REVIEW ARTICLE

Molecular and physiological approaches to maize improvement for drought tolerance

Wesley B. Bruce[1], Gregory O. Edmeades and Thomas C. Barker

Pioneer Hi-Bred International, Inc., 7300 NW 62nd Ave., Johnston, IA 50131–1004, USA

Abstract

Average maize yields have increased steadily over the years in the USA and yet the variations in harvestable yield have also markedly increased. Much of the increase in yield variability can be attributed to (1) varying environmental stress conditions; (2) improved nitrogen inputs and better weed control; and (3) continuing sensitivity of different maize lines to the variation in input supply, especially rainfall. Drought stress alone can account for a significant percentage of average yield losses. Yet despite variable environments, new commercially available maize hybrids continue to be produced each year with ever-increasing harvestable yield. Since many factors contribute to high plant performance under water deficits, efforts are being made to elucidate the nature of water-stress tolerance in an attempt to improve maize hybrids further. Such factors include better partitioning of biomass to the developing ear resulting in faster spikelet growth and improved reproductive success. An emphasis on faster spikelet growth rate may result in a reduction in the number of spikelets formed on the ear that facilitates overall seed set by reducing water and carbon constraints per spikelet. To understand the molecular mechanisms for drought tolerance in improved maize lines better, a variety of genomic tools are being used. Newer molecular markers and comprehensive gene expression profiling methods provide opportunities to direct the continued breeding of genotypes that provide stable grain yield under widely varied environmental conditions.

Key words: Maize, harvestable yield, drought stress, spikelet growth.

Introduction

Drought, like many other environmental stresses, has adverse effects on crop yield. Low water availability is one of the major causes for crop yield reductions affecting the majority of the farmed regions around the world. As water resources for agronomic uses become more limiting, the development of drought-tolerant lines becomes increasingly more important. Evidence of variations for available water within a field (Kitchen *et al.*, 1999) imply a need for a reasonable level of drought tolerance in all maize hybrids since farmers typically grow only one hybrid in any given field. Seed companies strive to produce newer hybrids and varieties with enhanced tolerance to stressful growing conditions. Over the years, maize breeders have aimed to generate hybrids with higher grain yield potential, better grain yield stability and improved grain traits for end-users (Duvick, 1997). Understanding the nature of the higher grain potential and enhanced yield stability especially in stress-prone environments will provide opportunities to improve the breeding process.

Although other comprehensive reviews on drought response in plants are available (Bartels and Nelson, 1994; Ingram and Bartels, 1996; Holmberg and Bülow, 1998), this review will focus primarily on the institutional and commercial seed industry's perspective for improving drought stress-tolerant maize lines. An historical perspective of the genetic improvements and other factors on overall maize yield increases will first be discussed. Since maize yields have been steadily rising in the last few decades, the nature of these improvements has directed the current selection strategies in commercial and institutional breeding programmes. Following the historical perspective, a description of some of the variations in the conventional breeding methods and uses of managed

environments will be provided. A discussion will ensue of genomic-related methods and tools that will provide further understanding of the molecular nature of stress improvements and undoubtedly shape the future of breeding strategies.

Historical perspective on maize breeding and improvements

Grain yield is a complex trait and is dependent on many factors including vigorous growth, adequate water and nutrient supplies, enhanced solar radiation interception and conversion to chemical energy, and improved genetics (Russell, 1991). Despite its complexities, nearly all of the commercial breeding programmes today include yield as a selection parameter. However, because of the complex nature of grain yield, a better understanding of the components that contribute to higher yields should improve the selection process. Current experiments have been designed to probe the nature of the historical improvements in maize lines. Not too surprisingly, many of the recent improvements seem to involve greater resistance to biotic and abiotic stresses as opposed to improved yield production under ideal growing conditions.

Although maize production in the US showed a net increase in yield during the past century, the rate of increase in grain yield has not been steady (Fig. 1). The changes in the rate of yield increase were due to a variety of factors. Nearly half of the yield enhancement was due to widespread mechanization, better farm management and inputs, and increasing planting densities. Genetic improvements contributed the other half
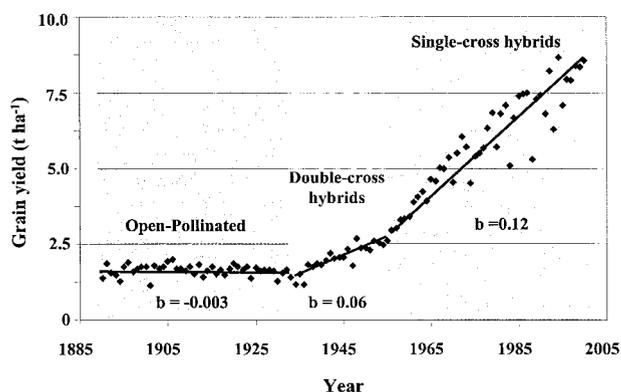

Fig. 1. Average US maize yields from the years 1890 to 2000. Slope values were calculated on three portions of the data, 1890 to 1934, 1935 to 1955 and 1960 to 2000. Standard errors on the regressed lines were 187, 200 and 612 kg ha^{-1} for the three periods, respectively. Open-pollinated varieties refer to lines used from populations that were pollinated without regard to line preferences. Double-cross hybrids were produced by making two sets of crosses whose progenies then were used as parents for the final hybrid line. Single cross hybrids were produced from two parental inbred lines. Source of data: US Department of Agriculture.

of the production increase (Duvick, 2001). The era from the 1890s to the 1930s included the use of primarily open-pollinated varieties. During this era, there was little change in the average grain yield with time. The increased use of hybrids during the 1930s and onward helped fuel the first real change in maize production. From the 1930s to the 1950s, double-cross hybrids along with improvements in farming practices contributed to small increases in grain yield. The 2-fold increase in production following the 1960s involved farmers' primary use of single-cross hybrids with superior genetic backgrounds, along with steady improvements in crop agronomy.

