

Genetic Engineering for Modern Agriculture: Challenges and Perspectives

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Abstract

Abiotic stress conditions such as drought, heat, or salinity cause extensive losses to agricultural production worldwide. Progress in generating transgenic crops with enhanced tolerance to abiotic stresses has nevertheless been slow. The complex field environment with its heterogenic conditions, abiotic stress combinations, and global climatic changes are but a few of the challenges facing modern agriculture. A combination of approaches will likely be needed to significantly improve the abiotic stress tolerance of crops in the field. These will include mechanistic understanding and subsequent utilization of stress response and stress acclimation networks, with careful attention to field growth conditions, extensive testing in the laboratory, greenhouse, and the field; the use of innovative approaches that take into consideration the genetic background and physiology of different crops; the use of enzymes and proteins from other organisms; and the integration of QTL mapping and other genetic and breeding tools.

Contents	
INTRODUCTION	444
CHALLENGES IN MODERN AGRICULTURE: CLIMATIC CHANGES AND ABIOTIC STRESS	444
Climate Change and Global Warming	444
Climate Change Effects on Plant Growth and Development	445
THE FIELD ENVIRONMENT, STRESS COMBINATION, AND PERSPECTIVES FOR STUDYING OF ABIOTIC STRESS	446
Field Conditions and Their Relevance to Laboratory Studies of Abiotic Stress	446
Abiotic Stress Combinations	448
CURRENT ACHIEVEMENTS IN ABIOTIC STRESS RESEARCH AND THEIR RELEVANCE TO AGRICULTURE	451
Regulatory Networks	451
Sensing of Stress	451
Retrograde and Systemic Signaling	452
Epigenetic Control	452
Small RNAs	453
QTL Analysis and Breeding	453
Strategies for Transgene Expression	454
FUTURE DIRECTIONS IN ABIOTIC STRESS RESEARCH ..	454
Novel Sources of Transgenes	454
Overcoming Genetic Programming	455

INTRODUCTION

Current and predicted climatic conditions, such as prolonged drought and heat episodes, pose a serious challenge for agricultural production worldwide, affecting plant growth and yield, and causing annual losses estimated at billions

of dollars (17, 82). Transgenic crops provide a promising avenue to reduce yield losses, improve growth, and provide a secure food supply for a growing world population (67, 68). The acclimation of plants to abiotic stress conditions is a complex and coordinated response involving hundreds of genes. These responses are also affected by interactions between different environmental factors and the developmental stage of the plant and could result in shortened life cycle, reduced or aborted seed production, or accelerated senescence. Here we review some of the critical challenges facing modern agriculture, discuss different considerations for the development of crops with enhanced tolerance to field conditions, and review recent achievements in the study of abiotic stress.

CHALLENGES IN MODERN AGRICULTURE: CLIMATIC CHANGES AND ABIOTIC STRESS

Climate Change and Global Warming

Climate change and global warming are generating rapid changes in temperature that are not matched by any global temperature increase of the past 50 million years (55, 60). Atmospheric CO₂ concentrations increased significantly in the past two centuries, rising from about 270 μmol.mol⁻¹ in 1750 to current concentrations larger than 385 μmol.mol⁻¹ (55, 65). This increase in atmospheric CO₂ has been accompanied by a coincident increase in the even more potent forcing gases methane, ozone, and nitrous oxide such that combined ambient greenhouse gas concentrations are now expected to exceed concentrations of 550 μmol.mol⁻¹ by 2050 (18, 101). The increase in greenhouse gases contributes to the greenhouse effect, leading to global warming, and average annual mean warming increases of 3°–5°C in the next 50–100 years have been projected (55). Although models differ considerably in their projections of local climate changes, they tend to agree in their predictions of increased frequencies of heatwaves, tropical cyclones, floods, and prolonged drought episodes (12). Agricultural

regions of our planet are likely to be differentially affected by climate change. Average surface temperatures in the Northern Hemisphere, for example, have been estimated to rise between 2–3°C by 2050 and by as much as 6.5°C by the end of the century (139). Because of the increased temperatures, projections for the western United States include earlier snowmelt, leading to reduced ice and decreased water storage in the spring. Climate models tend to simplify observed crop responses to climate change variables at both plot and field levels, reducing the levels of confidence in regional and global projections (134). Even though climate models vary in their predictions on the intensity of the changes in temperature, precipitation, and other variables affecting global climate, there is a general consensus supporting the notion that changes in atmospheric CO₂ concentrations, increase in ambient temperatures, and regional changes in annual precipitations will significantly influence future agricultural production.

Climate Change Effects on Plant Growth and Development

The increase in atmospheric CO₂ concentrations will stimulate photosynthesis and possibly lead to increased plant productivity and yields (55, 97, 134). Under optimal growth conditions, rising CO₂ concentrations will increase net photosynthetic carbon assimilation in C3 plants with a concomitant increase in yield because Rubisco is not CO₂ saturated at current atmospheric CO₂ concentrations and because CO₂ inhibits the oxygenation reactions and photorespiration (69). On the other hand, in C4 plants the high concentration of CO₂ inside the bundle sheath would prevent a significant increase in photosynthetic activity. Nevertheless, at elevated CO₂ concentrations the water status of C4 plants under drought conditions was improved, resulting in greater photosynthesis and biomass accumulation (66). Recent evidence from FACE (free-air concentration enrichment) experiments (3) have provided clear evidence that carbon gains are greater in C3

plants grown in high CO₂ concentrations. In crop FACE experiments, different CO₂ conditions are imposed on crops growing in large fields under well-managed farm conditions and as close as possible to field growing conditions. FACE experiments have established that C4 photosynthesis is not directly stimulated by higher CO₂ concentrations (66).

