Genomic approaches to plant stress tolerance

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Past efforts to improve plant tolerance to drought, high salinity and low-temperature through breeding and genetic engineering have had limited success owing to the genetic complexity of stress responses. Progress is now anticipated through comparative genomics studies of an evolutionarily diverse set of model organisms, and through the use of techniques such as high-throughput analysis of expressed sequence tags, large-scale parallel analysis of gene expression, targeted or random mutagenesis, and gain-of-function or mutant complementation. The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their roles in stress adaptation (obtained by the use of functional genomics) will provide the basis of effective engineering strategies leading to greater stress tolerance.

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Abbreviations

ABA	abscisic acid
EST	expressed sequence tag
GFP	green fluorescent protein
LUC	firefly luciferase
MAP	mitogen-activated protein
mRNP	messenger ribonucleoprotein
NEST	nuclear expressed sequence tag
ORF	open reading frame
QTL	quantitative trait locus
SAGE	serial analysis of gene expression
SOS	salt overly sensitive

Introduction

Environmental factors that impose water-deficit stress, such as drought, salinity and temperature extremes, place major limits on plant productivity [1]. To overcome these limitations and improve production efficiency in the face of a burgeoning world population, more stress tolerant crops must be developed [2•]. Traditional breeding strategies that have attempted to utilize genetic variation arising from varietal germplasm, interspecific or intergeneric hybridization, induced mutations and somaclonal variation of cell and tissue cultures have met with only limited success; very few new plant introductions with improved stress resistance under field conditions have resulted [3]. Traditional approaches are limited by the complexity of stress tolerance traits, low genetic variance of yield components under stress conditions and the lack of efficient selection techniques [4–7]. Furthermore, quantitative trait loci (QTLs) that are linked to tolerance at one stage in development can differ from those linked to tolerance at other stages [8•]. Once identified, desirable QTLs can require extensive breeding to restore desirable traits along with the introgressed tolerance trait. Nonetheless, marker-assisted selection of specific secondary traits that are indirectly related to yield (e.g. the interval between anthesis and silking [4,5], osmotic adjustment [9], membrane stability [7] or physiological tolerance indices [6]) might prove increasingly useful as the resolution of the genetic and physical chromosome maps of the major crops improves. This strategy could be used in combination with 'pyramiding' strategies or consecutive selection for, and accumulation of, physiological yield-component traits [3].

Genetic engineering of tolerance traits

In contrast with traditional breeding and marker-assisted selection programs, the direct introduction of a small number of genes by genetic engineering seems to be a more attractive and rapid approach to improving stress tolerance. Present engineering strategies rely on the transfer of one or several genes that encode either biochemical pathways or endpoints of signaling pathways that are controlled by a constitutively active promoter. These gene products protect, either directly or indirectly, against environmental stresses (Table 1) [10•,11,12,13•]. Engineered overexpression of biosynthetic enzymes for osmoprotectants [13•,14,15••,16], scavengers of reactive oxygen species [13•,17•] and stress-induced proteins (e.g. cold-regulated [COR] or late embryogenesis abundant [LEA]) [18,19] are among the approaches reported.

Ion transport and maintenance of ion homeostasis can profoundly effect plant growth and productivity [20•], a point that is well illustrated by the recent demonstration that the moderate overexpression of a homologous cDNA encoding a sodium/proton antiporter can confer improved salinity tolerance on *Arabidopsis* (21••). Halophytes might also have evolved distinct stress-recognition or signaling pathways, and regulatory controls that confer stress protection ([22••]; BJ Barkla, R Vera-Estrella, J Camacho-Emiterio, O Pantoja, personal communication). Alternatively, 'regulon' engineering with stress-specific transcription factors, which control the expression of a set of stress-adaptive proteins, has been used to improve salinity, drought, or freezing tolerance ([23•,24•,25,26••]; MA Villalobos, G Iturriaga, personal communication). Similarly, the expression of components of stress signaling pathways (e.g. constitutively active yeast calcineurin) has been used to achieve biochemical 'pathway' engineering involving multiple targets for salinity stress tolerance by improving ion homeostasis [27]. The success of these approaches has generally been limited by a lack of understanding of metabolic flux,

Table 1

The complexity of stress adaptation: major targets for engineered stress tolerance.

