Genes That Are Uniquely Stress Regulated in Salt Overly Sensitive (*sos*) Mutants[1]

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Repetitive rounds of differential subtraction screening, followed by nucleotide sequence determination and northern-blot analysis, identified 84 salt-regulated (160 mM NaCl for 4 h) genes in Arabidopsis wild-type (Col-0 *gl1*) seedlings. Probes corresponding to these 84 genes and *ACP1*, *RD22BP1*, *MYB2*, *STZ*, and *PAL* were included in an analysis of salt responsive gene expression profiles in *gl1* and the salt-hypersensitive mutant *sos3*. Six of 89 genes were expressed differentially in wild-type and *sos3* seedlings; steady-state mRNA abundance of five genes (*AD06C08*/unknown, *AD05E05*/vegetative storage protein 2 [*VSP2*], *AD05B11*/S-adenosyl-L-Met:salicylic acid carboxyl methyltransferase [*SAMT*], *AD03D05*/cold regulated 6.6/inducible2 [*COR6.6/KIN2*], and salt tolerance zinc finger [*STZ*]) was induced and the abundance of one gene (*AD05C10*/circadian rhythm-RNA binding1 [*CCR1*]) was reduced in wild-type plants after salt treatment. The expression of *CCR1*, *SAMT*, *COR6.6/KIN2*, and *STZ* was higher in *sos3* than in wild type, and *VSP2* and *AD06C08*/unknown was lower in the mutant. Salt-induced expression of *VSP2* in *sos1* was similar to wild type, and *AD06C08*/unknown, *CCR1*, *SAMT*, *COR6.6/KIN2*, and *STZ* were similar to *sos3*. *VSP2* is regulated presumably by *SOS2/3* independent of *SOS1*, whereas the expression of the others is *SOS1* dependent. *AD06C08*/unknown and *VSP2* are postulated to be effectors of salt tolerance whereas *CCR1*, *SAMT*, *COR6.6/KIN2*, and *STZ* are determinants that must be negatively regulated during salt adaptation. The pivotal function of the SOS signal pathway to mediate ion homeostasis and salt tolerance implicates *AD06C08*/unknown, *VSP2*, *SAMT*, *6.6/KIN2*, *STZ*, and *CCR1* as determinates that are involved in salt adaptation.

High soil salinity, which is caused typically by NaCl, results in ion toxicity and hyperosmotic stress leading to numerous pathologies including generation of reactive oxygen species (ROS) and programmed cell death (Niu et al., 1995; Zhu et al., 1997; Hasegawa et al., 2000b). Salt tolerance determinants are categorized either as effectors that directly modulate stress etiology or attenuate stress effects, or as regulatory molecules that are involved in stress perception, signal transduction, or modulation of effector function. Enzymes that catalyze rate-limiting steps in the biosynthesis of compatible solutes or osmoprotectants, e.g. sugar alcohol, quaternary ammonium, and tertiary sulfonium compounds, are categorical examples of osmotic stress tolerance effectors (Hanson et al., 1994; Ishitani et al., 1997; Yoshiba et al., 1997; Nelson et al., 1998; Hasegawa et al., 2000b). Other effectors include proteins that protect membrane integrity, control water or ion homeostasis, and ROS scavenging (Bray, 1994; Ingram and Bartels, 1996; Hasegawa et al., 2000b). Determinant function of some effectors has been confirmed because expression in transgenic plants enhances salt tolerance sufficiency (Hasegawa et al., 2000b).

Regulatory determinants include transcription factors and signal pathway intermediates that posttranscriptionally activate effectors (Hasegawa et al., 2000b). Basic Leu zipper motif, MYB and MYC, and zinc finger transcription factors, including rd22BP1 (MYC), AtMYB2 (MYB), DREB1A, and DREB2A (AP2 domain), and ALFIN1 (zinc finger), interact with promoters of osmotic-regulated genes (Abe et al., 1997; Liu et al., 1998; Hasegawa et al., 2000b). The osmotic stress tolerance function of DREB1A in Arabidopsis (Kasuga et al., 1999) and ALFIN1 in alfalfa (*Medicago sativa*; Winicov, 2000) has been confirmed by ectopic expression in transgenic plants. Regulatory intermediates that modulate plant salt stress responses include SOS3 (Ca^{2+}-binding protein), SOS2 (Suc nonfermenting-like) kinase, Ca^{2+}-dependent protein kinases, and mitogen-activated protein kinases (Sheen, 1996; Halfter et al., 2000; Kovtun et al., 2000). Additional signal intermediates have been implicated in the plant response to salt (Hwang and Goodman, 1995; Mizoguchi et al., 1996; Mikami et al., 1998; Piao et al., 1999; Hasegawa et al., 2000b).

The Zhu laboratory recently has pioneered identification of salt tolerance determinants using forward

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genetics in the plant model Arabidopsis (Hasegawa et al., 2000a; Sanders, 2000; Zhu, 2000). This effort has identified three complementation groups of ion hypersensitive (salt overly sensitive [*sos1-sos3*]) mutants. Genetic and physiological data indicate that SOS3, SOS2, and SOS1 are components of a signal pathway that regulates ion homeostasis and salt tolerance and their functions are Ca^{2+} dependent. Positional cloning revealed that *SOS1* encodes a putative plasma membrane Na^+/H^+ antiporter, *SOS2* encodes a Suc non-fermenting-like (SNF) kinase, and *SOS3* encodes a Ca^{2+}-binding protein with sequence similarity to the regulatory subunit of calcineurin and neuronal Ca^{2+} sensors (Liu and Zhu, 1998; Liu et al., 2000; Shi et al., 2000). Molecular interaction and complementation analyses indicate that SOS3 is required for activation of SOS2 that regulates *SOS1* transcription (Halfter et al., 2000; Shi et al., 2000), further confirming that the order of the signal pathway is SOS3 → SOS2 → SOS1 (Hasegawa et al., 2000a; Sanders, 2000; Zhu, 2000).

Herein is described a functional dissection of the plant salt stress response by analysis of gene expression controlled by the SOS signal pathway. Differential subtraction and northern analysis identified 84 salt-regulated genes, the majority of which have not been annotated to be salt responsive in Arabidopsis. Comparison of salt regulation in wild type and *sos3* identified six genes that are controlled by the SOS signal pathway. Transcription of one gene (*VSP2*) is controlled as an output from the SOS3/SOS2 pathway, similar to *SOS1* (Shi et al., 2000), whereas regulation of five other genes (*AD06C08, CCR1*, *SAMT*, *COR6.6/KIN2*, and *STZ*) is dependent on both *SOS3* and *SOS2* as well. Because *SAMT*, *COR6.6/KIN2*, and *STZ* are induced by NaCl, negative control by *SOS3* indicates that the SOS pathway functions to reduce the numerous signals induced by salt to those that function more specifically, to mediate processes like ion homeostasis.

