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Molecular Improvement of Tropical Maize for Drought Stress Tolerance in Sub-Saharan Africa

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Molecular Improvement of Tropical Maize for Drought Stress Tolerance in Sub-Saharan Africa

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The C4 grass *Zea mays* (maize or corn) is the third most important food crop globally after wheat and rice in terms of production and the second most widespread genetically modified (GM) crop, after soybean. Its demand is predicted to increase by 45% by the year 2020. In sub-Saharan Africa, tropical maize has traditionally been the main staple of the diet, 95% of the maize grown is consumed directly as human food and as an important source of income for the resource—poor rural population. However, its growth, development and production are greatly affected by environmental stresses such as drought and salinization. In this respect, food security in tropical sub-Saharan Africa is increasingly dependent on continuous improvement of tropical maize through conventional breeding involving improved germplasm, greater input of fertilizers, irrigation, and production of two or more crops per year on the same piece of land. Integration of advances in biotechnology, genomic research, and molecular marker applications with conventional plant breeding practices opens tremendous avenues for genetic modifications and fundamental research in tropical maize. The ability to transfer genes into this agronomically important crop might enable improvement of the species with respect to enhanced characteristics, such as enriched nutritional quality, high yield, resistance to herbicides, diseases, viruses, and insects, and tolerance to drought, salt, and flooding. These improvements in tropical maize will ultimately enhance global food production and human health. Molecular approaches to modulate drought stress tolerance are discussed for sub-Saharan Africa, but widely applicable to other tropical genotypes in Central and Latin America. This review highlights abiotic constraints that affect growth, development and production of tropical maize and subsequently focuses on the mechanisms that regulate drought stress tolerance in maize. Biotechnological approaches to manage abiotic stress tolerance in maize will be discussed. The current status of tropical maize transformation using *Agrobacterium* as a vehicle for DNA transfer is emphasized. This review also addresses the present status of genetically modified organisms (GMOs) regulation in sub-Saharan Africa.

Keywords tropical maize, drought stress, conventional breeding, genetic engineering

I. INTRODUCTION

A. Tropical Maize

Tropical maize refers to corn (*Zea mays* L.) genotypes that are widely grown in tropical and sub-tropical regions including sub-Saharan Africa and Central and Latin America. Maize or corn is the domesticated form of teosinte, a wild grass occurring naturally in isolated patches of central Mexico. Maize was introduced into Africa by the Portuguese at the beginning of the 16th century (McCann, 2005). In developing countries, tropical white maize tends to be more important than yellow maize that represents the bulk of maize grown worldwide (FAO, 1997) (Figure 1), although they are biologically and genetically similar. Sequence alignment of the *ZmPARP1* gene from the B73 temperate and the CML216 tropical inbred genotypes showed no variation in structure (S. Anami, unpublished results). The difference in appearance is due to the presence or absence of a carotene pigment in the yellow maize and tropical white maize, respectively. Indeed, the *Carotenoid Cleavage Dioxygenase 1*

(*CCD1*) locus that underlies the white cap1 (*wc1*) phenotype plays a significant role in reducing the carotenoid pool in tropical white maize (Kandianis *et al.*, 2008). Repression of *MtCCD1* in mycorrhizal roots of *Medicago truncatula* causes a significant accumulation of C₂₇ apocarotenoid with a concomitant color change to a clearly more intense yellow-orange root color (Floss *et al.*, 2008), indicating that temperate maize genotypes have an abundant C₂₇ apocarotenoid chromophore. However, at the genome level, tropical and temperate maize might differ significantly. Genome sequencing programs comparing the temperate B73, used as sequencing model, and an ancient tropical maize: Palomero the Palomero Toluqueno, member of the central and northern highlands group that produces short individuals (140–180 cm) and grows at elevations above 2000 m, estimated a reduction of 22% in the genome size of the tropical maize (Martinez de la Vega *et al.*, 2008). The comparison was based on the assembly of 199.2 Mb in close to 220,000 gene-enriched contigs averaging 0.9 kb in size in the tropical maize and suggested few variations in the abundance of repetitive elements in the intergenic regions, which makes tropical maize an attractive model for comparative studies of the maize genome evolution. In addition, large-scale sequencing efforts in the B73 genotype might not be sufficient to fully understand the maize genome organization and to identify all functional units available in the domesticated gene pool. Therefore, besides the B73 genotype, the Palomero Toluqueno tropical genotype has been chosen for genome sequencing (Martinez de la Vega *et al.*, 2008).

The International Maize and Wheat Improvement Center (CIMMYT) is the world's largest repository of tropical and semitropical maize germplasms, with more than 23,000 maize land race accessions ([www.cimmyt.org.](http://www.cimmyt.org/)). Extensive conventional breeding programs of tropical maize in a number of countries in sub-Saharan Africa have identified genotypes with putatively superior features that have been tested, maintained and nutured through seed propagation until sufficient stock could be commercially released. The selected repositories of tropical germplasm are kept by the respective National Agricultural Research Institutes (NARIs) in these countries. Several maize lines selected by the CIMMYT and NARIs can continue reproductive growth under inadequate water conditions, an important factor for grain production under drought stress. Some of the tropical land races selected for drought resistance by farmers in the tropics delay flowering under drought stress and others rapidly recover after stress (Hayano Kanashiro *et al.*, 2008).

Tropical maize germplasm at CIMMYT has very diverse origins and breeders have followed a strategy that intercrosses single germplasm with a broad genetic base, formed usually by cultivars with a larger variability than that of the temperate synthetic materials (Warburton *et al.*, 2005). Unlike their temperate counterparts that had been developed by the advanced cycle pedigree breeding method producing very distinct groups of maize lines with reduced genetic variation within each group, but maximum heterotic response, tropical maize has been obtained through modified full or half-sib recurrent selection to



FIG. 1. Tropical white maize cob compared to temperate maize cob that is predominantly yellow in color.

possess high yield potential and yield stability under a wide variety of production conditions and environments in the developing world (Vasal *et al.*, 1997). Because tropical maize is adapted a day length of 12–13 hours, the flowering occurs much later in longer days typical of the temperate regions. This characteristic of tropical maize causes the plant to grow very tall and become herbaceous making breeding and selection difficult.

Restriction Fragment Length Polymorphism marker analysis (Benchimol *et al.*, 2000; Warburton *et al.*, 2005) showed that tropical germplasm is genetically more diverse than temperate maize, suggesting that tropical inbred lines possess broad adaptations and might be a source of new alleles for identifying heterotic groups and facilitate selection of breeding materials. The use of amplified length polymorphism and microsatellite markers for grouping tropical lines into genetically similar clusters revealed that the lines were genetically polymorphic and the genetic distance correlated with the performance of phenotypic traits (heterosis) of their F1 populations (Glaubitz *et al.*, 2008; Kiula *et al.*, 2008). The genetic and phenotypic diversity present in tropical maize can be attributed to an effective population size, a high degree of out crossing and approximately 0.5 million years of recombination in its progenitor teosinte lineage (Timmermans *et al.*, 2004).

B. Tropical Maize as a Major Crop for Human Consumption

Maize has many desirable qualities making it a major food source for humans for several millennia. It is easy to culture from single plants or small nurseries to hundreds of hectares. The pollen-bearing inflorescence (tassel) atop the plant is handily separated from the female inflorescence (ear) along the culm, so that both can easily be manipulated, removed, or bagged, whereas the harvested ear is readily labeled, scanned, and stored as a progeny unit. In sub-Saharan Africa, tropical maize has been

customarily the main staple of the diet and its consumption exceeds its production. The whole grain, either mature or immature, can be roasted or cooked and served as food. Dried kernels or grains can be processed by dry milling to give maize flour that eventually is used to prepare “ugali,” as called in Kenya or “porridge,” upon mixing with hot water, and serves as the main raw material to produce the nonconventional local brews known in Kenya as “Changaa” and “Busaa.” These drinks are revered in local communities and are part and parcel of traditional ceremonies, including marriages, circumcision, and burials.

In the 21st century, there are concerns about global warming due to greenhouse gas emission, soaring oil prices, energy security, and the recognition that the global crude oil reserve is finite and its depletion occurring much faster than previously predicted. In addition, the environmental deterioration resulting from the overconsumption of petroleum-derived products, is threatening the sustainability of human society (Schubert, 2006). Therefore, an intensified quest for alternative sources of bioenergy has engulfed the world. Ethanol, a reliable, renewable and affordable source of carbon in its chemically reduced form that can support future economic developments without negative impact on the environment is believed to be one of the best alternatives to fossil fuel, but needs to dramatically increase its production capacity. Indeed, ethanol and biodiesel are predominantly produced from corn grain, sugar cane and soybean oil. Approximately 5 billion gallons of corn ethanol were produced in the USA in 2006, which is equivalent to only 3.6% of the total volume of gasoline consumed in that year, whereas similarly, the 100 million gallons of biodiesel produced accounted for <0.2% of the total diesel used domestically (Yacobucci and Schnepf, 2007).

In this respect, tropical maize has been regarded as the “bio-fuel emperor” due to its double advantage as a source of both starch (seed) and cellulose (stover). However, a sustainable

biofuel industry has to rely utterly on non-food crops. Given that tropical maize is used as food in sub-Saharan Africa and acclaimed as an emperor for the biofuel industry, separate land demarcation and distribution chains for tropical maize bioenergy and food production need to be organized and its production has to increase.

