BREEDING FOR A BETTER INDUSTRY: NEW BREEDING TECHNIQUES

By

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KEYWORDS: Molecular Markers, Genetic Modification, Transcription Analysis, Sugarcane Breeding.

Abstract

INVESTMENT in new technologies that compliment or extend traditional plant breeding methods is beginning to impact on variety improvement programs. This paper will seek to highlight the more important of these, and illustrate their current or potential future application in contributing to the sustainability of sugarcane-based industries. DNA molecular markers are being used routinely in some programs for managing germplasm collections, correcting pedigree information and checking the integrity of new hybrid combinations. Molecular breeding using markers putatively linked to stem-borer resistance is being used in South Africa to improve the efficiency of traditional breeding for this important pest, and a larger project to identify and test markers associated with yield components and cane quality is underway. The large database of more than 33 000 genes expressed in sugarcane developed by the SUCEST program in Brazil has identified markers located within genes of known function, and has the potential to be a very valuable additional resource for molecular breeding. The SUCEST database is also providing information on genes that are potential targets for genetic modification to alter sucrose accumulation, carbohydrate metabolism and other traits. Transgenic sugarcane modified for herbicide, insect and viral resistance has been produced in various programs, but no varieties have been commercialised due to intellectual property rights, and fears of consumer pressure. Potential also exists to exploit sugarcane’s high biomass yield by using it as a ‘factory’ to produce biopolymers, pharmaceuticals and other high value products in conjunction or as an alternative to sugar. In order to capitalise on this potential, strategic decisions will need to be made at Industry level, and partnerships sought with Life Science companies already investing in this area. The increasing development of ‘gene-chip’ technology, both for analysing expressed genes and for use in high-throughput marker systems is likely to facilitate the application of gene expression and marker data in variety improvement in the future. It must be remembered, however, that sound breeding programs provide the platform through which the potential of new technologies can be realised.

Introduction

Classical breeding, which involves making selection and crossing decisions based on individuals phenotype or trait value, has provided the world’s sugar industries with high yielding varieties best adapted to their respective growing conditions.

There is, however, constant pressure to develop improved cultivars with respect to the major agronomic traits of sucrose content, cane yield, resistance to pests, diseases and abiotic stresses, upright growth habit, etc. Apart from the main product of sucrose, there may also be the need to improve traits for secondary products such as fibre content or fibre quality.

As the number of traits increases, the probability of identifying genotypes that fulfil all criteria decreases, and hence breeding efficiency also decreases. Breeding programs have catered for this by testing large numbers of genotypes in early stages of selection.

However, in most, if not all, programs, resource constraints mean that programs cannot be expanded when additional criteria are added to the traits required in commercial varieties. In order then to maintain or improve breeding efficiency, new methods are required to increase the chances of producing and identifying superior varieties. This paper will attempt to briefly describe the technologies currently
under development, and illustrate how these are being targeted to different components contributing to the economic value of sugarcane varieties.

The new technologies fall into two broad classes. The first class includes genomic and gene-expression technologies that are refinements or extensions to conventional breeding, and include molecular markers associated with trait variation, and basic genomic information that improves our understanding of the genetics of sugarcane and aids decision-making. Some of these methods are already being implemented in breeding programs.

The second class involves techniques providing additional traits or novel products to sugarcane through genetic engineering. Although the technology for these approaches is well developed, issues around intellectual property, as well as public perception, have postponed the deployment of these technologies on a commercial scale in sugarcane.

The adoption of new technologies into sugarcane research programs has been largely through collaboration between sugarcane research institutes, along with university and industry partners. The International Consortium for Sugarcane Biotechnology (ICSB) has played an important role in this process. The ICSB was founded in 1991, and since then has provided funding of more than $4.3 million to 22 research projects in the areas of molecular markers and mapping, transgenic sugarcane, sugarcane diseases and the development of technological resources.

The consortium is an excellent example of how a collaborative approach to research can leverage funding from a number of organisations to conduct projects that could not be completed by any group acting alone. Along with ISSCT, the ICSB has also been instrumental in providing a forum for discussing strategic issues in developing technology for sugarcane improvement, and has been a significant contributor to the developments that will be described below.

