The SAC Domain-Containing Protein Gene Family in Arabidopsis[1]

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The SAC domain was first identified in the yeast (*Saccharomyces cerevisiae*) Sac1p phosphoinositide phosphatase protein and subsequently found in a number of proteins from yeast and animals. The SAC domain is approximately 400 amino acids in length and is characterized by seven conserved motifs. The SAC domains of several proteins have been recently demonstrated to possess phosphoinositide phosphatase activities. Sac1p has been shown to regulate the levels of various phosphoinositides in the phosphoinositide pool and affect diverse cellular functions such as actin cytoskeleton organization, Golgi function, and maintenance of vacuole morphology. The Arabidopsis genome contains a total of nine genes encoding SAC domain-containing proteins (AtSACs). The SAC domains of the AtSACs possess the conserved amino acid motifs that are believed to be important for the phosphoinositide phosphatase activities of yeast and animal SAC domain proteins. AtSACs can be divided into three subgroups based on their sequence similarities, hydropathy profiles, and phylogenetic relationship. Gene expression analysis demonstrated that the *AtSAC* genes exhibited differential expression patterns in different organs and, in particular, the *AtSAC6* gene was predominantly expressed in flowers. Moreover, the expression of the *AtSAC6* gene was highly induced by salinity. These results provide a foundation for future studies on the elucidation of the cellular functions of SAC domain-containing proteins in Arabidopsis.

Phosphoinositides are a group of phospholipids that differ from each other by the presence or absence of a phosphate group on the 3-, 4-, or 5-hydroxyl position of the inositol head group of phosphatidylinositol (PI). They exist as seven forms consisting of PI 3-monophosphate [PI(3)P], PI 4-monophosphate [PI(4)P], PI 5-monophosphate, PI 3,4-bisphosphate,, PI 3,5-bisphosphate [PI(3,5)P_2], PI 4,5-bisphosphate [PI(4,5)P_2], and PI 3,4,5-triphosphate [PI(3,4,5)P_3]. PI(4,5)P_2 is known to be the precursor of the second messengers inositol 1,4,5-triphosphate and diacylglycerol, which are important in the activation of protein kinase C and the release of intracellular calcium (Toker, 1998). Recent studies in yeast (*Saccharomyces cerevisiae*) and animals have demonstrated that phosphoinositides themselves are key players in cellular processes such as the organization of actin cytoskeleton, modulation of vesicle trafficking, maintenance of vacuole morphology, regulation of lipid storage, and activation of proteins such as phosphoinositide-dependent kinase 1 and phospholipase D (Takenawa and Itoh, 2001). The metabolism of phosphoinositides is regulated by kinases and phosphatases that phosphorylate and dephosphorylate them, respectively. In addition, phospholipases such as phosphoinositide-specific phospholipase Cs can also regulate the metabolism of phosphoinositides.

Phosphoinositide phosphatases and inositol polyphosphate phosphatases are traditionally classified based on the position of the phosphate that they hydrolyze, namely 1-, 3-, 4-, or 5-phosphatase (Takenawa and Itoh, 2001). Among these phosphatases, 5-phosphatases comprise a large family that is further divided into four types according to their substrate specificity. Except for the type I 5-phosphatases that only hydrolyze water-soluble inositol polyphosphate, the other three types of 5-phosphatases are capable of hydrolyzing phosphoinositides (Takenawa and Itoh, 2001). Recently, a novel group of phosphatases called SAC domain phosphatases have been identified in yeast and animals, and they were shown to hydrolyze phosphates on multiple positions of the inositol head group of phosphoinositides (Hughes et al., 2000a). The SAC domain was first discovered in the yeast phosphoinositide phosphatase Sac1p, which was identified in screens for "suppressor of actin" mutations (Novick et al., 1989) and suppressors of the defects caused by mutations of the Sec14 PI/phosphatidylcholine transfer protein (Cleves et al., 1989). The SAC domain was subsequently found in several other proteins from yeast and animals. Based on the features of the amino acid sequences outside the SAC domains, the SAC domain-containing proteins have been grouped into two classes (Hughes et al., 2000a). One class is represented by synaptojanins in which the N-terminal localized SAC domain is linked to a C-terminal localized type II 5-phosphatase domain. This class includes human synaptojanin 1 and synaptojanin 2 and yeast synaptojanin-like proteins Inp51p, Inp52p, and Inp53p. The other class is represented by Sac1p in

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which the SAC domain is linked to a C-terminal region without any recognizable domains. This class includes yeast Sac1p and Fig4p, human (*Homo sapiens*) hSac1, hSac2, and hSac3, and rat (*Rattus norvegicus*) rSac1. The C-terminal regions of the proteins in this class are different in length and unique in their amino acid sequences.

The association of phosphoinositide phosphatase activities with the SAC domains has been revealed recently through the studies of human synaptojanins and the yeast synaptojanin homologs Inp52p and Inp53p. It was discovered that these proteins possess a second phosphatase activity in addition to the type II 5-phosphatase activity and that this second activity resides in the SAC domain (Guo et al., 1999). The SAC domains of Sac1p and rSac1 were also shown to exhibit phosphoinositide phosphatase activities. These SAC domains are capable of hydrolyzing phosphates from PI(3)P, PI(4)P, and PI(3,5)P_2 (Hughes et al., 2000b; Nemoto et al., 2000). Another SAC domain-containing protein, hSac2, has been demonstrated to exhibit 5-phosphatase activity specific for PI(4,5)P_2 and PI(3,4,5)P_3 (Minagawa et al., 2001).

The SAC domains are approximately 400 amino acid residues in length and defined by seven conserved motifs that are believed to be important for their phosphatase activities. The highly conserved sequence RXNCXDCLDRTN in motif VI is proposed to be the catalytic core of the SAC domain phosphatases (Hughes et al., 2000a). The $CX_5R(T/S)$ motif within this sequence is also found in a number of metal-independent protein phosphatases and inositol polyphosphate phosphatases and is known to be a phosphatase catalytic site (Hughes et al., 2000a). The $CX_5R(T/S)$ motif is absent from the SAC domain of the yeast synaptojanin-like protein Inp51p, which is thought to be the cause of the lack of phosphatase activity of the Inp51p SAC domain (Guo et al., 1999). Furthermore, mutations of the first conserved Asp residue in the RXNCXDCLDRTN sequence as seen in the yeast *sac1-8* and *sac1-22* mutant alleles were demonstrated to inactivate the Sac1p functions (Kearns et al., 1997).

The cellular functions of SAC domain-containing proteins are best characterized in Sac1p. Sac1p is an integral membrane protein localized primarily in the endoplasmic reticulum (Whitters et al., 1993; Foti et al., 2001). Mutational analysis has demonstrated that Sac1p is mainly involved in the hydrolysis of phosphate from PI(4)P in vivo. Mutations of Sac1p caused a predominant increase in the PI(4)P level, which led to alterations in vacuole morphology, Golgi function, actin cytoskeleton organization, and regulation of lipid storage (Foti et al., 2001). The yeast Sac1p-like protein Fig4p was required for the proper actin organization and cellular morphogenesis during mating (Erdman et al., 1998), but it is not known whether Fig4p exhibits any phosphoinositide phosphatase activities. Although several other SAC domain-containing proteins from animals possess phosphoinositide phosphatase activities in vitro, their cellular functions remain unknown (Nemoto et al., 2000; Minagawa et al., 2001).

