

Abiotic Stress and Plant Genome Evolution. Search for New Models

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The remarkable ability of plants to adapt to many different adverse environments is a fascinating process. Research into the physiology and metabolism of so-called extremophiles not only fosters better understanding of the evolutionary processes that have created the diversity of life as it exists on earth, but also has economic implications for agricultural biotechnology and the development of novel products. The capacity to sequence genomes and the availability of novel molecular tools have now catapulted biological research into eras of genomics and post-genomics, creating an opportunity to apply genomic techniques to extremophile models. This has led plant scientists to search for such models among the relatives of *Arabidopsis* (*Arabidopsis thaliana*), the most universally used species in molecular plant research owing to its many technical advantages and the wealth of available biological information.

A workshop held in Paris in September 2004 united scientists from the United States, Canada, Japan, Israel, and Europe under the header Integrating International Research on Plant Abiotic Stress Tolerance Using *Arabidopsis* Relative Model Systems (ARMS): *Thellungiella halophila*. The aim of this Biotechnology and Biological Sciences Research Council (BBSRC)-funded meeting, coorganized by Anna Amtmann (University of Glasgow, UK) and Arnould Savouré (University of Paris VI, France), was to explore the use of *Thellungiella* as a model extremophile and to develop strategies for its development by the international community.

STRESS TOLERANCE

T. halophila (salt cress; synonymous to *Thellungiella salsuginea*; Al-Shebaz et al., 1999) is a close relative of *Arabidopsis*, with a genome size approximately twice that of *Arabidopsis*. In sharp contrast with *Arabidopsis*, *Thellungiella* tolerates extreme cold, drought, and salinity (Bressan et al., 2001; Inan et al., 2004; Taji et al., 2004). Arie Altman (Hebrew University, Jerusalem)

reported maintenance of constant water contents in *Thellungiella* treated with 600 mM NaCl that allowed protection of *Thellungiella* shoot meristems from desiccation, supporting fast recovery from extreme stress. Several groups at the meeting confirmed these observations.

ION TRANSPORT

There was a general consensus among conferees that differential salt tolerance of the two species is mirrored by differential ion homeostasis. In contrast with *Arabidopsis*, *Thellungiella* restricts Na⁺ accumulation in the shoots while maintaining superior uptake capacity for K⁺ (Inan et al., 2004; Volkov et al., 2004). Participants agreed that the study of ion transporters and their regulation will be a central topic of future *Thellungiella* research.

Ion transport pathways in *Thellungiella* responsible for Na⁺ and K⁺ uptake are studied in the laboratory of Anna Amtmann (Volkov et al., 2004) using electrophysiological techniques. Ion channels in *Thellungiella* root cells exhibit higher K⁺/Na⁺ selectivity than the respective channels in *Arabidopsis*. Also, x-ray microanalysis of ions in root sections showed a clear gradient of K⁺ and Na⁺ from epidermal tissues to the central cylinder (Arie Altman). Results reported by Wieland Fricke (University of Paisley, UK), using single-cell sampling techniques, indicate that *Thellungiella* and *Arabidopsis* differ in both uptake and release of K⁺ into/from leaf epidermal cells in high salinity. Participants agreed that future research will have to take into account tissue and membrane localization of individual transporter genes. It is anticipated that tissue-specific transporters for uptake of K⁺, export of Na⁺, and compartmentation of both ions play important roles in *Thellungiella*'s exceptional ability to control cellular and tissue ion homeostasis. Important differences in both the transporters themselves and their regulatory components between *Thellungiella* and *Arabidopsis* are likely to make significant contributions to the dazzling difference in salt tolerance of the two species. Several groups at the meeting presented plans and ongoing work to investigate these characteristics of *Thellungiella*, including the role of regulatory 14-3-3 proteins by Bert DeBoer's group and glycogen synthase

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kinases by Eric Souer's group (Vrije University, Amsterdam). Jian-Kang Zhu (University of California, Riverside, CA), Hans Bohnert (University of Illinois at Urbana-Champaign, IL), and José Pardo (INRA-CSIC, Seville, Spain) reported on efforts to examine physiological, biochemical, and genetic properties of the SOS1 and NHX proteins from *Thellungiella*, utilizing full-length clones and RNA interference (RNAi) lines for genes in the SOS signaling pathway (Zhu, 2003).

BIOCHEMISTRY

Another important aspect of salt tolerance is the accumulation of compatible solutes in the cytoplasm to osmotically balance the ions that accumulate in the vacuole during salt adaptation (Inan et al., 2004). *Thellungiella* is a dramatic Pro accumulator (Inan et al., 2004; Taji et al., 2004) and provides an exciting opportunity to study unique aspects of Pro-related signaling pathways in determining fitness under extreme environmental stress conditions. Possible new regulatory components that are responsible for the exceptional ability of *Thellungiella* to accumulate Pro were reported by several labs, including a possible role of phospholipase D (Arnould Savouré's group) and the roles of abscisic acid and jasmonate (Alain Bouchereau and colleagues, University of Rennes, France). Results of metabolite profiling for a range of compounds were reported by Elizabeth Weretilnyk (McMaster University, Hamilton, Canada) for the Yukon ecotype using plants exposed to salt or reduced watering in growth chambers and, for comparison, plants harvested at field sites in the Yukon. Such analyses will complement existing functional genomics methodologies.

