Identification of plant stress-responsive determinants in arabidopsis by large-scale forward genetic screens

Hisashi Koiwa1,*, Ray A. Bressan2 and Paul M. Hasegawa2

1 Department of Horticultural Science and Vegetable and Fruit Improvement Center, 2133 Texas A&M University, College Station, TX 77843-2133, USA
2 Center for Plant Environmental Stress Physiology, 625 Agriculture Mall Drive, Purdue University, West Lafayette, IN 47907-2010, USA

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Abstract

All plants sense and adapt to adverse environmental conditions, however, crop plants exhibit less genetic diversity for abiotic stress tolerance than do wild relatives indicating that a genetic basis exists for stress adaptability. Model plant genetic systems and the plethora of molecular genetic resources that are currently available are greatly enhancing our ability to identify abiotic stress-responsive genetic determinants. Forward genetic screens of T-DNA mutagenized Arabidopsis thaliana populations in the genetic background of ecotypes C24RD29a-LUC and Col-0 gl1 sos3-1 were carried out to begin an exhaustive search for such determinants. The C24RD29a-LUC screens identified mutants with altered salt/osmotic stress sensitivity or mutants with altered expression of the salt/osmotic/cold/ABA-responsive RD29a gene. Also, mutations that alter the NaCl sensitivity of sos3-1 were screened for potential genetic suppressors or enhancers of salt-stress responses mediated by SOS3. In total, more than 250,000 independent insertion lines were screened and greater than 200 individual mutants that exhibited altered stress/ABA responses were recovered. Although several of these mutants have been reported, most have not yet been studied in detail. Notable examples include novel alleles of SOS1 and mutations to genes encoding the STT3a subunit of the oligosaccharyltransferase, syntaxin, RNA polymerase II CTD phosphatases, transcription factors, ABA biosynthetic enzyme, Na+ transporter HKT1, and SUMO E3 ligase. The stress-specific phenotypes of mutations to genes that are involved in many basic cellular functions provide indication of the wide range of control mechanisms in cellular homeostasis that are involved in stress adaptation.

Key words: CCD imaging, osmotic stress, RNA polymerase II, salinity, T-DNA tagging, transcription.

Introduction

Severe climatic changes throughout millions of years have resulted in the evolution of flora that exhibit substantial genetic diversity for adaptation to environmental perturbations. However, domestication of plants over the millennia has narrowed the genetic diversity that exists in crop species (Buckler et al., 2001; Wright et al., 2005). Presumably, selection for high yield potential has resulted in the loss of alleles with significant contributions to environmental stress adaptability. Abiotic stresses, particularly reduced water availability or quality and temperature extremes, are substantial constraints to crop production (Boyer, 1982) even with the highly sophisticated intensive management practices of modern day agriculture. In fact, agricultural management practices aimed at maximizing production dating back to the dawn of civilization have been a primary cause of many global salinity problems that have rendered land unusable for crop production or have challenged sustainable irrigation (Flowers, 2004). The increasing loss of land for agricultural use because of aridity and/or salinity problems makes the task of agriculturalists to increase food production in order to meet the world population demands more difficult. Genetic tolerance to these abiotic stresses that is able to sustain crop yield capacity is a requisite to sustain food security.

Plants respond to water quantity and quality deficiencies and temperature extremes by initiating adaptive responses...
that are necessary to alleviate primary and secondary effects caused by these stresses in order to sustain their survival, growth and development (Thomashow, 1999; Hasegawa et al., 2000; Iba, 2002; Zhu, 2002; Bohnert et al., 2005). The exact stress sensory mechanisms are yet to be resolved, but it is clear that perception leads to activation of signal transduction pathways that control adaptive responses (Stockinger et al., 1997; Liu et al., 1998; Xiong and Zhu, 2001, Zhu, 2002). The signalling components and their target genes are the focus of intense research efforts. The task is daunting because responses to abiotic stresses have overlapping etiologies and therefore involve several signalling systems (Xiong and Zhu, 2001; Seki et al., 2003). It is clear that abiotic stress-responsive signal pathways are also modulated and amplified by hormonal and other signals, and connect to growth and development pathways. Signalling in these complex biological systems requires continuous modulation that necessitates precise positive and negative regulatory cues throughout the signal system. Because of this complexity, a robust molecular genetic model plant system like Arabidopsis thaliana is necessary for the functional dissection of the adaptive response system. Even though arabidopsis is not a particularly stress-tolerant plant, it does have the ability, like all plants, to sense and respond adaptively to abiotic stresses (Hasegawa et al., 2000; Zhu, 2002, 2003) allowing its formidable molecular genetic attributes to be utilized to some degree.