In plant cells, all phosphoinositides except PI(3,4,5)P_3 have been identified. Several lines of evidence suggest that as in yeast, phosphoinositides in plants may regulate many cellular activities such as vesicle trafficking (Matsuoka et al., 1995; Kim et al., 2001), pollen tube growth (Kost et al., 1999), and responses to stress and hormonal treatments (Mikami et al., 1998; Meijer et al., 1999, 2001; Pical et al., 1999; DeWald et al., 2001). A number of kinases and phospholipase Cs involved in the metabolism of phosphoinositides have been characterized in plants (Stevenson et al., 2000). A recent genome analysis has revealed that the Arabidopsis genome contains large families of phosphoinositide kinases and phosphoinositide-specific phospholipase Cs (Müller-Röber and Pical, 2002). In contrast, much less is known about phosphoinositide phosphatases in plants. The only phosphatases characterized are two inositol polyphosphate phosphatases (Quintero et al., 1996; Berdy et al., 2001; Xiong et al., 2001) and one Tyr phosphatase that was shown to hydrolyze PI(3,4,5)P_3 (Gupta et al., 2002), a substrate that has not been identified in plants. No studies have been described regarding the genes encoding SAC domain phosphatases and their possible functions in plants.

During the studies of the molecular mechanisms controlling fiber cell differentiation in Arabidopsis, we found that mutation of a SAC domain-containing protein in the *fra7* mutant caused alterations in cell wall synthesis and cell morphogenesis (R. Zhong and Z.-H. Ye, unpublished data). This finding suggests that the FRA7 SAC protein plays an essential role in plant cellular functions. Because SAC domain-containing proteins have not been characterized previously in plants, this prompted us to analyze the genes encoding these proteins in the Arabidopsis genome in comparison with those from yeast and animals. In this report, we show that the Arabidopsis genome contains nine SAC domain-containing proteins, all of which belong to the class of Sac1p-like SAC proteins. We provide sequence information for all nine *AtSAC* cDNAs and confirm the exon-intron organization of the *AtSAC* genes. We present sequence analysis data showing that the AtSAC proteins can be divided into three subgroups based on their sequence homology and phylogenetic relationship. We further demonstrate that the *AtSAC* genes are differentially expressed in different organs and that the expression of the *AtSAC6* gene is highly induced in response to salt treatment. The results presented in this report provide a foundation for further investigation of the cellular functions of SAC domain-containing proteins in plants.

RESULTS

Identification of a Family of SAC Domain-Containing Protein Genes in Arabidopsis

In the course of investigating the molecular mechanisms controlling fiber cell formation in Arabidopsis, we found that mutation of a SAC domain-containing protein gene in the *fra7* mutant led to a number of cellular defects (R. Zhong and Z.-H. Ye, unpublished data). This indicates that SAC domain-containing proteins may play important roles in plant cellular processes. In an attempt to identify SAC domain-containing proteins in Arabidopsis, a search of the Arabidopsis genome sequence was performed with the SAC domain sequence of Sac1p. This search identified a total of nine genes encoding putative proteins with a domain showing high sequence similarity (55%–69%) with the SAC domain of Sac1p. These predicted proteins do not contain any other recognizable domains except the SAC domain; therefore, they are tentatively named as Arabidopsis SAC domain-containing proteins (AtSAC; Table I). All of these proteins were annotated as unknown proteins by the Arabidopsis genome sequencing project except ATEM1.8, which was annotated as "putative transmembrane protein G5p." (Comella et al., 1999) To confirm the exon-intron organization of the *AtSAC* genes, we isolated and sequenced all nine *AtSAC* cDNAs. Their sequences were confirmed by comparison with the corresponding genomic sequences. These cDNAs were deposited in GenBank and their accession numbers and predicted protein length are shown in Table I.

Comparison of the cDNA sequences with their genomic DNA sequences showed that the annotated exon-intron structures of the *AtSAC1*, *AtSAC2*, *AtSAC3*, and *AtSAC5* genes are correct, but a few errors were found in the annotated positions of exons and introns in the *AtSAC4*, *AtSAC6*, *AtSAC7*, *AtsAC8*, and *AtSAC9* genes. Based on our analysis, the corrected exon-intron organization of the *AtSAC* genes is presented in Figure 1. It was found that the exon-intron organization of *AtSAC1* to *AtSAC5* is similar with each of them having sixteen exons, indicating that they might have arisen from the same ancestral gene. This is also true with *AtSAC6* to *AtSAC8*, each with 20 exons. *AtSAC9* has 13 exons, which is unique among all the *AtSAC* genes. It was interesting to note that several *AtSAC* genes utilize unconventional splicing sites in their introns. These include the seventh intron (AT-AC) and 14th intron (GC-AG) of the *AtSAC6* gene, the seventh intron (AT-AC) of the *AtSAC7* gene, and the seventh intron (AT-AA) and 17th intron (GC-AG) of the *AtSAC8* gene. The AT-AC introns have been reported as a minor class of introns in both animals and plants (Brown and Simpson, 1998). The unconventional 5′-splicing site GC in the GC-AG introns is also present in several myrosinase genes in Arabidopsis (Xue and Rask, 1995).

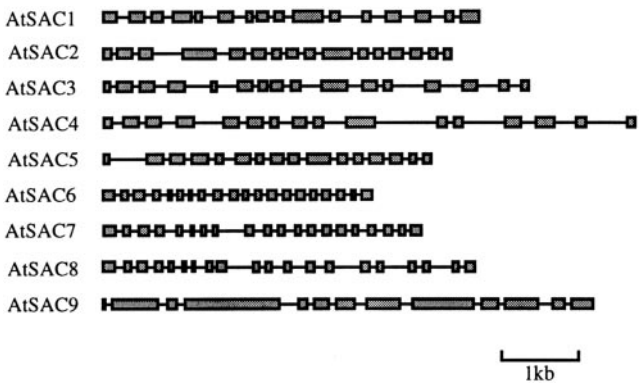

Figure 1. The exon-intron organization of the *AtSAC* genes. The positions of the exons (gray boxes) and introns (lines) of individual *AtSAC* genes were confirmed by comparison of the cDNAs with their corresponding genomic DNA sequences. The *AtSAC1* to *AtSAC5* genes have 16 exons, the *AtSAC6* to *AtSAC8* genes have 20 exons, and the *AtSAC9* gene has 13 exons.