C. Molecular Breeding to Increase Tropical Maize Production

Limited rain for agricultural land and water resources, expanding population on agricultural land, and environmental and biotic stresses, all result in a growing demand for improving both quality and quantity of tropical maize. Tropical and sub-tropical maize production in sub-Saharan Africa increasingly depends on continuous improvement through conventional breeding that relies on multilocation testing of progenies in target environments with emphasis on high and stable yield (Chimenti *et al.*, 2006), which has been historically effective. This sector has been boosted by the highly dynamic Alliance for the Green Revolution in Africa (AGRA) (www.agra-alliance.org) that focuses on the agricultural "value chain" from seeds, soil health, and water, to markets, agricultural education, and policy in a region bypassed by the green revolution. Despite the immense investments by AGRA, this participatory and comprehensive approach fails to recognize that conventional breeding programs are slow and that, in most cases, the drought-tolerant plants obtained through these programs show an inverse relationship with yield (Ceccarelli, 1987). Furthermore, the available genetic resources or germplasms from gene banks might lack the genes coding for major disease and pest resistance and abiotic stress tolerance that are necessary to develop crops adapted to changing weather and environments. In addition, variability is limited in the available germplasm of crop plants and relies on measuring phenotypes to identify individuals with the highest breeding value. The effects of environment, genotype by environment interaction and measurement errors, however, hamper the progress in phenotypic selection.

Traditional breeding strategies that involve selection for visible phenotypes in plants have generated very few crop varieties with improved tolerance to abiotic stresses. Contrary to the classical breeding approach, integration of the latest advances in biotechnology, genomic research, and molecular marker applications with conventional plant breeding practices has created the foundation for molecular plant breeding, an interdisciplinary science that is revolutionizing the 21st century crop improvement (Moose and Mumm, 2008) (Figure 2), and has been particularly successful on a global scale, e.g. the development of hybrid maize (Duvick, 2001), the introduction of wheat (*Triticum aestivum*) and rice (*Oryza sativa*) varieties that spearheaded the Green Revolution (Evenson and Golin, 2003), and the recent commercialization of insect- and herbicide-resistant transgenic crops (James, 2007). These and many other products of plant breeding have contributed to the numerous benefits so-

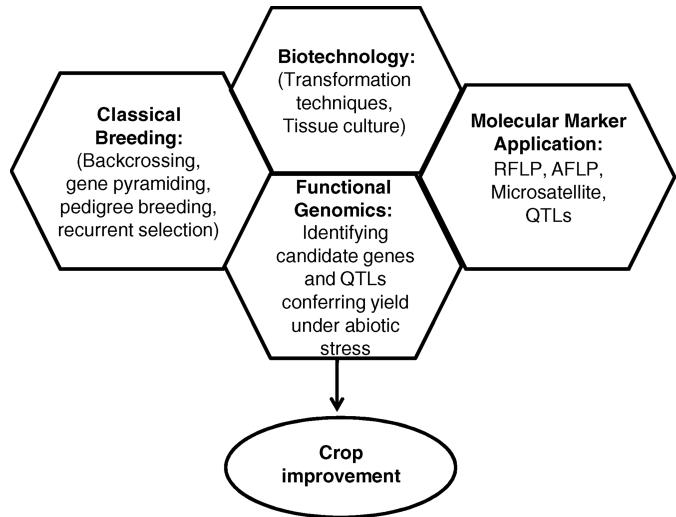


FIG. 2. Crop improvement by integration of classical breeding with molecular tools (modified from Sreenivasulu *et al.*, 2007).

society has received from increased sustainable supplies of carbon that may be harvested as food, feed, forests, fiber, and fuel.

With molecular plant breeding, the genetic diversity existing in tropical maize could be expanded by using exotic germplasm with desirable characteristics to enhance its production. Surveys of molecular marker alleles and nucleotide sequence variation have resulted in basic information about genetic diversity among geographically distributed land races (Sibov *et al.*, 2003; Oliveira *et al.*, 2004). The results greatly improved estimates on the number of loci, allelic effects, and gene action controlling traits of interest. In addition, such information enriches investigations of plant evolution and comparative genomics, contributes to our understanding of population structure, provides empirical measures of genetic responses to selection, and also serves to identify and maintain reservoirs of genetic variability for future mining of beneficial alleles (Slade *et al.*, 2005). Furthermore, with knowledge of genetic relationships among tropical germplasm sources parents might be chosen for production of hybrids or improved populations (Collard and Mackill, 2008). Therefore, variation between species and accessions in abiotic stress tolerance should be studied and the identified tolerant accessions/wild species should be introgressed into elite genomes. Such breeding material could be used in -omics technology to explore regulatory mechanisms controlling yield under stress conditions. Information about quantitative trait loci (QTLs) in tropical maize can be applied to increase heritability and favorable gene action to design optimum transgenic strategies for crop improvement. Marker-assisted selection also accelerates the use of transgenes in commercial cultivars, typically achieved through marker-assisted backcrossing.

In contrast, the use of plant biotechnology approaches generates new genetic diversity that often extends beyond species boundaries and facilitates the molecular stacking of transgenes as a dominant trait or a suite of traits into a single locus (Frizzi

et al., 2008). Excellent examples in corn are the production of insecticidal toxin proteins from *Bacillus thuringiensis* (*Bt*) to reduce the damage caused by larvae of the European corn borer *Ostrinia nubilalis*, the expression of a transcription factor that increases drought tolerance (Nelson *et al.*, 2007), and alterations in the balance between levels of the GLOSSY15 transcription factor and its repressor microRNA172 to delay flowering in maize hybrids (Lauter *et al.*, 2005). Therefore, increasing tropical maize production in sub-Saharan Africa will largely depend on strategies exploiting the gene pool to impart abiotic stress tolerance and to identify these stress-tolerant gene combinations via functional genomic approaches. Thereafter, the identified genes will have to be introduced into tropical maize. Alternatively, the QTLs or genes conferring stress tolerance could be used as molecular markers in marker-assisted breeding programs in order to improve yield stability.

II. ABIOTIC STRESS: PHYSIOLOGY AND MOLECULAR PATHWAYS

The production of tropical maize in sub-Saharan Africa is severely limited by a large number of biotic (arthropods, diseases and weeds) and abiotic (drought and mineral toxicity constraints). However, we will not deal with the biotic constraints although their solution is key to enhancing yield (reviewed by Gressel *et al.*, 2004).

Abiotic stresses, such as drought, salinity, extreme temperatures, oxidative stress and heavy metals, have a major impact on successful establishment, growth, development, reproduction and productivity of crop plants. The stresses cause a number of deleterious effects including reduced cellular osmotic potential, inhibition of cell division and expansion, reduced membrane integrity, impaired cellular function and disruption of ion homeostasis (Kant *et al.*, 2008). Thus, abiotic stresses are the major causes of yield losses in cultivated crops worldwide that result from a combination of different stresses during the growing season as well as of periodically occurring extreme weather conditions. This situation deteriorates as water resources become insufficient and soil salinity spread widely (Vinocur and Altman, 2005).

The effects of drought and salinity are linked because they both disrupt the plant water status (Verslues *et al.*, 2006) and occur through decreased availability of water, altered ion content and inefficient water uptake due to salinity. The two stresses are common in sub-Saharan Africa and might cause salinization of more than 50% of all arable land by 2050 (Wang *et al.*, 2003). Drought stress has an impact throughout the growth cycle of maize, with the greatest yield reduction and sterility when stress occurs during flowering and pollination (Figure 3) because silking or the onset of the reproductive stage is the most sensitive stage and might result in 100% yield loss when drought is coupled with higher temperature.

Water deficit stress during the vegetative growth phases in maize typically reduces plant and leaf sizes, resulting in grain

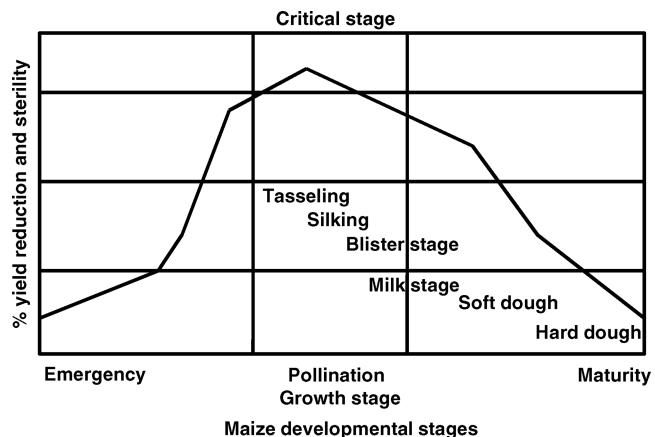


FIG. 3. Impact of drought stress at different stages of maize development (modified from Rhoades and Bennett, 1990).

loss through reduction in kernel numbers. Drought stress during tasseling and silking stages decreases ear size and potential yield, whereas water deficit stress around flowering and pollination delays silking until the pollen shed is nearly or completely finished, reduces silk length and inhibits embryo development after pollination. Furthermore, moisture stress interferes with synchronization of pollen shed and silk emergence. In addition, during periods of high temperature, low relative humidity, and inadequate soil moisture, exposed silk might desiccate and become nonreceptive to pollen germination. Late-stage drought stress, during the grain-filling period, can frequently lead to low yield by reduced kernel size as well as increased rates of kernel abortion during the first two weeks following pollination. In addition, drought during this period provokes leaf dying, shortens grain filling period and increases lodging. Consequently, when plants are exposed to abiotic stresses, they activate a diverse set of physiological and biochemical defense systems for survival and to sustain growth (Valliyodan and Nguyen, 2006), culminating in the re-establishment of the cellular homeostasis. Dehydration and salinization stresses also cause cellular damage that are manifested primarily as osmotic stresses, resulting in disruption of cellular aqueous and ionic equilibriums (Cattivelli *et al.*, 2008). In addition, hundreds of genes and their products respond to these stresses at transcriptional and translational levels (Umezawa *et al.*, 2006). Tolerance to drought stress is a very complex trait that has hampered the direct selection for grain yield under water-stressed conditions, which is attributed to the polygenic nature of the abiotic stress response (Vij and Tyagi, 2007), low heritability of the drought trait, epistasis, significant genotype-by-environment (GxE) interaction and QTLs-by-environment (QTLxE) interaction. The unpredictable nature of most periods of drought encountered in sub-Saharan Africa corroborated by gaps in our understanding of drought biology explains the slow progress in yield improvement in drought-prone environments. In addition, the plant response to each single abiotic stress is unique and, thus, the response to multiple stresses will also differ, making it difficult to control and engineer.

