much less broad, prohibiting patents on "natural living beings, in whole or in part, and biological material, including the genome or germplasm of any natural living being, when found in nature or isolated therefrom, and natural biological processes (19)." It is not clear whether this is intended to preclude a patent on the process of inserting a specific new gene into a plant; if such a patent is permitted, the patent holder has some control over the plant itself, for the plant may be a direct product of the process, and therefore infringing under Article 42 of the Brazilian Act. The exception is certainly intended to permit some of the patents described above, such as those on transformation methods. Thus, there have already been patents sought in Brazil on certain uses of Bt isolates (Mycogen) and of other pest control genes (Monsanto) as well as on certain processes for making transgenic maize (DeKalb) (20).

Uruguay apparently has no exception in the biological area (21). Chile has a very narrow one, prohibiting protection of plant varieties and of animal breeds, but not apparently restricting protection of biological processes or genes or (possibly) of organisms containing a specific gene (22). The patent law provision prohibiting the patentability of a variety may be read as permitting such claims on organisms containing a specific gene, because the claims do not cover a "variety." This is the way a similar provision had been interpreted in Europe — but the European Patent Office Board of Appeals recently applied the opposite interpretation (23).

The multinational response

With PBR, a line can be protected, and this includes a transgenic line, but PBR provides no way that a firm can prevent use of the material for breeding or prevent the backcrossing of new genes from the firm's proprietary materials into other materials. The latter can be prohibited only if plants can be patented under the regular patent law with claims that reach, for example, all plants transformed with a particular gene and the progeny of those plants. Such claims will not be available in Argentina, and possibly not in Brazil or Chile.

PBR alone has not significantly encouraged wheat breeding in the United States, presumably because of the right of farmers to reuse harvested seed (and, until recently, to exchange it). But a breeder has many more approaches available. Transformation mechanisms and constructs presumably can be patented, except in Argentina. Thus, actions by a competitor to transform plants or build the constructs for doing so can probably be prohibited, again except in Argentina. A firm can certainly slow competitors through management of access to parental lines of hybrids and perhaps seek, as do U.S. firms, (using contracts whose enforceability is not at all clear) to obtain contractual commitments from farmers not to reuse material and not to reuse it for breeding purposes. They may use terminator technology, which would significantly complicate the use of marketed product for breeding (and perhaps make it impossible).
They might also seek to exercise of intellectual property rights in market nations. However, given the character of the export markets for wheat, it is unlikely that, in the short term, there will be issues of infringement in importing markets. The areas of most importance to the seed industry are the major wheat-growing, i.e. exporting markets; it is in some of these that strong patent protection will be available and used. The market issues will come in the future as the seed firms push successfully for stronger protection in the Southern Cone or push for or obtain protection in the market nations as part of a pattern of protecting their competitive positions. At that point, exporters in nations competing with those of the Southern Cone might obtain patents in specific market nations, with a goal of protecting their position in those nations against competition by unlicensed products from the Southern Cone.

These legal issues will probably not keep the multinationals out. The multinationals will, however, certainly push for stronger protection in the basic patent laws — and they will argue that this is essential to protect their positions in competing against exporting nations that have stronger patent regimes. They may even threaten to withhold technology in order to support this argument, or, alternatively, may argue that they should not provide certain technologies because of fear of losing them within the Southern Cone market. But they will probably still bring significant technology.

Implications

For agriculture

As the multinational firms enter the transgenic wheat seed market, they will come with seeds that are better than those now available, and at prices that are higher than the current prices, but that leave the farmer a significant portion of the benefit of the improved seeds. But there is a very serious possibility that this multinational industry will consist of a small number of firms holding a monopoly (or small oligopoly) position on transgenic seeds. It may therefore have substantial control over the effective price of these seeds. The social need to protect the smaller farmer against the oligopolistic price strongly supports the need to ensure the availability of public sector (transgenic if legally possible, otherwise traditional) varieties available at a reasonable price. In the presence of such competition, the farmer can have the best of both worlds, sophisticated multinational technology at a reasonable price.

The Southern Cone nations may choose to improve their intellectual property systems to be responsive to the new technologies. At present, there is little encouragement for national biotechnological innovations (whether made in the public or private sectors). Hence, there are strong reasons for the national governments to consider stronger protection within their regular patent system for biotechnological innovations. Such legal changes may, however, simply strengthen and give advantage to the multinationals.
Here is where the nations could wisely tailor their patent systems, and it may be possible for them to shape the technical standards for patentability in a way that relatively strengthens the position of smaller, newer firms (which will sometimes be local firms), while still complying with TRIPS. Another approach is to shape competition laws in a way that maintains a strong defense against monopoly in the seed supply sector. The basic oligopoly is evolving at the global level beyond the control of Southern Cone nations; these nations may, however, still be able, for example, to impose protective terms on the takeover of a local firm, or to use compulsory licensing in response to monopolistic practices, and of course to restrain prices. All can be carried out in a way that is consistent with TRIPS, which has provisions supporting both competition laws and compulsory licensing (24).

For the research community

In this emerging world, the developing world public agricultural research sector (including the CGIAR institutions and the national research institutions) faces the question of defining its research program and intellectual property strategy to reflect the changes deriving from the entry of the multinationals.

In addition to the factors described above, the sector must also take into account the United Nations Convention on Biodiversity. This Convention supports the rights of nations to restrict the export of germplasm deriving from within their borders, and to condition that export on the recipient’s acceptance of an obligation to reimburse the source nation in the event that a significant commercial development arises from the exported material. The Brazilian legislature has been considering a law implementing this concept. Considering that wheat is an internationally grown and researched product, not native to the Southern Cone, it is unlikely that there will be serious genetic resource concerns over its exchange within the Southern Cone, although there be issues of technical compliance with legislation that inadvertently covers the exchange of such material. A principle of absolutely free exchange would probably be wisest here, and might usefully be implemented in a regional treaty regime. There will be more significant issues with respect to genetic material that may be inserted into wheat and may derive from a completely different organism or region. In such an event, the nation in which the wheat is grown may be asked to provide compensation to the nation from which the inserted gene was derived. International law demands this with respect to material provided under an appropriate material transfer agreement. International law does not require such compensation for material provided to the international research community before the entry into force of the Convention on Biodiversity at the end of 1993, although negotiating proposals have been put forward that would create some form of retroactive right.

In the overall legal situation and for the near future, it is clear that the Southern Cone public sector need pay little attention to restraints imposed by the intellectual property
system. It must, of course, give appropriate credit to nations providing genetic resources. But, it can use material protected by PBR in its breeding programs and can freely use the imported genetic material, unless that material was subject to a specific agreement. Moreover, under current law, Argentine researchers, and, depending on particular patents, Brazilian and Chilean researchers, need not be concerned about infringing patents, nor need they be concerned about producing new lines that might infringe patents.

