Learning from the Arabidopsis Experience. The Next Gene Search Paradigm

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PLANT MODELS

Many species have become conscripted for studies in plant biology, with the choices usually driven by considerations such as genetic potential, developmental complexity, or biochemical exclusivity or by a combination of these features. Also, species have been chosen for possessing characteristics of a primarily economic interest, such as the synthesis of storage compounds, for example, and this may be combined with morphological and developmental attributes such as fruit or seed development. Maize (*Zea mays*) and tomato (*Lycopersicon esculentum*) may be cited as established models in this category. Many models in plant biology research fall into a category that derives its rationale from commercial value. They have been favored primarily because of the species' nature as an agricultural commodity and therefore possess a bounty of characteristics of primary interest. In no small part, priorities in research funding in plant biology, based on perceived immediate benefits, have led to a concentration on these species. It is remarkable, however, that species with no intrinsic commercial value chosen at least in part for experimental expediency or for unique developmental or phenotypic characters have been indispensable prerequisites for fundamental breakthroughs, providing correlative application potential for the crop-type models. Indeed, expounding the importance of choosing an appropriate organism to facilitate the study of biological phenomena is akin to carrying owls to Athens.

The development of modern concepts of genetics, successful to an extent that a heightened attention to the progress of genetic studies has become part of our everyday culture and political awareness, started with the development of a few models such as *Escherichia coli* and its phages, yeast (*Saccharomyces cerevisiae*), *Drosophila melanogaster*, and corn. Interesting is how important Mendel viewed the choice of organisms. While working his way through several plant models, Mendel noted, "The selection of the plant group which is to serve for experiments of this kind must be made with all possible care if it is desired to avoid from the outset every risk of questionable results" (Orel, 1996; Henig, 2000). The advantages of the self-fertilizing pea (*Pisum sativum*) plants, combined with Mendel's quantitative training in physics, greatly facilitated advancement through scrupulous analysis and visionary interpretation, leading to the hypotheses of inherited "factors" (Lander and Weinberg, 2000). Along this road, plant model organisms have—in the past and continuing to this date—been instrumental in revealing many important principles of genetics. Plant models have seminally aided our knowledge of chromosome structure, division and genome organization, paramutation and gene mimicry, gene silencing, and, certainly, DNA transposition.

ARABIDOPSIS BECOMES THE PREEMINENT MODEL

The earliest Arabidopsis research is associated with the names of Friedrich Laibach (1900s), with pioneering work on chromosome structure and function, and Erna Rheinholz (1940s), with mutational genetic experimentation (Glass, 1951; Rédei, 1992). The latter studies resulted in the first report of Arabidopsis mutants and revealed the wide array of phenotypes that were controlled by single genes. Thus, the foundation was laid for the use of an inconspicuous weed as the primary model for plant genetics and biology research of the future. The adoption of Arabidopsis as a plant genetic model has since played a crucial role in our understanding of plant genes and their biological functions (Somerville, 2000; Meinke et al., 1998). Arabidopsis represents the quintessential model system chosen exclusively for its experimental attributes. Significantly, Arabidopsis possesses no redeeming agricultural features, which might explain the reluctance of its widespread acceptance until the 1980s. Its ascent to glory since has been based on an inspired and visionary interaction, rarely encountered, between scientists and administrators of funding agencies (National Science Foundation, 1990). The features of Arabidopsis that first attracted genetics researchers, comparable with the *D. melanogaster*

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model, were small size, high fecundity, and a rapid life cycle. Not unlike *D. melanogaster*, these qualities have allowed for the compaction of space and the time needed for experiments. After the advent of molecular genetics and the cloning of genes, small genome size became, for some time, another important explicitly helpful attribute of Arabidopsis and pointed the way to its choice as the first plant genome to be completely sequenced. Also, the ability to transform Arabidopsis evolved from stages of considerable difficulty to the present situation that can be described as almost effortless (Bent, 2000), and this ease of transformation has placed Arabidopsis, in this respect, in an advantageous position over many other important model systems including animal models. This has led to the development of large-scale forward and reverse genetic screens to identify the function of unprecedented numbers of genes (Maes et al., 1999; Weigel et al., 2000; Young et al., 2001).