Notably the variance for US maize yield averages were small until about the 1970s (Fig. 1). The low variation during the early years was most likely due to reduced nitrogen inputs and poor weed control, while reflecting that hybrids was more genetically diverse. However, between the 1960s and 2000, the variance in grain yield increased significantly (nearly tripling from the previous era) suggesting a greater volatility in average maize production. This fluctuation in grain yield may primarily be due to limited water supply and unfavourable temperatures since weed control and N deficiency have been effectively removed as production constraints. Global climate change may also have been a factor in recent years (de Vries, 2000). It is generally accepted that modern hybrids show an increased level of stress tolerance that counters the potential water limitations (Castleberry *et al.*, 1984; Duvick, 1997; Tollenaar and Wu, 1999) with significantly improved levels of productivity.

Direct comparisons of yield and associated traits of modern hybrids with their predecessors should provide a better understanding of the nature of the improvements in recent releases. Duvick (1997) examined the performance of 36 maize hybrids commercially released at intervals between 1934 and 1991. He showed that the newer hybrids produced higher yields than the older lines in four different environments that included both hot, dry conditions and wet, cool conditions. Duvick concluded that the improvements in yield over the years were indeed partly due to improved tolerance to abiotic stresses. Interestingly, he showed that the change in hybrid yield potential on a per plant basis from older to newer lines was not significant. Thus the increases were due to better performance under higher planting densities. Imposing higher plant densities generates symptoms of stress responses in maize and leads to a reduction of yield on a per plant basis (Modarres *et al.*, 1998; Tollenaar and Wu, 1999; Andrade *et al.*, 1999). However, this reduction typically is compensated by the increase in the plant numbers per unit area that increases net yield.

Improvements in heterosis or hybrid vigour *per se* have not contributed to greater yields (Duvick, 2001). Studies show that heterosis as measured by the difference between the yields of the hybrid and the mid-point of the

two parents has not increased since the 1950s. Instead the parental inbred lines have improved in yield due to breeding selection at about the same rate as the hybrids themselves (Duvick, 1999). Thus selection for improved yield in parental lines and for improved yield stability in their hybrid progenies appears to be the key to past success. Selection strategies to extend and improve these gains in the future require a better understanding of the components contributing to yield and the employment of managed stress environments that improve the efficiency of the breeding process targeting improved yield stability.

Improvement of maize for drought tolerance

Natural and breeder-induced selection pressures on maize in an unpredictable rainfall environment

When maize encounters water deficits, there is a decline in photosynthesis per plant. This can be due to a reduction in light interception as leaf expansion is reduced or as leaves senesce, and to reductions in C fixation per unit leaf area as stomates close or as photo-oxidation damages the photosynthetic mechanism. The accumulation of abscisic acid (ABA) may enhance survival but reduces productivity (Leung and Giraudat, 1998; Mugo *et al.*, 2000). Ability to protect cellular membranes and enzymes from stress and to recover from water deficits will also enhance a crop's capacity to survive and produce grain. In turn, the ability of the plant to capture water from a drying soil through deeper or more thorough root exploration or through osmotic adjustment should stabilize and increase the flow of assimilates to developing grains. A recent report links osmotic adjustment to stable grain yield under the transient stresses that are often encountered in wide-area testing of modern maize hybrids (Lemcoff *et al.*, 1998). Because maize evolved in the tropics where nitrogen is inherently deficient, natural selection has favoured the early uptake of N from the soil, its storage in leaves as photosynthetic enzymes, and its subsequent remobilization to developing grain during grain filling. When the grain sink size is increased by selection this can sometimes accelerate foliar senescence as the demands for N by the grain exceed capacity for uptake from a dry soil (Chapman and Edmeades, 1999).

Many of these mechanisms favour survival but may have limited value in enhancing grain yield under a level of water stress that varies spatially and across seasons. Hybrids usually yield better than varieties under drought with heterosis acting as an important source of stress tolerance (Blum, 1997). Obviously, yield potential is important in determining yield under moderate stress. It becomes much less so when yields fall below 50–60% of potential (Bänziger and Lafitte, 1997), when stress-adaptive secondary traits assume a real significance.

Grain yield is normally highly correlated with the kernel number per unit area and per plant rather than with weight per kernel (Bolaños and Edmeades, 1996; Edmeades *et al.*, 1999; Andrade *et al.*, 1999). Factors affecting grain set under drought, therefore, are of special interest in commercial breeding endeavours. Because maize is an out-crossing species, pollen must move from the anthers at the top of the plant to the exposed silks of the same and surrounding plants. This process is risky because pollen and the delicate stigmatic tissue are exposed to a desiccating environment. One universal phenomenon observed when maize flowers are under drought is the delay of silking in relation to pollen shed, giving rise to the anthesis-silking interval (ASI) whose duration is highly correlated with kernel set (DuPlessis and Dijkhuis, 1967; Edmeades *et al.*, 2000a). Under such conditions, pollen can arrive after it has desiccated, when silks have withered or senesced (Bassetti and Westgate, 1993a, b) or after ovaries have exhausted their starch reserves (Saini and Westgate, 2000; Zinselmeier *et al.*, 2000). The flux of currently formed assimilates to the developing ear is a powerful determinant of ear growth, suggesting that stored reserves in the stem and ear shank have little to do with reproductive success (Westgate and Boyer, 1985; Schussler and Westgate, 1995). Despite temperate germplasm's long history of improvement by selfing under conditions of high plant density and occasional exposure to heat and drought, delayed silking (increasing ASI) under severe drought stress can still be evident and thus negatively affect grain yield (Fig. 2).