RESULTS

Identification of Expressed Sequence Tags (ESTs) Corresponding to Salt-Regulated Genes

A combination of approaches was used to identify stress-regulated transcriptional outputs from the SOS pathway on the premise that these are salt tolerance determinants. A population of ESTs representing salt-regulated transcripts was identified by screening, through three rounds, a subtracted cDNA library prepared from wild-type (Col-0 *gl-1*) seedlings treated for 4 h without or with 160 mM NaCl. Differential dot-blot hybridization, and sequence and northern-blot analyses identified unique ESTs (salt-regulated ESTs [SREs]) that detected salt-regulated transcripts (Table I). The second and third rounds of screening eliminated highly repetitive clones from the initial subtracted library, including those ESTs identified during the first round of screening. The differential subtractive chain selection protocol was used in the third round (Luo et al., 1999) to further enrich for less abundant cDNAs in the library. The different rounds of screening led to identification of 84 nonredundant ESTs that hybridize to salt-regulated transcripts based on northern-blot analysis (Table II). The SREs represent the greatest number that has been used in one study to define the transcriptional response of plants to salt stress. These results identify genes whose expression is most likely controlled by transcriptional activation, although other factors such as salt stress-dependent mRNA stability might contribute also to steady-state transcript abundance.

Database comparisons of SREs using Blast programs determined that the corresponding encoded proteins included those involved in primary metabolism, cell wall synthesis or degradation, other cellular functions, transport or nutrient assimilation, signaling, and defensive responses (Table II). The SREs were compared with Arabidopsis genome data using Blastn. Blastp analysis was performed on any SRE ORF, without predicted function, that was identified in an Arabidopsis database. Blastx/Blastp analysis was performed on SRE sequences that were unannotated as an ORF.

Several of the salt-responsive genes identified in this evaluation encode components of octadecanoid signaling through jasmonic acid (Table II, lipid signaling responses). The plant hormone appears to be derived from hydrolysis of membrane phospholipids (Koiwa et al., 1997). Triacyl glycerol lipase (AD03B08) can release free linolenic acid from phospholipids that is then oxidized by lipoxygenase (AD04G12) and cyclized by allene oxide synthase and allene oxide cyclase (AD04D07) to 12-oxo-phytodienoic acid. Two

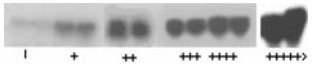
Table I. *Arabidopsis (Col-1 gl1) SREs identified after successive rounds of differential subtraction*

Salt regulation was based on detection of altered steady-state transcript abundance after NaCl treatment.

Round	Dot Blot	Sequenced	Unique Clones	Salt Regulated
First	1,000	137	34	25
Second	6,000	157	91	42
Third	3,000	320	80	17
Total	10,000	614	205	84

Table II. *Arabidopsis (Col-0 and gl1) ESTs corresponding to salt-responsive genes determined by northern-blot analysis*

Total RNA was isolated from seedlings transferred to medium without or with 160 mM NaCl for 4 h SREs were subjected to Blastn analysis and illustrated are annotations based on the information from the Arabidopsis genome sequence project: chromosome no., section on chromosomes 2 and 4, or BAC clone identification no. (for chromosomes 1, 3, and 5), and gene/protein accession nos. Highlighted in bold are SREs that detected transcripts differentially regulated in *gl1* and *sos3*. Transcript abundance is rated based on the scale illustrated.



GenBank Accession No.	EST No.	Chromosome	BAC Clone No.	geneID	proteinID	Annotation on Arabidopsis Gene/Homolog[a]	Score	E Value	Comments	gl-1 Control	gl-1 Salt
							bits				
Primary metabolism											
Photosynthesis											
BE844959	AD04H11	3	F5N5	ATU05218	AAA21570.1	ATP sulfurylase	492	e-138		++++	+++++
BE845179	AD08A06	2	25	At2g05100	AAD31358.1	Putative chlorophyll *a/b*-binding protein	789	0.0		–	++
BE845351	AD10C12	4	69	AT4g28750	CAB81463.1	Photosystem I subunit PSI-E-like protein	513	e-144		++++	+++++
BE845356	AD10D06	1	F12A21	F12A21.11	AAF26934.1	Putative PSBY emblCAA11248	728	0.0		++	+++
Carbohydrate											
BE844849	AD03C01	4	7	dl4575c	CAB46051.1	Putative β-amylase	1078	0.0	Gene duplication?	–	+
BE844998	AD05E02	3	45 T22E16	AT4g17090 T22E16.70	CAB80980.1 CAB75899.1	2-Oxoglutarate dehydrogenase, E1subunit-like protein	242	2e-63		+	++
Amino acid											
BE844834	AD03A02	4	59	AT4g23600	CAB79315.1	Tyrosine transaminase-like protein	605	e-172		+	+++++>
BE844976	AD05B09	1	F15I1	F15I1.19	AAD25783.1	Strong similarity to gblS77096 aldehyde dehydrogenase homolog from *Brassica napus* and is a member of PFI00171 aldehyde dehydrogenase family	137	5e-31	Proline degradation?	+	+++
BE845063	AD06C01	3	T21L8	T21L8.90	CAB51206.1	Glutamine-dependent asparagine synthetase	210	4e-53		+	+++
BE845111	AD06G04	4	82	AT4g34710	CAB80188.1	Arginine decarboxylase SPE2	809	0.0	Polyamine synthesis	–	++++
Others											
BE844989	AD05D03	5	K24M7	–	–	Not annotated	55.4	6e-08	Cytochrome p450	–	++
						pirllA29368 Prostaglandin omega-hydroxylase (EC 1.14.15.-) cytochrome P450 4A4, rabbit[a]	48	e-05			
Cellular function											
Cell wall											
BE844904	AD04B12	5	MMI9	AB019235.1	BAA97199.1	Ripening related, protein like; contains similarity to pectinesterase	712	0.0		+	+++
BE845087	AD06E01	5	F7K24	–	–	Not annotated	339	3e-92		+	++
						pirllT11610 Probable cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) CPRD14 (*Vigna unguiculata*)[a]	191	2e-48			