Class of target	Examples	Possible mode(s) of action
Osmoprotectants	Amino acids (proline, ectoine) Dimethyl sulfonium compounds (glycine betaine, DMSP) Polyols (mannitol, D-ononitol, sorbitol) Sugars (sucrose, trehalose, fructan)	Osmotic adjustment; protein/membrane protection; reactive (OH·) scavenging
Reactive oxygen scavengers	Enzymatic (catalase, Fe/Mn superoxide dismutase, ascorbate peroxidase; glutathione cycle enzymes: glutathione S-transferase, glutathione peroxidase; gamma-glutamylcysteine synthetase, alternative oxidase) Non-enzymatic (ascorbate, flavones, carotenoids, anthocyanins)	Detoxification of reactive oxygen species
Stress proteins	Late embyogenesis abundant proteins	Unknown, protein stabilization, water binding/ slow desiccation rates; chaperones; protein/ membrane stabilization; ion sequestration
Heat shock proteins	Various heat-, cold-, salt-shock proteins in several subcellular compartments	Reversal/prevention of protein unfolding; translational modulation
Ion/proton transporters	High-affinity K^+ transporter; low-affinity K^+ channels; plasma membrane, pre-vacuolar, vacuolar and organellar proton ATPases and ion transporters (H^+/ATPase; Na^+/H^+ antiporters)	K^+/Na^+ uptake and transport; establishment of proton gradients; removal and sequestration of (toxic) ions from the cytoplasm and organelles
Membrane fluidity	Fatty acid desaturases	Increased amounts of dienoic and fluidity; chilling tolerance
Water status	Aquaporins or water channels (solute facilitators: urea, glycerol, CO_2, possibly others and including ions); CO_2 concentration	Regulation of AQP amount differentially in tonoplast and plasma membrane; regulation of membrane location; stomatal behavior
Signaling components	Homologs of histidine kinases (AtRR1/2); MAP kinases (PsMAPK, HOG); Ca^{2+}-dependent protein kinases; SNF1/kinases; protein phosphatases (ABI1/2); CNA/B signaling systems; Ca^{2+} sensors (SOS3); inositol kinases	Ca^{2+}-sensors/phosphorylation mediated signal transduction
Control of transcription	Transcription factors: EREBP/AP2 (DREB, CBF); zinc finger TF (Alfin 1); Myb (AtMyb2, CpMyb10)	Upregulation/activation of transcription
Growth regulators	Altered biosynthetic pathways or conjugate levels for abscisic acid, cytokinins and/or brassinosteroids	Changes in hormone homeostasis

ABI, abscisic-acid-insensitive; AP2, APETELA2; AQP, aquaporin; AMPK1, AMP-activated protein kinase; AtMyb, *Arabidopsis thaliana* myeloblastosis (helix-loop-helix) transcription factor; AtRR1, *A. thaliana* two-component response regulators; CBF, C-repeat/DRE binding factor; CNA/B, calcineurin A/B; CpMyb, *C. plantagineum* myeloblastosis (helix-loop-helix) transcription factor; DMSP, dimethylsulfoniopropionate; DREB, dehydration-responsive element (DRE) binding protein; EREBP, ethylene-responsive element binding protein; HOG, high osmolarity glycerol; PsMAPK, *Pisum sativum* mitogen-activated protein kinase; SNF1, sucrose non-fermenting 1; TF, transcription factor.