Nevertheless, the public sector may wish to modify its research focus, in order to allocate resources effectively. For example, assuming the multinationals begin, as is likely, by selling hybrid transgenic seed to the most productive farmers, the public sector system may choose, should there be a substantial national community of poorer wheat farmers, to concentrate its efforts on the needs of that community. This is unlikely to be of concern to the multinationals. Similarly, if the multinational’s primary interest in selling the seed is to gain a market for a herbicide, there is little reason for it to be concerned if the local public sector uses such technology, for this will increase the market size. In situation such as these, the private sector and the public sector will, in no way, be in opposition.

The more difficult issues arise if the public sector maintains a role in the core commercial varieties. Some of these issues will be issues of cooperation. If the public sector concentrates on the biology of specific diseases or stresses endemic to the Southern Cone, for example, it can develop new technology, and share that technology with the global industry. It is in this context that the multinationals are most likely in turn to share their more advanced technologies, perhaps under a requirement that certain of the products be distributed only in the form of hybrids.

The thoughtful negotiation of such cooperative arrangements is one of the most important issues facing the public international agricultural research sector. The details always differ from transaction to transaction and always require specific political, scientific, and legal judgment. Three points are worth making in this summary, however:

1. Planning is crucial. There should be a scientific plan (almost a business plan) so that the parties are agreed on the objectives, on who provides what resources when, and on the way the results of the joint project will be beneficial to each party and (for the public sector party) to the national or international public interest. This plan must also allocate responsibility for tasks such as financing the acquisition of patent rights or obtaining biosafety approvals.

2. In today’s agricultural biotechnology world, no one can assume that a patent in any jurisdiction is valid or will continue to be valid, or that a technology can be practiced without infringing other patents. There is too much uncertainty and too much litigation. And there is always the possibility that a third party will later obtain a patent which the parties to the collaboration cannot avoid infringing.
The risks in this area, which affect both research and marketing, must be faced and allocated. They cannot be assumed away.

(3) After the plan is thoughtfully designed, with the risks in mind, rights to the technology that is to be developed under the joint project should be allocated according to the goals of the party. For example, should the private party obtain full rights in developed nation markets? Should the public party obtain full rights to use the technology within the Southern Cone, perhaps with a royalty for sales in the national market? Or should the private party be permitted to use the technology within the Southern Cone, but perhaps with some restrictions on the prices that can be charged to farmers. There are many alternatives; the object is to be fair in light of the contributions by and goals of each party.

Finally, it should be noted that public sector may have broader goals, and may, for example, wish to ensure that high quality varieties are available to the Southern Cone farmer at competitive prices. This may pit its position against that of the multinationals — and the public sector may then find itself in the future in a patent battle against the multinationals. This is of particular concern should the nations of the Southern Cone (or market nations) choose to strengthen their patent laws. In order to prepare for this contingency, the public sector should consider obtaining some blocking patents (in developed-world markets) as a bargaining chip to obtain freedom of action in international license negotiations. To have a strong position in the future, the public sector research establishment must begin considering filings now. For bargaining chips, the most useful patents are those which the multinationals will practice and the most useful place to obtain patents is the developed world, for such patents can provide the basis for an exchange in which freedom to use multinational patents in the Southern Cone is obtained in return for the multinational’s freedom to use public sector patents in important global seed markets. The need is for bargaining chips, not for commercialization of inventions, so this is a very different patenting strategy than that used to gain revenue. And to keep the expense down, it is useful to proceed only on a few of their most fundamental inventions that come alone. This is an unfortunate waste of resources, but a necessary hedge.

References


9. “Jury affirms Monsanto patent was infringed by Novartis and Mycogen; Monsanto will ask judge to set aside ruling that excuses infringement,” PR Newswire, June 30, 1998.


18. “The following shall not be patentable:

(b) all biological and genetic material existing in nature or derived therefrom in biological processes associated with animal, plant and human reproduction, including genetic processes applied to the said material that are capable of bringing about the normal, free duplication thereof in the same way as in nature.”

Law No. 24.481 as amended by Law No. 24.572 of March 20, 1996.


20. Uso de isolados de *Bacillus thuringiensis* para controlar pragas na família dos afidideos, Payne *et al.*, (Mycogen), application PI9305617-6 (Aug 6, 1993); Proceso de controle de insetos, Brown *et al.* (Monsanto), application PI9406586-1 (March 2, 1994); Transformação estável de células de milho através de eletroporação, Krzyzek *et al.*, (Dekalb), application PI9107265-4 (June 21, 1994).

21. This is based on secondary sources; I’ve been unable to find a copy of Uruguay’s statute at Stanford.


24. Articles 40 and 31 respectively.
Resumen

Propiedad Intelectual y el Mejoramiento de Trigo en el Cono Sur

Esta exposición explorará las implicancias de las tendencias actuales en los derechos de propiedad intelectual para los programas de mejoramiento de trigo en el Cono Sur y sugiere respuestas apropiadas de las instituciones del sector público. En primer lugar serán revisados los formularios de solicitud de propiedad intelectual, incluyendo tanto la protección de variedades vegetales como la ley de patentes, y serán brevemente descriptas las tendencias más relevantes de las leyes de patentes de EE.UU. y Europa. Esto incluirá una serie de ejemplos de diferentes tipos de patentes que están siendo otorgadas y que pretenden cubrir semillas de trigo, mejoramiento o producción relevante y técnicas de investigación. La relevancia de estas patentes para el mejoramiento de trigo y la producción en el Cono Sur será explorada, incluyendo la consideración de factores con impacto en la industria de producción de semillas en el Cono Sur, y adicionalmente, los impactos en la exportación de trigo hacia los mercados en los cuales estas patentes están vigentes. Finalmente se explorarán las respuestas apropiadas para los mejoradores del sector público y privado en el Cono Sur. Las alternativas incluyen el diseño de programas de mejoramiento en el Cono Sur que eviten infracciones, obteniendo licencias o interviniendo en acuerdos cooperativos con las empresas que ocupan posiciones de liderazgo y teniendo un portafolio de patentes para propósitos de negociación.
Opportunities for Utilization of Biotechnology in INIA's Wheat Breeding Program in Uruguay

Martha Francis and Fabián Capdevielle*

Abstract

The application of biotechnologies in plant breeding programs are primarily concentrated in those areas that impact wheat productivity and quality, such as:

1) genetic maps and identification of molecular markers linked to agronomic traits
2) improvement techniques for maximizing the expression of the potential of available germplasm in the short term
3) methods of analysis to evaluate and transfer the genetic information to practical breeding tools
4) upgrading and reduction of costs associated with molecular diagnostics
5) expression of introduced genes through genetic engineering and development of strategies that allow sustainable use of transgenics crops

An increasing amount of information aimed to increase the resolution on complex traits (yield, industrial quality, durable resistance to pathogens) is becoming available from the Advanced Research Institute (ARI’s) associated with global programs. In this scenario the main challenge is to develop biotechnology-based tools to be used by the breeding programs with an efficient cost/benefit relationship.

Strategically, the use of biotechnologies in the plant breeding programs will focus on the improvement of current selection programs, and enhancing the integration of new transgenic genotypes into the locally adapted germplasm.