A number of cDNAs corresponding to several *AtSAC* genes have been deposited previously in GenBank by the Arabidopsis cDNA sequencing groups. However, except for the cDNAs corresponding to *AtSAC2* (AY050432), *AtSAC5* (AY093760), and *AtSAC8* (U72504 and AY080659), we found a number of errors in the nucleotide sequences of other deposited cDNAs (AY080802, AY094477, AY080794, and

Table I. *SAC gene family members in Arabidopsis*

Gene Name (The Arabidopsis Information Resource)	AGI Gene Code	cDNA GenBank Accession No.	Predicted Protein Length (No. of Amino Acid)	Chromosomal Location (Genetic Distance [cM]/Physical Distance [Mbp])	Gene Family Name
F12K8.3[a]	At1g22620	AY227244	912	I (35.2/8)	AtSAC1
MAG2.20	At3g14205	AY227245	808	III (20.2/4.7)	AtSAC2
F7K15.70	At3g43220	AY227246	818	III (66.1/15.3)	AtSAC3
F22D1.10	At5g20840	AY227247	831	V (36.9/7.1)	AtSAC4
F28G4.21	At1g17340	AY227248	785	I (26.1/5.9)	AtSAC5
K2A18.9	At5g66020	AY227249	593	V (135.8/26.12)	AtSAC6
F26O13.100	At3g51460	AY227250	597	III (82.6/19.11)	AtSAC7
ATEM1.8	At3g51830	AY227251	588	III (83.1/19.23)	AtSAC8
F24G16.40	At3g59770	AY227252	1,630	III (95.7/22.11)	AtSAC9

[a] F12K8.3 is the same as T22J18.20. This gene is located in the overlapping region of bacterial artificial chromosome clones F12K8 and T22J18.

AF360267) by aligning them with their corresponding genomic sequences and the cDNA sequences we obtained. The cDNA sequence (AF412116) corresponding to *AtSAC9* only contains 1,541 nucleotides of the 5′ end of the *AtSAC9* cDNA sequence without a stop codon. Several expressed sequence tag (EST) sequences (AV787547, AV554769, BG459338, and AV546002) corresponding to small segments of the *AtSAC9* cDNA have been found in the GenBank database. No EST or cDNA sequences corresponding to *AtSAC6* had been deposited previously in GenBank.

Sequence Analysis of the SAC Domains of the AtSAC Proteins

The SAC domains of yeast and animal proteins are approximately 400 amino acids in length and contain seven conserved motifs. To examine in detail the motif organization in the SAC domains of the AtSAC proteins, we compared the SAC domain sequences between Sac1p and the AtSAC proteins. The SAC domains of Sac1p and other yeast and animal proteins share overall 18% to 43% sequence identity and 38% to 58% sequence similarity (Hughes et al., 2000a). A similar level of sequence identity is seen in the SAC domains between Sac1p and AtSACs. The SAC domains of the AtSAC proteins exhibit 22% to 35% sequence identity and 55% to 69% similarity with that of Sac1p (Table II; Fig. 2). Among the AtSAC proteins, pair-wise comparisons revealed that the SAC domains share 20% to 90% sequence identity and 45% to 97% similarity (Table II).

Sequence analysis showed that the SAC domains of all of the AtSAC proteins except AtSAC9 contain all seven conserved motifs found in Sac1p (Fig. 2). These conserved motifs were believed to be important for the functions of the SAC domains of yeast and animal proteins (Hughes et al., 2000). The SAC domain of AtSAC9 appears to lack motif VII. However, the putative catalytic core sequence RXNCXDCLDRTN located in motif VI is completely conserved among the AtSAC proteins except for a one-amino acid change seen in AtSAC9 (Fig. 2). This suggests that the AtSAC proteins may possess similar SAC domain functions as their yeast and animal counterparts.

It was intriguing to find that although the SAC domains of other AtSAC proteins contain all seven conserved motifs, AtSAC9 seemed to lack motif VII. Surprisingly, in its place is a putative WW domain (amino acid residues 509–543; Fig. 3D). WW domains have been shown to be involved in protein-protein interactions by recognizing Pro-containing ligands (Ilsley et al., 2002), and they are considered to be the smallest protein domain involved in protein-protein interactions. The WW domains are composed of approximately 35 amino acids that contain two signature Trp (W) residues spaced 20 to 22 residues apart. The putative WW domain of AtSAC9 shares all features typical for known WW domains, such as the two Trp residues spaced 22 residues apart, and the presence of other conserved residues including the essential aromatic doublet and Pro (Fig. 3D). None of the other AtSAC proteins or Sac1p contains a putative WW domain. The functional significance of the putative WW domain in AtSAC9 remains to be investigated.

Significant sequence similarity (24%–43% identity and 53%–66% similarity) between the AtSACs (except AtSAC9) and Sac1p is still seen in the seventy amino acid residues flanking motif VII, suggesting that this portion of the sequence may still belong to the SAC domain. However, the rest of the C-terminal sequences of the AtSACs did not show any similarity with Sac1p, synaptojanins, or any other phospoinositide phosphatases. In fact, they do not show any sequence similarity to any known proteins in the GenBank database. Therefore, all nine AtSAC proteins contain a SAC domain and a unique C-terminal region (Fig. 3, A–C), suggesting that they belong to the class of Sac1p-like SAC proteins and that no synaptojanin-like proteins are present in the Arabidopsis genome.

Table II. *Identity and similarity of the SAC domains of AtSACs in comparison with that of yeast Sac1p*

SAC Domain Proteins	Identity/Similarity[a]									
	Sac1p	AtSAC1	AtSAC2	AtSAC3	AtSAC4	AtSAC5	AtSAC6	AtSAC7	AtSAC8	AtSAC9
						%				
Sac1p	—	55.2	58.2	57.2	57.8	60.7	68.6	67.4	65.4	55.4
AtSAC1	25.8	—	79.2	76.2	75.0	71.8	55.2	53.5	54.0	55.2
AtSAC2	26.9	53.1	—	82.3	82.3	79.0	55.4	55.7	56.7	53.4
AtSAC3	27.1	53.8	62.6	—	93.9	75.2	53.7	53.9	57.0	54.2
AtSAC4	27.1	53.0	62.1	82.3	—	75.7	54.2	45.0	55.2	54.7
AtSAC5	28.1	47.2	53.8	52.6	51.1	—	58.1	52.1	56.5	57.6
AtSAC6	34.3	26.9	30.0	28.8	28.0	29.0	—	97.3	80.3	51.4
AtSAC7	35.2	26.0	29.6	27.5	27.3	26.1	89.7	—	80.9	51.4
AtSAC8	34.6	25.9	26.9	26.4	25.9	27.9	49.1	50.6	—	48.6
AtSAC9	22.4	24.4	25.1	24.2	25.7	28.5	19.8	20.6	23.7	—

[a] The sequences from the beginning of motif I to the end of motif VII of the SAC domains were used for comparison. Values in the lower left portion represent identity, and values in the upper right portion represent similarity.

