Markers, genomics and post-genomics approaches - will they assist in selecting for drought tolerance?

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Abstract

Molecular markers, genomics and post-genomics platforms offer unprecedented opportunities to identify, select and clone genes influencing drought tolerance. The dissection of the genetic basis of quantitative traits into their single components, the so-called QTLs (Quantitative Trait Loci) provides a more direct access to valuable genetic diversity for important physiological processes that regulate the adaptive response to drought. This, in turn, enables us to deploy marker-assisted selection (MAS) for enhancing crops' performance. However, despite the impressive progress in molecular techniques and the large number of QTLs described to influence yield in drought-stressed crops, the overall impact of MAS and other applications of genomics on the release of drought-tolerant cultivars has so far been marginal. QTL discovery should be viewed as the first step of a longer process aimed at identifying and isolating the underlying molecular polymorphism of the functional variation revealed through QTL analysis. The cloning of major QTLs will offer additional opportunities for a more effective exploitation of the allelic richness present in germplasm collections. A deeper integration of MAS with conventional breeding methodologies and other interdisciplinary approaches will enhance our capacity to improve drought tolerance.

Media summary

Genomics provides novel opportunities to unravel the genetic basis of drought tolerance and manipulate its genetic determinants (QTLs) through marker-assisted selection to obtain improved cultivars.

Keywords

Drought, genomics, QTL, marker-assisted selection, microarray, candidate gene.

Introduction

Among abiotic stresses, drought is the most important one from an economic standpoint and likely the most resilient to breeders' efforts. Traditionally, the multifaceted nature of drought coupled with an incomplete knowledge of the genetic and physiological bases of yield in water-limited conditions have hindered breeding progress in such conditions (Ceccarelli and Grando 1996; Boyer 1996; Passioura 1996, 2002). Of crucial importance is tailoring cultivars able to optimize water harvest and/or water use efficiency (WUE) while maximizing final yield in relation to the prevailing dynamics of the drought episodes, their severity and seasonal occurrence in the target environment, all factors that should be duly considered during the entire selection process (Blum 1988; Richards 1996; Bacon 2004). To overcome the low response to a direct selection for yield under drought conditions, substantial efforts have targeted the manipulation of morpho-physiological traits influencing drought resistance through an escape, avoidance and/or tolerance mechanism (Ludlow and Muchow 1990; Blum 1996). However, this indirect selection strategy has only been successful in a limited number of cases, a notable example being represented by the recent release of the wheat cvs. Drysdale (www.csiro.au/drysdale) and Rees, which have been selected for the unique dryland conditions of the Mediterranean-like environments of eastern Australia using carbon isotope discrimination, a molecular signature which provides an indirect measure of WUE (Farquhar et al. 1989; Richards et al. 2002). As compared to conventional approaches, the advent of molecular markers has enabled us to dissect quantitative traits into their single genetic components, i.e. the quantitative trait loci (QTLs; Dudley 1993; Tanksley 1993; Lee 1995; Beavis and Kein 1996; Quarrie 1996; Prioul et al. 1997; Tuberosa et al. 2002b), and to assist the selection and pyramiding of the beneficial QTL alleles through marker-assisted breeding (MAB; Ribaut et al. 2002). Importantly, MAB reduces or eliminates altogether the reliance on environmental conditions during the selection phase, a major hindrance to conventional breeding when dealing with traits influenced by drought (e.g. anthesis-silking interval in maize; Ribaut et al. 2004). During the past decade, an increasing number of studies have strived to map QTLs affecting drought-related traits and yield in major crops exposed to water deficit (for an updated list, see www.plantstress.com). In a limited number of cases, marker-assisted selection (MAS) has been deployed to manipulate the target traits and to ameliorate tolerance to drought

(Mackill et al. 1999; Crouch and Serraj 2002; Ribaut et al. 2002; Saxena and O'Toole 2002). More recently, bioinformatics (Bray 2002) and the deluge of information generated by the post-genomics platforms (Tuberosa et al. 2002a; Hazen and Kay 2003) have added new dimensions for deciphering the role and function of genes governing the response to drought. Despite all these impressive and fascinating technological breakthroughs, the overall impact of MAS and genomics on the release of drought-resilient cultivars has so far been disappointingly negligible. Herein, we critically analyze the reasons for this limited applicative impact and review the potentiality of a number of emerging post-genomics platforms for more effectively selecting drought-tolerant cultivars.

Prioritizing the choice of target traits and QTLs
The improvement of drought tolerance relies on the manipulation of the traits that limit yield and their accurate phenotyping under the prevailing field conditions being targeted. This issue is particularly crucial for the identification of QTLs of traits categorized as adaptive (e.g. accumulation of osmolytes or other metabolites in response to cellular dehydration) as compared to constitutive traits (e,g., root elongation rate). Notwithstanding that all traits, even those constitutively expressed, show a varying degree of plasticity in response to environmental cues (Via et al. 1995) and influence crops' adaptation to drought (Blum 2002), the ranking of the genotypes of a mapping population scored for a constitutive trait under different water regimes will be less affected by environmental and/or developmental factors. Indeed, one of the major difficulties in enhancing drought tolerance through MAS relates to the high QTL x environment (Q x E) interaction shown by the majority of QTLs in trials conducted under varying water regimes and/or during different seasons (Ribaut et al. 2002). A major advantage of constitutive traits is that their phenotyping does not require conditions of programmed drought stress as needed for adaptive traits.

