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meeting report

Plants, genes and ions

Workshop on the molecular basis of ionic homeostasis and salt tolerance in plants

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A meeting on the molecular basis of ionic homeostasis and salt tolerance in plants took place in Madrid, Spain, October 22–24, 2001. This meeting was organized by Eduardo Blumwald (Davis, CA) and Alonso Rodriguez-Navarro (Madrid, Spain) at the Centre for International Meetings on Biology ('Instituto Juan March de Estudios e Investigaciones').

Introduction

Ionic homeostasis is a fundamental cellular phenomenon. All living cells maintain an intracellular ionic composition compatible with their constituent molecules, and this requires the regulation of multiple membrane transporters and signal transduction pathways. Other biophysical parameters such as turgor and electrical potential are also part of this essential regulation. How ionic homeostasis is achieved, however, is not completely understood. Although most transporters have already been identified, their physiological function is only starting to be demonstrated and the receptors and most components of the regulatory pathways that effect ionic homeostasis remain unknown. In the case of plants, this problem is related to mineral nutrition and salinity tolerance, both of which have great relevance for agriculture. In fact, as demonstrated by this meeting, salinity stress has been one of the keys to opening the black box of ionic homeostasis in general. Another has been the novel molecular genetics of the plant *Arabidopsis thaliana*. Of course, other approaches have also contributed to our present understanding of ion homeostasis in plants and were represented at the meeting. For further details, see Blumwald (2000), Hasegawa *et al.* (2000), Bohnert *et al.* (2001), Serrano and Rodriguez-Navarro (2001) and Zhu (2001).

Some physiology of salt tolerance

Salt stress is an important threat to the future of agriculture in many productive areas of the planet. In countries such as Australia and Pakistan, salinity is already a national concern, as it was in the past in ancient Mesopotamia. Areas of California and the Mediterranean region are also threatened.

The greatest challenges faced by plants cultivated in the presence of excess salt are osmotic regulation, ion transport and toxicity and oxidative stress. The signal transduction pathways that are involved are not completely understood but include calcium-activated protein kinases, stress-activated MAP (mitogen-activated protein) kinases and the hormone abscisic acid (ABA) (Figure 1). Different speakers discussed these physiological aspects. Salinity stress results in increased production of reactive oxygen species, and detoxifying enzymes such as ascorbate peroxidase and glutathione peroxidase (G. Ben-Hayyim, Bet Dagan, Israel) are important components of the cellular response to salt stress. Plants rarely experience stress from a single environmental source, and multi-stress interactions were demonstrated by salt-stressed plants whose state was exacerbated by the resulting increase in their uptake of toxic boron and cadmium (A. Laüchli, Davis, CA). Genetic studies in wheat have demonstrated the important role of Na^+ exclusion from the shoot in salt tolerance. This Na^+ exclusion trait may be explained by K^+/Na^+ discrimination during xylem loading and the genes responsible are being approached by molecular markers (R. Munns, Canberra, Australia). It is probable that these will encode components of cation uptake and efflux systems. The synthesis of organic molecules known as 'osmolytes' is an important stress response for osmotic adjustment and the stabilization of cellular structures. A.D. Hanson (Gainesville, FL) described a very

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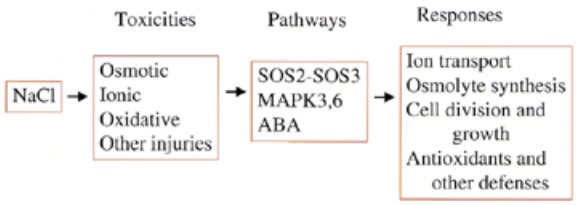

Fig. 1. Plant responses to salt stress start with the perception of the osmotic, ionic oxidative and other injuries through signal transduction pathways involving calcium-activated protein kinases like SOS2–SOS3, MAP kinases like AtMPK3,6 and ABA. Responses are effected by transcription factors like DREBs/CBFs.

ambitious, long-term project on the metabolic engineering of glycine betaine, one of the most effective osmoprotectants and naturally found in salt- or drought-tolerant plants like corn or sugarbeet. Manipulating the synthetic pathway in plants, such as tobacco, that are unable to accumulate this osmolyte is proving difficult, however, because successive bottlenecks need to be overcome. For example, once the conversion of choline into glycine betaine was increased by overexpression of chloroplast choline monooxygenase and betaine aldehyde dehydrogenase, the limiting role of choline synthesis and uptake by the chloroplast became apparent and only a modest accumulation of glycine betaine was obtained.

Targets of salt toxicity

A major gap in the understanding of salt toxicity is the nature of the targets at the cellular level. The cell division cycle is one such target and the activity of the cyclin-dependent kinase (CDK) complex is decreased in salt-stressed Arabidopsis roots. Reduced expression of cyclins, inhibitory phosphorylation of the threonine–tyrosine pair at the regulatory loop of CDK and increased expression of the CDK inhibitor ICK1 all contribute to the salt inhibition of this crucial kinase. This inhibition is important for salt adaptation because transgenic Arabidopsis plants expressing a constitutively active CDK, although more resistant in the short term, are hypersensitive during prolonged salt stress (G.T.S. Beemster, Gent, Belgium). Another important target of salt toxicity seems to be RNA processing, because overexpression of serine-arginine-rich (SR) proteins involved in this phenomenon improves the salt tolerance of both yeast and Arabidopsis (O. Vicente, Valencia, Spain).