POST-ARABIDOPSIS GENOMICS

We have entered the era of post-Arabidopsis genome sequence research. In essence, this means that we must begin to think about what direction research should take after some functional information is known about all genes in the Arabidopsis ecotype Columbia genome. One rationale for adding new models is now the desire to harness more evolutionary variation and ecological breadth of traits but at the same time retain as many as possible of the advantages that make Arabidopsis so attractive (Pigliucci, 1998). It is becoming increasingly clear that the genetics of some traits are refractive to studies using Arabidopsis, owing to the evolutionary position occupied by the Arabidopsis genome. The evolutionary history of Arabidopsis, reflected in specific genes and alleles and their hardwired interactions, is such that even the vast arsenal of Arabidopsis-based molecular tools cannot be used exclusively on Arabidopsis to fully understand a number of important traits. In other words, critical genes affecting at least some important biological traits may be absent altogether or exist in the Arabidopsis genome in forms that have evolved to function in other ways. For example, genes that are crucial in determining traits such as perennial growth, the development of salt glands, or genes for nodulation may be altogether absent. Also, an Arabidopsis gene that is known to function in resistance to a specific pathogen may still resemble closely a gene from tomato that evolved to control resistance to a very different pathogen. An important future goal will be to identify those critical genes by choosing and utilizing appropriate genomes (plants) that display important traits that are not obvious or easy to measure in the commonly studied ecotypes of Arabidopsis.

Expansion of the genomics tools in other important model species such as rice (*Oryza sativa*) and maize will facilitate the search for gene functions outside the evolutionary position of the Arabidopsis genome. Certainly, the completion of the sequencing of the rice genome will offer the opportunity to obtain functional information about many genes that have evolved in Arabidopsis beyond our ability to recognize easily in other species, and thereby assign function to them simply by comparison to Arabidopsis sequences (Bevan and Murphy, 1999).

These genomes or expressed sequence tag (EST) databases will facilitate direct comparisons between the phenotypes of gene knockouts of seemingly related or identical genes from different species. Such information will be crucial to the analysis of sequences similar enough to know that they are related, but not similar enough to be confident that they have the same or even similar functions. Comparisons of these knockouts will provide bountiful information on the evolution of biological function and the basis of ecological adaptation of genes that have diverged during the separation of species. The limiting factor in obtaining these important comparisons will be the ability to obtain gene knockouts in specific genes of different plant species that do not have available the molecular genetic tools of Arabidopsis, in particular, ease of transformation and availability of tagged mutant collections for reverse genetic screens. However, RNA interference technology (Citovsky, 1999; Chuang and Meyerowitz, 2000; DiSerio et al., 2001; Vaucheret and Fagard, 2001) should prove very useful for producing specific mutants in various species even when transformation for mutant generation is inefficient. Yet for several species that have served as genetic models such as tomato, maize, barley (*Hordeum vulgare*), rice, snapdragon (*Antirrhinum majus*), and others, there are certainly many traits where mutants are already available, and corresponding gene knockouts in Arabidopsis might easily be found for a comparison of phenotypes.

Since emerging EST collections and expression profiles show us already that there is much more variance in expressed genes in the plant world than anticipated, it is becoming increasingly imperative that we tap different genetic resources. Along with EST databases for many more crop plants and even exotic species with important traits that are missing in both crops and Arabidopsis, we will also eventually need gene knockout collections in many of these species. Tomato, rice, and maize knockout collections, for example, will not be sufficient.

Certainly, many of our other model plant systems will continue to serve as sources of important information about the function of unique genes. However, the Arabidopsis model, and the powerful tools associated with it, has presented a sort of "gold standard" for model systems. Our commentary is about Mendel's notion on the choice of models. The immense value of the Arabidopsis model comes with the rec-

ognition that Arabidopsis has certain limitations, and the community of plant scientists is certainly aware of these, especially the fact that Arabidopsis overtly lacks many traits of interest. Then what is next? We argue here for models that include as much as possible the well-known advantages of Arabidopsis but have the ecophysiological, developmental and biochemical backgrounds, and lifestyles of interest to many who have not yet fallen under the Arabidopsis spell. In essence, we implore the recruiting of more Arabidopsis ecotypes, which may be found in environments as diverse as possible. Also, we suggest that certain relatives of Arabidopsis in the crucifer family could provide superior models. Searches for such potential models have already begun (http://vanilla.ice.mpg.de/departments/Gen/wild.htm), and they should continue in earnest.

