



Integration of genomics approach with traditional breeding towards improving abiotic stress adaptation: drought and aluminum toxicity as case studies

Manabu Ishitani^{a,*}, Idupulapati Rao^a, Peter Wenzl^b, Steve Beebe^a, Joe Tohme^a

^aInternational Center for Tropical Agriculture (CIAT), A. A. 6713 Cali, Colombia

^bCenter for the Application of Molecular Biology to International Agriculture (CAMBIA), Canberra ACT 2601, Australia

Abstract

Traditional breeding efforts are expected to be greatly enhanced through collaborative approaches incorporating functional, comparative and structural genomics. Potential benefits of combining genomic tools with traditional breeding have been a source of widespread interest and resulted in numerous efforts to achieve the desired synergy among disciplines. The International Center for Tropical Agriculture (CIAT) is applying functional genomics by focusing on characterizing genetic diversity for crop improvement in common bean (*Phaseolus vulgaris* L.), cassava (*Manihot esculenta* Crantz), tropical grasses, and upland rice (*Oriza sativa* L.). This article reviews how CIAT combines genomic approaches, plant breeding, and physiology to understand and exploit underlying genetic mechanisms of abiotic stress adaptation for crop improvement. The overall CIAT strategy combines both bottom-up (gene to phenotype) and top-down (phenotype to gene) approaches by using gene pools as sources for breeding tools. The strategy offers broad benefits by combining not only in-house crop knowledge, but publicly available knowledge from well-studied model plants such as arabidopsis [*Arabidopsis thaliana* (L.) Heynh.]. Successfully applying functional genomics in trait gene discovery requires diverse genetic resources, crop phenotyping, genomics tools integrated with bioinformatics and proof of gene function *in planta* (proof of concept). In applying genomic approaches to crop improvement, two major gaps remain. The first gap lies in understanding the desired phenotypic trait of crops in the field and enhancing that knowledge through genomics. The second gap concerns mechanisms for applying genomic information to obtain improved crop phenotypes. A further challenge is to effectively combine different genomic approaches, integrating information to maximize crop improvement efforts. Research at CIAT on drought tolerance in common bean and aluminum resistance in tropical forage grasses (*Brachiaria* spp.) is used to illustrate the opportunities and constraints in breeding for adaptation to abiotic stresses. © 2004 Elsevier B.V. All rights reserved.

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1. Introduction

The completion of the *Arabidopsis thaliana* genome sequence in 2000 ([Arabidopsis Genome](#)

* Corresponding author. Tel.: +1 650 833 6625;

fax: +1 650 833 6626.

E-mail address: m.ishitani@cgiar.org (M. Ishitani).

Initiative, 2000), followed by the nearly completed genome sequence for rice in 2002 (Goff et al., 2002; Yu et al., 2002), has caused much excitement among plant researchers. Rapidly following these landmark efforts are advances in characterization of genomes of other crops including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and legumes such as soybean [*Glycine max* (L.) Merr.] and barril medic (*Medicago truncatula* Gaertner; Ware et al., 2002; Lunde et al., 2003; Shoemaker et al., 2002; Young et al., 2003). The accumulating information allows plant researchers to explore new paradigms to address fundamental and practical questions in a multidisciplinary manner. Although new research fields such as metabolomics have emerged as post-genomic era technologies (Phelps et al., 2002), challenges still lie ahead in answering how genomics will aid in crop improvement from a practical standpoint (Osterlund and Paterson, 2002).

CIAT (<http://www.ciat.cgiar.org/>) has formed a biotechnology team aimed at improving varieties of beans, cassava, upland rice and tropical forages for priority traits. The team currently includes specialists in breeding, genetics, cellular and molecular biology, pathology, plant physiology, entomology and statistics. The Center is equipped with state of the art technology including cDNA microarray facilities for large-scale gene expression analyses, tissue culture-cryo conservation for genetic conservation and molecular marker facilities for genotyping.

Under field conditions, crop plants often experience abiotic stresses including water deficits, high temperatures, salinity, and aluminum (Al), low soil fertility, and mechanical wounding. Such stresses can reduce yields to 20% or less of potential yields (Boyer, 1982; Wang et al., 2003). Several excellent reviews in plant abiotic stress responses are available (Chaves et al., 2002; Griffiths and Parry, 2002; Munns, 2002; Zhu and Xiong, 2002; Rao and Cramer, 2003).

