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# Unraveling abiotic stress tolerance mechanisms – getting genomics going

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Homeostasis, a set-value for metabolism under optimal conditions, is rarely achieved by plants because of the cost exerted by external stress factors: climatic, biotic, and nutrient imbalances. Among these, stresses caused by abiotic conditions, such as temperature extremes (freezing, cold and heat), water availability (drought and ion excess) and ion toxicity (salinity and heavy metals), have been difficult to dissect because defense responses to abiotic factors require regulatory changes to the activation of multiple genes and pathways. Genomics technologies that have emerged during the past decade have been useful in addressing, in an integrated fashion, the multigenicity of the plant abiotic stress response through genome sequences; cell-, organ-, tissue- and stress-specific transcript collections; transcript, protein and metabolite profiles and their dynamic changes; protein interactions; and mutant screens.

## Addresses

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## Introduction

The application of genomics-type technologies is beginning to have an impact, enhancing our understanding of plant responses to external factors – abiotic stresses — that disturb the homeostatic equilibrium. Here, we understand the term genomics as any technology that, preferably in a high-throughput genome-focused fashion, promises insights and answers on how plant genes, proteins, protein activities, and metabolite type and flux respond to external factors. We discuss recent progress and address views on how to develop further research. Understanding the mechanisms that govern tolerance explains, foremost, ecological diversity and species distribution, and provides important information about the performance of field crops. The resulting ability to modify

tolerance would carry the potential for increasing yield stability under stressful conditions.

Until recently, the prevalent strategy of the molecular genetic approach could be termed the ‘candidate gene-by-gene’ approach. This approach aims to dissect single genes in many pathways in order to determine a gene’s place in the stress-response cascade and to gauge its contribution to tolerance acquisition [1••]. Much of the work has been guided by physiological and biochemical knowledge accumulated in earlier studies.

A second view and approach has become possible through genomics. This places emphasis on integrated analysis of stress-dependent behavior by the entire plant. With this view gaining strength, genomics can become a bridge to whole-plant physiology, agronomy and crop breeding. The functional genomics strategy begins to tie together physiological and phenotypic observations with information on gene complement, transcription and transcript regulation, the behavior of proteins, protein complexes and pathways, evolutionary adaptive diversification, and mutated or engineered phenotypic characters. We present progress along this systems-oriented road. Results on inter- and intra-species genetic diversity in stress responses will become increasingly useful in helping us to understand how species, ecotypes, or breeding lines in crop species can either mount a successful defense or succumb to abiotic stress.

Recent reviews have comprehensively addressed various aspects that are relevant in the context of plant stress responses [1••,2••,3–6,7•,8]. The topic of the engineering of abiotic stress tolerance has been covered expertly, highlighting the relative success of the over- or under-expression of single genes by targeting genes, proteins or enzymatic reactions that had, over many years, been indicated as important components of stress tolerance or sensitivity. Vinocur and Altman’s review [1••] also brought about a shift in emphasis: away from focusing on the end-points of response chains to engineering genes governing upstream reactions that affect entire pathways and groups of genes. An important aspect, not only in the field of plant stress, are the capacities to generate reactive oxygen species as molecules for signaling across membranes, to convert radical-based signals by alterations of the redox state of intracellular sensors, to modify and route proteins, and to prevent radical chemistry becoming a runaway signal for cell death [2••]. Other reviews have addressed stress-dependent signaling pathways and cross-talk between different abiotic stresses [3–5], compared

stress-specific transcript profiles, and linked, beginning with the *Arabidopsis thaliana* model, stress-dependent regulation of transcription to promoter structure [6]. Lucid examples of progress are the advances made in understanding the transcriptional regulation of heat shock responses during stress and recovery and the mechanisms necessary to acquire tolerance of extreme temperatures [7,8].