The purpose of this presentation is to design a practical approach to utilize these technologies by the plant breeders and biotechnologists of INIA. Our goal is to build a bridge that unites the challenges from the emerging technologies with the demands from plant breeding programs in Uruguay. The information provided by the invited experts is considered essential to do this “case study” regarding the application of biotechnology to the Wheat Breeding program in Uruguay.

* Unidad de Biotecnología, INIA. Estación Experimental Las Brujas Ruta 48, Km. 10, Rincón del Colorado, CP 90200, Canelones, URUGUAY, corresponding author email: mfrancis@inia.org.uy
Introduction

The main objective of this presentation is to review the present status of biotechnology applications becoming available for breeding purposes in wheat, based on different aspects discussed at this workshop. Several issues to be taken into consideration while integrating biotechnology into INIA’s Wheat Breeding Program in Uruguay will also be discussed.

The joint organization of this workshop by CIMMYT and INIA, provides us the opportunity to receive “first hand” the contribution of many recognized researchers, for a complete up to-date “state of art” regarding the application of biotechnology in wheat breeding. During these days it has also been possible to work with and share the experience of scientists that are leading their respective fields.

Based on the information obtained during the seminar, it has been possible to develop a case-study approach for INIA’s Wheat Program, considering emerging technologies and the demands faced by the wheat-breeding program in Uruguay. There is still a very large gap between the technical feasibility of some biotechnologies already available and their practical implementation within an ongoing breeding program facing more challenges every day. Consequently, there is a need for a well-defined framework of evaluation and assessment of biotechnologies specifically designed to improve the efficiency of the wheat breeding program in identifying and use of gene sources to address specific breeding objectives. This, in turn, requires every possible effort to keep building better communication among local Biotechnology Unit, Breeding Programs and the International Research Community, in order to be able to adopt the emerging technology as soon as possible and apply them to solve the problems of wheat production in Uruguay.

Status of biotechnology applications in a wheat breeding program:

The case of Uruguay

According to the survey presented by Dr. Mohan Kohli, one of the major limitations (besides cost and complexities) for the application of the biotechnology in the wheat breeding program comes from the researchers themselves. Lack of communication between researchers in biotechnology and plant breeders has been detected in several countries.

From another perspective, maybe the major reason why the biotechnology-breeding synergy is progressing slowly in wheat is its low-value compared to cotton, rice, and soybeans; and the issue, yet to be addressed remains “whether costly genetic approach to wheat can be justified or not”. Another reason is that wheat has an extremely large
Opportunities for Utilization of Biotechnology in INIA's Wheat Breeding Program in Uruguay

153

genome of 42 chromosomes with three different sets: A, B and D (Röder et al., 1998). Improving wheat through biotechnology will be a far more complex and time-consuming challenge because of the size of its genome. While biotechnology will not be the only tool for improving wheat, still it can allow breeders to solve specific limitations and introduce new traits by genetic engineering.

It is important to consider different factors that currently affect the national wheat production and commercialization processes, and be aware of the multiple demands coming from the consumers, industry, and of course, farmers. Only within this particular context, it may be possible to design the best research strategy for biotechnology to play an important role in the improvement of INIA's wheat breeding program.

Recently, the industry is consistently moving towards producing "differential" products. Consequently, there is an increasing demand for quality. From this point, the program needs to focus on those characters that are involved in the breadmaking quality. Meanwhile, the problems related with increased productivity and reduced yield losses will continue to be major components of the economic analysis of crop production.

In order to develop a strategy to use biotechnology in wheat breeding in Uruguay, one has to face additional challenges related with the application of emerging technologies presented at this workshop. In reality, there is neither a demand from the industry, or from the consumers to use the new tools. It is probably caused by the misinformation about the possible impact that the emerging technologies can cause in a developing country like Uruguay.

The scientists of the developing countries must work actively with other players in the production chain to inform the consumers, industry, farmers, breeders and decision-makers, about the new possibilities of biotechnology. But this must not be only from the technical standpoint, but should also include information about social and economic impact analysis. It is a key aspect for the successful application of biotechnology to productive systems.

Recently, Dr. Norman Borlaug, known to many as the "Father of the Green Revolution," reviewed this issue at the "Gene Technology Forum" organized by Kasetsart University, Thailand. He said biotechnology can help mankind meet its future food and fiber needs in the coming centuries — but only "if science is permitted to work as it should be." "There seems to be a growing fear of science, per se, as the pace of technological change increases," Borlaug lamented. He criticized "misinformed environmentalists" in the more developed nations for creating a backlash against science, technology and industry. "While the affluent nations can certainly afford to adopt elitist positions, and pay more for food produced by the so-called 'natural' methods," he said, "one billion chronically undernourished people of the low-income, food-deficit nations cannot."
The implementation of biotechnology in the Wheat Breeding Program in Uruguay, must be within the framework of the mission of INIA as the National Agricultural Research Institute. Consequently it should contribute to the national agricultural system, by generating, incorporating and adapting knowledge and technology, according to growers’ demands, considering national policies, sustainability, the agri-industrial process and the consumers.

INIA has a long-term experience in conventional plant breeding. The Experiment Station “La Estanzuela” has been headquarter for one of the oldest Wheat breeding programs in Latin America, started by Dr. Alberto Boerger 85 years ago. This program has been the main source of new wheat varieties. Research efforts are concentrated in conventional breeding using field and laboratory facilities for variety development. The wheat breeding program has been mainly focused on agronomic traits affecting grain production, disease resistance, yield stability, grain quality and adaptation to the cropping systems more representative of the Uruguayan wheat environment. Presently, new varieties are protected by INIA according to the UPOV Convention and commercial agreements are made through licensing for seed multiplication and distribution.

On the other hand, INIA has established a core-group of researchers with agronomic background and specialization in biotechnology at Central Biotechnology Unit located at “Las Brujas” Experimental Station. They have access to modern laboratories and equipment facilities for conducting research in Plant Tissue Culture and Molecular Biology (Capdevielle et al., 1996).

Considering the available human resources and facilities and what is needed, there is clear demand to build up technical capacity in molecular breeding techniques to contribute to the development of improved germplasm. This will allow the networking abilities required to design and operate marker assisted selection schemes on a practical level, to be able to succeed in the application of these emerging technologies, one should not forget to build solid technical bases to contribute information towards the following aspects: marketing of biotechnology-based products, public perception of the role of biotechnology in Uruguay, enforcing of Intellectual Property Rights related with emerging technologies and interaction with the industry as mentioned by Dr. Barton.