FULL USE OF THE ARABIDOPSIS GERMPLASM

The most obvious germplasm that is available to explore for traits absent from the commonly used Arabidopsis ecotypes is, of course, the reservoir of additional Arabidopsis ecotypes, because they carry the important experimental attributes needed for rapid and efficient genetic studies. Alonso-Blanco and Koornneef (2000) have already pointed out the limited availability of traits within the surprisingly narrow genetic diversity of the commonly studied ecotypes. Indeed, almost all studies using Arabidopsis have been restrained to very few ecotypes that are also closely related (Rédei, 1992). Even if genes controlling certain traits are present in the widely studied ecotypes, identifying many of these genes can be hampered by focusing genetic screens on only a few ecotypes because genes in any particular genome may be redundant (have overlapping functions) or may be silent (already nonfunctional). A good example of such a phenomenon is the difference in the induction of early flowering by vernalization of laboratory versus natural ecotypes of Arabidopsis that is controlled by apparent functional and nonfunctional alleles of FLC and FRI loci (Michaels and Amasino, 1999). Therefore, we emphatically agree with Alonso-Blanco and Koornneef (2000), who pointed to the considerable benefit that would accrue from including a broader genetic range of Arabidopsis ecotypes in the search for gene functions. Use of this wider germplasm base for both map-based and insertion mutagenesis-based gene identification will be a task for the near future. One may be certain that there will be great rewards because of the different life styles of many ecotypes. The large potential benefit of such a widening of the genetic base is now being recognized, and even different species of Arabidopsis are gaining attention (http://ukcrop.net.agr/; http://vanilla.ice.mpg.de/departments/Gen/wild.htm).

The question is about which ecotypes and related species could be targeted. Although much more information is needed to help answer this question, some efforts to characterize Arabidopsis-related species are under way. The genus Arabidopsis is composed of approximately 10 diploid species. At least two other genera exist with species that are closely related to Arabidopsis including the *Arabis* group that is centered in Eurasia and the North American *Boechera* group (previously classified as *Arabis*). Although these relatives of Arabidopsis offer some unusual characteristics, such as a perennial life cycle, many also have undesirable features, from a molecular genetics perspective, notably self-incompatibility. Nevertheless, such germplasm within the Cruciferae are already being exploited and tested for use in the identification of genes controlling characteristics not accessible in Arabidopsis germplasm. Several laboratories are working to establish recombinant inbred lines, linkage maps, and bacteria artificial chromosome clone libraries of Arabidopsis lyrata and other closely related species (http://ukcrop.net.agr/; http://vanilla.ice.mpg.de/departments/Gen/wild.htm).

NOT ARABIDOPSIS BUT STILL "ALL IN THE FAMILY"

We seek genes that control important characteristics for life under stress that may be absent, or at least are functionally challenged, in Arabidopsis. There are no known ecotypes of Arabidopsis with extreme tolerance to any abiotic stresses. Even so, genes that characterize plant "extremophiles" may actually be lurking close to the Arabidopsis home and be more accessible than previously thought. The crucifer (Brassicaceae) family constitutes a large and widely distributed group of plants. Over 3,000 species inhabit all continents except Antarctica. More importantly, crucifers have colonized virtually all types of environments including arctic, subarctic, tropical, subtropical, arid, true desert, temperate, alpine, marsh, aquatic, coastal, and high altitude. In addition, crucifers have colonized many different edaphic environments (Rollins, 1993). Because of this family's extremely wide distribution within vastly different climates and ecological settings, virtually all of the important environmental adaptations made by plants certainly are displayed by family members. In addition, a cornucopia of growth and developmental features are represented. Just the roughly 700 Cruciferae species native to North America display vast differences in both root and shoot architecture, floral and reproductive structure and development, leaf morphology, fruit structure, size and texture, seed number, size, and morphology, as well as numerous other traits (Rollins, 1993). Traits such as requirements for stratification, vernalization, differences in growth patterns including perennialism, and many others with great potential importance to agriculture can be found within this family. The degree of ge-