This paper reviews strategies for integrating genomic research with traditional crop improvement efforts as applied to abiotic stresses, particularly emphasizing phenotyping prior to applying large scale functional genomic approaches. Examples to illustrate this integration are drawn from CIAT research on common bean and *Brachiaria* spp. forage grasses, with the key role of physiology discussed.

2. CIAT strategies for use of genomics approach toward crop improvement

Major activities for genetic improvement of crops at CIAT still involve a traditional breeding approach, including screening of germplasm for new traits, creating new crosses to recombine sources of variation in new genotypes. CIAT's comparative advantages in these activities include: (1) large holdings of diverse germplasm; (2) linkages to a broad network of collaborators in developing countries as well as in advanced institutes; and (3) relatively low operational cost. These advantages are also invaluable for applying genomics to crop improvement. However, various issues need to be addressed in the application of genomics: identifying an appropriate role for genomics in the breeding process, assessing the feasibility specific genomics-based approaches and finally, how to implement genomics approaches. Initially, the main role for genomics likely is to identify alleles contributing to traits of interest (e.g., quantitative trait loci or "QTLs") and in facilitating screening of populations by providing molecular markers. Excellent reviews for QTL studies are provided by Osterlund and Paterson (2002) and Morgante and Salamini (2003).

The feasibility of applying genomic approaches varies with each crop species depending upon available sequence data or expected level of homology with sequenced species, ease of transformation, and the specific breeding objectives, among other factors. However, building infrastructure for functional genomics capabilities remains costly. Further, tools such as a large population of expressed sequenced tags (ESTs) and access to a large-scale gene expression analysis tool (e.g., a microarray facility) can rapidly become obsolete. Therefore, CIAT applies multiple approaches to ensure access to tools by linking to a broad network of collaborators in advanced research institutions including public and private sectors. This is essential to ensure that projects using genomic approaches remain current in their research procedures. For instance, common bean has a limited number of ESTs (575 registered in NCBI database: <http://www.ncbi.nlm.nih.gov/dbEST/>). However, genomics tools from other legume crops such as soybean can serve as alternate sources (<http://soybean.ccg.umn.edu>) while tools for com-

mon bean are being developed. Alternatively, if a trait is well characterized phenotypically and physiologically in a crop, large-scale genomics tools may be unnecessary. Instead, targeted genomics tools (e.g., a cell-specific EST collection rather than genome-wide EST collection) may be more useful. This is one reason why, as we argue below, plant phenotyping is essential for application of functional genomics.

There are several approaches to confirm the function of genes, including plant transformation and examining phenotypes of lines containing different alleles for the gene(s) of interest. Ability to transform a crop is the preferred method for providing “proof of concept” for promising genomics approaches (Meagher, 2002; Wang et al., 2003) since transformation allows confirmation of the function of candidate genes. For crops such as common bean, where a reliable transformation system is lacking, the ‘proof of concept’ approach cannot be applied directly, and testing has to be conducted in species such as Arabidopsis and rice, which have high transformation efficiencies. If tests are successful, the candidate gene(s) can be transferred using conventional breeding methods combined with marker-assisted selection.

3. The CIAT strategy for increased abiotic stress tolerance in crops

The overall CIAT strategy for studying abiotic stress tolerance uses a top-down approach (phenotype to gene) by beginning with trait analyses in multiple environments (Fig. 1). This involves analyzing crop phenotypes for stress physiology (e.g., looking for traits related to water use efficiency and photosynthesis related parameters) and agronomic traits (e.g., high yield, grain quality) in different locations under different growth environments. A broad range of environments is essential to cover the full range of genetic expression and identify phenotypic traits for further exploration. Fortunately (or perhaps unfortunately), locations representing wide-ranging levels of water deficits that reduce yields of common bean exist in many regions, but impacts are especially severe in north central Mexico, northeast Brazil, and southern Africa (Thung and Rao, 1999; Rao, 2001; Terán and Singh, 2002; Broughton et al., 2003). Crop phenotyping is a key preparative step preceding trait gene discovery by functional genomics approaches (Nguyen and Blum, 2004). Phenotyping allows establishing screening environments and procedures,