Plant stress adaptation responses include dynamic transcriptome changes that are presumed to play an important role in the co-ordination of the many different molecular responses responsible for cellular and organismal homeostasis. In arabidopsis, Responsive TO Dehydration (RD) and Cold-Regulated (COR) genes are major members of the osmotically regulated transcriptome and both abscisic acid (ABA)-independent and -dependent pathways are involved in their control (Yamaguchi-Shinozaki and Shinozaki, 2005). ABA mediates gene expression through a series of pathways that culminate in the interaction of basic leucine zipper transcription factors (ABF/AREBs) with ABREs (ABA-responsive elements) (Choi et al., 1999; Uno et al., 2000) that exist in the promoters of several RD and COR genes. RD or COR gene activation by low temperature or desiccation that is independent of ABA occurs through the interaction of other transcription factors called CBF/DREB DNA-binding proteins with the C-repeat/DRE type cis-elements (Stockinger et al., 1997; Liu et al., 1998). Overexpression of CBF/DREB proteins in transgenic arabidopsis plants induces ectopic expression of RD/COR genes and confers desiccation and cold tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). The arabidopsis RD29α promoter contains both DRE and ABRE cis elements and is thus a convergence point for these two pathways that link ABA and osmotic stress signalling (Narusaka et al., 2003). The RD29α promoter and luciferase (LUC) reporter system has been used for extensive genetic analyses resulting in the identification of numerous genes that positively and negatively control osmotic stress signalling (Ishitani et al., 1997, 1998; Gong et al., 2002; Guo et al., 2002; Koiwa et al., 2002; Lee et al., 1999, 2001, 2002; Xiong et al., 1999b, 2001a, b, c, 2002a, b).

The Salt-Overly-Sensitive (SOS) signalling pathway is a major regulatory cascade that controls Na+ homeostasis in response to high salinity. A forward genetic screen for mutations that cause NaCl hypersensitivity of arabidopsis seedlings led to the identification of the first major components of the pathway SOS1, SOS2, and SOS3 (Zhu, 2002; Chinnusamy et al., 2005). The plasma membrane SOS1 Na+/H+ antiporter is a principal target of the pathway (Shi et al., 2000, 2003). SOS1 is the primary transport system responsible for cellular Na+ efflux (Zhu, 2003) and controls Na+ loading into the xylem of the root thereby restricting accumulation of the toxic ion in the shoot (Shi et al., 2002). Furthermore, SOS1 is localized in the epidermis, particularly in the root tip where ion exclusion is a primary mechanism for the salinity tolerance of cells that are critical for growth and differentiation and have an underdeveloped vacuole where Na+ could otherwise be transported for detoxification (Shi et al., 2002). Current models indicate that the EF hand Ca2+ binding protein, SOS3, is activated in a localized microdomain by a Ca2+ transient that is induced by NaCl stress. Ca2+ binding by SOS3 facilitates its interaction with the SNF-like kinase SOS2 resulting in its activation through the repression of autoinhibition (Guo et al., 2001; Halfter et al., 2000). Activated SOS2 is then recruited to the plasmamembrane and phosphorylates SOS1 leading to activation of its Na+/H+ antiporter activity (Quintero et al., 2002). Furthermore, SOS1 has also been suggested to function as a Na+ sensor and is known to mediate control of target gene expression (Zhu, 2003).

As a part of the plant stress genome project (National Science Foundation Plant Genome Award DBI-98-13360), T-DNA populations were generated in the genetic backgrounds of C24 RD29α::LUC and Col-0 sos3-1. A forward genetic screen of the C24 RD29α::LUC population identified mutations that alter the responsiveness to NaCl, cold, and ABA while a screen of the Col-0 gil sos3-1 population identified suppressor and enhancer mutations of sos3-1 NaCl hypersensitivity. Herein the results of these forward genetic screens for stress-tolerance determinants are summarized.

**Materials and methods**

**Biological materials**

Dr Jian-Kang Zhu and Dr Richard Walden kindly provided Arabidopsis thaliana Col-0 gil sos3-1 (Liu and Zhu, 1997) and C24 RD29α::LUC (Ishitani et al., 1997), and Agrobacterium tumefaciens GV3101 pMP90RK (Hayashi et al., 1992), respectively.
**Plasmid materials**