Target traits
Yield improvements through conventional approaches have been achieved with limited knowledge of the morpho-physiological determinants of drought tolerance. The limited success in improving drought resistance through molecular approaches relates to the difficulty in identifying the key determinants of yield under drought conditions in the field (Blum 1988; Ludlow and Muchow 1990; Boyer 1996; Turner 1997; Passioura 2002; Nguyen and Blum 2004; Tuberosa 2004). As an example, a greater capacity of the root meristem to adjust osmotically at a given water potential will have a positive impact on final yield only if deeper roots can extract additional moisture from the soil. However, when moisture is unavailable in deeper soil, a condition quite common to many drought-prone environments (e.g. the Mediterranean basin), growing deeper roots will not provide any advantage to the crop and may even influence negatively its final yield due to an excessive partitioning of photosynthates to the root. Roots show a high degree of plasticity in terms of response to environmental factors, especially to the availability of water and nutrients (Gahoonia and Nielsen 1997). To a varying degree, this plasticity is under genetic control (O'Toole and Bland 1987; Nguyen et al. 1997; Babu et al 2001; Tuberosa et al. 2002c, 2003b), with each genotype being characterized by its own response to different environmental cues. This is the case for most cereal plants where the phasic development of adventitious roots (Hochholdinger et al. 2004) determines the root system profile and water extraction (Blum and Arkin 1984). The first critical step is thus to identify genes/QTLs playing a major role in governing genetic variability for tolerance to drought. Under this aspect, the dilemma faced by the "molecular" breeder is somehow similar to that faced by conventional breeders, although the focus is shifted from whole traits (e.g. breeding for adaptive vs. constitutive traits) to the effects of their genetic determinants, such as estimating the magnitude of QTL effects and the Q x E interaction. In this context, priority should be given to QTLs characterized by a limited interaction with the water-regime, or other environmental variables, and by a consistent pleiotropic effect on yield. The second, and perhaps more critical step from an applicative perspective, is to verify to what extent the effect of the beneficial QTL allele is consistent in the genetic backgrounds to be improved.

A major shortcoming of any QTL study is the low accuracy in detecting the real number of QTLs affecting the genetic variation of the investigated traits (Charcosset and Gallais 1996; Xu 2003), particularly with populations of less than 150-200 progenies, which is the case in the majority of QTL studies reported so far. In a simulation study applied to experimental data, Beavis (1994) showed that with populations of ca. 100-200 progenies, only a modest fraction of QTLs were identified; furthermore, the effect of each single QTL was usually overestimated. Another study showed that with fewer than 500

progenies and independently from marker density, it is very difficult to detect QTLs of small effect (Beavis 1998). These predictions were substantiated in experiments carried out with maize mapping populations sufficiently large (> 400 progenies) to allow for meaningful subsamplings (Melchinger et al. 1998; Openshaw and Frascaroli 1997). The effectiveness of detecting QTLs using the values of single environments and their mean has been evaluated in a number of studies. Inconsistency in QTL detection across environments has been reported by a number of authors (Paterson et al. 1991; Mather et al. 1997; Wang et al. 1999; Landi et al. 2002b). Additionally, most of the current methods of QTL analysis, in terms of experimental design, population dimension and statistical approach are inadequate for an effective detection of epistatic QTL interactions, which may represent a strong source of variation for complex traits such are those affecting drought tolerance. Given their potential for impact on response to selection, empirical investigations to quantify the importance of epistasis and gene-by-environment interactions for trait phenotypes is considered to be an important component of the design and optimization of any marker-assisted selection strategy (Podlich et al. 2004). Statistical methods to detect epistatic QTLs that have been recently proposed (Kao and Zheng 2002) are waiting for field-experiment validation. With maize as a model species, computer simulation carried out by Bernardo (2001) showed that gene information is most useful in selection when few loci (e.g. 10) control the trait. With many loci (> 50), the least squares estimates of gene effects become imprecise. Overall, the results of the simulation indicated that the typical reductionist approach pursued through QTL discovery only has limited potential for enhancing selection for quantitative traits in hybrid crops of the trait of interest.

One additional reason for the limited applicative results of the QTL approach is because the choice of the parental lines used for QTL discovery has prevalently been based on their differences for the morpho-physiological traits of interest rather than their agronomic value. This approach maximizes the opportunities to identifying major QTLs but does not guarantee any real progress when MAS is applied to introgress the beneficial QTL alleles, also because such alleles, or those with even more beneficial effects, may have already been fixed in the elite germplasm. In view of this, the negligible impact that genomics has made so far on the release of drought-tolerant cvs. should come as no surprise. Therefore, QTL discovery is only the first step of a longer process aimed at identifying and isolating the underlying molecular cause at the sequence level (i.e. polymorphism) of the functional variation revealed by QTL analysis, i.e. the so-called QTNs (Quantitative Trait Nucleotides; Morgante and Salamini 2003). The cloning of the sequence underpinning the functional basis of the QTL will allow us to characterize and manipulate more precisely the available germplasm for the type of alleles present at the target QTL. Clearly, the number of drought-related QTLs suitable for a positional cloning approach is very limited, and such QTLs are the exception rather than the rule. However, a careful choice of the target traits and parental lines will facilitate the identification of major QTLs with a consistent effect across water regimes. Indeed, the widespread notion that drought is inevitably a complex trait has been recently challenged. According to Blum (2002), the complexity of drought tolerance can be greatly reduced if two major points are considered: 1) a number of plant traits crucial for the control of plant water status and yield under drought are constitutive and not stress-adaptive; 2) plant water status, more than plant function, controls crops' performance under drought. However, because the adaptation to severe drought (i.e. when plant survival is threatened) involves molecular pathways specifically regulated under such extreme conditions, the targeted manipulation of adaptively-expressed genes/QTLs will contribute further possibilities for the fine-tuning of crops' survival in arid environments.