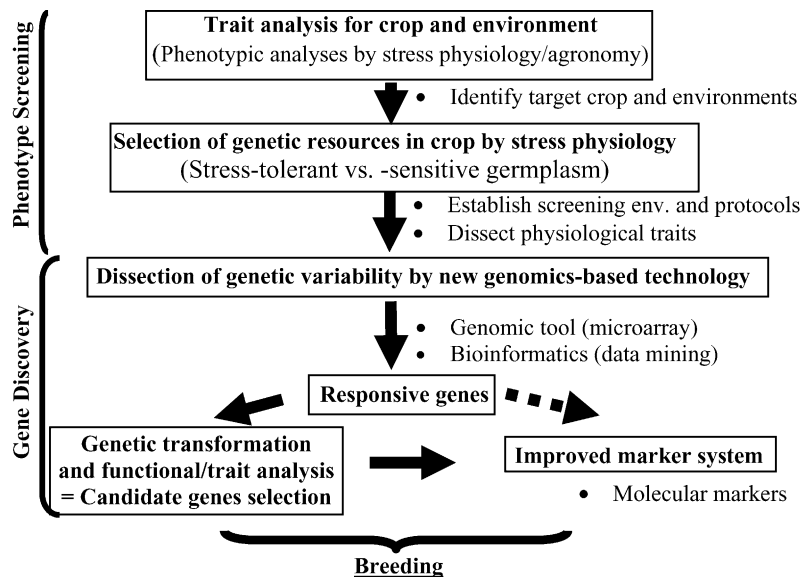


Fig. 1. Overall CIAT crop improvement program strategy for developing abiotic stress tolerance. CIAT utilizes physiological and agronomic research to analyze crop traits and select promising genetic resources, then integrates this research with genomics-based approaches to identify candidate genes for crop improvement.

including identifying appropriate stress levels for differentiating genotypes and developmental stages most responsive to a given stress. Phenotypic and physiological traits in the target crop are then dissected further. Genetic resources in the crop are selected based on phenotyping, which reveals phenotypic and physiological traits required for a particular stress environment (e.g., stress-tolerant versus stress-sensitive germplasm). Genomics-based technologies are then applied to dissect the genetic variability of the selected genetic resources. Technologies from functional genomics (i.e., microarrays) are used because they can analyze large numbers of genes in parallel, thus providing an overall picture of how genes are regulated under stress conditions. This differs from a structural genomics approach (i.e., mapping) in which one or a few genes are studied to determine their location on individual chromosomes.

With a functional genomics approach, thousands of genes are screened using mRNA expression profiling and sequence information coupled with bioinformatics. This allows us to select a group of candidate genes that are putatively involved in expression of phenotypic and physiological traits. These genes can then be examined for “proof of concept” by genetic transformation (over-expression and/or knock-out of the genes) followed by trait analysis (Meagher, 2002; Wang et al., 2003) and for creating molecular markers to be tested in further physiological studies and through breeding programs.

For genomic strategies to successfully support crop improvement, key components are required. Availability of diverse genetic resources allows us to identify superior alleles for genes controlling traits of interest, and an active breeding program is essential to ensure that these genes can be recombined and selected in combinations that result in agronomically useful populations or lines (Hoisington et al., 1999). Access to physiological expertise and an understanding of phenotypic and physiological aspects of traits of interest are also essential (Mifflin, 2000; Bruce et al., 2002).

Stress-responsive genes, and non-stress responsive genes (e.g., cell cycle genes expressed in roots), are applicable in breeding for desirable traits. Sources of these genes will come from both in-house research and publicly available information from model plants such as *arabidopsis* (Bressan et al., 2001; Hall et al., 2002).

For example, if candidate genes are identified for a trait in *arabidopsis*, these genes could be useful resources for genes (comparative genomics) that can be applied as new molecular markers (structural genomics). Thus, our breeding program (e.g., creation of molecular markers) will be advanced by this synergy of different genomic approaches.