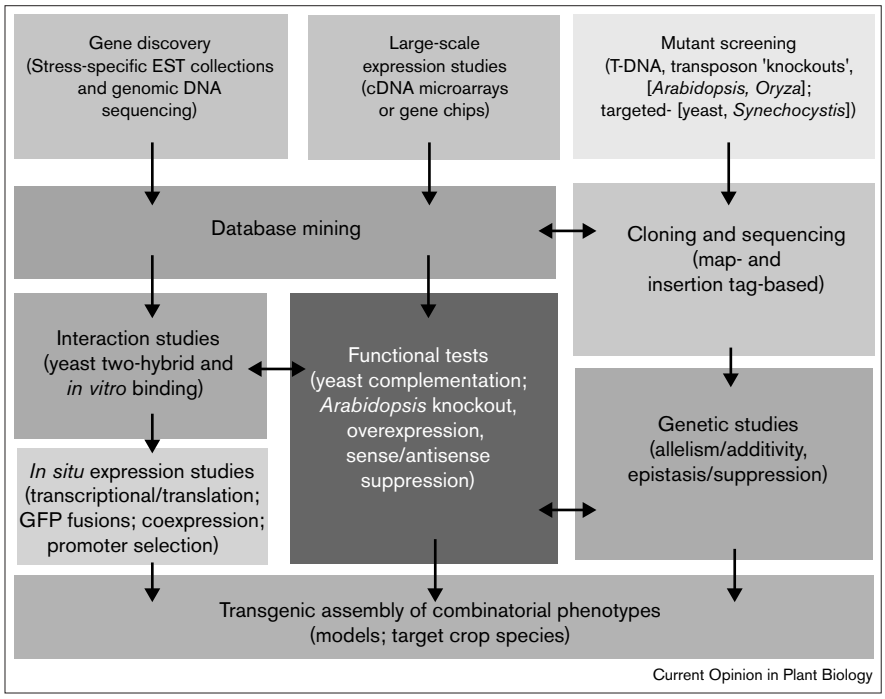
compartmentation and function [11,15••,26••]. A more complete understanding of the complexity and interplay of osmotic, desiccation and temperature tolerance mechanisms, and their corresponding signaling pathways, is therefore needed and will come from integrative, whole-genome studies [28,29].

Gene discovery in glycophytes

The first step towards cataloging and categorizing genetically complex abiotic stress responses is the rapid discovery of genes by the large-scale partial sequencing of randomly selected cDNA clones or expressed sequence tags (ESTs) (Figure 1). Extensive EST collections already exist for *Arabidopsis* [30] and rice [31]. Large-scale EST sequencing initiatives are also well under way for various crop species [32•] including cotton, *Medicago truncatula*, maize, soybean, tomato and sorghum and also for Loblolly pine (http://www.nsf.gov/bio/pubs/awards/genome99.htm). The number of tags available in the rapidly growing EST collections in the public domain can be followed at the dbEST section of GenBank (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). These sequencing efforts have generated collections in which more than half of the total gene complement (i.e. ~28,000 genes) is represented (as estimated from the gene content of the entirely sequenced chromosome 2 in *Arabidopsis* [33]). The collections are, however, biased towards high to moderate abundance classes that are derived from different tissues,

Figure 1

Functional genomics strategy from gene discovery to evaluation of stress tolerance phenotypes.



organs or cells; different developmental states; various external stimuli such as heat-shock or nitrogen starvation; and treatments with plant growth regulators (e.g. 6-benzyladenine or gibberellin). In contrast, relatively few studies have focused specifically on ESTs from plants that have been exposed to environmental stresses.