Table II. *Continued*

GenBank Accession No.	EST No.	Chromosome	BAC Clone No.	geneID	proteinID	Annotation on Arabidopsis Gene/Homolog[a]	Score	E Value	Comments	gl-1 Control	gl-1 Salt
							bits				
BE845121	AD06H02	4	6	AT4g02330	CAB80726.1	Strong similarity to similar to pectinesterase, contains pectinesterase signatures AA407-414	424	e-118		+	+++
Vesicle/protein trafficing											
BE845046	AD06A05	5	F17I14	F17I14_270	CAB89377.1	Periaxin-like protein	856	0.0	PDZ domain	+	+++
BE845048	AD06A07	1	F23N19	F23N19.7	AAF19550.1	Similar to vacuolar processing enzyme	385	e-106	Protease	–	+
BE845064	AD06C02	2	189	At2g34250	AAC27401.1	Putative protein transport protein SEC61 alpha subunit	216	6e-55	Vesicle transport	–	+++
BE845086	AD06D12	1	T25N20	T25N20.17	AAF79733.1	Putative transport protein	155	2e-36	Vesicle transport	+	++++
						refINP_062761.1I SEC23B (*Saccharomyces cerevisiae*)[a]	692	0.0			
Other cellular functions											
BE844934	AD04F04	4	67	AT4g27400	CAB81391.1	Putative protein: similarity to Arabidopsis nap gene, PID:e1234813	749	0.0	Cell division and expansion	–	+++
BE844985	**AD05C10**	4	90	AT4g39260	CAB80589.1	Gly-rich protein (clone AtGRP8): Contains eukaryotic putative RNA-binding region RNP-1 signature AA47-54	59.7	e-08	Ccr1	+++	+
BE845050	AD06A09	1	F1P2	F1P2.100	CAB61981.1	Putative protein: similarity to PIT1, Arabidopsis, GB: AF130849	480	e-134	Plant growth	–	+++++
BE845059	AD06B07	4	63	AT4g25630	CAB81373.1	Fibrillarin-like protein: strong similarity to probable fibrillarin (Sb21) mRNA, *Picea mariana*, AF051216	412	e-114	Pre-rRNA processing	–	++
BE845095	AD06E12	?	?	ATHATJ	AAB86799.1	Chaperone protein (atj)	509	e-143	Chaperon	–	+
BE845137	AD07C05	2	210	At2g38860	AAC79625.1	Unknown protein, splO59413IPFPI_PYRHO PROTEASE I (*Pyrococcus horikoshii*)[a]	157 64	4e-37 3e-09	Protease	+	+++
BE845172	AD07H10	2	185	At2g33210	AAC04902.1	Mitochondrial chaperonin (HSP60)	186	4e-46	Chaperon	+	+++
BE845190	AD08B06	2	21	At2g04460	AAD25832.1	Putative retroelement pol polyprotein	638	0.0	Transposon	+++	+
BE845392	AD10H03	4	14	AT4g05050	CAB81047.1	Contains similarity to Pfam family PF00240, ubiquitin family	1086	0.0		++	+++
Transporters/nutrient uptake											
BE844897	AD04B05	5	F9D12	F9D12.17	AAC26243.1	Contains similarity to sugar transporters	424	4e-32	Transporter	–	++++
BE844907	AD04C05	5	F14F18	F14F18_180	CAB87674.1	Putative protein, splQ96250IATP3_ARATH ATP SYNTHASE GAMMA CHAIN, MITOCHONDRIAL PRECURSOR[a]	995 35.2	0.0 1.6	Transporter?	–	+
BE844924	AD04E04	2	91	At2g15620	AAD17406.1	Ferredoxin-nitrite reductase	406	e-112	Nitrogen	+	++
BE845007	AD05E11	1	T17F3	T17F3.10	AAF07386.1	Putative peptide transporter	609	e-173	Transporter	–	+++
BE845058	AD06B06	5	F8M21	F8M21_130	CAB89334.1	Putative protein: similarity to amino acid transport protein, Arabidopsis, EMBL U39783	527	e-148	Transporter	–	+
BE845182	AD08A09	1	F5I6	F5I6.6	AAF27688.1	Putative sulfate transporter	234	e-60	Transporter	–	+++

Table II. *Continued*

GenBank Accession No.	EST No.	Chromosome	BAC Clone No.	geneID	proteinID	Annotation on Arabidopsis Gene/Homolog[a]	Score	E Value	Comments	gl-1 Control	gl-1 Salt
							bits				
BE845347	AD10C07	5	MUG13	AB021934	BAA74589.1	Nicotianamine synthase	541	e-152	Iron uptake	–	+++
BE845350	AD10C11	3	F4P12	F4P12_210	CAB67658.1	ABC transporter-like protein	5866	0.0	Transporter	–	+
Signaling											
General signal components											
BE844894	AD04B01	2	106	At2g18190	AAD31347.1	Putative AAA-type ATPase	884	0.0		+	+++
BE844899	AD04B07	1	T10O24	T10O24.2	AAD39582.1	Hypothetical protein, dbjIBAB11554.1I (AB011479), contains similarity to bHLH DNA-binding protein: gene_id:MNA5.5 (Arabidopsis)[a]	1085 36.7	2.9e-185 0.16	Transcription	–	+++
BE844973	AD05B05	5	MJC20	MJC20.11	BAB08434.1	Contains similarity to unknown protein (AF117897), rab11-binding protein (*Bos taurus*)[a]	507 187	e-142 4e-46	WD repeat	+	+++
BE845012	AD05F04	3	MMM17	MMM17.7	BAB01914.1	Casein kinase-like protein	353	4e-96		+++	++++
BE845028	AD05G10	3	T5P19	T5P19_160	CAB88054.1	Putative protein: similarity to TATA-binding protein-binding protein, ABT1: *Mus musculus*, EMBL AB021860	291	e-77	Transcription	+	++
BE845052	AD06A11	5	MQN23	MQN23.23	BAB11664.1	G protein-coupled receptor-like protein	377	e-103		+++	+++++
BE845077	AD06D03	3	F24M12	F24M12.170	CAB62635.1	Putative protein: similarity to lin-10 protein: *Rattus norwegicus*, PIR: JE0239	268	2e-70	Receptor targeting	+	++
BE845082	AD06D08	5	MAC9	MAC9.10	BAB10078.1	Transcription factor-like protein	317	2e-85	Transcription	+	+++
BE845106	AD06F11	1	F24B9	F24B9.4	AAF75068.1	Contains similarity to a protein kinase gblD88207	139	9e-33		+	+++
BE845234	AD08G06	3	F26F24	F26F24.8	AAF86997.1	Hypothetical protein, emblCAB92072.1I (AL121575), dJ914N13.2.1 (cofactor required for Sp1 transcriptional activation, subunit 3; 130kd; CRSP130, DRIP130, SUR2, and KIAA1216; isoform 1) (*Homo sapiens*)	274 84.3	2e-72 9e-15		–	+
BE845243	AD08H07	2	77	At2g13790	AAD28318.1	Putative receptor-like protein kinase	184	7e-46		–	++
BE845394	AD10H07	3	MGL6	MGL6.10	BAB00069.1	Translationally controlled tumor protein like	559	e-157	Ca2+ binding	+++	+++++
Lipid signaling/response											
BE844846	AD03B08	3	T8P19	T8P19.200	CAB62358.1	Putative protein, splP24484ILIP2_MORSP LIPASE 2 (TRIACYLGLYCEROL LIPASE)[a]	1176 58.6	0.0 9e-08	Lipase	+	+++
BE844917	AD04D07	3	K13N2	K13N2.11	BAA95764.1	Unknown, emblCAB95731.1I (AJ272026) allene oxide cyclase (*Lycopersicon esculentum*)[a]	472 249	e-132 3e-65	JA synthesis	+	++++
BE844950	AD04G12	3	T14D7	T14D3.80	CAB72152.1	Lipoxygenase AtLOX2	432	e-120		+	+++++>
BE845001	**AD05E05**	5	?	AB006778	BAA33447.1	Vsp2 gene for vegetative storage protein	509	e-143		–	+++