A brief catalogue of technologies

The main technologies with application in breeding, and their terminologies, are briefly described:

DNA markers or molecular markers

These are DNA fragments that show a size difference due to sequence variation between different genotypes. Markers can be used for DNA fingerprinting and to produce maps of the relative chromosomal position of the markers. If the sequence variation detected is associated with phenotypic variation, it is possible that a gene involved in trait expression has been ‘marked’, and that a quantitative trait locus (QTL), or allele at the locus (QTA) has been identified. In this case, the markers can be used as a breeding tool to predict the likely phenotype of individuals with different marker combinations. Common methods of producing markers include RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism, Vos et al., 1995) and SSR (simple sequence repeats).

Molecular cytology

Several techniques are available to study the number of chromosomes and their structure. Genomic in situ hybridisation (GISH) reveals the parental origin of different chromosomes in hybrids between species, e.g. between S. officinarum and S. spontaneum in sugarcane hybrids. Fluorescence in situ hybridisation (FISH) reveals the location of single DNA fragments, i.e. individual genes, on chromosomes. Flow cytometry is a technique to automatically measure the DNA content of individual cells, which can then be used to estimate the number of chromosomes.

Genetic modification

Apart from inserting new genes to be expressed, genetic engineering can also be used to ‘switch-off’ or down-regulate native genes existing in the plant. Anti-sense technology involves the insertion of DNA with a complementary sequence to the gene to be down-regulated. This prevents some or all of the RNA transcribed from the native gene from being translated into protein or enzyme. RNAi or RNA-interference is a new technology used to silence native genes through the introduction of small RNA molecules. The interfering RNAs induce the plant’s natural gene-control mechanisms to degrade the RNA transcribed from the native gene, resulting in no gene product being expressed.

Transcription analysis

These methods aim at revealing the genes expressed in plants and comparing gene expression between different tissues, e.g. leaf vs stem, or under different conditions, e.g. high vs low water availability. Expressed sequence tags (ESTs) are DNA sequences or genes expressed in the plant at the time of sampling. Comparing the sequences obtained against large international databases can reveal the gene identity; e.g. the gene coding for the enzyme sucrose synthase. Micro-arrays (or macro-arrays) are large numbers of ESTs that are spotted onto glass slides (or nylon membranes). These can be probed with DNA
from other plants to estimate the level of transcript expression of those ESTs or genes in large numbers of individuals.

**Molecular housekeeping: Technologies for managing germplasm collections and germplasm introgression**

Sugarcane breeding requires the maintenance of large germplasm collections of ancestral varieties and parents used for crossing, which are kept for many years and need regular re-planting. This introduces the potential for planting and labelling mistakes, which can have serious consequences. Molecular markers are being used routinely in a number of different breeding programs to genetically fingerprint sugarcane varieties, and this is perhaps the widest use of new technology in breeding at present.

In an ICSB-funded project, 250 SSR primers have been developed (Cordeiro et al., 2000) and are being used in conjunction with other marker systems (AFLP and RFLP) for management of germplasm collections, and verification of pedigree information (e.g. Piperidis et al., 2001). At the South African Sugar Research Institute, SASRI (previously SASEX), SSR markers have been used to test the parentage of a disputed cross (Hack et al., 2001), and to identify several mislabelled genotypes in the germplasm collection. In a complex pedigree, AFLP markers were able to confirm the parents of many of the genotypes in the study, but showed also that some relationships were incorrect (Butterfield, unpublished). This could be either because the pedigree is incorrect or because the parent clones in the germplasm collection are mislabelled, and additional comparative fingerprinting work is being done to resolve this issue.

New cytological techniques for studying chromosomes are also playing a role in breeding, particularly in germplasm introgression. Genomic in situ hybridisation (GISH), in combination with molecular markers, has been used to identify true hybrids between sugarcane and *Erianthus* (D'Hont et al., 1995; Piperidis and D'Hont, 2001), and GISH has also been used to determine the relative contribution of *S. officinarum* and *S. spontaneum* chromosomes in commercial hybrids (D'Hont et al., 1996; Piperidis and D'Hont, 2001). On a more applied level, in South Africa, flow cytometry is being used to estimate chromosome numbers during introgression breeding to identify F1 hybrids with high chromosome number, and in Australia is being used to characterise chromosome number in germplasm collections of wild *Saccharum* material (Piperidis, pers. commun.).