Initial attempts to identify stress-specific transcripts using EST approaches were conducted in glycophytic vascular model plant species that were exposed to salinity stress. The random sequencing of 780 ESTs from rice cell-suspension cultures that were exposed to salinity (or nitrogen-starvation stress) revealed that salinity stress induced the expression of several enzymes related to glycolysis and the tricarboxylic acid cycle, which contribute to ATP production [34]. The sequencing of 220 randomly chosen ESTs from a subtracted *Arabidopsis* cDNA library identified 15 osmotic-stress-induced genes that had early, late or continuous patterns of expression, and which were induced 2–50-fold by exposure to osmotic stress [35]. The scarcity of ESTs that are derived from cDNAs of stressed tissues of glycophytes suggests that stress-relevant transcripts are under-represented or absent from existing EST collections. In an attempt to redress this deficiency, large-scale EST sequencing is now in progress using tissue-specific and developmental-stage-specific cDNA libraries generated from the RNA of salinity-stressed *Arabidopsis* and rice. The cDNA libraries investigated are listed at http://www.biochem.arizona.edu/BOHNERT/functgenomics/front2.html. Current EST data sets can be browsed and searched on-line at the Stress Functional Genomics Consortium website (http://stress-genomics.org/). As part of the gene discovery effort in maize, EST collections are also being established from libraries of cDNA that has been prepared from salt-stressed roots and shoots (http://www.zmdb.iastate.edu/zmdb/EST_project.html).

Gene discovery in stress-tolerant models

Although functional adaptation mechanisms are likely to be largely conserved among glycophytes (Table 1), halophytic organisms have evolved additional structural or regulatory differences that account for their ability to withstand severe osmotic or ionic stress ([22••]; BJ Barkla, R Vera-Estrella, J Camacho-Emiterio, O Pantoja, personal communication). To identify these potential differences, major EST sequencing efforts have been initiated for the halophyte *Mesembryanthemum crystallinum* and the halotolerant green alga *Dunaliella salina* [36]. Comparative sampling of approximately equal numbers (~1200) of ESTs from the leaf tissue of well-watered and salinity-stressed *M. crystallinum* revealed that the stressed plants expressed ~15% more functionally unknown genes than the unstressed plants [37]. This finding supports the notion that ESTs that are related to salinity stress are under-represented in the current non-redundant GenBank database. Furthermore, only 13% of the non-redundant ESTs in this relatively small *M. crystallinum* data set are expressed in both well-watered and salt-stressed plants, thus highlighting the dramatic alteration in gene-expression profile that accompanies stress treatment. Sampling differences between unstressed and stressed plants also revealed pronounced downregulation of transcript abundance for components of the photosynthetic apparatus and a concomitant upregulation of constituents involved in either

proteome restructuring (e.g. proteases and ubiquitinases) or adaptation to osmotic and dehydration stress.

Some bryophytes, such as *Tortula ruralis* [38], and vascular plants, such as the resurrection plants, *Craterostigma plantagineum* [39•], *Selaginella lepidophylla* [40] and *Sporobolus stapfianus* [41], have evolved tolerance of desiccation in their vegetative tissues. *T. ruralis* gametophytes rely on a constitutive protection system, coupled with an active rehydration-induced recovery mechanism, to restrict damage during rehydration. During slow drying, large (>150 kDa) messenger ribonucleoprotein (mRNP) particles form in the vegetative cells and permit the rapid restoration of protein synthesis following rehydration, thereby facilitating the survival of the desiccated tissues [38]. Sequencing of a limited sample of 152 ESTs from a library of cDNA obtained from polysomal mRNP fractions of a desiccated moss, *T. ruralis*, showed that the majority (~70%) of the ESTs represented novel sequences. The sequencing of such EST collections should help to define the range of gene products that are essential for cellular repair and recovery after vegetative desiccation [42••]. EST collections have also been initiated for *Physcomitrella patens*, a moss model system that has efficient gene targeting [43]. To identify genes that are associated with desiccation tolerance, 169 ESTs were characterized from *P. patens* protonema following treatment with abscisic acid (ABA). Most of the ESTs (69%) shared homology with known sequences, although many of the clones encoded proteins that are induced as part of the heat tolerance, cold acclimation, oxidative stress adaptation or xenobiotic detoxification responses [43].