## Models

To some degree, the advent of genomics has supplanted the necessity to focus abiotic stress biology on species that show natural tolerance. The identification of stress-relevant genes and pathways has been facilitated by increasing numbers of tools and resources in the traditional models. These encompass genome sequences, including bacterial artificial chromosome (BAC) libraries and BAC-end sequences; expressed sequence tag (EST) and cDNA collections; transcript profiling platforms; sophisticated technologies for the real-time non-destructive localization of proteins; and sensors for and profiles of metabolites. However, the genomic resources and results developed any model system will also guide work towards finding the distinguishing characters of the traditional stress-biology models. Evolutionary and comparative functional studies will probe the knowledge gained from work with *A. thaliana* or rice, their ecotypes, breeding lines and close relatives, and even unrelated species (Table 1). Plant stress biology has already profited from comparisons between *A. thaliana* and the closely related crucifer *Thellungiella halophila* (*Thellungiella salsuginea*), a species with extreme cold and freezing, drought, and salinity tolerance [9–12]. *Thellungiella* appears to be ‘stress-anticipating’ because it shows elevated pre-stress expression of genes, some of which are paralogous versions and alleles that might be stress-inducible in *A. thaliana*, and constitutively elevated metabolites that

act either as signaling molecules or osmolytes [11,12]. Among *A. thaliana* ecotypes, those adapted to stressful environments will probably show differences in stress responses that are similar to the differences in development that have been documented [13]. A comparison of transcriptional regulation in five *A. thaliana* accessions revealed genes that have distinct expression patterns, which identified differences in a number of characters, including stress responses [14]. In this respect, the suitability of reference genes for transcript normalization (as shown for *A. thaliana* [15]) or of other markers that identify metabolic state should receive special attention.

Increased attention to experimental protocols will be important for meaningful comparisons across plant families and orders. Details of phenotypes, growth history and developmental stage must be recorded, and the (species- or line-specific) sub-lethal levels of treatments imposed should be controlled and verified by recording physiological parameters. We also make a case for the use of ‘shock’ stress abiotic treatments followed by a time course that will reveal a species’ capacity for acclimation. Thus, the succession of sensing, signaling, transcriptional and post-transcriptional programs can be sampled. Analyses after gradual, long-term stress imposition, which tends to measure the relative success of acclimation and recovery, will be useful once parameters of the response bandwidth have been established. Armed with information about species- or line-specific stress-response competence, the available tools can then be used to instruct studies on plants grown in the field.

## Genomics technologies

Essential requisites for abiotic stress tolerance are being assembled on a genome-wide scale in an anthological way rather than by hypothesis-driven experimentation. These data will be necessary, however, to allow the further

**Table 1**

### Genomics approaches, tools and platforms for global analyses.

Examples of genomes, approaches, tools and platforms used for comparative sequencing of model species and their close relatives that have contrasting stress tolerance.

<b>Genomes</b>	<i>Lycopersicon esculentum</i> versus <i>Lycopersicon pennellii</i>	[81]
	<i>Hordeum vulgare</i> versus <i>Hordeum spontaneum</i>	[79]
	<i>A. thaliana</i> versus <i>T. halophila</i> ( <i>T. salsuginea</i> )	[11,12]
	<i>Zea mays</i> versus <i>Zea mays</i> ssp. <i>parviglumis</i>	[80]
	<i>A. thaliana</i> ecotypes	[13,14]
<b>Transcriptomes</b>	Condition-specific transcription (in responses to drought, cold/heat and salinity, and for ROS scavenging capacity)	[26]
	cDNA library generation, microarray observations	[18–20,22,27,28]
	Global analysis of spatial and temporal location dynamics	[16*,30,32*,35*]
	Co-regulation/regulon identity	[56]
	SAGE	[22,23]
<b>Protein dynamics</b>	Protein presence and dynamic change	[54**,61,62,63**]
	Enzyme activity control	[36*]
<b>Metabolome</b>	Metabolite identification and profiling	[44–47,50,51*]
	Flux parameters	[52,53]

elucidation of abiotic stress circuits, providing the foundation for detailed studies. In general categories, the existing anthology of data has been obtained from reverse genetics [16\*,17], cDNA libraries, ESTs and serial analysis of gene expression (SAGE)-derived transcript populations [18–23], profiling of stress-responsive transcripts, including coverage of nutritional stresses [24–31], and fluorescence imaging *in vivo* [32\*,33,34,35\*,36\*,37,38].