**Molecular breeding: Technologies to assist in breeding and selection**

The main objective of molecular breeding is to identify markers associated with important traits or quantitative trait alleles (QTAs), so that crossing and selection decisions can be based both on genotype and phenotype, rather than phenotype alone. The segregation pattern of markers can be used to determine if they are linked on the same chromosome or appear on different chromosomes, and a genetic map can be developed to establish the relative position of markers on the different sugarcane chromosomes. Genetic maps, and QTAs for important traits identified on those maps, may then be used to determine which genotypes are likely to have specific traits, and this information can be used in breeding and selection.

The first genetic map constructed within the *Saccharum* complex was for the wild species *S. spontaneum* (Al-Janabi et al., 1993; da Silva et al., 1993). This was the first project funded after the formal creation of the ICSB, and was followed by mapping and QTL discovery in *S. officinarum* (Mudge et al., 1996), also funded by the ICSB. These projects used small numbers of markers (<300), but demonstrated that mapping and QTL detection was possible in the complex sugarcane genome using single-dose genetic markers, as described by Wu et al. (1992). This early success led to a larger ICSB-funded mapping and QTL analysis project involving two *S. spontaneum* and two *S. officinarum* genotypes (Ming et al., 1998, 2001), and the mapping of the first commercial sugarcane variety - R570 - by CIRAD (Grivet et al., 1996; Hoarau et al., 2001). Although these maps have not been directly applicable in breeding programs, they have provided valuable information regarding the genome, which has improved our understanding of the potential for molecular breeding in sugarcane.

Maps derived from bi-parental crosses or selfed populations can be difficult to apply directly in breeding programs, where many individuals are used as parents. In order to provide molecular data that can be applied directly, new methods of detecting QTAs by association and mapping markers through linkage disequilibrium (LD) have been developed (Butterfield, 2005a, and unpublished data). Linkage disequilibrium—the persistence of ancestral marker linkages or chromosome segments in modern genotypes—is likely to be high in sugarcane due to the few ancestral clones that contribute to most modern germplasm and the relatively few generations of breeding, and this has been demonstrated by Jaiinoo et al. (1999). At SASRI, markers associated with resistance to the stalk borer *Eldana saccharina* have been used in the breeding program since 1992. Initial work using a population of 78 parental genotypes used in the
breeding program and based on a small set of markers (275) identified a set of 3 QTAs ascribing up to 30% of the phenotypic resistance to eldana (Butterfield et al., 2004). Expanding the data set to over 1000 markers has produced a set of 6 markers ascribing more than 65% of the observed phenotype. A population of 53 parent genotypes used in a recurrent breeding program for eldana resistance has been genotyped at 10 marker loci. Specific crosses based on the marker genotype of parent varieties have been designed to maximise the chance of recovering offspring with favourable marker combinations. Although no selection of progeny based on markers has been done yet, progeny of specific crosses will be used to validate the efficiency of markers used once phenotypic data on eldana resistance are available.

As breeding needs to be done on many traits simultaneously, molecular breeding will be of far more value if markers are available for several important traits. A new project currently funded by the ICSB is aimed at developing LD maps and identifying QTAs in two sugarcane populations for traits involved in cane-quality and cane-yield components. Results from this project will show whether association mapping can detect QTAs for complex traits such as yield components and, based on the size of the marker effects detected, enable comparisons to be made between the expected gains from marker-assisted breeding and conventional breeding.

The approaches described above use anonymous DNA markers that detect genome-wide sequence variation. The Brazilian SUCEST project (see below) has, however, identified more than 33,000 unique genes expressed in sugarcane (Vettore et al., 2003), and SSR sequences have been identified in over 2000 of them. These SSRs are being used to map the genes in which they occur in a bi-parental cross (Pinto et al., 2004) and, if variation within the identified genes is associated with phenotypic variation, the microsatellite markers have the potential to be a powerful tool in molecular breeding by identifying desirable alleles at genes with known function.

**Genetic modification: Technologies to create novel genetic variation beyond the capacity of conventional breeding**

Genetic transformation offers the potential to introduce new genes into sugarcane, to produce new phenotypes not possible through conventional breeding. Well-known examples of this are the introduction of herbicide resistance, and also of resistance to insect pests by introducing the bacterial-derived bt toxins. For many years, genetic transformation of sugarcane has been a reality in different laboratories around the world.