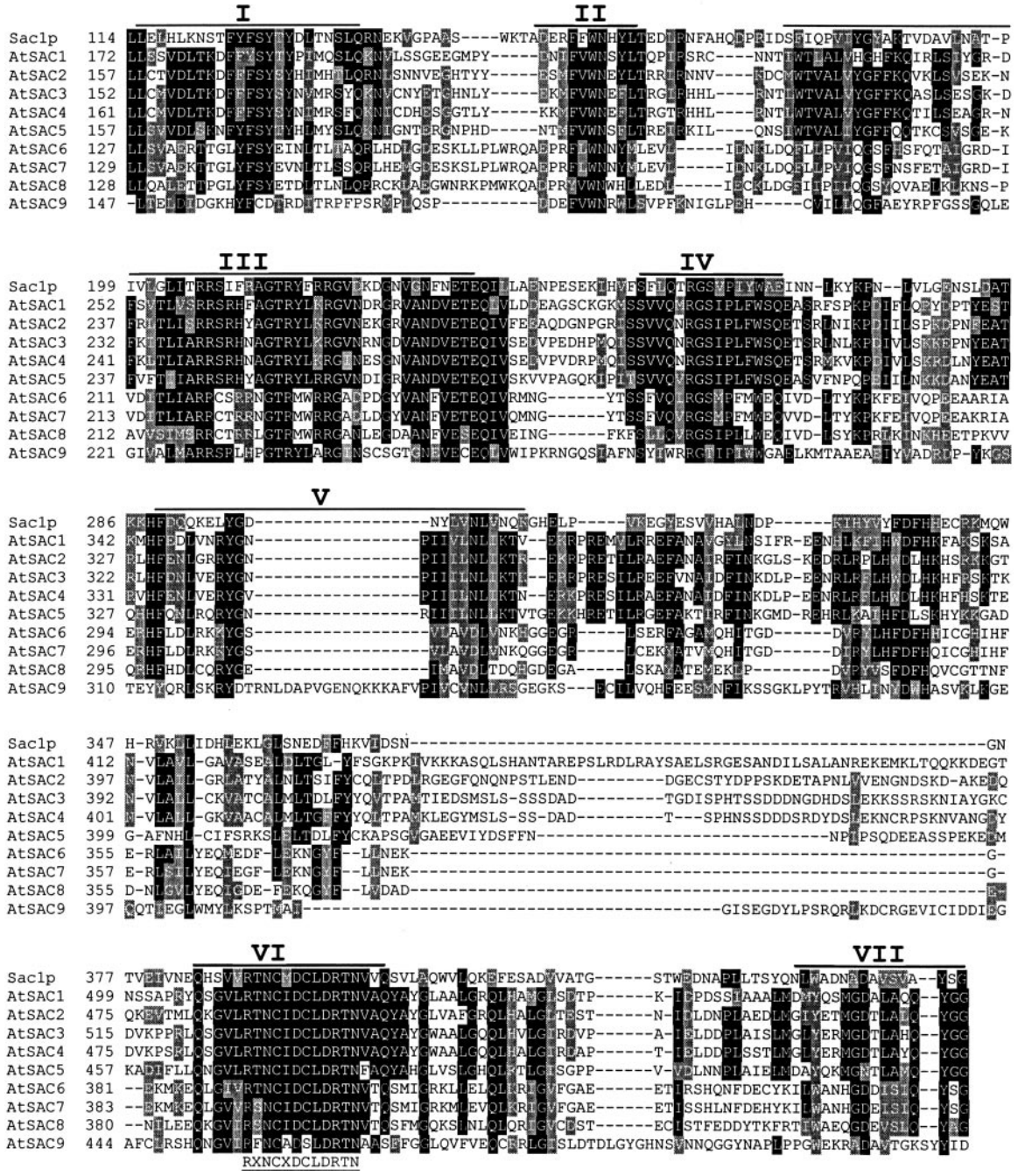

Figure 2. Sequence alignment of the SAC domains of Sac1p and AtSACs. The amino acid sequences from the beginning of motif I to the end of motif VII in the SAC domains were used for alignment with the ClustalW 1.8 program. The numbers shown at the left of the individual sequences are the positions of amino acid residues in the corresponding proteins. Gaps (marked with dashes) were introduced to maximize the sequence alignment. Identical and similar amino acid residues are shaded with black and gray, respectively. The seven conserved motifs defined for the SAC domains of yeast and animal proteins (Hughes et al., 2000) were indicated with the solid lines above the sequences. The putative catalytic core sequence of the SAC domain phosphatase is located in motif VI, and its consensus is shown below the sequences.

The AtSAC Protein Family Can Be Divided into Three Subgroups

Sequence analyses suggested that the AtSAC proteins fall into three subgroups. Subgroup I consists of AtSAC1 to AtSAC5, subgroup II consists of AtSAC6 to AtSAC8, and subgroup III is composed of AtSAC9. The SAC domains share 20% to 30% sequence identity between the subgroups (Table II), but higher sequence identity is seen within the individual subgroups. SAC domains within subgroup I share 50%

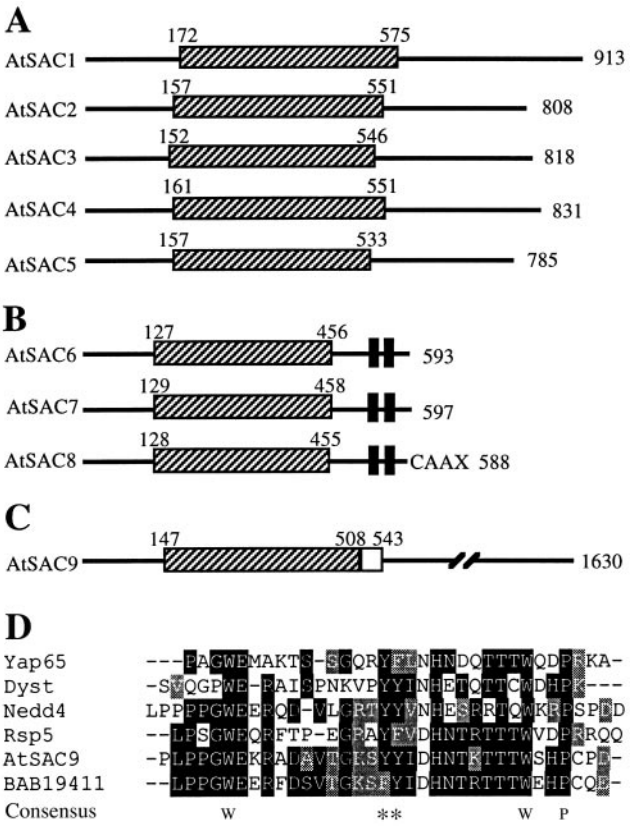

Figure 3. Diagrams of the domain organization of AtSACs. The amino acid length of the deduced AtSAC proteins is shown at the right ends of individual proteins. The hatched boxes represent the SAC domains starting from the beginning of motif I to the end of motif VII. The numbers above the boxes denote the amino acid positions at the beginning and the end of the SAC domains. A, Subgroup I proteins AtSAC1 to AtSAC5 have C-terminal sequences without any recognizable domains. B, Subgroup II proteins AtSAC6 to AtSAC8 have C-terminal sequences that contain two putative transmembrane helices (black bars). The C terminus of AtSAC8 has a putative CAAX motif that is known to be a prenylation site. C, Subgroup III protein AtSAC9 contains a long stretch of C-terminal sequence with 1,122 amino acids. A putative WW domain (amino acids 508–543, marked with a white box) is located right after the SAC domain. D, Sequence alignment of the putative WW domains of AtSAC9 (amino acids 509–543) and rice (*Oryza sativa*) SAC protein BAB194111 (amino acids 343–376) with the WW domains of mouse (*Mus musculus*) YAP65, human dystrophin (Dyst), mouse Nedd4, and yeast Rep5. Identical and similar amino acids are shaded in black and gray, respectively. The consensus for the WW domains is shown below the sequences. The two asterisks denote the essential aromatic doublet.

to 82% sequence identity, and those within subgroup II share 49% to 90% sequence identity (Table II).