4. CIAT Genomic Platform: identify gene function and interaction for use in breeding

An important component of the CIAT program is to also work from the bottom-up, i.e., from the gene to the phenotype. To identify candidate genes responsible for a specific trait, a method is required for selecting candidate genes from the large pool of genes available from genetic resources. For example, the rice genome is thought to contain more than 28,000 genes (Goff et al., 2002; Yu et al., 2002; Kikuchi et al., 2003), a number that prohibits analyzing them individually. Beginning with this large gene pool, genetic data available for crop plants will be filtered first by phenotypic, physiological, and biochemical screening as outlined above and in Fig. 2.

Even after filtering, many genes are expected to respond to a particular stress (Oztur et al., 2002; Shinozaki et al., 2003). However, with the combination of the phenotypic and physiological analyses,

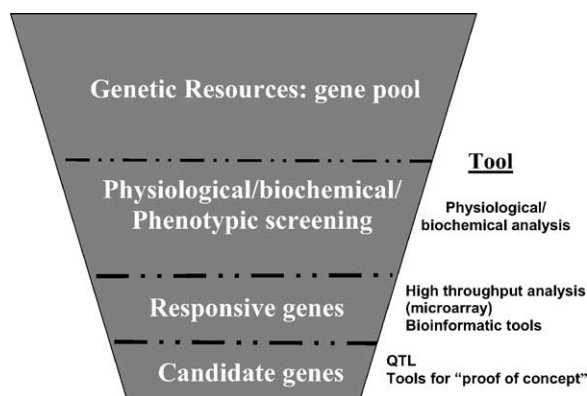


Fig. 2. Filtration of genes for traits. From an initially large pool of genetic resources, physiological/biochemical analyses are followed by identification of responsive genes and subsequent selection of candidate genes by a combination of QTL analysis and testing to confirm gene function (proof of concept).

efforts can focus on a specific category of genes (e.g., function-specific or tissue-specific genes) of interest within the pool of responsive genes. For instance, the focus can be on genes that are responsive in root tissue if the phenotypic or physiological data suggest that roots are the site of action for traits thought to confer resistance or tolerance.

The most promising genes are then assessed for associations with existing genetic information, such as from quantitative trait loci (Morgante and Salamini, 2003). Following filtering and further selection, the reduced pool of candidate genes can be examined by manipulating the gene expression in plants to determine whether the desired trait is expressed in the target test environment as described before. Verification of function is required because most genes that respond to an abiotic stress will not be genes that increase stress tolerance. For such work, a large population of gene knock-out lines will be of great utility (Hirochika, 2001). This “proof of concept” research enables us to identify genes responsible for a specific crop trait (Meagher, 2002; Wang et al., 2003).

5. CIAT abiotic stress research: Two case studies

Two case studies are presented to demonstrate how CIAT uses both a top-down and bottom-up approach to integrate functional genomics, traditional breeding, and physiology in ongoing activities to improve abiotic stress adaptation in common bean and *Brachiaria* species. In bean, we have successfully bred drought tolerant varieties with input from plant physiologists. Several, but limited, genomic tools are available for bean, and gene discovery research began in 2003 (Ishitani and Tohme, 2003). In *Brachiaria*, germplasm was identified that is well adapted to the complex of stresses associated with acid soils. Much progress has been made in our physiological understanding, and genomic studies were initiated in 2002.

5.1. Progress in bean breeding for drought tolerance – 25 years of work

CIAT began working in the 1970s toward developing more drought tolerant varieties of common bean by screening advanced lines selected for other traits

(Broughton et al., 2003). The line BAT 477, among others, showed intermediate tolerance to drought stress. In the 1980s, physiological studies linked this tolerance to greater root growth under water deficit conditions (Sponchiado et al., 1989) and further showed that genetic control of this trait was expressed in roots, not shoots (White and Castillo, 1992). Additional genetic sources for drought tolerance were also identified in the 1980s, especially from Mexican varieties. During the 1990s, a combination of genetic sources was used to develop varieties with higher tolerance and additional mechanism (e.g., photosynthate mobilization from leaves and stems to developing seeds) for drought-stress tolerance were identified (Rao, 2001). By 2002, these drought-tolerant varieties were being tested in the field and parallel to this work, attempts to integrate genomic techniques with traditional breeding were initiated. Five cycles of conventional breeding were required to obtain the drought tolerant varieties of high yielding red and black beans now available. The best families in terms of yield under drought conditions include lines from five pedigrees (Table 1; S. Beebe, unpublished data).

