Genetic Analysis of Salt-Tolerant Mutants in *Arabidopsis thaliana*

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Manuscript received July 28, 1999
Accepted for publication September 20, 1999

ABSTRACT

Stress caused by the increased salinity of irrigated fields impairs plant growth and is one of the major constraints that limits crop productivity in many important agricultural areas. As a contribution to solving such agronomic problems, we have carried out a large-scale screening for *Arabidopsis thaliana* mutants induced on different genetic backgrounds by EMS treatment, fast neutron bombardment, or T-DNA insertions. From the 675,500 seeds we screened, 17 mutant lines were isolated, all but one of which yielded 25–70% germination levels on 250 mm NaCl medium, a condition in which their ancestor ecotypes are unable to germinate. Monogenic recessive inheritance of NaCl-tolerant germination was displayed with incomplete penetrance by all the selected mutants, which fell into five complementation groups. These were named *SALOBREÑO* (*SAÑ*) and mapped relative to polymorphic microsatellites, the map positions of three of them suggesting that they are novel genes. Strains carrying mutations in the *SAÑ1-SAÑ4* genes display similar responses to both ionic effects and osmotic pressure, their germination being NaCl and mannitol tolerant but KCl and Na_2SO_4 sensitive. In addition, NaCl-, KCl-, and mannitol-tolerant as well as abscisic-acid-insensitive germination was displayed by *sañ5*, whose genetic and molecular characterization indicates that it carries an extremely hypomorphic or null allele of the *ABI4* gene, its deduced protein product lacking the APETALA2 DNA binding domain.

FOLLOWING centuries of irrigation and extensive exploitation, many important agricultural areas of our planet have suffered changes in the chemical composition of their soils. The accumulation of salts is one such modification, salinity being one of the major constraints limiting plant growth in some of the most productive agricultural regions of the world (Boyer 1982). Mechanisms underlying salt tolerance have been studied by different experimental approaches, whose results indicate that plants have evolved a wide spectrum of mechanisms to cope with salt stress, the most complex of which involve multiple interactions. Examples of such mechanisms include some developmental processes (*e.g.*, variations in flowering time), structural features (such as leaf surface permeability), and physiological processes (such as ion partitioning to vacuoles). A fourth adaptative resource, consisting of metabolic responses, is likely to involve fewer gene products than the above-mentioned phenomena and is therefore assumed to be more amenable to analysis (McCue and Hanson 1990). Examples of processes of the last type are the synthesis and accumulation of osmoprotective low-molecular-weight metabolites, called compatible solutes or osmolytes, such as glycine-betaine, proline, polyols, or fructans (Yancey *et al.* 1982; Paul and Cockburn 1989; Delauny and Verma 1993; Hendry 1993; Hanson *et al.* 1994; Stoop *et al.* 1996), the detoxification of radical oxygen species (Bohnert and Sheveleva 1998), the regulation of potassium acquisition (Rubio *et al.* 1995), and the transcriptional activation of genes coding for osmoprotective proteins like dehydrins and late embryogenic abundant (LEA) proteins, which are normally synthesized during seed maturation (Bray 1993), or osmotin (Singh *et al.* 1985).

With the avowed goal of obtaining engineered crop variants able to cope with salinity, much effort has been devoted to the better understanding and the eventual manipulation of plant responses to salt stress (Greenway and Munns 1980; Bohnert and Sheveleva 1998; Holmberg and Bülow 1998). One of the strategies followed focuses on the identification of genes whose transcriptional levels increase in response to salinity (Urao *et al.* 1993, 1994; Hirayama *et al.* 1995; Mizoguchi *et al.* 1995; Savouré *et al.* 1995; Rentsch *et al.* 1996; Hong *et al.* 1997; Qiang *et al.* 1998; Roosens *et al.* 1998) or proteins whose activity rises (López *et al.* 1996) or that are accumulated (Singh *et al.* 1985) under high salt concentrations. Most NaCl-responsive genes are also induced by abscisic acid (ABA), a plant hormone that modulates stress responses (Hetherington and Quatrano 1991). Although many genes are induced by salt, only a few of them seem to be essential for salt tolerance (Serrano and Gaxiola 1994).

As previously mentioned, it is known that under saline conditions some plants accumulate osmoprotective metabolites (Yancey *et al.* 1982). The engineered expres-

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sion of genes pertaining to pathways producing osmolytes in nonaccumulator plants (Tarczynski *et al.* 1993; Kishor *et al.* 1995; Thomas *et al.* 1995; Lilius *et al.* 1996; Hayashi *et al.* 1997; Karakas *et al.* 1997; Sheveleva *et al.* 1997) or of genes encoding LEA proteins (Xu *et al.* 1996) have led to some increases in plant salt tolerance. However, it has yet to be shown whether transfer of the above-mentioned transgenes to crop plants will enable them to successfully cope with salinity.

A third strategy is based on the use of unicellular organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, as models to study salt tolerance, identifying key genes for salt resistance and attempting to transfer them into plant genomes or to isolate their orthologs in plants (Serrano and Gaxiola 1994). However, the evolutionary distance between unicellular organisms and higher plants might be a serious setback when trying to obtain high expression, at the right time and cellular localization, of foreign genes in plants (Holmberg and Bülow 1998).

Another approach consists of isolating mutant plants with reduced or increased sensitivity to salt. In the glycophyte *Arabidopsis thaliana*, a few mutants capable of germination in highly saline conditions have been isolated (Saleki *et al.* 1993; Werner and Finkelstein 1995), although none of the damaged genes has yet been cloned. In addition, salt-hypersensitive *sos* (*salt overly sensitive*) Arabidopsis mutants, unable to grow on low-K^+ culture medium, have recently been isolated (Wu *et al.* 1996; Liu and Zhu 1997; Zhu *et al.* 1998), allowing the cloning of the *SOS3* gene, whose product shares homology with the calcineurin B subunit of yeast and neuronal calcium sensors of animals (Liu and Zhu 1998).

Due to the advantages it has for genetic and molecular analysis, we have chosen the model system *A. thaliana* in an attempt to isolate variants capable of germinating in the presence of high salt concentrations. We present here the results of a large-scale screening for *A. thaliana* mutants able to germinate on high NaCl concentrations.

MATERIALS AND METHODS

Plant material and growth conditions: *A. thaliana* (L.) Heynh. M_2 seeds derived from either ethyl methanesulfonate or fast-neutron mutagenesis were supplied by Lehle Seeds (Round Rock, TX). T_4 seeds of lines carrying T-DNA insertions (obtained in the laboratory of K. Feldmann) and F_5 seeds of lines carrying *Ac/Ds* insertions (obtained in the laboratory of C. Dean) were provided by the Nottingham *Arabidopsis* Stock Centre (NASC, Nottingham, UK).

Sterile (in 150-mm petri dishes containing 100 ml agar medium) and nonsterile (in pots containing a 1:1:1 mixture of perlite, vermiculite, and sphagnum moss) cultures were performed at 20 ± 1°, 60–70% relative humidity and continuous illumination of 7000 lux as described in Ponce *et al.* (1998).

Salt-tolerant growth was tested by sowing mutant and wild-type seeds on either nonsupplemented or 50 mm NaCl agar medium, at a density of 100 seeds per plate. A total of 30 stressed or nonstressed plants from each wild-type and mutant line were collected 3 wk after sowing. Fresh weight was determined immediately after harvest and dry weight after desiccation at 65° overnight.

Mutant isolation and germination assays: To screen for mutants, seeds were sown on agar medium supplemented with 250 mm NaCl. Such high density sowings consisted of spreading ~5000 seeds (80 mg) per 150-mm petri dish, with the help of 6 ml of top agar (5 g $liter^{-1}$). For all other germination assays, low density sowings were performed by plating seeds in a water suspension, using a Pasteur pipette, at a density of 100 regularly spaced seeds per plate, in 150-mm petri dishes filled with 100 ml of agar medium supplemented with NaCl (0–250 mm), KCl (0–150 mm), Na_2SO_4 (0–150 mm), mannitol (0–500 mm), or ABA (2.5–50 μm). We considered as salt resistant only those seedlings that displayed green and fully expanded cotyledons up to a maximum of 2 wk after sowing.

Genetic analysis: Crosses were performed under a binocular microscope by transferring pollen from mature anthers to the stigmas of previously emasculated flowers. F_1 and inbred F_2 seeds from backcrosses between mutants and their wild-type ancestors were plated on agar medium containing 200 or 250 mm NaCl. In mutants carrying T-DNA insertions, the number of independent inserts was established by sowing F_2 seeds from backcrosses between mutants and their wild-type ancestors on agar medium supplemented with kanamycin (50 μg ml^{-1}). Allelism tests were performed by intercrossing independently isolated homozygous mutants and sowing the resulting F_1 seeds on media supplemented with 200 or 250 mm NaCl.

Individuals homozygous for representative alleles of each *SAÑ* complementation group were outcrossed to ecotypes that were polymorphic to those from which they were obtained, and their F_2 progeny were sown on 200 mm NaCl medium. F_2 seedlings displaying the salt-resistance recessive mutant phenotype were transferred to medium without NaCl and their genomic DNA extracted so that linkage to polymorphic simple sequence length polymorphism (SSLP) markers (Bell and Ecker 1994) could be tested. DNA purification, PCR amplifications, and SSLP analysis were performed following the high-throughput method described in Ponce *et al.* (1999), which is based on multiplex PCR and fluorescent semiautomated detection of amplified microsatellites. Kosambi's mapping function (Kosambi 1944; Koornneef and Stam 1992) was used to convert raw recombination percentages into map distances in centimorgans.

PCR amplifications: Synthetic oligonucleotides were bought from Perkin-Elmer Applied Biosystems UK. The sequences of those used to amplify and sequence a segment of the *ABI4* gene were as follows: ABI4F, 5′-GGACCCTTTAGCTTCCC AAC-3′; ABI4R, 5′-GACCGACCTTAGGGATGCTC-3′; and ABI4R-2, 5′-CCGTATAGGTAAACGGCAGC-3′; their homologous regions in the *ABI4* gene (Finkelstein *et al.* 1998) are 153-172, 1190-1170, and 458-439, respectively. Oligonucleotides used to test for the presence of T-DNA insertions were the following: LB1, 5′-GCTGCAGTAATAGGCAAGGCGTACT GCG-3′; pBR322-2, 5′-AAGTGCCACCTGACGTCTAAG-3′; RB2, 5′-CGGAATTCTCAGCCTGTGATGACCTTGC-3′; and pBR322-1, 5′-ACCTGTCCTACGAGTTGCATG-3′; their homologous regions in the T-DNA-based construct pGV3850:1003 (Velten and Schell 1985) are 737-760, 3123-3103, 16345-16326, and 13917-13937, respectively. PCR amplifications were performed in Perkin-Elmer (Norwalk, CT) 2400 thermocyclers, using 0.2-ml thin-walled tubes in 25-μl mixtures containing 1 μl of genomic DNA (≈300 ng) as a template, 0.5 units of BioTaq enzyme (Bioline), 10 pmol of each oligonucleotide, and 0.2 mm deoxynucleotide mixture. The thermocycling program started with an initial denaturation of 2 min

at 94°, followed by 35 cycles of 30 sec at 94°, 15 sec at 55°, and 2.5 min at 72°, and a final 10 min incubation at 72°. A total of 2.5 μl of each reaction mix was run in ethidium-bromide-stained 1% agarose gels.