Varieties with herbicide (Snyman et al., 1998; Falco et al., 2000) and insect (Nutt et al., 1999; Braga et al., 2001) resistance have been produced, as well as with resistance to sugarcane mosaic virus (Joyce et al., 1998; Ingelbrecht et al., 1999) and sugarcane yellow leaf virus (Rangel et al., 2003).

To date, none of these have been commercialised due either to intellectual property considerations or concerns over public perception, and these constraints on deployment are likely to continue in the near future.

The ease with which many sugarcane genotypes can now be transformed, and the identification of the sequences of thousands of genes that this plant expresses (see below) raise the possibility of altering the expression of specific genes in the plant and identifying the effects this modification has on the plant’s phenotype.

The development of RNAi and anti-sense technology allows for gene down-regulation and, at Copersucar, the anti-sense expression of a single gene involved in flower development has produced non-flowering plants of a sugarcane variety that otherwise flowers heavily every year. Down-regulation and over-expression of genes involved in carbohydrate metabolism is also being pursued at Copersucar, SASRI and other groups, with the aim of increasing the content of sucrose and other metabolites.

Recently, sugarcane has become the target for production of novel products such as proteins with pharmaceutical properties (Anon., 2003) and as a producer of biopolymers (Brumbley et al., 2004). Vegetative propagation, the absence of flowering in most commercial varieties, the production of a large biomass, the large amount of carbon partitioned into sucrose (up to 42% of the stalk dry weight), and the mobile pool of hexose sugars through most of its life cycle are among the characteristics that make sugarcane suitable to these approaches.

It should also be appreciated, however, that many other crops also are being considered for the production of novel products, some of which may have advantages as biofactories over sugarcane. For example, Tate & Lyle and Dupont recently announced a joint venture (DuPont Tate & Lyle BioProducts) to produce industrial chemicals for use in textiles and plastics from renewable resources such as maize (Anon., 2004). It is likely that if sugarcane industries want to exploit these possibilities, partnerships will need to be made with the Life Science Corporates or established or start-up Biotech companies.
Transcription analysis: Tools to understand the sugarcane genome, and to identify gene targets for molecular breeding and genetic modification

An understanding of the genes and their products involved in sugarcane growth and response to differing environments can play an important role in identifying genes and genomic regions that can be targeted for modification by either conventional breeding and selection, or by transgenesis.

Initial studies to investigate DNA sequences expressed in sugarcane focused on identifying genes that are differentially expressed in the stem, with emphasis on those involved in carbohydrate partitioning and metabolism (Carson and Botha, 2000; Casu et al., 2001). Subsequently, the Brazilian SUCEST project, involving 74 sequencing and data mining groups, has produced 237 954 ESTs derived from 26 cDNA libraries representing different tissues under various developmental conditions (Telles et al., 2001, Vettore et al., 2003). This has allowed expression studies to be conducted in many aspects of plant differentiation, growth and development, trying to understand their behaviour under different biotic and abiotic stresses, and to compare genotypes that have different yields and sugar accumulation capabilities. A whole volume of Genetics and Molecular Biology (Volume 24, issues 1–4, 2001) was dedicated to results from this program. The data from SUCEST continues to contribute very significantly to our understanding of sugarcane molecular biology, as well as identifying genes to be targeted for up- and down-regulation through transgenesis, and by identifying new markers that have potential for molecular breeding.

Future prospects

Our industry is just entering the age where the new technologies emerging in the past 20 years are starting to be applied in sugarcane breeding and variety improvement. Each technology has the potential to significantly improve the economic viability of growing and processing sugarcane, as long as the barriers to routine application can be overcome.

One of the hurdles to routine use of molecular markers in breeding is the cost and throughput of current marker systems (viz. AFLP and SSR) across many genotypes. A pilot project is currently being funded through the ICSB to develop a ‘gene-chip’ for high-throughput, automated marker analysis, with the company Diversity Array Technologies Pty Ltd, Canberra. DArT chips are already being used in other crops (Wenzl et al., 2004) and, if shown to be useful in sugarcane, will provide a platform for the routine screening of germplasm for QTA discovery and gene mapping, as well as for marker assisted selection. Another challenge will be how to integrate molecular information into conventional breeding programs to maximise breeding efficiency in the short term, without compromising genetic variation and breeding success in the long term (Butterfield, 2005b).

