Plant salt tolerance

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Soil salinity is a major abiotic stress in plant agriculture worldwide. This has led to research into salt tolerance with the aim of improving crop plants. However, salt tolerance might have much wider implications because transgenic salt-tolerant plants often also tolerate other stresses including chilling, freezing, heat and drought. Unfortunately, suitable genetic model systems have been hard to find. A recently discovered halophytic plant species, *Thellungiella halophila***, now promises to help in the detection of new tolerance determinants and operating pathways in a model system that is not limited to** *Arabidopsis* **traits or ecotype variations.**

Soil salinity existed long before humans and agriculture but the problem has been aggravated by agricultural practices such as irrigation. Today, ~20% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity[1]. High concentrations of salts cause ion imbalance and hyperosmotic stress in plants. As a consequence of these primary effects, secondary stresses such as oxidative damage often occur.

In this article, salt-tolerance mechanisms in plants are discussed in connection with the different uses of plant genetic modification to improve salt tolerance in various ways. Multiple genetic model systems that are crucial for elucidating tolerance mechanisms are presented in a review that aims to fit various reported salt tolerance determinants, regulations and genetic improvements into a proposed network of pathways. The intention is not simply to compile facts but rather to present some ideas about salt tolerance studies in the hope of stimulating new discussion in this field.

Aspects of plant salt tolerance

High salt stress disrupts homeostasis in water potential and ion distribution. This disruption of homeostasis occurs at both the cellular and the whole-plant levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death. To achieve salt tolerance, three interconnected aspects of plant activities are important (Fig. 1). First, damage must be prevented or alleviated. Second, homeostatic conditions must be re-established in the new, stressful environment. Third, growth must resume, albeit at a reduced rate.

Detoxification

The nature of the damage that high salt concentrations inflict on plants is not entirely clear. The integrity of cellular membranes, the activities of various enzymes, nutrient acquisition and function of photosynthetic apparatus are all known to be prone to the toxic effects of high salt stress. An important cause of damage might be reactive oxygen species (ROS) generated by salt stress. Plants subjected to salt stress display complex molecular responses including the production of stress proteins and compatible osmolytes[2]. Many of the osmolytes and stress proteins with unknown functions probably detoxify plants by scavenging ROS or prevent them from damaging cellular structures.

Most of the transgenic improvements in plant salt tolerance reported to date have been achieved through this detoxification strategy. This is obvious in the case of transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases[3,4]. More recent engineering with the regulatory protein NPK1, a mitogen-activated protein (MAP) kinase, is another good example; this protein kinase appears to mediate oxidative stress responses[5]. Support for the importance of oxidative protection in salt tolerance also comes from the characterization of the *Arabidopsis* mutant *pst1*, which has a mutation in an as yet unknown putative negative regulator of oxidative stress responses[6]. The *pst1* mutant plants are more resistant to high salt concentrations, and this is correlated with an increased capacity to tolerate oxidative stress.

Engineering with osmolytes such as mannitol, fructans, trehalose, ononitol, proline, glycinebetaine and ectoine also probably works through oxidative detoxification. These osmolytes are active in scavenging ROS (Ref. 7). In addition, targeted production of the osmolytes in the chloroplast by placing a signal sequence in front of the engineered enzymes, results in better protection[7]. This is consistent with the notion that chloroplasts are the primary site of ROS production. Another reason for believing that osmolytes act in this way is that the levels of osmolytes in the transgenic plants are generally too low to be significant in osmotic adjustment. Finally, the osmolyte-producing transgenic plants are improved in tolerance not only to salts but also to various other stresses such as chilling, freezing, heat and drought, which also generate ROS (Ref. 8). This is demonstrated clearly in glycinebetaine-producing plants[9,10]: *Arabidopsis* plants transformed with a modified bacterial gene for choline oxidase accumulated glycinebetaine in chloroplasts and were more tolerant to salt, cold and heat stress than wild-type plants[11].