RT-PCR: Three-week-old plants were transferred from agar plates to flasks containing 30 ml of liquid medium consisting of 2.2 g liter^{-1} Murashige and Skoog basal medium (Sigma, St. Louis), 10 g liter^{-1} sucrose, and 0.5 g liter^{-1} MES, the pH being adjusted to 6.00 with KOH before autoclaving. The flasks were shaken at 125 rpm and after 1 day, an appropriate volume of 5 m NaCl was added to reach a 250 mm NaCl concentration. Whole plants were collected for RNA isolation 14 hr later, immediately frozen in liquid nitrogen, and stored at −80°. Each sample of plant material was weighed immediately prior to being homogenized in 1.5-ml Eppendorf tubes containing 500 μl of Trizol (GIBCO/BRL, Gaithersburg, MD) and incubated for 5 min at room temperature. RNA was chloroform-extracted, isopropyl-alcohol-precipitated, and resuspended in H_2O. Genomic DNA was removed by adding 5 units of DNase I (GIBCO/BRL) and incubated for 30 min at 37° and then for 10 min at 70°, to inactivate the enzyme. RNA was ethanol precipitated and resuspended in 50 μl water. First-strand cDNA synthesis was performed with 200 units of Super-Script II (GIBCO/BRL) reverse transcriptase, in a 20-μl reaction mixture containing 0.5 mm of each dNTP, 10 mm DTT, 40 units of RNaseOUT, and 10× $p(dN)_6$ (Boehringer Mannheim, Indianapolis) primers. Total RNA (1 μg) was used as template, after being incubated 5 min at 65° and then immediately put on ice. Samples were incubated for 10 min at 25° and then for 1 hr at 42°. The enzyme was inactivated by heating the reaction mixture for 15 min at 70°.

Primer sets used to RT-PCR-amplify messages from the *APK2a*, *P5CS-1*, and *MYB2* genes were the following [values in parentheses indicate positions in the corresponding cDNA (Ito *et al.* 1997) or gene (Urao *et al.* 1993; Savouré *et al.* 1995) sequences]: APK2aForward, 5′-CATCTGTTACGTTGT TCCACG-3′ (111-131); APK2aReverse, 5′-AATGGCCCAAAAC TACTATCTG-3′ (343-322); P5CS-1Forward, 5′-TAGGAGCAC TGTGTGAACAG-3′ (500-520); P5CS-1Reverse, 5′-TTCACCA GAAGTTGAGCTGC-3′ (1202-1182); MYB2Forward, 5′-TGCT CGTTGGAACCACATCG-3′ (1108-1127); MYB2Reverse, 5′-GGTGATCATTGACTCCACTTG-3′ (1681-1661). Each of these oligonucleotide pairs included a forward primer labeled with HEX phosphoramidite and was designed to span at least one intron to distinguish between genomic DNA and cDNA amplification products. PCR amplifications were performed in 5-μl reaction mixes of 200 μm for each dNTP and 2 mm $MgCl_2$, containing 0.2 units of BioTaq enzyme (Bioline), 0.5 μl of 10× reaction buffer (Bioline), and 0.5 μl of the 20-μl cDNA solution obtained from each sample of plant material. The final concentration of each oligonucleotide in the reaction mixture was 0.06 μm, which was reached by taking 1.2 μl from a master mixture containing the oligonucleotides listed in Table 1, each at a concentration of 0.25 μm. The thermocycling program started with an initial 1.5-min denaturation step at 94°, followed by 35 cycles (30 sec at 94°, 15 sec at 55°, 1.5 min at 70°), and a final 7 min incubation at 70°.

Electrophoreses were carried out in a Perkin-Elmer ABI PRISM 377 DNA sequencer, using a 4.25% acrylamide-bisacrylamide (29:1)/6 m urea gel, with 36-cm well-to-read glass plates. DNA fragment analysis was performed using the GENESCAN 2.1 software (Applied Biosystems) as described in the manufacturer's manual. Peak sizes were calculated using the local Southern method sizing option. For gel analysis, 1 μl of loading buffer, comprising a 5:1:1 mixture of deionized formamide, 50 mg ml^{-1} blue dextran, and 4 nm GeneScan-500 (TAMRA) internal size standard (Perkin-Elmer Applied Biosystems) were combined with 0.6 μl of PCR mixture. Samples were heated at 94° for 3 min immediately prior to gel loading and run for 3 hr, selecting the GS 36C-2400 module.

Southern blot analysis: Genomic DNA from mutants derived from T-DNA mutagenesis was isolated, digested, electrophoresed, transferred to membranes, and hybridized with digoxigenin-labeled probes as described in Ponce *et al.* (1998). Probes were made from either left (LB) or right (RB) border T-DNA segments included in constructs pBSH23 (with a 3.2-kb insert corresponding to RB) and pBSH10 (with a 6.5-kb insert corresponding to LB), both provided by the *Arabidopsis* Biological Resource Center.

Sequencing: PCR amplification products were subjected to direct sequencing without further cloning steps. Sequencing reactions were carried out with ABI PRISM dye terminator cycle sequencing kits according to the instructions of the manufacturer. Electrophoreses were performed on a Perkin-Elmer ABI PRISM 377 DNA sequencer.

RESULTS

Isolation of *salobreño* mutants: We performed a large-scale screening of ~675,500 seeds of *A. thaliana* that originated from >65,000 parental lines of different genetic backgrounds, subjected to different mutagenesis procedures (Table 1), with the aim of isolating mutants capable of germinating on agar medium supplemented with 250 mm NaCl, a concentration that completely abolishes expansion and green pigmentation of wild-type cotyledons. Seeds were sown at high density as described in materials and methods, and seedlings displaying fully expanded green cotyledons ~2 wk after sowing were transferred to agar medium without NaCl. In contrast to such putative mutants, the remaining seeds remained apparently dormant or showed radicle emergence but no further growth. A total of 578 putative mutant seedlings were isolated in this way and transferred to soil 5–6 wk after germination. Only 53 plants survived and yielded M_3 or T_5 viable progeny, which was collected for further studies.

We also tested whether the effect of high NaCl concentrations on wild-type seed germination was reversible. With this aim, wild-type seeds that apparently had not germinated after 10 days of exposure to 200 mm NaCl medium were transferred to agar medium not supplemented with salt. Of such seeds, 84% became seedlings with fully expanded green cotyledons 7 days later.

Study of the heritability of the mutant phenotypes: After their isolation, the first step of the characterization of our mutants was aimed at ascertaining phenotype heritability. Salt resistance of the progeny of each putative mutant was tested by sowing its inbred seeds at low density (see materials and methods) on petri dishes with agar medium supplemented with 250 mm NaCl. The salt-resistant germination phenotype was displayed by the progeny of 36 of the 53 putative mutant lines that were viable and fertile, although penetrance was incomplete in all instances. Seedlings of such mutants were able to survive up to 2 wk after sowing on 250 mm

TABLE 1

Selection of Arabidopsis mutants displaying salt-tolerant germination

Genetic background	Mutagen	Mutagenized lines	Screened seeds (generation)	Putative mutants	Viable mutants	
					Obtained	Studied in this work
L*er*	EMS[a]	12,000	100,000 (M_2)	65	5	1 (*sañ3-1*)
L*er*	Fast neutrons[a]	2,500	20,000 (M_2)	25	2	1 (*sañ1-4*)
Ws-2	T-DNA[b]	6,480	130,000 (T_3)	49	12	9 (*sañ1-1a* to *sañ1-1g*, *sañ2*, and *sañ4-2*)
Col	Fast neutrons[a]	45,452	363,000 (M_2)	439	34	6 (*sañ1-2*, *sañ1-3*, *sañ3-2*, *sañ4-1a*, *sañ4-1b*, and *sañ5*)
L*er*	Maize Ac/Ds system[c]	—	62,500 (F_5)	0	0	0
Total		66,432	675,500	578	53	17

[a] Mutagenesis performed by Lehle seeds.
[b] Mutagenesis performed in the laboratory of K. Feldmann (Feldmann and Marks 1987).
[c] Mutagenesis performed in the laboratory of C. Dean (Bancroft *et al.* 1992).

NaCl medium, displaying expanded green cotyledons, which became bleached when left longer on that salt concentration. We selected 16 of the 36 M_2 mutant lines for further characterization, those with the highest germination levels on 250 mm NaCl (ranging from 25 to 70%). One additional line, T07c, obtained from T-DNA mutagenesis, was shown to be kanamycin resistant but unable to germinate on 250 mm NaCl. It was also studied because it reached higher germination levels on 100 and 150 mm NaCl than its wild-type ancestor. Among those mutant lines, which were named *salobreño* (*sañ*; from the Spanish word for salty land), 9 derived from T-DNA insertional mutagenesis, 7 from physical mutagenesis by fast neutron bombardment, and the remaining 1 was obtained from chemical mutagenesis by ethyl methanesulfonate (EMS) treatment. As commented below, mutants isolated from the same parental group were considered possible candidates to be identical.

Determination of the inheritance patterns of Salobreño phenotypes: Mutants were backcrossed to their ancestor ecotypes as well as outcrossed to at least one ecotype other than that from which they originated, the F_1 progeny being sown on 250 mm NaCl medium. After establishing that all the studied phenotypes in such F_1 progenies had been inherited as recessive traits (data not shown), F_2 seeds were obtained by selfing F_1 individuals grown on medium not supplemented with NaCl. Germination of 100% was obtained when ~100 F_2 seeds from each cross were sown on medium not supplemented with NaCl. Additional F_2 progeny were sown on 200 mm NaCl, the number of putative homozygous recessive individuals being calculated on the basis of the expected number of salt-resistant seeds and the average penetrance already known for each mutant line on that salt concentration (Table 2). Under the above assumptions, a 3:1 (wild type:mutant) phenotypic segregation ratio, corresponding to a monogenic recessive transmission pattern, was found likely for the F_2 progeny of all backcrosses, except those involving the mutant lines N1a and T20, which were later named *sañ1-2* and *sañ4-2* (see below). In addition, the penetrance of a given mutant phenotype was found to depend upon its genetic background and to change in the F_2 progeny from outcrosses involving a mutant and an ecotype different from its wild-type ancestor. It must be taken into account that the ecotypes L*er*, Col, and Ws-2 differ in their sensitivities to NaCl, as described below and illustrated in Figure 1.

Complementation analysis: To determine the number of genes identified in our search, homozygous recessive mutant individuals were intercrossed and their F_1 progeny sown on 250 mm NaCl medium (Table 3). As previously mentioned (Table 1), 9 of the 17 mutants under study derived from T-DNA mutagenesis, 7 of which were finally named *sañ1-1a* to *sañ1-1g*, since they were thought to represent the same mutation because they had been obtained from the same T_4 pool of screened seeds. Two lines of evidence confirmed such a suspicion. First, both their inbred seeds and F_1 seeds from their intercrosses displayed very similar germination percentages on 200 mm NaCl. Second, gel blots of restricted genomic DNA, probed with either left border or right border of T-DNA, showed an identical pattern of bands for all these 7 T-DNA-tagged mutant lines, as has to be expected from genotypically identical individuals (data not shown). From these mutants, we elected *sañ1-1a* for further studies. Similarly, the mutant lines *sañ4-1a* and *sañ4-1b* were obtained from the same pool of M_2 seeds derived from fast neutron mutagenesis and thus were suspected of representing the same mutation. They presented quite similar germination percentages on salt-supplemented media and did not complement. Mapping data confirmed that these two mutants were identical, since both were likely to represent the rare event of viable translocation (see below).