Dr Detlef Weigel kindly provided the T-DNA activation tagging plasmid pSK015, (Weigel and Ahn, 2000). Plasmids p1933-apx encoding the arabidopsis ubiquitin promoter and pUA58X containing the superpromoter were provided by Dr Phillip Mullineaux and Dr Stan Gelvin (Ni et al., 1995), respectively. Dp620 containing the bar gene and the potato pinII terminator was provided by Pioneer Hybred. The pBIIB-HYG plasmid (Becker, 1990) was provided by Dr Xiaomu Niu. To construct pSuperTag2, a 0.25 kbp BamHI-HindIII fragment was excised from p1933-apx, blunted and cloned into pBC, which was digested with SpeI and then blunted to produce pBC-Ubi. A 0.88 kbp BamHI-EcoRI fragment containing the bar ORF-PinII terminator was cloned into the BamHI and EcoRI sites of pBluescript to produce pBS-bar. The ubiquitin promoter was excised from pBC-Ubi by NotI and BamHI restriction and ligated into the NotI and BamHI sites of pBS-bar that are upstream of the bar ORF to produce pBS-Ubi-bar. A 1.2 kbp HindIII-XbaI fragment containing the superpromoter was cloned into pBC to produce pBC-super. pBS-super was then digested with HindIII, blunted, and digested with XbaI to excise the superpromoter. pBS-Ubi-bar was digested by NotI, blunted, digested with XbaI, and ligated to the superpromoter fragment to produce pBSSP-Ubi-bar, pBS-Super-Ubi-bar was cleaved with XbaI and ligated with a 3.7 kbp XbaI fragment of pSK015 containing the T-DNA border sequence and a replication origin for Agrobacterium to produce pSuperTag. Because an ATG codon existed in the ubiquitin promoter, pSuperTag was digested with EcoRI, blunted, digested with HindIII, and ligated with a 0.88 kbp fragment from pBS-bar, which was digested with BamHI, blunted with mung bean nuclease, and digested with HindIII, to produce pSuperTag2 (pSPT2, Fig. 1). pSPT2 and pSK015 were introduced into Agrobacterium tumefaciens GV3101 pMP90RK that harboured the binary vector pSK015 by immersion of inflorescences, or spraying with an Agrobacterium suspension. Sprayed or immersed plants were then kept in a closed environment to maintain a high humidity so that floral surfaces remained wet for 24 h. Plants were retransformed 2 weeks later and grown to maturity in the greenhouse. Phosphinothricin (Liberty/Finale) resistant T1 seedlings were identified after successive rounds of herbicide (50 μg ml⁻¹) spraying (usually for 3 consecutive days followed by a week without spraying. This cycle was usually repeated 2–3 times. Seeds were collected from surviving plants and maintained in pools of 10–50 plants each.

**Root growth screening**

Sensitivity of root growth was determined using a modified root-bending growth assay (Liu and Zhu, 1997). Seed germination plates were prepared by placing hydrated cellophane membranes (Bio-Rad) that were autoclave sterilized onto solid medium containing 1× MS salt, B5 vitamins, 3% sucrose, 1.6% agar (germination medium). About 200 T2 seeds per 10-line pool were surface-sterilized in 70% ethanol for 20 s and then in 10% laundry bleach (NaCl hypochlorite) solution for 5 min, and rinsed three times with sterilized distilled water. Sterilized seeds were resuspended in 200 μl of a 0.1% agar (Sigma) solution. A drop of Agribrom solution (1500 ppm) was added to the seed suspension, and seeds were stratified at 4 °C in the dark for 4 d before plating. Individual seeds were sown along a level horizontal line onto the surface of the cellophane membrane. Plates were oriented vertically (180°) with the seed line parallel to the shelf surface in a growth room (25 °C, 16/8 h light/dark cycle). After 7 or 8 d (roots 7–10 mm in length), seedlings were transferred by placing the cellophane membrane directly onto fresh medium containing 160 mM NaCl. Plates were then placed upside-down in a vertical position in a growth room. After an additional 7 d, the seedlings were evaluated for root growth in the opposite direction (180° bend) from the growth that occurred prior to transfer to NaCl.

**Luciferase imaging**

Luminescence imaging was performed as described (Xiong et al., 1999a) using a CCD system (RoperScientific, NJ). Seeds were surface-sterilized as above, inoculated onto medium containing 1× MS salt, 3% sucrose, and 0.6% agar, and kept under a 16/8 h light/dark cycle for 1 week. The optimum conditions to induce RD29a-LUC expression were established previously (Ishitani et al., 1997). Briefly, seedlings were exposed to 0 °C in the dark for 24–48 h, or sprayed with 100 μM (+)ABA and maintained under light for 3 or 4 h or placed onto filter paper moistened with solution containing 1× MS salts, 3% sucrose, and 300 mM NaCl and maintained in the laboratory for 4 h. For imaging, seedlings were sprayed with an aqueous solution of 1 nM luciferin and 0.1% Triton X100 and incubated for 5 min in the dark. A luminescence image was acquired using a digital dip or spray method (Clough and Bent, 1998; Chung et al., 2000).