This new approach will be useful to add value in the process of releasing new varieties quickly and also create a new inter-phase of Breeding & Biotech Services. The new orientation to promote the incorporation of biotechnology based tools in wheat breeding programs must be designed to monitor the innovations globally and to capture the advanced tools in order to build up a sustainable capacity. In the past, time was spent studying the objectives within a plan for a particular development, but now the new paradigms associated with the innovations through emerging technologies need to be extensively applied to restructure the development of new objectives.
Use of biotechnology for wheat improvement

During the workshop, an extensive update on different applications of biotechnology for wheat improvement has been discussed. The major areas are summarized as follows:

- Production of DH-homozygous lines from selected crosses
- Genetic dissection of productive traits (QTLs)
- Use of map-based molecular markers for agronomic traits
- Scale-up of molecular diagnostics for marker assisted selection (MAS)
- Development techniques for transgenics

Doubled haploids vs. other techniques for production of homozygous line

The production of pure lines by conventional breeding methods take several generations, and the application of biotechnology can have a significant impact on reducing the time required for the release of new cultivars.

The information presented here by Dr. Kazi indicates that CIMMYT has developed suitable protocols using wide-pollination techniques. Currently, the productions of DH-homozygous lines from selected crosses apply standard protocols using maize pollen, and this appears to be a ready-to-use technology for application in the breeding programs. However, there is still a need for assessing this technology within the genotypic base of INIA's germplasm. A study of the cost / benefit relationship is needed in order to get a practical value of the DH-production in each specific laboratory.

Map-based molecular markers for agronomic traits

Dr. Sorrells proposed the use of molecular markers in fingerprinting, parental selection, monitoring homozygosity for selection of loci controlling traits that are difficult or costly to evaluate and selection against donor genome in backcrosses. The isolation and characterization of microsatellites from hexaploid wheat provide detectable markers for agronomically important genes and quantitatively inherited traits that facilitate their handling in segregating breeding populations (Röder et al., 1998).

The application of microsatellites, located on different chromosomes, used to analyze a set of 105 bread wheat varieties from Argentina, is also being used for the molecular characterization of the wheat cultivars released and protected in Argentina.
Large numbers of molecular markers are now available for all major crop species and generally markers can be found that are closely linked to genes of interest. There are several possible methods for implementation of MAS in a conventional breeding program and in general, the same principles apply that are used for other selection techniques. Backcrossing desirable alleles into elite genotypes is generally regarded as the most efficient approach and can be modified to suit the specific breeding goals and resources available. Integration of conventional and MAS using index and/or culling selection (multiple stage selection) may be useful for specific traits and germplasm provided population sizes can be kept small.

Although marker assisted selection is gradually being incorporated into conventional wheat breeding programs; given its high cost MAS is currently only considered to select loci of traits that are otherwise difficult or costly to evaluate. The effective use of the MAS will be dependant on several factors such as the efficient screening for disease resistance, identification of sources of resistance genes, the relevance of major genes vs. QTL’s, the possibility of combining strategies for durable disease resistance, the access to map-based molecular markers for agronomic traits, the use of marker-assisted selection for pyramiding resistance genes, the implementation of accelerated backcrossing to recover recurrent parent and the opportunities for selection in the absence of pathogens. Markers can increase selection efficiencies when breeding for disease resistance; which has been successfully done in the cases of Fusarium Head Blight (FHB), and Tan Spot, as shown by Dr. Jim Anderson.

One of the possible uses of MAS in the wheat breeding program in Uruguay will be to apply it to improve disease resistance. The incidence of leaf rust in selected wheat cultivars, has been studied by Silvia Germán (German, 1996), and there is the possibility to use molecular markers linked to leaf rust resistance genes (Procunier et al., 1995, Schachermayr, 1994), to evaluate the new materials.

Dr. Sorrels mentioned that among the recent accomplishments of biotechnology applied to the Triticeae is the completion of molecular genetics framework maps of all the wheat and barley chromosomes. In addition, the demonstration of the genetic transformation of wheat, barley, and rye establishes that direct gene manipulation in crop improvement is possible. The application of these technologies can require significant resources in the developmental stages. However, it may not be necessary to repeat all developments for every crop. The exciting finding that the genomes of all cereals share extensive genetic similarity means that scientific advances in other cereals, such as rice and corn, can often be utilized by wheat and barley researchers.

A majority of economically important plant traits, such as grain or forage yield, can be classified as multigenic or quantitative. Even traits considered to be more simply inherited, such as disease resistance, may be “semi-quantitative” for which trait expression is governed by several genes (e.g., a major gene plus several modifiers).
During the past century, both plant and animal geneticists have obtained convincing evidence that Mendelian principles apply to quantitative as well as to qualitative traits. This evidence has also shaped the general model that embraces the multiple factor hypothesis for quantitative traits. If one agrees that Mendelian principles apply to quantitative traits, it becomes necessary to define the concept of a QTL. Most geneticists and breeders consider QTLs to be chromosomal locations of individual genes or groups of genes that influence complex traits. (William at this workshop).

Although it is often assumed that a QTL represents a single genetic determinant (or factor), there are examples of individual QTLs that have been resolved into multiple genetic factors by recombination. For the manipulation of a vast majority of QTLs in plant breeding programs, it must not be important to determine whether the QTL represents a single genetic factor or a cluster of tightly linked genes. Several of the important traits that must be manipulated by plant breeders are more simply inherited than grain yield but still may involve the expression of several genes. For example, disease and insect resistance frequently is controlled by only one or a few genetics factors.

In most cases, the first steps in a marker-based introgression program are the identification and mapping of the genes (more realistically, chromosomal segments) targeted for transfer to the desired line or strain. Breeders may not need to know the locations of their targeted QTLs with very great accuracy. Marker-assisted selection against unwanted chromosomal regions from the donor (reducing linkage drag) will expedite the introgression process.

The selection for breadmaking quality, involving MAS for high protein content and introgression of hardness alleles presented by Dr. Dubcovsky, and manipulation of gluten strength by Dr. Anderson are successful examples. Utilization of molecular diagnostics for MAS based on fingerprinting and of genetic variation for speeding up the breeding cycle by selection of parental genotypes, determination of allelic contribution, and genealogical evaluation of markers associated with agronomic traits were other aspects discussed here.

The identification of large chromosomal segments (QTLs) that have an effect on a trait is only the beginning of a long and arduous process to determine the underlying genes controlling the trait of interest. Marker-facilitated techniques provide valuable tools for the rapid transfer of known trait variability from one individual or population to another. However, to precisely manipulate a trait and/or to create variability that does not exist in natural populations, it is necessary to understand the structure and function of all genes involved in the expression of that trait. This requires a highly coordinated effort that includes large scale sequencing efforts, gene annotation, highly integrated genetic and physical mapping, and studies of syntenic relationships. This massive amount of information must then be carefully brought together into a comprehensive,
interactive, accurate, highly accessible, and user-friendly database. This is when genomics becomes involved.

Genomics is a whole-genome approach where one focus is on developing dense physical and genetic maps. It relies on high throughput technologies that have been advanced for human and microbial genome sequencing projects. The massive quantities of data generated require robust databases, data mining, and analysis tools, which are collectively termed bioinformatics. This genomics-bioinformatics area can be a very productive field for designing and testing the statistical and molecular tools allowing to integrate the large databases already available from both the agronomic and molecular fields into useful selection tools.

For the development of an efficient strategy to use marker-assisted selection in Uruguay, it is necessary to manage the cost/benefit relationship of the implementation of these techniques.

