The *Arabidopsis* SUPPRESSOR OF AUXIN RESISTANCE Proteins Are Nucleoporins with an Important Role in Hormone Signaling and Development

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Nucleocytoplasmic transport of macromolecules is regulated by a large multisubunit complex called the nuclear pore complex (NPC). Although this complex is well characterized in animals and fungi, there is relatively little information on the NPC in plants. The suppressor of auxin resistance1 (sar1) and sar3 mutants were identified as suppressors of the auxin-resistant1 (axr1) mutant. Molecular characterization of these genes reveals that they encode proteins with similarity to vertebrate nucleoporins, subunits of the NPC. Furthermore, a SAR3–green fluorescent protein fusion protein localizes to the nuclear membrane, indicating that SAR1 and SAR3 are *Arabidopsis thaliana* nucleoporins. Plants deficient in either protein exhibit pleiotropic growth defects that are further accentuated in sar1 sar3 double mutants. Both sar1 and sar3 mutations affect the localization of the transcriptional repressor AXR3/INDOLE ACETIC ACID17, providing a likely explanation for suppression of the phenotype conferred by axr1. In addition, sar1 sar3 plants accumulate polyadenylated RNA within the nucleus, indicating that SAR1 and SAR3 are required for mRNA export. Our results demonstrate the important role of the plant NPC in hormone signaling and development.

INTRODUCTION

Nucleocytoplasmic transport is an essential process in eukaryotic organisms (Gorlich and Kutay, 1999; Weis, 2003). Protein and RNA molecules move between the nuclear and cytoplasmic compartments through pores that lie at invaginations of the nuclear membrane. The nuclear pore is composed of a set of membrane-bound anchor proteins and a protein complex that lies within the space occupied by the pore (Hetzer et al., 2005). This nuclear pore complex (NPC) is a large conglomerate composed of protein subcomplexes that are repeated in eightfold radial symmetry around a central channel (Vasu and Forbes, 2001). The NPC has been studied in some detail in both animals and yeast but is poorly characterized in plants. Many general features of the NPC, as well as the constituent protein complexes, are conserved among all eukaryotes that have been investigated (Bapteste et al., 2005). Recent studies have begun to define which protein subcomplexes are responsible for the movement of specific molecules into and out of the nucleus. One such complex, called the NUP107–120 complex in animals and the NUP84 complex in yeast, plays a central role in NPC function (Siniossoglou et al., 2000; Bai et al., 2004; Liodice et al., 2004).

Comprehensive analysis of the NUP107–120 complex has defined at least nine members (Lutzmann et al., 2002; Liodice et al., 2004). Its central importance is highlighted by the fact that when the complex is depleted from *Xenopus laevis* egg extracts, the reconstituted nuclei are devoid of nuclear pores (Harel et al., 2003; Walther et al., 2003). Furthermore, mutation of individual members of this complex in human cell lines and in yeast results in smaller nuclei that have severe defects in mRNA export (Siniossoglou et al., 1996; Vasu et al., 2001; Bai et al., 2004).

Transport of molecules through the NPC is mediated by karyopherin proteins. Individual members of this large protein family can facilitate nuclear import (importins) or export (exportins), and their activity requires interaction with the small GTPase Ran (Harel and Meier, 2005). The *Arabidopsis thaliana* proteome contains proteins similar to many of those involved in metazoan and yeast nucleocytoplasmic transport, including karyopherin proteins. The HASTY and PAUSED genes encode plant homologs of exportin5 and exportinT, respectively (Bollman et al., 2003; Hunter et al., 2003; Li and Chen, 2003). HASTY interacts with the small GTPase Ran (Harel and Forbes, 2004; Mossmannparast and Pemberton, 2004).