Genetic variation for drought tolerance: directed selection in tropical germplasm

For the past 25 years, CIMMYT has made a concerted attempt to select for improved drought tolerance in tropical germplasm. Detailed accounts of this have been published elsewhere (Bolaños and Edmeades, 1993a, b; Edmeades *et al.*, 1993, 1999, 2000b), and only highlights
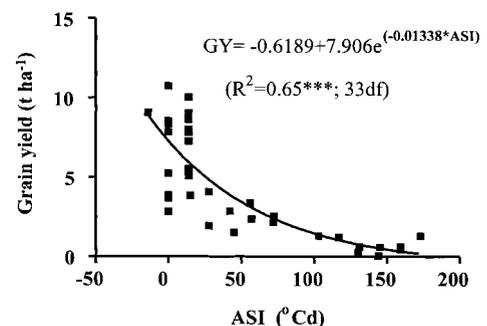
$$GY = -0.6189 + 7.906e^{(-0.01338*ASI)}$$

$$(R^2 = 0.65***; 33df)$$

Fig. 2. Relationship between grain yield and anthesis-silking interval (ASI) in 12 elite temperate maize hybrids when grown under varying levels of water stress at flowering, Kekaha, Hawaii, 2000. GY refers to grain yield.

will be given here and pointers for methods that have applicability in a commercial context.

Selection procedures

Selection began in the elite lowland tropical white dent population, Tuxpeño Sequía, in 1975, and was extended to five other elite tropical populations in 1986. These populations underwent 2–8 cycles of recurrent selection among full-sib and/or S_1 families in rain-free crop seasons at Tlaltizapán or Obregón, Mexico, where the timing and the intensity of stress can be managed by irrigation. Each of 250–600 families was grown in single-row plots under well-watered (WW), intermediate stress (IS; water withdrawn during late flowering and grain filling), and severe stress (SS; no water applied from 3 weeks before silking onwards). Selection of the best 50–80 families for recombination was based on an ideo-type having high grain yield, small ASI and a low level of leaf senescence (staygreen) under SS and IS, and adequate yield, small tassels and upright leaves under well-watered conditions. Each of the managed environments was used to expose genetic variation for specific traits: WW for yield potential, IS for lower leaf senescence and grain yield, and SS for tassel blasting, barrenness and ASI.

Progress from selection

Improvements in performance under drought have averaged 126 kg ha^{-1} cycle^{-1}. Similar gains were also observed under low N (Table 1), suggesting a common mechanism of tolerance to stress at flowering. These improved sources also exhibited tolerance to drought when transferred to southern Africa. The superiority of local hybrids under unstressed conditions, however, made the introgression of Mexican sources into local germplasm necessary. This, in combination with screening under managed stress levels, has led more recently to maize hybrids with superior and stable performance across a wide range of growing environments in southern and eastern Africa, and the development of an open-pollinated variety that shows a 35% superiority over commercial hybrid controls under moderate to severe levels of water stress (Bänziger *et al.*, 2000a).

Further evidence of the robustness of gains has been obtained by testing original and advanced selection cycles of these populations in 16 environments differing in water availability but distinct from those where selection took place. Data from locations ranging from Georgia to Hawaii indicated that gains were similar (132 kg ha^{-1} cycle^{-1}; Table 2) to those observed in evaluations

Table 1. *Effects of selection for drought tolerance on gains per selection cycle in four maize populations when evaluated at 3–6 water-stressed (SS) sites, at 5–8 well-watered (WW) sites, or at two low N sites*

Locations were in Mexico (Mex.) or outside (Int.). *, **, ns: significant rate of change per selection cycle at $P < 0.01$, $P < 0.05$, or $P > 0.05$, respectively (Beck *et al.*, 1996; Bänziger *et al.*, 1999a, b).

Population	Yield (kg ha^{-1})			Anthesis WW (d)	ASI[a] SS (d)	Ears plant^{-1} SS
	SS	WW	Low N			
Evaluation 1988 to 1991						
Tuxpeño Seq. (Mex.)	100**	125**		−0.40**		
Tuxpeño Seq. (Int.)	52ns	101**		−0.24**		
Evaluation 1992 to 1994						
La Posta Seq. (Mex.)	229**	53ns	233	−0.52**	−1.18**	0.07**
Pool 26 Seq. (Mex.)	288**	177**	207	−0.93**	−1.50**	0.08**
Tuxpeño Seq. (Mex.)	80**	38**	86	−0.32**	−0.44**	0.02**
Pool 18 Seq. (Mex.)	146**	126**	190		−2.13**	0.05**
Mean	149	103	179	−0.48	−1.31	0.06

[a]Anthesis-silking interval.

Table 2. *Comparison of gains due to selection in four tropical populations when evaluated in 16 environments differing in available water and in photoperiod*

Gains at the 3 t ha^{-1} and 6 t ha^{-1} yield levels are predicted from linear regression of yield of each selection on mean yield of all entries in each environment. All fitted regressions were significant to $P < 0.01$.

Population comparison	Gain at 3 t ha^{-1} level (kg ha^{-1} cycle^{-1})	Gain at 6 t ha^{-1} level (kg ha^{-1} cycle^{-1})	Mean gain (kg ha^{-1} cycle^{-1})
TSC$_0$ versus C$_6$	22	118	62
TS6C$_0$ versus C$_4$	136	92	117
La Posta C$_0$ versus C$_6$	173	182	177
Pool 18 C$_0$ versus C$_5$	198	138	173
Mean	132	132	133

conducted largely at the sites where selection had occurred. Thus, although tropical germplasm is generally poorly adapted in temperate sites, the gains from selection for drought tolerance showed little interaction with the environment, were remarkably similar at varying yield levels, and indicated that the selection methodology is robust and useful in temperate areas as well.

Changes associated with selection

Gains in yield were largely due to reduced barrenness under drought (Table 1), and not to increased biomass production (Bolaños and Edmeades, 1993a; Edmeades *et al.*, 1999). Reduced barrenness under stress was associated with more rapid ear growth (Edmeades *et al.*, 1993). Anthesis date became slightly earlier (Table 1). Root biomass in the top 50 cm of soil declined by 35%, but there was no change in any trait indicative of plant water status (e.g. predawn or noon water potential; osmotic adjustment; canopy temperature, water extraction profiles) (Bolaños *et al.*, 1993). Recurrent selection also reduced the final number of spikelets (the maternal structure containing the ovule) per ear by 2.5% per cycle in one population (Edmeades *et al.*, 1993), a change thought to be associated with selection for early vigorous silking under drought. Thus fewer spikelets were formed that grew more rapidly and were ultimately more successful in forming grain, especially under conditions of drought at flowering. A dominant symptom of these changes was a reduction in ASI. The quantitative trait loci (QTL) for this trait have been identified and used in marker-assisted backcrossing (Ribaut *et al.*, 1999). The mechanisms leading to improved yield under drought also appear to hold under low N (Table 1).