Genetic engineering

Transformation technology has been demonstrated in wheat and barley. New and valuable genes can be introduced into cereals via transformation that are not available through traditional breeding practices. However, Dr. Pellegrineschi pointed out a number of impediments that slow the widespread use of this technology which requires too much expertise for routine use, and is influenced by differences in the genotypes.

In this field, the main focus of a practical wheat breeding program may concentrate on designing strategies for the sustainable use of transgenic varieties (when available and with a clearly-defined market), and transfer of transgenic genotypes into adapted germplasm. The evaluation of a particular trait transferred into local and regional germplasm can be done through collaborative agreements, following biosafety rules according with international guidelines.

Multidisciplinary approach for the innovation

The large amount of information that needs to be put together for the successful application of these emerging technologies, makes it necessary to create a new modality of team work to conduct such a project. This includes the promotion of multidisciplinary teams including plant breeders together with experts in plant physiology, functional genomics, environmental sciences, plant pathology and genetic engineering.
This sort of team work can be promoted not only through direct contact of different research specialists within a particular Lab or Research station, but also through networking at the international level with other centers sharing the same research goals. One of the important disease problem for wheat worldwide is the increasing incidence of FHB. This case can become an example of how such a networking centered on biotechnology applications to breeding can be used. This provides a potential case for application of two complementary techniques, the development and use of molecular markers for resistance genes from well-known genetic sources, and the production of transgenic wheat expressing antifungal enzymes.

Both approaches have been envisaged by the Consortium of Research Centers in the US and Canada, where most of the available techniques can be readily introduced into a wheat breeding program following the appropriate evaluation procedures. Based on this research initiatives, DNA markers are being utilized to aid in the identification of FHB resistance genes in wheat. One marker that explained about 25% of the variation in scab resistance in a population segregating for FHB resistance was derived from the Chinese cultivar, Sumai 3. Using the DNA marker to select for the presence of specific resistance gene(s) would facilitate the elimination of susceptible materials earlier in the evaluation process and enrich the resource-intensive FHB screening nurseries with more resistant materials.

The design of application of biotechnology in a breeding program needs to be planned carefully and each project must give a clear added value, have realistic objectives within a set of activities, including the specific tasks for each member in a multidisciplinary team.

Through biotechnology, and especially functional genomics, it is anticipated that it will be possible to influence both the yield and quality of cereal crops. Given the global nature of wheat consumption and trade, broad consumer acceptance of the technology is important. While respecting public concerns, it should be possible to explain the benefits to the consumers. Furthermore, this will potentially result in greater segmentation of the wheat crop, generating specialty wheat varieties for specific processing industries.

During the planning of INIA's mid-term activities (Indicative Program of Medium Term, 1997-2001), the proposed activities for wheat biotechnology included the use of molecular markers for disease resistance (rusts). Pursuing the strategic partnership with research centers worldwide, it has been possible to introduce and validate PCR-based molecular markers for specific rust resistance genes, which are presently being tested for marker-assisted application within the wheat breeding program.

One major contribution of this workshop is the identification of several practical combinations of valuable sources of information, generated in advanced laboratories,
and validation techniques using local germplasm, to incorporate biotechnology into a breeding program. Furthermore, these strategic agreements among Agricultural Research Institutes on an international level will prove to be a cornerstone in the life sciences industry millennium.

References


Resumen

Oportunidades para Utilización de Biotecnología en el Programa de Mejoramiento de Trigo en Uruguay; como:

Los mayores esfuerzos para la incorporación de biotecnología en los programas de mejoramiento se ubican en áreas con diferentes impactos en la productividad y calidad de trigo:

1) mapas genéticos e identificación de marcadores asociados a caracteres agronómicos
2) prácticas de mejoramiento para expresar en el menor tiempo posible todo el potencial del germoplasma utilizado
3) métodos de análisis que permitan evaluar y transferir dicha información a herramientas prácticas de selección
4) escalamiento y reducción de los costos asociados con diagnósticos moleculares
5) expresión de genes introducidos por ingeniería genética y desarrollo de estrategias que permitan un uso sostenible de cultivos transgénicos

El contexto actual a nivel internacional se caracteriza por una oferta creciente en la información generada por centros de investigación avanzada a nivel mundial, frecuentemente asociados en programas globales, para aumentar el grado de resolución sobre caracteres complejos (rendimiento, calidad industrial, resistencia durable a patógenos). En este escenario el mayor desafío actual es desarrollar herramientas basadas en dicha información genética que puedan ser usadas por los programas de mejoramiento en forma operativa y con una relación costo/beneficio satisfactoria.

Desde el punto de vista del planeamiento estratégico que deberían realizar los programas de mejoramiento que aspiran a utilizar un enfoque biotecnológico, el aporte fundamental de la Biotecnología estará determinando por su contribución al mejoramiento de los sistemas de selección en uso, así como por su capacidad para acelerar la integración de los nuevos genotipos transgénicos dentro del germoplasma adaptado a condiciones locales.

El objetivo de esta presentación es establecer una aproximación práctica sobre este tema, desde el punto de vista de los mejoradores y biotecnólogos de INIA. Nuestro objetivo es construir un puente que una los desafíos de las tecnologías emergentes con las demandas de los programas de mejoramiento en Uruguay. La información aportada por los especialistas invitados será considerada como un aporte para este "estudio de caso" sobre la aplicación de la Biotecnología al Programa de Mejoramiento del Trigo en INIA-Uruguay.
Closing Remarks

Uruguay enjoys a long tradition in wheat breeding that started in 1914 with the creation of the “National Plant Breeding and Seeds Institute, La Estanzuela” under the directorship of Dr. Alberto Boerger. Since then, the breeders have worked successfully to supply our farmers with improved varieties adapted to local environmental conditions.

The participation of CIMMYT in Uruguay has been very active in introduction and evaluation of wheat germplasm which has not only helped our country but also the other countries of the Southern Cone region. Its fruitful work has widened to new horizons that include adoption of new methodologies. At the present time, the advances in the scientific knowledge and the development of biotechnology has created an impact in the world through production of novel crop varieties.

It is with pleasure that we highlight the close relationship between INIA -and its predecessor CIAAB- and CIMMYT, started over 25 years ago and transformed in an interinstitutional agreement since 1994, to assist nationally and regionally. At present, we are in the process of revitalizing it with new and more ambitious objectives.

The “International Workshop on Application of Biotechnologies to Wheat Breeding” has been jointly conducted within the scope of INIA-CIMMYT agreement. It clearly focuses on how both institutes are working to generate information for the breeders regarding recent breakthroughs in scientific knowledge and development of biotechnologies applied to agriculture.

The workshop has been honored by the participation of outstanding scientists specialized in various fields of emerging biotechnologies in wheat breeding, such as: genetic engineering, production of doubled haploids to speed the creation and release of new varieties, use of molecular markers for fingerprinting and marker-assisted selection of agronomic and quality traits. These show us the paths being taken by biotechnology that will assist in the production of superior wheat varieties.