The results of such a complementation analysis revealed that 16 of the studied mutants carried mutations falling into four complementation groups, which were

TABLE 2

Germination on 200 mm NaCl medium of the F_2 progeny of crosses involving *sañ* mutants and wild-type individuals

Cross (♀ × ♂)	Genetic background of the mutant strain	F_2 seeds		Penetrance (%)		χ^2 (3:1)
		Sown	Germinated	Observed	Expected	
Ws-2 × T78e (*sañ1-1a*)	Ws-2	198	25	50.5	58.8 ± 16.1	0.69
Col × T78e (*sañ1-1a*)		360	81	90.0		*17.40*
Col × N1a (*sañ1-2*)	Col	201	40	79.6	50.4 ± 24.1	*8.60*
L*er* × N1a (*sañ1-2*)		415	77	74.2		*12.05*
Col × N20a (*sañ1-3*)	Col	439	76	69.2	83.4 ± 4.8	2.94
L*er* × N20a (*sañ1-3*)		415	72	69.4		2.70
L*er* × N39 (*sañ1-4*)	L*er*	208	41	78.8	65.7 ± 16.5	1.33
Col × N39 (*sañ1-4*)		284	78	109.9		*23.90*
L*er* × E47a (*sañ3-1*)	L*er*	206	39	75.7	69.6 ± 10.8	0.20
Col × E47a (*sañ3-1*)		685	147	85.8		*7.20*
Col × N6a (*sañ3-2*)	Col	186	31	66.6	73.4 ± 10.4	0.21
L*er* × N6a (*sañ3-2*)		363	58	63.9		1.10
Col × N10a (*sañ4-1a*)	Col	197	39	79.2	84.8 ± 3.5	0.17
L*er* × N10a (*sañ4-1a*)		467	97	83.1		0.03
Col × N10e (*sañ4-1b*)	Col	202	41	81.1	76.6 ± 0.5	0.08
L*er* × N10e (*sañ4-1b*)		645	121	75.0		0.07
Ws-2 × T20 (*sañ4-2*)	Ws-2	312	60	76.9	59.8 ± 6.1	*4.05*
L*er* × T20 (*sañ4-2*)		303	46	60.7		0.00
Col × T20 (*sañ4-2*)		305	64	83.9		*8.10*
Col × N3c (*sañ5*)	Col	107	21	78.5	81.0 ± 9.2	0.00
L*er* × N3c (*sañ5*)		207	28	54.1		*7.70*

All mutant strains, which are noted according to their protocol numbers, were homozygous. Allele names indicated in parentheses were given after complementation analysis (see Table 4). ♀, pollen recipient; ♂, pollen donor. The χ^2 values represent the fit of the data to an expected 3:1 (wild type:mutant) phenotypic segregation and were obtained after calculating the number of expected germinated seedlings, multiplying the number of seeds sown by 0.25 and by the penetrance of the mutant phenotype in its original genetic background at that NaCl concentration. Numbers in italics indicate that the null hypothesis tested was rejected.

named *SAÑ1* to *SAÑ4*. The remaining mutant line was not crossed to others due to its insensitivity to ABA, a phenotypic trait not shared by the remaining mutants, which suggested that it carried a mutation in a different locus. This hypothesis was confirmed by mapping data that showed that the line defined a new locus, initially named *SAÑ5* and later found to be an allele of the *ABI4* gene (see below). The *SAÑ1* gene was represented by four mutant alleles of different parental origin and *SAÑ3* and *SAÑ4* by two, whereas *SAÑ2* had only one.

Characterization of mutants derived from insertional mutagenesis: To determine if any of the mutants derived from T-DNA mutagenesis were tagged, T_5 progeny from selfed plants were sown on agar medium supplemented with kanamycin. It was found that all T78e (carrying the *sañ1-1* mutation) sibling-isolates and T07c (*sañ2*) plants were kanamycin resistant (Kan^R). However, the strain T20 (*sañ4-2*) was shown to be kanamycin sensitive (Kan^S), and no products were obtained in PCR amplifications performed with its genomic DNA as a template and synthetic oligonucleotides designed to amplify the left or the right T-DNA borders. Gel blots of digested T20 genomic DNA, probed with either LB or RB probes, also failed to detect the presence of any T-DNA insert (data not shown).

To determine the number of T-DNA insertions in T78e and T07c mutant lines, these mutant lines were reciprocally crossed to their wild-type ancestor (Ws-2), all their F_1 seedlings being Kan^R. The segregation of the latter phenotype was tested in F_2 families derived from crosses of T78e (7 families) and T07c (12 families) by Ws-2. In the first case, phenotypic segregation data of 2 families fit a 15:1 ratio (Kan^R:Kan^S), while for the remaining 5 families a 63:1 ratio was found likely. These data are consistent with the presence of three unlinked T-DNA-tagged loci in T78e, two of them in a homozygous and one in a heterozygous condition. As regards T07c, segregation of all the studied F_2 families fit a 15:1 ratio, suggesting the presence in its genome of two independent T-DNA insertions, both homozygous. This line was not studied further, however, because of its weak salt-tolerant phenotype.

Linkage between the *sañ1-1* salt-resistant allele and the kanamycin resistance marker was further analyzed by sowing F_2 seeds obtained from crosses between T78e and Ws-2, on 200 mm NaCl, and selecting seedlings with

TABLE 3

Complementation testing of *salobreño* mutants

♀	♂									
	sañ1-1a	*sañ1-2*	*sañ1-3*	*sañ1-4*	*sañ2*	*sañ3-1*	*sañ3-2*	*sañ4-1a*	*sañ4-1b*	*sañ4-2*
T78e (*sañ1-1a*)	60.3(794)	NP	NP	NP	NP	NP	NP	NP	NP	NP
N1a (*sañ1-2*)	24.0(167)	26.2(316)	40.0(60)	66.6(21)	0.0(108)	0.0(47)	0.0(29)	0.0(23)	0.0(23)	NP
N20a (*sañ1-3*)	30.6(51)	65.0(77)	57.2(331)	58.0(81)	0.0(58)	0.0(62)	0.0(56)	0.0(108)	0.0(74)	0.0(40)
N39 (*sañ1-4*)	59.1(66)	76.1(138)	41.2(51)	32.0(473)	0.0(21)	0.0(147)	0.0(87)	0.0(27)	0.0(5)	0.0(12)
T07c (*sañ2*)	0.0(36)	NP	NP	NP	53.0(150)	NP	NP	NP	NP	NP
E47a (*sañ3-1*)	0.0(64)	10.0(34)	0.0(90)	0.0(193)	0.0(54)	40.6(561)	10.0(20)	0.0(25)	0(8)	0.0(50)
N6a (*sañ3-2*)	0.0(121)	0.0(54)	0.0(41)	0.0(44)	0.0(128)	42.0(222)	37.4(174)	0.0(37)	0(39)	0.0(62)
N10a (*sañ4-1a*)	0.0(51)	0.0(42)	0.0(49)	0.0(40)	0.0(70)	0.0(28)	0.0(38)	70.0(261)	53.0(115)	34.3(51)
N10e (*sañ4-1b*)	0.0(19)	0.0(77)	10.0(192)	0.0(39)	0.0(11)	0.0(32)	0.0(16)	20.2(183)	40.5(351)	NS
T20 (*sañ4-2*)	UC	UC	0.0(14)	UC	UC	UC	0.0(29)	35.1(37)	24.0(46)	41.0(994)

♀, pollen recipient; ♂, pollen donor. Homozygous mutant lines were intercrossed and their F_1 progeny sown on 250 mm NaCl agar medium, with the exception of T78e (*sañ1-1a*) and T07c (*sañ2*) seeds, which were sown, respectively, on 200 and 150 mm. Numbers indicate germination percentages, those in parentheses indicating the absolute number of analyzed seeds. UC, unsuccessful cross; NS, nonviable seeds; NP, cross not performed.

fully expanded green cotyledons to grow until their inbred progeny were collected. Seeds from 16 F_3 families were sown on medium supplemented with either 200 mm NaCl or 50 μg ml^{-1} kanamycin. All the studied F_3 families showed germination percentages on NaCl-supplemented medium similar to those of their *sañ1-1* ancestor. However, different Kan^R:Kan^S ratios were obtained (15:1 and 3:1 segregations, as well as 1 family with no resistant individuals), allowing us to discard linkage between salt resistance and the Kan^R marker, as well as to conclude that *sañ1-1* is an untagged mutation.

Mapping of *salobreño* mutants: To map the *sañ* mutations, homozygous mutants in a Col background (*sañ1-2*, *sañ1-3*, *sañ3-2*, *sañ4-1a*, *sañ4-1b*, and *sañ5*) were outcrossed to wild-type L*er* plants, whereas mutants in L*er* (*sañ1-4* and *sañ3-1*) and Ws-2 (*sañ1-1a*, *sañ2*, and *sañ4-2*) backgrounds were outcrossed to wild-type Col individuals. F_2 progenies were sown on agar media supplemented with 200 mm NaCl (to map the salt-resistance loci) or 3 μm ABA (to map the ABA insensitivity mutation) and seedlings displaying green, fully expanded cotyledons (phenotypically recessive F_2 individuals) were selected and transferred to nonsupplemented agar medium 6–8 days after sowing in order to extract their genomic DNA 2 wk later. Map positions were determined by testing linkage to SSLP markers (Bell and Ecker 1994; Ponce *et al.* 1999). All alleles of *SAÑ1* were shown to be on chromosome 5, 7.3 ± 1.6 cM below the MBK5 marker. Linkage to nga129 (30.6 ± 3.5 cM away from *SAÑ1*) had to be scored in F_2 individuals obtained from T78e (in a Ws-2 background) by Col crosses, because of the small degree of polymorphism for this marker between the ecotypes L*er* and Col. Linkage analysis for *sañ2* could not be assessed due to the weakness of the mutant phenotype of T07c, which hindered the selection of true phenotypically mutant F_2 individuals. The *SAÑ3* gene was found to be on chromosome 1, 8.4 ± 4.1 cM below T27k12-Sp6 and 11.6 ± 3.1 cM above AthGENEA, as well as 35.2 ± 4.7 cM below AthZFPG. As regards *SAÑ4*, all mutant lines carrying *sañ4* alleles were subjected to linkage analysis. Unexpectedly, overall data for *sañ4-1a* and *sañ4-1b* showed linkage to markers on chromosome 1, 3.0 ± 1.5 cM below AthZFPG and 8.3 ± 5.8 cM above T27k12-Sp6, as well as to markers on chromosome 5, 8.7 ± 2.7 cM below nga139 and 11.1 ± 3.0 cM above AthPHYC, which indicated that both mutant lines represented a single allele, which corresponds to a translocation involving chromosomes 1 and 5. However, for the remaining allele, *sañ4-2*, we only found linkage to markers on chromosome 1, 11.7 ± 3.6 cM above AthZFPG and 24.9 ± 4.7 cM below AthACS. Finally, the *sañ5* mutation was mapped near the lower telomere of chromosome 2, at a very short distance from nga168, since only one recombinant event was found between these loci after scoring 104 chromosomes.