Both greenhouse and FACE experiments aimed at assessing the effects of elevated atmospheric CO₂ on evapotranspiration (ET) demonstrated a decrease in stomatal conductance (g_s) in potato, rice, wheat, and soybean, with a consistent decrease in ET ranging from 5% to 20%, depending on species and location (66). The CO₂-induced reduction in ET would improve water use efficiency of most crops, contributing to a better tolerance to water deficit. However, a decrease in ET would increase leaf temperatures, thereby possibly reducing photosynthesis (120). Climate change factors, such as drought and increased temperatures, projected for the near future may often limit and even decrease any yield increase brought about by high atmospheric CO₂ concentrations. Brief periods of high temperature of a few days above those permissive for the formation of reproductive organs and the development of sinks such as seeds and fruits can have serious yield detriments. For example, a short episode of high temperature during anthesis can greatly reduce grain production in cereals (135, 143). Because carbon supply increases in plants growing at elevated CO₂, it could be possible to utilize the increased carbon acquisition to sustain an increased sink development (increase in fruit or seed). Nonetheless, a recent analysis of the protein content in food crops showed that at elevated CO₂ concentrations there is a 10%–15% reduction in grain protein content (129), due to the nitrogen acquisition gap at elevated CO₂ (4; excluding legumes). The possibility of partitioning a greater portion of the photosynthate into carbon-rich metabolites associated with stress tolerance has been discussed (4). Thus, carbon-rich osmolytes, such as pinnitol, mannitol, trehalose, etc., could contribute to the stabilization of protein structures

Free-air concentration enrichment (FACE): experimentally enriching the atmosphere-enveloping portions of a field with controlled amounts of carbon dioxide without using chambers or walls

during water deficit and the scavenging of reactive oxygen species (ROS) during stress (14, 81). Nevertheless, more research would be needed to overcome intracellular transport constraints and attain proper compartmentation (i.e., cytosol versus chloroplast) of the osmolytes (7, 108). Although experiments performed in controlled environmental conditions indicated an effect of elevated CO₂ concentrations on flowering of both short-day and long-day species, FACE experiments suggested that CO₂ concentrations have little or no effect on flowering time in either C₄ or C₃ species (30, 122; see, however, 19).

THE FIELD ENVIRONMENT, STRESS COMBINATION, AND PERSPECTIVES FOR STUDYING OF ABIOTIC STRESS

Field Conditions and Their Relevance to Laboratory Studies of Abiotic Stress

The main abiotic stresses that affect plants in the field are being extensively studied (20, 25, 85). They include drought, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone and anaerobic stresses (e.g., 2, 10, 22, 51, 88, 138). Nevertheless, field conditions are unlike the controlled conditions used in the laboratory. For example, within any given field, large fluctuations in drought, salinity, extremes of temperature, or anaerobic conditions can occur (44, 103). As a consequence, a large degree of heterogeneity between the stress levels that impact different plants in the same field can be present. This heterogeneity, in turn, can affect plant performance and yield. Abiotic stress-induced nonuniform flowering in differing parts of the field can, for example, cause significant reductions in yield (145).

In addition to heterogeneity in stressful conditions in differing parts of a given field, the simultaneous occurrence of different abiotic stresses should also be addressed (82; see below). Abiotic stresses such as drought and salinity, salinity and heat, and distinct combinations of drought and temperature, or high light

intensity are common to many agricultural areas around the globe and could affect plant productivity. It was recently shown that the response of plants to a combination of drought and heat stress is unique and cannot be directly extrapolated from the response of plants to drought or heat stress applied individually (64, 106, 107, 126). Similar findings were also reported for a combination of heat and high light intensity (49), and heat and salinity (61). Because different abiotic stresses are most likely to occur simultaneously under field conditions, a greater attempt must be made to mimic these conditions in laboratory studies (82). It is expected that a large number of distinct stress combinations will occur under field conditions in different areas of the world, and it is likely that the same principles reported with drought and heat (64, 106, 107, 126), heat and high light intensity (49), and heat and salinity (61) will apply to the co-occurrence of these stresses as well (see below).

The timing of the abiotic stress event with respect to the developmental stage of the plant should also be addressed (118). Although plants can differ in their sensitivity to various abiotic stresses during different developmental stages including germination, vegetative growth, reproductive cycle, and senescence, from a strictly agronomic point of view there appears to be only one main consideration: How would this interaction between stress and development affect overall yield? Germinating seedlings, for example, can be rapidly replaced by the farmer if damaged by an abiotic stress event, but a fully mature field ready to flower, or in the midst of its reproductive cycle, cannot. Most crops are highly sensitive to abiotic stresses during flowering, with devastating effects on yield (11, 54, 113). In contrast, most laboratory studies, especially those performed with *Arabidopsis*, do not address the effects of abiotic stress on seed productivity. As indicated above, stress events could also cause poststress premature flowering in a field, significantly reducing productivity and yield (145). The interactions between abiotic stress events and plant productivity are perhaps the most critical for agricultural

productivity and should be taken into consideration when conducting abiotic stress experiments.