Table II. *Continued*

GenBank Accession No.	EST No.	Chromosome	BAC Clone No.	geneID	proteinID	Annotation on Arabidopsis Gene/Homolog[a]	Score	E Value	Comments	gl-1 Control	gl-1 Salt
							bits				
BE845091	AD06E08	5	MTI20	MTI20.3	BAB08850.1	Lipid transfer protein; glossy1 homolog	414	e-114		–	+++
BE845100	AD06F05	3	T02O04	T02O04.11	AAB63638.1	Jasmonate-inducible protein isolog, myrosinase binding protein like	466	e-130		++	++++
Hormone related											
BE844978	**AD05B11**	3	F28D10	F28D10_50	CAC03536.1	AtPP-like protein: prokaryotic membrane lipoprotein lipid attachment site AA199-209 (AF133053), S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (*Clarkia breweri*)[a]	706 129	0.0 6e-29	SA	–	++
BE845401	AD05C05	?	?	AF183827	AAF22295.1	Beta-glucosidase homolog (BG1)	3515	0.0		–	++++
BE845084	AD06D10	3	T10D17	T10D17_90	CAB88998.1	Nitrilase 2	202	e-50		+	+++
Cell death											
BE844851	AD03C06	5	MQL5	MQL5.19	BAA97167.1	Palmitoyl-protein thioesterase precursor like	422	e-117	Anti-apoptosis?	+	++
BE844958	AD04H10	3	F28D10	F28D10_70	CAC03538.1	Lethal leaf-spot 1 homolog Lls1: contains prokaryotic membrane lipoprotein lipid attachment site AA150-160	472	e-132	Dioxygenase	+	++
Defense response											
Defense proteins											
BE844875	AD03G09	1	F22K20	F22K20.19	AAC00625.1	Alcohol dehydrogenase	486	e-136		+	+++
BE844896	AD04B03	5	MSG15	MSG15.3	BAB11043.1	Mandelonitrile lyase-like protein	519	e-146	Cyanogenesis	–	++
BE844926	AD04E06	3	MDB19	MDB19.5	BAB02775.1	Contains similarity to endo-1,3-1,4-beta-D-glucanase gene_id:MDB19.5	414	e-115		+	+++
BE844972	AD05B03	1	F5F19	F5F19.6	AAD12691.1	Similar to gblY09437 myrosinase binding protein from *B. napus*	194	8e-49		–	+
BE844990	AD05D04	1	F24B9	F24B9.34	AAF75098.1	Metallothionein	605	e-172		++++	+++++
BE845403	AD06E03	1	F15K9	F15K9.17	AAC72119.1	Strong similarity to gblD14550 extracellular dermal glycoprotein (EDGP) precursor from *Daucus carota*	745	0.0		+	++
BE845117	AD06G10	1	F14N23	F14N23.25	AAD32887.1	Similar to glutathione S-transferase TSI-1 (gil2190992)	763	0.0	Cyanogenesis	+	+++++
BE845152	AD07F07	2	236	At2g43620	AAB64044.1	Putative endochitinase	549	e-154		+	++++
BE845153	AD07F08	5	MOJ9	MOJ9.4	BAB11145.1	Polygalacturonase-inhibiting protein	509	e-142		–	++++
Cor/RD proteins											
BE844850	AD03C02	4	73	AT4g30650	CAB79783.1	Strong similarity to low temperature and salt-responsive protein LTI6A, Arabidopsis	383	e-105		+	+++
BE844854	**AD03D05**	5	F1N13	F1N13_110	CAC01796.1	Cold-regulated protein COR6.6 (KIN2)	341	2e-92		–	+++++>
BE844855	AD03D11	2	F14N22	At2g42540	AAD22999.1	Cold-regulated protein cor15a precursor	519	e-14		–	+++++>
BE844865	AD03F02	5	K24M7	D13044	BAA02376.1	Desiccation-responsive rd29A	371	e-101		–	+++++>
BE845022	AD05G04	5	T14C9	ATHRD22	BAA01546.1	rd22 Gene	448	e-124		–	+++++
BE845049	AD06A08	1	F15M15	F5M15.21	AAF79613.1	Similar to cold-regulated protein cor47	127	4e-28		?	?