A problem frequently encountered in interpreting QTL effects under drought conditions in the field is due to the confounding effect that differences in the water status of the plants may have on the value of the morpho-physiological traits being investigated. Therefore, a correct interpretation of QTL effects on morpho-physiological traits influenced by water status (e.g. concentration of ABA and osmolytes) and, more importantly, the interpretation of their pleiotropic effects on yield, requires careful consideration of this aspect. This is easier said than done, since an accurate measurement of the water status in the large number of plants typically included in a mapping population is a rather daunting task, also considering the sizeable and rapid changes that can occur in the water status of the plant as a result of the fluctuations in the evapotranspirative demand during the day. For this category of traits, a more accurate evaluation of a set of genotypes can be obtained under controlled conditions which allow for a better control of daily fluctuations in the water status of the plants. As an example, exposing plants to a given concentration of osmolytes (e.g. mannitol solution) provides an opportunity to evaluate the capacity to accumulate ABA at a given level of water deficit. An additional source of inaccurate estimates of QTL effects may be related

to the small plot size frequently adopted to evaluate mapping populations. This condition exacerbates competition for water, nutrients and light between adjacent plots, which may lead to overestimating the effect on yield of the QTLs for morpho-physiological traits (e.g. early vigor, root architecture, leaf angle, anthesis date, etc.) affecting the level of competition. In this case, even if a QTL is consistently detected in several field trials, once the beneficial allele is introgressed and evaluated in larger plots of genetically homogeneous material, its overall effects on yield may be consistently reduced or even negative (e.g. an excessive root mass).

Harnessing beneficial alleles from wild relatives
Domestication has drastically reduced the level of genetic variability present in crops compared to their wild counterparts, an aspect probably even more relevant for those drought-related traits with a substantial role in survival under natural conditions. To overcome this limitation, Tanksley and Nelson (1996) have advocated the use of advanced backcross quantitative trait locus analysis (AB-QTL), an approach that offers the opportunity to quickly discover and exploit beneficial QTL alleles present in wild germplasm. The AB-QTL approach relies on the evaluation of backcross (BC) families between an elite variety used as recurrent parent and a donor accession, usually a wild species sexually-compatible with the crop. Preferably, QTL analysis is delayed until the BC_2 generation and after selecting in BC_1 against characteristics with a negative effect on yield (e.g. ear shattering in cereals). The validity of AB-QTL has already been proven in tomato (Tanksley et al. 1996) and rice (Xiao et al. 1998; Moncada et al. 2001). Wild barley (*Hordeum spontaneum*) is a source of valuable alleles for traits which influence resistance to abiotic stresses (Grando and Ceccarelli 1995; Forster et al. 2000, 2004; Baum et al. 2003). An *H. vulgare* x *H. spontaneum* backcross population was investigated in three Mediterranean countries under rainfed conditions to identify beneficial QTL alleles contributed by the wild parent (Talamè et al. 2004). Among the 81 putative QTLs found to influence heading date, plant height, ear length, ear extrusion, grain yield and/or 1000-grain weight, in 43 cases (53%) the wild parental line contributed the alleles increasing the traits' value. As to grain yield, although the majority (65%) of the favourable QTL alleles were those of *H. vulgar*e, at six QTLs the alleles increasing grain yield were contributed by *H. spontaneum*. These results are encouraging as to the possibility of using AB-QTL as a germplasm enhancement strategy for identifying wild progenitor alleles capable of improving yield of the related crop cultivated under arid conditions. This approach may be particularly valuable for the identification of beneficial wild alleles improving survival under extreme drought, an unlikely occurrence in most agricultural systems, but one that can have disastrous socio-economic consequences in marginal agro-ecosystems prone to prolonged droughts. Ideally, introgression of such beneficial alleles (examples provided by Humphreys and Thomas 1993; Johnson et al. 2000; Gororo et al. 2002) should bear no negative consequences under more favourable conditions.

QTL characterization and validation
The accurate characterization and validation of a QTL requires its isogenization, usually carried out through MAS. The evaluation of testcrosses obtained by crossing a number of tester lines with pairs of NILs contrasted at a QTL region allows us to more accurately determine the consistency of the QTL effect and its applicative value. Additionally, once pairs of congenic strains have been derived for different QTLs, crossing schemes can be devised to test for epistatic interactions between the isogenized QTLs. Although the derivation of congenic strains through MAS does not lead to short-term applications, it is an essential step to "Mendelize" single QTLs and towards their positional cloning. A number of NILs have been obtained for QTLs of traits relevant for drought tolerance (Tuinstra et al. 1998; Shen et al. 2001; Landi et al. 2002a, 2004; Price et al. 2002; Sanchez et al. 2002) and several others are presently being derived. In maize, MAS has been deployed to derive pairs of near-isogenic backcross-derived lines (BDLs) differing for the parental alleles at a major QTL (*root-ABA1*) on bin 2.04 near *csu133* that has been shown to consistently affect root architecture as well as ABA concentration (Tuberosa et al. 1998; Quarrie et al 1999; Sanguineti et al. 1999; Landi et al. 2004). A field evaluation conducted under well-watered and water-stressed conditions during two consecutive seasons indicated that each pair of *root-ABA1* BDLs differed significantly and markedly for L-ABA, thus confirming the effectiveness of the MAS (Landi et al. 2004). Furthermore, the BDLs and derived near-isogenic hybrids showed significant differences for root lodging, root mass and brace root angle (Giuliani 2004; Landi et al. 2004), supporting the previously suggested hypothesis that the two parental lines differ for root architecture (Sanguineti et al. 1999). A more systematic approach to generate a series of NILs covering the whole genome, irrespectively from the investigated trait, is provided by the construction of a series of lines, each carrying

a small portion (usually ca. 15-30 cM) of a donor genome in an otherwise common genetic background. An important example of the effectiveness of this approach for gene/QTL discovery and cloning has already been provided in tomato (Eshed and Zamir 1994).