Although relatively few studies have been performed, the mechanism of nucleocytoplasmic transport appears to be conserved between plants and other eukaryotes (Merkle, 2003; Meier, 2005). The *Arabidopsis thaliana* proteome contains proteins similar to many of those involved in metazoan and yeast nucleocytoplasmic transport, including karyopherin proteins. The HASTY and PAUSED genes encode plant homologs of exportin5 and exportinT, respectively (Bollman et al., 2003; Hunter et al., 2003; Li and Chen, 2003). HASTY interacts with the small GTPase Ran in a yeast two-hybrid assay, localizes to the nuclear periphery, and is involved in nuclear processing of certain microRNAs (miRNAs) (Bollman et al., 2003; Park et al., 2005). Expression of PAUSED in *Saccharomyces cerevisiae* rescues a deficiency in exportinT, whereas paused mutant plants accumulate transfer RNAs in the nucleus (Hunter et al., 2003; Park et al., 2005). These results indicate that HASTY and PAUSED play a role in the nuclear export of RNA. Plants deficient for either of these proteins exhibit a pleiotropic phenotype that is characterized by...
defects in phase change (Telfer and Poethig, 1998; Hunter et al., 2003; Li and Chen, 2003). The precise explanation for this phenotype is not known, although it is presumably related to a defect in RNA processing and transport.

The LOS4 protein is a DEAD box RNA helicase that is localized to the nuclear rim (Gong et al., 2005). Accumulation of poly(A) RNA in the nuclei of los4 plants suggests that it is required for the movement of mRNA into the cytoplasm (Gong et al., 2005). Interestingly, like the hasty and paused mutants, los4 mutants exhibit early flowering.

The Arabidopsis MOS3 and MOS6 (for MODIFIER OF SNC1) genes were identified from a mutant screen for suppressors of gain-of-function snc1 (for suppressor of npr1-1, constitutive) plants (Palma et al., 2005; Zhang and Li, 2005). SNC1 is a disease resistance gene that when mutated in a predicted regulatory region leads to constitutive activation of the disease resistance response. MOS3 is a homolog of vertebrate nucleoporin NUP96, and consistent with its proposed role, MOS3–green fluorescent protein (GFP) localizes to the nuclear periphery (Zhang and Li, 2005). Human NUP96 and its yeast homolog NUP145C are members of the NUP107–120 subcomplex, suggesting that this large subcomplex is also present in plants. The MOS6 gene encodes the importin AtImp9 (Palma et al., 2005). Single mos3 and mos6 mutants exhibit increased susceptibility to certain pathogens (Palma et al., 2005; Zhang and Li, 2005). It is unclear at present how the NPC and the nuclear transport machinery specifically affect the disease resistance response. However mos3 and/or mos6 may alter the transport of disease-specific molecules or reduce activity in other functions of the putative plant NUP107–120 complex (Palma et al., 2005; Zhang and Li, 2005).

The Arabidopsis auxin-resistant1 (axr1) mutants were originally isolated in a screen for auxin-resistant seedlings (Lincoln et al., 1990). Subsequent analysis demonstrated that AXR1 is a subunit in the RUB-activating enzyme, the first enzyme in a pathway that conjugates the ubiquitin-related protein RUB to members of the cullin family (del Pozo and Estelle, 1999; del Pozo et al., 2002; Parry and Estelle, 2004). Cullin proteins are components of several distinct families of ubiquitin protein ligases (E3), including the well-characterized SCF complexes (Deshales, 1999). In general, E3 enzymes promote the attachment of ubiquitin to diverse proteins, typically resulting in their degradation by the proteasome (Moon et al., 2004). The axr1 mutants are auxin-resistant because the RUB conjugation pathway is required for the normal function of the E3 SCFTR1 and related SCFs (del Pozo et al., 2002). These SCFs promote the auxin-dependent degradation of a family of transcriptional repressors called the Aux/IAA proteins (Gray et al., 1999, 2001). This degradation is likely to occur within the nucleus, because both SCFTR1 and the Aux/IAA proteins are present in this compartment. In axr1 plants, the Aux/IAA accumulate, presumably in the nucleus, and repress auxin-regulated transcription, resulting in decreased auxin response.