Table II. *Continued*

GenBank Accession No.	EST No.	Chromosome	BAC Clone No.	geneID	proteinID	Annotation on Arabidopsis Gene/Homolog[a]	Score	E Value	Comments	gl-1 Control	gl-1 Salt
							bits				
Anthocyanin synthesis											
BE844845	AD03B06	5	MOP10	MOP10.14	BAB11549.1	Leucoanthocyanidin dioxygenase-like protein	664	0.0		–	+++++>
BE844892	AD04A11	2	206	At2g38240	AAC27173.1	Putative anthocyanidin synthase	203	4e-87		–	++++
BE845101	AD06F06	4	T29A15	AT4g27560	CAB38268.1	Strong similarity to UDP rhamnose-anthocyanidin-3-glucoside rhamnosyltransferase, *Petunia hybrida*	837	0.0		+	++
Antioxidation											
BE844884	AD04A02	5	F5O24	AB035137	BAA86999.1	Blue copper-binding protein	494	e-138	Anti-oxidation	+	++++
Unknown											
BE844860	AD03E08	3	T19D11	–	–	Not annotated, dbj|BAB02819.1| (AB024036), dbj|BAA87936.1: gene_id:MQC12.15, similar to unknown	135 46.1	4e-31 5e-05		–	+++
BE844992	AD05D06	5	K3M16	K3M16_30	CAC01890.1	Hypothetical protein	777	0.0		–	++++
BE845016	AD05F09	3	F14O13	F14O13.28	BAB03026.1	Similar to unknown protein	476	e-133		+	++
BE845069	AD06C07	1	F22O13	F22O13.29	AAF99773.1	Unknown protein	690	0.0		+	++
BE845070	**AD06C08**	3	F16J14	–	–	Not annotated	910	0.0		+	++
						emb|CAB86422.1| (AL138648) putative protein (Arabidopsis)[a]	78.8	4e-14			
BE845097	AD06F02	?	?	–	–	Not annotated				+	++++
BE845355	AD10D05	1	F24J8	–	–	Not annotated	775	0.0	Transporter	–	++++
						gb|AAF28474.1|AF173553_1 (AF173553) V-ATPase 110-kD integral membrane subunit (*Manduca sexta*)[a]	29.7	16			
BE845376	AD10F07	1	T27G7	–	–	Not annotated	579	e-164		+	+++
						sp|Q08180|ICCR_DROME IRREGULAR CHIASM C-ROUGHEST PROTEIN PRECURSOR (IRREC PROTEIN)[a]	32.1	3.7			

[a] Blastp analysis was performed on any Arabidopsis open reading frame (ORF) that corresponded to an SRE that is not functionally annotated. Any SRE sequence that did not match an Arabidopsis ORF was subjected to Blastx/Blastp analysis.

SREs were annotated previously as jasmonic acid regulated, VSP2 (AD05E05) and AD06F05. Furthermore, the genes of a number of SREs are involved in plant defense and may be regulated by the octadecanoid signal pathway. Some of these genes have been shown to express upon dehydration in tomato (*Lycopersicon esculentum*; Reymond et al., 2000). Several abscisic acid (ABA)-responsive SREs are included in Table II and the plant hormone is a potentiator of octadecanoid signaling.

Genes Differentially Regulated by Salt in Wild Type and *sos3*

The salt regulated expression profile of *SRE* transcripts, as well as that of previously characterized stress-regulated genes (*ACP1*, *RD22BP1*, *MYB2*, *STZ*, and *PAL*), revealed that most are controlled similarly in wild type and *sos3*. Six of the salt-responsive genes, including *STZ* (Lippuner et al., 1996), were differentially regulated in wild type and *sos3* (Fig. 1). Transcript abundance of two was lower (*AD06C08*/unknown and *AD05E05*/vegetative storage protein2 [*VSP2*]) and of four was higher (encoding *AD05C10*/cold-circadian rhythm-RNA binding1 [*CCR1*], *STZ*/salt tolerance zinc finger [not shown], *AD05B11*/*S*-adenosyl-L-Met: salicyclic acid carboxyl methyltransferase [*SAMT*], *AD03D05*/cold regulated/cold inducible [*COR6.6/KIN2*]) in the salt-sensitive mutant. *SAMT* and *COR6.6/KIN2* transcript abundance was slightly elevated in *sos3* but the steady-state mRNA levels were hyper-induced by salt treatment. *CCR1* was the only gene for which transcript abundance is reduced by salt treatment.

Salt regulation of *VSP2* is similar in wild type and *sos1* indicating that transcriptional activation is not dependent on *SOS1* (Fig. 2). Methyl jasmonate (MeJA) induces *VSP2* transcript abundance in wild type and *sos3* (not shown). The SOS3/2 pathway and the hormone seem to regulate *VSP2* independently. Signal pathways often converge to regulate transcription of key effectors involved in cellular adaptation to environmental perturbation (Rep et al., 2000). *VSP2* is a member of a two-gene family (87% nucleotide sequence identity over the coding region) that encodes a protein with similarity to soybean VSPs (Berger et al., 1995; Utsugi et al., 1998), which are vacuolar-localized glycoproteins with acid phosphatase activity (Mason and Mullet, 1990). These proteins are presumed to be amino acid sinks during water deficit but are important reduced nitrogen sources after stress relief (Mason and Mullet, 1990).

CCR1, *STZ*, *SAMT*, *COR6.6/KIN2*, and *AD06C08*/unknown have similar expression profiles in *sos1*, *sos2*, and *sos3*, implicating these as transcriptional outputs requiring all components of the SOS pathway. *CCR1* encodes a Gly-rich RNA-binding protein implicated in posttranscriptional regulation. *CCR1* and *CCR2* comprise a two-gene family, and their expression is regulated by a diurnal circadian clock (Carpenter et al., 1994; Heintzen et al., 1997; Kreps and Simon, 1997). *CCR1* or *CCR2* expression negatively regulates either gene and this feedback loop presumably facilitates diurnal oscillation controlled by the master circadian clock (Heintzen et al., 1997). *CCR1* and *CCR2* steady-state mRNA levels are induced by cold but *CCR1* transcript is negatively regulated by ABA and dehydration, whereas *CCR2* expression is induced by dehydration (Carpenter et al., 1994). *CCR1* transcript abundance was down-regulated in *sos3* indicating that the SOS pathway is at least another negative regulator that controls *CCR1* expression downstream of the circadian rhythm clock (Heintzen et al., 1997; Kreps and Simon, 1997). These results indicate that control of circadian oscillations may be required during the salt stress response.

COR6.6//KIN2 and *SAMT* are both implicated in plant stress responses. *COR6.6/KIN2* is linked in tandem to its homolog *KIN1* (95% nucleotide sequence identity in the coding region) and both encode hydrophilic peptides that are boiling soluble but of unknown function (Thomashow, 1999). *KIN1* and *2* transcript abundance is cold and ABA induced (Kurkela and Franck, 1990; Kurkela and Borg-Franck, 1992; Thomashow, 1999) but *KIN2* and not *KIN1* expression is modulated positively by drought and salt (Kurkela and Borg-Franck, 1992). SAMT catalyzes the formation of methylsalicylate from salicylic acid using *S*-adenosyl-L-Met as the methyl donor (Ross et al., 1999). The volatile ester is implicated as a pollinator attractant and a signal in plant defense mediated by salicylic acid (Ross et al., 1999; Dudareva et al., 2000).