Sensitivity of *salobreño* mutants to salts and mannitol:

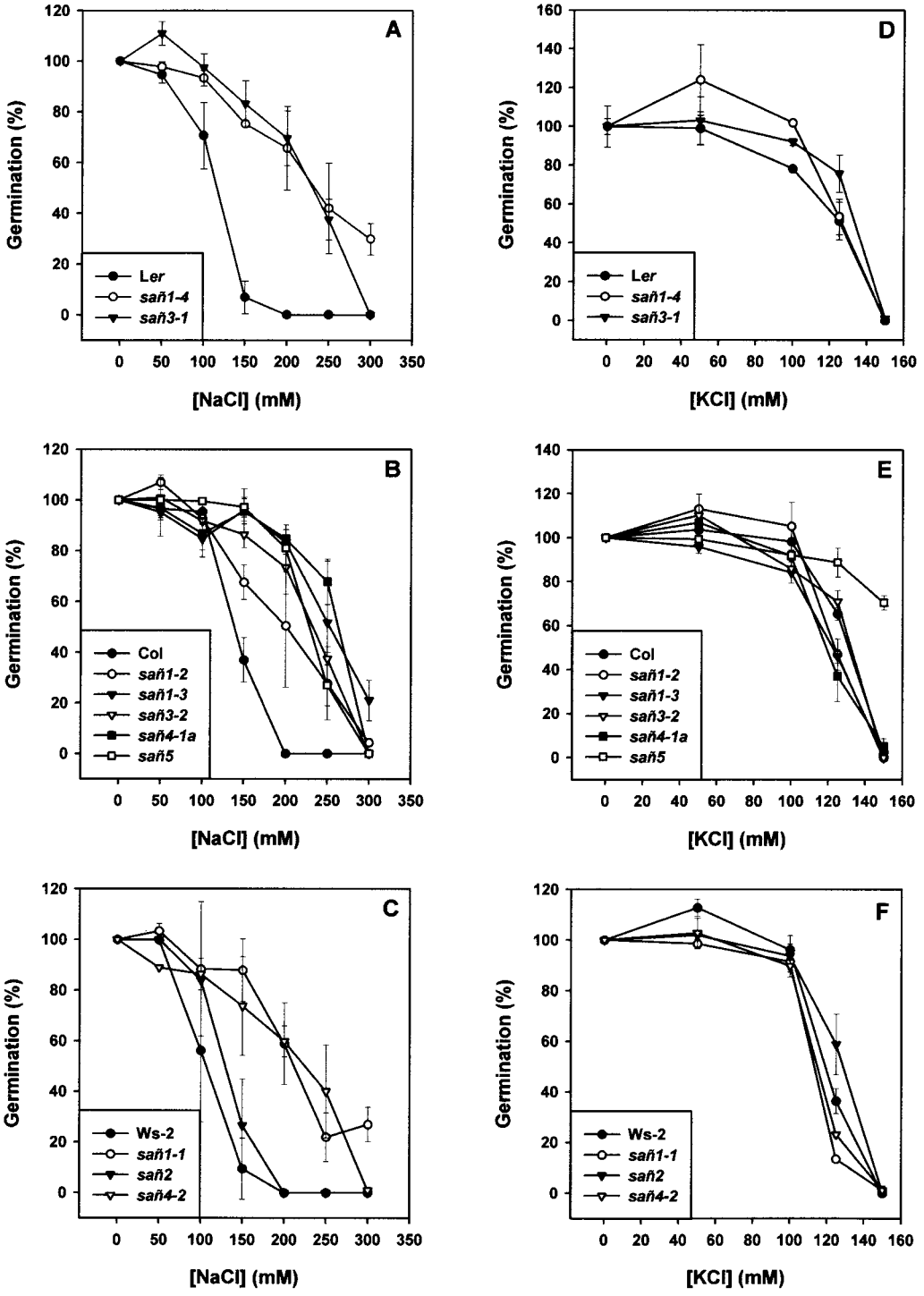

Figure 1.—(A–I) Ion toxicity and (J–L) effects of osmotic stress on wild-type and mutant Arabidopsis lines. Each graph represents the germination pattern of several mutants and their ancestor ecotype in various NaCl (A–C), KCl (D–F), Na_2SO_4 (G–I), and mannitol (J–L) concentrations, scored 10 days after sowing. Germination patterns of each mutant line were obtained after calculating the percentages of germinated seedlings on supplemented media and referring them to values reached for the same strain on nonsupplemented media. Error bars represent standard deviations. Each dot corresponds to the average of three or more replicates of 50–100 seeds. L*er*, Landsberg *erecta*; Col, Columbia; Ws-2, Wassilewskija.

To determine the ability of our mutants to germinate on NaCl, mutant seeds were sown on different concentrations of this salt (Figure 1, A–C). Since mutant lines had been obtained in three different genetic backgrounds (L*er*, Col, and Ws-2), we used seeds of the corresponding wild-type ancestor as controls in each experiment. Cotyledon expansion was completely inhibited on 200 mm NaCl in the three ecotypes studied. On 150 mm NaCl, however, 40% of Col seeds and <10% of Ws-2 or L*er* seeds displayed expanded green cotyledons (Figure 1, A–C). In contrast, all mutants except *sañ2* germinated on 250 mm and showed reduced sensitivity to NaCl, producing fully expanded green cotyledons. Moreover, some *sañ1* seeds (Figure 1, A–C) were able to germinate even on 300 mm NaCl, a salt concentration higher than that used to screen for mutants.

To determine whether *sañ* mutants were resistant to specific ions or osmotic stress, seeds were sown in media

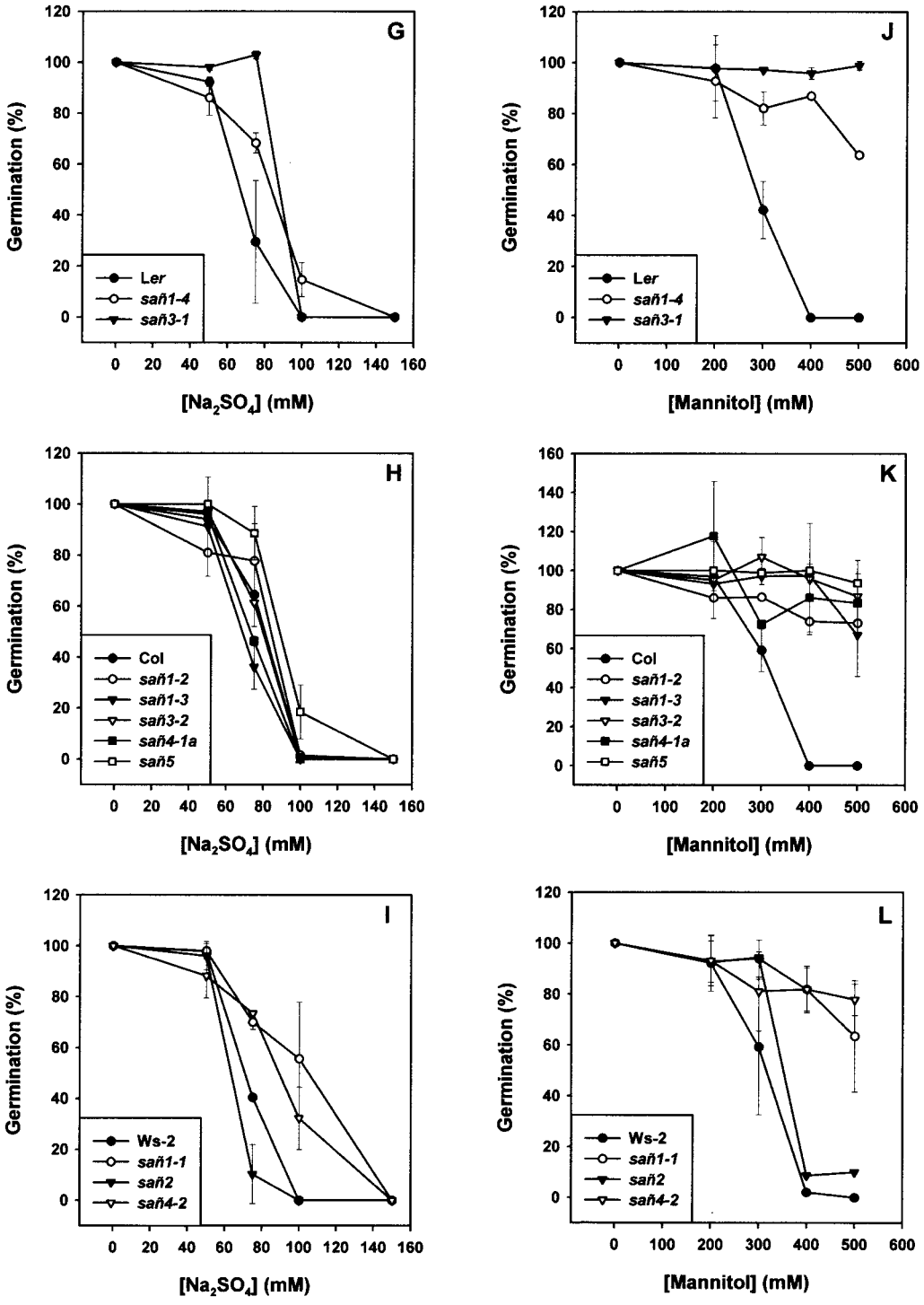

Figure 1.—*Continued.*

containing different salts or mannitol, respectively. In the first case, we tested whether the ability of *sañ* mutants to germinate on NaCl was due to an altered response to Na^+ or Cl^- ions by sowing mutant and wild-type seeds on different concentrations of KCl (Figure 1, D–F) or Na_2SO_4 (Figure 1, G–I). In the former salt, no significant differences were found between mutants in L*er* and Ws-2 backgrounds and their wild-type ancestors (Figure 1, D and F). On the other hand, *sañ5* was the only mutant to be clearly more insensitive to KCl than its wild-type ancestor at the germination stage (Figure 1E). However, 25.6% of *sañ1-4* and 44.7% of *sañ3-1* seedlings that showed expanded cotyledons 10 days after sowing on 125 mm KCl survived and produced up to four vegetative leaves 11 days later, whereas none of the L*er* seedlings survived, the latter displaying bleached cotyledons and no visible leaf primordia. As regards mutants in a Col background, only the seedlings of *sañ3-2* showed a

TABLE 4

Effect of NaCl on the growth of *sañ* mutants

	Fresh weight (mg)			Dry weight (mg)		
	[NaCl] (mm)		Weight loss	[NaCl] (mm)		Weight loss
Strain	0	50	(%)	0	50	(%)
L*er*	33.0 ± 2.8	22.9 ± 0.6	30.6	2.7 ± 0.3	2.3 ± 0.0	14.0
sañ3-1	30.4 ± 0.5	18.5 ± 3.8	39.1	2.5 ± 0.2	1.7 ± 0.1	33.1
Col	23.6 ± 1.4	22.3 ± 1.4	5.5	2.1 ± 0.2	1.8 ± 0.0	11.5
sañ3-2	24.9 ± 0.5	25.2 ± 0.8	−1.0	2.3 ± 0.0	2.5 ± 0.0	−6.7
sañ4-1a	18.3 ± 0.4	13.7 ± 0.4	25.7	1.9 ± 0.2	1.7 ± 0.0	11.1
sañ5	29.1 ± 6.3	21.7 ± 1.5	25.4	2.4 ± 0.6	2.1 ± 0.2	14.2
Ws-2	25.0 ± 2.3	20.0 ± 1.9	20.0	2.2 ± 0.2	1.9 ± 0.1	14.0
sañ1-1a	20.0 ± 0.0	18.7 ± 0.7	6.5	2.0 ± 0.0	2.1 ± 0.1	−4.5
sañ2	35.3 ± 0.9	22.0 ± 0.9	37.9	2.3 ± 0.2	2.4 ± 0.1	−5.2

Values are the average ±SD of at least two replicates of 15 plants each.

higher survival rate (55%) than the wild type (20.5%), the former displaying up to four vegetative leaves 3 wk after sowing on 125 mm KCl medium. No differences were found between the Ws-2 ecotype and Ws-2-derived mutants when sown in KCl-enriched media. In addition, *sañ1-1*, *sañ1-4*, *sañ4-2*, and *sañ5* showed better germination than their wild-type counterpart on 75 and 100 mm Na_2SO_4 media, the germination of their wild-type ancestors being completely abolished in the higher concentration. At the remaining salt concentrations studied, the germination patterns were undistinguishable from those of the wild-type strains (Figure 1, G–I).