In contrast with bryophytes, *Craterostigma plantagineum*, *Selaginella lepidophylla* and *Sporobolus stapfianus* use one or more mechanisms, which are induced by ABA and/or drying, to accumulate molecules, such as LEA proteins and sugars (e.g. sucrose, raffinose or trehalose), that are involved in the establishment of cellular protection prior to desiccation. Bockel, Salamini and Bartels [39•] used differential, subtractive or cold-plaque screening of 200 cDNA clones from *C. plantagineum* leaves that had been either dried for 1 h or totally dried down [39•]. One half of the sequences showed no significant similarity to those in public databases; of those sequences with a predicted function, 6% and 58% were upregulated or transiently upregulated by dehydration, respectively, whereas 35.8% were downregulated by dehydration [39•]. Using cDNA clones from *S. stapfianus*, genes encoding abundant drought-induced proteins that are correlated with desiccation tolerance, or low-abundance transcripts that encode gene products not previously associated with drought stress, have been isolated by differential screening [41] or by cold-plaque hybridization procedures [44•], respectively. Hence, resurrection plants may possess unique gene complements or regulatory processes that contribute to desiccation tolerance. Furthermore, this hypothesis is supported by an earlier proteomic comparison of *S. stapfianus* with a closely related desiccation-sensitive species, *S. pyramidalis*, that revealed a set of 12 novel proteins that are probably associated with desiccation tolerance [45].

High-throughput stress-specific gene expression analysis

In *Arabidopsis*, the precise function of approximately half of all predicted protein-coding genes deduced from amino acid sequence information remains unknown [29,30,33]. In the absence of other information, differential expression patterns often provide clues to gene function and are an important criterion for exploiting EST resources on a large scale [46••]. Analysis of variation in the frequency of individual tags reveals the differential expression of the corresponding genes, but this 'digital northern' approach identifies only the most abundant, significantly upregulated or downregulated genes. We can gain confidence that differences in EST frequency, particularly for rare transcripts, are significant only by increasing the sampling size of the EST collections [47]. Alternatives, such as serial analysis of gene expression (SAGE), have been developed for rapidly quantifying the occurrence of large numbers of transcripts in a particular population. With a 9–12-base size for each tag, SAGE unambiguously identifies individual transcripts, yet improves the efficiency (up to 40-fold) of generating extremely large EST databases by sequencing multiple tags within a single clone [48]. Another alternative, called nuclear expressed sequence tag (NEST) analysis, combines fluorescence-assisted nucleus sorting and cDNA generation (based on the expression of nucleus-targeted green fluorescent protein [GFP], which is controlled by a cell-specific promoter) from the RNA of isolated nuclei [49••]. The RNA from such preparations accurately reflects nuclear transcript abundance, avoiding the influence of post-transcriptional turnover in the cytosol. Cell-specific cDNAs can be characterized by differential-display reverse transcriptase-mediated PCR or by EST analysis. In tobacco, approximately 25% of salinity-induced transcripts identified by NEST analysis show significant homology to functionally unknown genes (C-P Song, DW Galbraith, personal communication).

To obtain novel insights into gene function and the regulatory control of biological processes that are associated with stress responses to drought, salinity or freezing, cDNA microarrays offer a high-throughput approach to obtaining comprehensive gene expression profiles [50,51,52•]. High-throughput parallel gene expression monitoring, using cDNA microarray-based methods, has been used to examine gene expression patterns in tissues including root, leaf and flowers at two different stages of development [53,54], and under dark and light conditions [55]. Large-scale cDNA microarray analyses of the expression profiles of genes that respond to salinity-stress are underway for *M. crystallinum* (M Cushman *et al*., unpublished data), rice (S Kawasaki *et al*., unpublished data), and

Arabidopsis (M Deyholos, D Galbraith, personal communication). Although these analyses will assess only a small fraction of the entire gene complement, until more comprehensive EST collections are available, they will provide an important starting point for prioritizing unknown genes for further functional analysis.