Division of the AtSAC protein family into three subgroups was supported by the features of amino acid sequences outside of the SAC domains. First, the length of the C-terminal regions after the SAC domains clearly divided the AtSAC proteins into three subgroups. Members of subgroup I, AtSAC1 to AtSAC5, contain C-terminal sequences with a range of 252 to 338 amino acid residues (Fig. 3A). Members of subgroup II, AtSAC6 to AtSAC8, have C-terminal sequences consisting of 133 to 137 amino acid residues (Fig. 3B). The only member of subgroup III, AtSAC9, differs from the other AtSAC proteins by having a long stretch of C-terminal sequence consisting of approximately 1,100 residues (Fig. 3C).

Second, the hydropathy profiles of members in the same subgroups revealed similar hydrophobic and hydrophilic patterns within the SAC domains, but the patterns were different between the subgroups (Fig. 4, A–C). No potential transmembrane helices were evident in any members of subgroups I and III (Fig. 4, A and C). However, the C-terminal regions of members in subgroup II contain two putative transmembrane helices (Fig. 4B). In addition, sequence analysis using the PROSITE database of protein families and domains (http://us.expasy.org/prosite/) identified a putative prenylation site, i.e. a typical CAAX motif, at the C-terminal end of AtSAC8 (Fig. 3B). It has been shown that the CAAX motif is a signal for the addition of a farnesyl or a geranylgeranyl group to the Cys residue and that lipid modification of some proteins has been demonstrated to be responsible for their membrane association (Zhang and Casey, 1996). The possible biological significance of the putative CAAX motif in AtSAC8 remains to be studied.

The relatedness among the AtSAC proteins was further supported by phylogenetic analysis of the SAC domain sequences indicating the divergence of the three subgroups of AtSAC proteins (Fig. 4D). These results suggest that the *AtSAC* gene family evolved and diverged into three distinct subgroups. This was also reflected in their exon-intron organization (Fig. 1) in which members in the same subgroup have the same exon-intron arrangement. As shown in the phylogenetic analysis (Fig. 4D), within subgroup I, AtSAC3 and AtSAC4 had the highest relatedness, and within subgroup II, AtSAC6 and AtSAC7 exhibited the highest relatedness. This relatedness was reflected in their sequence identities throughout their whole length at both the protein level and the cDNA level. AtSAC3 and AtSAC4 share 76% amino acid and 80% nucleotide sequence identity throughout their entire length. AtSAC6 and AtSAC7 share 85% amino acid and 85% nucleotide sequence identity. It was interesting to note that subgroup I AtSACs were more closely related to Fig4p and hSAC3 than to other subgroups of AtSACs, and that subgroup II AtSACs were more closely related to Sac1p and synaptojanins (Fig. 4D).

To determine whether the *AtSAC* gene family was the result of genome duplication events, we analyzed their chromosomal locations. The *AtSAC* genes are distributed on different regions of chromosomes I, III, and V (Table I). *AtSAC1* and *AtSAC5* are located on the upper arm of chromosome I. They appeared not to be tightly linked. *AtSAC4* and *AtSAC6* were found on the upper and lower arms of chromosome

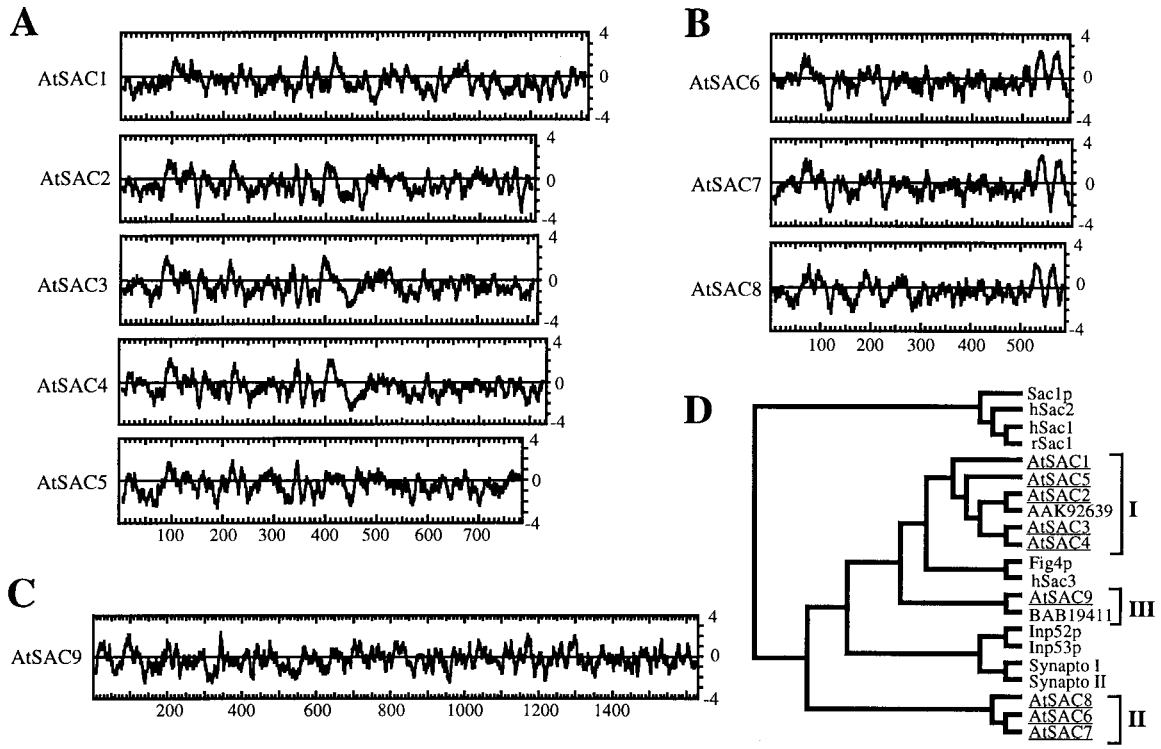

Figure 4. Hydropathy profiles of AtSACs and phylogenetic analysis of the SAC proteins. The amino acid sequences of the deduced AtSAC proteins were analyzed for their hydropathy profiles with the Kyte-Doolittle method using the DNA Strider program. The positive numbers indicate hydrophobicity, and the negative numbers denote hydrophilicity. A, Subgroup I AtSAC proteins exhibit a similar hydrophobic and hydrophilic pattern within the SAC domains and do not have any potential transmembrane helices. B, Subgroup II AtSAC proteins show a similar hydrophobic and hydrophilic pattern and contain two potential transmembrane helices in their C-terminal regions. C, Subgroup III AtSAC9 protein has a distinct hydropathy profile and does not show any potential transmembrane helices. D, Phylogenetic tree of AtSACs and other SAC proteins. The phylogenetic relationship of the SAC proteins was analyzed based on the amino acid sequences of the SAC domains. The SAC domain sequences are from Arabidopsis (AtSAC1 to AtSAC9), rice (AAK92639 and BAB19411), yeast (Sac1p, Fig4p, Inp52p, and Inp53p), human (hSac1, hSac2, hSac3, Synapto I, and Synapto II), and rat (rSac1).