A. Physiological, Morphological and Metabolic Changes in Plants upon Drought Stress

Drought stress limits plant growth and crop productivity significantly (Araus *et al.*, 2002). However, in certain tolerant crop plants, morphological, physiological and metabolic changes occur in response to drought that allow the plant to avoid water loss by continuous water uptake at reduced water potential or to tolerate a reduced tissue water content. In the field, either concurrently or at different times through the growing season, plants might experience several distinct abiotic stresses (Tester and Bacic, 2005) whose detrimental effect on plant performance is at least partly caused by disruption of the plant water status through decreased availability, altered ion content and water uptake due to salinity.

1. Physiological Changes

Plants react to stress by consuming large quantities of energy in the form of ATP (De Block *et al.*, 2005) that can no longer be used for vital physiological processes, such as growth and carbon fixation in photosynthesis. The effects of moderate stress over extended periods or short-term episodes of extreme stress can completely drain the plant's energy reserves, resulting in irreversible damage to the plant or even death. However, plants have certain biochemical and metabolic adaptations that facilitate their survival in stress-induced depletion of ATP. The "metabolic flexibility" protects plants by activating first a pyrophosphate required for converting poly (ADP-ribose) into ATP molecules (Petermann *et al.*, 2003; Hakmé *et al.*, 2008) that are important when cellular ATP pools are diminished during stresses; secondly, alternative glycolytic reactions bypass ATP-requiring steps, and finally the salvage pathways are activated to mitigate against the impacts of depleted ATP (Dobrota, 2006).

Adaptive traits, such as osmotic adjustment, dehydration tolerance, and reduction of photosynthetic activity arise in response to water deficit. Reduction in photosynthetic activity invariably leads to 'respiratory flexibility' due to several coordinated events, such as stomatal closure and the decreased production of photosynthetic enzymes. Furthermore, the photosynthetic parameters, such as electron transport rate, carboxylation efficiency, respiration rate and intrinsic water use efficiency, correlate with stomatal conductance. Also photophosphorylation, regeneration and activity of ribulose-1,5-bisphosphate have been shown to be impaired under drought (Medrano *et al.*, 2002).

At the cellular level, drought stress-associated physiological changes include turgor loss and changes in membrane fluidity and composition. The physiological changes that occur during the plant's response to water stress have been correlated with rapid translocation of abscisic acid (ABA) in the transpiration stream and increase in ABA concentration in plant organs (Alvarez *et al.*, 2008).

During drought stress, the xylem vessels give up contents (including their ABA) to the leaf apoplast, thereby increasing the hormone concentration in this compartment. ABA is car-

ried with the transpiration stream inside the leaf around and/or through the mesophyll cells so that it reaches the stomatal guard cells of the epidermis, which contain ABA receptors with external (and possibly internal) loci in their plasma membranes. Once bound, the hormone induces an internal signal transduction cascade, usually involving increases in both externally and internally sourced cytoplasmic calcium, which eventually reduces the osmotic potential of guard cells via loss of K^+ and Cl^- with stomatal closure, as a consequence (for review, Wasilewska *et al.*, 2008).

2. Morphological Changes

Plants exposed to sublethal abiotic stress conditions exhibit a broad range of morphogenic responses that include inhibition of cell elongation, localized stimulation of cell division and alterations in cell differentiation status (Potters *et al.*, 2007). As such, abiotic stress stimuli negatively affect plant growth and development through the arrest of the cell cycle machinery. For instance, the Oryza;EL2 gene, an inhibitor of the cyclin dependent kinase (CDK) was transcriptionally induced by abiotic stress treatments (Peres *et al.*, 2007), indicating that Oryza;EL2 coordinates stress perception and cell cycle progression. Abiotic stress perception activates signaling cascades that stimulate cell cycle checkpoints, resulting in an impaired G1-to-S transition, slowing down of DNA replication, and/or delayed entry into mitosis (Kadota *et al.*, 2005). Water stress induces meristem shortening in leaves of wheat and maize and prolongs the cell cycle duration as a result of reduced CDK activity (Granier *et al.*, 2000), whereas salinity inhibits *Arabidopsis* root growth by reducing the pool of dividing cells in the meristem (West *et al.*, 2004). In plant tissues, water potential and content are maintained close to the unstressed level by increasing uptake or limiting loss, so that loss and uptake rates of water remain balanced. Such a balance is achieved in the short term mainly by developmental and morphological traits, such as stomatal closure that is paralleled by a decreased photosynthetic rate (Lawlor, 2002). Indeed, stomatal closure in response to drought stress restricts CO_2 entry into leaves, thereby decreasing CO_2 assimilation and water loss from the leaves, and affecting mesophyll metabolism (Parry *et al.*, 2002).

In the longer term, changes in root and shoot growth, leading to increased root/shoot ratio, tissue water storage capacity, cuticle thickness and water permeability are also potentially important, of which changes in root growth to maximize water uptake are the most crucial for crop plants (Verslues *et al.*, 2006). Highly water-stressed corn plants respond by leaf rolling early in the day. The stress-induced morphogenic response is postulated to be part of a general acclimation strategy, whereby plant growth is redirected to diminish stress exposure.

3. Proteomic and Metabolic Changes

By proteomic analysis of the maize xylem sap, abundance of cationic peroxidases was found, which might induce lignin biosynthesis in the xylem vessels and induce cell wall stiffening

that might strengthen the xylem vessels and prevent any further cell expansion either to better withstand the tension occurring during water stress or to restrict water loss from internal tissues (Alvarez *et al.*, 2008). Furthermore, in water-stressed plants, the levels of many amino acids in the sap increase transiently and a number of amino acids also accumulate only under severe water stress.

Metabolic changes associated with drought stress include modifications in solute concentration and protein–protein and protein–lipid interactions (Valliyodan and Nguyen, 2006). The plant defense response to drought stress is associated with the synthesis of osmoprotectants, osmolytes or compatible solutes. Production of phytoalexins, activation of the general phenylpropanoid pathway and induction of lignin biosynthesis have evolved as adaptation mechanisms to water deficit. Salicylic acid, methyl salicylate, jasmonic acid, methyl jasmonate and other small molecules produced as a result of stress can also serve as signaling molecules activating systemic defense and acclimation responses (Shulaev *et al.*, 2008), whereas others protect plants from oxidative damage associated with a variety of stresses, such as ascorbic acid, glutathione, tocopherols, anthocyanins and carotenoids by scavenging the generated active oxygen intermediates.

Knowledge on the physiological and biochemical responses to severe water deficit conditions has been exploited to engineer drought stress tolerance in plants. Multiple transgenic approaches include the use of compounds, such as polyols mannitol (Tarczynski *et al.*, 1993) and sorbitol (Abebe *et al.*, 2003); dimethylsulfonium compounds, such as dimethylsulfoniopropionate, glycine betaine (Chen and Murata, 2002); sugars, such as sucrose, trehalose (Garg *et al.*, 2002), galactinol (Taji *et al.*, 2002), ononitol (Sheveleva *et al.*, 1997) and fructan (Pilon-Smits *et al.*, 1995); or amino acids such as proline (Kishor *et al.*, 1995) and ectoine that serve as osmolytes and osmoprotectants.