Marker-assisted selection for improving drought tolerance
Although simulation studies have outlined the advantages of utilizing MAS to improve quantitative traits (Lande and Thompson 1990; Hospital and Charcosset 1997), only a few studies have demonstrated the possibility of using molecular markers for improving quantitative traits (Tanksley et al. 1996; Mohan et al. 1997; Shen et al. 2001; Ribaut et al. 2002, 2004). A number of factors have contributed to the inability to successfully deploy MAS for improving drought tolerance and other complex traits. Perhaps the major difficulty has been the detection of major QTLs and an accurate estimate of their effects. In a recent study presenting a new and more effective approach for MAS of complex traits, Podlick et al. (2004) have underlined that most evaluations of mapping and MAS strategies have assumed that QTLs act independently (i.e. no interaction with other genes and/or environment), an assumption that has led Bernardo (2001) to suggest that MAS has little if any power over traditional phenotypic selection. The approach to MAS has been to develop accurate estimates of QTL effects within a relatively narrow reference population and use those estimates in the application of marker-based selection. The approach so far utilized for MAS relies on the assumption that the effects of the desirable QTL alleles will not vary after a number of selection cycles. However, the value of QTL alleles can differ depending on the genetic structure of the current set of germplasm in the breeding program (Wade 2002). To overcome this problems, Podlick et al. (2004) have suggested the "Mapping As You Go " (MAYG) approach, namely a mapping-MAS strategy which partially accounts for the presence of epistasis and G x E interaction by implementing MAS such that the estimated values of QTL alleles can evolve as the current germplasm evolves over cycles of selection. The MAYG method operates by cyclically reestimating the value of QTL alleles each time a new set of germplasm is created during the breeding process. The effectiveness of the MAYG method for a range of genetic models has been estimated through simulation (Podlich et al. 2004).

In maize, the first large-scale application of MAS to ameliorate drought tolerance occurred at CIMMYT, where in 1994 two parallel projects were initiated to improve the performance of elite lines and open-pollinated populations. In this case, MAS was deployed to introgress QTL alleles for reducing the anthesis-silking interval (ASI), i.e. the interval between the extrusion of the anthers and the extrusion of the silks. ASI is consistently and negatively associated with grain yield under conditions of water deficit (Bolaños and Edmeades 1996; Ribaut et al. 1997b, 2002). The availability of molecular markers linked to the QTLs for ASI allows for a more effective selection under drought as well as when drought fails to occur at flowering (Ribaut et al. 2002, 2004). Using the line CML247 as the recurrent parent and Ac7643 as the drought-tolerant donor, Ribaut and co-workers started a backcross-MAS (BC-MAS) project based on the manipulation of five QTLs affecting ASI. CML247, an elite line with high yield *per se* under well-watered conditions, is drought susceptible and shows long ASI under drought. The QTL regions carrying alleles for short ASI were introgressed through MAS from Ac7643 into CML247. A number of lines (ca. 70) derived through BC-MAS were crossed with two testers and were evaluated for three consecutive years under several water regimes. Under severe stress conditions that reduced yield by at least 80%, the selected lines were superior to the unselected control. However, this advantage decreased at a lower stress intensity, and disappeared for a stress that reduced yield less than 40%. Across the water-limited trials, a few genotypes consistently outperformed the controls. Under well-watered conditions the selected lines did not show any yield penalty when compared to the control lines. Notwithstanding the success of this BC-MAS experiment, Ribaut et al. (2002) pointed out that QTL manipulation to improve germplasm for polygenic traits has a number of limitations, the most distinct being the inability to predict the phenotype of any given genotype based on its allelic composition, a constraint particularly important when epistatic interactions influence the target trait (Podlick et al. 2004). Another limitation of MAS pertains to the high cost associated with QTL discovery and validation, as well as the release of superior lines. At CIMMYT, a new strategy to overcome these limitations has been implemented for improving the cost-effectiveness of MAS and its capacity to deliver improved germplasm (Ribaut and Betran 1999). This new method, named single large-scale MAS (SLS-MAS), combines conventional breeding with the use of MAS conducted at early generations for selecting plants with fixed, favorable alleles at target QTLs. The first step of SLS-MAS entails the selection of a number of elite lines with a high level of allelic complementarity (e.g. belonging to different heterotic groups) and optimal expression of the target traits.

This choice can be based on phenotypic data for the target traits obtained with conventional approaches (e.g. performance *per se* and in diallel crossing) complemented with molecular data on the genetic distances (e.g. fingerprinting profiles) of the lines. In the second phase, agronomically valuable QTL alleles are identified through the evaluation of mapping populations derived from the cross of the elite lines and testers with poor performance for the target traits. Subsequently, the lines are intercrossed to develop large segregating populations from which plants homozygous for desirable alleles at target QTLs are selected in the F_2-F_3 generations. Due to the large number of plants analysed, the cost-effectiveness of this approach relies on the utilization of PCR-based markers (Ribaut et al. 1997a). During the third and final step, pedigree selection based on the characteristics of the target environment and local needs is carried out to develop new elite lines. The distinctive feature of SLS-MAS is that MAS is deployed only once and at an early generation to fix the desired alleles at target QTLs. Although this approach reduces the chances of undesired recombination between the QTL and the flanking markers, it requires the selection of a minimum number of plants to guarantee a sufficient allelic variability at non-selected loci. SLS-MAS has been adopted to improve drought tolerance in germplasm adapted to eastern and southern Africa and the first results will soon be available (Ribaut et al. 2004).

Major MAS efforts to ameliorate drought tolerance are underway also at IRRI for rice (reviewed in Saxena and O'Toole 2002), ICRISAT for pearl millet and sorghum (Crouch and Serraj 2002; Hash et al. 2003) and, more recently, at ICARDA for durum wheat (Nachit and Elouafi 2004). Due to the key role played under rainfed conditions by roots in determining rice yield (Nguyen et al. 1997; Hemamalini et al. 2000; Babu et al. 2001; Venuprasad et al. 2002), MAS for root depth has been deployed at IRRI in an attempt to tailor genotypes better adapted to the range of environments where rice is cultivated (Champoux et al. 1995; Yadav et al. 1997; Mackill et al. 1999). After identifying QTLs affecting root parameters in a population derived from IR64 x Azucena, Shen et al. (2001) used MAS to transfer into IR64 the Azucena allele for deeper roots at four QTLs (on chromosomes 1, 2, 7 and 9). Three backcross progenies were selected solely on the basis of their genotypes at the marker loci in the target regions up to the BC_3F_2 generation. Twenty-nine selected BC_3F_3 NILs were developed and compared to IR64 for the target root traits and three non-target traits. The phenotypic evaluation of root traits indicated that the backcross-MAS for root traits was successful for QTL regions on chromosomes 1, 7 and 9, but not for the QTL on chromosome 2. This result prompted a reanalysis of the initial data with a more sophisticated software package which revealed two linked QTLs with opposite effects in this region (Shen et al. 2001).