V, respectively. *AtSAC2* is located on the upper arm of chromosome III. *AtSAC3*, *AtSAC7*, *AtSAC8*, and *AtSAC9* are distributed on the lower arm of chromosome III. *AtSAC7* and *AtSAC8* are tightly clustered with a spacing of only 123 kb. Analysis of the chromosomal locations of the *AtSAC* genes with the Genome and Redundancy Viewer at MIPS (http://mips.gsf.de/proj/thal/db/gv/gv_frame.html) revealed that *AtSAC6* and *AtSAC7* reside in a small duplication block in the lower arms of chromosomes V and III, indicating that they evolved through genome duplication. Other *AtSAC* genes did not appear to be located in any chromosomal duplication segments. However, it is reasonable to suggest that *AtSAC3* and *AtSAC4* might also have arisen from gene duplication because their proteins exhibit 76% amino acid sequence identity.

Presence of SAC Domain-Containing Protein Genes in Other Plant Species

A search of the GenBank database showed that in addition to Arabidopsis, several other plant species contain genes encoding proteins with high sequence similarity to the AtSAC proteins. Noticeably, the rice genome has two SAC-like genes, one of which encodes a protein (AAK92639) with similar amino acid length and high sequence similarity to members in the AtSAC subgroup I (Fig. 4D), which contains all seven conserved motifs in its SAC domain (data not shown). The other rice SAC-like gene encodes a protein (BAB19411) with similar amino acid length and sequence similarity to the subgroup III member AtSAC9 (Fig. 4D). The AAK92639 protein has the highest sequence identity with AtSAC2, sharing 55% identity and 75% similarity throughout their sequence. The BAB19411 protein and AtSAC9 share 51% identity and 64% similarity throughout their sequence. Like AtSAC9, the BAB19411 protein also contains a putative WW domain right after motif VI of the SAC domain (Fig. 3D).

A search of the EST sequences in the GenBank database revealed that the deduced amino acid sequences of three ESTs from maize (*Zea mays*; accession nos. AY111870, AY109595, and AY104833) have

high sequence similarities (48%–65%) with the subgroup I AtSACs. However, these sequences are not full-length cDNAs, and they contain many uncertain nucleotides; therefore, they were not incorporated into the phylogenetic analysis. It is important to note that the deduced partial amino acid sequences of all three maize ESTs revealed the presence of motifs VI and VII of the SAC domain, and one of them also contained motif V (data not shown). This analysis indicates that their corresponding genes most likely encode proteins with a complete SAC domain. These results suggest that the SAC domain-containing proteins may be present in diverse plant species and likely play important roles in plant cellular processes.

Expression Patterns of the *AtSAC* Genes in Different Organs

To examine the expression patterns of the *AtSAC* genes in plant organs, we performed semiquantitative reverse transcription (RT)-PCR analysis with different tissues, including young seedlings, flowers, leaves, roots, and stems of different ages. This analysis showed that the *AtSAC6* gene was predominantly expressed in flowers with little expression in other organs (Fig. 5). Although the other *AtSAC* genes were expressed in all organs examined, they exhibited differential expression patterns (Fig. 5). For example, several *AtSAC* genes including, *AtSAC2-5* and *AtSAC8*, showed a relatively higher level of expression in young seedlings than in other tissues. The *AtSAC1* gene exhibited a relatively higher expression level in both young elongating and nonelongating stems. All *AtSAC* genes had a relatively lower level of expression in mature leaves. They also exhibited a lower expression level in roots from 8-week-old plants with the exception of the *AtSAC1* and *AtSAC9* genes. The control ubiquitin gene was expressed at similar levels in different organs (Fig. 5). These results indicate that several *AtSAC* genes are differentially expressed and may play dominant roles in particular organs.

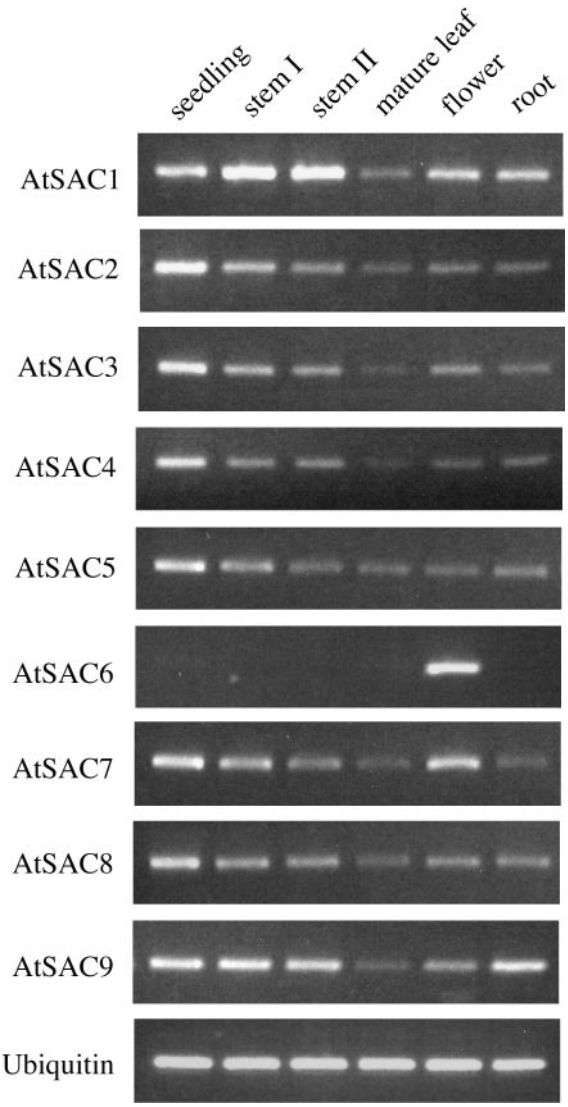

Figure 5. Expression of the *AtSAC* genes in Arabidopsis organs. Gene-specific primers were used to detect the expression levels of *AtSACs* in different organs using semiquantitative RT-PCR. The expression level of a ubiquitin gene was used as an internal control. The seedlings used were 2 weeks old. Mature leaves were from 6-week-old plants. Flowers and mature roots were from 8-week-old plants. Stems I and II were from 4- and 8-week-old plants, respectively.