A vital mission of computational stress genomics is identifying genes that respond to diverse environmental stimuli. Regulation, which is determined by chromatin structure, the binding of transcription factors and *cis*-regulatory DNA sequences, can be inferred computationally by mining transcript profiles and the regional structure and distribution of short response elements in corresponding promoter regions. Several databases for the analysis of plant promoters exist (e.g. <http://www.dna.affrc.go.jp/PLACE> and <http://intra.psb.ugent.be:8080/PlantCARE>). Reconciling stress-regulated genes with promoter structures, and then extracting rules about regulation in a cell and tissue context can, in principle, rely on the increasing number of microarray datasets but genome-wide analyses are still few. These analyses have focused on regulatory networks for which response elements, such as dehydration-responsive element binding protein (DREB) or abscisic-acid-responsive element (ABRE) in plants, or cAMP-responsive element binding protein (CREB) in yeast, are well known [39,40]. The relative paucity of results suggests that additional techniques for the identification of promoter-binding proteins will have to be applied: chromatin immunoprecipitation, protein binding to synthetically generated promoters, and *in vivo* recordings of promoter activity with fluorescent markers [41,42]. Cell-, tissue- and treatment-specific transcript profiles from ablated or laser dissected cells or sorted fluorescently painted cells will be essential on the way towards generating a stressed plant *in silico* [43].

Some integration of approaches has been achieved by combining transcript analysis and metabolite profiling, reflecting the rationale that downstream results of altered transcription might be most easily documented in this way [1\*\*,12,44–46]. The growing attention to metabolite analysis is obvious in the development of metabolite sensors and databases that unite metabolite and transcript profiling [47,48\*,49,50,51\*,52,53]. A recognized deficiency at present is the lack of data on metabolite flux parameters (discussed before in this series [52]), which might be alleviated by the introduction of powerful new instruments and protocols for NMR imaging [51\*]. In contrast, high-throughput protein analyses on the stress proteome are few. Instead, analyses have concentrated on the behavior of individual proteins or protein families [54\*\*,55]. The requirement for the annotation and integration of data has stimulated work on databases and statistical methods [48\*]. Although not specific to the abiotic stress

responses, these tools are essential for the analysis of plant stress data. For example, a superior clustering method, fuzzy k-means [56], assumes that genes could be a part of several pathways, at different weights, and therefore provides flexibility in data analysis that is superior to that provided by classical data clustering. Figure 1 exemplifies how genomics-type technologies can use natural genetic diversity and allele mining to generate phenotypes in model species. Various profiling platforms can assemble information on abiotic-stress-dependent mechanisms that must then be incorporated by bioinformatics into a plant-wide stress database to generate new phenotypes, and to allow the information to be exported to crop species in an iterative fashion.