To further understand the function of the AXR1 protein, we have screened for extragenic suppressors of the axr1 mutants. Previously, we described the characterization of a second-site suppressor of axr1 called suppressor of auxin resistance1 (sar1) (Cernac et al., 1997). Here, we report the identification of another suppressor named sar3 and the molecular characterization of both SAR1 and SAR3. Surprisingly, we find that SAR1 and SAR3 encode putative nucleoporins of the NUP107–120 subcomplex. Our results suggest that defects in this complex restore partial auxin sensitivity to axr1 mutants by affecting the translocation of the Aux/IAA proteins into the nucleus. Furthermore, the loss of both SAR1 and SAR3 results in a severe growth phenotype and the accumulation of poly(A)− RNA in the nucleus.

RESULTS

Identification of New Suppressors of the axr1 Mutant

The axr1 mutants are auxin-resistant and exhibit a pleiotropic phenotype consistent with an overall reduction in auxin response (Lincoln et al., 1990; Leyser et al., 1993). In a previous report, we described the isolation of the sar1 mutant (Cernac et al., 1997). This recessive mutation restores auxin response to the roots of axr1 seedlings. Subsequent analysis indicated that the sar1 mutation suppresses most aspects of the phenotype conferred by axr1. In addition, sar1 mutants have a novel phenotype that is independent of AXR1 (Cernac et al., 1997).

To identify additional axr1 suppressors, we performed a new screen, this time focusing on the axr1 hypocotyl phenotype. When grown at 29°C in the light, ecotype Columbia (Col-0) seedlings have an elongated hypocotyl compared with seedlings grown at a lower temperature (Gray et al., 1998). By contrast, axr1 seedlings do not exhibit this auxin-dependent response. We grew mutagenized axr1-3 seedlings at the higher temperature and identified 25 lines with taller hypocotyls. Genetic analysis revealed that two lines, isolated from independent pools of mutagenized seeds, carried recessive alleles of one locus, and we called these lines sar3-1 and sar3-2.

The sar3-1 Mutation Suppresses Most Aspects of the Phenotype Conferred by axr1 and Results in Early Flowering

Consistent with our mutant screen, the hypocotyls of sar3-1 axr1-3 plants are longer than those of axr1-3 plants when grown at 29°C (Figure 1A). Furthermore, sar3-1 partially suppresses other aspects of the phenotype conferred by axr1, including auxin-resistant root growth and a decrease in auxin-induced lateral root formation (Figure 1B). The roots of axr1 mutants are also resistant to methyl jasmonate (Tiryaki and Staswick, 2002), and sar3-1 partially restores this response (see Supplemental Figure 1 online).

In addition to defects in auxin growth responses, the axr1 mutants are deficient in auxin-regulated transcription, including expression of the Aux/IAA genes (Abel et al., 1995; Timpte et al., 1995). To determine whether sar3-1 affects auxin induction of the Aux/IAA genes, we measured IAA1 and IAA5 RNA levels in response to auxin. The data in Figure 1C show that sar3-1 partially restores auxin induction of both IAA1 and IAA5 in the axr1-3 mutant. Similar results were obtained with the sar1 mutant (Cernac et al., 1997).

We next investigated the affects of sar3 on development in both axr1 and AXR1 backgrounds (Figure 1D, Table 1). In general, sar3 and sar3 axr1 plants are very similar in appearance. The primary root is shorter than in the wild type and produces fewer lateral roots (Table 1). Most striking, both single and double
mutants flower significantly earlier than wild-type plants and are much smaller and less robust throughout their life cycle (Figure 1D, Table 1). Overall, the phenotype conferred by sar3 is very similar to that conferred by sar1 (Cernac et al., 1997). These findings suggest that the two genes have related functions in hormone response and development.