A generalized strategy used by plants to cope with water deficit is drought escape (73), where drought-stressed plants complete their life cycle through rapid growth and early flowering, resulting in low plant productivity and inferior seed yields (145). Studies have demonstrated the occurrence of earlier flowering and early maturity of crop plants during the past 50 years and crop phenological events have advanced at rates ranging from 0.8 to 2.5 days per decade depending on location (30, 38, 77). Although increased atmospheric CO₂ concentrations would not likely affect flowering time in either C3 or C4 plants (122), studies have shown that season length has increased with warmer winter and spring temperatures (30). Thus, the increase in atmospheric temperature during this century will extend the growing season of many different crops and influence plant phenology. The extension of the grain-filling period could improve yields, and this improvement would be dependent on nutrient acquisition from the soil, efficient mobilization of nutrients from the sources to the sinks, and slow rates of leaf senescence. Nevertheless, two separate aspects of climate change (increased ambient temperature, and frequent drought episodes) could act antagonistically on yield during an extended growing season due to stress-induced leaf senescence. Thus, efforts toward developing transgenic plants with decreased stress-induced leaf abscission rates could render varieties able to tolerate dryer and extended growth periods. There is some experimental support to this notion: It has been shown that the cytokinin-induced delay of senescence in transgenic plants expressing IPT (isopentenyl transferase; a critical step in cytokinin production) under the control of a maturation- and stress-induced promoter resulted in increased drought tolerance, yield, and growth (104, 105).

All stress events are typically followed by recovery, although only a few studies have focused on the molecular, biochemical, or metabolic

events that accompany recovery from stress (21). Moreover, when laboratory experiments on abiotic stress responses in plants included recovery, they mostly mimicked a single stress event followed by a single recovery period, with very few exceptions (75). In contrast, in the field multiple cycles of stress and recovery typically occur over the growth period of the plant (21, 39, 99), and the acclimation to these stresses and relief cycles could be very different from the acclimation to a single stress event such as that studied in the laboratory. It could, for example, involve epigenetic changes and/or hormonal memory, situations that are unlike those provided by a single stress scenario.

Another key difference between laboratory studies and field conditions is the intensity and duration of the stress. In the field drought conditions are generated gradually during a period of several days and plants do not experience a sudden water stress. Thus, artificial soil mixtures containing a high content of peat moss, vermiculite, or high organic matter should be avoided because they cannot reproduce natural soil drying conditions. Similarly, results obtained in laboratory experiments where plants are grown under hydroponic conditions should be corroborated, at least, with results of greenhouse experiments that attempt to reproduce field conditions. Conditions of water deficiency similar to those occurring in the field can be mimicked in the laboratory by growing plants under limited daily amounts of water rather than by withholding water altogether (e.g., 104). The root:shoot ratio has been shown to be an important determinant in the ability of plants to respond to environmental stress in general and to salt and drought in particular (85), and in the field roots play critical roles in the plant strategy for stress avoidance (78, 85). Laboratory experiments should utilize large pots in order to facilitate root growth and a relative high root:shoot ratio and small pots should be avoided. A similar principle can be applied to all other abiotic stresses studied in the laboratory including heat, cold, and anaerobic stresses. It is mostly unknown at present whether the typical standards used to study abiotic stress in the

IPT: isopentenyl transferase

MS: Murashige and Skoog medium

QTL: quantitative trait loci

TILLING: targeting induced local lesions in genomes

laboratory will elicit plant responses similar to those seen under field conditions.

An additional consideration that is mostly neglected by abiotic stress studies in the laboratory is the relationship between abiotic stress and plant nutrition. For example, improved nitrogen use efficiency, which represents nitrogen uptake efficiency and nitrogen utilization efficiency, can correlate with improved drought tolerance. In addition, the potassium content of the soil can determine the degree of salinity stress affecting the plant (117, 149). As a consequence, researchers who use K^+ -rich growth medium, such as MS (Murashige and Skoog) (86), together with salinity stress are subjecting the plants to a much lower degree of salt toxicity than those using an MS-based growth medium with controlled levels of K^+ and other nutrients (121). A distinction should also be made between soil salinity that is mostly used in laboratory studies (i.e., NaCl) and soil sodicity that occurs in large areas of our globe (98, 102). The high sodium nature of sodicity can come in the form of many salts including chlorides, sulfates, carbonates, and bicarbonates of calcium magnesium, sodium, potassium, and high levels of boron and/or selenium, and have a high pH value. Plants developed by genetic engineering to tolerate salinity (i.e., NaCl) under controlled growth conditions in the laboratory might therefore not be suitable for tolerating sodicity in the field. Because the nutrient content and/or pH of the soil or media can have a dramatic effect on the degree and mode of action of the abiotic stress applied, these parameters should be taken into consideration when studying the abiotic stress response of plants under laboratory conditions (149).

Plant biologists have long acknowledged the importance of breeding for tolerance to abiotic stresses and stress combination (e.g., 50). The genetic characterization of segregating populations of various crop species facilitated the identification of QTLs (quantitative trait loci) associated with root growth (154), early flowering (130), drought (36, 133), etc. Even so, few of these QTLs were successfully used for breeding programs. The most important limitation

of stress-related QTLs is that they are dependent on the environmental conditions to which they were characterized (high $G \times E$ interaction) (28). Other constraints are that the different QTLs associated with stress-related traits (water use efficiency, osmotic potential, etc.) can explain only a low percentage of the variation of the phenotype and that the effects of a favorable allele could not be transferable due to epistatic interactions (93). The challenge is to identify QTLs of major effect that are independent of the particular genetic background and clone the genes in the QTL. Functional analysis of the genes can be significantly aided through the application of reverse genetics approaches such as RNA interference (RNAi) and by screening TILLING libraries (76) in order to characterize the individual gene function(s). Emphasis should be given to forward genetics studies where the identified genes can be expressed in genotypes that have been already selected for their adaptation to stressful environmental conditions.

In light of the complex nature of the field environment as described above, and the interactions between abiotic stress and plant development, a better attempt should be made to reproduce field conditions in the laboratory. In addition, genetically modified plants should be tested under experimental conditions that represent the various combinations of restrictive conditions that occur in the field environment (82).