Although some programs do have transgenic sugarcane with herbicide, insect or virus resistance, there are still significant obstacles in the path of commercialisation. The costs of securing intellectual property rights and registering new transgenic varieties through the health, safety, environmental and legislative regulations required for deployment may be a significant factor in the deployment of transgenic sugarcane for sugar production. Public resistance to GM crops for human consumption is another issue that is difficult to quantify, and, although acceptance is likely to occur in the long term, it cannot be predicted when consumers and customers will begin to accept GM sugar in the international market.

Gene expression studies have also been limited by the costs involved in the production, hybridisation and analysis of macro- and micro-arrays. However, the development of new technologies such as oligonucleotide arrays produced by Affymetrix and other companies, and the availability of sugarcane ESTs in public databases should allow the construction of gene chips containing, if not all, at least most of the genes that are expressed in a sugarcane plant. These tools, in conjunction with well-defined greenhouse and field experiments, will certainly bring in the future a much better understanding of the genetics of sugarcane, knowledge that can be used to design the sugarcane varieties of the future.

Because the crop is vegetatively propagated and offers no potential returns to private seed companies, sugarcane research does not attract the interest of the large life-science companies, with their large research budgets. Despite this, good progress has been made in developing, and now implementing, technologies that are starting to impact on variety improvement programs. This has happened largely by collaborative efforts and interaction through the ICSB and ISSCT with financial support from the sugar industry, and provides a model of what is achievable through collaborative research. With continued industry backing, the next phase of ensuring that these new technologies start delivering tangible benefits will be an exciting challenge. This challenge is not only for scientists working in research and variety development, but also for field, factory and industry managers who are responsible for capitalising on the economic potential of improved varieties. Making the leap from a solely sugar-based industry to one exploiting other sugarcane components such as biomass, fibre, alcohol or novel products produced through
genetic transformation will require strategic decisions and investment at the industry level, rather than the scientific. It will also require collaboration between technologists working in variety development and those in processing and engineering, if the opportunities afforded by new methodologies are to contribute to the long-term financial viability of sugarcane-based industries.

In summary, new technologies are already assisting traditional breeding programs in managing germplasm collections and ensuring the correct identity of desired parent combinations. Molecular markers are also being used as tools to increase the efficiency of breeding for specific traits such as pest resistance, although this approach still needs to be validated. In addition to these methods that compliment traditional breeding, new technologies in gene discovery and genetic modification offer the potential to re-engineer sugarcane for alternative uses. In the allure of new and ‘sexy’ technologies, it must not be forgotten, however, that a sound and well-managed breeding program is the foundation on which any potential improvements through technology is based. We must make sure that in the race of economic sustainability, the cart does not overtake the horse.

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L’AMÉLIORATION VARIÉTALE POUR UNE INDUSTRIE PLUS PERFORMANTE : LES NOUVELLES TECHNIQUES
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MOTS CLÉS: Marqueurs Moléculaires, Modification Génétique, Analyse de Transcription, Amélioration Variétale de la Canne a Sucre.