The sar1 and sar3 Mutations Partially Suppress the rce1 Mutation

The AXR1 protein is a subunit of the heterodimeric E1 enzyme that functions in the RUB conjugation pathway. The next step in the pathway is a RUB-conjugating enzyme (E2) called RCE1 (del Pozo and Estelle, 1999; Dharmasiri et al., 2003). Like axr1, mutations in rce1 lead to multiple auxin-related defects (Dharmasiri et al., 2003). To determine whether the SAR genes have a general effect on RUB conjugation, we generated sar1 rce1 and sar3 rce1 double mutants and characterized their phenotypes. Both sar1 and sar3 partially suppress auxin resistance conferred by rce1 (Figure 2A). The sar3 rce1 plants are more sensitive to auxin than are sar1 rce1 plants, indicating that sar3 suppresses this phenotype of rce1 better than sar1.

Mutant rce1 plants are shorter than wild-type plants (Dharmasiri et al., 2003), and both sar1-1 and sar3-1 partially suppress this defect (Figure 2B). In general, both the sar1 rce1 and sar3 rce1 mutants are similar to the sar1 and sar3 single mutants, with a

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reduction in floral bud size, a decrease in stem thickness, and a decrease in silique size (see Supplemental Figure 2 online). The only known substrates of the RUB modification pathway are the cullin proteins. In the *axr1* and *rce1* mutants, the level of RUB-modified CUL1 is reduced, resulting in a decrease in SCF function (del Pozo et al., 2002; Dharmasiri et al., 2003). To determine whether the *sar1* and *sar3* mutations directly affect the RUB conjugation pathway, we examined the level of RUB-CUL1 in *sar1-1* and *sar3-1* plants. As shown previously, the relative level of RUB-modified CUL1 is drastically decreased in the *rce1* mutant (Figure 2C) (Dharmasiri et al., 2003). Neither the *sar1-1* nor the *sar3-1* mutation increased the level of RUB-CUL1 in an *rce1* background (Figure 2C). Thus, suppression by *sar1* and *sar3* does not directly involve changes in the RUB conjugation pathway.

**SAR3 Is Related to Vertebrate NUP96**

To gain further insight into the function of SAR3, we cloned the gene using a map-based strategy. We crossed *sar3-1* (Col-0) with ecotype Landsberg *erecta* and recovered homozygous *sar3* plants from the resulting F2 population. After an analysis of 680 plants, the *sar3* mutation was mapped to a 140-kb region at the bottom of chromosome 1 on BAC F23A5. While this work was in progress, we learned that an early-flowering mutant called *precoz* (*pre*) had also been mapped to the same region (C. Alonso-Blanco, I. Ausin, L. Ruiz-Garcia, and J.M. Martinez-Zapater, unpublished data) and encoded the At1g10860 protein. Because both *sar3* and *pre* are early-flowering, we sequenced the At1g10860 gene from *sar3-1* and *sar3-2* plants. This analysis revealed the presence of a single base pair deletion at position 1103 within the predicted second exon of the *sar3-1* gene. The identical lesion was detected in *sar3-2*, even though these lines were isolated independently. This change introduces a premature stop codon 150 bp downstream from the site of the mutation (Figure 3A). To verify that this mutation is responsible for the phenotype conferred by *sar3-1*, we obtained a T-DNA insertion line (sar3-3) from the Salk Institute Genomic Laboratory T-DNA collection (SALK_109959) (Alonso et al., 2003). The T-DNA insertion is within intron 4 at position 3570 with respect to the ATG. Like *sar3-1*, *sar3-3* plants have a shorter root than wild-type plants and flower early. In addition, the aerial phenotype of *sar3-3* plants is indistinguishable from that of *sar3-1*, confirming that *At1g10860* is *SAR3* (Figure 1C). This gene was also identified in a screen for suppressors of the *snc1* mutation and designated *MOS3* (Zhang and Li, 2005).

To determine the pattern of *SAR3* gene expression, we performed RT-PCR using RNA from a variety of tissues. We found that *SAR3* is expressed in representative tissues throughout the plant (Figure 3B). We also determined the effects of the *sar3-1* and *sar3-3* mutations on the *SAR3* transcript. Figure 3B shows that the *sar3-1* mutant produces a full-length transcript, whereas in *sar3-3* we detected a partial transcript. Because both alleles may produce a truncated protein, it is not clear whether either mutation is a functional null.