Secondary traits in selection

Grain yield under stressed conditions is usually the primary trait for selection. A suitable secondary trait is (1) genetically associated with grain yield under drought; (2) highly heritable; (3) stable and feasible to measure; (4) not associated with yield loss under ideal growing conditions (Edmeades *et al.*, 2001). Very few proposed secondary traits meet these criteria. Using selection theory, Bänziger and Lafitte showed that the use of secondary traits plus yield improved selection gains for maize yield under low N by 20% versus selection for yield alone with the gains increasing as N deficiency intensified (Bänziger and Lafitte, 1997). Experience in CIMMYT and at Pioneer Hi-Bred indicates that key secondary traits under drought are reduced barrenness, ASI, staygreen, and to a lesser extent, epinasty or leaf rolling under drought (Bänziger *et al.*, 2000b). Other traits such as changes in the root systems will only be

worth adding when they have been field-tested and have met these criteria.

Root systems obviously play an important role in water acquisition for plants and are a significant component of tolerance to water-deficit stress (Barker and Varughese, 1992; Weerathaworn *et al.*, 1992; McCully, 1999). In rice, some groups have shown the significance of certain root attributes that contribute to higher yields in low water stress-prone environments and identified the corresponding QTL (Hemamalini *et al.*, 2000; Zheng *et al.*, 2000). Others have made progress in using markers in breeding modifications of root attributes in rice that correlate well with improved tolerance to dry environments (Mackill *et al.*, 1999). It is apparent from such studies including the recurrent selection described above that shifting root biomass from the upper soil zones to deeper regions would enhance the chances of encountering new water sources. And this shift in biomass would need to occur in response to drying soils.

In an attempt to show a relationship between early seedling root development and effects of drought on yield, a Pioneer study was recently conducted in Chile using recombinant inbred lines (RILs) from a B73×Mo17 population differing in seedling root traits (TC Barker and I Saab, unpublished data). Sixteen RILs classified as having poor early root development lines showed reduced adventitious and lateral root development. Ten vigorous early root development lines exhibited extensive adventitious and lateral root development at the 2-week-old stage. As shown in Fig. 3, the class of RILs with poorer early root development yielded better than the class with more vigorous early root development. These data are reminiscent of the observation of improved grain yield under drought stress that was accompanied by reduced root biomass (Bolaños *et al.*, 1993). It is likely that vigorous root growth may come a cost to grain production despite the improved advantage of water acquisition in dry soils. Defining early seedling traits correlating with stress tolerance would benefit the selection process in breeding programmes by reducing input resources and time to develop new germplasm.
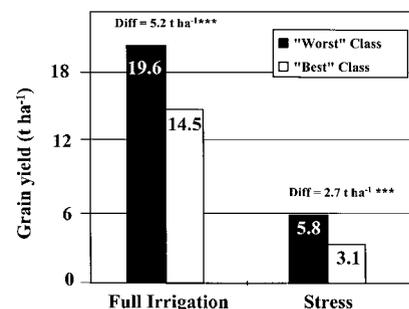

Fig. 3. Seedling root traits associated with drought tolerance. The 'worst' class of RILs showed relatively poor early root growth whereas the 'best' class represents the most vigorous lines in early root growth.

Genetic variation for drought tolerance in modern temperate germplasm

Duvick showed that the grain yield of temperate maize hybrids released from the period 1930 through 1991 in mildly stressed environments increased 53 kg ha^{-1} year^{-1} and is accompanied by a significant decrease in ASI of approximately 0.04 d year^{-1}, an increase in harvest index of 0.1% year^{-1}, and an increase in ears per plant of 0.002 year^{-1} (Duvick, 1997). When Jensen studied these hybrids under well-watered conditions, however, he reported genetic gains of 84 kg ha^{-1} year^{-1}, suggesting that the selection process from 1930 to 1991 had favoured well-watered environments (Jensen, 1994).

The apparent increase in stress tolerance in modern temperate maize germplasm (e.g. Castleberry *et al.*, 1984; Duvick, 1984, 1997; Tollenaar and Wu, 1999) has been attributed to a number of factors: (1) the occurrence of heat and drought in nurseries with no available irrigation; (2) the use of high plant densities during hybrid line development; (3) large-scale broad-area testing that includes the use of winter nurseries; and (4) the use of stable high-yielding progenitors to form the next crop of parental inbred lines. Further enhancements of stable, drought-tolerant, inbred lines at Pioneer have been made using side-by-side comparisons under rainfed and irrigated plots of elite×elite F_2 populations (rather than broad-based populations), and the acceptance that drought tolerance does not automatically imply lower yield potential. Early research endorsed the use of ASI, staygreen, heat tolerance, and reduced kernel abortion as selection criteria. And the analysis of drought tolerance depended heavily on performance stability estimates that were based on yields from broad-area testing, often using data from >300 environments (Jensen and Cavalieri, 1983; Jensen, 1994).

Is there a genotype×drought level interaction in modern temperate germplasm? There is ample evidence that the ranking for grain yield can change significantly between water regimes (e.g. 3346 versus 33J56, Fig. 4) and that the performance stability of leading hybrids differs in important ways. Evidence exists showing maize is unusually susceptible to drought around the flowering period, and variation in this susceptibility could give rise to genotype×drought interactions (Shaw, 1977). Based on preliminary data using more recent hybrids (Fig. 5), the drought-tolerant hybrid 3223, unlike the partially susceptible hybrid 3245, does not show this historical vulnerability to drought at flowering. In this study, water was withdrawn for 2 week periods starting 3 weeks and 1 week before 50% of the test plants were undergoing anthesis, and compared with the well-watered control (P Tausend, G Edmeades, unpublished results). Yields in hybrid 3223 were not reduced by exposure to stress near flowering compared with 3245

(Fig. 5). Both hybrids reached the 50% anthesis stage within 2 d of each other, but the stress was lifted within 2–3 d of this time in 3223 that flowered later of the two, and this may have prevented significant kernel abortion. Nonetheless, the ASI of 3245 was 117 °Cd versus 0 °Cd for 3223, an ample demonstration of contrasting hybrid behaviour under stress.