Improvements provided by proteins such as barley HVA1 (Ref. 12) and CBF/DREBs (Ref. 13) in transgenic plants might also be credited to the detoxifying effect of the expressed protein or its downstream target proteins. One hallmark of the

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Fig. 1. The three aspects of salt tolerance in plants (homeostasis, detoxification and growth control) and the pathways that interconnect them; homeostasis is broken down into ionic and osmotic homeostasis. The SOS pathway mediates ionic homeostasis and Na$^+$ tolerance. A mitogen-activated-protein kinase (MAPK) cascade similar to the yeast HOG1 pathways is proposed to mediate osmotic homeostasis. The two primary stresses, ionic and osmotic stresses, cause damage or secondary stresses such as oxidation. Lea-type stress proteins such as RD29A are proposed to function in the detoxification or alleviation of damages. CBF/DREB transcription factors mediate some of the stress protein gene expression in response to secondary stresses caused by high salt concentrations, cold, drought or abscisic acid (ABA). The ionic homeostasis, osmotic homeostasis and detoxification pathways are proposed to feed actively into cell division and expansion regulation to control plant growth.
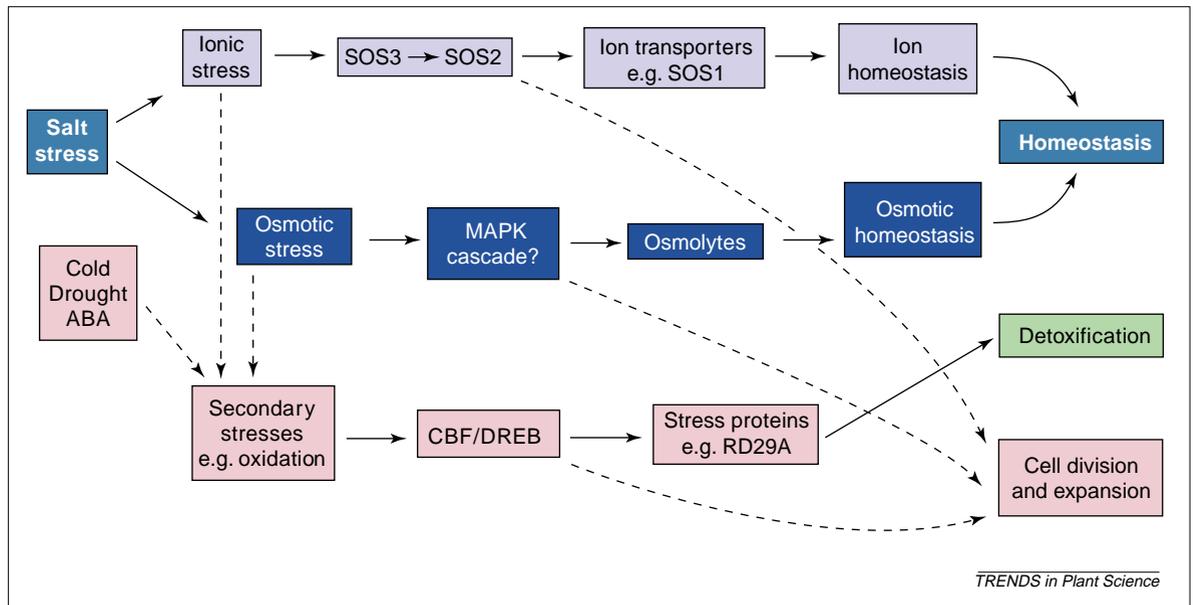
detoxification effect is its lack of specificity; that is, the transgenic plants have increased tolerance not only to high salts but also to drought, cold and, in some cases, even heat shock. For example, transgenic plants overexpressing CBF/DREB proteins have improved tolerance to drought, salinity and freezing stress[13]. The CBF/DREB transcription factors can bind to the DRE/CRT element that is found in the promoters of some stress-responsive genes[13,14]. Therefore, ectopic expression of these transcription factors leads to the expression of their target genes regardless of stress. The biochemical function of the target stress-responsive genes, which can be loosely considered as leas or dehydrins, is not known. One of them, COR15A, seems to interact with cellular membranes to discourage the formation of harmful membrane structures[15].

Homeostasis
Another strategy for achieving greater tolerance is to help plants to re-establish homeostasis in stressful environments. Both ionic and osmotic homeostasis must be restored. Various ion transporters are the terminal determinants of ionic homeostasis. Because Na$^+$ inhibits many enzymes, it is important to prevent Na$^+$ accumulating to a high level in the cytoplasm or in organelles other than the vacuole. To this end, Na$^+$ entry should be prevented or reduced. Nonselective cation channels (NSCs) are proposed to mediate Na$^+$ entry into plant cells[16], although their molecular identities are not yet known. Transporters such as HKT1 and LCT1 have been shown to be permeable to Na$^+$ in oocytes and yeasts[17,18]. Therefore, these gene products, which were originally isolated as K$^+$ transporters, might also mediate Na$^+$ influx into plant cells. An important goal of salt tolerance studies has been to determine which transporter(s) function in Na$^+$ entry into plant cells to find a way to block Na$^+$ influx and thus to achieve increased salt tolerance.