The response of *sañ* mutants to osmotic stress was analyzed by sowing mutant and wild-type seeds on media with different concentrations of the osmoticum mannitol (Figure 1, J–L). All mutants except *sañ2* were clearly less sensitive than their corresponding ecotype ancestors, germination being higher than 60% on 400 mm mannitol, a concentration that completely inhibited germination of the wild types. Altogether, these results point toward our mutants being tolerant both to ionic effects and osmotic pressure, as produced by Cl^- or Na^+, and mannitol, respectively.

Effect of *sañ* mutations on plant growth: To determine whether *sañ* mutations conferred some degree of salt resistance during developmental stages other than germination, and whether they displayed some degree of salt dependence, sowings were made in medium supplemented with 50 mm NaCl, a concentration already shown to be permissive for the germination and growth of mutants and wild-type individuals. These tests were performed with at least one mutant line from each complementation group. The fresh and dry weights of such stressed plants are shown in Table 4, together with those of nonstressed plants. The presence of NaCl decreased both fresh and dry weight in all ecotypes, the least affected by salt being Col. As regards the studied mutants, the fresh and dry weights of *sañ3-2* were not reduced after exposure to NaCl, whereas *sañ4-1a* and *sañ5* showed a fivefold greater decrease in fresh weight compared with their ancestor ecotype.

Transcription of salt-stress-induced genes in *sañ* mutants: A large number of genes are known to respond to salt stress at the transcriptional level (Zhu *et al.* 1997). To test their expression in *sañ* mutants, we elected the salinity-inducible *P5CS-1* gene, which encodes the Δ^1-pyrroline-5-carboxylate synthetase enzyme, involved in proline biosynthesis (Savouré *et al.* 1995), and the *MYB2* gene, whose product is a MYB-related transcription factor putatively controlling drought and salinity stress responses (Urao *et al.* 1993). We used as a control the *APK2a* gene, encoding a putative serine/threonine protein kinase that is strongly expressed in Arabidopsis leaves (Ito *et al.* 1997). RT-PCR experiments were performed on total RNA obtained from mutant and wild-type individuals and grown on 250 mm NaCl-supplemented liquid media. RT-PCR amplification products of the expected sizes were obtained, their electrophoregram peak heights indicating that the expression of *P5CS-1* and *MYB2* genes was induced in wild-type and mutant lines in response to NaCl, whereas *APK2a* expression was nearly identical in stressed and nonstressed plants (data not shown). When compared to their wild-type ancestors, *P5CS-1* showed similar levels of enhanced expression in response to NaCl in all mutants except *sañ3-2* and *sañ4-1a*. The former showed a weak induction whereas no increased expression was observed in the second. As regards *MYB2*, the increase in expression in response to 250 mm NaCl was similar to that shown by wild type in all mutants except *sañ3-2*, whose level was about half of its ancestor's ecotype.

Sensitivity of *sañ* mutants to abscisic acid: Various plant responses to environmental stresses, salinity included, are regulated by ABA (Chandler and Robertson 1994). It has been reported that some *abi* (ABA-insensitive) mutants exhibit better germination than

their wild-type ancestors on high NaCl concentrations, probably due to a disturbance in the signaling pathway of this hormone (Werner and Finkelstein 1995). To determine whether any of our mutants was ABA insensitive, we sowed them on 2.5 μm ABA, a concentration that strongly reduces wild-type seed germination. Only *sañ5* germinated in these conditions, showing a level of germination very similar to that reached by seeds sown on medium without ABA (≈100%), indicating the complete penetrance of the phenotype. Analysis of F_1 progeny from crosses between *sañ5* and its wild-type ancestor (Col) showed that the insensitivity to ABA was a recessive trait. When F_2 seeds were sown on 5 μm ABA agar medium, a 3:1 phenotypic segregation ratio (wild type:mutant, $\chi^2 = 0.02$; $n = 107$) was found likely. These results demonstrate that the ABA-resistant phenotype of *sañ5* is due to a recessive mutation in a single locus.

The *sañ5* strain carries an extremely hypomorphic or null allele of the *ABI4* gene: As previously mentioned, we found that the abscisic-acid-insensitive mutation carried by the *sañ5* strain is closely linked to the nga168 marker. Since Finkelstein (1994) provided evidence of tight linkage between nga168 and the *ABI4* gene (no recombinants found after studying 86 chromosomes), we attempted to determine whether *sañ5* carries an allele of *abi4*. Germination of the F_1 progeny of a *sañ5* × *abi4* cross was shown to be ABA insensitive and salt resistant when sown on either 3 μm ABA or 175 mm NaCl medium, respectively. Furthermore, *abi4*/*abi4*, *abi4*/*sañ5*, and *sañ5*/*sañ5* individuals yielded, respectively, 1.2, 13.6, and 81.0% germination on 200 mm NaCl. No seed of their wild-type ancestor, Col, was capable of germination under these conditions. In addition, the dose response for ABA inhibition of germination shows that *sañ5* is much less sensitive than *abi4* (Figure 2). These results point to *sañ5* carrying a null or extremely hypomorphic allele of the *ABI4* gene, whereas the *abi4* mutation is probably a weak hypomorphic allele. The mutation carried by the *sañ5* strain will be named *abi4-2* in what follows.

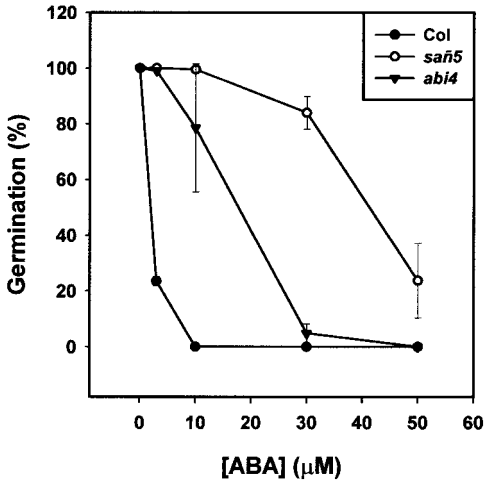

Figure 2.—Effects of ABA on the germination of *sañ5* (*abi4-2*) and *abi4* mutants. Data are means of two replicates of 50–100 seeds scored 12 days after sowing.

Cosegregation between salt- and ABA-resistant phenotypes of *sañ5* (*abi4-2*) was tested by sowing F_2 seeds obtained from crosses between the mutant and Col on 3 μm ABA medium and selecting ABA-insensitive seedlings to collect their inbred progeny. Seeds from 38 F_3 families were sown on 175 mm NaCl or 3 μm ABA, and all were ABA insensitive and NaCl resistant, which indicated cosegregation of the phenotypes under study.

In addition, we designed two oligonucleotides spanning the entire coding region of the *ABI4* gene (Finkelstein *et al.* 1998) and performed PCR amplifications using genomic DNA of *sañ5* (*abi4-2*), Col, L*er*, and Ws-2 as a template, a molecule of the expected 1037-bp size being synthesized from all the templates used. A third oligonucleotide, which was homologous to sequences within the gene, was designed and both chains of the coding region of the *ABI4* gene were sequenced in Col, *abi4*/*abi4*, *abi4-2*/*abi4-2*, and *abi4*/*abi4-2* individuals, the results unequivocally indicating that *abi4-2* (*sañ5*) carries a single base pair deletion in position 277 of the transcription unit. This results in a frameshift that disrupts the C-terminal half of its deduced protein product, which in turn would be a truncated protein with 109 amino acids instead of the 328 of its wild-type counterpart (Figure 3A). The predicted protein product of the *abi4-2* allele would lack the AP2 domain, which is presumed to be involved in DNA binding, as

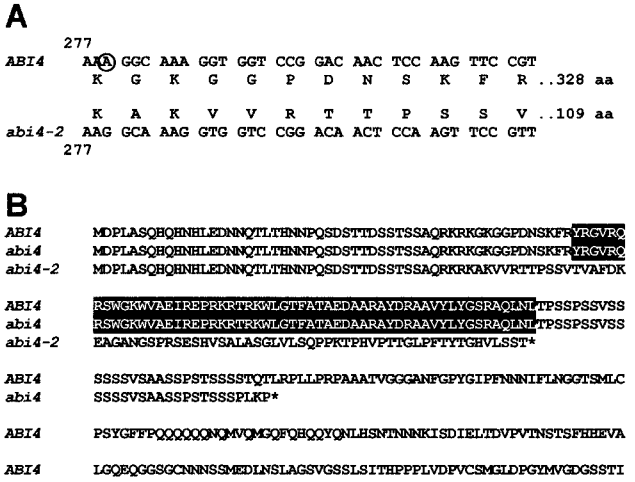

Figure 3.—Sequence of the *abi4-2* (*sañ5*) mutant allele of the *ABI4* gene. (A) Nucleotide and predicted amino acid sequences of the *abi4-2* mutant and *ABI4* wild-type alleles. Only the genomic region close to the 1-bp deletion in *abi4-2* is shown. A circle indicates the nucleotide deleted in *abi4-2*. (B) Alignment of the amino acid sequences of the protein products of the wild-type *ABI4* allele and the mutant *abi4-2* and *abi4* alleles of the *ABI4* gene. The APETALA2 domain is shaded.

well as domains putatively required for transcription activation (Figure 3B).

Effects of *abi4-2* (*sañ5*) mutation on vegetative growth: The sensitivity of *sañ5* mutant seedlings to ABA and NaCl was tested by determining their fresh weight 11 days after transfer from agar medium to media supplemented with 10 μm ABA, or 100 or 150 mm NaCl. Seedlings were transferred 4, 7, and 10 days after sowing on nonsupplemented medium to determine which stage (if any) of seedling growth is more sensitive to ABA or NaCl. *sañ5* seedlings were less sensitive than their wild-type ancestor to ABA inhibition of growth, the greatest differences being observed in seedlings transferred 4 days after sowing (Figure 4A). In contrast, *sañ5* and Col seedlings displayed similar levels of sensitivity on NaCl-supplemented media (Figure 4, B and C).