**Arabidopsis transformation and herbicide selection of T1 plants**

Seeds dried to a moisture content of ~5% were stratified for 5–10 d at 4 °C in the dark and then sown onto the soil surface. Alternatively, seeds were sown onto moist soil surface and stratified at 4 °C in the dark for 3–10 d. Seeds were germinated in high humidity obtained by using a mist system or by covering the soil with a transparent plastic cover and subsequently grown in the greenhouse. After plants produced a primary inflorescence stalk, the stalk was removed to enhance development of secondary inflorescences. Plants with inflorescences at a stage just prior to anthesis were used for transformation (Clough and Bent, 1998; Chung et al., 2000). Arabidopsis plants were transformed with Agrobacterium tumefaciens GV3101 pMP90RK that harboured the binary vector pSK015 by immersion of inflorescences, or spraying with an Agrobacterium suspension. Sprayed or immersed plants were then kept in a closed environment to maintain a high humidity so that floral surfaces remained wet for 24 h. Plants were retransformed 2 weeks later and grown to maturity in the greenhouse. Phosphinothricin (Liberty/Finale) resistant T1 seedlings were identified after successive rounds of herbicide (50 μg ml⁻¹) spraying (usually for 3 consecutive days followed by a week without spraying. This cycle was usually repeated 2–3 times. Seeds were collected from surviving plants and maintained in pools of 10–50 plants each.

![Fig. 1. Map of pSuperTag2. The T-DNA region contains a synthetic superpromoter that drives transcription of the genome fragment adjacent to the right border region, pBluescript plasmid, and bar gene regulated by Arabidopsis ubiquitin promoter and potato pinII terminator. The XbaI-XbaI fragment containing Vir region and T-DNA border sequence is identical to pSK015.](http://jxb.oxfordjournals.org/)

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LUC expression was established previously (Ishitani et al., 1997). Briefly, seedlings were exposed to 0 °C in the dark for 24–48 h, or sprayed with 100 μM (+)ABA and maintained under light for 3 or 4 h or placed onto filter paper moistened with solution containing 1× MS salts, 3% sucrose, and 300 mM NaCl and maintained in the laboratory for 4 h. For imaging, seedlings were sprayed with an aqueous solution of 1 nM luciferin and 0.1% Triton X100 and incubated for 5 min in the dark. A luminescence image was acquired using a digital dip or spray method (Clough and Bent, 1998; Chung et al., 2000). Arabidopsis plants were transformed with Agrobacterium tumefaciens GV3101 pMP90RK that harboured the binary vector pSK015 by immersion of inflorescences, or spraying with an Agrobacterium suspension. Sprayed or immersed plants were then kept in a closed environment to maintain a high humidity so that floral surfaces remained wet for 24 h. Plants were retransformed 2 weeks later and grown to maturity in the greenhouse. Phosphinothricin (Liberty/Finale) resistant T1 seedlings were identified after successive rounds of herbicide (50 μg ml⁻¹) spraying (usually for 3 consecutive days followed by a week without spraying. This cycle was usually repeated 2–3 times. Seeds were collected from surviving plants and maintained in pools of 10–50 plants each.
by a 5 min exposure to the CCD system and analysed using WinView software.

sos3-1 suppressor and enhancer screening

Individual T2 seeds (200 seeds per 10-line pool) were sown onto cellophane membranes that were placed onto solid germination medium. For identification of suppressor and enhancer mutations of sos3-1 NaCl sensitivity, cellophane membranes with seedlings were transferred to medium with 120 mM NaCl. Pools were considered to contain salt-hypersensitive seedlings harbouring mutations if this NaCl treatment resulted in rapid bleaching and lethality. In order to rescue the seedlings, these pools were re-screened on medium containing 75 mM NaCl. Suppressor mutations were identified based on identification of seedlings that were NaCl-tolerant relative to sos3-1 seedlings. Salt sensitivity was determined based on root tip growth and anthocyanin production in leaves. Putative mutant seedlings were transferred to medium without NaCl.

Germination screening for salt tolerance

Seeds were sown onto medium containing 1× MS salt, 2% sucrose, 145 mM NaCl, and 1% agar (pH 5.7). After sowing, the seeds were stratified for 2–4 d at 4°C. Germination was assessed visually after 14 d.

Thermal asymmetric interlaced PCR (TAIL-PCR)

Flanking sequence of inserted T-DNA from selected mutants was determined by TAIL-PCR analysis (Rus et al., 2001; Koiwa et al., 2002; Zhu et al., 2002). Genomic DNA was extracted from leaves of 2–3-week-old plants (Edwards et al., 1991). TAIL-PCR analysis with pSKI015 was performed using a set of nested left border primers (LB) and complementary strand degenerate primers (Rus et al., 2001; Koiwa et al., 2002; Zhu et al., 2002). TAIL-PCR products were sequenced after gel purification or after cloning into pBluescript-T vector prepared as described (Marchuk et al., 1990).