B. Reactive Oxygen Species (ROS) Signaling in Plants under Abiotic Stress

Another aspect of dehydration tolerance is control of the level ROS or limitation of the damage caused by ROS. Drought stress invariably leads to oxidative stress in the plant cells due to higher leakage of electrons toward O₂ during energy-generating processes, photosynthesis and respiration with ROS generation as a consequence, mainly in the chloroplasts, peroxisomes, and mitochondria (Van Breusegem and Dat, 2006). Abiotic stresses signal the generation of ROS, particularly O[•] and H₂O₂, by deregulating electron transport in chloroplasts and mitochondria and by activating the plasma membrane-bound NADPH oxidases, the cell wall-bound NAD(P)H oxidase-peroxidase, and the amine oxidases (Papadakis and Roubelakis-Angelakis, 2005). ROS function as signaling molecules in eukaryotes, triggering specific downstream responses (Mittler *et al.*, 2004). When the increase in ROS is relatively small, the housekeeping antioxidant capacity is recruited to reset the original balance

between ROS production and scavenging, thus reestablishing the redox homeostasis. In this respect, H₂O₂ produced directly or by superoxide dismutase (SOD) induces the expression of antioxidant genes, such as ascorbate peroxidase and catalase (CAT), that detoxify ROS (Apel and Hirt, 2004). Efficient scavenging of ROS plays a significant role in the osmotic stress tolerance of plants, although its exact contribution remains unknown (Hasegawa *et al.*, 2000). Under abiotic stresses, such as temperature extremes, water deficit, or salt stress, the carbon fixation rate is inadequate, increasing photoinhibition potentially directing the photosystem toward overproduction of superoxide radicals and H₂O₂ (Foyer and Noctor, 2005). Similarly, during ozone exposure, ROS are generated following the entry of ozone through the stomata and its conversion in the leaf apoplast, eventually leading to the formation of hypersensitive response-like lesions. Ozone treatment of *Arabidopsis* leads to activation and nuclear translocation of MPK3 and MPK6 (Ahlfors *et al.*, 2004), whereas RNAi lines silenced for either of these MAPKs are hypersensitive to ozone. The accumulation of ROS in the apoplast results from an increased activity of apoplastic peroxidases, amine oxidases, and an NADPH-oxidase complex coupled to a decrease in cellular ROS-scavenging capacity (Van Breusegem and Dat, 2006).

Upon abiotic stress, the ROS production could affect gene expression by first activating ROS sensors to induce signaling cascades that ultimately impinge on gene expression. Alternatively, components of the signaling pathways could be directly oxidized by ROS or ROS might alter gene expression by targeting and modifying the activity of transcription factors (Apel and Hirt, 2004) (Figure 4). The OXI1 kinase acts as a ROS sensor in plants to activate MPK3 and MPK6 (Rentel and Knight, 2004), and OMTK1 plays a mitogen-activated protein kinase scaffolding role and activates H₂O₂-induced cell death in plants (Nakagami *et al.*, 2004).

In detached leaves of *Arabidopsis*, AtMPK4 and AtMPK6 are activated by drought, indicating an important role of these two kinases in a general response to hyperosmotic stresses (Ichimura *et al.*, 2000). Activation of the MAPKs can facilitate their translocation to the nucleus where they can phosphorylate and activate transcription factors, thereby modulating gene expression (Zhang *et al.*, 2006). Through the (MAPK) cascade, extracellular stimuli are transduced into intracellular responses in all eukaryotic cells (Jonak *et al.*, 2002). Thus, redox-dependent plant stress tolerance involves a range of signaling molecules, but this interactive regulatory network is just starting to be elucidated (Van Breusegem and Dat, 2006).

C. ABA-Dependent Signaling upon Drought

Drought stress induces *de novo* synthesis of the phytohormone ABA that plays an important role in the adaptation of vegetative tissues to abiotic stresses, such as drought and high salinity, by promoting stomatal closure in guard cells (Shinozaki *et al.*, 2003). Many ABA-inducible genes contain

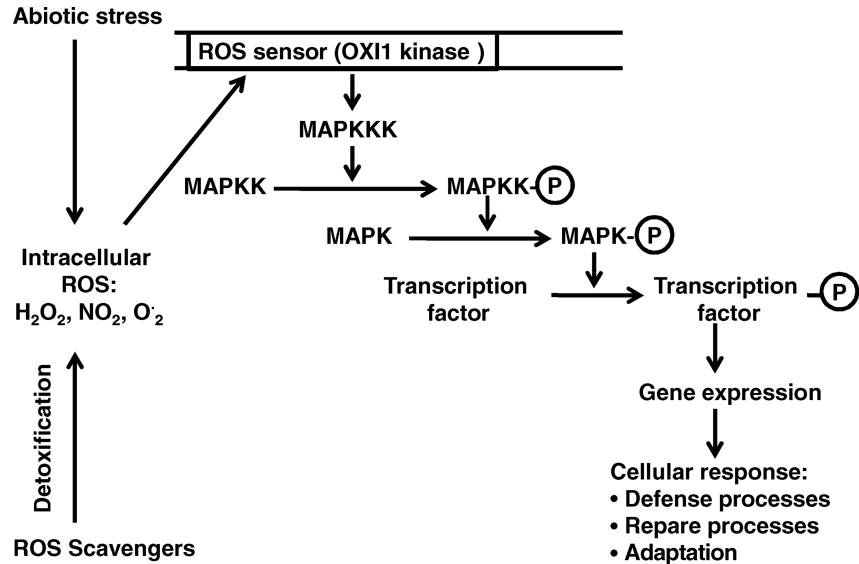


FIG. 4. Cellular ROS signaling in plants. Abiotic stress activates the production of intracellular ROS. When the increase in ROS is relatively small, the housekeeping antioxidant capacity is recruited to reset the original balance between ROS production and scavenging, thus reestablishing the redox homeostasis. Otherwise, ROS is sensed by membrane-localized kinases that eventually activate the MAPK. MAPK regulates gene expression by altering the transcription factor activity through phosphorylation of serine and threonine residues, whereas ROS is regulated by oxidation of cysteine residues (modified from Apel and Hirt, 2004).

a conserved, ABA-responsive, *cis*-acting element, designated ABRE (PyACGTGGC) in their promoter regions. Reversible protein phosphorylation is an early and centrally regulated event in ABA signal transduction, at least in the guard cells. Upon drought stress, the ABA-responsive 42-kDa kinases are activated, thereby phosphorylating the conserved regions of AREB/ABFs. Several type-2 SNF1-related protein kinases (SnRK2-type) such as ABA-activated protein kinase (AAPK) (Li *et al.*, 2008) and OST1/SRK2E in *Arabidopsis* (Mustilli *et al.*, 2002) were reported as AAPKs. All these kinases phosphorylate *in vitro* a motif in the so-called Constant (C) subdomains found among basic-leucine zipper (b-ZIP) transcription factors, including ABA Responsive Element Binding protein (AREB)1, AREB2, and ABI5 (Furihata *et al.*, 2006). Some b-ZIP transcription factors may also be the targets of calcium-dependent protein kinases (CPKs). Transgenic *Arabidopsis* plants over-expressing the active form of AREB1 showed ABA hypersensitivity and enhanced drought tolerance by activating its downstream genes, such as RD29B. Thus, AREBs/ABFs regulate ABA-mediated ABRE-dependent gene expression that enhances drought tolerance in vegetative tissues, whereas phosphorylation/dephosphorylation plays an important role in the activation of the AREB/ABF proteins (Figure 5).

D. ABA-Independent Signaling upon Drought

Advancement in plant genomics and systems biology, including the availability of the complete genome sequences of both *Arabidopsis* and rice and that yet to be released of maize, has offered an unprecedented opportunity to identify regulatory genes and networks that control drought stress tolerance. Changes in gene expression play an important role in plant drought stress

response, and many stress-induced genes are known or presumed to play roles in drought resistance. For many of these genes, the hormone ABA is a key signaling intermediate controlling their expression either in an ABA-dependent or ABA-independent manner (Figure 5), as shown largely by the analysis of ABA-deficient and ABA-insensitive mutants in *Arabidopsis* (Koornneef *et al.*, 1998).

Genetic engineering of plants for tolerance to extreme abiotic stresses could be achieved by the regulated expression of stress-induced transcription factors, which, in turn, would regulate the expression of a large number of relevant downstream genes (Yamaguchi-Shinozaki and Shinozaki, 2005). The best stress-responsive transcription factors are the C-repeat-binding factor (CBF)/dehydration-responsive element-binding (DREB) proteins that belong to the AP2/ethylene-responsive element binding protein family (Yamaguchi-Shinozaki and Shinozaki, 2005; Qin *et al.*, 2007). These factors enhance or modulate the expression of genes with a CBF/DRE box in their promoters and define a major stress tolerance pathway, in addition to the ABA biosynthesis/response pathway. On the other hand, the promoter of the *EARLY RESPONSE TO DEHYDRATION1* (*ERD1*) gene contains *cis*-acting element(s) involved in ABA-independent stress-responsive gene expression (Nakashima *et al.*, 1997). Among transcription factors reported to enhance drought stress tolerance in cereal crops are the *Arabidopsis HARDY* gene (Karaba *et al.*, 2007) and a member of the NF-Y family of transcription factors (Nelson *et al.*, 2007). Additional transcriptional regulators are under investigation, such as NAC transcription factors that bind a drought-responsive *cis* element (Tran *et al.*, 2004), and the *SALT TOLERANCE* (*SAT*) gene *MBF1α* (Kim *et al.*, 2007), which are promising candidates for enhancing drought stress tolerance in crop plants.