DISCUSSION

A genetic approach to the manipulation of salt tolerance in the glycophyte *Arabidopsis thaliana*: The last 15 years have seen a large number of studies performed with the aim of understanding and controlling plant salt tolerance, many of them based on the assumption that it is possible to increase the salt tolerance of crop species by transferring into their genomes wild-type, constitutively expressed, or hypermorphic alleles of genes from other plant species, yeasts, or bacteria. An alternative approach involves searching for hypomorphic or null mutations in genes endogenous to a glycophyte, non-salt-tolerant plant species.

For *A. thaliana*, as for other model organisms, there is a large inventory of mutants expressing different types of tolerance or resistance, such as those that are insensitive to auxin (Maher and Martindale 1980; Estelle and Somerville 1987; Wilson *et al.* 1990; Hobbie and Estelle 1995), gibberellin (Koornneef *et al.* 1985), cytokinin (Su and Howell 1992; Deikman and Ulrich 1995), ethylene (Bleecker *et al.* 1988; Hua *et al.* 1995; Sakai *et al.* 1998), or ABA (Koornneef *et al.* 1984; Finkelstein 1994), or those tolerant to different biotic or abiotic stress agents, such as freezing (Xin and Browse 1998), aluminum (Degenhardt *et al.* 1998; Larsen *et al.* 1998), cesium (Maathuis and Sanders 1996), chlorate (Oostindiër-Braaksma and Feenstra 1973; Wilkinson and Crawford 1991; Lin and Cheng 1997), and several herbicides (Mourad *et al.* 1994; Li and Last 1996). It is therefore surprising that only a few mutations conferring salt tolerance have been identified in Arabidopsis: these include three nonallelic EMS-induced *RS* mutants (Saleki *et al.* 1993), and the γ-ray-induced alleles of the *rss* gene (Werner and Finkelstein 1995). None of these genes has been cloned.

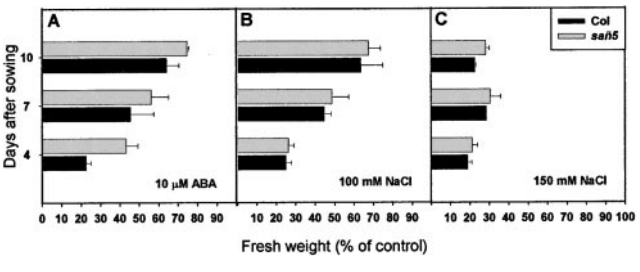

Figure 4.—Effects of ABA and NaCl on the growth of the *abi4-2* (*sañ5*) mutant. Each graph represents the fresh weight of plants transferred to (A) ABA- or (B and C) NaCl-supplemented media, at different times after sowing, expressed as a percentage of the fresh weight of those transferred to nonsupplemented media. Values are the mean of two replicates of 15 plants each. Error bars represent standard deviations.

We have attempted to isolate *A. thaliana* variants capable of germinating in highly saline conditions. We first compared the ability of 102 wild-type strains of *A. thaliana* to germinate in saline conditions, finding a broad spectrum of germination percentages (V. Quesada, M. R. Ponce and J. L. Micol, unpublished results). Such observations led us to expect that mutagenesis performed on different ecotypes would produce mutants with altered responses to salt, whose phenotypes would depend to some extent on their genetic background. Consequently, we performed a large-scale screening for mutants displaying salt-tolerant germination, using different genetic backgrounds as well as different mutagenesis procedures. Seventeen mutants were isolated and studied, all of them being crossed to their wild-type ancestor in a reciprocal manner and pairwise to each other to determine patterns of inheritance and allelism, respectively. The results of the genetic analysis show that the *sañ* mutants display monogenic phenotypes and carry 10 genuinely different recessive mutations that fall into five complementation groups.

Salt-tolerant germination of F_2 seeds from crosses between *sañ* mutants and ecotypes was shown to depend on genetic background, the Col genetic background being more tolerant to NaCl than the others. On the other hand, among the 10 mutations identified, 5 were obtained on a Col genetic background, 2 on L*er*, and 3 on Ws-2. Consequently, the ratio of identified mutations to mutagenized lines was 1/9090, 1/7250, and 1/2160 for the genetic backgrounds Col, L*er*, and Ws-2, respectively. As regards the relative efficiency of the mutagens used, and considering solely the 10 mutations that were unequivocally different, one out every 7992 M_1 seeds exposed to fast neutron bombardment produced a salt-tolerant M_2 individual, whereas the corresponding values for T-DNA and EMS were 1/2170 and 1/12,000, respectively. Since null alleles are usually obtained from fast neutrons and T-DNA mutagenesis, whereas EMS typically causes hypomorphic mutations, the greater efficiency of fast neutrons and T-DNA compared to EMS might be explained by the high stringency of the screening, which would only have allowed the selection of seeds affected by the complete absence of some function that is critical to prevent germination on

a highly saline medium. The *abi4-2* (*sañ5*) and *abi4* alleles of the *ABI4* gene might be mentioned as an example, since the former, obtained in this work, is a null mutation that confers extreme insensitivity to ABA and resistance to NaCl. Such phenotypic traits are weak in the hypomorphic *abi4* mutant, which would have been lost in the highly restrictive conditions employed in our screening.

Degree of saturation of the Arabidopsis genome reached in the mutant screening: Since noncomplementing mutants isolated from the same parental group were initially suspected and later demonstrated to be identical, it is clear that we studied 17 mutants that correspond to only 10 genuinely different mutations, 4 of them being alleles of *SAÑ1*, 2 of *SAÑ3* and *SAÑ4*, and a single allele of the remaining genes. Hence, the average number of alleles obtained per gene is $10/5 = 2$. Assuming that all the genes are equally mutable, the occurrence of mutations in a mutagenesis should follow Poisson's distribution (Jürgens *et al.* 1991; Berná *et al.* 1999). It follows that the class of genes not represented among our mutants would be almost one-seventh (13.5%) of those capable of salt-tolerant germination on 250 mm NaCl, since $m = 2$, $f(0) = e^{-m} = e^{-2} = 0.135 = 13.5\%$. Hence, we approached but did not reach saturation of the genome, in spite of the large number of seeds screened (675,500). A likely explanation for this fact is that we studied only the mutants whose germination levels were >25% on 250 mm NaCl medium, while the other 19 mutant lines displaying a heritable salt-tolerant germination phenotype were not subjected to genetic analysis because of their low penetrance of <24%. On the other hand, the vast majority of the 578 putative mutant seedlings initially isolated in our screening died or were unable to yield viable progeny after being transferred to soil not supplemented with salt. The alleles or genes not identified in our search, such as *rss* (Werner and Finkelstein 1995), might be among the 19 mutant lines not yet studied. In addition, the 525 M_3 or T_4 putative mutants that did not complete their life cycle might correspond not only to lethal alleles but also to mutations that cause increased stress in a transfer from high- to low-salt media.

Speculations on the function of *SAÑ* genes: In the opinion of some authors, the distinctive feature of tolerance to abiotic stress factors, salt in particular, is its polygenic nature, which makes it difficult to dissect in molecular terms (Zhu *et al.* 1997). Nevertheless, several mutations in individual genes that confer tolerance or sensitivity to NaCl have been identified and studied in recent years. It has been proposed that these mutations affect genes whose products participate in processes such as ion exclusion (Abel 1969), the transduction or perception of stress signals (Werner and Finkelstein 1995; Liu and Zhu 1998), and the accumulation of proline or other osmolytes (Kueh and Bright 1982; Sumarayati *et al.* 1992) or of specific ions (Saleki *et al.* 1993) such as osmotic regulators.

The germination patterns of our mutants were quite different from those of their wild-type ancestors, both on NaCl and mannitol, and to a lesser extent on Na_2SO_4-supplemented medium. Differences were extreme when media were supplemented with mannitol, and no mutant except *sañ5* (*abi4-2*) was able to germinate on 150 mm KCl. Taken together, these results indicate that during germination our mutants exhibit reduced sensitivity to Na^+ and Cl^- as well as to the osmotic stress produced by mannitol, all but *sañ5* (*abi4-2*) being sensitive to K^+. Nevertheless, *sañ1-4*, *sañ3-1*, and *sañ3-2* exhibited some growth differences compared to their wild-type ancestor on 125 mm KCl, in which they were able to survive more than 3 wk, whereas all the wild-type plants died. The results suggest that these mutations confer some degree of tolerance to K^+, which is not noticed at germination but which is manifested during growth.

Exposure to high NaCl concentrations does not cause irreversible damage to wild-type seeds, since they can germinate and the resulting seedlings normally grow after being transferred to a nonsaline culture medium. Thus, the inhibition of germination by saline stress might be considered as an adaptative response. Hence, it is likely that some of the *sañ* mutations affect genes whose products are elements of stress signal transduction pathways, which would inhibit germination in situations of saline stress. The perturbation of some such functions would give rise to the salt-tolerant germination phenotype. In fact, the product of the only *A. thaliana* gene that has been cloned from a mutant with a phenotype of altered sensitivity to NaCl (*SOS3*; Liu and Zhu 1998) is a homologue of the calcineurin B subunit of *S. cerevisiae*, a protein involved in a signaling pathway that is specifically activated in the presence of high NaCl concentrations. In *S. cerevisiae*, the search for NaCl-sensitive mutants has led to the characterization of some transduction pathways of osmotic stress signals, one of which is that of calcineurin. Another is a phosphorylation cascade involving homologues of the MAPK, MAPKK, and MAPKKK proteins (Brewster *et al.* 1993). The SHO1 protein (Maeda *et al.* 1995) and the SLN1-SSK1 two-component system (Maeda *et al.* 1994) are receptors that perceive osmolarity changes in the medium and activate the signal transduction pathway. The conservation of the function of the *SOS3* gene from yeast to plants, as well as the identification in *A. thaliana* of an ortholog of the *SLN1* gene of *S. cerevisiae* (ATHK1; Urao *et al.*, unpublished results; quoted in Shinozaki and Yamaguchi-Shinozaki 1997) suggest that other components of the osmotic stress signaling pathways in yeast could be present in *A. thaliana* and are candidates to be affected by some of the *sañ* mutations.

Other functions that could be disturbed in the *sañ* mutants are those related to the maintenance of ionic

homeostasis. The specific insensitivity to sodium displayed by the *sañ* mutants, the only exception being *sañ5* (*abi4-2*), suggests that they could be affected in genes whose products regulate its concentration, their normal function being to prevent excessive increases in the intracellular levels of Na^+ and to inhibit germination under NaCl stress. It has been described that HKT1, a wheat root high-affinity K^+ transporter, mediates Na^+ uptake instead of K^+ uptake in the presence of high NaCl concentrations. Transgenic yeasts transformed with mutant alleles of the *HKT1* gene, whose products are unable to transport Na^+, were able to grow on medium supplemented with NaCl concentrations that completely inhibited wild-type growth (Rubio *et al.* 1995). On the other hand, the insensitivity of *sañ* mutants to the nonionic solute mannitol is not easy to explain solely on the basis of alterations in ionic homeostasis controls. Nevertheless, an *A. thaliana* mutant has been described (*sos1*; Zhu *et al.* 1997) that is sensitive to both mannitol and NaCl, confirming the existence of genes that are involved in the acquisition of tolerance to both substances.