netic variation can be appreciated by a quick examination of the startling illustrations of trichome diversity given by Rollins (1993) in his treatise on the "Cruciferae of Continental North America". The varying life styles found within this family imply complex alterations between the genomes and hint at an enormous amount of genetic diversity, not only allelic variability but also evolutionary divergence in terms of sensing and response connectivity. The Cruciferae family thus represents a storehouse of many potential plant models with not only specific traits of interest but also other needed experimental features that would allow rapid experimental progress. The most important experimental features needed would be the crucial traits of Arabidopsis. Many Cruciferae are reasonably small and produce copious amounts of seeds in a relatively short life cycle. However, features that allow a rapid route to identify the genes responsible for natural trait variations or mutation-induced variant genes are of paramount importance in these potential models. The two main routes to connect phenotypes with specific genes are map-based cloning and insertion-tagging mutagenesis.

Map-Based Cloning of Genes in Wild Relatives of Arabidopsis

Crossing even closely related family members with the Columbia ecotype is not very feasible since crucifers that are as closely related as species within the genus Arabidopsis usually vary in chromosome number (Koch et al., 1999; http://ukcrop.net.agr/; http://vanilla.ice.mpg.de/departments/Gen/wild.htm). However, many species with special characteristics within the Cruciferae will probably be represented by a number of sexually compatible ecotypes possessing polymorphic DNA markers. In addition, it is possible that sequence similarity and synteny with the known genome of Arabidopsis would greatly facilitate gene-cloning strategies. Very good colinearity has been found to exist between several Cruciferae members. Even though more variations in microsynteny are common, the high degree of gene sequence identity and general colinearity between Arabidopsis and different Cruciferae species will allow the expedient use of the Arabidopsis genome sequence to aid in mapping loci in other Cruciferae species (Schmidt et al., 2001). *Barbarea verna*, for example, is being used as a model biennial plant with an absolute vernalization requirement (http://www.wfu.edu/~taguebw/) in attempts to map genes controlling this trait.

Tagging Genes from Wild Relatives of Arabidopsis

Genes controlling unusual phenotypes in crucifer species could potentially be identified also by an insertional mutagenesis strategy. This would depend primarily on the feasibility of efficient genetic transformation of these species. Bent (2000) has outlined many factors controlling transformation efficiency in Arabidopsis and concluded that ovule structure and development timing are the most crucial. This may actually be a benefit because the structure and development of the fruit and associated tissues have been primary criteria for classification of the Cruciferae (Rollins, 1993). Therefore, it is a reasonable assumption that the anatomical and developmental characteristics affecting easy transformation have been substantially conserved in many members of the family. In fact, other members of the Cruciferae family have been transformed (Bent, 2000). In addition, Arabidopsis mutants that affect fruit set and maturation, such as Crabclaw (Bent, 2000), greatly influence transformability. These observations suggest that even Cruciferae members that lack highly efficient transformation potential may be sufficiently transformable to introduce genetic changes that will increase this efficiency to an acceptable level. Transposable elements could be used in another strategy to overcome the lack of transformation efficiency by increasing the number of insertion mutations resulting from each primary transformation with an insertion element. This strategy has been used with some success for tomato (Meissner et al., 1997). Several crucifers with important traits of interest may be amenable to insertion mutagenesis, but there is not yet any information available about this possibility (Bent, 2000). We have located two members of the genus *Thellungiella,* salt cress (*T. halophila*) and *T. parvula*, that are extremely salt tolerant, and at least one of these, salt cress, is an excellent candidate to serve as a test case for a trait-specific crucifer model system.