Managed environments for phenotypic screening methods

The efficient identification of genotypes with improved abiotic stress tolerance requires careful management of drought stress to expose genetic variations in phenotypes such as ASI and fecundity. Much of the progress in crop improvement over the past century is a direct result of careful site selection for testing (Bramel-Cox *et al.*, 1991). In selecting new germplasm, Pioneer relies in part on a drought tolerance scoring that is based on performance stability analyses, observations by sales and research
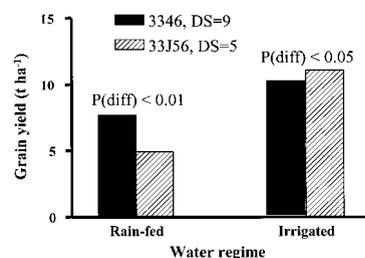
Fig. 4. A comparison of two modern temperate maize hybrids, 3346 and 33J56 grown under rain-fed versus irrigated conditions at York, Nebraska, 2000. The rain-fed conditions resulted in D1 to D2 stage of drought (refers to <65–70% of normal rainfall during the previous three months among other moisture indices) during the peak flowering time as defined by the National Drought Mitigation Centre, Lincoln, Nebraska (http://enso.unl.edu/monitor/). 'DS' refers to a drought score based on a scale of 1 for highly susceptible to 9 highly tolerant to water-deficit effects.
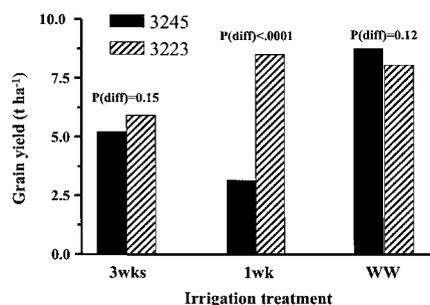
Fig. 5. Grain yield of two hybrids with contrasting susceptibility to drought stress. Hybrid 3245 (susceptible, drought score = 5) and 3223 (tolerant, drought score = 8) were subjected to 2 weeks of water withdrawal starting at 3 weeks and 1 week before 50% anthesis where indicated, and compared with a well-watered (WW) control in Kekaha, Hawaii, 2000 (P Tausend, G Edmeades, unpublished results).

staff, and many direct side-by-side comparisons in a large number of locations. However, using side-by-side comparisons for drought tolerance screening, while an efficient means of comparing genotypes under more normal conditions, depends on an absence of rain during critical periods to be effective and can be unpredictable. Therefore test environments need to be established where the probability of drought stress is high, where timing, duration and intensity of stress can be modulated by irrigation, and where there are obvious similarities to the area of adaptation (AOA) of the germplasm under examination. Arid locations in the US and in the southern hemisphere that have access to reliable irrigation and are exposed to 14–16 h photoperiods during the crop season are currently under evaluation. Issues involving such locations are also being addressed and include methods of appropriately weighting data from these sites and integrating these values into broad-area testing data ultimately to improve hybrid selection. Such locations will provide a high level of reliability for field trials, yet the questions of how representative is the stress and what the level of genotype × environment interactions is across locations still need to be answered.

Managed-stress environments play an essential role in assuring reproducible stress conditions targeted at specific growth stages. The use of modern experimental designs, such as alpha lattices or row and column designs, can further increase selection efficiency (Yau, 1997). New techniques relating to environmental classification, crop modelling and the identification of the target population of environments (TPE) the crop encounters over time suggest that considerable gains can be expected in this area (Chapman *et al.*, 2000).

Applying stress for selection

Three important elements of drought characterization for successful breeding of stress tolerance are timing, duration and intensity of the stress. Most periods of stress encountered in the major grain-growing areas worldwide are transient, unpredictable, and imprecisely measured, leading to difficulties in breeding efforts for drought tolerance (Zavala-Garcia *et al.*, 1992). As an example of this challenge, a recent series of trials conducted at Pioneer involved test environments that were split-blocks of full-irrigation versus water-deficit stress. The intensity of the stress condition was aimed at producing a 50% yield reduction created by an imposed water deficit during flowering. Twelve locations were planted in 1999 and 16 were planted in 2000 all in the United States. None of the 1999 locations and only two of the 16 locations in 2000 produced a 50% yield reduction. Thus, the overall target intensity stress level was rarely achieved. Furthermore, of the 17 locations that did show a 20% or better yield reduction (five locations in 1999, 12 in 2000), six different combinations of timing and duration of stress were encountered. These results underscore the difficult nature of obtaining reliable, reproducible stress environments in the field where seasonal rainfall can occur at any time.

Generic environmental classifications do not describe the timing, duration or the intensity of the stress more precisely. The limitations of such classifications are exemplified using the same series of trials conducted at Pioneer as described above. One of the trials that was planted in Weslaco, Texas, in 2000 generated mean yields for 11 hybrids of 5.1 and 3.0 t ha^{-1} under full irrigation and water-deficit stress during flowering, respectively, resulting in a yield loss of approximately 40%. In a Chile 1999–2000 trial using the same 11 hybrids, the yield loss from stress at flowering was 62%. However, the mean yields in Chile under full irrigation and water-deficit stress were 19.75 and 7.48 t ha^{-1}, respectively. Thus the yield under full irrigation at Weslaco was significantly less than the yield of the water-deficit stress condition in Chile. One of the primary differences in these two environments is the average ambient temperature, both in the day and night. Temperature among other factors may explain the differences in hybrid performances in the two environments despite the fact that the intensity and timing criteria of drought stress in these two trials were met. Such results again demonstrate the need for better control of stress parameters to provide the necessary reproducible environments for selection of new hybrids.