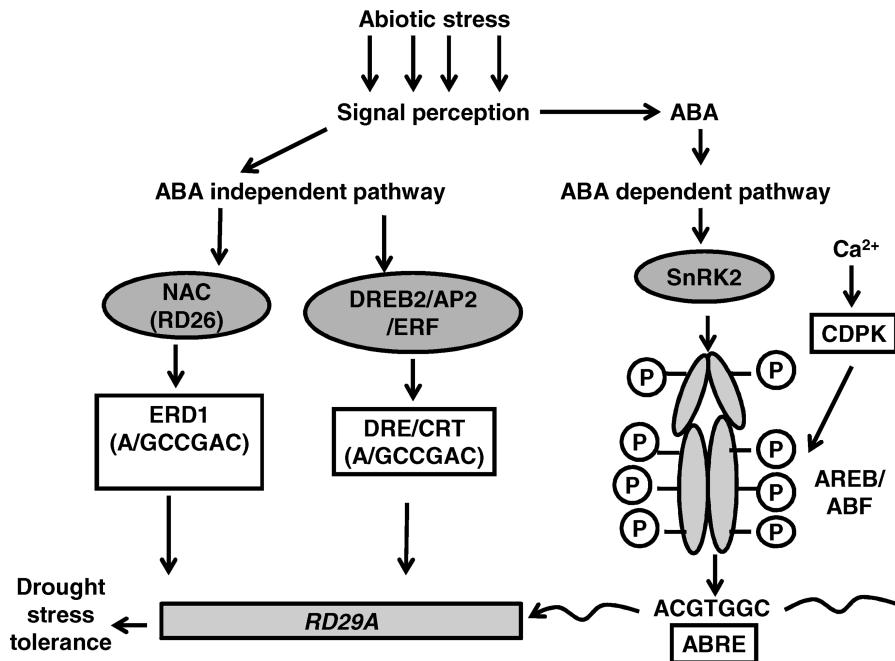


FIG. 5. ABA-dependent and ABA-independent signaling in plants. DREB2 and NAC signal transduction pathways are induced in response to drought stress and are involved in the expression of downstream target genes responsive to drought stress both in *Arabidopsis* and maize. The NAC transcription factor and DREB2 are involved in *ERD1* and *RD29A* gene expression, respectively. SnRK2 can interact physically and phosphorylate b-ZIP transcriptional activators that might also be phosphorylated by certain CPKs, which recognize similar or even identical C-domain motifs as the SnRK2s. The *RD29* gene contains both ABRE and DRE/DRT *cis* elements in its promoter. DRE, drought-responsive; ABA, Abscisic acid (modified from Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Wasilewska *et al.*, 2008).

These positive reports on the use of transcription factors to improve drought resistance in model and crop plants are based on laboratory and greenhouse conditions rather than field conditions; hence, the use of transcription factors in enhancing drought tolerance in crop plants should be considered with caution. Therefore, there is a need for understanding the basic molecular mechanisms influencing drought tolerance and grain yield under field stress conditions.

III. EMERGING PATHWAYS FOR ENGINEERING STRESS TOLERANCE IN TROPICAL CROPS

Crop yield can be improved by increasing general stress tolerance against, for instance, cold nights, high temperatures at noon, or nutrient depletion. By enhancing the tolerance to multiple stresses, plants become less sensitive to adverse environmental changes with positive impact on the overall yield. The urgent need for rational approaches to develop crop plants with increased abiotic stress tolerance has led to an impressive body of work in the area of plant genetics, plant physiology, plant biochemistry and plant molecular biology and resulted in the first transgenic maize plants with enhanced drought tolerance (Quan *et al.*, 2004; Shou *et al.*, 2004; Nelson *et al.*, 2007).

Although by using functional genomics approaches in maize, regulatory pathways involved in abiotic stress response have been dissected and shown to enhance abiotic stress tolerance under laboratory and greenhouse conditions by activating stress-

responsive signal transduction and downstream transcription factor genes in transgenic plants, their performance is rather poor in the fields where plants are exposed to a combination of abiotic stresses for short or long periods. Apparently, the expression of any single gene under a strong constitutive promoter might not give sustained tolerance to abiotic stresses in field conditions because of serious implications with respect to energy loss or other deleterious effects. Hence, it is equally important to focus on new approaches that integrate developed knowledge as an outcome of functional genomics into real knowledge-based breeding programs via genomics-assisted breeding to develop stable populations conferring both stress tolerance and yield stability under field conditions.

Drought induces structural, physiological and molecular abnormalities in the processes leading to the development of the gametes and seriously influences the success of fertilization because dysfunctional male and female gametophytes are produced, even when fertilization itself takes place under optimum environmental conditions. The grain-filling period is also of considerable importance in determining yield (Barnabás *et al.*, 2008). Therefore, to maximize the yield in cereals, all breeding or genetic manipulation approaches have to converge finally at fertilization and early grain filling.

Understanding these developmental processes is of utmost importance for the future, and modern genomics approaches might help considerably in identifying the most sensitive molecular processes during reproduction, and making them amenable

to genetic improvement. For instance, such genomics approaches have been successful in engineering glycine betaine (Quan *et al.*, 2004), and the NF-Y transcriptional regulator (Nelson *et al.*, 2007) in maize. In addition, the maintenance of energy homeostasis in response to drought under constant global climate change has been demonstrated to be effective in sustaining broad stress tolerance in plants (De Block *et al.*, 2005). In addition, epigenetic regulation in abiotic stress response is a promising new route. The precise control of chromatin modification might play a central role in regulating gene expression in response to environmental cues because the switch between permissive and repressive chromatin allows alteration of gene expression in response to environmental changes (Tsuiji *et al.*, 2006; Sokol *et al.*, 2007). As sessile organisms, plants cannot choose their living environments. In this respect, some plants survive under very stressful conditions and have developed rapid responses for their adaptation and survival. Therefore, a great potential exists to exploit the abilities of extremophilic plants for crop improvement.

A. Enhancing Glycine Betaine Synthesis

The acclimation of a plant to a constantly changing environment involves the accumulation of compatible solutes in the cytoplasm to maintain turgor pressure and stabilize the structures and functions of certain macromolecules during water stress (Papageorgiou and Murata, 1995). Representative compatible solutes include certain polyols, sugars, amino acids, betaines and related compounds.

Glycine betaine, an amphoteric quaternary amine, plays an important role in enhancing plants tolerance to a variety of abiotic stresses (Sakamoto and Murata, 2002). Although different maize varieties differ in their capacity to accumulate glycine betaine (Brunk *et al.*, 1989), a positive correlation has been found between the level of endogenous glycine betaine and the degree of salt tolerance. Therefore, enhancing glycine betaine synthesis might potentially improve stress tolerance in maize.

Metabolic engineering has allowed the introduction into maize of biosynthetic pathways of glycine betaine from micro-organisms. Indeed, maize accumulated higher levels of glycine betaine when transformed with the *beta* gene from *Escherichia coli* that encodes choline dehydrogenase, a key enzyme in the biosynthesis of glycine betaine (Quan *et al.*, 2004). The transgenic maize plants were more tolerant to drought stress at germination and young seedling stage. Most importantly, their grain yield had improved significantly when compared to that of wild-type plants after drought treatment. However, drought tolerance was only measured at the seedling stage and under greenhouse conditions, while the most critical stage for drought is the reproductive period and grain filling. In this respect, current research efforts should be focused on the elucidation of the mechanisms by which glycine betaine protects the cellular machinery *in vivo* and how, as a result, it enhances the tolerance of whole plants to environmental stress in field conditions. In addition,

the improvement of drought tolerance by increased osmoprotection (for a review, see Quan *et al.*, 2004; Vasil, 2007) could provide suitable strategies to protect reproductive organs from short-term environmental extremes.

B. Engineering Transcription Factors and Signaling Components

Transcription factors have been used to elicit multiple biochemical and developmental pathways that regulate drought tolerance, thereby improving performance during drought under laboratory and greenhouse conditions (Umezawa *et al.*, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Recently, drought tolerance in transgenic maize plants under field conditions has been enhanced through overexpression of NF-YB (Nelson *et al.*, 2007), which is part of a ubiquitous heterotrimeric transcription factor composed of three distinct subunits NF-YA (HAP2), NF-YB (HAP3), and NF-YC (HAP5) (Mantovani, 1999). The NF-Y complex is also known as the HAP or the CAAT complex that acts in concert with other regulatory factors to modulate gene expression in a highly controlled manner. The *Arabidopsis* NF-YA5 and NF-YB1 transcripts are strongly induced by drought stress in an ABA-dependent manner and regulated both transcriptionally and post-transcriptionally by miRNA (Nelson *et al.*, 2007; Li *et al.*, 2008). The overexpression of a maize CAAT box transcription factor (*ZmNFYB2*) imparted significant tolerance to drought, resulting in increased yield. Most importantly, in field trials, the transgenic lines gave higher grain yields than control lines under drought conditions.

Engineering upstream signaling components of drought stress pathways might be another promising way to obtain drought stress tolerance. Indeed, constitutive expression of a tobacco MAPKKK, *NPK1*, in maize enhanced drought tolerance, as demonstrated by higher photosynthesis rates and higher kernel weight in the transgenic plants than those of the non-transgenic controls under greenhouse dehydration conditions (Shou *et al.*, 2004).