Abiotic Stress Combinations

Plant acclimation to a particular abiotic stress condition requires a response tailored to the precise environmental condition that the plant encounters (20, 25, 87, 136). Although some overlap is expected, biochemical, physiological, and molecular events triggered by a specific environmental stress condition would mostly differ from those activated by a different set of abiotic parameters (24, 32, 40, 106, 107). In addition to the differences that exist between the acclimation of plants to various abiotic conditions, different stresses, when combined, might

actually require antagonistic responses (82, 131, 151). For example, during heat stress, plants increase their stomatal conductance in order to cool their leaves by transpiration. However, if the heat stress occurred simultaneously with drought, plants would not be able to open their stomata and their leaf temperature would be 2°–5°C higher (106, 107). Salinity or heavy metal stress might pose a similar problem when combined with heat stress because enhanced transpiration could result in enhanced uptake of salt or heavy metals (61, 142). High light intensity could prove problematic to plants subjected to drought or cold stress (47). Under these conditions the dark reactions are inhibited owing to the low temperature or insufficient availability of CO₂, and the high photosynthetic energy absorbed by the plant, owing to the high light intensities, enhances oxygen reduction and thus ROS production (81, 84). On the other hand, some stress combinations might have beneficial effects on plants, when compared to each of the individual stresses applied separately. Drought stress, for example, would cause a reduction in stomatal conductance, thereby enhancing the tolerance of plants to ozone stress (70, 90). Because energy and resources are required for the process of plant acclimation, nutrient deprivation could pose a serious problem to plants attempting to cope with stress (78, 140). Likewise, limited availability of key micronutrients such as iron, copper, zinc, or manganese, required for the function of different detoxifying enzymes such as copper/zinc, iron, and manganese superoxide dismutases, or certain peroxidases (94, 100) could result in an enhanced oxidative stress in plants subjected to diverse abiotic stresses (81). The acclimation of plants to a combination of different abiotic stresses would, therefore, require a well-tailored response customized to each of the individual stress conditions involved, as well as to the need to adjust for some of the antagonistic or synergistic aspects of stress combination (82).

Drought and heat stress represent an excellent example of two distinct abiotic stress conditions that occur in the field simultaneously (50, 82, 83, 95, 115). This combination was found

to have a significantly higher detrimental effect on the growth and productivity of maize, barley, sorghum, and different grasses and plants than if each of the several stresses was applied individually (1, 29, 37, 50, 58, 115, 116, 150). A comparison of all major U.S. weather disasters between 1980 and 2004 indicates that a combination of drought and heat stress caused an excess of \$120 billion in damages. In contrast, over the same period, drought not accompanied by heat stress caused some \$20 billion in damages (82). Physiological characterization of plants subjected to simultaneous drought and heat stress revealed that the stress combination has several unique aspects combining high respiration with low photosynthesis, low stomatal conductance, and high leaf temperature (106, 107). Drought and heat stress combination was found to involve the conversion of malate to pyruvate generating NADPH and CO₂, which is possibly recycled into the Calvin–Benson cycle and thereby alleviates the effects of stress on photosynthesis (64). The source of malate for this reaction is starch breakdown that, coupled with energy production in the mitochondria, might play an important role in plant metabolism during a combined drought and heat stress (64, 107). Transcriptome profiling studies of plants subjected to drought and heat combination support the physiological and metabolic analysis of this stress combination and suggest that it requires a unique acclimation response involving over 770 transcripts, not altered by drought or heat stress (107). Similar changes in metabolite and protein accumulation were also found, with several unique metabolites and at least 53 different proteins accumulating specifically during the stress combination (64, 107). In addition, at least one plant gene, cytosolic ascorbate peroxidase 1 (*Apx1*), was found to be specifically required for the tolerance of *Arabidopsis* plants to drought and heat stress combination (64). A recent study that examined the response of sunflower plants to a combination of heat and high light intensity stress supported the results obtained during the exposure of *Arabidopsis* plants to a drought and heat combination and identified a large number of genes that specifically

responded to the stress combination (49). In addition, the activity of different antioxidative enzymes was found to be particularly effected by a combination of drought and temperature stress (61).

The extent of damage caused to agriculture by stress combination underscores the need to develop crops with enhanced tolerance to a combination of abiotic stresses (82). Drawing upon the limited physiological, molecular, and metabolic studies performed with plants simultaneously subjected to two distinct abiotic stresses, it is not sufficient to study each of the individual stresses separately (49, 61, 64, 106, 107). The particular stress combination should be handled as a new state of abiotic stress in plants that requires a new type of acclimation response (82).

Figure 1 summarizes many of the combinations of environmental conditions that could have a significant effect on agricultural

production (the “stress matrix”). Stress interactions that have a deleterious effect on crop productivity include drought and heat, salinity and heat, ozone and salinity, ozone and heat, nutrient stress and drought, nutrient stress and salinity, UV and heat, UV and drought, and high light intensity combined with heat, drought, or chilling (29, 41, 47, 48, 50, 58, 78, 100, 114, 115, 116, 137, 140, 141, 142). Environmental interactions that do not have a deleterious effect on yield and could actually have a beneficial impact on the effects of each other include drought and ozone, ozone and UV, and high CO₂ combined with drought, ozone, or high light (4, 18, 90, 124, 144). Perhaps the most studied interactions presented in **Figure 1** are those of different abiotic stresses with pests or pathogens (i.e., biotic stress). In some instances, it was reported that a particular abiotic stress condition enhanced the tolerance of plants to pathogen attack (16, 92, 110, 114). However, in most cases prolonged exposure of plants to abiotic stress conditions, such as drought or salinity, resulted in weakening of plant defenses and enhanced susceptibility to pests or pathogens (5, 6, 46, 114, 147). In contrast to the biotic-abiotic axis, most of the abiotic stress combinations presented in **Figure 1** have received little attention. The experience of farmers and breeders should be used as a valuable guide and resource to plant biologists trying to address a specific stress combination that is pertinent to their crop of interest or region. In addition, different plants or crops specifically developed by individual breeding programs might have varying degrees of sensitivity to distinct abiotic stress combinations. Major U.S. crops, including corn and soybean, are especially vulnerable to a combination of drought and heat stress during their reproductive cycle. In contrast, trees and a range of crops from northern hemispheres such as Sweden or Canada are routinely subjected to a combination of cold stress and high light intensity (82). The global climatic changes causing increased CO₂, ozone, and UV stresses together with high average temperatures are also becoming major factors in stress combination research (4, 18). Although