In chickpea, MAS is being deployed at ICRISAT to introgress QTL alleles associated with a large root size into elite germplasm (Saxena et al. 2002). Terminal drought can curtail chickpea yield from 20% to more than 50%. Hence, a deep root system capable of extracting additional soil moisture should positively impact yield in drought-prone areas. At ICRISAT, similar projects for using MAS to improve drought tolerance are underway also for groundnut and pigeonpea (Crouch and Serraj 2002). Among the major crops, cotton presents the peculiarity that large acreages are grown under both irrigated and rainfed conditions, thus making G x E interactions of even greater importance than usual in designing crop-improvement strategies. In a recent study carried out at different water regimes, Paterson et al. (2003) described 17 QTLs affecting fiber quality only in the water-limited treatment, while only two were specific to the well-watered treatment. These results suggest that improvement of fiber quality in cotton under water stress may be even more difficult than improvement of this already complex trait under well-watered conditions, thus reducing the expected rate of genetic gain. To overcome these difficulties, Paterson et al. (2003) suggested deploying MAS based on a number of markers found to be associated to the QTLs discovered in their study.

One factor that will greatly affect the extent to which MAS will be more routinely exploited relates to its cost-effectiveness as compared to conventional breeding practices. Morris et al. (2003) compared the cost-effectiveness of MAS and conventional maize breeding for the introgression of an elite allele at a single dominant gene into an elite maize line. In this particular case, neither method showed clear superiority in terms of both cost and speed: conventional breeding schemes are less expensive, but MAS-based breeding schemes are faster. These findings may question to some extent the validity of integrating MAS within conventional breeding programs. High-throughput genotyping based on the scoring of markers that do not require the use of gels (Salvi et al. 2001) coupled with quick DNA extraction protocols are needed to streamline MAS, lower the associated costs and to make MAS more widely applicable.

Climbing the genomics ladder: from QTLs to genes
QTL cloning should be viewed as an essential entry point towards a more effective exploitation of sequence variability at selected target loci and to unlock the allelic richness present in germplasm collections (Tanksley and McCouch 1997). Although QTL cloning is well advanced in mammals (Korstanje and Paigen 2002), only ten QTLs have so far been cloned in plants through positional cloning by refining their map position to a small physical region and then deploying different approaches to validate the involvement of a specific gene at the target region. A few more QTLs have been either fine-mapped (Salvi et al. 2002) or functionally linked to specific candidate genes (Pelleschi et al. 1999; Vagujfalvi et al. 2003). Among the above-mentioned QTLs, those controlling flowering time (Salvi et al. 2003; Doi et al. 2004) may have relevant implications towards the improvement of tolerance to drought, although in this case conventional breeding is rather effective, given the high heritability of this trait. On a similar line, efforts are underway in maize to positionally clone *root-ABA1*, a QTL on bin 2.04 which in the Os420 x IABO78 background has been shown to influence consistently root architecture, root lodging and ABA concentration (Giuliani 2004).

Until now, two approaches have been deployed for the molecular dissection of a QTL: positional cloning and association mapping. Both approaches seek an association between polymorphisms at marker loci and variability in the target quantitative trait and exploit linkage disequilibrium (LD) to identify the most promising candidate gene/s for the subsequent validation phase. Both positional cloning and association mapping benefit from the map information of candidate genes and mutants in the species under investigation and in closely related species. The large number of drought-induced genes so far described (Hazen et al. 2003) is a valuable source for the construction of functional maps that can provide clues for the identification of candidate genes for drought/related QTLs. Specific efforts toward enriching linkage maps with function-specific genes, sometimes described also as functional maps, have been undertaken and utilized for QTL analysis (Andersen and Lubberstedt 2003; Gupta and Rustgi 2004).

Positional cloning
The first step required for positional cloning of a major QTL is the production of a large secondary cross in a nearly isogenic background where only the target QTL segregates. The large number of plants (ca. 1,500 or more) in the segregating population allows for the recovery of a sufficiently high number of recombination events in the target region, an essential prerequisite to achieve the desired level of map resolution. Another important prerequisite is the availability of markers in the target region. Bulk segregant analysis (Salvi et al. 2002) and comparative mapping based on synteny with model species and also among cultivated crops (Ware et al. 2002; Bennetzen and Ma 2003; Sorrels et al. 2003) are valuable sources of additional markers. Microarray analysis can provide clues toward the identification of polymorphisms influencing the level of expression of genes in the target region, and, possibly, of the gene/s underlying the QTL (Hazen and Kay 2003). Following the fine mapping, the markers more tightly linked to the QTL are used to anchor the genetic region to a physical chromosome region. For *Arabidopsis* and rice, the anchoring can now be extended to the complete genome sequence, thus making positional cloning of Mendelian loci a routine practice and strongly facilitating QTL cloning (Yano and Sasaki 1997; Borewitz and Chory 2004; Yazaki et al. 2004). When the genome sequence is not available, genomic libraries (prevalently based on BAC clones) are screened. Polymorphic genes or genomic sequences that are found to be completely linked with the QTL are then functionally tested with a number of different approaches (e.g. genetic engineering, identification of knockouts, etc.). On this line, gene replacement based on homologous recombination, although not yet widely applicable in higher plants, is the ultimate approach (Terada et al. 2002; Greenberg et al. 2003). Other procedures such as RNAi (Waterhouse and Helliwell 2003) and TILLING (Targeted Induced Local Lesions IN Genomes; McCallum et al. 2000; Stemple 2004) allow for a genome-wide functional screening applicable to almost any species.