Expression of the *AtSAC* Genes in Response to Stress Treatments

It has been demonstrated that the metabolism of phosphoinositides in plants is regulated by hormones and various stress treatments (Mikami et al., 1998; Meijer et al., 1999, 2001; Pical et al., 1999; DeWald et al., 2001). To understand whether some of the *AtSAC* genes may be involved in hormonal or stress response, we investigated their expression levels in response to hormone and stress treatments. Two-week-old seedlings were treated with plant hormones including auxin, cytokinin, GA, and abscisic acid, or incubated under various conditions including dark, cold, salt, and wounding. The expression levels of the *AtSAC* genes in these treated samples were examined by semiquantitative RT-PCR. The expression of the *AtSAC* genes was not noticeably altered by any hormonal treatment (data not shown). The expression of the *AtSAC6* gene was highly induced by salt treatment (Fig. 6), indicating that it may play a role in salt stress response. Several other *AtSAC* genes exhibited minor changes in their expression under certain treatments (Fig. 6). The expression of the control ubiquitin gene did not show any detectable changes under these treatments.

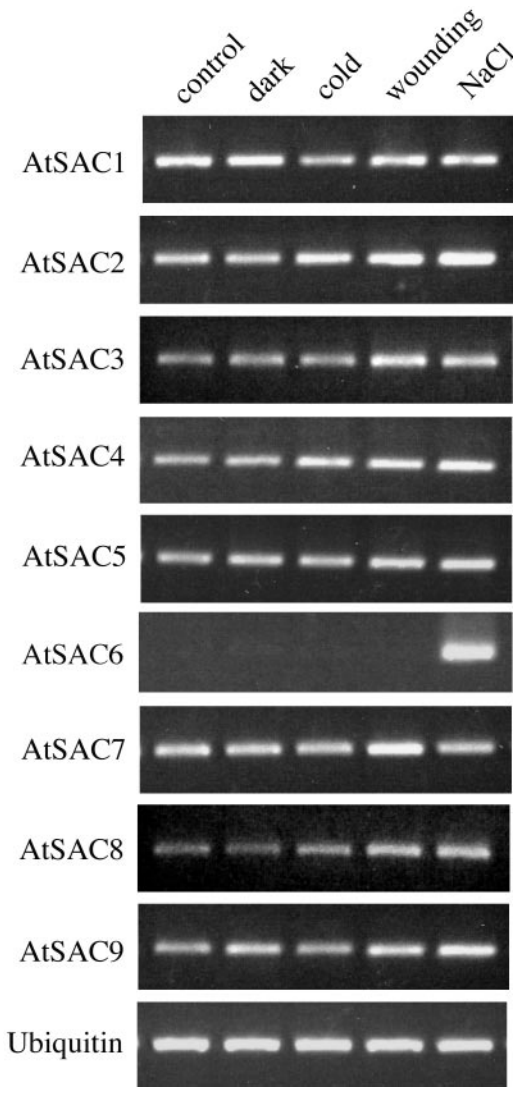

Figure 6. Expression of the *AtSAC* genes in response to dark, cold, wounding, and salt treatment. Two-week-old seedlings were incubated in the dark, cold (4°C), or treated with salt (250 mM NaCl) for 5 h before being harvested for RNA isolation. For the wounding treatment, seedlings were cut into small pieces and incubated in Murashige and Skoog medium for 5 h. The expression levels of the *AtSAC* genes were determined using semiquantitative RT-PCR. The expression of a ubiquitin gene was used as an internal control.

DISCUSSION

The Arabidopsis Genome Contains a Family of SAC Genes

The Arabidopsis genome appears to have more members of the SAC gene family than yeast or human. Although nine SAC genes were identified in Arabidopsis, five are present in yeast (Hughes et al., 2000a) and five in human (Minagawa et al., 2001). Interestingly, whereas both the yeast and human genomes contain genes encoding proteins belonging to the two classes of SAC domain-containing proteins, the Arabidopsis genome appears not to have any genes encoding the synaptojanin-like proteins. Currently, it is not known whether AtSAC proteins possess any phosphoinositide phosphatase activities. Because the SAC domains of the AtSACs (except AtSAC9) contain all seven conserved motifs believed to be important for the phosphatase activities of yeast and animal SAC proteins, it is conceivable that the SAC domains of the AtSACs may function as phosphoinositide phosphatases. Definite proof of such an activity awaits the biochemical and functional characterization of the AtSAC proteins.

The SAC domains of several proteins from yeast and human have been demonstrated to exhibit different specificities toward different phosphoinositides. For example, the SAC domains of synaptojanins, Sac1p and rSac1, hydrolyzed PI(3)P, PI(4)P, and PI(3,5)P_2 in vitro (Guo et al., 1999; Hughes et al., 2000b; Nemoto et al., 2000), whereas hSac2 possessed a 5-phosphatase activity toward PI(4,5)P_2 and PI(3,4,5)P_3 (Minagawa et al., 2001). In plant cells, six forms of phosphoinositides have been detected (Braun et al., 1999; Meijer et al., 1999, 2001; Xue et al., 1999; DeWald et al., 2001; Kim et al., 2001; Westergren et al., 2001; Müller-Röber and Pical, 2002). The fact that the Arabidopsis genome contains nine SAC genes belonging to three subgroups suggests that different AtSACs might possess different substrate specificities, and, therefore, they may regulate the metabolism of different phosphoinositides in the phosphoinositide pool, which in turn influences diverse cellular processes.

It is also possible that different members of the AtSAC protein family may play roles in different subcellular compartments. It has been shown that Sac1p and rSac1 contain two putative transmembrane helices in their C-terminal regions and that these transmembrane helices are essential for their proper subcellular locations (Whitters et al., 1993; Nemoto et al., 2000; Foti et al., 2001). Both of these proteins are integral membrane proteins localized in the endoplasmic reticulum, and deletion of the transmembrane helices in Sac1p rendered a loss of its cellular functions inside the cells (Foti et al., 2001). Although the subcellular locations of the AtSAC proteins are currently unknown, hydropathy analysis revealed that subgroup II AtSACs contain two putative transmembrane helices (Fig. 4B). The presence of these transmembrane helices suggests that these proteins may be integral membrane proteins and, as in Sac1p, the location of these proteins in the membranes may be essential for their cellular functions. In contrast, members of subgroups I and III do not contain any potential transmembrane helices, suggesting that their subcellular locations might be different from those of subgroup II proteins. It is noted that the C terminus of AtSAC8 contains a putative CAAX motif. The CAAX motif has been shown to be a signal for prenylation of the Cys residue (Zhang and Casey, 1996), which is responsible for the mem-

brane association of several phosphatases including the human type I inositol-1,4,5-triphosphate 5-phosphatase (Smedt et al., 1996) and the human type II inositol polyphosphate 5-phosphatase (Matzaris et al., 1998). It will be important to determine the subcellular locations of the AtSAC proteins to elucidate their biological functions.

The functional significance of the C-terminal regions of AtSAC subgroups I and III is not clear. They may be important for proper subcellular localization of these proteins, or they may be involved in protein-protein interactions, which could be crucial for the biological functions of AtSACs. It is interesting to note that AtSAC9 has a long C-terminal region with about 1,100 amino acid residues, and the rice genome also contains an AtSAC9 homolog with nearly identical numbers of residues. The C-terminal regions of AtSAC9 and the rice AtSAC9 homolog contain a putative WW domain that is known to be involved in protein-protein interactions (Ilsley et al., 2002). To fully understand the functions of AtSAC proteins, it will be important to dissect the functions of the C-terminal regions in addition to the activities of the SAC domains.