Our working hypothesis is that when soil fertility is limiting, expression of drought tolerance requires that plants also possess tolerance to low soil fertility, expressed as early vigor and good root development (Beebe et al., in press). Thus, multiple stresses represent a particularly complex challenge for crop improvement, but the common denominator for tolerance to both low soil fertility and drought is the vigor

Table 1
Grain yield (kg ha^{-1}) under water deficit (rainfed) conditions of the best families of drought-adapted common bean with small red seed from a nursery of 100 entries grown at CIAT, Palmira, Colombia

Pedigree of family or name	Grain yield
Family	
(SEA21 \times RAB623) \times 9653-16B	1520
(TAR4 \times SEA18) \times Tio Canela	1350
(RAB651 \times Tio Canela) \times (RAB608 \times SEA15)	1310
(SEA21 \times RAB623) \times 9653-16B	1250
Line or crop	
Cowpea	1520
SEA5	1060
Tio Canela	450
DOR 390	230
LSD (0.05)	460

of the root system of the plant. This suggests an area of research that could be especially fruitful for both physiology and genomics, including comparisons among sister species such as cowpea [*Vigna unguiculata* (L.) Walp.], that are better adapted to various edaphic stresses.

A second trait that contributes both to drought and low soil fertility tolerance is the capacity of the plant to translocate biomass into grain. Translocation of photosynthate from vegetative structures to seed is limited by abiotic stresses, and varies considerably between bean varieties. Improved translocation capacity translates into better grain filling, superior grain quality and higher yields (Rao, 2001). Interestingly, roots again appear to play a major role in translocation capacity (Rao, 2001). Therefore, a key question is whether beans have inherently weak roots, and if so, why?

Currently, we are working in three areas to improve yield in common bean (Fig. 3). The first goal is to

incorporate abiotic stress resistance as a breeding objective into ongoing programs for beans by identifying contrasting genotypes, using inter-specific crosses, and evaluating selected genotypes with farmers. Genetic improvement is being accelerated by application of molecular markers (Broughton et al., 2003; Morgante and Salamini, 2003). This involves developing bean populations that are segregating for stress tolerance and identifying molecular markers linked to QTLs contributing to overall stress tolerance. We are continuing research to understand the phenotypic and physiological basis of drought adaptation in beans. This will aid in dissecting the overall stress tolerance in bean into identifiable individual physiological components, especially for segregation patterns of phenotypic and physiological mechanisms in bean populations segregating for stress tolerance. The phenotypic and physiological components are prerequisites for trait gene discovery as described before.