Seeds germinated in the presence of salt synthesize ABA, which may serve to inhibit growth under hostile conditions. This property allows mutants in the pathways for ABA biosynthesis and perception to be isolated and was used by P. L. Rodriguez (Valencia, Spain) to clone the *ABA2* biosynthetic gene. This is the latest ABA biosynthetic gene to be identified and it corresponds to an alcohol dehydrogenase, catalysing the oxidation of xanthoxin to ABA-aldehyde. As with constitutively active CDK mutants, ABA deficient or insensitive mutants are only more salt resistant in the short term (germination assay), being hypersensitive during prolonged salt stress.

Sodium and potassium transporters

In order to survive extreme saline conditions, plants must maintain a high cytoplasmic K^+/Na^+ ratio and therefore must be efficient at K^+ uptake in a high Na^+ background and be able to exclude or remove Na^+ from the cytoplasm. H. Sentenac (Montpellier, France) presented a systematic study of the different isoforms of the K^+ channels of Arabidopsis. The approaches used included the characterization of knock-out mutants, determination of the expression patterns in plant tissues and expression of recombinant proteins in yeast and insect cells. The inward rectifier AKT1 is a major route of K^+ uptake from soil by root epidermis. The outward rectifier SKOR present at the root stele largely mediates xylem loading of K^+. In guard cells the inward rectifiers KAT1 and KAT2 participate in stomata opening, whereas the outward rectifier GORK is required for stomata closing. The pollen-specific AKT5 (inward rectifier) is required for pollen tube development. Other isoforms that are less well characterized include the phloem-specific AKT2 and AKT3, the flower-specific AKT6 and KC1. These channels are highly selective for K^+ over Na^+ and therefore are unlikely to mediate Na^+ transport during salt stress.

One major pathway for Na^+ uptake is blocked by external calcium and occurs via non-selective cation channels. D. Sanders (York, UK) presented evidence for members of the cyclic-nucleotide-gated channel (CNGC) family, specifically Arabidopsis CNGC1 and CNGC3, being mediators of such activity. Expression of CNGC3 in yeast increased Na^+ uptake, whereas an Arabidopsis knock-out mutant in CNGC1 was more tolerant to moderate Na^+ concentrations.

The other, calcium-insensitive, pathway for Na^+ uptake was clarified by the poster of A. Rus (West Lafayette, IN), winner of the special poster award of the workshop, and also discussed in the presentation by R.A. Bressan (West Lafayette, IN). His laboratory is collaborating with those of P.M. Hasegawa (West Lafayette, IN), J.-K. Zhu (Tucson, AZ) and M. Reddy (Bhavanagar, India) to perform a screen for suppressor mutations of the salt-sensitive Arabidopsis *sos3* mutant, which hyperaccumulates Na^+. From >65 000 T-DNA-tagged lines derived from the *sos3* mutant, two null alleles of the HKT1 transporter were identified as suppressors of both the Na^+ sensitivity and Na^+ hyperaccumulation of the *sos3* mutant. These results constitute the *in vivo* demonstration that HKT1 is an entry system for Na^+. Whether SOS3 simply modulates the SOS1 Na^+ efflux system or also the HKT1 influx system is still unclear.

E. Blumwald (Davis, CA) presented data on transgenic tomato and rapeseed (Canola) plants overexpressing the Arabidopsis NHX1 vacuolar Na^+–H^+ antiporter, and the resulting salt tolerance was impressive (Zhang and Blumwald, 2001; Zhang *et al.*, 2001). However, it was argued by some participants that the resistance observed, compared with the control plants, may have been dependent on the salinization regime, as a gradual increase in salinization resulted in greater tolerance of the transgenic plants, which had not always been observed in other laboratories.

A. Rodriguez-Navarro (Madrid, Spain) discussed the ENA-like cation extrusion ATPase, which is a major determinant of salt tolerance in fungi but is absent from plants. Members of the family exhibit different relative activities with Na^+ and K^+ as the pumped cations. The natural history of the enzyme suggests that

it was originally a K^+-extrusion pump of fungi associated with plants and therefore exposed to high external K^+ concentrations. The most Na^+-specific members, such as the *Neurospora crassa* ENA-like ATPase, could be useful tools to improve salt tolerance in plants when specifically expressed in root epithelia.

Evaluating transgene impact on salt tolerance

A group of participants led by A. D. Hanson took the initiative to define a series of rules, which should be followed in order to more accurately evaluate the impact of transgenes in stress tolerance and to allow a consistent comparison between tolerance evaluations performed in different laboratories on various genotypes.

(i) Establish that the stress-tolerant phenotype of interest is shown by several, e.g. 10, independent transgenic lines. this avoids being misled by insertional mutagenesis, positional effects and somaclonal variation.

(ii) A comparable number of control lines should be used. In addition to untransformed controls, plants transformed with the empty vector should be used.