C. Higher Energy Use Efficiency

An emerging metabolic engineering strategy to broaden stress tolerance in plants by maintaining energy homeostasis (bioenergetic flexibility) under stress conditions has been proposed (De Block *et al.*, 2005). 'Bioenergetic flexibility' that plays an important role in plant acclimation to stress is an imperative target (Dobrota, 2006). In general, stresses cause high energy consumption and enhance the respiration with a concomitant production of ROS (Rizhsky *et al.*, 2002; Tiwari *et al.*, 2002). When the stresses are extreme or persistent, an energy threshold is reached at which the damage caused by the stress can no longer be repaired; as a consequence, cells, tissues or, ultimately, the whole plant die, partially due to a breakdown in the NAD⁺ pool caused by the enhanced activity of the poly(ADP-ribose) polymerase (PARP), that uses NAD⁺ as a substrate to synthesize polymers of the ADP-ribose and

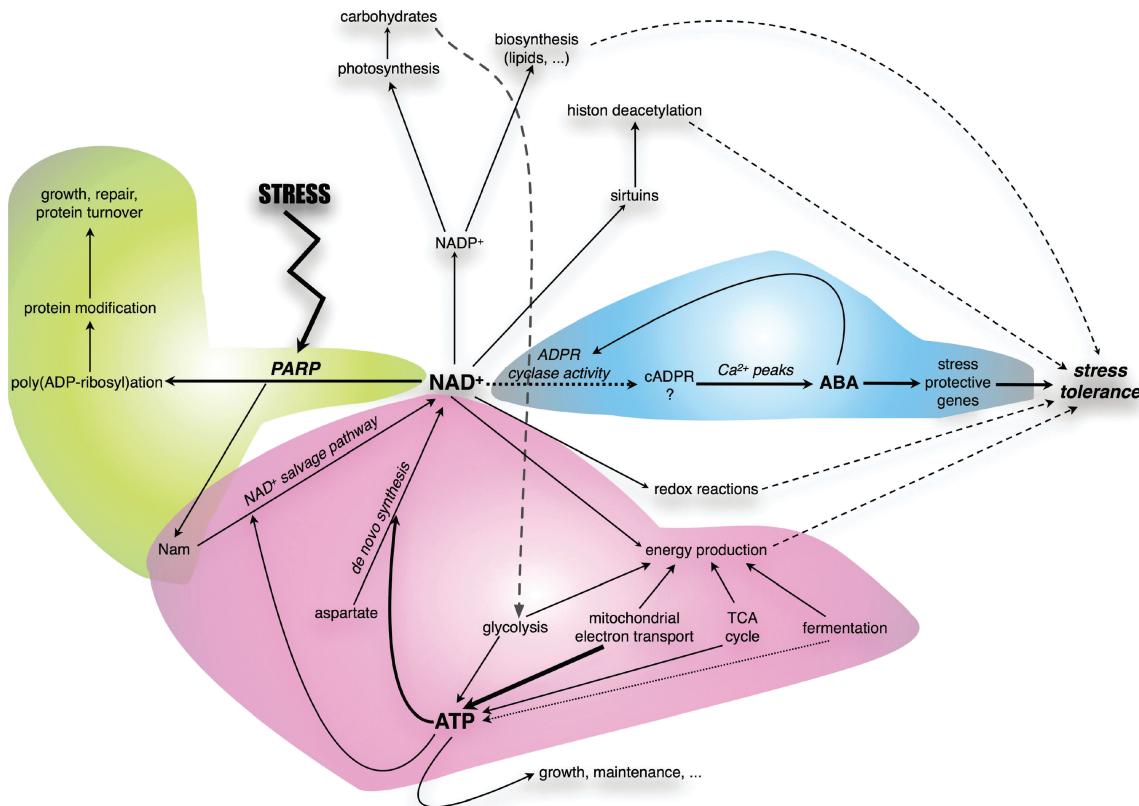


FIG. 6. Central role of NAD⁺ in plant cell metabolism and relation to stress tolerance. When stress is imposed on a cell, PARP is induced. The activated PARP enzymes consume large amounts of NAD⁺ for the synthesis of poly(ADP-ribose) polymers (green). In this process, nicotinamide (Nam) is formed as a breakdown product. As a consequence of the NAD⁺ consumption, the signaling pathway for the ABA-dependent induction of stress-protective genes is disturbed (blue). Moreover, the resynthesis of NAD⁺ requires high amounts of ATP, resulting in an energy depletion of the cell (pink). The *de novo* synthesis of one molecule of NAD⁺ from aspartate requires five molecules of ATP, while the recycling of Nam in the salvage pathway requires only three. Breakdown of NAD⁺ has many other consequences for the cell, such as disturbance of redox reactions and lower NADP⁺ concentrations affecting photosynthesis (light and carbon reactions) and biosynthesis (modified from Vanderauwera *et al.*, 2007).

nicotinamide (Figure 6). When the PARP activity was reduced by RNAi gene silencing in *Arabidopsis* and *Brassica*, cell death was inhibited and the plant became tolerant to a broad range of abiotic stresses (De Block *et al.*, 2005). Such plants with low poly(ADP-ribosyl)ation activity maintained their energy homeostasis under stress conditions by reducing the NAD⁺ breakdown and, consequently, the energy consumption, preventing plants from excessive mitochondrion respiration and the formation of ROS. De Block *et al.* (2005) attributed the observed broad-spectrum stress-resistant phenotypes of transgenic *Arabidopsis* solely to a sustained energy homeostasis due to reduced NAD⁺ consumption. A genome-wide transcript analysis of stressed PARP2-deficient transgenic *Arabidopsis* (hpAt-PARP2) plants (Vanderauwera *et al.*, 2007) revealed that the induction of specific ABA signaling pathways steered increased levels of the cyclic nucleotide ADP-ribose (cADPR). Therefore, engineering crop plants for high NAD⁺ regeneration, for example, by an efficient upregulation of the NAD⁺ salvage pathway or by a reduced NAD⁺ consumption under stress conditions, is a valuable approach to enhance overall stress tolerance in crops because the strategy has been very effective in the model plant

Arabidopsis and in crops such as canola, corn and cotton under field conditions (M. De Block, unpublished results).

D. Epigenetics in Abiotic Stress Regulation

Epigenetics are mitotically and meiotically heritable changes in gene expression through biochemical processes that do not entail a change in DNA sequence, but instead modify the physical accessibility to the genome (Zhang, 2008). During DNA replication, transcription and DNA repair in eukaryotes, the cellular machineries performing these tasks need to gain access to the DNA that is packaged into chromatin in the nucleus. The accessibility of the genome can be changed by covalent, post-translational modifications of the N-terminal tails of the histones, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation (Nelissen *et al.*, 2007). These histone modifications reversibly alter the accessibility to the genome by the transition from heterochromatin to euchromatin and vice versa. The combinatorial nature of the histone modifications reveals a “histone code” that is thought to incorporate intra- and extracellular signals to regulate how the information from the DNA code becomes accessible.

Abiotic stresses have been shown to cause significant epigenetic modifications inducing a number of protein-encoding genes that enhance stress tolerance in plants (Madlung and Comai, 2004; Chen and Tian, 2007; Deng *et al.*, 2007). The stress tolerance conferred to plants through chromatin modifications upon abiotic stress represents a novel, compelling approach toward engineering improved plant production in suboptimal growth conditions. Therefore, epigenetic control of developmental transitions and morphogenetic processes needs to be further explored (Reyes *et al.*, 2002).

A number of histone acetylases and deacetylases (HDAC) are present in the plant genome, among which the plant-specific HD2 subfamily. The *Arabidopsis* HD2 protein, AtHD2C, is involved in modulating ABA and abiotic stress responses in plants (Sridha and Wu, 2006). Upon high salinity, exogenous ABA and cold stress, both *Arabidopsis* and tobacco cells show dynamic and reversible histone modifications, as manifested by rapid transient upregulation of histone H3 Ser10 phosphorylation, H3 phosphoacetylation as well as histone H4 acetylation (Sokol *et al.*, 2007). Furthermore, Tsuji *et al.* (2006) have demonstrated that flooding stress in rice induces reversible histone modifications from a dimethylated to a trimethylated state of the Lys4 residues of the histone H3 proteins (H3-K4s) at both the 5'- and 3'-coding regions of the alcohol dehydrogenase 1 (*ADH1*) and pyruvate decarboxylase (*PDC1*) genes.

Reversible histone modifications, such as acetylation/deacetylation, are of critical importance to make DNA available for transcription or to repress transcription. A well studied case is the vernalization-dependent deacetylation induced by the *VERNALIZATION INSENSITIVE 3* (*VIN3*) gene encoding a PHD finger protein and, hence, inactivation of the *FLOWERING LOCUS C* (*FLC*) gene (Sung and Amasino, 2004). Indeed, the acetylation of histone H4 and H2B in response to amino acid deprivation has been shown to involve the activating transcription factor 2 (ATF2), suggesting that ATF2 promotes the modification of the chromatin structure to enhance the transcription of a number of amino acid-regulated genes (Bruhat *et al.*, 2007). Furthermore, abiotic stress leads to genetically programmed responses resulting in stress tolerance, including transposon activation, transposition, and structural genome changes (Madlung and Comai, 2004).

Histone acetyl transferase (HAT) complexes are postulated to act as an interface for integrating stimuli (light) to alter transcriptional activity in plants (Offermann *et al.*, 2006). The maize C4-specific gene encoding phosphoenolpyruvate carboxylase (C4-PEPC), which has a role in primary CO₂ fixation during photosynthesis and is regulated by both light and nitrogen availability, is highly expressed in the mesophyll cells of green, light-grown leaf tissue compared to etiolated tissue. The C4-PEPC promoter becomes highly acetylated at the N-terminal tails of histones H3 and H4 of the 5' region of the gene during light-induced activation of transcription. Thus, illumination can alter the acetylation status of a promoter in the absence of a change in transcription. Reversible histone modifi-

cation and gene transcription are cooperatively regulated in response to changing light environments (Guo *et al.*, 2008), hence, chromatin remodeling is a key process in acclimatization and adaptation.