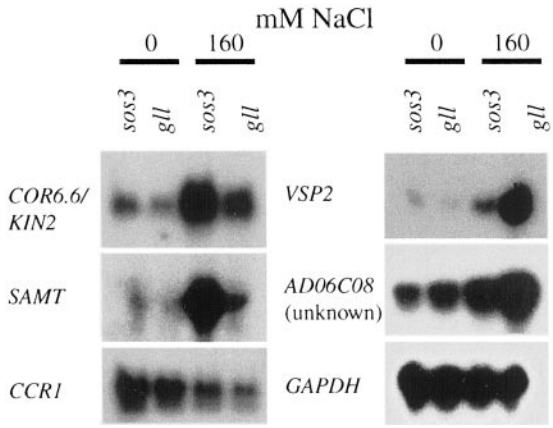


Figure 1. Salt-responsive gene expression that is dependent on the SOS pathway in *Arabidopsis*. Genes that are differentially regulated in wild-type (Col-0 *gl1*) and *sos3*. Total RNA (40 μg) from seedlings grown in liquid culture (14 d) and treated without or with 160 mM NaCl for 4 h. The northern blot was hybridized with ^{32}P-labeled probe corresponding to: *COR6.6/KIN2*, *SAMT*, *CCR1*, *VSP2*, and *AD06C08* (unknown). *AtGAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) is the control.

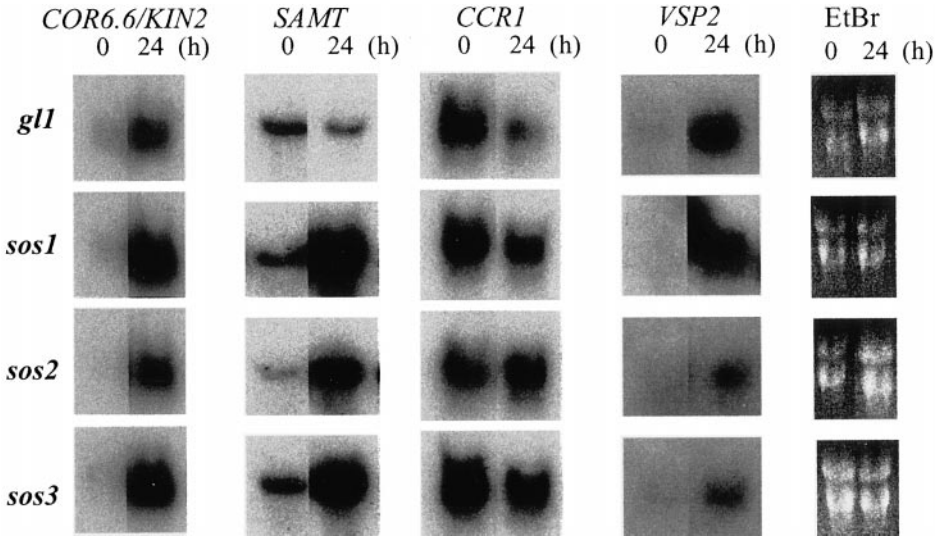


Figure 2. Comparative expression of genes dependent on the SOS pathway in wild type (Col-0 and *gl1*) and *sos1*, *sos2*, and *sos3*. Illustrated is the northern blot of steady-state mRNA levels of *COR*6.6/*KIN*2, *SAMT*, *CCR1*, and VSP2 in plants without (0 h) or 160 mM NaCl (24 h). Ethidium bromide staining was used to monitor RNA loading.

DISCUSSION

Many salt-regulated genes are responsive also to other biotic or abiotic perturbations, indicating that these stresses have common etiologies, e.g. water deficit, and cold are both osmotic stresses, occur simultaneous in the environment or elicit similar pathologies (Bohnert et al., 1995; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 1997; Hasegawa et al., 2000b). Furthermore, osmotic and ionic stresses induce secondary cellular perturbations that arise from ROS, elicitors from degradation of cell wall and plasma membrane macromolecules, or wounding, which initiate signal transduction pathways that modulate other plant defensive processes (Hasegawa et al., 2000b). In fact, many of the proteins encoded by the 84 salt-responsive genes identified in this study can be categorized as functional outputs from these different signal cascades (Table II). Most of these genes were not known previously to be modulated as a part of the Arabidopsis salt response. It is interesting that even after subtractive hybridization only approximately 13% of the ESTs (84/614) were unique and detected salt-regulated transcripts.

Six of 89 genes examined were differentially responsive to NaCl in wild type and *sos3*, implicating the SOS pathway in their transcriptional regulation. *AD06C08*/unknown and *VSP2* are induced and *CCR1*, *STZ*, *SAMT*, and *COR6.6/KIN2* are controlled negatively by SOS3. *CCR1* expression was lower in wild type after NaCl treatment, whereas the message abundance of the others was salt induced. From these results, a model for the SOS pathway regulation of these genes is illustrated in Figure 3 (Zhu, 2000). Salt regulated expression of *VSP2* is the same in wild type and *sos1* defining this gene as a transcriptional output from the SOS pathway that does not require *SOS1*. This supports the premise that SOS3 and SOS2 are signal intermediates and SOS1 is an effector of Na^+ homeostasis (Shi et al., 2000). However, salt regulation of *AD06C08*/unknown, *CCR1*, *STZ*, *SAMT*, and *COR6.6/KIN2* is dependent on *SOS2* and *SOS1*, perhaps implicating a signaling function for SOS1. Genes encoding an enzyme catalyzing the penultimate step in Pro biosynthesis (*P5CS*) and a putative transcription factor (*AtMYB*) are hyper-induced by

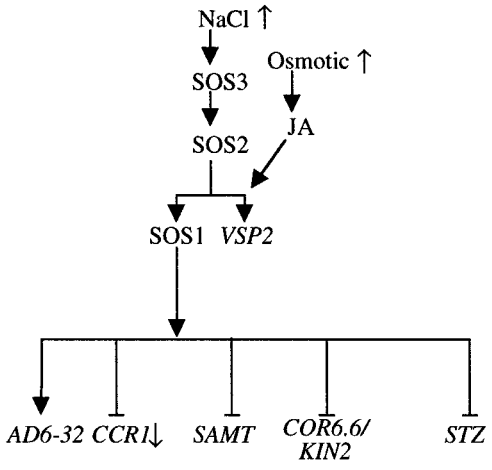


Figure 3. Illustrated is a model depicting the SOS pathway regulation of salt responsive genes. Hypersaline conditions activate the SOS (SOS3 → SOS2 → SOS1) signal pathway (Zhu, 2000) and transcript abundance of *AD0608* (unknown), *VSP2* (vegetative storage protein 2, *AD05E05*), *SAMT* (S-adenosyl-L-Met:salicylic acid carboxyl methyltransferase, *AD05B11*), *COR6.6/KIN2* (cold regulated 6.6/inducible 2, *AD03D05* [*COR6.6/KIN2*], and *STZ* [salt tolerance zinc finger], Lippuner et al., 1996) increase and of CCR1 (circadian rhythm-RNA binding1, *AD05C10*) decreases in Arabidopsis seedlings. Positive (↓) or negative (⊥) regulation by the SOS pathway is indicated.

salt in *sos1* compared with wild type (Liu and Zhu, 1997). Furthermore, some transport proteins include sensor domains or function in association with sensors (Ozcan et al., 1998; Heinisch et al., 1999; Sabirov et al., 1999) as could the putative Na^+/H^+ antiporter SOS1 (Shi et al., 2000).