One approach to functional identification of Na$^+$ influx mechanisms would be to screen for additional second-site mutations that partially suppress the *sos2* or *sos3* salt-hypersensitive phenotypes. Because the *sos2* and *sos3* mutants of *Arabidopsis* have increased Na$^+$ accumulation[19], mutations in Na$^+$ influx transporters might partially alleviate their salt hypersensitivity.

Any Na$^+$ that manages to get into cells can be stored in the vacuole or exported out of the cell. Na$^+$ compartmentation is an economical means of preventing Na$^+$ toxicity in the cytosol because the Na$^+$ can be used as an osmolyte in the vacuole to help to achieve osmotic homeostasis. Many naturally salt-tolerant plants (halophytes) rely on this strategy[20]. Na$^+$–H$^+$ antiport activities were detected in tonoplast vesicle preparations years ago[21]. However, the molecular nature of the antiporters was not revealed until recently, when several ESTs and, later, genomic sequences with similarities to microbial and animal Na$^+$–H$^+$ antiporters appeared in the GenBank database as a result of EST sequencing and the *Arabidopsis* Genome Initiative. Several groups then characterized these Na$^+$–H$^+$ antiporter sequences[22–24]. The vacuolar Na$^+$–H$^+$ antiporters appear to form a multigene family that might show different temporal or spatial expression of the various isoforms. The importance of the vacuolar transporters for plant salt tolerance is underscored by the finding that overexpression of one of them, AtNHX1, appeared to improve plant salt tolerance substantially[22].

In addition to Na$^+$ influx control and vacuolar compartmentation, Na$^+$ efflux is also important in maintaining a low Na$^+$ concentration in the cytoplasm. Unlike animal cells, which have Na$^+$–K$^+$ ATPases, or fungal and perhaps some algal cells, which have Na$^+$ ATPases for Na$^+$ efflux, plant cells do not appear to contain Na$^+$ ATPases. By contrast, Na$^+$–H$^+$ antiport activities have been detected in

plasma-membrane-enriched membrane vesicles[25]. Recently, the *SOS1* gene was shown to encode a putative plasma membrane Na$^+$–H$^+$ antiporter[26]. Mutations in *SOS1* render *Arabidopsis* plants extremely sensitive to Na$^+$ stress. Overexpression of *SOS1* lowers shoot Na$^+$ content and improves salt tolerance in *Arabidopsis* plants and callus tissues (H. Shi and J-K. Zhu, unpublished).

As well as maintaining ionic homeostasis in the cell cytosol, plants under salt stress also need to establish water or osmotic homeostasis. Plants accumulate various compatible osmolytes in the cytosol, thus lowering the osmotic potential to sustain water absorption from saline soil solutions[2]. As discussed earlier, some of the organic osmolytes have also been shown to help to protect cellular structures by detoxifying ROS. Water channel proteins might be involved in controlling the speed of water flux across cellular membranes under salt stress[27].

Growth regulation

Salt stress, like many other abiotic stresses, inhibits plant growth. Slower growth is an adaptive feature for plant survival under stress because it allows plants to rely on multiple resources (e.g. building blocks and energy) to combat stress. In nature, the extent of salt or drought tolerance often appears to be inversely related to growth rate. One cause of growth-rate reduction under stress is inadequate photosynthesis owing to stomatal closure and consequently limited carbon dioxide uptake. More importantly, however, stress might inhibit cell division and expansion directly. The connection between stress signalling and control of cell division and expansion needs to be better understood. Even mild stresses could result in slower growth and significant loss of plant productivity. Some plants might be so responsive to stress that they 'panic' and almost cease growing when only mild stress occurs. By contrast, some plants are probably not responsive enough and so run the risk of dying by continuing to grow when stress is already serious. Fine tuning this responsiveness could potentially improve productivity under salt or drought stress.

A potentially important link between stress and cell division was revealed by induction of *ICK1* in *Arabidopsis* by abscisic acid[28]. ICK1, a cyclin-dependent-protein-kinase inhibitor, might hinder cell division by reducing the activities of cyclin-dependent protein kinases that help to drive the cell cycle. Salt and water stress might inhibit cell division by causing the accumulation of abscisic acid, which, in turn, induces *ICK1*. These stresses probably also influence cell division through transcriptional and/or post-transcriptional regulation of other components of the cell cycle machinery. The link between salt or water stress and cell expansion control has not been examined carefully. Because of the important roles of several hormones in regulating cell elongation, it would not be surprising if stress inhibits cell

expansion by reducing the concentration of growth-promoting hormones such as auxin, cytokinin, gibberellins and brassinolides.