Chromatin assembly requires a negatively charged histone chaperone to shield the basic histones from the acidic DNA (reviewed in Tyler, 2002). Recently, Castiglioni *et al.* (2008) have shown that constitutive expression of two members of a family of bacterial RNA chaperones *E. coli* CspA and *B. subtilis* CspB, confer abiotic stress tolerance in transgenic *Arabidopsis*, rice, and maize, suggesting that chaperones could play a significant role in making DNA accessible for transcription under environmental conditions. One component of the chromatin assembly machinery is an ATP-dependent chromatin remodeling multi-subunit complex (Fyodorov and Kadonaga, 2001) that alters the chromatin structure by locally disrupting or altering the topology of the DNA. In a detailed molecular genetic study, Mlynárová *et al.* (2007) demonstrated the role of the *Arabidopsis* *AtCHRI2*, a plant SNF2/Brahma-type chromatin remodeling gene, in growth control upon the perception of environmental stimuli helping plants to cope with adverse conditions.

The HAT complex Elongator has recently been identified in plants (Nelissen *et al.*, 2005; Falcone *et al.*, 2007) and mutations in each of the six structural components revealed that Elongator has a positive effect on the cell proliferation rate during organ growth in *Arabidopsis* (Nelissen *et al.*, 2005). *elo* mutants have a narrow leaf phenotype indicating that leaf size and shape are under chromatin control. Stress-related pathways were affected in an *elo2* allele, which was an EMS mutant with ABA-oversensitive and drought-tolerant phenotype, showing that the Elongator complex is required for growth under normal and adverse conditions. Therefore, Elongator is postulated to act as an adaptive mechanism to regulate growth in response to abiotic stimuli.

E. Extremophilic Plants

The remarkable ability of plants to adapt to many different adverse environments is a fascinating process. Sub-Saharan Africa is endowed with extreme environments, including very high mountains, hot springs, saline lakes and seas and deserts, presenting a wide variety of features and characteristics. The life processes occurring within these environments are equally diverse, not only depending on stress factors (e.g., temperature, pressure, pH and chemicals), but also on the type of life forms, ranging from microbes to higher species. In coping with the extreme environments, extremophilic plants have demonstrated their ability to adapt and metabolize under high environmental stress.

Research on the physiology and metabolism of so-called extremophiles not only fosters better understanding of the evolutionary processes that have created the diversity of life as it exists on earth, but also has economic implications for the agri-

cultural biotechnology and the development of novel products. The capacity to sequence genomes and the availability of novel molecular tools have now catapulted biological research into genomics and post-genomics eras, creating an opportunity to apply genomics techniques to extremophile models (Amtmann *et al.*, 2005). For instance, *Thellungiella salsuginea*, a close relative of *Arabidopsis* with a genome size approximately twice that of *Arabidopsis*, tolerates extreme cold, drought, and salinity (Taji *et al.*, 2004) and maintains a constant water content when treated with 600 mM NaCl, allowing *Thellungiella* shoot meristems to be protected from desiccation and supporting fast recovery from extreme stress. Therefore, by molecular cloning some of these abilities, such as tolerance to extreme temperature, drought, salt, heavy metal and enhanced UV stability, might be transferred from extremophilic plants to crop plants including tropical maize in the near future.

IV. TRANSGENIC RESEARCH IN TROPICAL MAIZE

A. *Agrobacterium* Transformation of Tropical Maize: State of the Art

The biotechnological approach to engineer abiotic stress tolerance implies the availability of an efficient plant transformation method. The production of genetically transformed plants depends both on the ability to integrate foreign genes into target cells and the efficiency with which plants are regenerated. The use of the *Agrobacterium*-mediated gene delivery system has several superior advantages over the biolistic particle bombardment: it results in a greater proportion of stable integration with fewer rearrangements of long molecules of DNA with defined ends (Cheng *et al.*, 2004), leads to a low-copy number transgenic events (Ishida *et al.*, 1996), offers the possibility of transferring large DNA segments into recipient cells, is highly efficient (Zhao *et al.*, 1998) and its recombinant DNA is inheritable. Moreover, the *Agrobacterium*-mediated gene delivery system represents a simple and low-cost technology. Since the first temperate maize transformation by *Agrobacterium tumefaciens* carrying a 'super binary vector' (Ischida *et al.*, 1996), various factors that influence *Agrobacterium* T-DNA delivery and plant regeneration in tissue culture have been investigated and modified in a protocol for efficiently introducing genes into maize (Frame *et al.*, 2002, 2006; Shrawat and Lörz., 2006). These laboratory protocols have been developed for genotypes adapted to temperate zones and the focus on the transformation potential of maize germplasm adapted to tropical regions, where productivity is often low due to abiotic stresses has received little attention (Rascón-Cruz *et al.*, 2004). Maize transformation with the preferred *Agrobacterium*-based system has been reported in a limited number of genotypes, such as A188 and its hybrid Hi-II (A188xB73), adapted to the temperate zones (Frame *et al.*, 2002, 2006; Shrawat and Lörz., 2006), but was not readily applicable to germplasms adapted to tropical regions. It would be a time-consuming and costly procedure to introduce transgenes from temperate genotypes into local tropical maize varieties

by backcrossing (Lupotto *et al.*, 2004). In addition, the recovery of progeny with both the transgenic trait and other suitable agronomic traits is often difficult due to incompatible heterotic groups and poor combination ability (O'Kennedy *et al.*, 2001).

Tropical inbred maize lines have been reported to be recalcitrant to transformation, mainly as a result of their inherent limitations associated with resistance to *Agrobacterium* infection and recalcitrance to *in vitro* regeneration (Shrawat and Lörz, 2006). To date, only scanty information on the transformation of tropical maize through microprojectile/DNA bombardment and *Agrobacterium*-mediated system has been published. Such information has mainly been reported by the CMMYT (Bohorova *et al.*, 1999) and universities in Mexico (Valdez-Ortiz *et al.*, 2007). Genetically transformed tropical maize plants have been obtained by biolistic transformation with immature embryos and shoot tips (O'Connor-Sánchez *et al.*, 2002) as explants, but particle bombardment is not favored because multiple transgenic DNA fragments are integrated into the genome. Furthermore, considerable variations are seen in stability, integration and expression of the introduced transgene (Kohli *et al.*, 1999). The system is also known to be traumatic to the cells, messy and expensive due to the need for special equipments, making further analysis of the introduced trait complex to evaluate.

Bohorova *et al.* (1995) tested the regeneration ability of tropical plants and obtained plantlets from embryogenic type-II calli. Similar results have been reported by Prioli and Silva (1989) and Carvalho *et al.* (1997) and serve as a basis for the development of the genetic engineering technology for tropical maize inbred genotypes. The *Agrobacterium* system has been successfully used on embryos wounded by particle bombardment and sonication, and transgenic tropical maize lines have been obtained (Valdez-Ortiz *et al.*, 2007). Furthermore, a key point in the various *Agrobacterium*-mediated gene delivery protocols is the choice of an explant that consists of actively dividing, embryonic cells, such as scutella-induced calli.

To enhance the capacity for public-sector maize transformation and effectively broaden the *Agrobacterium*-mediated transformation to tropical maize germplasm, scientists at the Biosafety Level II Plant Transformation Facility at Kenyatta University (Kenya), embarked on an ambitious program to improve the regeneration (Oduor *et al.*, 2006; Binott *et al.*, 2008; Ombori *et al.*, 2008) and transformation (Anami *et al.*, 2008) of diverse tropical maize lines by using *Agrobacterium tumefaciens*. A number of inbred and hybrid tropical-adapted genotypes have been identified capable of producing type-II calli from immature embryos that can easily regenerate into plants. Regeneration of tropical maize has been improved by optimizing the age of the immature embryo explant, adjusting the sucrose and 2,4-D auxin concentrations upon callus initiation from immature embryos and removing the hormones upon shoot induction (Oduor *et al.*, 2006; Binott *et al.*, 2008). For some of the inbred genotypes, an *Agrobacterium* transformation step was integrated into the regeneration procedure; the subsequent selection on herbicide-containing medium resulted in putative

primary transformants (Anami *et al.*, 2008). Further research at the Kenyatta Plant Transformation Facility is aimed at testing the transgenic plants for drought stress tolerance both in the greenhouse and in the field.

Transgenic plants obtained by *Agrobacterium* infection contain non-T-DNA regions from the vector backbone or even bacterial chromosomal DNA at low frequency (Kononov *et al.*, 1997), which needs to be avoided in transgenic product development.

B. Biosafety Framework for Genetically Modified Organisms (GMOs) in Sub-Saharan Africa

The first commercially successful genetically engineered agricultural crops were launched more than a decade ago (Castle *et al.*, 2006). These first products were largely based on simple monogenic traits, such as herbicide tolerance and insect resistance (Century *et al.*, 2008). The biotechnology industry is expected to deliver a second generation of transgenic products for more challenging traits related to yield and yield stability, which are under complex polygenic control (Gutterson and Zhang, 2004; Salmeron and Herrera-Estrella, 2006).