Genetic analysis and molecular characterization of T-DNA insertion alleles

Primary screens were conducted on T2 progeny (segregating population) and designed to ensure 95% probability of identifying phenotypes caused by recessive mutations, i.e., minimum of 20 seedlings per line. T3 progeny from individual T2 plants were obtained and phenotypes were re-evaluated to identify homozygous lines. Genetically confirmed homozygous mutant plants were evaluated for co-segregation of herbicide resistance, backcrossed to the wild type, genotypically characterized to identify the flanking sequence and evaluated for the expression of the T-DNA insertion allele. Cause and effect relationship between phenotype and the presence of an insertion allele was established by co-segregation analysis, multiple allele analysis, RNAi gene silencing, and/or genetic complementation.

Allelic homozygosity was confirmed by genotyping using a PCR-based detection analysis. PCR primers that specifically detected either the wild type or T-DNA insertion allele were designed for the analysis. To detect a SOSI allele with a T-DNA insertion, PCR was performed with the LB3 primer and a gene-specific primer (SOS1F: GTGTCAAGTGTCGAACAGA) while the wild-type allele was detected using only gene-specific primers (SOS1F, SOS1R: TCGGA-GAAATCGATTCTCAACAGAT).

Co-segregation of the phenotype and the T-DNA allele was also evaluated using T2 segregating seedlings reconstituted from backcrosses to the wild type.

After identification of the T-DNA insertion flanking sequence, publicly available populations were monitored for potential additional mutant alleles. The SIGnAL T-DNA express database provided the most instances of a second allele. Plants were genotyped and those that were homozygous for the T-DNA allele were used for further phenotypic analysis.

Gene silencing of wild-type plants using RNAi was also used to phenocopy the mutants originally isolated by screening. The RNAi vector pFGC1008 was obtained from Dr R Jorgensen (University of Arizona). A 500–1000 bp cDNA fragment was inserted into pFGC1008 according to the recommended protocol (http://www.chromdb.org/). The resulting RNAi construct was introduced into A. tumefaciens GV3101 and used for Arabidopsis transformation as described above. T2 seeds were germinated on medium containing 20 μg ml−1 hygromycin B and transformed seedlings were identified. Phenotypic analysis occurred in the T1 and/or T2 generation.

Binary vectors were used to express genomic fragments or cDNAs in order to assess genetic complementation. Transgene expression was driven either using the CaMV 35S or the innate endogenous promoter.

Results

Preparation of T-DNA tagged populations

Approximately 180 000 and 100 000 independent T-DNA insertion mutant lines of C24 RD29a::LUC lines and Col-0 gl1 sos3-1, respectively, were obtained using the activation tagging plasmid pSK1015. Several mutant phenotypes that were identified in the C24 RD29a::LUC T1 generation were not evident in the T2 generation. This may indicate silencing of the 35S enhancer (Weigel and Ahn, 2000), which may occur more frequently in the C24 than in the Col background. The forward genetic screens for altered abiotic stress responsiveness of the T2 populations identified, in almost all instances, recessive mutations. Additional tagging lines were generated with pSupertag2 in various mutant or transgenic backgrounds (Table 1). pSupertag2 activation is achieved using the superpromoter instead of the 4× 35S enhancer repeats, and also allows transcription of the DNA sequences that flank the right border of the inserted T-DNA when using TAIL-PCR to identify insertions responsible for identified phenotypes. The pSK1015 vector cannot be used for TAIL-PCR with primers from its right border because of the 4× repeated 35S enhancer sequences. The transformation efficiency achieved with both mutagenesis vectors varied from 0.1% to 2% with a 1% frequency being typical. Although activation tagging vectors were used to generate these lines, only a few mutant phenotypes were observed from the T2 population screens that were the result of dominant alleles. Screening for altered stress tolerance was not conducted with T1 populations, so most mutant phenotypes found at the T1 stage were visually obvious morphological or development mutants and were not checked for dominant or recessive inheritance.