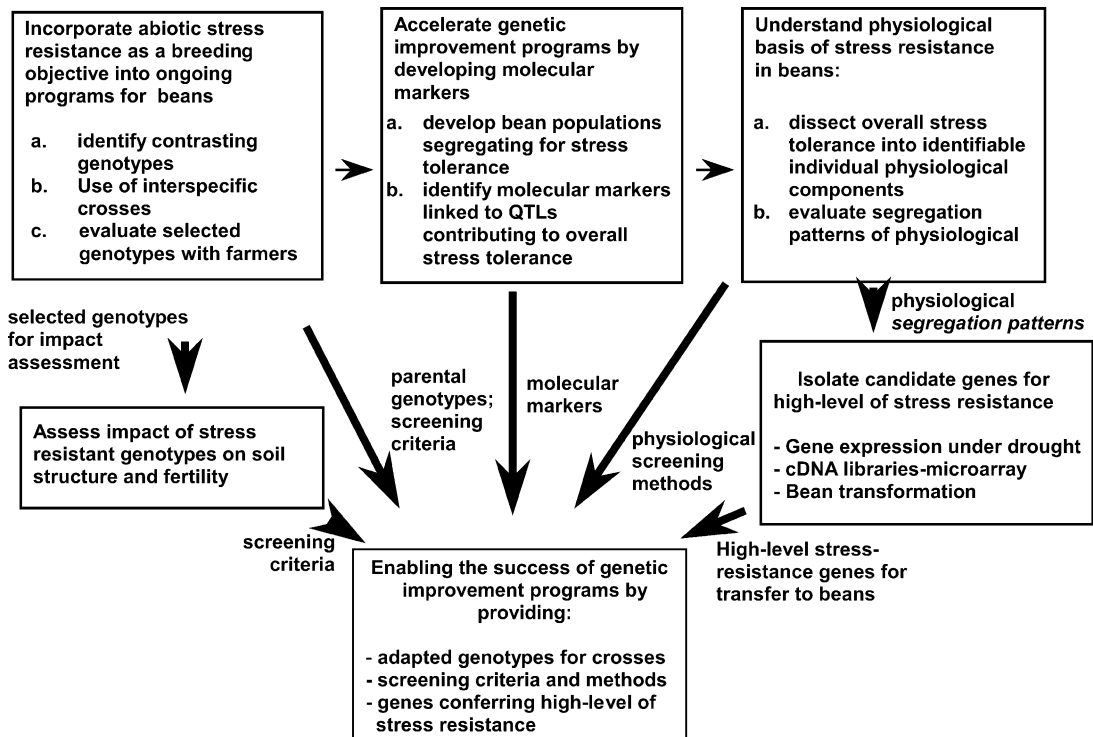


Fig. 3. Where will future increases in yield come from? CIAT plans to increase yield in bean crops by an intensive program including understanding the physiological basis of drought in beans, incorporating abiotic stress resistance as a breeding objective, and development of molecular markers for stress-tolerant genes.

Due to limited genomic resources in common bean, we are beginning extensive preliminary molecular analyses before applying large-scale genomic techniques, such as cDNA microarray. A major effort is underway to select 100–200 genes from species such as arabidopsis. The selected genes should represent 12 functional categories based on prior evidence suggesting the genes are involved in stress response or metabolic pathways. Corresponding bean genes will be examined through polymerase chain reaction (PCR)-based technology using conserved gene sequences. This characterization of expression has two purposes. First, these genes will give a snapshot of the molecular response in bean to drought stress (e.g., tolerant versus susceptible lines). This should indicate which category of genes to focus on for the expression of the phenotypic and physiological trait. Second, and more importantly, expression analyses of this subset of genes, particularly using a set of house-keeping genes, will provide a selection screen that will allow us to reduce variability of field-grown plant materials that will be used for more comprehensive gene expression analyses.

One example of genes under consideration is the *DREB* genes, originally isolated from arabidopsis as transcription factors and thought to be master switches for stress responses (Kasuga et al., 1999; Fowler and Thomashow, 2002). Our preliminary work suggested there are at least three *DREB*-like genes in common bean (Galindo et al., 2003). The *DREB* research will help us to answer the following questions: (1) do the common bean *DREB* genes have the same function as in other plants? and (2) will these *DREB* genes serve as robust molecular markers for stress adaptation both in the field and in laboratory studies? To characterize the function of the *DREB* genes, DNA binding activity of gene products will be tested using yeast-one hybrid system, and the increase of stress tolerance will be examined with transgenic approaches (Kasuga et al., 1999; Fowler and Thomashow, 2002).

Research to develop additional molecular markers continues parallel to these efforts. In 2003, the core collections of *P. coccineus* and *P. polyanthus* were evaluated with AFLP markers, demonstrating that very few differences in genetic structure exist between these two species, although Mexican and Guatemalan accessions of *P. coccineus* separate slightly, and an ecotype of *P. polyanthus* exists in South America (S.

Beebe, unpublished data). This research will contribute to genetic improvement by identifying adapted genotypes for crosses, screening criteria and methods, and genes conferring high-levels of stress resistance.

5.2. *Brachiaria*: an important tropical forage grass with limited genomic tools

Brachiaria, a forage grass of African origin, is widely sown in the Americas (e.g., 40–50 million hectares in Brazil). All commercial *Brachiaria* cultivars, with the exception of the diploid *B. ruziziensis* Germain & Evrard., are polyploid apomicts. Breeding efforts started in the mid-1980s when scientists at CIAT initiated work to recombine edaphic adaptation of *B. decumbens* Stapf. with the spittlebug resistance of *B. brizantha* (Hochst. ex A. Rich.) Stapf. Because of their apomictic mode of reproduction, the two species were first used to pollinate sexually reproducing, tetraploidized *B. ruziziensis*. The hybrids created are being recombined to achieve the desired combination of traits (Miles et al., in press). Apart from a limited number of microsatellite and EST markers, developed at CIAT during 2000–2001, there are currently no genomic tools that aid genetic improvement of *Brachiaria*.