Comprehensive genome-wide surveys of stress-responsive gene expression using microarrays are, however, currently possible in single-celled model organisms, including *Synechocystis* sp. PCC 6803 and *Saccharomyces cerevisiae*, whose entire genome sequence is known. A recent analysis of yeast cells exposed to hyperosmotic shock (1 M NaCl for 0–90 min) revealed that ~300 transcripts (~5% of all open reading frames [ORFs]) showed a > two-fold increase in transcript abundance, whereas ~200 genes were downregulated to a similar extent (J Yale *et al*., unpublished data). Genes involved in energy metabolism, ion homeostasis, cell defense, chaperone functions and transport facilitation were most strongly upregulated. These analyses are expected to provide the first functional information about the role of unknown ORFs in cellular stress adaptation processes. Closer analysis of expression data sets has also indicated that a number of these upregulated ORFs in *S. cerevisiae* have counterparts in *Synechocystis* (R Burnap, unpublished data), *Aspergillus nidulans* (R Prade, personal communication), and *M. crystallinum* after salt stress (JC Cushman, unpublished data; J Yale, HJ Bohnert, unpublished data). The comparisons among cyanobacteria, fungi and plants comprise an aggregate of genes that delineate cellular tolerance mechanisms.

Equally important for our understanding of cellular responses will be detailed surveys of gene expression profiles that give insight into how plants integrate stress responses in the context of development and a complex assortment of tissues (each with differential sensitivities or susceptibilities to different environmental stresses). Microarrays will also permit comparisons between one or more glycophytic (e.g. *Arabidopsis* and rice), halophytic (e.g. *M. crystallinum)* and desiccation-tolerant (e.g. *C. plantagineum*, *S. lepidophylla* and *S. stapfianus*) models, thereby permitting the identification of differences and similarities in expression patterns or gene complements that contribute to tolerance of specific stresses such as salinity, drought and temperature extremes. In addition, microarrays offer a rapid and comprehensive technique for identifying stress tolerance determinants by detecting transcripts whose expression patterns under stress conditions differ in mutants that are dysfunctional in biochemical-endpoint or signaling-pathway components from those in the wild-type (Figure 1). Analyses of the *cnb1* and *hog1* yeast mutants, which are defective in a protein phosphatase 2B (calcineurin) involved in the signaling of ion homeostasis, and in a MAP kinase involved in high osmotic stress regulation, respectively, have revealed the target sets of endpoint genes for each of the important signaling pathway components that are defective in these mutants (T Matsumoto *et al*., unpublished data). Expression patterns alone will not, however, reveal the functions of unknown stress-regulated genes in yeast and plant ESTs.

Forward and reverse genetics

Intelligent engineering of regulatory circuits will require detailed knowledge of signaling hierarchies and the impact of metabolic changes involved in stress responses. Mutant screens for salinity-hypersensitive *Arabidopsis* (e.g. 'salt overly sensitive' [SOS]) led to the discovery of important and novel structural and signaling components that are critical for stress tolerance. One such mutation, *SOS3*, was found to encode a calcineurin B-like Ca^{2+}-binding protein defective in Ca^{2+}-binding properties that is essential for K^+ nutrition and K^+/Na^+ selectivity in the presence of large concentrations of Na^+ ions [56••]. Interestingly, SOS3 has recently been shown to interact with the product of a second *SOS* locus, *SOS2*, that encodes a sucrose nonfermenting/AMP-activated protein kinase (SNF1/AMPK)-protein kinase involved in the control of Na^+/K^+ homeostasis (U Halfter, M Ishitani, J Liu, JK Zhu, personal communication). Conversely, the isolation of *Arabidopsis* mutants with improved tolerance of freezing or salinity has revealed novel regulatory genes for proline biosynthesis and breakdown [57], and active oxygen detoxification [58•], respectively. Antisense approaches aimed at dissecting the roles of key adaptive enzymes, such as Δ^1-pyrroline-5-carboxylate synthetase, have also uncovered functional roles of proteins that are unrelated to stress tolerance [59].