Commercial hybrid development within an AOA is based on at least three performance parameters: (1) the net mean yield across a wide array of environments; (2) yield stability, as determined by the regression of yield on an environmental index, or other statistical methods; (3) paired comparisons of the experimental hybrid versus key commercial checks grown in the same trials. There are several limitations to this approach in terms of drought stress characterization. First, several seasons and many locations, which are resource intensive, are necessary to develop confidence in the yield stability measurements. Depending upon the nature of the environmental stresses encountered during the pre-commercial testing, it is likely that other forms of stress, more extreme stress levels or combinations of the two will be encountered once the hybrid is commercialized. Second, standard stability regressions do not distinguish between low yields due to low moisture levels and pathogens, poor soil fertility, excess water, weed competition, etc. unless the data are explicitly structured to take into account these other variables (Wicks and Carson, 1987).

Range of environmental controls versus correlation with TPE

A range of environments available for drought stress research can be described in terms of their suitability
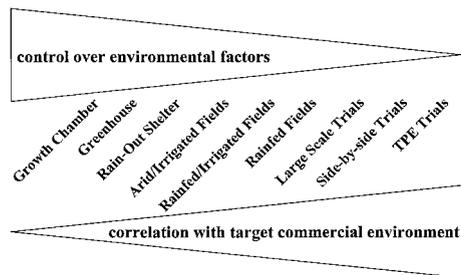
Fig. 6. Continuum of environments for drought tolerance screening. The control over environmental factors decreases from the use of growth chambers to target population environment (TPE) while the correlation of performance with target commercial environments increases.

to control the precision of stress treatments. Figure 6 illustrates a continuum of environments from the growth chamber to the TPE. Growth chambers and greenhouses allow very precise control of the timing, duration and intensity of stress control, but they are least like the TPE that are close representations of the farmers' fields, but lack any control of environmental factors. For intermediate control of drought stress, a rain shelter (NeSmith and Ritchie, 1992) would allow crops to grow in more natural rooting environments, yet the light, air movement and other microclimatic conditions are still somewhat artificial. Moreover, it would be prohibitively expensive to conduct large-scale progeny testing or general yield testing under such artificial shelters.

Arid environments such as the central valley of Chile are more economical for larger scale testing than rain shelters. In such locations, the lack of rainfall and available irrigation systems allow for fairly precise control of drought-stress treatments. The requirements of timing and duration of stress can be met in the arid Chilean environment and based on previous experiences with such SS treatments, a greater than 50% yield reduction can be induced. However, a current challenge in using such locations is to understand the nature of hybrid performance between arid environments (like Chile) and the target AOA where the controls of water-deficit stress are less rigorous, but performance is more relative to the TPE.

In a commercial breeding programme, hybrids are ultimately evaluated in environments most similar to farmers' fields, typically done in strip tests or side-by-side comparisons. These evaluations generally require multiple seasons and a large number of sample environments to develop confidence in the performance of a hybrid in terms of stress response, yield potential and yield stability. Statistical procedures such as estimating genetic correlations among environments, analysis of genotype × environment interaction, and stability analyses can help improve the process of establishing the reliability of hybrid yield prediction from one environment to others (Cooper and DeLacy, 1994).

Understanding the molecular events to direct novel genetic improvements

Marker assisted breeding (MAS) methods are becoming increasingly useful in the development of newer germplasm with improved stress tolerance (Hoisington *et al.*, 1996; Quarrie *et al.*, 1999; Hemamalini *et al.*, 2000). To clarify the genetic network involved in stress tolerances further, key agronomic traits, such as grain yield, need to be categorized into individual components to reduce complex analyses (Jacobs and Pearson, 1991; Modarres *et al.*, 1998; Tollenaar and Wu, 1999). In maize, it is helpful to consider both the number and size of the kernels generated on the ear, since these are determined at different stages in the life cycle of the crop (Bolaños and Edmeades, 1996; Edmeades *et al.*, 1999; Andrade *et al.*, 1999). Factors affecting kernel development thus become the main research focus in improving yield and yield stability in newer germplasm.

The target genes that play central roles in developing kernels can also be used to screen for variations in a population showing a continuum of sensitivity to drought stress. Likewise, transgenic approaches can exploit the appropriate modifications of target genes, ultimately to improve drought tolerance (Bartels and Nelson, 1994; Ingram and Bartels, 1996; Xu *et al.*, 1996). Some methods of identifying key genes rely on physiological and biochemical examination whereby certain pathways that are rate-limiting under stress conditions can be easily identified (Bartels and Nelson, 1994; Leung and Giraudat, 1998). Other more high-throughput methods such as genome map-based and functional genomics approaches can provide indications of less obvious pathways that are nonetheless just as important (Seki *et al.*, 2001; Sun *et al.*, 2001). The combination of several such methods is expected to reduce the tens of thousands of candidate genes to a few target genes.

Functional genomic tools

To identify the less obvious gene networks that are responding to stress, more massively parallel and sensitive methods are necessary. The advent of whole genomic-related technology is providing the necessary tools to identify the key gene networks that respond to drought stress and relating their regulation to adaptive events occurring during stress. Differential display was one of the earliest methods of parallel screening for differences in the levels of cDNA fragments generated from mRNA isolated from samples between experimental treatments (Liang and Pardee, 1992). This technology depends on semi-random PCR using degenerate primers and gel-based separation of the amplified cDNA fragments. Other methods were subsequently developed using polynucleotides bound to miniature solid supports (i.e. GeneChips, cDNA microarrays) hybridized with complex probes

either as one or both treatments simultaneously (Schena *et al.*, 1995; Chee *et al.*, 1996). Despite the robustness of the GeneChip technology, such methods can only rely on available clones or sequence information. However, comprehensive sequence information of transcribed genes or whole genomes for many of the important crops are just now becoming readily available. Alternative methods, such as open-ended technologies, use modifications and improvements of the original differential display technology. One such platform (designed by CuraGen Corporation, New Haven, CT, USA) proved useful in identifying the regulation of known and novel genes associated with particular phenotypic traits or a gene network (Shimkets *et al.*, 1999; Bruce *et al.*, 2000). This technology does not necessarily rely on sequence information to uncover candidate genes. However, having a comprehensive sequence database of the expressed genes for desired organisms would benefit the open-ended technology and further improve the identification of candidate genes. With this in mind, both public institutions and private companies have invested heavily in producing a comprehensive expressed sequence tag database for several important crops (Gai *et al.*, 2000).