Association mapping
QTL discovery through association mapping (Flint-Garcia et al. 2003) relies on the molecular and phenotypic characterization of unrelated accessions. The analysis evaluates the difference in allele frequency in case-control samples, or, preferably when dealing with complex traits, the change in the mean of the investigated traits caused by allele substitution. In plants, the interest lies in the possibility of performing QTL analysis and cloning without the costly and time-consuming production of large experimental populations (Morgante and Salamini 2003). Factors influencing the applicability of

association mapping are the level of LD, availability and cost of molecular markers, and knowledge of population structure. Strategies of association mapping are strongly influenced by the level of LD present in the target population. Populations characterized by a higher level of LD are more suitable for gene/QTL discovery, particularly when the panel of accessions has been profiled at a limited number of loci. The validation of a candidate gene, particularly if its sequence is available, requires the utilization of panels with much lower LD, hence a much higher level of genetic resolution. In barley, association mapping has already been applied to identify chromosome regions influencing tolerance to salt stress (Pakniyat et al. 1997). A similar effort is presently underway in durum wheat through an EU-funded project aimed at improving WUE of durum wheat cultivated in the Mediterranean basin (project acronym IDuWUE; http://137.204.42.130/iduwue/html) and will soon be implemented on a wider scale within the recently launched initiatives of the Challenge Program Generation on biodiversity (http://www.generationcp.org). A major point to consider is related to the fact that the chromosome region underlying a particular QTL may be a non-coding sequence, in some cases separated by several kb from the effector gene modulating the QTL effect, as recently shown in maize for *Vgt1*, a QTL for flowering time (Salvi et al. 2003). As recognized by Rafalski and Morgante (2004), the identification of regulatory regions often quite distant from the effector genes indicates that the selection of a candidate sequence to be tested for association mapping with a phenotype is not a trivial undertaking if the genomic scan aims to be comprehensive. Indeed, it is likely that a relevant portion of the genetic variability observed for drought-related traits lies in regulation of gene expression, rather than in functional differences of the encoded products.

"Omics" and beyond
Once a major QTL is mapped, the next logical step is to identify suitable candidate sequences accounting for its effects, validate their role and proceed with a more direct manipulation using the gene itself as marker for MAS within the same species and/or through genetic engineering in other species (Tuberosa and Coraggio 2004). The identification of candidate genes and the elucidation of their role can be facilitated combining QTL analysis with different sources of information and technological platforms (Pfleiger et al. 2001; Wayne and McIntyre 2002). The recent progress in the mass-scale profiling of the transcriptome, proteome and metabolome (i.e. the so-called "omics") offers the possibility of investigating the concerted response of thousands of genes to drought and other stresses. High-throughput profiling of mRNAs has been applied to investigate the changes in gene expression in response to dehydration in *Arabidopsis* (Oono et al. 2003; Seki et al. 2003; Kawaguchi et al. 2004), barley (Ozturk et al. 2002) and maize (Zinselmeier et al. 2002; Yu and Setter 2003). Additionally, it should be noted that the functional basis of a number of cloned plant QTLs relates to differences in the level of expression. Therefore, QTL discovery may in some cases be possible through a direct profiling approach applied to suitable genetic materials (Wayne and McIntyre 2002; Hazen and Kay 2003). An interesting application of transcriptome analysis is the identification of the so-called eQTLs, namely QTLs that influence the level of expression (hence the "e") of a particular gene. In this case, the analysis of the level of gene expression carried out on each progeny of a mapping population will identify eQTLs influencing the observed variability among progenies in mRNA level of the profiled genes (Tuberosa et al. 2003a). Circumstantial evidence regarding the importance of each ORF (Open Reading Frame) in governing variability for yield under conditions of drought can be obtained by comparing the map position of QTLs for yield with the map position of the ORF itself and the corresponding eQTLs. In plants, eQTLs have been reported for the first time in maize (Schadt et al. 2003) and in *Arabidopsis* (Singer et al. 2004). The eQTLs were found to map both at the gene loci for which expression was analysed (indicating allelic differences at *cis* regulatory regions) and at different chromosome positions (indicating allelic differences for trans-acting regulatory factors). Clearly, the cost for profiling a large number of RNA samples required to identify eQTLs is still prohibitive to conceive the routine application of this approach. Instead, transcriptome profiling is better suited for studies involving a limited number of samples extracted from congenic strains differing at key genomic regions (e.g. NILs, BDLs, etc.) and/or bulked samples obtained from the tails of mapping populations. Besides providing clues for the identification of candidate genes, transcriptome profiling of congenic QTL strains offers the opportunity of enriching the region with additional functional markers, provided that the different levels of expression of the investigated genes are caused by a sequence polymorphism (e.g. SNPs or insertion/deletions) that can be utilized for producing a useful marker. Under this respect, SNP (Single Nucleotide Polymporphism) markers are particularly valuable because their profiling can be automated. The interpretation of the results obtained from profiling experiments carried out under controlled environments should take in due consideration

the experimental conditions utilized to dehydrate the plant tissue. In a number of studies, a rather severe dehydration has been artificially imposed in a very short time, commonly in a few hours (Ozturk et al. 2002; reviewed in Hazen et al. 2003). These experimental conditions will certainly be more shocking and damaging at the cellular level if compared to similar levels of water deficit that plant tissues may undergo in the field, where dehydration unfolds over a prolonged time (commonly days or weeks), thus allowing for a more proper activation of the signaling cascade leading to beneficial adaptive responses at the morpho-physiological level (e.g. osmotic adjustment, early flowering, thickening of leaf cuticles, etc.). In barley, the changes in gene expression observed in excised leaves following a rapidly-induced dehydration have shown a low correlation (from 0.19 to 0.41) with those attained under a slower dehydration regime (in pots) which mimicked more closely field conditions (Talamè et al. unpublished). Furthermore, when dehydration unfolds over a prolonged period, the percentage of genes showing a significant up- or down-regulation decreases dramatically as compared to a rapid dehydration treatment (Helentjaris et al. 2003). Consequently, molecular results obtained under artificial conditions should be considered with great caution and duly validated prior to their deployment in a more applicative context.