Studies with transgenic overexpression of stress tolerance components have also implied that there are connections between stress and growth regulation. Constitutive overexpression of transgenes in plants does not generally seem to compromise plant growth, suggesting that energy is not limiting for plant growth under normal conditions. By contrast, the constitutive expression of several stress-related genes, including *CBF1*, *DREB1A*, *ATHB7* and yeast trehalose synthase, has been shown to cause slow growth of transgenic plants[13,29,30]. *CBF1*, *DREB1A* (Ref. 13) and *ATHB7* (Ref. 31) are cold- or drought-inducible genes that are not expressed under normal growth conditions. Their gene products or downstream target molecules probably actively feed into cell division and expansion machinery to result in growth inhibition and therefore they might represent 'stress signals'. In the case of transgenic plants that produce trehalose, trehalose itself might well be a signalling molecule for growth control as well as for stress tolerance.

Genetic engineering of salt tolerance via different strategies has shown promising results. An important question that arises is the relative importance of the different strategies. Various transgenic plants should be compared in the same laboratories under identical test conditions to identify the most useful genes. This would be important not only for field applications but also for addressing a fundamental question: is detoxification or homeostasis more important for tolerance? A good example of the relative utility of transgenes is a recent comparison of *CBF1* and *DREB1A* transgenic plants[32]. Both transgenes were reported to improve freezing tolerance significantly in *Arabidopsis* by activating the transcription of downstream stress-responsive genes in a similar fashion[33,34]. However, *CBF1* improved freezing tolerance by 1°C whereas *DREB1A* improved the same trait by >10°C. These results might suggest different underlying mechanisms for the operation of both transgenes, which should be studied in more detail.

Genetic model systems

Research on salt, drought and cold tolerance has suffered from a dearth of functional genetic analysis[2,35]. The plants that have been the favourite subjects of stress studies, such as tobacco, ice plant (*Mesembryanthemum crystallinum*) and tomato, are not amenable to molecular genetic analysis. As a result, most stress studies have been correlative in nature, usually comparing gene expression profiles between stressed and unstressed plants. In the absence of a good plant genetic model system, yeast has been used as an alternative model to study stress responses in plants[35].

However, *Arabidopsis* has emerged recently as an excellent model system to study plant salt tolerance[36].

Although it is not a crop plant, *Arabidopsis* has an obvious advantage over yeast in being a real plant. In addition, mechanisms of whole-plant integration cannot be studied in unicellular model organisms. The *Arabidopsis* system should enable researchers to dissect most of the stress responses in plants. However, some novel processes or mechanisms unique to naturally stress-tolerant plants could be more difficult to study with *Arabidopsis*. Hence, a genetic model system(s) will be needed that is based on naturally tolerant plants (halophytes and xerophytes).

Yeast model

The use of *Saccharomyces cerevisiae* has led to the discovery of two important pathways for salt stress tolerance in the unicellular fungus: the HOG1 pathways for adaptation to hyperosmotic stress and the calcineurin pathway for ionic stress[2]. Calcineurin is a Ca^{2+}- and calmodulin-dependent protein phosphatase consisting of a catalytic A subunit (CnA) and a regulatory B subunit (CnB)[37]. CnB has four high affinity EF-hand calcium-binding sites and full activation of CnA requires calcium–CnB and calcium–calmodulin complexes. In yeast, calcineurin regulates Na^+, K^+ and Ca^{2+} homeostasis and pheromone response[38,39]. Loss of function mutations in CnB make yeast cells more sensitive to Na^+ and Li^+ inhibition[38,39].

Calcineurin is required for the transcriptional induction of genes encoding Na^+ and Ca^{2+} ATPases and a cell wall β-1,3 glucan synthase[38,40]. A downstream zinc-finger transcription factor, CRZ1/TCN1, participates in the transcriptional induction of these genes[40]. Calcineurin regulates the nuclear localization of CRZ1/TCN1 through dephosphorylation[41]. The region of CRZ1 that is required for calcineurin-dependent regulation of its phosphorylation, localization and activity is similar to a portion of NF-AT, a family of mammalian transcription factors whose localization is also regulated by calcineurin. The initial input of the calcineurin pathway for ionic homeostasis is cytosolic Ca^{2+} signal generated by Na^+ stress. No potential biological sensor for Na^+ has yet been identified.