### Reverse genetics and RNA interference

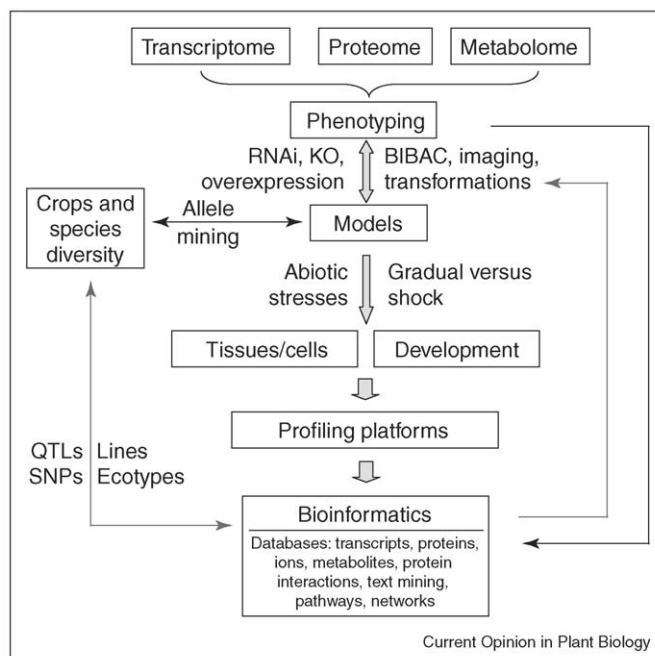
Pioneering work carried out in the 1990s (reviewed in [4]) introduced the concept of an *A. thaliana* lines that is able to express a reporter gene under the control of a stress-inducible promoter. Subsequent random mutagenesis of this transgenic reporter line generates individuals in which an alteration of the stress response can be observed *in vivo*. This approach has matured [16\*] and continues to produce potential new target genes for detailed analyses. Alternatively, improved imaging tools, such as non-destructive thermal imaging [17], provide a means to search for mutants that have, for example, altered stomatal control. The imaging of stresses that are associated with oxygen radicals and of the radical oxygen species that are produced during abiotic stress will add another important dimension to mutant screening. The central role of reactive oxygen species in abiotic stress signaling in *A. thaliana* has recently been highlighted by the identification of a redox-responsive zinc-finger protein, ZAT12 [31]. Manipulating ZAT12 expression indicated that this protein functions in a variety of abiotic stresses. Transcript profiling in overexpressing lines enhanced the expression of oxidative-stress- and light-stress-responding transcripts.

Another new tool depends on RNA interference (RNAi)-based reduction or elimination of specific transcripts or functions [32\*,57]. The RNAi technology, already widely used in plant biology, can relate the transcript levels of stress-responsive genes to the degree of stress tolerance, hence it is especially potent in quantitative abiotic stress studies. A collection of gene-specific sequence tags (GSTs) had been generated for at least 21 500 *Arabidopsis* genes [32\*] and as an application, hairpinRNA expressing lines have been constructed for 8136 different GSTs. When combined with appropriate screening strategies, these resources will greatly improve our knowledge on plant stress tolerance.

### Transcript populations and transcript profiling

EST sequencing, as important as it continues to be for providing a fast overview of abundant transcripts in a

Figure 1



Flow chart of stress systems biology. The chart connects the systems approach to the analysis of plant stress response pathways with gene mining and the transfer of knowledge from models to crops.

species, is being superseded by more sophisticated approaches. Among these are libraries that are enriched for full-length cDNAs [18,19], and normalized and subtracted cDNA libraries [20,21]. Such libraries can be made up of transcripts from multiple experimental conditions in which each RNA is converted into cDNA with an oligonucleotide tag attached that identifies a transcript population [21]. Especially with fully sequenced genomes, SAGE provides, with little effort, hundreds of thousands of tags with each tag identifying the nature and abundance of a transcript [22,23]. Conceivably, further advances in SAGE profiling could make this technique a viable alternative to other transcript profiling platforms.

Transcript profiling, using GeneChips or long-oligonucleotide array slides, provides important insights into the dynamics of the transcriptional changes that accompany abiotic stress treatments. While both array platforms provide comparable results, they are distinguished by their ease of handling, dynamic range, reproducibility, and sensitivity to nucleotide polymorphisms in the targets. Several contributions have reported on changes to transcript profiles under cold, drought, and high-salinity conditions, mainly in *A. thaliana* and rice [12,24–28], and the variety of species in which analyses focusing on stress responses have been undertaken is gradually increasing [28]. In addition, profiling studies have been directed towards understanding transcriptional regulation during

nutrient stresses [29,30] or on probing the effects of stresses on the transcriptome by eliminating regulatory genes that are involved in stress perception or signaling [57].