As reported previously, *SAR3/MOS3* is a unique gene in *Arabidopsis* and encodes a protein with similarity to human NUP96 (Zhang and Li, 2005). However, unlike that previous study, our analysis indicated that the N terminus of the SAR3/MOS3 protein is also related to the C terminus of a different human nucleoporin, NUP98. This finding can be explained by the fact that in vertebrates, both NUP96 and NUP98 are produced by the

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*Figure 2. The *sar1* and *sar3* Mutations Suppress Auxin Resistance Exhibited by *rce1* Plants. (A) Root elongation of wild-type and mutant seedlings grown on 0.16 μM 2,4-D. The experiment was performed as described for Figure 1B. Student’s *t* test indicated that the values for *rce1* and *sar1 rce1* are significantly different (*P < 0.05*). Error bars represent SE (*n* = 10 or greater). *Ler*, Landsberg *erecta*. (B) Phenotypes of 5-week-old wild-type and mutant plants. Bar = 2 cm. (C) Immunoblot using anti-CUL1 antibody on total protein extracted from floral tissue. Arrows indicate RUB-modified (top) and unmodified (bottom) forms of CUL1. The bottom gel shows an unknown nonspecific band used as a loading control.*
posttranslational processing of a larger precursor protein called NUP196. The N-terminal 864 amino acids of NUP196 form NUP98, and the C-terminal 920 amino acids form NUP96 (Fontoura et al., 1999). We found that SAR3/MOS3 shares 22% identity and 40% similarity with the C-terminal 1130 amino acids of NUP196 (Figure 3C). At its N terminus, SAR3/MOS3 is 45% identical to NUP196, and for a stretch of 336 amino acids in the center of the protein, the identity is >30% (Figure 3C). SAR3 appears to encompass the entire NUP96 protein sequence as well as the C-terminal 196 amino acids of NUP98. The region of similarity includes the well-conserved site of autoproteolysis (Figure 3C). SAR3 also shares sequence similarity with NUP145 from Saccharomyces cerevisiae (see Supplemental Figure 3 online). This protein is homologous with human NUP196 and also undergoes processing to generate two distinct nucleoporins called NUP145N and NUP145C (Teixeira et al., 1997, 1999; Rosenblum and Blobel, 1999). Although NUP96 and NUP145C have a low level of similarity (Fontoura et al., 1999), several studies have demonstrated that these nucleoporins reside within equivalent protein complexes and are likely to have similar functions (Vasu et al., 2001; Lutzmann et al., 2002).
NUP96 localizes to the nuclear periphery in human cell lines (Enninga et al., 2003). To determine whether SAR3 is also localized to the nuclear periphery, we generated transgenic plants that express a SAR3-GFP translational fusion under the control of the cauliflower mosaic virus 3SS promoter. We isolated 25 independent transgenic lines in a Col-0 background and observed no obvious phenotypic changes in these plants (Figure 4A). We then crossed the 3SS:SAR3-GFP transgene into sar3-1 plants and identified homozygous sar3-1 plants carrying the transgene among the F2 progeny. These plants have a nearly wild-type phenotype, confirming that SAR3-GFP is functional (Figure 4B). The localization of SAR3-GFP was assessed by confocal microscopy. The results shown in Figure 4B demonstrate that SAR3-GFP was clearly localized to the nuclear periphery in cells of the root tip and the root elongation zone (Figure 4B). Considered together with its similarity to NUP96, this GFP localization strongly suggests that SAR3 is present within the NPC. Similar results have been obtained with lines expressing a MOS3-GFP transgene (Zhang and Li, 2005).