Brachiaria decumbens performs well on infertile acid soils (Miles et al., in press). Such soils are a major constraint to agricultural production in Latin America. They tend to contain low levels of essential cations, nitrogen and plant-available phosphate, in combination with high levels of phytotoxic Al (Rao et al., 1993). Simulating this “stress syndrome” in nutrient solutions, we found that less-adapted *B. ruziziensis* became more sensitive to Al toxicity as the level of nutrients in the growth medium was reduced. By contrast, Al resistance of well-adapted *B. decumbens* was unaffected by nutrient deficiency (Wenzl et al., 2003a). The level of resistance of *B. decumbens* was considerably higher than that of Al-resistant cultivars of graminaceous crops (Wenzl et al., 2001; Miles et al., in press). These results suggested that *B. decumbens* was a good subject to study physiological adaptations to the various stress components of the “soil acidity syndrome.” CIAT developed a glasshouse screening method for vegetative *Brachiaria* propagules that quantifies both Al resistance and root growth vigor in the absence of nutrients. Using this

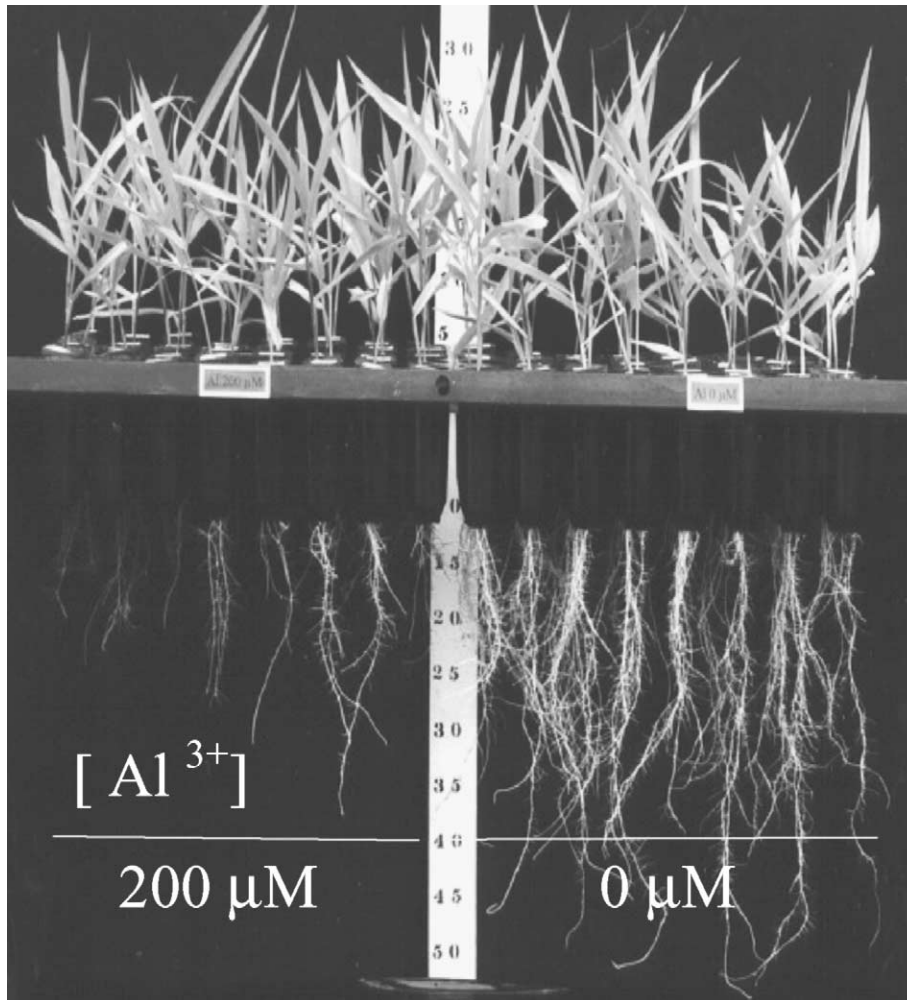


Fig. 4. Effect of elevated aluminum on roots of *B. ruziziensis* × *B. decumbens* hybrids under nutrient-limited conditions. Hybrids grown for 21 days in 200 μM Al^{3+} in nutrient solution show large differences in root growth, but no differences among hybrids were found in the 0 μM Al^{3+} control.