Yeast calcineurin has also been implicated in the regulation of K^+ transport systems under salt stress[38]. Under Na^+ stress, the K^+ uptake system is converted into a high affinity mode of K^+ transport that results in higher discrimination between K^+ and Na^+, thereby reducing the influx of Na^+. It has been proposed that calcineurin directly or indirectly regulates the phosphorylation status of TRK1, a high affinity K^+ transporter in yeast cells[38].

The presence of a calcineurin-like activity has only been implicated in plants. The phytohormone abscisic acid increases the cytosolic free Ca^{2+} concentration, which leads to the inactivation of inward rectifying K^+ channels and stomatal closure. A step that is sensitive to cyclosporin (a calcineurin-inhibitory drug) appears to be involved in the calcium regulation of inward K^+ channel activity[42]. It has also been shown that bovine brain calcineurin can modulate the activity of calcium-permeable slow vacuolar ion channels in stomatal guard cells[43].

The HOG1 pathways for osmotic homeostasis in yeast start with either the low osmolarity sensor SHO1 or the high osmolarity sensor SLN1, producing the two pathways SLN1→SSK1→SSK2 or SSK22→PBS2→HOG1 and SHO1→PBS2→HOG1. These two pathways converge at PBS2 (Ref. 44) and ultimately lead to transcriptional activation of glycerol biosynthetic genes to increase the glycerol concentration for osmotic balance. SLN1 and SSK1 are, respectively, the sensor and response regulator of a two-component system[45]. SHO1 is a transmembrane protein with a Src-homology 3 (SH3) domain, which interacts with PBS2 through its SH3-binding motif[46]. It is not clear whether SLN1 and SHO1 sense osmotic stress directly. Perhaps these proteins perceive turgor changes through their extracellular portions, which might interact with the cell wall. Downstream of the putative sensors is a MAP kinase pathway: SSK2/SSK22, PBS2 and HOG1, are a MAP kinase kinase kinase, a MAP kinase kinase and a MAP kinase, respectively[47].

Arabidopsis model

Except for gene replacement through homologous recombination, almost everything that can be done with yeast can now be done with *Arabidopsis*, including mutagenesis, mutant screening, positional cloning and gene tagging. The availability of the entire genome sequence makes the *Arabidopsis* system even more powerful and attractive. DNA microarrays and gene chips are becoming essential tools to identify transcriptional cascades and targets of regulatory pathways through expression profiling in various mutant backgrounds.

Application of the *Arabidopsis* model system has yielded a regulatory pathway for ionic homeostasis under salt stress[36]. The pathway was discovered through the cloning of the salt overly sensitive (*SOS*) genes. Mutations in the *SOS* genes render *Arabidopsis* plants more sensitive to Na^+ stress. The pathway begins with SOS3, a myristoylated protein with three EF hands for calcium binding[48,49]. SOS3 interacts physically with SOS2, which is a serine/threonine protein kinase[50,51]. One downstream target of SOS3–SOS2 kinase complex is SOS1 (Ref. 26), which is a plasma membrane Na^+–H^+ antiporter that exports Na^+ from the cell[26]. *SOS1* expression is upregulated by salt stress in wild-type *Arabidopsis* plants but this upregulation is reduced by *sos3* or *sos2* mutations[26]. It remains to be seen whether or not SOS3–SOS2 directly regulates the activities of SOS1 and other transporter through phosphorylation. Remaining components in the SOS pathway are expected to be identified by cloning additional *SOS* genes and screening for second site suppressor and enhancer mutations in the *sos* mutant backgrounds.
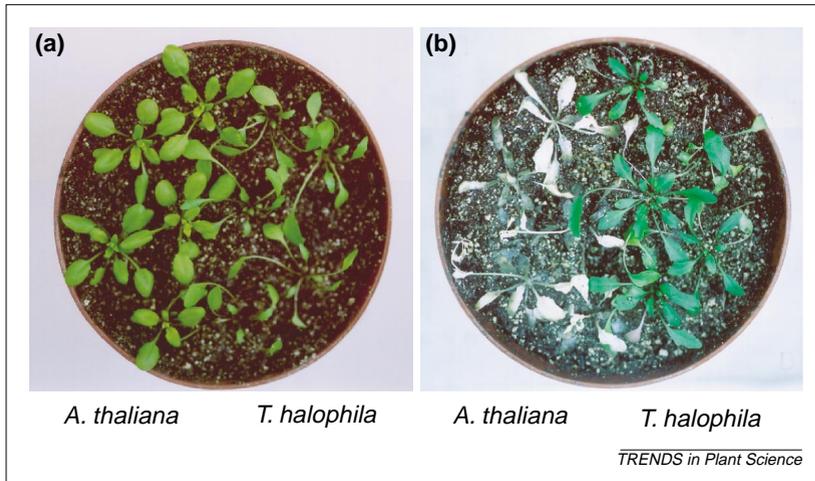
Fig. 2. *Thellungiella halophila*, a halophytic relative of *Arabidopsis*, tolerates high levels of salt stress. (a) Plants before NaCl treatment. (b) Plants after watering with 500 mM NaCl for 10 days. For comparison, *Arabidopsis* plants (left) were grown in the same pot as *T. halophila* (right).