CIMMYT technical staff -especially Dr. Mohan Kohli- and those of INIA have worked hard to assemble here the most outstanding scientists from CIMMYT, renowned universities and research centers. Their vast experience in the application of biotechnologies to wheat breeding ensured us of the success of this workshop.

The research results included in these proceedings constitute a valuable source of information for the wheat breeding programs to meet the needs of our countries.
participation of breeders, seed industry and technical staff from both public and private sectors demonstrates the growing interest drawn by the application of biotechnologies.

We extend our acknowledgements to all speakers and participants that made the organization of this workshop, under the INIA-CIMMYT agenda, possible. We also hope that this workshop may serve as the beginning of a closer cooperation in the development of this new field of research.

Ing. Agr., Pedro Bonino
President,
Board of Directors, INIA,
Montevideo, Uruguay
INTERNATIONAL WORKSHOP
Application of Biotechnologies to Wheat Breeding

LIST OF PARTICIPANTS
November 19-20, 1998
INIA La Estanzuela
Colonia-Uruguay

SPEAKERS

Dr. James A. Anderson
Assistant Professor, Department of Agronomy and Plant Genetics
University of Minnesota
411 Borlaug Hall
St. Paul, MN, U.S.A.
Postal Code: 55108
Phone: 001 612 6259763
Fax: 001 612 6251268
E-mail: ander319@tc.umn.edu

Dr. Olin D. Anderson
Research Geneticist, USDA
800 Buchanan St.
Albany, California, U.S.A.
Postal Code: 95616
Phone: 001 510 5595773
Fax: 001 510 5595777
E-mail: oandersn@pw.usda.gov

Dr. John Barton
Professor, Stanford Law School
Stanford, CA, U.S.A.
Postal Code: 94305
Phone: 001 650 7232691
Fax: 001 650 9616171
E-mail: jbarton@leland.stanford.edu

Dr. Jorge Dubcovsky
Professor, University of California
670 Bianco Court
Davis, U.S.A.
Postal Code: 95616
Phone: 001 530 7525159
Fax: 001 530 7534361
E-mail: jdubcovsky@ucdavis.edu

Dr. Martha Francis
Coordinator, Biotechnology Unit
INIA Las Brujas
Ruta 48, Km. 10
Rincón del Colorado
Canelones, Uruguay
Postal Code: 90200
Phone: 00598 23677641
Fax: 00598 23677609
E-mail: mfrancis@inia.org.uy

Dr. Man Mohan Kohli
Principle Scientist, CIMMYT
Andes 1365, Of. 314
Montevideo, Uruguay
Postal Code: 11100
Phone/Fax: 00598 2 9028522
E-mail: cimmyt@inia.org.uy
Lic. Maria Marcela Manifesto
Researcher, INTA Castelar
Las Cabañas y Reseros s/n
Castelar, Argentina
Postal Code: 1712
Phone: 0054 11 621 1819
Fax: 0054 11 621 6903
E-mail: mmanifes@cicv.gov.ar

Dr. Abdul Mujeeb-Kazi
Principle Scientist, CIMMYT
Apdo. Postal 6/641
Mexico D.F., Mexico
Postal Code: 06600
Phone: 0052 5 804 2004
Fax: 0052 5 804 7558
E-mail: m.kazi@cgiar.org

Dr. Alessandro Pellegrineschi
Associate Scientist, CIMMYT
Apdo. Postal 6/641
Mexico D.F., Mexico
Postal Code: 06600
Phone: 0052 5 804 2004
Fax: 0052 5 804 7558
E-mail: a.pellegrineschi@cgiar.org

Dr. Mark Earl Sorrells
Professor of Plant Breeding
University of Cornell
252 Emerson Hall Ithaca, NY, U.S.A.
Postal Code: 14853
Phone: 001 607 2558092
Fax: 001 607 2556683
E-mail: mes12@cornell.edu

Dr. Manilal William
Associate Scientist, CIMMYT
Apdo. Postal 6/641 Mexico D.F., Mexico
Postal Code: 06600
Phone: 0052 5 804 2004
Fax: 0052 5 804 7558
E-mail: m.william@cgiar.org

PARTICIPANTS

ARGENTINA

Ing. Agr. Pablo Bergada
Genetist, NIDERA S.A.
Paseo Colón 505, P.4
Buenos Aires, Argentina
Postal Code: 1063
Phone: 0054223 4642680
Fax: 0054223 4642680
E-mail: pbergada@nidera.com.ar

Biol. Hilda Buck
JOSE BUCK S.A.
La Dulce, Argentina
P.O. Box 23
Postal Code 7637
Phone/Fax: 0054 2262 434061
E-mail: bucksem@infovia.com.ar

Ing. Agr. Lizardo González
JOSE BUCK S.A.
La Dulce, Argentina
P.O. Box: 23
Postal Code: 7637
Phone/Fax: 0054 2262 434061
E-mail: bucksem@infovia.com.ar

Ing. Agr. Oscar Antonio Klein
Researcher, Criadero Klein S.A.
6634 PLA Buenos Aires, Argentina
Postal Code: 6634
Phone/Fax: 0054 1 34670148
E-mail: klein@ssdnet.com.ar

Ing. Agr. Sergio Luis Lassaga
Breeder, INTA Paraná, Argentina
Postal Code 3100
P.O. Box 128
Phone: 0054 11 43 975200
Fax: 0054 11 43 975155
E-mail: eparana@inta.gov.ar
Ing. Agr. Juan Ramón López
Wheat Breeder, INTA EEA Bordenave
Bordenave, Argentina
Postal Code: 8187
Phone/Fax: 0054 924 20621/22
E-mail: postmaster@eborde.inta.gov.ar

Ing. Agr. Martin Luis Luders
Head of Department
NOVARTIS ARGENTINA S.A.
Postal Code: 2605
P.O.Box: 25
Argentina
Phone: 0054 46290525
Fax: 0054 46225964
E-mail: martin.luders@seed.novartis.com

Ing. Agr. Néstor Machado
Plant Breeder, MONSANTO
9 de Julio, Argentina
Postal Code: 6500
P.O. Box: 126
Phone/Fax: 0054 317 30114
E-mail: nestor-machado@cargill.com

Ing. Agr. Héctor José Milisich
Wheat Breeder, INTA Paraná
Postal Code: 3100
P.O. Box: 128
Paraná, Argentina
Phone: 0054 11 43 975200
Fax: 0054 11 43 975155
E-mail: eparana@inta.gov.ar

Ing. Agr. Ruben Miranda
ACA
Estomba 458
Bahía Blanca, Argentina
Postal Code: 8000
Phone/Fax: 0054 11 4918295
E-mail: rmiranda@criba.edu.ar

Ing. Agr. Jorge Enrique Nisi
National Coordinator of Wheat
INTA Marcos Juárez
Córdoba, Argentina
Postal Code: 2580
Phone/Fax: 0054 3472 427171
E-mail: mjtrigo@inta.gov.ar