Ultimately, a systematic effort to mutagenize all stress-relevant genes is required to complement information obtained by gene discovery and expression profiling. To this end, functional analysis is under way for selected genes that participate in drought, salinity and low temperature stress-adaptive signaling and responses. The generation and screening of large T-DNA or transposon insertional mutant collections of *Arabidopsis* and rice will also provide essential resources for finding tagged mutations that lead to defective stress tolerance responses [60•,61–63]. These populations can be surveyed using both forward and reverse genetic screens to isolate 'knockout' mutants that are either tolerant of or hypersensitive to stress. Activation T-DNA (bialaphos resistance marker and 4X 35S enhancer) tagged collections are being generated in transgenic *Arabidopsis* backgrounds in order to isolate mutations that affect stress signaling. These transgenic plants express chimeric genes composed of promoters that are responsive to osmotic potential, cold, stress and ABA (e.g. RD29A) fused to the coding sequence of firefly luciferase (LUC) [64]. Luciferase enzyme activity is used to rapidly identify promoter activity, which, after mutagenesis of the plant line, may be enhanced, diminished or no longer dependent on activation by stress (Figure 1). This approach has revealed that ABA-dependent and ABA-independent signaling pathways share considerable cross talk, through both positive and negative interactions, to bring about stress-responsive gene expression [65,66]. So

far, about 90,000 T-DNA-tagged lines in the *RD29A-LUC* genetic background have been produced, and mutants with altered stress signaling or sensitivity have been isolated. The production of DNA pools for the tagged population is under way for the reverse genetic identification of mutants and will be made available through the *Arabidopsis* Stock Center at Ohio State University.

Targeted 'knock-out' and random-insertion stress-sensitive mutants are being generated in *Synechocystis* sp. PCC6803, yeast and *Aspergillus nidulans*. Selected mutant strains are complemented with expression libraries from *Arabidopsis*, rice, tobacco and *M. crystallinum* to isolate suppressors of stress-sensitive phenotypes. This approach has resulted in the isolation of plant orthologs of yeast protein kinases [67,68••,69], transcription factors [70], and signaling components (TK Matsumoto *et al.*, personal communication). Alternatively, evaluation of salt tolerance determinants for sufficiency can be performed by overexpression in wild-type transgenic plants [27] or by the suppression of salt-sensitive mutants of *Arabidopsis*. Finally, transcriptional or translational GFP-fusion constructs can be used to visualize the temporal and spatial expression patterns of individual genes and the subcellular location of gene products.

Conclusions

The genomic-scale EST and genome sequencing, and cDNA microarray analyses that are now under way promise to rapidly isolate and identify all candidate genes of the 'osmome', 'xerome' or 'thermome' — the gene complement essential for tolerance of osmotic potential, desiccation or temperature stresses. As outlined in Figure 1, the large datasets generated by these efforts will be integrated and comparisons made between different cellular and glycophytic, halophytic and xerophytic plant models to identify the cellular tolerance mechanisms that are evolutionarily conserved. Mining of these data will supply a systematic agenda for functional analysis with the use of tagged mutant collections, complementation and overexpression tests accompanied by microarray analyses to reveal hierarchical relationships between specific signaling components and downstream effector genes.

Understanding specific protein–protein interactions will require the construction of protein-linkage maps using yeast two-hybrid technologies. Approaches with proteomics will be necessary to clarify the structural predictions of genome sequence information and to assess the protein modifications and protein–ligand interactions that are relevant to stress tolerant phenotypes. Ultimately, the functional determination of all genes that participate in stress adaptation or tolerance reactions are expected to provide an integrated understanding of the biochemical and physiological basis of stress responses in plants. Armed with such information from established models, it will be possible to rationally manipulate and optimize tolerance traits for improved crop productivity well into the twenty-first century.

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