As previously mentioned, it is known that proline acts as osmolyte, and studies have described its accumulation in salt-tolerant mutants of *Nicotiana plumbaginifolia* obtained from protoplast cultures (Sumarayati *et al.* 1992) and in NaCl-resistant lines of *Brassica juncea* obtained *in vitro* (Kirti *et al.* 1991). Proline levels in *A. thaliana rss* mutants exposed to NaCl, on the other hand, were lower than those of the wild-type controls (Werner and Finkelstein 1995). We used RT-PCR to test for the expression of the *P5CS-1* gene, which plays a central role in proline biosynthesis, and found that only the *sañ4-1a* mutant presented a clearly different behavior from the remaining mutant and wild-type strains, since the gene was not induced after exposure of the mutant to NaCl 250 mm. This behavior is similar to that of the *rss* mutants and may be considered evidence of the impairment of some element required for the perception of saline stress, the consequence being the activation of proline synthesis.

The *abi4-2* (*sañ5*) mutation eliminates the AP2 domain of the ABI4 protein: We have already mentioned the considerable amount of information concerning involvement of ABA both in the final stages of seed maturation and in some responses to osmotic stress during vegetative development. Hence, ABA might be involved in the inhibition of the germination of wild-type *A. thaliana* seeds in the presence of high concentrations of NaCl or mannitol. Such a hypothesis is supported by the fact that the *abi1*, *abi2*, and *abi3* mutants show some degree of salt-tolerant germination (Werner and Finkelstein 1995). It is therefore reasonable to assume that disturbances of ABA signaling mechanisms could lead to a salt-tolerant germination phenotype. To test this hypothesis, we determined the germination patterns of *sañ* mutants on ABA-supplemented media. Only one mutant strain, *sañ5*, was found to be insensitive to ABA, and it was also able to germinate in the presence of high concentrations of mannitol, Na^+, Cl^-, SO_4^{2-}, and K^+ that completely inhibited the germination of its wild-type ancestor.

The map position of the ABA-insensitive mutation carried by *sañ5* that we obtained was very similar to that reported by Finkelstein (1994) for the *abi4* mutation, suggesting that we had identified a new allele of the *ABI4* gene, as was later confirmed through complementation analysis. The greater germination capacity in the presence of either NaCl or ABA of the *sañ5* seeds with respect to the *abi4* seeds indicated that we had identified a null or extremely hypomorphic allele of the *ABI4* gene. The product of this recently cloned gene (Finkelstein *et al.* 1998) is a protein of the family of transcription factors that includes an APETALA2 domain. When we sequenced the *abi4-2* (*sañ5*) allele of the *ABI4* gene, it was found that a single base-pair deletion causes a frameshift that eliminates the APETALA2 domain and generates a stop codon that removes the C-terminal half of the ABI4 protein. These results suggest that the protein product of *abi4-2* is not functional, supporting the idea of its complete lack of function.

As we mentioned previously, perturbations in perception mechanisms mediated by ABA yield alterations in stress responses. The disturbance of ABA signal transduction in *sañ5* (*abi4-2*), as a consequence of the lack of function of the *ABI4* gene, would allow seed germination in NaCl-enriched culture media. The observation that *sañ5* (*abi4-2*) seeds also germinate in media with high concentrations of other ions, such as K^+ and SO_4^{2-}, or of nonionic additives such as mannitol, suggests that ABI4 also participates in the inhibition of germination in response to these substances. The sensitivity to ABA displayed by the remaining *sañ* mutants suggests that the salt-tolerant germination phenotype might arise from the alteration of other mechanisms, independent of ABA perception.

We analyzed the effects of the *abi4-2* (*sañ5*) mutation on the inhibition of growth, one of the ABA-mediated stress responses during vegetative development. The *sañ5* (*abi4-2*) plants were sensitive to ABA and showed lower reductions in fresh weight than the wild-type controls. Such differences were maximal in the earliest stages studied. In addition, *sañ5* (*abi4-2*) individuals presented a greater sensitivity than their ancestor ecotype to the reduction of growth caused by a continuous exposure to 50 mm NaCl during the first 3 wk after germination. These results indicate that the ABA signaling pathway mediated by *ABI4* is not restricted to germination, in accordance with Finkelstein *et al.* (1998), who observed *ABI4* expression both in wild-type seeds and seedlings. Other *A. thaliana* genes involved in the synthesis of ABA or in the transduction of its signal regulate the expression of some salt-induced genes during vegetative development. Thus, the levels of the transcripts of

ATHB-7 (Söderman *et al.* 1996) in some ABA-deficient *aba1* alleles, and those of the *P5CS-1* and *P5CS-2* genes in the ABA-insensitive *abi1* mutant (Strizhov *et al.* 1997), are lower in NaCl-supplemented media than those of the wild-type control plants.

Perspectives on the genetic and molecular analysis of *sañ* mutants: With the exception of *sañ5* (*abi4-2*), the germinative behavior of our mutants resembles to a certain extent that of *RS* (Saleki *et al.* 1993) and *rss* (Werner and Finkelstein 1995) mutants, which suggests that they could be affected in related processes. *rss* alleles were shown to map on chromosome 1, linked to the *ADH* gene (17.4 cM away from the lower telomere), where no *sañ* mutation maps, indicating that they define different loci. The salt-hypersensitive *sos* mutants also map in positions different from those of the *SAÑ* loci. Since there is no published information on map positions of *RS* mutants, their intercrosses to *sañ* mutants will ascertain any degree of allelism.

The study of the new set of mutants presented here should contribute to a better understanding of salt perception and salt-tolerance mechanisms in plants. These mutants might provide valuable insight into the isolation of the damaged genes, since most of them were induced by fast neutrons and probably carry deletions, as has been demonstrated in the case of other mutants obtained by this mutagenesis procedure (Bruggemann *et al.* 1996). Low resolution mapping of *SAÑ* genes has allowed us to localize them in almost completely sequenced regions (*SAÑ1*) or regions that will be sequenced soon (*SAÑ3* and *SAÑ4*). Although we have made a molecular analysis of the *abi4-2* (*sañ5*) mutation, the nature of the others remains unknown. Attempts to positionally clone these genes are in progress.

Since *abi4-2* (*sañ5*) is a null alelle of the *ABI4* gene, it should be a useful tool for identifying genes that are regulated by *ABI4* by means of differential display, subtractive hybridization, or any other procedure that allows the visualization of differential gene expression between *sañ5* and wild-type individuals.

We thank P. Piqueras for sharing some unpublished results, H. Candela, A. Martínez-Laborda, J. M. Pérez-Pérez, P. Piqueras, P. Robles, A. Vera, and two anonymous referees for comments on the manuscript, S. Gerber and J. M. Serrano for their expert technical help, the Nottingham *Arabidopsis* Stock Centre and the *Arabidopsis* Biological Resource Center for providing seeds of ecotypes and clones, and K. Feldmann and C. Dean for making publicly available their tagged lines. V. Quesada was fellow of the Conselleria de Cultura, Educació i Ciència of the Generalitat Valenciana. This research was supported by grants from the Comisión Interministerial de Ciencia y Tecnología of Spain (BIO94-0253 and BIO97-1050).

LITERATURE CITED

Abel, G. H., 1969 Inheritance of the capacity for choride inclusion and exclusion by soybeans. Crop Sci. **9:** 697–698.

Bancroft, I., A. M. Bhatt, C. Sjodin, S. Scofield, J. D. G. Jones *et al.*, 1992 Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana*. Mol. Gen. Genet. **233:** 449–461.

Bell, D. J., and J. R. Ecker, 1994 Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics **19:** 137–144.

Berná, G., P. Robles and J. L. Micol, 1999 A mutational analysis of leaf morphogenesis in *Arabidopsis thaliana*. Genetics **152:** 729–742.

Bleecker, A. B., M. A. Estelle, C. Somerville and H. Kende, 1988 Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241:** 1086–1089.

Bohnert, H. J., and E. Sheveleva, 1998 Plant stress adaptations: making metabolism move. Curr. Biol. **1:** 267–274.

Boyer, J. S., 1982 Plant productivity and environment. Science **218:** 443–448.

Bray, E. A., 1993 Molecular responses to water deficit. Plant Physiol. **103:** 1035–1040.

Brewster, J. L., T. de Valoir, N. D. Dwyer, E. Winter and M. Gustin, 1993 An osmosensing signal transduction pathway in yeast. Science **259:** 1760–1763.

Bruggemann, E., K. Handwerger, C. Essex and G. Storz, 1996 Analysis of fast neutron-generated mutants at the *Arabidopsis thaliana HY4* locus. Plant J. **10:** 755–760.

Chandler, P. M., and M. Robertson, 1994 Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45:** 113–141.

Degenhardt, J., P. B. Larsen, S. H. Howell and L. V. Kochian, 1998 Aluminum resistance in the *Arabidopsis* mutant *alr-104* is caused by an aluminum-induced increase in rhizosphere pH. Plant Physiol. **117:** 19–27.

Deikman, J., and M. Ulrich, 1995 A novel cytokinin-resistant mutant of *Arabidopsis* with abbreviated shoot development. Planta **195:** 440–449.

Delauny, A. J., and D. P. S. Verma, 1993 Proline biosynthesis and osmoregulation in plants. Plant J. **4:** 215–223.

Estelle, M. A., and C. R. Somerville, 1987 Auxin resistant mutants of *Arabidopsis* with an altered morphology. Mol. Gen. Genet. **206:** 200–206.

Feldmann, K. A., and M. D. Marks, 1987 *Agrobacterium*-mediated transformation of germinating seed of *Arabidopsis thaliana*: a non tissue culture approach. Mol. Gen. Genet. **208:** 1–9.

Finkelstein, R. R., 1994 Mutations at two new *Arabidopsis ABA* response loci are similar to the *abi3* mutations. Plant J. **5:** 765–771.

Finkelstein, R. R., M. L. Wang, T. J. Lynch, S. Rao and H. M. Goodman, 1998 The *Arabidopsis* abscisic acid response locus *ABI4* encodes an APETALA2 domain protein. Plant Cell **10:** 1043–1054.

Greenway, H., and R. Munns, 1980 Mechanisms of salt tolerance in nonhalophytes. Annu. Rev. Plant Physiol. **31:** 149–190.

Hanson, A. D., B. Rathinasabapathi, J. Rivoal, M. Burnet, O. Dillon *et al.*, 1994 Osmoprotective compounds in the *Plumbaginaceae*: a natural experiment in metabolic engineering of stress tolerance. Proc. Natl. Acad. Sci. USA **91:** 306–310.

Hayashi, H., Alia, L. Mustardy, P. Deshnium, M. Ida *et al.*, 1997 Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase: accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J. **12:** 133–142.

Hendry, G. A. F., 1993 Evolutionary origins and natural functions of fructans: a climatological, biogeographic and mechanistic appraisal. New Phytol. **123:** 3–14.

Hetherington, A. M., and R. S. Quatrano, 1991 Mechanisms of action of abscisic acid at the cellular level. New Phytol. **119:** 9–32.

Hirayama, T., C. Ohto, T. Mizoguchi and K. Shinozaki, 1995 A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **92:** 3903–3907.

Hobbie, L., and M. Estelle, 1995 The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. Plant J. **7:** 211–220.

Holmberg, N., and L. Bülow, 1998 Improving stress tolerance in plants by gene transfer. Trends Plant Sci. **3:** 61–66.

Hong, S. W., J. H. Jon, J. M. Kwak and H. G. Nam, 1997 Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high salt, and cold treatments in *Arabidopsis thaliana*. Plant Physiol. **113:** 1203–1212.

Hua, J., C. Chang, Q. Sun and E. M. Meyerowitz, 1995 Ethylene insensitivity conferred by *Arabidopsis ERS* gene. Science **269:** 1712–1714.