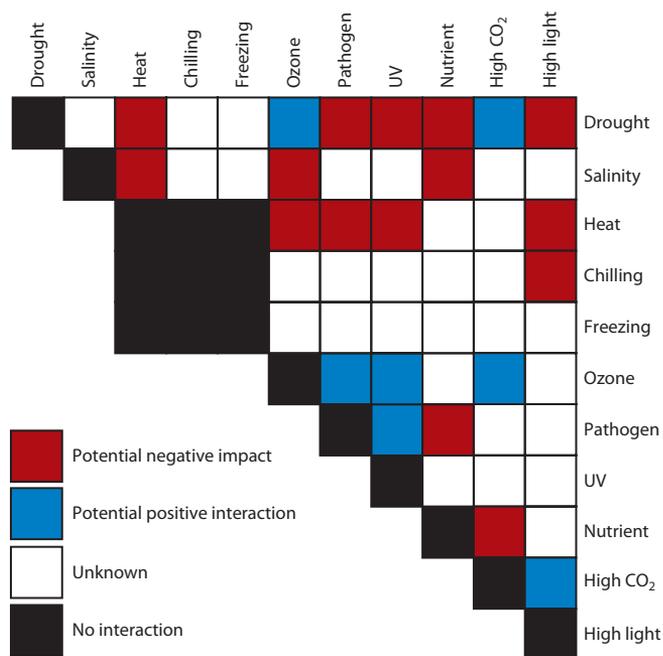


Figure 1

The stress matrix. Different combinations of potential environmental stresses that can affect crops in the field are shown in the form of a matrix. The matrix is color coded to indicate stress combinations that were studied with a range of crops and their overall effect on plant growth and yield. References for the individual studies are given in the text (adapted from Reference 82).

the effects of elevated concentrations of CO₂ are considered beneficial for crop resistance to abiotic stresses, when nutrients are not limited, care should be taken when assessing these effects in diverse areas of our globe and with different crops (4, 18).

CURRENT ACHIEVEMENTS IN ABIOTIC STRESS RESEARCH AND THEIR RELEVANCE TO AGRICULTURE

The central dogma of abiotic stress research in plants is to study how plants sense and acclimate to abiotic stress conditions, and then use this knowledge to develop plants and crops with enhanced tolerance to abiotic stresses. The development of new methodologies has been a major driving force in this research: For example, microarray technology have driven much of the research into transcriptional networks during abiotic stress, whole-genome sequencing and chromatin immunoprecipitation have driven research into epigenetic control of gene expression during stress, and metabolic profiling has driven research into metabolic networks and their role in stress tolerance.

Despite this enormous research endeavor, the roles of very few genes in enhancing abiotic stress tolerance have thus far been demonstrated under field conditions. Moreover, these genes were identified through extensive screening of transgenic lines in the laboratory and in the field using a pipeline approach by various biotech companies (89). Still unclear is whether massive screening of transgenic lines is superior to the central dogma approach in the development of future crops. Perhaps a comprehensive understanding of the plant acclimation process would be needed before efficient molecular tools to enhance crop tolerance to abiotic stress could be designed. Of course, such understanding should take into account many of the field conditions described above. Several promising avenues of research have been described in recent years. These include gene networks and upstream regulators of abiotic stress, the role of retrograde signaling and the balancing of stress

and energy signaling, epigenetic control of gene expression during stress, and metabolomics and systems biology approaches.

Regulatory Networks

Transcriptional regulatory networks and upstream regulators in response to abiotic stress have been classified into regulons. These include the *CBF/DREB* regulon that is mainly involved in cold stress responses, is controlled by ICE1/HOS1 and SIZ1, and involves Zat10 and Zat12; the *AREB/ABF* regulon that is mainly involved in ABA, drought, and salinity responses is controlled by Snf1-related protein kinases and has a cross talk interaction with the *CBF/DREB* regulon via CBF4/DREB1D; the *NAC/ZF-HD* regulon that is ABA-independent and is involved in drought and salinity responses; the *MYC/MYB* regulon that is ABA-dependent and could be activated by different abiotic stresses; and additional networks such as the *HSF* and *WRKY* that have a broad function in many biotic and abiotic stresses (23, 26, 40, 87, 112, 123).

A network of upstream regulatory genes controls the transcriptional regulatory networks and includes different proteins that integrate calcium signaling with protein phosphorylation to decode particularized stress signals and activate acclimation responses (e.g., 57, 72, 91, 132, 123, 152). This network includes histidine kinases (HKs); receptor-like kinases; mitogen-activated protein kinases (MAPK cascades); calcium-dependent protein kinases (CDPKs); and different calcium channels, pumps, and calcium binding proteins such as calmodulin (CaM) and calcineurin B-like proteins (CBLs). Unfortunately, the link between stress perception and the extensive networks of calcium, ROS, and protein phosphorylation signaling is largely unknown and only a few new studies have begun to unravel it (see below).