SALT CRESS: A SALINITY TOLERANCE MODEL SYSTEM FROM THE CRUCIFERAE

Many, too many, species have been used to examine salinity response physiology, but the names associated with genetic or molecular genetic studies in salinity stress research are few. As a selection, they identify some Chenopodiaceae (*Atriplex* species, sugar beet [*Beta vulgaris*], *Salicornia* species, *Suaeda* species), Poaceae (e.g. *Distichlis* species), and a few Caryophyllaceae (e.g. *Mesembryanthemum crystallinum*). For *M. crystallinum*, large transcript collections and a coherent set of supportive data on growth, development, and salinity stress responses exist, and while this is less the case for sugar beet, the ability to transform sugar beet has recently been reported (Adams et al., 1998; Zhang et al., 2001). As we have said, the usefulness of any halophyte model species today must be evaluated by balancing its trait of primary interest (salinity tolerance) against the collection of molecular genetics techniques that characterize Arabidopsis. Although Arabidopsis is a typical glycophyte that is not very salt tolerant, technological advantages of this model plant have been compelling

in its use to study salinity tolerance. Indeed, important advances in understanding the bases of salt tolerance have been made using Arabidopsis, and a number of recent studies suggest that it may contain versions of many important genes that one might find in halophytic, salt-resistant, or salt-loving plants that affect tolerance (Zhu et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1999; Zhu, 2000, 2001). It is now hypothesized that halophytes use salt tolerance effectors and regulatory pathways very similar to those in glycophytes and that subtle differences in their regulation can account for large variations in salt sensitivity (Hasegawa et al., 2000a, 2000b; Zhu, 2000). Many investigators began to realize that to directly test this hypothesis, genes responsible for tolerance mechanisms operating in halophytes must be discovered through functional genetic analysis and the novelty of their functions (compared with their glycophyte versions) subsequently determined. As we have argued so far, this would require the use of a halophytic model system that provides experimental expediency similar to that of Arabidopsis. That is, a model would be needed that had (a) desirable life history traits, i.e. small size, short life cycle, self-pollination, and high seed number, and (b) favorable genetic traits such as self-fertilization, a small genome, efficient transformation, and mutagenesis. One halophytic plant species that meets all of these criteria is salt cress. We suggest that this plant in the Cruciferae family can serve as an appropriate test species to determine whether crucifer models can be developed to search for genes that control important traits not associated with Arabidopsis. If salt cress becomes a successful model, eventually other trait-specific models from the Cruciferae may be found and exploited.

Although salt cress is a close relative of Arabidopsis, it is not in the Arabidopsis genus, and having seven chromosomes, cannot be crossed successfully with Arabidopsis despite having been considered synonymous with Arabidopsis in the past (Al-Shehbaz and O'Kane, 1995; Al-Shehbaz et al., 1999). The life cycle of salt cress, 2 to 2.5 months, is similar to that of Arabidopsis. Salt cress resembles Arabidopsis in development, size, and structure, but there are several distinguishing developmental differences between the two species. For example, compared with Arabidopsis (Columbia ecotype), salt cress leaves are more elongated and serrated, with longer petioles (Fig. 1). Salt cress has an obligate vernalization requirement in order to flower, in contrast to Arabidopsis, where low temperature simply accelerates flowering. The minimal vernalization time required for salt cress is approximately 3 weeks. Once rosette plants of salt cress are vernalized, they bolt rapidly, often producing multiple inflorescences that are morphologically similar to those of Arabidopsis (Fig. 1). As in Arabidopsis, salt cress flowers consist of four green sepals, four white petals, six stamens,