The SAR1 Gene Encodes Another Nucleoporin, NUP160

Previously, we had mapped SAR1 to chromosome 1 between markers nga280 and nga248 (Cernac et al., 1997). Further fine-mapping resolved this interval to a 180-kb region that includes At1g33410, predicted to encode a protein related to the vertebrate nucleoporin NUP160. Given the similarity between the phenotypes conferred by sar3 and sar1, this gene was a good candidate for SAR1. Sequence analysis of At1g33410 in sar1-1 plants revealed a point mutation at position 3403 that introduces a stop codon in the 11th exon of the gene (Figure 5A). Our previous study identified two additional sar1 alleles. The sar1-2 mutation has a phenotype similar to that of sar1-1, whereas sar1-3 has a weaker phenotype (Cernac et al., 1997). We sequenced At1g33410 in these lines and found that sar1-2 contains a mutation at the junction between intron 4 and exon 5, whereas sar1-3 has a mutation within intron 21 of the gene. We subsequently obtained a T-DNA allele with an insertion within At1g33410 (sar1-4) from the Salk collection (SALK_126801) (Alonso et al., 2003). We used PCR to identify a line homozygous for sar1-4 and subsequently verified by sequencing that the T-DNA insertion is within exon 17 of the gene at position 5652 with respect to the ATG. The sar1-4 mutant displays the same aerial phenotype as sar1-1 (Figure 5B), indicating that the defect in At1g33410 is responsible for the phenotype conferred by sar1.

Like SAR3, the SAR1 gene is expressed throughout the plant (Figure 5C). RT-PCR analysis shows that the sar1-1 allele produces normal levels of transcript. However, in sar1-4 plants, we could identify an RT-PCR product only using primers that are 5’ of the T-DNA insertion site (Figure 5C). Because it is possible that sar1-4 produces a truncated protein, it is not clear whether any of the sar1 alleles are nulls.

SAR1 is a large protein of 1500 amino acids that is not related to any other Arabidopsis protein. Along its entire length, SAR1 exhibits 14% identity and 31% similarity with the vertebrate nucleoporin NUP160. However, a C-terminal stretch of 400 amino acids in these two proteins shares 23% identity and 40% similarity (Figure 5D). These results, together with the similarity between the phenotypes conferred by sar3 and sar1, suggest that SAR1 is the Arabidopsis homolog of NUP160. A recent analysis of the NPC among various eukaryotes also proposed that At1g33410 is a homolog of human NUP160 (Bapteste et al., 2005).

Defects in Both SAR1 and SAR3 Result in a Severe Phenotype

To learn more about the genetic and functional relationships between the SAR1 and SAR3 proteins, we generated sar1 sar3 double mutant plants. Because we had difficulty generating a fertile double mutant line, we initially identified plants that were homozygous for sar1 and heterozygous for sar3. These plants were similar to sar1 or sar3 single mutants (Figure 6A). Among the progeny of these plants, we identified sar1 sar3 double mutants with a variety of significant growth defects. The phenotype of the double mutants was somewhat variable, but for the purpose of description, we divided them into two broad classes of approximately equal size. Class A plants lack pigment and die as seedlings. The most severely affected seedlings have two cotyledon-like structures but do not develop leaves or a root (Figure 6C). Other class A seedlings develop very small leaves.

![Figure 4](image-url)

**Figure 4.** SAR3-GFP Is Localized to the Nuclear Periphery.

(A) Rosettes of 25-d-old wild-type and sar3-1 plants with or without the 3SS:SAR3-GFP transgene. Bars = 2 cm.

(B) Confocal images of cells from the root tip (left) and the root elongation zone (right) from Col-0 3SS:SAR3-GFP. Green signal represents GFP expression, and cell walls are stained with propidium iodide.
but do not form a root and remain extremely small (Figure 6C). Class B plants develop a small root and misshapen leaves but remain small and undergo very early floral transition (Figure 6B). Typically, the primary inflorescence is similar to the wild type in appearance, but subsequent inflorescences are much shorter, giving the plants a short, bushy appearance (Figure 6D). These plants produce little or no seed.