Metabolic events in drought response

For maize, the critical developmental period of determining grain yield centres on flowering and early seed development (Claassen and Shaw, 1970; Shaw, 1977). Numerous studies have examined components of stress response during the flowering stage of development (Edmeades *et al.*, 1993; Bolaños and Edmeades, 1996; Zinselmeier *et al.*, 1999, 2000). A recent study showed that a steady flow of photoassimilates plays an important role in both ovule and seed development (Zinselmeier *et al.*, 1999). Interruption of this flow due to dehydration, for instance, causes reductions in key carbon metabolic enzyme activities. Earlier studies suggested that water deficit effects might influence acid invertase activity and cause partial reductions in the flux of photoassimilates suitable for growth to the developing ear (Schussler and Westgate, 1995). More recently, Zinselmeier *et al.* demonstrated that acid invertase indeed has a central role in providing the necessary sugars for the developing ear and that its activity is sensitive to drought effects (Zinselmeier *et al.*, 1999, 2000). The insoluble form of acid invertase activity correlated well with ovary dry mass accumulation and final kernel number between well-watered and water-stressed plants, with or without supplemental sucrose (Zinselmeier *et al.*, 2000). These results suggest that increased acid invertase activity is important for the continuing development of ovaries just prior to pollination and kernels after pollination.

Molecular and genetic studies of drought stress in model plant systems

Many of the changes due to dehydration stress in plants include the accumulation of a variety of sugars, proline and glycine betaine in addition to changes in protein levels (Bartels and Nelson, 1994; Ingram and Bartels, 1996; Verslues and Sharp, 1999; Zinselmeier *et al.*, 1999). These changes are associated with osmotic adjustment and the protection of membranes from damage as cell contents desiccate. Gene expression is also affected by changes in available water and may indicate some of the pathways important for adaptive responses to stress. ABA is produced to high levels in response to drought (Ingram and Bartels, 1996; Leung and Giraudat, 1998). Exogenous ABA treatments can also affect the expression of dehydration-regulated genes suggesting that ABA acts as signal transducer for response to water-deficit stress (Leung and Giraudat, 1998). However, other drought-induced genes do not respond to ABA treatments suggesting an ABA-independent pathway and a greater complexity in the drought response phenomenon (Gosti *et al.*, 1995; Shinozaki and Yamaguchi-Shinozaki, 1996). As noted above, high levels of tissue ABA may reduce productivity even though they may contribute to survival.

In *Arabidopsis*, numerous genes that respond to dehydration stress were identified and have been categorized as *rd* (responsive to dehydration) and *erd* (early response to dehydration) (Shinozaki and Yamaguchi-Shinozaki, 1996). In addition, Shinozaki and Yamaguchi-Shinozaki describe the potential existence of four ABA-independent pathways and one ABA-dependent pathway in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki, 1996). Drought-stress is 'sensed' by an as yet unknown set of receptors that initiate a signal cascade with or without ABA and thus affect gene expression of dehydration-responsive genes through activation of a set of particular transcription factors. Several of the genes induced in these pathways have been identified and include the rd29B and bZIP transcription factors that regulate rd29B, AREB1, 2 and 3 (Uno *et al.*, 2000). Still the nature of the initial drought-sensitive receptors and activation of particular transcription factors has yet to be elucidated.

Certain molecules are produced or accumulate to detectable levels during water-deficits suggesting a protective role for the plants. Polyamines, for example, are thought to play a role in plant growth and development especially under stressful growing conditions (Ben-Hayyim *et al.*, 1994). Using differential display, a key and possibly rate-limiting enzyme, *S*-adenosylmethionine decarboxylase (SAMDC) involved in polyamine biosynthesis was recently shown to be regulated by various stresses including drought and ABA treatments

(Li and Chen, 2000). Proline dehydrogenase (PDH), the first enzyme in the proline oxidation pathway, was shown to be down-regulated in maize during drought stress but not affected by ABA treatments suggesting ABA-independent, drought-mediated regulation (Dallmier and Stewart, 1992). Proline was postulated also to have a protective function during water deficits similar to polyamines, but the exact mechanism is unknown. These data suggest that SAMDC and PDH could be included as targets for screening of drought resistance in desired crops.

A very recent study involved the monitoring of cold- and drought-affected gene expression in *Arabidopsis* using cDNA microarray technology (Seki *et al.*, 2001). From 1300 randomly chosen full-length cDNA clones, 44 were shown to modulate 2-fold or greater at the mRNA level after a 2 h drought stress treatment. Interestingly, a subset of the 44 genes show mRNA level modulation due to 2 h cold treatment suggesting similarities in the signalling to gene expression control (Shinozaki and Yamaguchi-Shinozaki, 1996; Seki *et al.*, 2001). However, further knowledge of the biological context of these genes such as spatial and temporal regulations will need to be defined. Similar expression profiling strategies can be applied to breeding lines that show contrasting drought sensitivities.

Mutants have been identified that are especially sensitive to drought effects and may illuminate some of the underlying mechanisms of the stress response and tolerance. For example, several of the non-allelic mutations designated *Wilty* in maize exhibit chronic wilting mostly on the top leaves under mild drought conditions (Neuffer *et al.*, 1997, and references therein). These mutants do not seem to be affected in ABA sensing and it has been concluded that the corresponding genes control vascular element development (Rock and Ng, 1999). It is possible that modern maize lines differ more subtly in vascular development that regulate water flow and contribute to differential tolerances to water stress. Mutations that affect ABA sensing and response in maize and other model plants (McCarty, 1995; Leung and Giraudat, 1998) may also uncover classes of genes whose variations in a population (with a continuum of sensitivities to drought) could provide gene targets for associating with tolerant lines.