Root growth screening and mutant identification

A total of 93 840 independent T2 progeny in the C24RD29a-LUC background were screened on medium with 160 mM NaCl. This required phenotypic analysis of about 1.9 million seedlings (20 individuals of each line) in order to
ensure a high probability of recovering recessive mutations from pools each with 10 independent lines. The previously established basal media for the screening includes 1 x MS and 3% sucrose (Liu and Zhu, 1997). Use of this basal media in screening facilitated the isolation of salt-sensitive mutants that are impaired in the osmotic-stress response, because MS salts and sucrose contributed to increase the osmotic strength of the media without imposing too strong an ion toxicity. Ninety salt/osmotic stress-sensitive mutants were confirmed based on genetic inheritance of the phenotype in the T3 and further generations. This represents about 5% of the putative mutants identified in the initial screen of T2 populations. The 90 mutants were categorized into two groups (Fig. 2). Group 1 mutants (48 lines) exhibited growth that was the same as or very similar to wild type on medium without NaCl. Group 2 mutants (42 lines) exhibited greater than 50% growth reduction relative to wild type on medium without NaCl.

Among Group 1 mutants were two independent alleles of SOS1 (sos1-14, sos1-15) (Fig. 3, and not shown). Several NaCl-sensitive mutants were also sensitive to non-ionic osmotic stress imposed by the addition of mannitol to the medium. Root tip growth of mutants sensitive to both NaCl and mannitol was less inhibited by other stress than mutants that were sensitive only to NaCl stress. The IC50s for NaCl/mannitol stress-sensitive mutants were greater than 100 mM solute equivalents compared with IC50s of 50–75 mM solute equivalents for the mutants that are sensitive to NaCl only. In addition, NaCl/mannitol stress-sensitive mutants usually hyper-responded to a high concentration of KCl, but their response to LiCl unlike most NaCl-sensitive mutants was similar to wild type. The NaCl/mannitol-sensitive mutants usually exhibited altered stomatal functions as well. Group 1 mutants were found to result from mutations in loci encoding proteins that are involved in a wide range of cellular functions including vesicular trafficking (syntaxin), protein glycosylation (oligosaccharyltransferase) and folding (tetratrico peptide repeat protein), signalling (kinase associated protein phosphatase, transcription factors, fatty acid elongase), and organic solute transport (Major Facilitator Superfamily) (Zhu, 2002; Koiwa et al., 2003; unpublished results).

Group 2 mutants were not characterized extensively because they exhibit growth alterations that are not specific to osmotic stress.

Salt-tolerance screening

Approximately 11 000 C24Rd29a::LUC T2 lines were screened for enhanced capacity for germination and growth on medium containing 145 mM NaCl, and two genetically stable salt-tolerant mutants were isolated. One, sto1, was characterized and its salt-tolerance phenotype was found...
to result from a T-DNA insertion in the NCED3 gene that encodes an isoform of 9-cis-epoxycarotenoid dioxygenase, an enzyme that catalyses a critical step in ABA biosynthesis in response to osmotic stress (Ruggiero et al., 2004).

Seedlings of sto1 are restricted in capacity for stomatal closing and were also susceptible to dehydration when grown outside the culture dishes where screening was performed.

Screening for suppressor or enhancer mutations of sos3-1 NaCl sensitivity
Seedlings of sos3-1 are NaCl hypersensitive and exhibit K+ deficiency when the external Ca2+ concentration is sub-optimal (Liu and Zhu, 1997). T2 progeny of 70 000 T-DNA tagged lines of Col-0 gl1 sos3-1 were screened for an increase or a further decrease in salt tolerance when grown in medium containing 120 mM NaCl. Putative mutants were identified based primarily on the criteria of shoot and root growth and shoot anthocyanin accumulation in response to NaCl. Eighty-nine lines were isolated that exhibited a genetically stable suppressed or enhanced sos3-1 phenotype. Of these, 16 mutations that suppressed the NaCl sensitive phenotype of sos3-1 were studied further. The strongest suppressor lines were determined to be allelic with mutations in AtHKT1, the gene encoding a transporter that mediates Na+ influx into cells and Na+ homeostasis in planta (Rus et al., 2001).

Physiological analysis of the hkt1 phenotype revealed that, in addition, to HKT there is a Na+ uptake system(s) that is inhibited by mM levels of Ca2+ ext, but is operational at sub-mM concentrations of the ion (Rus et al., 2001, 2004). Most data reported implicate non-selective cation channels (NSCC)/voltage-insensitive channels (VIC) as the molecular entities responsible for this Ca2+-sensitive Na+ uptake system. There are 20 genes annotated in the arabidopsis database for NSCCs (Demidchik et al., 2002). Although the NSCCs are categorized as non-selective, those that have been characterized do exhibit some transport specificity (Hua et al., 2003). To identify any specific NSCCs that may be responsible for Na+ uptake in planta, 70 000 Col gl1 sos3-1 T-DNA insertion lines were screened on medium with 75 mM NaCl and no added Ca2+ ions. Interestingly, this screen did not result in the identification of mutations that can substantially suppress the NaCl-sensitive phenotype of sos3-1. Although three were found and confirmed to very modestly suppress the sos3-1 NaCl-sensitive phenotype. Mutations in a number of genes that encode NSCCs, including one that mediates Na+ conductance (Hua et al., 2003) have been reported not to cause lethality, suggesting that NSCC family members have overlapping function. It is possible that regulatory genes control several NSCCs that mediate Na+ influx and this study’s screen, that
was insufficient to saturate the arabidopsis genome, failed to identify such a regulatory component.