Résumé
L’INVESTISSEMENT dans les nouvelles technologies qui viennent compléter ou s’ajouter aux méthodes traditionnelles de génétique commence à produire un impact sur les programmes d’amélioration variétale. Cet article cherchera à mettre en évidence les technologies les plus importantes et à illustrer leur potentiel, présent et futur, pour contribuer à la durabilité des industries de la canne à sucre. Les marqueurs moléculaires sont couramment utilisés dans certains programmes pour gérer les collections de germoplasme,corriger des informations sur la généalogie et vérifier l’intégrité de nouveaux hybrides. L’amélioration variétale moléculaire utilisant des marqueurs putatifs liés à la résistance au foreur des tiges est utilisée en Afrique du Sud pour parfaire l’efficacité de la méthode traditionnelle pour ce ravageur important. Un projet plus vaste visant à identifier et à tester des marqueurs associés aux composantes du rendement et à la qualité de la canne est en cours. La base de données de plus de 33 000 gènes exprimés dans la canne à sucre, développée par le programme SUCEST au Brésil, a identifié des marqueurs situés à l’intérieur des gènes dont les rôles sont connus, et pourrait être une ressource additionnelle précieuse pour l’amélioration variétale moléculaire. La base de données SUCEST fournit aussi des informations sur des gènes qui sont des cibles potentielles pour la modification génétique visant à altérer l’accumulation de saccharose, le métabolisme des carbohydrides et d’autres caractères. La canne à sucre transgénique modifiée pour la résistance aux herbicides, insectes et virus a été produite sous différents programmes mais aucune variété n’a été commercialisée en raison des droits de propriété intellectuelle et par crainte de pression de la part des consommateurs. Il existe aussi un potentiel pour exploiter le fort taux de biomasse de la canne à sucre en l’utilisant comme une ‘usine’ pour produire des biopolymères, des produits pharmaceutiques et d’autres produits à forte valeur ajoutée soit conjointement avec le sucre ou comme produits alternatifs. Pour pouvoir tirer profit de ce potentiel, des décisions stratégiques devront être prises au niveau de l’industrie et des collaborations recherchées avec des compagnies de Bio-Science qui investissent déjà dans ce domaine. Le développement accru de la technologie ‘gene-chip’, aussi bien pour l’analyse des gènes exprimés que pour l’utilisation de systèmes de marqueurs à forte capacité de production, va probablement faciliter l’application des données sur l’expression des gènes et sur les marqueurs dans l’amélioration variétale dans le futur. Toutefois, il ne faut pas oublier que des programmes d’amélioration variétale solides serviront de base pour que le potentiel de ces nouvelles technologies puisse être réalisé.
MEJORAMIENTO GENÉTICO PARA UNA MEJOR INDUSTRIA: NUEVAS TÉCNICAS DE CRUZAMIENTOS

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Resumen

LAS inversiones en nuevas tecnologías que complementan o extienden los métodos tradicionales están empezando a impactar en los programas de mejoramiento de variedades. Este documento busca señalar las más importantes de éstas e ilustrar sus actuales o futuras aplicaciones potenciales al contribuir a la sostenibilidad de las industrias basadas en la caña de azúcar. Los marcadores moleculares de AND se están usando rutinariamente en algunos programas para el manejo de colecciones de germoplasma, correcciones de información de pedigree y el chequeo de integridad de nuevas combinaciones de híbridos. El mejoramiento molecular usando marcadores directamente ligados a la resistencia del Barfenador del Tallo es empleado en Sudáfrica para mejorar la eficiencia del mejoramiento tradicional ante ésta importante plaga y un proyecto más grande para identificar y probar marcadores asociados a componentes de rendimiento y calidad de caña está en camino. La gran base de datos de más de 33,000 genes presentes en la caña de azúcar, desarrollada por el programa SUCEST en Brasil ha identificado a marcadores localizados en genes con funciones conocidas, y tiene el potencial de ser un recurso adicional muy valioso para el mejoramiento molecular. La base de datos SUCEST también está proveyendo información sobre genes que son sujetos potenciales de modificación genética para alterar la acumulación de sacarosa, metabolismo de carbohidratos y otros aspectos. Caña de azúcar modificada transgénicamente para resistencia a herbicidas, insectos y virus han sido producidas en varios programas, pero ninguna variedad ha sido comercializada debido a derechos de propiedad intelectual y a temor ante la presión de los consumidores. El potencial también existe para aprovechar el alto rendimiento en biomasa de la caña de azúcar usándola como una ‘fábrica’ para producir biopolímeros, farmacéuticos y otros productos con alto valor, en conjunto o como alternativa para el azúcar. Para poder capitalizar este potencial, se deberán tomar decisiones estratégicas a nivel de industria y buscar asociarse con compañías de ciencias de la vida que ya se encuentren invirtiendo en esta área. El creciente desarrollo de la tecnología ‘geno-chip’, para analizar genes expresados y para uso en sistemas marcadores de alta respuesta, tenderá a facilitar la aplicación de la expresión genética y de los datos de los marcadores en el mejoramiento de variedades a futuro. Sin embargo, debe recordarse que sólidos programas de mejoramiento proveen la plataforma a través de la cual se puede implementar el potencial de nuevas tecnologías.