The chemical composition of leaf cuticular waxes from *Arabidopsis* and two *Thellungiella* ecotypes has been analyzed (Teusink et al., 2002), and Hui Zhang (Shandong Normal University, Jinan, China) and colleagues generated *Thellungiella* lines with altered wax production using RNAi technology. Apart from their importance for research into epicuticular wax function in stress tolerance, these results are encouraging from a technical point of view as they show that RNAi strategies are applicable to *Thellungiella*.

GENE EXPRESSION

High DNA sequence homology allows for the use of *Arabidopsis* microarray platforms for expression profiling of *Thellungiella*. Full-length cDNA arrays (Seki et al., 2002) and arrays based on long oligonucleotides (Maathuis et al., 2003) have been used with good results: 70% to 90% of the probes produced significant signal intensity (communicated by Anna Amtmann, Hans Bohnert, and Teruaki Taji, Riken Institute, Tsukuba, Japan). By contrast, arrays employing very short oligonucleotide probes based on the *Arabidopsis* genome sequence are not suitable (approximately 20%

signal; Volkov et al., 2004). Initial transcript profiling (Inan et al., 2004; Taji et al., 2004) revealed that a similar level of salinity induces fewer genes in *Thellungiella* than in *Arabidopsis*. However, under salt-free conditions, *Thellungiella* orthologs of some stress-related *Arabidopsis* genes show high base levels of expression. Conclusions from these studies and additional results presented in Paris are, first, that salt stress-induced gene expression in *Arabidopsis* might reflect a panic attack rather than specific stress acclimation, and, second, that comparative analysis of gene expression in tolerant and sensitive species can identify genes that are at the basis of stress adaptation. Both points emphasize the tremendous potential of extremophile ARMS for understanding plant stress tolerance.

Arabidopsis arrays deliver information about close homologs of *Arabidopsis* genes, but only a *Thellungiella* genome sequence and *Thellungiella*-specific transcript profiles can identify the novel stress-responsive genes that have evolved in this species. Barbara Moffatt (University of Waterloo, Canada) reported on the isolation of >3,000 nonredundant expressed sequence tags (ESTs) from cDNA libraries of the Yukon ecotype and spotting them onto slides. Hybridizations with this *Thellungiella* array have identified several species-specific genes that are regulated by salt, drought, and/or cold. These genes are being examined using genetic and molecular analyses. An analysis by cDNA-AFLP of about 3,000 transcripts in the laboratory of Erik Souer yielded 17 *Thellungiella* genes that were either up- or down-regulated by salt stress.

GENOMIC TOOLS

What promotes *Thellungiella* from a mere curiosity to a model system is its amenability to molecular techniques and, hence, its accessibility for genomic and post-genomic approaches. Indispensable molecular tools for *Thellungiella* are under construction in the United States, Canada, China, and Japan. Altogether 1,800 EST sequences from the Shandong ecotype have been deposited at the National Center for Biotechnology Information under the names *T. halophila* and *T. salsuginea* (Wang et al., 2004), and their similarity to *Arabidopsis* sequences has been evaluated (Inan et al., 2004). More cDNA libraries from stressed plants have been created and were reported at the meeting. For example, Barbara Moffatt's group constructed three cDNA libraries from cold-, salt-, and drought-treated *Thellungiella*, Yukon ecotype, as well as four libraries from PCR subtraction (two for cold, one for drought, and one for salt stress). A total of 6,200 clones were sequenced, revealing approximately 50% redundancy. Teruaki Taji communicated progress on the production of full-length cDNA libraries from *Thellungiella* grown under salt, cold, and abscisic acid treatments. Added sequence tags allow tracing clones back to the original treatment sample. These libraries will be available to interested researchers.

Jian-Kang Zhu introduced work of several groups in China active in *Thellungiella* research and outlined new genomic tools available, including a BIBAC2 expression library (Qi Xie, Chinese Academy of Sciences, Beijing) and libraries in GATEWAY vectors for overexpression and green fluorescent protein labeling of *Thellungiella* proteins in *Arabidopsis* (Chengbin Xiang, University of Science and Technology, Anhui, China). Transgenic *Arabidopsis* lines are being screened for increased salt tolerance and patterns of green fluorescent protein expression. Ethylmethane sulfonate and T-DNA insertion mutant lines of *Thellungiella* have been created by Jian-Kang Zhu and Ray Bressan (Purdue University, West Lafayette, IN). Ongoing screens include germination and growth on salt, rescue from salt, growth on the Pro analog azetidine, and lack of vernalization requirement. These lines are available to interested researchers for screening projects.

A collection of promoter regions of stress-induced *Thellungiella* genes and RNAi lines for assessment of stress relevance were produced by Hans Bohnert's group. Some BAC sequences have now been obtained by Hans Bohnert and Jian-Kang Zhu. Comparison with the *Arabidopsis* genome indicates syntenic regions that show insertions absent from the *Arabidopsis* genome.