Nine mutants exhibited a sos3-1 suppressed phenotype that was less dramatic than that caused by the hkt1 alleles and was only evident on medium containing lower concentrations of NaCl. One of these is a mutation at the AtSIZ1 locus, which encodes a SUMO E3 ligase (Miura et al., 2005). Mutations of SIZ1 cause sos3-1 seedlings to exhibit not only suppression of NaCl sensitivity but also hyper-responsiveness to Pi starvation based on its modified root architecture and altered expression of target genes of Pi starvation. In addition, 30 flavonoid-deficient mutants were identified, eight with transparent testa and 22 that have normal coloured testa, but fail to accumulate anthocyanins in response to some but not all stresses that normally induce anthocyanin accumulation (M Van Oosten, unpublished data). One dominant mutation resulted in morphological differences that include substantially increased biomass, narrow leaves and large primary inflorescences relative to wild type (H Koiwa, unpublished results).

Thirty-eight mutations enhanced the NaCl sensitivity of sos3-1 seedlings, which is indicative of a function in a NaCl-response pathway or a tolerance mechanism that is at least partly independent of sos3-1. Characterization of two of the mutants has implicated them in the control of cellular redox and adenylate balance (PM Hasegawa, unpublished results).

** Luciferase imaging analysis**

The C24RD29a-LUC population was also screened for mutations that alter the expression of the RD29a-LUC transgene in response to cold, ABA, and NaCl treatments. T2 progeny of 54,200 T-DNA tagged C24RD29a-LUC lines were evaluated. Thirty-one mutant lines with altered luciferase expression were identified. Included were two individual lines harbouring independent mutations in two of the four members of a gene family that encodes isoforms of C-terminal domain (CTD) phosphatases (CPLs) (Koiwa et al., 2002). AtCPLs dephosphorylate conserved heptad repeats (YSPTSPS) in the C-terminal domain of the RNA polymerase II largest subunit (Koiwa et al., 2004). Mutations in cpl1 sensitize RD29a expression in response to cold and NaCl stresses and ABA treatment, whereas the cpl3 mutation confers only an ABA-dependent RD29a hyper-responsiveness to plants. This indicates that CTD-phosphorylation and dephosphorylation specifically control different aspects of stress signal outputs. Mutation of hos9-1 (for high expression of osmotically responsive genes) results in the hyperactivation of the RD29a promoter under low temperature, but not in response to ABA or salinity stress treatments (Zhu et al., 2004). The HOS9 gene encodes a nuclear-localized homeodomain transcription factor that facilitates freezing tolerance. Microarray analysis of hos9-1 indicated that HOS9 regulates a freezing tolerance transcriptome that functions independently of CBF (Zhu et al., 2004). HOS10/MYB8 also hyperactivates the RD29a promoter and is required for cold acclimation. The HOS10 gene may also control an ABA biosynthetic pathway that is necessary for gene expression under cold- and osmotic-stress conditions (Zhu et al., 2005).

**Discussion**

Over the last decade, vast insight about plant growth and development and other plant processes has been gained because of the molecular genetic approaches and resources that are available for the study of *Arabidopsis thaliana* biology. Genes involved in numerous cellular and whole-plant phenotypes have been identified and characterized through the analysis of mutants that were generated using physicochemical or biological mutagens, such as EMS, fast-neutron, T-DNA, and transposons. Analysis of gene function has been expedited by the completion of the arabidopsis genome sequencing project and the subsequent creation of databases with annotations of gene products (Somerville and Somerville, 1999; Borevitz and Ecker, 2004). More recently, the availability of indexed insertion mutant collections has facilitated the dissection of genetic interactions between combinations of genes (Borevitz and Ecker, 2004). These resources and capacities are being used increasingly to identify and characterize the functions of genes that impact the capacity of plants to respond and adjust to various environmental perturbations.

A T-DNA tagging strategy using arabidopsis was used to evaluate plant responses to cold, osmotic, and salinity stresses by generating mutations in several different genetic backgrounds (Table 1). RD29a reporter gene system-based screening was used to identify many mutants that lack an obvious visual phenotype under normal growth conditions. However, several of these exhibited impaired survival and growth capabilities (reduced in most lines) under various stress treatments (Koiwa et al., 2003, 2004; Zhu et al., 2002, 2005).