The experimental evidence presented here indicates that the SOS pathway controls expression of only a few salt stress-specific tolerance determinant genes among the numerous genes (six of 89 in this study) that are regulated in the plant response to NaCl treatment (Zhu et al., 1997). This is similar to the paradigm that has been established recently for the salt stress response of the unicellular eukaryote yeast (*Saccharomyces cerevisiae*). Genome-wide array analysis determined that osmotic upshock causes a rapid and multi-fold increase in mRNA of between 186 and 1,359 genes and reduced transcript abundance of more than 100 genes depending on the severity of osmotic shock, the osmotic agent (NaCl or sorbitol), and time after treatment (Posas et al., 2000; Rep et al., 2000; J. Yale and H.J. Bohnert, unpublished data). Salt-induced expression of most is either partially or completely controlled by the high osmolarity glycerol and mitogen-activated protein kinase pathway. The yeast calcineurin pathway is analogous to the Arabidopsis SOS pathway, controls ion homeostasis and is essential for salt tolerance in yeast (Mendoza et al., 1994, 1996), and affects expression of many fewer osmotic responsive genes (T.K. Matsumoto, unpublished data). Like the SOS pathway, calcineurin regulates expression of genes that encode salt tolerance effectors such as Na^+ efflux transporters (Mendoza et al., 1994; Shi et al., 2000; Matusmoto et al., unpublished data).

It is conceivable that signal transduction through the SOS pathway that mediates salt tolerance may have a substantial component that involves posttranscriptional activation of salt tolerance effectors, particularly over the time span (minimum of 4 h) of the salt treatment used in experiments reported here. Plant survival in severe stress likely requires very immediate cellular responses, whereas transcriptional regulation may be sufficient for stress recovery and adaptation. Notwithstanding, salt induces transcriptional activation of genes in yeast within minutes (Posas et al., 2000; Rep et al., 2000). Genes that are transiently induced or weakly expressed further complicate inference of function from expression profile analysis. The majority of yeast genes induced by mild salt shock exhibit transient expression (Posas et al., 2000). Determinant gene transcript abundance differences in wild type and *sos3* may be insufficient for the resolution limits of the subtraction protocols, yet are biologically meaningful to salt stress adaptation.

It is interesting that the SOS pathway negatively controls the expression of four salt-regulated genes (*SAMT*, *C0R 6.6/KIN2*, *STZ*, and *CCR1*), three (*SAMT*, C0R6.6/KIN2, and *STZ*) of which are induced by NaCl treatment. So, salt tolerance determinants include genes that must be repressed, at least temporally, during the plant stress response. Negatively regulated genes may include those that contribute to growth arrest necessary during the period of adjustment or ameliorate other etiologies that occur coincidentally with salt in the native environment of the organism. *CCR1* and *COR6.6/KIN2* are cold induced, whereas *SAMT* is implicated in plant defense against pathogens indicating their principal function is not in salt adaptation. Together, this suggests that a function of the SOS pathway is to discriminate against the myriad of stress signals that are elicited by salt and to focus the capacity of the plant to cope with the principal etiology; in this instance, ion dis-equilibrium. Furthermore, the SOS pathway may coordinate temporal gene expression to focus the availability of effectors, as required, during stress perception, amelioration, or adaptation. Confirmation that the genes identified in this study encode tolerance determinants awaits molecular genetic confirmation by loss- or gain-of-function experimentation.

MATERIALS AND METHODS

Plant Material

Arabidopsis (ecotype Columbia-0 *gl1*) and *sos1*, *sos2*, and *sos3* were wild-type and salt-hypersensitive genotypes, respectively (provided by Dr. Jian-Kang Zhu, University of Arizona). Seeds were surface disinfected and stratified for 2 d at 4°C. Seeds were germinated in liquid medium (Murashige and Skoog salts [Murashige and Skoog, 1962] and 3% [w/v] Suc [pH 5.8]) in 250-mL flasks on a gyratory shaker (80–100 rpm) under low-intensity Cool White fluorescent illumination (light/dark:16/8 h daily) at 22°C to 24°C. After 14 d, seedlings were transferred to fresh medium without or with 160 mM NaCl for the time interval indicated. Seedlings were harvested, frozen in liquid nitrogen, and stored at −80°C.

Construction of Subtraction Libraries

Total RNA was isolated as described by Gong et al. (1997). mRNA was isolated using the Poly(A)$^+$ RNA purification kit (CLONTECH, Palo Alto, CA). The PCR-Select cDNA subtraction kit (K1804-1, CLONTECH) was used to obtain subtracted cDNA libraries.

Subtraction of cDNAs Obtained from mRNA of Salt-Treated *sos3* and *gl1*

Subtractive hybridization was used to identify cDNAs corresponding to salt regulated genes differentially expressed in *sos3*. Both forward and reverse subtractive hybridizations were performed with salt-treated (160 mM NaCl, 4 h) seedlings. The forward subtraction used tester cDNA obtained from mRNA of *gl1* and driver cDNA from *sos3*. In the reverse subtraction, the tester cDNA was ob-

tained from *sos3* and driver cDNA from *gl1*. Driver cDNAs are the reference and are targets for elimination during subtraction leaving unique tester cDNAs. Forward and reverse subtractive hybridization was meant to identify salt-responsive genes that are specifically regulated in *sos3*.

Subtraction of cDNAs Obtained from *gl1* after Treatment without or with NaCl

This subtraction was intended for identification of genes differentially regulated by salt in *gl1*. The forward subtraction tester cDNA was obtained from mRNA of *gl1* seedlings 4 h after transfer to medium with 160 mM NaCl and driver cDNA from *gl1* plants grown in medium without salt. In the reverse subtraction, tester and driver cDNA was obtained from *gl1* grown without and with 160 mM NaCl, respectively.