The *AtSAC* Genes Exhibit Differential Expression Patterns

Gene expression analysis suggests that different AtSACs may play specific roles in particular organs or tissues. It is apparent that the *AtSAC6* gene is predominantly expressed in flowers, suggesting that the AtSAC6 protein may play a role mainly in flowers. Although other *AtSAC* genes showed overlapping expression profiles, they exhibited a differential expression pattern among different organs. Further investigation on the roles of individual AtSAC proteins in different organs and tissues is critical to our understanding of the functions of AtSAC proteins in plant growth and development. Putative T-DNA insertion lines for several *AtSAC* genes are available from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) stock center, and they will be valuable tools for the functional study of AtSAC proteins.

It is intriguing to discover that the *AtSAC6* gene was highly induced in response to salt treatment, suggesting that it may be involved in salt stress response. It has been reported that salt or hyperosmotic stress alters the levels of phosphoinositides in plant cells (Meijer et al., 1999, 2001; Pical et al., 1999; DeWald et al., 2001). In addition, mutation of an inositol polyphosphate 1-phophatase caused a defect in tolerance to various stresses including salinity (Xiong et al., 2001). It will be interesting to investigate the functional roles of AtSAC6 in response to salt stress.

MATERIALS AND METHODS

Sequence Analysis

The SAC domain-containing protein genes in Arabidopsis were identified by searching public databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and The Arabidopsis Information Resource (http://www.Arabidopsis.org/Blast/). The SAC domain sequences were aligned using the ClustalW 1.8 program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The ALIGN program (http://www2.igh.cnrs.fr/bin/align-guess.cgi) was used to perform pair-wise comparisons to determine the sequence identity and similarity between the SAC domains. The hydropathy profiles of the amino acid sequences of the AtSAC proteins were generated with the Kyte-Doolittle method using the DNA Strider program. For analysis of the phylogenetic relationship of the SAC domain-containing proteins, the SAC domain sequences from various proteins were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/), and the resulting alignment parameters were used to generate the phylogenetic tree using TREEVIEW (Page, 1996).

cDNA Isolation and Sequencing

The full-length *AtSAC* cDNAs were PCR amplified from an Arabidopsis cDNA library constructed with stem mRNA (Zhong and Ye, 1999) or from first strand cDNA made from flower mRNA for the *AtSAC6* cDNA. The cDNAs were sequenced using a population of the PCR products, and their sequences were confirmed by comparison with their corresponding genomic sequences. The DNA sequencing was performed using a dye-based cycle sequencing kit (Applied Biosystems, Foster City, CA).

Plant Growth Conditions

Arabidopsis plants (ecotype Columbia) were grown in a greenhouse. Two-week-old seedlings, leaves from 6-week-old plants, roots and flowers from 8-week-old plants, and stems from 4- or 6-week-old plants were collected for total RNA isolation.

Two-week-old Arabidopsis seedlings grown in tissue culture on Murashige and Skoog medium were used for hormonal and stress treatments. All of the treatments were done for 5 h. The seedlings were immersed in Murashige and Skoog medium (control), Murashige and Skoog medium with 50 μM hormones (naphthylacetic acid, GA, benzyladenine, or abscisic acid) or 250 mM NaCl (Hsieh and Goodman, 2002). For the cold treatment, the seedlings in the Murashige and Skoog medium were incubated at 4°C. For the wounding treatment, the seedlings were cut into small pieces with a razor blade and incubated in the Murashige and Skoog medium. For the dark treatment, the seedlings in the Murashige and Skoog medium were placed in the darkness.

Gene Expression Analysis

Total RNA was isolated from various tissues using a Qiagen RNA isolation kit (Qiagen, Valencia, CA). One microgram of the purified RNA was first treated with DNase I to remove any potential genomic DNA contamination and then used for first strand cDNA synthesis by RT. One-twelfth of the synthesized first strand cDNA was used for PCR amplification of the *AtSAC* cDNAs with gene-specific primers. The primers used span three or more introns, and the size of the PCR products using cDNA or genomic DNA as templates could be distinguished clearly with the differences ranging from 400 to 1,400 bp. No genomic DNA was amplified in the RT-PCR reactions. Therefore, the RT-PCR results represented mRNA levels. The gene-specific primers were designed based on unique sequences in the *AtSAC* cDNAs, and their sequences are as follows: AtSAC1, 5′-CACGACACAGTCTAGAGAGTTTCT-3′ and 5′-GAACACATTCACAAACATGCAACC-3′; AtSAC2, 5′-ATAGTAAGGATGCTAAGGAAGATC-3′ and 5′-CTTTCTACTGTTGGAACCAACTGT-3′; AtSAC3, 5′-TAGAGAGTCAATCCTACGAGAAGA-3′ and 5′-ATATGGCTCACATGCTGTAATGGT-3′; AtSAC4, 5′-CAGGAGACCTCACGGATGAAAGTT-3′ and 5′-TTGCAACATTCTTACTTGGGCGGC-3′; AtSAC5, 5′-AAGGCAATCCATTTTGATCTAAGC-3′ and 5′-AGAGGAACTGCCTTGAAACATCTG-3′; AtSAC6, 5′-ATGGTGAGTAGGCTCAAGATACAC-3′ and 5′-ACGAGTGCCGCGACAGCTACACTG-3′; AtSAC7, 5′-GCGCTTATGCGAGAAGTATGCTAC-3′ and 5′-ACCAGTGCAGCCATGCCCA-

CGCAA-3′; AtSAC8, 5′-AATCGTAAGCCTATGTGGAAGCAG-3′ and 5′-GTTGTGATCCAATGGGCTGGAGTT-3′; and AtSAC9, 5′-AGTGTGATTCTACTCGTTAGTCCA-3′ and 5′-TCAGACACTTGAAAGGCTAGTCCA-3′. The PCR was performed for variable cycles to determine the logarithmic phase of amplifications for all of the samples. It was concluded that 27 cycles of amplification for all of the samples falls into the logarithmic phase. Therefore, 27 cycles of PCRs were used to examine the expression of all *AtSAC* genes except the *AtSAC6* gene for which 35 cycles were used in an attempt to detect its expression in other tissues besides flowers. To reveal the relative difference in the levels of gene expression under stress treatments, one or two fewer cycles were used in the RT-PCR reactions. The RT-PCR reactions were repeated three times, and identical results were obtained. The expression of a ubiquitin gene was used as an internal control for determining the RT-PCR amplification efficiency among different samples.

GenBank Accession Numbers

The GenBank accession numbers for the sequences described in this article are BAB19411 and AAK92639 (rice [*Oryza sativa*] SAC proteins), NP_014074 (Fig4P), X51672 (*Sac*Ip), NM_053798 (rSac1), NM_014016 (hSac1), NM_014937 (hSac2), NM_014845 (hSac3), NP_014293 (Inp52), NP_014752 (Inp53), NM_003895 (Synapto I), NM_003898 (Synapto II), NM_009534 (Yap65), NP_011051.1 (Rsp5), NP_004014.1 (Dyst), and I83196 (Nedd4).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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