This study’s screens included a total of 250,000 independent insertion lines. Given the c. 125 Mbp genome size of arabidopsis (Borevitz and Ecker, 2004) and an average of 1.5 T-DNA insertions/transformant (Feldmann, 1991), theoretically the lines screened represented a population of T-DNA insertions at every 0.6–1.7 kbp in the genome. The selection for herbicide (bialaphos) resistance carried by the mutagenic T-DNA and used to identify T1 lines was extremely efficient as any mutants that failed to carry a copy of the marker gene were not identified, although, in a few instances, its function was lost in the T2 generation. The cellophane membrane transfer and CCD imaging systems were highly robust and contributed to a streamlined high throughput screening for mutant phenotypes. It is intriguing to note that very few dominant gain-of-function mutations have been identified that alter responses to abiotic stresses. In some instances, this may...
have been due to the very specific nature of the screens, such as for suppressors of sos3. However, in the RD29a:: LUC screens, no lines were identified that exhibited a phenotype that was the result of activated expression of CBF/DREB or ABF/ABREB transcription factors which are known to activate the RD29a gene (Narusaka et al., 2003). The frequency of apparent dominant morphological mutants in the T1 generation was also not very high, averaging about one in 400. Since most of the screening was conducted with the C24 ecotype, perhaps it possesses greater capability to silence 35S enhancer repeats that were used for most of the activation T-DNA tagging. Indeed, recent studies indicate that a large proportion of T-DNA and 35S enhancer insertions are silenced in transgenic plants due to its chromosomal location and multiple T-DNA insertions (Chalfun-Junior et al., 2003; Francis and Spiker, 2005). These obstacles may be overcome in the future by using mutant backgrounds that lack a transcriptional and post-transcriptional gene silencing capacity (Sung et al., 2003; Baulcombe, 2004; Matzke et al., 2004).

Dysfunctional alleles were generated by insertions in the 5′ or 3′ UTR as well as insertions as far upstream as 600 bp from the transcription start. Mutated alleles that caused phenotype changes sometimes resulted from knockout and knockdown expression as well as production of modified transcripts or proteins. Further, a number of the mutations that substantially affect plant responses to abiotic stresses occurred in individual members of gene families. Such cases indicated that functional redundancy often could not be predicted from DNA sequences (Bioinformatics). Double mutant analysis confirmed that, in some instances, gene families did have essential functions, i.e. mutations in both members of a family caused lethality (Koiwa et al., 2004).

These studies have shown that T-DNA-tagging followed by phenotypic screens (forward genetics) allows the expeditious identification of genes involved in cold, osmotic, and salinity stress and ABA-mediated gene expression. Confirmation of the linkage between the T-DNA insertion locus and a mutant phenotype can be confirmed by a comparative analysis with insertion mutants available from indexed collections such as the SALK collection. Furthermore, these genomic scale resources greatly facilitate the functional analysis of gene family members by reverse genetics. Large-scale screening has revealed some interesting characteristics of stress-tolerance mutants. For example, mutants impaired in stress and ABA responses were found to be the result of altered loci encoding proteins involved in very global control systems such as glycosylation, vesicular transport, sumoylation, and RNA synthesis and processing which has also been observed by others (CPL, SAD, etc.) and these cellular systems were previously considered to be associated with general cellular function and not with specific phenotypes.

As previously mentioned, mutants with reduced tolerance to both ionic and non-ionic stress often exhibited altered stomatal function. The high through-put forward genetic approach should eventually allow larger screens capable of almost saturating the genome for one or more mutant alleles at nearly every locus. It is, therefore, within present technical capabilities to identify, with reasonable effort, all of the loci contributing to any particular phenotype. From such a comprehensive collection of phenotype altering loci, particular key loci that impact different physiological mechanisms involved in a trait of interest may then be recognized. For example, the loci most required and/or with the highest sufficiency for osmotic adjustment and for stomatal control during adaptation to limited water availability could be identified by genome saturating loss of function screens. After identifying such key loci, the challenge will be to locate superior (gain of function) alleles of these loci. The most promising sources of these superior alleles would be trait-specific (e.g. NaCl-stress tolerant) Arabidopsis Relative Model Systems (ARMS) (Inan et al., 2004) and germplasm sources (probably secondary and even tertiary gene pools) of specific crop species that exhibit the precise trait of interest. Eventually, effective alleles of the most important loci for a trait, such as salt tolerance, could be pyramided into crops by either genetic transformation or zero distance marker-assisted breeding using the identified important loci as zero distance markers.

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References