(iii) For progeny of primary transformants, work with single-insert lines. After selfing (self-fertilization), compare the homozygotes and the azygotes (each one-quarter of progeny).

(iv) Carry out stress tests using standard statistical blind comparisons, in which the researcher is unaware of the plant's identities.

Signal transduction pathways

J. Sheen (Boston, MA) illustrated the use of the Arabidopsis protoplast transient expression assay to dissect MAP kinase cascades. One common second messenger produced by diverse stress stimuli, H_2O_2, activates a cascade composed of AtANP1 (a MAP kinase kinase kinase) and AtMPK3,6 (two MAK kinases). This pathway represses auxin-inducible genes (*GH3* and *ER7*) and induces stress defence genes (*GST6* and *HSP18*). Truncation of the regulatory domain of AtANP1 creates a constitutively active kinase, which, upon expression in transgenic plants, improves the tolerance to multiple stresses such as cold, heat, drought and salinity.

This cascade was also investigated by K. Shinozaki (Tsukuba, Japan), who placed MKK4,5 (two MAP kinase kinases) between the two steps defined above. Shinozaki also described recent work on ATHK1, a two-component histidine kinase homologous to the yeast osmosensor Sln1, which is able to complement the *sln1* mutation. Sln1 is a negative regulator of the HOG1 MAP kinase pathway, which is counteracted by osmotic stress. Using the yeast system, Shinozaki isolated a dominant negative mutation of ATHK1, which, upon expression in transgenic Arabidopsis, caused a constitutive stress response involving many genes such as *rd22* and *MYB2*, improved tolerance to drought, salt and cold and resulted in some growth retardation. However, the regulation of the AtANP1 pathway by ATHK1 has still not been demonstrated.

One important mechanism of signal transduction during salt stress is the activation of the SOS1 plasma membrane Na^+–H^+ antiporter by the SOS2–SOS3 protein kinase complex. Zhu described the mechanism of regulation of the SOS2 protein kinase subunit by the calcium-binding SOS3 subunit. A 21 amino acid region within the regulatory domain of SOS2 (designated as the FISL motif) is both necessary and sufficient for binding to SOS3, and deletion of this motif results in constitutively active SOS2.

J.M. Pardo (Sevilla, Spain) utilized the yeast model system to characterize different isoforms (NHX1, NHX2 and NHX5) of the Arabidopsis vacuolar Na^+–H^+ antiporters. He also utilized yeast to characterize the plasma membrane SOS1 Na^+–H^+ antiporter identified by Zhu. The most impressive result, which allowed Pardo to win the special 'wine award' of the meeting, was the reconstitution of the SOS1 regulatory pathway in yeast (a collaboration with Zhu). The activity of the antiporter increased dramatically upon co-expression of the SOS2–SOS3 protein kinase activating system and the resulting phosphorylation of the SOS1 protein.

The yeast model system was used once again to show that, through its regulation of the K^+ transporter Trk1 and Trk2, the protein phosphatase Ppz1 is a key determinant of pH homeostasis, cell cycle progression and cell wall integrity (R. Serrano, Valencia, Spain). This highlights a connection between ionic homeostasis and other fundamental cell processes that can be extrapolated to plants.

Genomic approaches

High-throughput analysis systems are now replacing the classical gene-by-gene approaches in studies of gene expression and function. H.J. Bohnert (Urbana, IL) reported a genome-wide analysis of transcriptional responses to salinity stress in organisms ranging from yeast to higher plants. About 8% of all transcripts are responsive in every organism (500–3000 genes). In rice, where the analysis was most complete, it seems that the early response (in the first hour) to salt stress is very important for tolerance. It includes many transcripts required for signal transduction pathways and is more apparent in salt-tolerant varieties such as Pokkali than in salt-sensitive ones such as IR64 or IR29.

The number of T-DNA-tagged Arabidopsis mutant lines generated by the laboratory of Bressan and Hasegawa is reaching genome saturation (>300 000 lines). Now the collection is being screened for salt tolerance/sensitivity phenotypes, promising an exhaustive genetic analysis of the phenomenon. In addition, >60 000 T-DNA-tagged lines have been generated in a background expressing an rd29::luc fusion, with the stress-responsive rd29 promoter driving luciferase expression. Mutants with altered regulation of the rd29 promoter are currently being isolated.

Conclusions and perspectives

From a retrospective point of view, the use of Arabidopsis for a genetic analysis of salt tolerance has enabled a breakthrough in our understanding of the transporters and signalling pathways that determine ionic homeostasis in plants. The pioneering effort initiated by Zhu was widely recognized during the meeting, and it was clear that the massive collections of T-DNA-tagged mutants generated by Bressan and Hasegawa contain all the

necessary elements to eventually solve the remaining ionic homeostasis questions.

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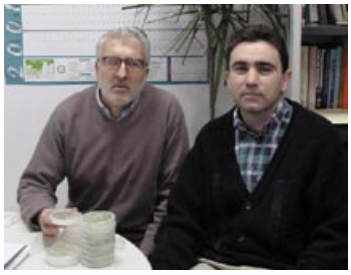

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