Sensing of Stress

Sensing of abiotic stresses could be mediated via different routes. The sensor molecule could

Pipeline: large-scale analysis of hundreds of genes expressed in transgenic plants and tested for tolerance to abiotic stress

be physically affected by the stress, for example, as a membrane protein the receptor could be affected by changes in membrane fluidity/rigidity or separation of the membrane from the cell wall. In contrast, the sensor could be activated by indirect changes in plant metabolism that result from stress such as metabolic changes, accumulation of ROS, release of ATP, or reduced energy levels (e.g., 9, 35, 84, 111, 117, 127). It was recently shown that energy depletion in *Arabidopsis* during abiotic stress is directly linked to the activation of abiotic stress responses via SnRK1 (SNF1-related kinase 1; 8). This upstream regulator triggers extensive transcriptional changes involving over 600 genes and contributes to the restoration of cellular homeostasis and cellular survival (8). The large number of transcripts involved in the various metabolic and other acclimation responses controlled by SnRK1 demonstrates that this protein functions as a key regulator of abiotic stress responses in plants. Another putative stress receptor that appears to function at a high level along the osmotic stress response signaling pathway is ATHK1. It was recently proposed that this plasma membrane histidine kinase functions through a phosphorelay mechanism together with ARR3/ARR4 and/or ARR8/ARR9 to activate ABA-dependent and ABA-independent responses involved in osmotic stress and seed desiccation tolerance, and controls the expression of about 400 target genes (146). Another protein that was recently shown to function as an upstream regulator of salinity responses in *Medicago truncatula* is a novel leucine-rich repeat receptor kinase (*Srlk*; 31). However, RNAi or TILLING mutants in *Srlk* failed to suppress root growth in response to salinity stress and had lower expression of salinity response transcripts. These findings indicate that *Srlk* is involved in sensing of salinity stress and reveal an interesting mode for salinity adaptation in *Medicago*.

Retrograde and Systemic Signaling

Retrograde signaling from the chloroplast or mitochondria to the nucleus has also been

proposed to mediate abiotic stress perception (96). Many abiotic stress conditions will influence chloroplast or mitochondria metabolism and could generate signals such as overreduction of the electron transport chain, enhanced accumulation of ROS, or altered redox potential that will, in turn, trigger nuclear gene expression and acclimation responses. Signaling from the chloroplast to the nuclei was recently shown to be mediated by *Gun1* and *Abi4* and to regulate a large number of nuclear transcripts (63). This pathway was also shown to be important for heat stress acclimation and could be involved in sensing of other stresses (80). Retrograde signaling could also mediate the response to high light intensity stress (96, 109). Using a luciferase reporter gene fused to the promoter of the ascorbate peroxidase 2 (*Apx2*) gene, it was previously reported that a local high light intensity stress can trigger a plant-wide systemic acclimation response (59). This systemic response was recently shown to enhance the tolerance to oxidative stress and to involve the zinc finger protein *Zat10* (109). Rapid systemic responses to such abiotic stress conditions as heat, cold, salinity, and high light intensity were recently reported to be mediated by an auto-propagating wave of ROS that travels at a rate of ~ 8.4 cm min^{-1} and is dependent on the presence of the respiratory burst oxidase *RbohD* gene (79). The rapid rates of systemic signals detected with luciferase imaging suggest that many of the responses to abiotic stresses might occur at a much faster rate than previously thought. It is therefore possible that many of the GeneChip® studies for abiotic stress, present for example in GENEVESTIGATOR (45), lack key early time points from their analysis and these should be taken into consideration when designing future studies.

Epigenetic Control

A very exciting area in abiotic stress research has emerged in recent years focusing on epigenetic factors that mediate responses to and memory of different abiotic stresses (25, 52, 56). Chromatin immunoprecipitation of DNA

cross-linked to modified histones coupled with next-generation sequencing technology, as well as shotgun bisulfite sequencing, has opened the way for genome-wide analyses of changes in epigenome state. Stable or heritable DNA methylation and histone modifications can therefore be linked with abiotic stresses and show how plants use these mechanisms for long-term memory. Of particular interest to the characterization of abiotic stress under field conditions is the control of flowering time during abiotic stress. Mutations in some of the genes involved in epigenetic processes during stress were shown to cause changes in flowering times (25). For example, late flowering of the freezing-sensitive *Arabidopsis* mutant *hos15* was shown to result from deacetylation of the flowering genes *SOC* and *FT* (153). The flowering repressor *FLC* (a MADS-box protein) is epigenetically repressed during vernalization, allowing the acquisition of the competence to flower after exposure to prolonged low temperatures (33). This process was shown to involve numerous proteins with potential to alter chromatin remodeling including *VIN3*, *FCA*, and *FPA* (25). In addition, it was recently shown that *VIN3* is also responsive to hypoxic conditions, suggesting that other abiotic stresses might affect flowering time via modifications of this pathway (15). Because transition from the vegetative to the reproductive stage in plants is heavily controlled by epigenetic mechanisms, more studies are needed to examine how these mechanisms are altered by differing abiotic stresses. Such understanding could lead to better control of stress-induced early flowering under field growth conditions.

Small RNAs

In addition to chromatin remodeling, and partially responsible for some types for transcriptional suppression, the involvement of small RNAs in abiotic stress responses has received increased attention recently (71, 125). Small RNAs belong to at least two groups: microRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs). miRNAs and siRNAs

can cause posttranscriptional gene silencing via RISC (RNA-induced silencing complex)-mediated degradation of mRNA in the cytosol. In addition, siRNA can suppress gene expression by altering chromatin properties in the nuclei via RITS (RNA-induced transcriptional signaling; 125). The involvement of small RNAs in suppressing protein translation during stress has also been proposed (125). Small RNAs such as miR398, 393, 395, and 399, as well as siRNAs such as *SRO5-P5CDH* and *ATGB2*, were shown to control gene expression during abiotic stresses including cold, nutrient, dehydration, salinity, and oxidative stresses (71, 125). Small RNAs were also implicated in the control of flowering time. For example, overexpression of miR159 and 319 causes delayed flowering, and overexpression of miR172, which targets an AP2 transcription factor, results in early flowering (125). A key question, of course, is how the expression of small RNAs is regulated during abiotic stress. Unraveling the mode of small RNA expression during abiotic stress will allow better control of gene expression during stress and the improvement of crop stress tolerance. However, this task is difficult because of the large number of potential small RNAs that exist in the genome of different plants.