The precision of global expression profiling is now such that it can report with high confidence stress-dependent changes in transcript abundance that had previously been observed in isolated experiments [12]. The consolidation and integration of individual transcript-profiling results (for which the establishment of data standards will be necessary) into a species-wide and, later, a plant-wide database would provide control and a high level of confidence. The transcript profiles for *A. thaliana* collated in AtGenExpress (<http://www.arabidopsis.org/info/expression/ATGenExpress.jsp>) form one such database. Nonetheless, we have insufficient information about stress-dependent, cell-specific expression patterns (for example, in guard cells, cells in the vasculature or shoot and root meristems) and current technologies do not readily distinguish the presence of different splicing isoforms or tell us how resident transcripts might be utilized in translation [58].

### Imaging of protein location and metabolites

One way to advance stress genomics is provided by imaging tools [33,34,35,36,37,47]. Random fusions of cDNAs with green fluorescent protein (GFP) are one approach [34], which has revealed unexpected

complexities of expression and dynamic changes in cells. Meanwhile, a classical approach, involving a calcium (aequorin) reporter combined with a yellow fluorescent protein (YFP), has been used to monitor cell-type-specific responses to drought, salt and cold in roots. Different imaging applications, such as fluorescence imaging to report ion flux [35<sup>\*</sup>] or metabolite pool dynamics [47], have become available. The more widespread, preferably high-throughput, use of imaging would provide a new dimension to abiotic stress analyses because it would allow us to examine the tissue- and cell-specific action of adaptive protective mechanisms that are not revealed by whole-plant genomics.

### Protein profiling

The importance of protein profiling has long been acknowledged in plant abiotic stress studies. Previous studies have provided useful information on individual enzymes or transporters, measuring their stress-dependent changes in quantity and activity. The results of such studies have formed the current hypotheses on stress-responsive networks, in which protein modifications, protein–protein interactions, stress-dependent protein movements, *de novo* synthesis, and controlled degradation play significant roles, literally spanning the time and space of the stress response. The need for protein studies is also underscored, for example, by the recognition that rapid calcium spikes (which may be modulated in space, amplitude and frequency) lead to protein modifications that precede transcript changes [33]. Consequently, large-scale high-throughput proteome analyses must be integrated with transcriptome and metabolome analyses if we are to obtain a comprehensive understanding of the stress response.

In reality, large-scale proteome studies are still limited for several reasons, expertly summarized in [59]. At present, there are two major approaches to proteome profiling. The first and traditional approach uses two-dimensional gel separation (2D-PAGE) and mass spectrometry to measure changes in protein quantity [60,61]. Although in excess of 1000 proteins can be readily identified, few stress-dependent changes in the quantities of these proteins have been identified, possibly due to problems in isolation or quantification or the inability to recognize protein modification. A second approach focuses on specific modifications of the proteome, such as membrane protein phosphorylation [62] or populations of nitrosylated proteins [63<sup>\*\*</sup>]. Unexpected, yet illuminating results have emerged from this approach, such as very divergent phosphorylation sites of receptor-like-kinases from the same subfamily or the discovery of candidates for NO signaling pathways. (Note that no stress treatments were imposed in the ‘possibility’-type studies that used the second approach; there could only be more possibilities if stress conditions were used.)

Unlike transcriptome analyses, for which mature platforms exist, proteome analyses require novel, specific tool boxes [54<sup>\*\*</sup>,55]. A specific protein chip has been developed for large-scale kinase assays to study the potential substrates of AtMPK3 and AtMPK6, the two mitogen-associated protein kinases (MAPKs) known for their involvement in various stress responses [62]. The identified substrates contained a large number of ribosomal proteins. One interpretation and hypothesis would assume that, when under stress, the MAPKs might directly affect mRNA-loading by changing the phosphorylation state of ribosomal proteins.