Expression profiling analysis of stress response in maize

Probing breeding populations that show improved traits during environmental stress with genomic tools is expected to uncover important gene networks involved in drought tolerance. Recently, scientists at Pioneer applied cDNA microarraying analysis to discover maize genes expressed in the ear that are changing due to water stress during flowering (Sun *et al.*, 2001). They focused on ear

growth effects due to low water stress in one particular hybrid. The cDNA for selections of maize genes corresponding to cell cycle, known stress responses, carbohydrate metabolism, and ABA responsive genes were included on the microarrays. Of the cell cycle genes, they noted three general patterns of gene expression modulations: (1) an increase in mRNA levels during the period of stress; (2) a decrease in mRNA during the stress followed by an increase during subsequent recovery; (3) an increase in mRNA only during the recovery period. These results suggest distinct functions for members of the cell cycle regulation gene family during dehydration and recovery. Expression modulations in other genes included decreases in water channel aquaporins and β-glucosidase (*Glu1*) during the stress period, but an increase in gene expression during recovery. Similar patterns of expression in response to water-deficit stress were also noted for several other genes. The results of this study provide compelling leads for further analysis.

Since hybrids are being planted at ever-higher population densities, the stresses imposed by high density merit examination (Modarres *et al.*, 1998; Tollenaar and Wu, 1999). Higher planting densities obviously create greater competition for input resources such as solar radiation capture and nutrient and water acquisition in densely packed neighbouring root systems (Scheiner *et al.*, 2000). Increasing planting densities leads to less available water per plant and an acceleration of the occurrence of water stress symptoms as dry spells lengthen (Tollenaar and Wu, 1999). Shown in Fig. 7, expression profiling data of whole root mRNA from two era-related hybrids (one released in the 1960s and the other in the 1990s) grown under high versus low planting densities showed differential responses in several genes that included water-stress related proteins (WB Bruce, unpublished results). The newer hybrid responded with more stress-related genes showing differential expression to the higher planting densities than the older hybrid suggesting that such gene expression modulations may correlate well with better adaptation to stressful environments. It is probable that lines that are selected for better performance in higher planting densities show improved tolerance to drought conditions and vice versa, especially if processes such as those affecting kernel set when photosynthesis is reduced are affected by both stresses.

Summary

Historically, maize improvements in grain yield have been due to a variety of factors, one of which is greater tolerance to environmental stresses. This improvement has been attributed to enhanced performance of parental inbred lines in part due to tolerance to water-deficit stress. For maize, the period during pollination and
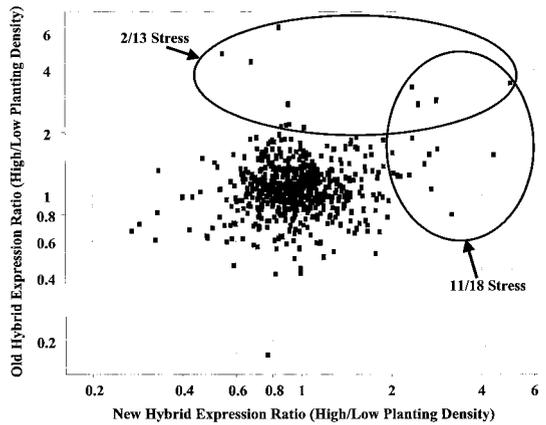
Fig. 7. RNA profiling of root from older versus newer hybrid planted under two population densities. RNA from three replicate whole root samples from 3394 (1990s era hybrid) and 3306 (1960s era hybrid) grown at planting densities of 8.65 and 1.0 plants m^{-2} were isolated at flowering, labelled and probed as described (Chee *et al.*, 1996) on Affymetrix GeneChips containing oligonucleotides representing 1501 non-redundant maize cDNA's. Following normalization (Chee *et al.*, 1996) and removal of data with signals of less than 40 units, ratios of the average intensities for samples from high planting density to low planting density were calculated and plotted for both hybrids. GeneChip entries showing a 2-fold or better positive increase due to higher planting density are indicated by the ovals. The numbers of drought stress-related entries that are similar to those previously reported (Seki *et al.*, 2001) out of the total within the ovals are indicated.

early grain filling is the most sensitive to water stress as compared with preflowering and late grain-filling growth stages. Studies involving recurrent selection in tropical germplasm clearly showed that the gains under water deficit could occur at no cost to yield in unstressed environments, in part because partitioning to the ear and harvest index were increased in all environments. As a result of selection for improved performance in water-limited environments, the tropical germplasm showed reduced final number of spikelets, early vigorous silking and reduced ASI. Several modified secondary traits were also apparent from the selection process and included mild increase in staygreen, and reduction in barrenness, root biomass and leaf rolling.

To achieve efficient selection for drought tolerance, the selection process must include managed environments where the timing, duration and intensity of the water-deficit stress can be controlled. Breeding programmes can take advantage of a variety of environments from growth chambers to the TPE, but should consider the trade-offs of controlling the stress with the need to mimic the environments of farmers' fields. Careful classifications of environments and measurements of factors affecting the environments are also needed to help reduce the current resources dedicated to wide-area testing in stress-related breeding programmes.

Newer genomic-related tools are being used to facilitate the selection of tolerant lines. These tools include associated DNA markers, known orthologous stress-related genes and newly identified genes based on more conventional physiological and biochemical approaches. RNA-profiling experiments to date have identified key target genes that respond to stress inputs and are targets for screening variations in the breeding population. Ultimately, approaches described here will provide a better knowledge of the nature of improved hybrid performance in stressed environments and generate faster, cheaper and more available breeding germplasm for drought tolerance.

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