Deciphering gene function can also be facilitated by information gathered through the profiling of the proteome and metabolome, which, as compared to the transcriptome, are functionally "closer" to the observed phenotypic traits used to select for drought tolerance. In rice, more than 2,000 proteins were detected reproducibly in drought-stressed and well-watered leaves (Salekdeh et al. 2002). Among the 1,000 proteins that were reliably quantified, 42 changed significantly in abundance and/or position. The three most marked changes were identified for the actin depolymerizing factor, the chloroplastic glutathione-dependent dehydroascorbate reductase and a homologue of the S-like ribonucleases. Profiling the proteome of a mapping population offers the opportunity to identify QTLs influencing protein quantity (PQLs, Protein Quantity Loci; Damerval et al. 1994; de Vienne et al. 1999; Thiellement et al. 1999; Zivy and de Vienne 2000; Pflieger et al. 2001; Consoli et al. 2002). Co-localization of a PQL with its protein-coding locus would indicate that allelic differences at that locus influence the expression of the protein, whereas co-localization between a PQL and a QTL for a different trait would suggest an association between the candidate protein and trait variation (de Vienne et al. 1999; Pelleschi et al. 1999). In maize, Jeanneau et al. (2002) have shown that under conditions of mild water stress the *Asr1* gene, a putative transcription factor, co-localizes with a PQL for its protein (ASR1) and a QTL for ASI and leaf senescence. Based on these findings, it was hypothesized that the *Asr1* polymorphism is responsible for the presence or absence of the ASR1 protein, which would pleiotropically affect the other responsive traits; trangenesis confirmed the validity of the hypothesis (Jeanneau et al. 2002). Metabolome profiling is aimed at the identification and quantification of all metabolites in a given biological sample. Metabolic databases provide a valuable framework to predict biochemical pathways and products given a certain genotype and to reveal the phenotype of specific mutations (Fell 2001), thus contributing to a more comprehensive view of the functional characteristics under investigation. With the present technology, ca. 2,000 different metabolites can be profiled in a single sample (Fiehn 2002). When applied to a mapping population, metabolome profiling can be used to identify QTLs regulating the level of a particular metabolite and verify its coincidence with QTLs for yield and/or genes involved in metabolic pathways. In maize, the changes occurred during a drought episode in the level of sugars and starch and other key metabolites in the reproductive organs and in the growing kernel have been investigated (Zinselmeier et al. 1999) and QTLs for invertase activity have been described in a population subjected to drought stress (Pelleschi et al. 1999). The number of QTLs for invertase activity detected under drought (nine in total) was more than twice the number detected under well-watered conditions (four in total), an indirect indication of the important role of this enzyme under drought conditions. One QTL common to both treatments was located near *Ivr2*, an invertase-encoding gene on chromosome bin 5.03. Drought produced an early stimulation of acid-soluble invertase activity in adult leaves, whereas the activity of the cell wall invertase was found to be unaffected. This response was closely related to the mRNA level for only one (*Ivr2*) of the invertase genes.

Future challenges and opportunities
Among the traits more amenable to MAS root architecture and photosynthetic efficiency are interesting targets, in view of the difficulties of manipulating these traits through conventional approaches and also considering that crops' biomass can only be increased through a greater water use, a more efficient utilization of water and/or a more efficient CO_2 fixation. An example is provided by the identification of QTLs regulating root penetration through the hardpans in the soil which are frequently found in rice

growing areas (Price et al. 2000). The presence of a hardpan limits root growth, hence the amount of soil that roots can explore for extracting moisture and nutrients. A high penetration capacity is also beneficial when the crop is grown in hard soil. Additional traits whose selection might be facilitated by MAS are osmotic adjustment, relocation of stem reserves and stay green. QTLs for these traits have already been described (reviewed in Tuberosa and Salvi 2004). Given the negative association usually found between the capacity to relocate photosynthates to the sinks and the capacity of the leaf to stay green, QTL analysis may lead to the identification of alleles with a more balanced effect on these two traits and thus more beneficial to the overall capacity of the plant to withstand drought over a range of water regimes.

A recent study in which QTL information was linked to crop modelling has shown that QTL analysis removes part of the random errors of measured model input parameters and that this information can successfully be coupled with crop models to replace measured parameters (Tardieu 2003). The QTL-based modelling overcomes the limitations in designing ideotypes by using models that ignore the inheritance of model input traits. On the other hand, crop modelling can potentially be a powerful tool to resolve G x E interactions as well as the genetic basis of traits' plasticity (Via et al. 1995; Chapman et al. 2003). For this approach to be effective, there is a need to develop crop models that are capable of predicting yield differences among genotypes in a population under various environmental conditions (Tardieu 2003). An interesting example on how an ecophysiological model and QTL analysis can be integrated to investigate the genetic basis of leaf growth in response to drought and other environmental factors has been recently provided by Reymond et al. (2003), who have identified QTLs affecting leaf elongation rate in maize as a function of water vapor pressure difference, soil water status and meristem temperature. Of the several QTLs identified, most were specific for their response to only one variable. Remarkably, a model based on the combined QTL effects predicted 74% of the variability for leaf elongation rate measured among a random sample of recombinant inbred lines of the mapping population used for QTL detection (Reymond et al. 2003). However, the validity of such models should also be tested when more environmental variables vary simultaneously, the condition typically encountered by crops in the field. Therefore, crop modelling based on QTLs for morpho-physiological traits responding to drought may help us to more appropriately address and resolve G x E interactions and to identify the relevant genetic determinants. In turn, this will offer novel opportunities for collaboration between breeders and modellers to define the best ideotypes (Yin et al. 2003) and may assist dissecting yield into characters under more simple genetic control (Asseng et al. 2003).