Like the calcineurin pathway in yeast, calcium has been proposed as a second messenger for the SOS pathway[36]. The initial receptor for Na^+ has not been identified in any system. SOS1 is predicted to have a long cytosolic tail in addition to its transmembrane domains[26]. Its overall topology is reminiscent of sensor proteins such as the glucose sensors Snf3 and Rgt2 (Ref. 52), and EIN2, which was proposed to be a sensor for unknown ions involved in ethylene signalling[53]. Future studies should reveal whether SOS1 can serve as a Na^+ sensor as well.

More work is needed regarding the second messenger calcium. Drought stress, like salt stress, generates transient calcium signals[54]. Is there specificity in the Ca^{2+} signal generated by salt stress? How is the specificity achieved? SOS3, being a myristoylated protein, might be targeted to certain microdomains of membranes, perhaps near a Ca^{2+} channel, because a high level of Ca^{2+} is necessary to activate the SOS pathway[49].

Although plants might use pathways similar to the yeast HOG1 for osmotic regulation, none of the components has been identified genetically in *Arabidopsis*. An SLN1-like histidine protein kinase[55] as well as several protein kinases with similarities to MAPK components[56] have been implicated in osmotic stress sensing or signalling, largely based on their osmotic, stress-regulated transcript accumulation. Their involvement in osmotic stress signalling remains to be determined through functional genetic analysis. Genetic screens using reporter gene expression have yielded many mutations thought to be in components of the osmotic homeostasis or detoxification pathways[57].

A halophytic model system?

Although *Arabidopsis* is a typical glycophyte in being not particularly salt tolerant, various pieces of indirect evidence suggest that it might contain most, if not all, of the salt tolerance genes one might find in halophytes[36]. It is hypothesized that halophytes generally use similar salt tolerance effectors and regulatory pathways that have been found in glycophytes, but that subtle differences in regulation account for large variations in tolerance or sensitivity[36]. To test this hypothesis directly, the salt tolerance mechanisms operating in halophytes must be discovered. Several halophytes have been used extensively in physiological and molecular biological investigations[58,59]. However, none of these plants is a suitable genetic model system. To be a genetic model system, a plant must have desirable life history traits (small size, short life cycle, ability to self-pollinate and high seed number) and also certain genetic traits (small genome and easy transformation and mutagenesis).

The recent discovery of the halophytic plant species, *Thellungiella halophila*, which is native to the seashore saline soils of eastern China, and meets all the criteria for being a genetic model system (Fig. 2) (H. Zhang and J-K. Zhu, unpublished), shows that nature should not be neglected as a valuable resource. *T. halophila* has the added advantage that it is a close relative of *Arabidopsis* (>90% nucleotide identity in cDNA sequences), shares a similar morphology and life history. As such, the gene arrangement and sequences of *T. halophila* might be quite similar to those of *Arabidopsis*. Its genome size is slightly less than twice that of *Arabidopsis*. Importantly, *T. halophila* plants can be transformed by the floral dipping method, which is commonly used in *Arabidopsis*. With this ease of transformation, it would be possible to generate hundreds of thousands of T-DNA insertion lines of this halophyte and then to identify and clone (using the T-DNA as a tag) mutations affecting salt tolerance. Thus, thorough and systematic genetic analysis in *T. halophila* has the potential to discover salt tolerance determinants and pathways operating in a halophyte.

The same criteria as described above might be applied to find good genetic model plants that are drought or freezing tolerant, or have any other important agronomic traits. These new model plants should bring about a post-*Arabidopsis* revolution because one would not be limited to studying traits possessed by *Arabidopsis* or its ecotype variations.

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