The development of techniques for generating GMOs has provoked mixed reactions in sub-Saharan Africa related to global biosafety issues. The extreme pro-genetic engineering groups tend to catalogue the potential benefits of the technology and often dismiss any concerns about potential risks. They tend to portray biotechnology as the panacea to combat food insecurity in Africa. In contrast, the anti-biotechnology activists, who see no evident benefits and associate the technology with nothing but total danger and risks, would like to ban the development, commercialization and application of the technology. Nevertheless, the pertinent issue is how to regulate the GMO technology, to optimize benefits while preventing risks. In many countries, GMOs are introduced according to strict regulations to ensure that only products that have been safety tested in relation to human health and the environment are marketed. In this respect, transboundary movement is regulated internationally by the Cartagena Protocol on Biosafety (CPB) as part of the Convention on Biological Diversity (CBD). It seeks to save biodiversity of natural ecosystems from risks posed by the deliberate release of GMOs into the environment, sustainable use of genetic resources, and fair and equitable sharing of the benefit arising from the use of these resources. However, a number of countries in sub-Saharan Africa have still to implement the CPB-CBD, despite being signatories and still lack the coherent regulatory instruments and institutions for risk management in relation to genetic engineering as well as the capacity to design and implement science policies. When rules are formulated and adopted by governments, the institutional arrangements for the enforcement of the regulatory procedures are weak. As such, there is no consensus on how best to respond to global developments in genetic engineering and, particularly, whether to allow the development and import of GMOs.

In this regard, the African Union (AU) is addressing the issues of safety regulation in biotechnology at the continental level. The African Heads of State and governments in the 74th Ordinary Session of the AU Council of Ministers held in 2002 in Lusaka (Zambia) endorsed the African Model Law on Biosafety (www.africa-union.org). This law proceeds on the assumption that the measures of the CPB are minimal: hence, based on their sovereign rights, African States could adopt more rigorous standards on the subject. The African Model Law on Safety in Biotechnology strives to device additional measures, such as labelling among others, that are not dealt with fully in the protocol but are required to ensure its full implementation. African governments have recognized the importance of regional cooperation to address possibilities and the range of issues associated with biotechnology and genetic modification. These regional economic communities (RECs) include the Southern Africa Development Community (SADC), Common Market for Eastern and Southern Africa (COMESA), Economic Community of West African States (ECOWAS), East African Community (ECA) and Economic Community of Central African States (ECCAS). Therefore, besides the African Model Law, the African Strategy on Biosafety has the objective of creating and strengthening regional centers of excellence in both modern biotechnology and biosafety, at least one in each of the African RECs. These centers will play an important role in building capacities for risk assessment, risk management, as well as GMO testing and bio-safety advice (communicated by Sarah Olelombo, Senior Policy officer, AU at the 1st All Africa Congress on Biotechnology, Nairobi, Kenya, September 2008).

In Africa, only 41 countries have signed and ratified the CBD, meaning that they have agreed to develop functional National Biosafety Frameworks (NBFs), a legal, technical, administrative and information management system set in place to address safety in the field of modern biotechnology to ensure that the development, handling, transport, use, transfer and release of any living modified organism are undertaken in a manner that prevents or reduces the risks on biological diversity and human health. Six countries in sub-Saharan Africa have fully functional NBFs (Figure 7), among which South Africa is the only country that has commercialized GMOs. Egypt and Burkina Faso have recently approved trade in biotech crops and their legislation will allow the commercialization of GM crops. Other countries in sub-Saharan Africa (Zimbabwe, Malawi, Uganda, Tanzania, Ghana, Nigeria, Tunisia, Morocco, and Mauritania) have an interim National Biosafety legislation that allows field trials on GM products (communicated by Francis Nang'ayo, Regulatory Affairs Manager, the African Agricultural Technology Foundation (AATF) at the 1st All Africa Congress on Biotechnology, Nairobi, Kenya, September 2008). On December 9, 2008, after rigorous and extensive stakeholder consultations since 2002, the Kenyan parliament passed overwhelmingly a national biosafety bill (www.merid.org/fs-agbiotech/more.php?id=7199) that aims at facilitating responsible research and trade in GM products through a transparent science-based and predictable

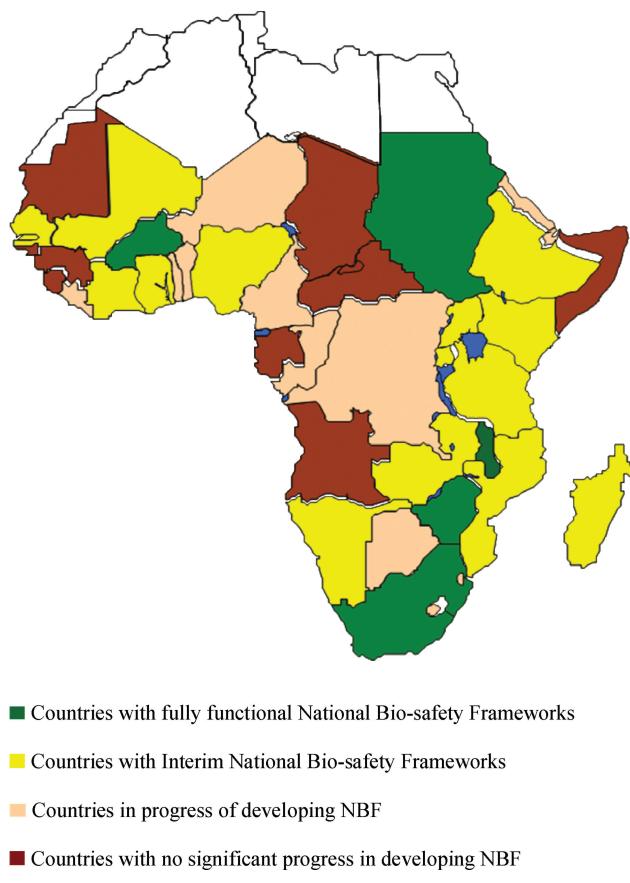


FIG. 7. Status of National Biosafety Frameworks in sub-Saharan Africa.

process. Given Kenya's strategic importance in Africa, this significant bill includes provisions for the establishment of a National Biosafety Authority that will be in charge of regulatory approvals and decision making to move on-going confined field trials to the next level of commercialization.

V. PERSPECTIVES

Advances in genetic engineering and biotechnology approaches focusing on single genes has been very successful in promoting revolutionary changes in agriculture, industry, nutrition, and even medicine. The relatively crude first-generation 'input trait' GM crops, perceived by many to offer benefits only to seed companies and farmers, has prompted several commentators and pressure groups to deem the technology a failure. Progress toward second-generation 'output trait' products with nutritional, environmental or other benefits that consumers can appreciate directly has been slow and will only accelerate when technologies have been adopted for the coordinated manipulation and introduction of multiple genes or traits into crop plants. Gene stacking or pyramiding potentially provides durable multitoxin resistance to particular pests or multiple resistances to different types of pathogens, perhaps in crops that are also herbicide tolerant. Similarly, the potential to introduce new metabolic

pathways into plants is enormous, and could lead to the development of plants able to grow in inhospitable environments, provide healthier foodstuffs or improved raw materials (Kebeish *et al.*, 2007). Therefore, interest in dissecting and analyzing complex metabolic pathways and the need to exploit the full potential of multigene traits for plant biotechnology are growing, mandating the development of new methods and tools for delivery and stacking of multiple genes in plant cells, including the design of novel transformation vectors (Dafny-Yelin and Tzfira, 2007).

In addition, the search for appropriate promoters that are specifically expressed in reproductive organs is important. When fused to interesting genes, the specific expression during gamete formation and/or fertilization might be sufficient to cope with drought stress because the reproductive phase is the most vulnerable. These promoters might be identified from transcriptome data or promoterome studies, and those with a specific activity will replace the constitutive ones as they are too demanding for the plant's energy balance. The generation of transgenic crop with multitraits (gene stacking) requires the use of multiple transgenes under the control of different promoters, necessitating the use of promoters from different crop plants, because of the advantage in reducing homology-based transcriptional gene silencing that might impair expression of one or more transgenes. Thus, for successful gene stacking in the same tissue of

a crop plant, the use of homologous or heterologous promoters with the same tissue specificity is required, but with little homology to circumvent transcriptional gene silencing (Furtado *et al.*, 2008).

Recently, miRNAs and siRNAs have been identified as components of stress response, revealing another level of gene regulation in plants. Understanding the post-transcriptional gene regulation by small RNAs under abiotic stress is crucial for improving stress tolerance in crop plants and will accelerate the use of the small RNAs within the regulatory networks that govern diverse physiological processes. Appropriate manipulation of miRNA target genes should help overcome post-transcriptional gene silencing and, thus, might lead to more efficient expression of engineered traits in transgenic plants (Sunkar *et al.*, 2007). Many genes have been shown to confer marginal improvements in stress tolerance when overexpressed in transgenic plants (Bartels and Sunkar, 2005). A combination of previously reported as well as novel approaches will be needed to increase plant abiotic stress resistance to levels high enough for field application. Such novel technologies include the manipulation of artificial miRNAs (amiRNAs) to improve plant stress tolerance (Alvarez *et al.*, 2006; Niu *et al.*, 2006; Schwab *et al.*, 2006; Khraiwesh *et al.*, 2008; Warthmann *et al.*, 2008). AmiRNAs represent a novel, feasible and highly specific approach for effective post-transcriptional gene silencing in plants that can effectively modulate agronomically important traits in varieties used in modern breeding programs. In addition, the approach is suited for candidate gene validation, comparative functional genomics between different varieties, and for improvement of agronomic performance.

Finally, both transcriptomics and metabolomics are important emerging technologies to understand the physiology of drought stress in plants in a holistic way, especially when performed in time course experiments and will lead to innovative approaches for enhancing stress tolerance in crops in the near future (Zhuang *et al.*, 2007; Fernandes *et al.*, 2008).

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