The subtracted libraries were subjected to two rounds of PCR amplification, the second using nested primers for adaptors 1 and 2R (CLONTECH). The PCR products were ligated into pT-Adv (CLONTECH), and transformed into *Escherichia coli*. The white colonies were isolated and inserts amplified by PCR.

Enrichment of Unique Salt-Regulated cDNAs in the Subtraction Library

A modification of the differential subtraction chain method (Luo et al., 1999) was used to enrich the subtracted library for unique salt-regulated cDNAs. The driver for this subtraction (driver 2) is a mixture of second PCR product from reverse subtraction (100 μL; tester DNA:*gl1* without salt, driver DNA:*gl1* with salt) and PCR products amplified from highly repetitive salt-induced cDNAs isolated from previous rounds of screening (0.5 μL of each). To remove adaptor sequences from driver 2, the mixture was digested at the restriction sites of adaptors 1 (*Sma*I and *Rsa*I) and 2R (*Eag*I and *Rsa*I) by consecutive restriction digestion using *Sma*I, and then *Eag*I + *Rsa*I. The digested DNA was extracted with phenol and phenol/chloroform, and then precipitated with ethanol. The precipitate was redissolved in 100 μL of water and passed through the Microcon YM-30 column (Amicon, Beverly, MA) to separate the DNA from Adaptor fragments. Driver 2 DNA was recovered in 20 μL of water and used as driver in the following PCR subtraction. About 10 times excess amount of driver 2 DNA (2 μL) was mixed with 1μL of forward subtraction library (driver and tester = *gl1* grown without and with 160 mM NaCl, respectively) in hybridization buffer {5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-HCl (pH 8.3), 12 mM NaCl, and 0.05 mM EDTA} in a total volume of 12 μL. The hybridization solution was denatured at 94°C for 5 min, and incubated at 72°C for 12 h.

After hybridization, the mixture was precipitated with ethanol, and digested with mung bean nuclease (Promega, Madison, WI) to remove adaptor sequence from products of the driver-tester hybridization. Adaptor sequences in tester-tester hybridization products are aligned with matching ends and these are not digested by mung bean nuclease. The mung bean nuclease digested mixture was passed through the Microcon column and purified DNA was rehybridized at 72°C. The mung bean nuclease digestion and DNA purification procedures were repeated. The DNA was amplified by PCR using nested primers for 1 and 2R, 11 cycles. The cloning of PCR products was as described as above.

Dot-Blot Analysis

For dot-blot analysis, cDNA inserts of forward subtraction library clones were individually amplified by PCR and 2 μL of PCR product was mixed with 2 μL of 0.6 M NaOH. Two microliters of the mixture was blotted onto each of two duplicate nylon membrane filters. Probes for dot-blot analysis were either forward or reverse subtraction products or cDNA of RNA from plants, as indicated. The products of forward and reverse subtraction were digested with *Sma*I, *Rsa*I, and *Eag*I to remove the adapter sequences and labeled with ^{32}P using the Ready-To-Go kit (Amersham Pharmacia Biotech, Piscataway, NJ). The labeled forward probes were hybridized to one membrane and the reverse probes to the duplicate.

Template Preparation, DNA Sequencing, and Data Analysis

Plasmid templates were prepared from selected bacterial colonies by 96-well alkaline lysis minipreps according to the manufacturer's instructions (Edge BioSystems, Inc., Gaithersburg, MD).

DNA sequencing reactions were conducted using DyeDeoxy "Terminator PRISM" mix (Perkin-Elmer-ABI, Foster City, CA) according to the manufacturer's instructions in a multiplate thin-wall 96-well microplate on an MJ Research PTC-100-96 (MJ Research, Watertown, MA) programmable thermal controller using the following profile: 96°C for 30 s, 45°C for 15 s, and 60°C for 4 min for 49 cycles. Unincorporated dye terminators were removed by passing reactions over a 96-well gel filtration block (Edge BioSystems). Recovered sequencing reaction products were analyzed on either an ABI 373A-XL Stretch or an ABI 3700 capillary array automated DNA sequencing system (Perkin-Elmer Applied BioSystems). Raw sequence data was analyzed using PHRED (Ewing and Green, 1998; Ewing et al., 1998) and Cross match to removal vector sequences. Additional vector sequence removal and editing was done manually using FACTURA software (Perkin-Elmer Applied BioSystems). Polished EST sequence files were assembled into singleton and contig files using PHRAP (P. Green, unpublished data). EST identities were determined by sequence comparison to the nonredundant GenBank database using BLASTN (BLAST 2.0) using default parameters (Altschul et al., 1997). In instances where an unannotated match was obtained, BLASTX searchers were conducted and sequence homology information was used to assign putative identities. All EST sequences reported here have been deposited in dbEST and can be browsed and retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov) under accession numbers BE844684 through BE845405.

Northern-Blot Analysis of Putative Clones

Total RNA was isolated from seedlings and 40 μg from each sample was separated on 1.2% (w/v) agarose formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham) as previously described (Gong et al., 1997). The cDNA insert of each clone was amplified by PCR using nested primers that hybridize to adaptors 1 and 2R, and purified from agarose gels using the Qiagen Gel Purification kit. The probes for *RD22BP1*, *MYB2*, *PAL*, *STZ*, and *ACP1* were obtained by PCR amplification using cDNA obtained from mRNA of salt-treated *gl1* as template; *RD22BP1* (AB000875), forward primer: 5′-ATGACGCTGTTGATGAGGAG-3′ and reverse primer: 5′-TTTCGGATTCTGGGTCTGAG-3′ (0.56 kb); *MYB2* (D14712), forward primer: 5′-GAAATGGAAGATTACGAGCG-3′ and reverse primer: 5′-TTAATTATACGAATACGATGTC-3′ (1.0 kb); *PAL* (L33677), forward primer: 5′-ATGGAGATT-AACGGGGCACAC-3′ and reverse primer: 5′-ACGT-TCACCGTTGGGACCAG-3′ (1.1 kb); STZ (X95573), ORF; and *ACP1* (AF009228), forward primer: 5′-CAA-AAGCCATTTTTCAAATTTCAAACTCAG and reverse primer 5′-GTTTTCAATGATAGTGAAGAAAGATG-TAC-AAC (0.83 kb). The purified PCR products were labeled using ^{32}P dCTP using the Ready-To-Go kit. Blot-blot hybridization and washes were as described (Gong et al., 1997). The blots were stripped by boiling in 0.5% (w/v) SDS solution for 3 min and were rehybridized with another probe.

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