QTL Analysis and Breeding

QTL analysis and traditional breeding have proven to be useful for the identification of genes responsible for biotic and abiotic stress tolerance in crops (27, 128). Thus, genes responsible for salinity tolerance were identified in wheat and rice; genes responsible for boron and aluminum toxicity were identified in wheat, sorghum, and barley; and genes responsible for tolerance to anaerobic stress were identified in rice (128). How the availability of next-generation sequencing and advanced metabolic profiling will impact this field and facilitate the cloning of more genes responsible for tolerance to abiotic stresses will be an interesting avenue to explore (62). The ability of these tools to cosequence or coscreen a large number of F2

siRNAs: small interfering RNAs

RISC: RNA-induced silencing complex

RITS: RNA-induced transcriptional signaling

or recombinant inbred lines coupled with statistical linkage analysis could open the way for a very rapid and new type of marker-assisted mapping at the genome or metabolome level.

Strategies for Transgene Expression

Strategies for the use of selected genes to improve tolerance to abiotic stresses in crops include gain- and loss-of-function approaches that target single genes at various levels. These genes could be enzymes, proteins, or regulatory genes such as transcription factors or MAPK. Tissue-specific, constitutive, or stress-inducible promoters have been used to express the selected genes in order to achieve maximum efficiency in stress protection with as few as possible negative effects on growth and productivity. Balancing energy requirements with acclimation appears to be a major challenge, and the identification of functional homologs of SnRK1 (8) in a range of crops could be a major breakthrough for this research. A better understanding of gene networks and regulons controlling individual stress and metabolic networks is required in order to truly balance energy, acclimation, and growth under stress conditions and during recovery. Such understanding will likely be achieved in the near future through system-level studies of stress responses in a variety of crops and model plants. A better understanding of field conditions, agricultural needs, classical plant physiology, as well as the attitude and objectives of agricultural business interests should be a major focus for plant molecular biologists in studying basic mechanisms of abiotic stress tolerance.

FUTURE DIRECTIONS IN ABIOTIC STRESS RESEARCH

Various strategies can be used to enhance the tolerance of plants to abiotic stress by genetic engineering. As described above, detailed understanding of the response of plants to abiotic stress is a prerequisite to the identification and use of upstream regulators to activate a balanced acclimation response that will enhance

the tolerance of plants to different stresses. The activation of this response could be facilitated during normal stress episodes in the field via the use of abiotic stress-response promoters, or triggered prior to the stress event using different chemicals combined with chemical-specific inducible promoters, a strategy similar to the priming used to alleviate biotic stresses (13). However, even if all of the plant's acclimation responses are activated, the plant might not be able to survive or produce sufficient yield under the abiotic stress because of natural limitations of the specific cultivar or plant and its genetic programming.

Novel Sources of Transgenes

One strategy that might enable plants to resist otherwise lethal abiotic stresses is to introduce genes from stress-adapted species such as desert and halotolerant plants, or organisms such as freezing-tolerant fish. The use of these specialized proteins, enzymes, or channels might give the crop plant the necessary additive advantage and enable it to resist far greater stress conditions than the nonmodified parental plant is able to. The large number of plant genome sequencing projects in progress, as well as the sequencing projects of other organisms from extreme environments, and even metagenomics projects, could well provide a rich source of genes for the manipulation of crop tolerance to abiotic stresses. A potentially interesting source of genes to enhance abiotic stress tolerance in crops may come from genes of unknown function, which account for 20%–40% of each new genome sequenced (42, 53). The majority of genes with unknown function were found to be species specific, suggesting that they could encode for stress adaptive mechanisms that are unique to different plants and other organisms (42, 43). A test of 42 ROS-response genes with unknown function in transgenic plants determined that most of these genes could enhance the tolerance of plants to oxidative stress and demonstrated that *Arabidopsis* plants could contain *Arabidopsis*- and/or *Brassica*-specific pathways for tolerance to oxidative stress (74). Thus

genes of unknown function could be a promising source of unique mechanisms for abiotic stress tolerance.

Overcoming Genetic Programming

A key consideration with respect to abiotic stress tolerance of annual crop plants is their genetic programming to undergo early flowering and accelerated senescence in response to stress. Although this tendency is ideal for survival in nature, it can have devastating effects on crop productivity. Overcoming this genetic programming by expression of a gene mediating cytokinin biosynthesis under the control of a drought stress-inducible promoter was recently shown to result in a dramatic increase in plant productivity under drought stress conditions (104, 105). Elucidating and controlling

the epigenetic mechanisms that regulate the transition from vegetative to reproductive phases and early flowering during stress could have similarly positive effects on plant productivity under stress. Because programmed cell death (PCD) is thought to be activated by different abiotic conditions and enhanced by ROS accumulation, as part of the genetic programming of annual plants, suppressing abiotic stress-induced PCD could also result in a similar enhancement of yield under stress (34, 148). Although suppressing senescence and PCD during stress might seem counterproductive, annual plants might have mechanisms to resist far greater stresses than previously thought, but they either do not activate these mechanisms, or use them only for the short period needed to generate seeds during stress-induced early flowering and senescence.