Genomics and bioinformatics allow us to investigate sequence colinearity in the main crops (Paterson et al. 1996; Bowers et al. 2003) and to compare their gene order and content with those of model species like *Arabidopsis* and rice whose genomes have been sequenced Although it has been shown that colinearity between *Arabidopsis* and rice (Devos et al. 1999), and between *Arabidopsis* and maize (van Buuren et al. 2002) has been eroded to such an extent that the *Arabidopsis* sequence does not seem to help the identification of related genes in cereals, comparative mapping between rice and the other grasses (Gale and Devos 1998; Ware et al. 2002) provides valuable opportunities to exploit high resolution colinear maps in order to facilitate the positional cloning of QTLs in cereals and/or identify candidate genes. However, chromosomal rearrangements may undermine the use of rice as a model for cross-species transfer of information in evolutionarily non-conserved regions (Sorrells et al. 2003). For dicot crops, opportunities are available for those species more closely related, in evolutionary terms, to *Arabidopsis*, whose contribution to the discovery of genes/QTLs influencing drought tolerance should not be disregarded. This is particularly true for the identification of genes/QTLs controlling the perception of dehydration and the activation of the signal-transduction cascade that leads to the adaptive response to drought. Additionally, significant variability has been detected for WUE (Nienhuis et al. 1994) and drought-adaptive characteristics of *Arabidopsi*s (Meyre et al. 2001).

In terms of experimental materials utilized for gene/QTL discovery, growing attention will be devoted to the exploitation of progenies derived from multiparental crosses and large panels of germplasm accessions with different levels of LD. The mapping populations so far utilized for QTL discovery have three major shortcomings: their size is usually small, only two alleles are explored at each single QTL locus in the biparental crosses and most QTL loci go undetected due to the presence of functionally similar alleles. Exploring more than two genotypes will increase the chances of identifying functional variability due to QTLs and selecting the most beneficial allele in terms of agronomic performance. On a similar line, useful information can also be obtained through the construction of comparative consensus

maps integrating the information of anchor markers and the results of different mapping populations within the same species (Khavkin and Coe 1997; Tuberosa et al. 2002b; Ribaut et al. 2004) and, based on synteny information, from different species (Paterson et al. 1996; Gale and Devos 1998). Preliminary, albeit limited, evidence suggests the possibility that clusters of QTLs may govern developmental features (Khavkin and Coe 1997), some of which influence adaptation to drought conditions. In maize, an example in this direction is provided by the region on chromosome bin 2.04 which has been shown to influence in different backgrounds a number of traits important for tolerance to drought (Lebreton et al. 1995; Tuberosa et al. 1998; Quarrie et al. 1999; Landi et al. 2004; Ribaut et al. 2004). Finally, the importance of an accurate phenotyping based upon good field practices and appropriate experimental designs coupled with the application of the most advanced software cannot be overemphasized for optimizing the discovery of QTLs and the characterization of their effects in different environments.

Conclusive remarks

A number of research consortia sponsored by governmental (e.g. the Challenge Program, EU-funded projects, etc.) as well as non-governmental agencies (e.g. the Rockefeller Foundation, etc.), together with a growing flow of molecular information are providing a new impetus to the applications of genomics toward the release of cvs. more tolerant to drought. These efforts will increasingly rely upon collaboration amongst different categories of scientists, avoiding that each category develops a vision of their roles in plant breeding from their own short-sighted perspective. The success and effectiveness of MAS in tailoring improved cvs. will depend on the identification of beneficial QTL alleles conferring drought tolerance and pyramiding such alleles in the correct combinations. To some extent, this could be regarded as an evolution of the so-called ideotype breeding, the main difference being that we now have more powerful tools for dissecting at a much higher level of resolution the genetic basis of the phenotype and for piecing back together the best QTL alleles, in a sort of molecular jigsaw puzzle. This new concept of "breeding by design" (Peleman and van der Voort 2003), although already applicable from a purely technical standpoint in a number of major crops, in the case of drought tolerance it is still a long way from being routinely applicable, in view of our incomplete understanding of the molecular basis of drought tolerance and, most importantly, the difficulty in predicting the phenotypic value of a new genotype tailored through MAS. Paraphrasing the title of a review on MAS (Young 1999), we remain cautiously optimistic as to the prospects of MAS for drought tolerance, more so today than a decade ago, when it was unclear to what extent QTLs could be cloned. For its far-reaching implications, QTL cloning should probably be viewed as one of the potentially most important contributions of molecular genetics to our future capacity to more effectively manipulate traits influencing drought tolerance, particularly considering that not long ago an authoritative review on drought tolerance indicated that the development of NILs would be restricted to traits only controlled by one or a few genes (Ludlow and Muchow 1990), a clear example of how difficult it is to predict technical breakthroughs. Similar considerations are valid for the profiling of gene expression, now possible at unprecedented levels on a genome-wide basis (Hazen and Kay 2003) and in terms of specific cell types (e.g. laser-capture microdissection; Nakazono et al. 2003). However, the deluge of information originated by these novel molecular platforms does not necessarily translate into knowledge on how to improve drought tolerance. It is unlikely that a single profiling experiment or a single QTL study will provide clear indications as to which molecular targets should be pursued to improve drought tolerance. On a more realistic ending note, the successful exploitation of MAS, genomics and the post-genomics platforms to enhance drought tolerance will only be possible within an interdisciplinary context and relying on a thorough field assessment of the selected materials in the target environments. The past decade has witnessed an increasing number of QTL studies and the first encouraging results in QTL cloning and post-genomics studies. This notwithstanding, we have barely scratched the surface of the genomic landscape governing drought tolerance and our capacity to effectively manipulate it remains rudimental. New and more informative high-throughput platforms (e.g. Hardenbol et al. 2003) capable of reducing the cost of molecular profiling coupled with an increasing capacity in genome sequencing hold great promise that genomics approaches will positively impact the release of more drought-resilient cultivars. Although it is not possible to predict to what extent MAS, genomics and post-genomics approaches will eventually affect conventional breeding practices in the developed and less-developed nations, we remain confident that future progress toward a more sustainable agriculture will be accelerated through a more systematic discovery of the function of the genes influencing yield under water-limited conditions and a deeper understanding of their interactions with the environment.

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