### Candidate genes and QTLs

Many of the genes that have been selected for transformation in order to enhance stress responses have functions at the end-points of putative tolerance pathways in a variety of functional categories [64–67]. In a large number of experiments, enhanced tolerance has been documented, at least under controlled conditions, but the transgenic plants often had slower growth rates. The examples of single-gene transfer chosen here, involving genes that have roles in trehalose and spermidine biosynthesis and the overexpression of alkali ion transporter, have to some degree been captured by high-throughput profiling studies. The engagement of these, and other genes, can credibly contribute to tolerance acquisition. It seems equally comprehensible, however, that the engineering of biochemical end-points in the highly regulated plant metabolic network will result in limited gains, and will result in unintended consequences, such as substrate imbalances or signaling disturbance. The second wave of engineering attempts presently targets a select group of genes that encode well-studied stress-responsive transcription factors. The most advanced work is on C-repeat-binding factors (CBFs) and ABRE-binding factors (ABFs) that control the expression of a set of downstream functions in stress acclimation [26,46,68<sup>\*</sup>,69,70]. The overexpression of these regulatory genes usually has a negative effect on plant growth, but the existence several DREB/CBF/ABF gene family members, which are distinguished by domains that provide functional redundancy, might indicate functional diversity within the family [68<sup>\*</sup>,69,70]. At least in one case, growth retardation and stress protection could be separated [68<sup>\*</sup>].

The use of transcription factors will require the engineering of both promoter and coding regions to dissect those elements that govern cross-talk between different abiotic and developmental programs. Thus, it can be imagined that the next higher level of gene expression control, sensing and signaling, could provide promising targets. Very recent studies begin to realize this next phase. The ectopic expression of genes that function in mRNA export [71], protein phosphorylation [67], and calcium sensing [72] has been reported to confer stress tolerance in *A. thaliana*.

Quantitative trait locus (QTL) analysis of those plants whose genome sequences have been entirely, or near completely, sequenced provides another, superior, source for candidate genes. Given the genetic diversity in ecotypes of *A. thaliana* and the many breeding populations and lines in rice and other crops, this approach has flourished [13,73,74]. Recently, a QTL for salt tolerance has been identified. The region could be narrowed to a single gene that encodes a sodium transporter of the HKT-type [75\*].

### Conclusions: fast-forward stress genomics – what next?

A new avenue has been opened by the detection of regulatory systems that depend on small non-coding RNAs in plants [76\*,77\*]. Some siRNAs have been shown to be stress-inducible [76\*]. In addition to affecting the translation process, these siRNAs might participate in the alternative splicing [78] of mRNAs and represent components of an additional level of regulation.

The host of genomics tools has provided a wealth of data and, already, a better understanding of the changes in cellular metabolism that are induced by abiotic stresses, but fewer results have been forthcoming with respect to the functioning of the whole plant. The conversion of the many data points into understanding is still incomplete. Integration and filtering of data and confirmation by independent means in combination with advanced bioinformatics tools will alleviate this deficit. An understanding of plants as a system of interacting functions will emerge, but a more immediate problem seems to be finding applications for all this knowledge.

Searching for and recording quantitative traits has the advantage of an unbiased approach. Also, one important lesson from comparative, functional genomics studies is the recognition by molecular means of enormous adaptive functional diversity in many characters, including stress tolerance. ‘Allele mining’, as it may be termed, can focus on close relatives of the established models for which sufficient genomic resources are available (Table 1). Tremendous genetic diversity that can be exploited exists in *Lycopersicon pennellii*, *Hordeum vulgare* ssp. *spontaneum*, and *T. halophila* (*salsuginea*), relatives of domesticated tomato, barley and *A. thaliana*, respectively [12,13,79–81]. With the *A. thaliana*–*Thellungiella* species combination, it is already possible to transfer, for example, BAC libraries or full-length cDNA populations from the stress-tolerant relative to the stress-sensitive model organism, and to follow this transfer by screening. Such strategies appear possible with several other species combinations, and should provide a way to harness the existing evolutionary adaptive diversity to develop stress-protected crops in which growth and yield are less compromised by abiotic stresses. The search for stress-tolerance alleles that retain growth and yield and the provision of the

knowledge to breeding programs present the real challenge for plant genomics.

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