Whereas most groups represented at the Paris meeting work with the Shandong ecotype originating from the seacoast of northeast China, Marilyn Griffith (University of Waterloo, Canada) and colleagues collected a *Thellungiella* ecotype from alkaline salt flats in the Yukon Territory of Canada. The habitat of ecotype Yukon is characterized by a semiarid climate with a very short growing season. The permafrost soils are saturated with calcium carbonate, magnesium sulfate, and sodium chloride and are deficient in essential macronutrients. This ecotype is also far more freezing tolerant than *Arabidopsis*, with LT_{50} values of -13°C for nonacclimated plants and -19°C when acclimated.

Hui Zhang, Ziyi Cao (China Agriculture University, Beijing), and Ray Bressan collected additional ecotypes that are being developed as separate lines. In addition to ecotypes Shandong and Yukon, the collection includes ecotypes from Colorado and Xinjiang and other provinces in China with extreme climate conditions, which should greatly facilitate map-based cloning of mutated genes. Ray Bressan reported that about 80% of EST loci examined between Shandong and Yukon ecotypes showed single nucleotide polymorphisms. Seeds of another recognized *Thellungiella* species, *Thellungiella parvula*, have been collected in Central Turkey and are available from the *Arabidopsis* Biological Resource Center in Columbus, Ohio. This species is as salt tolerant as *T. halophila (salsuginea)* but has distinct morphological features and shows additional flood tolerance. Physiological differences between *Thellungiella* ecotypes and species are currently being characterized, with particular emphasis on differences in germination and flowering times.

PRACTICAL ISSUES

Protocols for physiological experiments, plant maintenance, and molecular techniques were discussed at workshop sessions. *Thellungiella* exhibits the typical extremophile features that can pose difficulties when adapting protocols originally established for *Arabidopsis*. In particular, extended periods of germination and flowering, especially under nonoptimal conditions, have to be taken into account when carrying out phenotypic analysis and transformation. Ray Bressan discussed practical tips on plant health, stratification, and vernalization requirements and for the improvement of transformation rates. This and other practical information may be retrieved from (or contributed to) the Web site <http://thellungiella.org>. The participants recognized that the individual choice of experimental material (field or laboratory) and treatments (identical stress conditions or conditions that provoke similar stress) depended on the particular question investigated. The group decided on a list of parameters that should be considered and documented in future reports, to be available at the Web site. Workshops also identified knowledge gaps in *Thellungiella* characteristics, including physiological parameters (e.g. membrane potential, cellular morphology, seed viability) and accompanying factors (e.g. developmental stage, light, ionic background), that still await systematic evaluation.

CONCLUSION

Thellungiella research is well under way, generating results on physiology, gene expression, and biochemistry that distinguish this extremophile from its helpless cousin *Arabidopsis*, making it a valuable model for the study of abiotic stress tolerance. Genomic tools in place and being created will amplify its potential as an experimental system. Research efforts in the following areas should be intensified: (1) transporter genes and signaling pathways affecting ion homeostasis under salt stress; (2) the biochemistry and regulation of metabolic pathways leading to the synthesis of compatible solutes and maintenance of metabolic activity under stress; and (3) the genes involved in protein protection and injury repair during extreme salt shock, drought, and cold stress.

Essential resources needed for the immediate future are as follows. (1) Transcript libraries. In particular, tagged full-length libraries will facilitate the identification of *Arabidopsis*/*Thellungiella* paralogs and the discovery of novel alleles through reciprocal transformation studies. (2) Saturation mutagenesis of the genome. Collections of T-DNA insertion lines for forward and reverse genetic approaches to discover stress-related genes require consistent and efficient transformation protocols for *Thellungiella*. This will make possible the in planta functional dissection of genes through the exciting ability to exchange genes

and gene domains and promoters between the two species. (3) A genomic-scale effort to screen gain-of-function lines using BIBAC2 transformation of *Thellungiella* genes into *Arabidopsis*. (4) A genome sequence. To allow the plant science community to fully exploit the tremendous potential of this extremophile model, large-scale sequencing of the *Thellungiella* genome was considered a most urgent issue. The *Thellungiella* genome sequence could immediately be merged with the superior *Arabidopsis* tools and thus provide ample opportunity for comparative functional genomics, e.g. an immediate tool box of PCR markers for map-based cloning, for gene identification from insertion tag cloning, etc.

Jian-Kang Zhu emphasized the importance of data sharing within the scientific community as a requisite for progress, and all participants expressed their wish to intensify collaboration. As a result of the meeting, a Web page is developing (<http://thellungiella.org>) that will give users access to genomic tools, research data, and technical protocols. Future meetings, open to all interested, will be announced on this page. We invite the community to consider the *Thellungiella* model for their research and to take advantage of its obvious potential to deepen our understanding of plant genome evolution and abiotic stress responses. The Amalfi coast of Italy is under consideration for a follow-up meeting to begin to reduce stress on the participants.

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