Ing. Agr. Raúl Horacio Rodríguez
Wheat Breeder, INTA Balcarce
Ruta 226, Km. 73.5
Balcarce, Argentina
Postal Code: 7620
Phone: 0054 2266 22040/42
Fax: 0054 2266 21756
E-mail: msalaberry@inta.gov.ar

Ing. Agr. Carlos Sala
Research Manager, NIDER A S.A.
Paseo Colón 505, P.4
Capital Federal
Buenos Aires, Argentina
Postal Code: 1036
Phone: 005411 4346 8100
Fax: 005411 4346 8101
E-mail: agricola@nidera.com.ar

Ing. Agr. Enrique Y. Suárez
Director, Genetics Resource Group
INTA
De Los Reseros y Las Cabañas s/n
Castelar, Argentina
Postal Code: 1712
Phone: 0054 1 6211819
Fax: 0054 1 621 6903
E-mail: ysidro@cirn.inta.gov.ar
**BOLIVIA**

Ing. Mario Winsor Crespo Márquez  
Researcher, PROTRIGO  
Estación Experimental Tarata  
Cochabamba, Bolivia  
Postal Code: 3299  
Phone/Fax: 00591 4578020  
E-mail: protrigo@comteco.entelnet.bo

**BRASIL**

Ing. Ana Christina Albuquerque  
Researcher, EMBRAPA – Trigo  
BR 285, Km. 174  
Passo Fundo, RS, Brasil  
Postal Code: 99001-970  
P.O. Box: 451  
Phone: 0055543113444  
Fax: 0055543113617  
E-mail: azanatta@cnpt.embrapa.br

Ing. Cantidio N. Alves de Sousa  
Breeder, EMBRAPA Trigo  
BR 285, Km. 174  
Passo Fundo, Brasil  
Postal Code: 99001-970  
Phone: 005554311344  
Fax: 0055543113617  
E-mail: cantidio@cnpt.embrapa.br

Ing. Agr. Lizete Augustin  
Oat Researcher, UPF  
FAMV-Campus Barrio Sao José  
Passo Fundo, RS Brasil  
Postal Code: 99001-970  
Phone:0055543168168  
Fax: 0055543138152  
E-mail: augustin@upf.tche.br

Dr. Amarilis Barcellos  
Researcher, EMBRAPA – Trigo  
BR 285, Km. 174  
Passo Fundo, Brasil  
Postal Code: 99001-970  
Phone: 0055543113444  
Fax: 0055543113617  
E-mail: amarilis@cnpt.embrapa.br

Dr. Paulo Bertagnolli  
Researcher, EMBRAPA – Trigo  
BR 285, Km. 174  
Passo Fundo, Brasil  
P.O. Box: 451  
Postal Code: 99001-970  
Phone: 0055543113444  
Fax: 0055543113617  
E-mail: bertag@cnpt.embrapa.br

Ing. Francisco de Assis Franco  
COODETEC  
BR, 467, Km. 98  
85806-970 Cascavel, PR, Brasil  
Postal Code: 301  
Phone: 0055 45 2263536  
Fax: 0055 45 2263906  
E-mail: coodetec@certto.com.br

Ing. Maria de Fátima Grossi de Sá  
Researcher, EMBRAPA CENARGEN  
Sain. Parque Rural - Final W5 Norte  
Brasilia DF, Brasil  
Postal Code: 02372  
Phone: 0055 61 3403605  
Fax: 0055 61 3403624  
E-mail: fatimasa@cenargen.embrapa.br
Dr. Edson J. Iorczeski  
Researcher, EMBRAPA – Trigo  
BR 285, Km. 174  
Passo Fundo, Brasil  
Postal Code: 99001-970  
P.O. Box: 451  
Phone: 005554311344  
Fax: 0055543113617  
E-mail: edson@cnpt.embrapa.br

Dr. Euclydes Minella  
Breeder, EMBRAPA – Trigo  
BR 285, Km. 174  
Passo Fundo, Brasil  
Postal Code: 99001-970  
Phone: 0055543113444  
Fax: 0055543113617  
E-mail: eminella@cnpt.embrapa.br

Dr. Carlos R. Riede  
Wheat Breeder, IAPAR  
Rodovia Celso Garcia Cid, Km. 375  
Londrina, PR 96001-970, Brasil  
Postal Code: 481  
Phone: 0055 433762348  
Fax: 0055 433762101  
E-mail: crriede@pr.gov.br

Ing. Marco Antonio Rott de Oliveira  
Breeder, COODETEC  
BR, 467, Km. 98  
Cascavel, PR, Brasil  
Postal Code: 85806-970  
Phone: 0055 45 2263536  
Fax: 0055 45 2263906  
E-mail: marcorot@certto.com.br

Ing. Márcio Só e Silva  
Researcher, EMBRAPA – Trigo  
BR 285, Km. 174  
Passo Fundo RS, Brasil  
P.O. Box: 451  
E-mail: soesilva@cnpt.embrapa.br

Ing. Vanderlei Tonon  
Wheat Breeder, FUNDACEP  
RS 342, Km. 14  
Cruz Alta, Brasil  
P.O. Box: 10  
Phone/Fax: 0553227900  
E-mail: fundacep@azcomnet.com.br

CHILE

Ing. Agr. M. Sc. Mireya Aizize  
Plant Breeder, INIA La Platina  
Santa Rosa 11610  
Santiago, Chile  
Postal Code: 439/3  
Phone: 00562 5417223  
Fax: 00562 5417667  
E-mail: dgranger@platina.inia.cl

Dr. Hugo Campos de Quiroz  
Research Scientist, INIA Carillanca  
Temuco, Chile  
Postal Code: 58-0  
Phone: 005645215706  
Fax: 005645216112  
E-mail: hcampos@carillanca.inia.cl

Dr. Claudio Roberto Jobet  
Wheat Breeder, INIA Carillanca  
Temuco, Chile  
Postal Code: 58-D  
Phone: 0056 45215706  
Fax: 0056 45216112  
E-mail: cjobet@carillanca.inia.cl
Ing. Agr. Mario Arturo Mellado  
Wheat Breeder, INIA Quilamapu  
Chillán, Chile  
P.O. Box: 426  
Phone: 0056 2211177  
Fax: 0056 275406  
E-mail: mmellado@quilamapu.inia.cl

Dr. Ignacio Ramírez  
Plant Breeder, INIA La Platina  
Santa Rosa 11610, La Pintana  
Postal Code: 439/3 Santiago, Chile  
Phone: 00562 5417223  
Fax: 00562 5417667  
E-mail: iramirez@platina.inia.cl

URUGUAY

Dr. Tabaré Abadie  
Plant Breeder, Facultad de Agronomía  
Libertador 1676/1101  
Montevideo, Uruguay  
Phone: 00598 2 9011973  
E-mail: tabadie@inia.org.uy

Bach. Martín Arbelbide  
Facultad de Agronomía  
Plácido Ellauri 3429  
Montevideo, Uruguay  
Postal Code: 11300  
Phone: 00598 2 6220550  
Fax: 00598 2 5088718  
E-mail: martinar@internet.com.uy