SUMMARY POINTS

1. A better attempt should be made to reproduce field conditions in the laboratory, and genetically modified plants should be tested under experimental conditions that represent the different combinations of restrictive conditions in the field environment.
2. Stress combination should be handled as a new state of abiotic stress in plants that requires a new type of acclimation response.
3. The interactions between abiotic stress events and plant productivity are perhaps the most critical for agricultural production and should be considered when developing transgenic crops with superior field performance.
4. Severe yield losses can be caused by climate change resulting in brief periods of temperature of a few days above the limits associated with the formation of reproductive organs and the development of sinks such as seeds and fruits.

FUTURE ISSUES

1. Omics tools should be used to study abiotic stress response in the field with elite cultivars as model plants.
2. Researchers should learn how to regulate flowering time and control stress-induced early flowering.
3. Identification of key upstream regulators/sensors of stress acclimation and their use in enhancing abiotic stress tolerance is an important area of research.

4. Research is needed to protect reproductive tissues against abiotic stress, with a special focus on heat and drought stresses.
5. We should attempt to overcome the genetic programming of annual crops and suppress stress-induced facilitated life cycle and early senescence.
6. Novel sources of genes could enhance crop abiotic stress tolerance.

DISCLOSURE STATEMENT

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Contents

A Wandering Pathway in Plant Biology: From Wildflowers to Phototropins to Bacterial Virulence <i>Winslow R. Briggs</i>	1
Structure and Function of Plant Photoreceptors <i>Andreas Möglich, Xiaojing Yang, Rebecca A. Ayers, and Keith Moffat</i>	21
Auxin Biosynthesis and Its Role in Plant Development <i>Yunde Zhao</i>	49
Computational Morphodynamics: A Modeling Framework to Understand Plant Growth <i>Vijay Chickarmane, Adrienne H.K. Roeder, Paul T. Tarr, Alexandre Cunba, Cory Tobin, and Elliot M. Meyerowitz</i>	65
Female Gametophyte Development in Flowering Plants <i>Wei-Cai Yang, Dong-Qiao Shi, and Yan-Hong Chen</i>	89
Doomed Lovers: Mechanisms of Isolation and Incompatibility in Plants <i>Kirsten Bomblies</i>	109
Chloroplast RNA Metabolism <i>David B. Stern, Michel Goldschmidt-Clermont, and Maureen R. Hanson</i>	125
Protein Transport into Chloroplasts <i>Hsou-min Li and Chi-Chou Chiu</i>	157
The Regulation of Gene Expression Required for C ₄ Photosynthesis <i>Julian M. Hibberd and Sarah Covshoff</i>	181
Starch: Its Metabolism, Evolution, and Biotechnological Modification in Plants <i>Samuel C. Zeeman, Jens Kossmann, and Alison M. Smith</i>	209
Improving Photosynthetic Efficiency for Greater Yield <i>Xin-Guang Zhu, Stephen P. Long, and Donald R. Ort</i>	235
Hemicelluloses <i>Henrik Vibe Scheller and Peter Ulvskov</i>	263
Diversification of P450 Genes During Land Plant Evolution <i>Masabaru Mizutani and Daisaku Ohta</i>	291

Evolution in Action: Plants Resistant to Herbicides <i>Stephen B. Powles and Qin Yu</i>	317
Insights from the Comparison of Plant Genome Sequences <i>Andrew H. Paterson, Michael Freeling, Haibao Tang, and Xiyin Wang</i>	349
High-Throughput Characterization of Plant Gene Functions by Using Gain-of-Function Technology <i>Youichi Kondou, Mieko Higuchi, and Minami Matsui</i>	373
Histone Methylation in Higher Plants <i>Chunyan Liu, Falong Lu, Xia Cui, and Xiaofeng Cao</i>	395
Genetic and Molecular Basis of Rice Yield <i>Yongzhong Xing and Qifa Zhang</i>	421
Genetic Engineering for Modern Agriculture: Challenges and Perspectives <i>Ron Mittler and Eduardo Blumwald</i>	443
Metabolomics for Functional Genomics, Systems Biology, and Biotechnology <i>Kazuki Saito and Fumio Matsuda</i>	463
Quantitation in Mass-Spectrometry-Based Proteomics <i>Waltraud X. Schulze and Björn Usadel</i>	491
Metal Hyperaccumulation in Plants <i>Ute Krämer</i>	517
Arsenic as a Food Chain Contaminant: Mechanisms of Plant Uptake and Metabolism and Mitigation Strategies <i>Fang-Jie Zhao, Steve P. McGrath, and Andrew A. Meharg</i>	535
Guard Cell Signal Transduction Network: Advances in Understanding Abscisic Acid, CO ₂ , and Ca ²⁺ Signaling <i>Tae-Houn Kim, Maik Böbmer, Honghong Hu, Noriyuki Nishimura, and Julian I. Schroeder</i>	561
The Language of Calcium Signaling <i>Antony N. Dodd, Jörg Kudla, and Dale Sanders</i>	593
Mitogen-Activated Protein Kinase Signaling in Plants <i>Maria Cristina Suarez Rodriguez, Morten Petersen, and John Mundy</i>	621
Abscisic Acid: Emergence of a Core Signaling Network <i>Sean R. Cutler, Pedro L. Rodriguez, Ruth R. Finkelstein, and Suzanne R. Abrams</i>	651
Brassinosteroid Signal Transduction from Receptor Kinases to Transcription Factors <i>Tae-Wuk Kim and Zhi-Yong Wang</i>	681

Directional Gravity Sensing in Gravitropism
Miyo Terao Morita 705

Indexes

Cumulative Index of Contributing Authors, Volumes 51–61 721
Cumulative Index of Chapter Titles, Volumes 51–61 726

Errata

An online log of corrections to *Annual Review of Plant Biology* articles may be found at <http://plant.annualreviews.org>