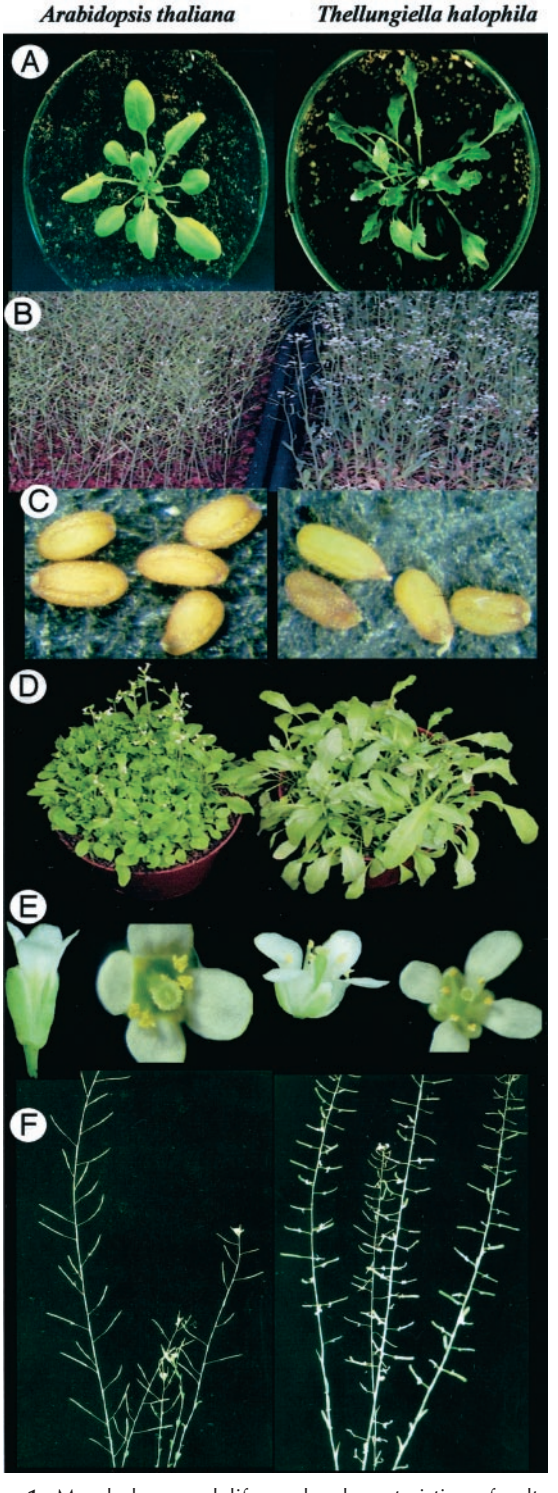

Figure 1. Morphology and life cycle characteristics of salt cress compared with Arabidopsis, Columbia ecotype. A, Both species form rosettes, but salt cress has a longer petiole and serrated leaves. B, Salt cress continues flowering later than Arabidopsis, producing similar seed yield of about 4,000 to 8,000 seeds/plant with nearly identical seeds (C). D, Salt cress plants will continue to grow and not flower until vernalized. Flower structure (E) and inflorescences (F) are also nearly identical, with siliques reaching maturity at about the same time, 4 to 6 weeks.

and one pistil. All stamens are of equal length in salt cress, whereas two different length classes are found in Arabidopsis. Salt cress is self-fertile and has slightly shorter siliques than Arabidopsis, but the plant is as prolific in seed yield as Arabidopsis. As many as 4,000 to 8,000 seeds can be collected from a single plant. Salt cress seeds are also slightly more elongated (Fig. 1).

Salt cress is able to withstand dramatic salinity shock up to 500 mM NaCl and grow in salt far in excess of the capability of Arabidopsis (Fig. 2). This plant does not produce salt glands or other complex morphological alterations either before or after salt adaptation. It appears that salt tolerance in salt cress is largely the result of basic biochemical and physiological mechanisms that can be subject to impact by individual gene mutations.

By using flow cytometry, we have found that salt cress has a relatively small genome of less than twice the size of the Arabidopsis genome. EST analyses of several hundred salt cress clones revealed averages of 90% and 95% identities between salt cress and Arabidopsis cDNA and amino acid sequences, respectively (J.-K. Zhu, unpublished data). We are pursuing three strategies to identify highly specialized genes that control the extreme salt tolerance of salt cress. First, a Transformation-competent artificial chromosome library of salt cress is being constructed to attempt the introduction of salt tolerance genes into Arabidopsis. Second, we have begun to search for divergent ecotypes of salt cress in hopes of developing a sufficient DNA polymorphism base to map natural alleles or induced gene mutations that affect salt tolerance. Finally, because salt cress can be transformed efficiently by dipping its inflorescences in an *Agrobacterium tumefaciens* suspension (Fig. 3), we have begun to establish a sufficiently large insertion tag collection of *Thellungiella thellungiella* mutants that can be screened for altered salt tolerance. Seeds of salt cress will soon be available from the Arabidopsis Biological Resource Center at Ohio State University (http://www.Arabidopsis.org/abrc/; Arabidopsis+@osu.edu).