Ito, T., N. Takahashi, Y. Shimura and K. Okada, 1997 A serine/threonine protein kinase gene isolated by an in vivo binding procedure using the *Arabidopsis* floral homeotic gene product, AGAMOUS. Plant Cell Physiol. **38:** 248–258.

Jürgens, G., U. Mayer, R. A. Torres Ruiz, T. Berleth and S. Miséra, 1991 Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development **1** (Suppl.): 27–38.

Karakas, B., P. Ozias-Akins, C. Stushnoff, M. Suefferheld and M. Rieger, 1997 Salinity and drought tolerance of mannitol-accumulating transgenic tobacco. Plant Cell Environ. **20:** 609–616.

Kirti, P. B., S. Hadi, P. A. Kumar and V. L. Chopra, 1991 Production of sodium-chloride-tolerant *Brassica juncea* plants by in vitro selection at the somatic embryo level. Theor. Appl. Genet. **83:** 233–237.

Kishor, P. B., Z. H. Kavi, M. Guo-Hua, A. Chein-An and D. P. S. Verma, 1995 Overexpression of proline-5-carboxylate synthase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol. **108:** 1387–1394.

Koornneef, M., and P. Stam, 1992 Genetic analysis, pp. 83–99 in *Methods in* Arabidopsis *Research*, edited by C. Koncz, N.-H. Chua and J. Schell. World Scientific, Singapore.

Koornneef, M., G. Reuling and C. M. Karssen, 1984 The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana.* Physiol. Plant **61:** 377–383.

Koornneef, M., A. Elgersma, C. J. Hanhart, E. P. Van Loenen-Martinet, L. Van Rijn *et al.*, 1985 A gibberellin insensitive mutant of *Arabidopsis thaliana.* Physiol. Plant **65:** 33–39.

Kosambi, D. D., 1944 The estimation of map distance from recombination values. Ann. Eugen. **12:** 172–175.

Kueh, J. S. H., and S. W. J. Bright, 1982 Biochemical and genetic analysis of three proline accumulating barley mutants. Plant Sci. Lett. **27:** 233–241.

Larsen, P. B., J. Degenhardt, C-Y. Tai, L. M. Stenzler, S. H. Howell *et al.*, 1998 Aluminum-resistant *Arabidopsis* mutants that exhibit altered patterns of aluminum accumulation and organic acid release from roots. Plant Physiol. **117:** 9–18.

Li, J., and R. L. Last, 1996 The *Arabidopsis thaliana trp5* mutant has a feedback-resistant anthranilate synthase and elevated soluble tryptophan. Plant Physiol. **110:** 51–59.

Lilius, G., N. Holmberg and L. Bülow, 1996 Enhanced NaCl stress tolerance in transgenic tobacco expressing bacterial choline dehydrogenase. Biotechnology **14:** 177–180.

Lin, Y., and C. L. Cheng, 1997 A chlorate-resistant mutant defective in the regulation of nitrate reductase gene expression in *Arabidopsis* defines a new *HY* locus. Plant Cell **9:** 21–35.

Liu, J., and J.-K. Zhu, 1997 An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. Proc. Natl. Acad. Sci. USA **94:** 14960–14964.

Liu, J., and J.-K. Zhu, 1998 A calcium sensor homolog required for plant salt tolerance. Science **280:** 1943–1945.

López, F., G. Vansuyt, F. Cassedelbart and P. Fourcroy, 1996 Ascorbate peroxidase-activity, not the messenger level, is enhanced in salt-stressed *Raphanus sativus* plants. Physiol. Plant **97:** 13–20.

Maathuis, F. J., and D. Sanders, 1996 Characterization of *csi52*, a Cs^+ resistant mutant of *Arabidopsis thaliana* altered in K^+ transport. Plant J. **10:** 579–589.

Maeda, T., S. M. Wurgler-Murphy and H. Saito, 1994 A two component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242–245.

Maeda, T., M. Takegawa and H. Saito, 1995 Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science **269:** 554–558.

Maher, E. P., and S. J. B. Martindale, 1980 Mutants of *Arabidopsis* with altered responses to auxins and gravity. Biochem. Genet. **18:** 1041–1053.

McCue, K. F., and A. D. Hanson, 1990 Drought and salt tolerance: towards understanding and application. Trends Biotechnol. **8:** 358–362.

Mizoguchi, T., N. Hayashida, K. Yamaguchi-Shinozaki, H. Kamada and K. Shinozaki, 1995 Two genes that encode ribosomal-protein S6 kinase homologs are induced by cold or salinity stress in *Arabidopsis thaliana.* FEBS Lett. **358:** 199–204.

Mourad, G., G. Haughn and J. King, 1994 Intragenic recombination in the *CSR1* locus of *Arabidopsis.* Mol. Gen. Genet. **243:** 178–184.

Oostindiër-Braaksma, F. J., and W. Feenstra, 1973 Isolation and characterization of chlorate-resistant mutants of *Arabidopsis thaliana.* Mutat. Res. **19:** 175–185.

Paul, M. J., and W. Cockburn, 1989 Pinitol, a compatible solute in *Mesembryanthemum crystallinum* L. J. Exp. Bot. **40:** 1093–1098.

Ponce, M. R., V. Quesada and J. L. Micol, 1998 Rapid discrimination of sequences flanking and within T-DNA insertions in the *Arabidopsis* genome. Plant J. **14:** 497–501.

Ponce, M. R., P. Robles and J. L. Micol, 1999 High-throughput genetic mapping in *Arabidopsis thaliana.* Mol. Gen. Genet. **261:** 408–415.

Qiang, X., H. H. Fu, R. Gupta and S. Luan, 1998 Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in *Arabidopsis.* Plant Cell **10:** 849–858.

Rentsch, D., B. Hirner, E. Schmelzer and W. B. Frommer, 1996 Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell **8:** 1437–1446.

Roosens, N. H. C. J., T. T. Thu, H. M. Iskandar and M. Jacobs, 1998 Isolation of the ornithine-δ-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana.* Plant Physiol. **117:** 263–271.

Rubio, F., W. Gassmann and J. I. Schroeder, 1995 Sodium-driven uptake by the plant potassium transporter HKT1 and mutations conferring salt-tolerance. Science **270:** 1660–1662.

Sakai, H., J. Hua, Q. G. Chen, C. Chang, L. J. Medrano *et al.*, 1998 *ETR2* is an *ETR1-like* gene involved in ethylene signalling in *Arabidopsis.* Proc. Natl. Acad. Sci. USA **95:** 5812–5817.

Saleki, R., P. G. Young and D. D. Lefebvre, 1993 Mutants of *Arabidopsis* capable of germination under saline conditions. Plant Physiol. **101:** 839–845.

Savouré, A., S. Jaoua, X-J. Hua, W. Ardiles, M. Van Montagu *et al.*, 1995 Isolation, characterization, and chromosomal location of a gene encoding the Δ^1-pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana.* FEBS Lett. **372:** 13–19.

Serrano, R., and R. Gaxiola, 1994 Microbial models and salt stress tolerance in plants. Crit. Rev. Plant Sci. **13:** 121–138.

Sheveleva, E., W. Chmara, H. J. Bohnert and R. Jensen, 1997 Increased salt and drought tolerance by d-ononitol production in transgenic *Nicotiana tabacum* L. Plant Physiol. **115:** 1211–1219.

Shinozaki, K., and K. Yamaguchi-Shinozaki, 1997 Gene expression and signal transduction in water-stress response. Plant Physiol. **115:** 327–334.

Singh, N. K., A. K. Handa, P. M. Hasegawa and R. A. Bressan, 1985 Proteins associated with adaptations of cultured tobacco cells to NaCl. Plant Physiol. **79:** 126–137.

Söderman, E., J. Mattsson and P. Engström, 1996 The *Arabidopsis* homeobox gene *ATHB-7* is induced by water deficit and by abscisic acid. Plant J. **10:** 375–381.

Stoop, J. M. H., J. D. Williamson and D. M. Pharr, 1996 Mannitol metabolism in plants: a method for coping with stress. Trends Plant Sci. **1:** 139–144.

Strizhov, N., E. Abraham, L. Okresz, S. Blickling, A. Zilberstein *et al.*, 1997 Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1*, *ABI1* and *AXR2* in *Arabidopsis.* Plant J. **12:** 557–569.

Su, W., and S. H. Howell, 1992 A single locus, *CKR1*, defines *Arabidopsis* mutants in which root growth is resistant to low concentrations of cytokinin. Plant Physiol. **99:** 1569–1574.

Sumarayati, S., I. Negrutiu and M. Jacobs, 1992 Characterization and regeneration of salt and water-stress mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viviani). Theor. Appl. Genet. **83:** 613–619.

Tarczynski, M. C., R. Jensen and H. J. Bohnert, 1993 Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science **259:** 508–510.

Thomas, J. C., M. Sepahi, B. Arendall and H. J. Bohnert, 1995 Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana.* Plant Cell Environ. **18:** 801–806.

Urao, T., K. Yamuguchi-Shinozaki, U. Satomi and K. Shinozaki, 1993 An *Arabidopsis myb* homolog is induced by dehydration

stress and its gene product binds to the conserved *MYB* recognition sequence. Plant Cell **5:** 1529–1539.

Urao, T., T. Katagiri, T. Mizoguchi, K. Yamuguchi-Shinozaki, N. Hayashida *et al.*, 1994 Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stress in *Arabidopsis thaliana*. Mol. Gen. Genet. **244:** 331–340.

Velten, J., and J. Schell, 1985 Selection-expression plasmid vectors for use in genetic transformation of higher plants. Nucleic Acids Res. **13:** 6981–6999.

Werner, J. E., and R. R. Finkelstein, 1995 *Arabidopsis* mutants with reduced response to NaCl and osmotic stress. Physiol. Plant **93:** 659–666.

Wilkinson, J. Q., and N. M. Crawford, 1991 Identification of the *Arabidopsis CHL3* gene as the nitrate reductase structural gene *NIA2*. Plant Cell **3:** 461–471.

Wilson, A. K., F. B. Pickett, J. C. Turner and M. Estelle, 1990 A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Mol. Gen. Genet. **222:** 377–383.

Wu, S-J., L. Ding and J-K. Zhu, 1996 *SOS1*, a genetic locus essential for salt tolerance and potassium acquisition. Plant Cell **8:** 617–627.

Xin, Z., and J. Browse, 1998 *eskimo1* mutants of *Arabidopsis* are constitutively freezing-tolerant. Proc. Natl. Acad. Sci. USA **95:** 7799–7804.

Xu, D., X. Duan, B. Wang, B. Hong, T-H. D. Ho *et al.*, 1996 Expression of late embryogenesis abundant protein gene *HVA1* confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol. **110:** 249–257.

Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus and G. N. Somero, 1982 Living with water stress: evolution of osmolyte systems. Science **217:** 1214–1222.

Zhu, J.-K., P. M. Hasegawa and R. A. Bressan, 1997 Molecular aspects of osmotic stress in plants. Crit. Rev. Plant Sci. **16:** 253–277.

Zhu, J.-K., J. Liu and L. Xiong, 1998 Genetic analysis of salt tolerance in *Arabidopsis*: evidence for a critical role of potassium nutrition. Plant Cell **10:** 1181–1191.

Communicating editor: V. Sundaresan