method, the genetic complexity underlying these two traits was characterized by studying their segregation pattern in a *B. ruziziensis* × *B. decumbens* population of 282 hybrids (Fig. 4). Both traits showed continuous variation, suggesting polygenic inheritance (Buitrago et al., 2003). QTL mapping based on simple sequence repeat (SSR) and amplified fragment-length polymorphism (AFLP) markers is currently underway.

In parallel, Al resistance is being dissected into physiological components in order to develop criteria for the isolation of candidate genes. Root apices of many plant species, including wheat, corn, buckwheat

and tobacco, secrete organic acids in response to exposure to Al. These are thought to bind and detoxify Al in the apoplast and rhizosphere (Matsumoto, 2000; Ma and Furukawa, 2003). Surprisingly, root apices of *B. decumbens* accumulated, rather than secreted, organic acids when exposed to Al (Wenzl et al., 2001, 2002). Thus, organic acids may contribute to Al resistance of *B. decumbens* by detoxifying Al ions within the apical symplasm. A comparison with less-adapted *B. ruziziensis*, however, suggested that this was probably not the mechanism responsible for the several-fold higher resistance level of *B. decumbens*

compared to *B. ruziziensis* (Wenzl et al., 2002). Recent experiments suggest that Al resistance in *B. decumbens* may be due to a hitherto uncharacterized, generic mechanism that is not only effective against Al but a range of other cationic toxins (Wenzl et al., 2003a). Rather than isolating candidate genes based on a physiological model, we therefore initiated experiments to isolate genes based on heterologous expression in yeast and a comparison of their expression patterns in root apices (Wenzl et al., 2003b).

Genes isolated from Al-stressed root apices of *B. decumbens* were expressed in yeast to identify genes that increase Al resistance of the heterologous host, an approach successfully used in previous studies on metal toxicity and resistance (Frommer and Ninnemann, 1995; Anoop et al., 2003). Preliminary results suggest that genes such as S-adenosylmethionine synthetase, cysteine proteases and β -1,3-glucanase are associated with Al resistance (Wenzl et al., 2003b), but this must be confirmed by further experiments.

For the approach based on differential gene expression, two groups of *B. ruziziensis* \times *B. decumbens* hybrids were selected for contrasting levels of Al resistance. We are currently analyzing gene expression patterns in root apices from the two bulks, using a combination of subtractive hybridization and microarray-based expression profiling (about 4000 subtracted cDNAs; Arango et al., 2003). The genes identified by their differential expression will be selected by criteria associated with gene function and existing QTL information in a different crop such as rice (Nguyen et al., 2002). The selected genes will be used to develop molecular markers to see co-segregation with the phenotype in the mapping population. This genetic approach of marker development and QTL will enhance the efficiency of the breeding program.

6. Conclusion

Considerable progress has been made building infrastructure for applying genomic approaches. These include one-dimensional genetic information (genome sequences), a large number of expressed sequence tags, and gene-knockout populations in several plant species of biological and agronomical importance. The information generated on plant genome and gene structure has drastically changed strategies

in plant research and is starting to impact plant breeding (Tabata, 2002). Comparative genomics utilizing the integrated information from different plants is expected to provide a common language to aid knowledge transfer among different species (McCouch, 1998; Cushman and Bohnert, 2000; Shimamoto and Kyojuka, 2002).

The complex molecular basis of tolerance to environmental stresses, often quantitative in nature, provides a challenge to plant breeding (Morgante and Salamini, 2003). Scientists at CIAT are incorporating functional genomics strategies into genetic improvement programs with other genomic (structural and comparative) approaches to dissect complex traits into manageable molecular components. Efforts are well underway in the case of Al resistance of *Brachiaria*, but more emphasis is needed on drought tolerance in common bean, particularly in the areas of physiology and biochemistry. We remain optimistic that functional genomics will contribute greatly to bridging the gap between phenotype and genotype in the context of breeding for complex traits such as adaptation to abiotic stresses.

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