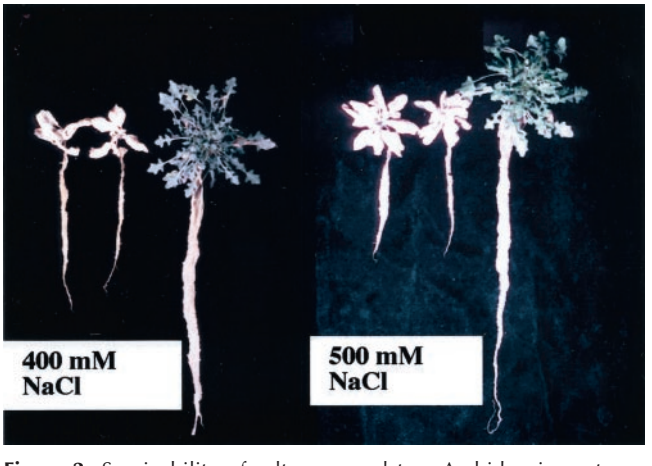

Figure 2. Survivability of salt cress and two Arabidopsis ecotypes (left to right, Columbia, Wassilewskija, and salt cress) in Turface hydroponic growth medium (quarter-strength Murashige and Skoog salts 16-h light:8-h dark photoperiod at 22°C) after increasing NaCl exposure from 0 mM to 500 mM in 100 mM increments every 5 d. Plants were harvested and pictures taken 10 d after reaching indicated NaCl concentration. Out of 36 plants of each type, no Arabidopsis plants survived 300 mM and higher NaCl, whereas 100% of salt cress plants survived and grew in all conditions including 500 mM NaCl.

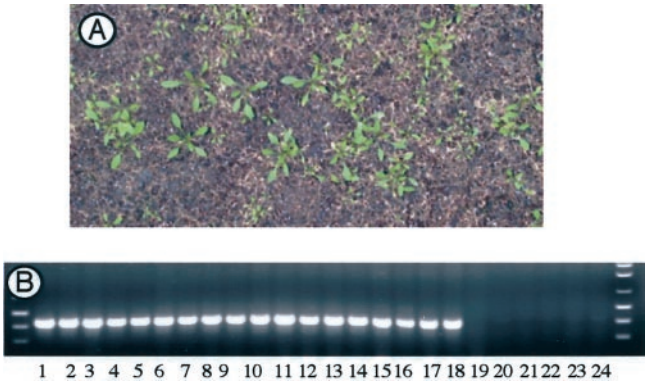

Figure 3. Appearance of bialaphos-tolerant salt cress seedlings transformed with vector pSKI1015 in GV3101. A, The picture was taken 10 d after treatment with 30 mg L^{-1} bialaphos. Transformation was confirmed by PCR identification of bialaphos marker gene in DNA from randomly chosen bialaphos-tolerant seedlings (lanes 1–18). B, PCR reaction of bialaphos-sensitive seedlings (lanes 19–24).

Genes involved in salt tolerance in Arabidopsis such as SOS1, -2, and -3 may be examined in many halophyte species to determine whether special alleles of such genes have evolved in halophytes. However, without a good halophyte genetic model like salt cress, we cannot access possible unique genes of halophytes that are involved in salt tolerance. In addition, salinity tolerance (and this applies equally for drought or ozone, or UV-B, or freezing, etc.) may in different classes and families of plants include evolutionary "inventions" that constitute novel adaptation strategies. The number of families, above, in which salinity tolerance, and abiotic stress tolerance in general, prevails is biased toward the class Caryophyllales in which only few crop species exist. Evolutionary divergence and adaptation to an extreme lifestyle in many plants in this class might have led to the appearance of novel gene combinations for the support of tolerance. This possibility is supported by the available *M. crystallinum* EST collection, which, when compared against the Arabidopsis genome, seems to include a number of transcripts that have no counterparts in this genome sequence. Eventually, genetic information obtained from this remarkable salt-tolerant crucifer will represent a key step in the discovery of genes involved in tolerance of other halophytes and glycophytes alike.

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