Lic. Andrea Branda  
Researcher, INIA Las Brujas  
Ruta 48, Km. 10  
Rincón del Colorado  
Canelones, Uruguay  
Postal Code: 90200  
Phone: 00598 2 367 7641  
Fax: 00598 2 367 7609  
E-mail: abranda@hotmail.com

Ing. Agr. Juan C. Caffarel  
National Variety Testing Program  
Winter Crops  
INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: jcc@inia.org.uy

Ing. Agr. Fabián Capdevielle  
Researcher Biotechnology Unit  
INIA Las Brujas  
Ruta 48, Km. 10  
Rincón del Colorado  
Canelones, Uruguay  
Postal Code: 90200  
Phone: 00598 2 367 7641  
Fax: 00598 2 367 7609  
E-mail: fabian@inia.org.uy

Q. F. Jacqueline Cea  
Micotoxins, LATU  
Av. Italia 6201  
Montevideo, Uruguay  
Postal Code: 11500  
Phone: 00598 2 6013724  
Fax: 00598 2 6018554  
E-mail: jcea@latu.org.uy

Ing. Agr. M. Sc. Sergio E. Ceretta  
Head National Variety Testing Program  
INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: ceretta@inia.org.uy
Ing. Agr. Federico Condón Priano
Researcher Genetic Resources
INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: fcondon@inia.org.uy

Ing. Agr. Martha Díaz
Phytopatologist, INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: martha@inia.org.uy

Ing. Agr. Roberto Díaz
Head of Department, Crops Research
INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: rdiaz@inia.org.uy

Ing. Agr. Enrique Estramil
Plant Breeder, Facultad de Agronomía
Avda. Garzón 780
Montevideo, Uruguay
Phone: 00598 2 3072201
Fax: 00598 2 3093004
E-mail: estraxy@biagro.edu.uy

Ing. Agr. Fernanda M. Gamba
Phytopatologist,
Facultad de Agronomía
Ruta 3, Km. 373
Paysandú, Uruguay
Postal Code: 60000
Phone: 00598 72 26250
Fax: 00598 72 27950
E-mail: dmattian@adinet.com.uy

Ing. Agr. Jorge Gari
Researcher, AGROSAN S.A.
Cno. Gral. Máximo Santos 1900
Montevideo, Uruguay
Postal Code: 12900
Phone: 00598 2 3059262
Fax: 00598 2 3093551
E-mail: agrosan@adinet.com.uy

Dr. Silvia Elisa Germán Faedo
Researcher, INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: sgerman@inia.org.uy

Ing. Agr. Carlos Gómez
Etchebarne
Researcher, INASE
Avda. Millán 4703
Montevideo, Uruguay
Postal Code: 12900
Phone: 00598 2 3097924/3097832
Fax: 0059823096053
E-mail: No
Ing. Agr. Wilson Hugo
Researcher, INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: whugo@inia.org.uy

Ing. Agr. Marcos Malosetti Zunin
Assistant, Facultad de Agronomía
Avda. Garzón 780
Montevideo, Uruguay
Postal Code: 12900
Phone: 00598 2 3072201
Fax: 00598 2 3093004
E-mail: marcos@biagro.edu.uy

Ing. Agr. Francisco Mandl
Researcher, INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: fmandl@inia.org.uy

Ing. Agr. Alejandro Peculio
Researcher, INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: pecu@le.inia.org.uy

Ing. Agr. Horacio Ozer Ami
Plant Breeder
Barrio INVE, Block D, Ap. 206
Paysandú, Uruguay
Postal Code: 60000
Phone: 00598 72 33438
Fax: 00598 72 28719
E-mail: ozer@adinet.com

Lic. Jorge E. Pereira Benitez
Genetic Professor,
Facultad de Agronomía
Avda. Garzón 780
Montevideo, Uruguay
Postal Code: 12900
Phone: 00598 2 3072201
Fax: 00598 2 3093004
E-mail: jpereira@biagro.edu.uy

Ing. Agr. Facundo Ponce De León
Researcher, INIA La Estanzuela
Ruta 50, Km. 11
Colonia, Uruguay
Postal Code: 70000
P.O. Box: 39173
Phone: 00598 52 24060
Fax: 00598 52 24061
E-mail: facundo@inia.org.uy

Juan Pedro Puignau
Communication Specialist
PROCISUR/IICA
Andes 1365, P.8
Montevideo, Uruguay
Postal Code: 11100
Phone: 00598 2 9020424
Fax: 00598 2 9002292
E-mail: jpuignau@procisur.org.uy
Ing. Agr. Martín Quincke  
Wheat Breeder, INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: mquincke@le.inia.org.uy

Ing. Qco. Alberto Varela  
Cereals Quality, LATU  
Av. Italia 6201  
Montevideo, Uruguay  
Postal Code: 11500  
Phone: 00598 2 6013732  
Fax: 00598 2 6018554  
E-mail: avarela@latu.org.uy

Lic. Biol. Silvina Stewart  
Phytopathologist  
INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: silvina@inia.org.uy

Ing. Agr. Rubén Verges  
Wheat Breeder, INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: verges@inia.org.uy

Ing. Agr. Mario Stoll  
Researcher, Biotechnology Unit  
INIA Las Brujas  
Ruta 48, Km. 10  
Rincón del Colorado  
Canelones, Uruguay  
Postal Code: 90200  
Phone: 00598 2 367 7641  
Fax: 00598 2 367 7609  
E-mail: mstoll@inia.org.uy

Ing. Agr. Diego Vilaró  
National Variety Testing Program  
INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: vilaro@inia.org.uy

ORGANIZING COMMITTEE

Dr. Man Mohan Kohli  
CIMMYT  
Andes 1365, Of. 314  
Montevideo, Uruguay  
Postal Code: 11100  
Phone/Fax: 00598 2 9028522  
E-mail: cimmyt@inia.org.uy
Dr. Martha Francis  
Coordinator, Biotechnology Unit  
INIA Las Brujas  
Ruta 48, Km. 10  
Rincón del Colorado  
Canelones, Uruguay  
Postal Code: 90200  
Phone: 00598 23677641  
Fax: 00598 23677609  
E-mail: mfrancis@inia.org.uy

Ing. Agr. José Silva  
Director, INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: jsilva@inia.org.uy

SECRETARIES

Karina Cabrera  
INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: kcabrera@inia.org.uy

Virginia Criado  
INIA La Estanzuela  
Ruta 50, Km. 11  
Colonia, Uruguay  
Postal Code: 70000  
P.O. Box: 39173  
Phone: 00598 52 24060  
Fax: 00598 52 24061  
E-mail: No

Sara Prieto  
CIMMYT  
Andes 1365, Of. 314  
Postal Code: 11100  
Montevideo, Uruguay  
Phone/Fax: 00598 2 9028522  
E-mail: cimmyt@inia.org.uy