An examination of sar1 sar3 flowers provides an explanation for the infertility of these plants. As shown in Figure 6E, the gynoecium is reduced in these flowers and the anthers are poorly developed, forming very few if any pollen grains. Furthermore, these plants have a severe defect in inflorescence meristem function. In wild-type plants, flowers develop at regular intervals along the stem with a spiral phyllotaxy (Figure 6F). By contrast, in sar1-1 and sar3-1 single mutants, 35 to 40% of the flowers are irregularly spaced, causing the siliques to form in groups (Figure 6G). In sar1 sar3 plants, the regular spacing of siliques is almost completely absent and ∼80% of the siliques are grouped together (Figure 6H). This is indicative of a severe defect in the timing of floral meristem initiation.

sar1 sar3 Plants Are Deficient in mRNA Export

In vertebrate and yeast cells, defects in the NUP107–120 complex result in the accumulation of mRNA within the nucleus (Fabre et al., 1994; Vasu et al., 2001). A similar defect was observed in the los4 mutant of Arabidopsis (Gong et al., 2005). To determine the effect of the sar mutants on mRNA localization, we performed in situ localization of poly(A) RNA (Fabre et al., 1994; Vasu et al., 2001; Boehmer et al., 2003; Gong et al., 2005). Small leaves (<5 mm) from wild-type and sar1 sar3 plants grown at 22°C were fixed, labeled with a fluorescein-labeled poly(T) probe, and examined by confocal microscopy. In Col-0 leaves, a faint fluorescent signal could be observed both in the elongated cells adjacent to the midvein and in the smaller cells between the midvein and the leaf margin (Figure 7). This is a similar result to that observed in leaves from the C24 Arabidopsis ecotype (Gong et al., 2005). In sar1 sar3 leaves, we observed a much stronger fluorescent signal in the nuclei of both cell types (Figure 7). These results indicate that mRNA accumulates to higher levels in the nuclei of sar1 sar3 cells than in wild-type cells.

Figure 5. SAR1 Is Similar to Vertebrate NUP160.

(A) Structure of the SAR1 gene. Boxes represent exons. Asterisks denote the positions of missense mutations in sar1-1, sar1-2, and sar1-3. The position of the T-DNA insertion in sar1-4 is represented by an inverted triangle. Lines A to C correspond to regions amplified from the cDNA by primers used in (C).

(B) Phenotypes of 5-week-old wild-type and sar1 plants. Bar = 10 cm.

(C) RT-PCR of SAR1 from a variety of tissue types and in sar1 mutant alleles using internal primers within the SAR1 and ACTIN2 genes. Tissues are as described for Figure 3B. Regions amplified by primers A to C are shown in (A).

(D) Alignment of amino acid sequence from SAR1 (residues 1028 to 1406) and human NUP160 (residues 962 to 1316). Black shading denotes identical residues.
Localization of the Aux/IAA Protein IAA17 Is Altered in sar1 and sar3 Mutants

The axr1 mutants are deficient in auxin response because reduced levels of RUB-CUL1 affect the function of SCFTIR1 and related SCF complexes. This defect results in the stabilization of the Aux/IAA proteins, repressors of auxin-regulated transcription (Dharmasiri and Estelle, 2004). To assess the effects of sar1-1 on Aux/IAA degradation, we introduced the HS:AXR3NT-GUS transgene into mutant plants. This construct has been used to examine

Figure 6. Loss of Both SAR1 and SAR3 Results in Severe Defects in Development.

(A) Phenotype of a 19-d-old plant homozygous for sar1-1 and heterozygous for sar3-1. Bar = 5 mm.
(B) Examples of class B sar1-1 sar3-1 mutants. These are the most robust double mutant plants. Bars = 2 mm.
(C) Examples of class A sar1-1 sar3-1 seedlings. Arrows in (i) and (ii) show positions of cotyledons, and asterisks in (ii) and (iii) show locations of abortive true leaves. Bars = 1 mm.
(D) Phenotype of a 45-d-old sar1-1 sar3-1 plant exhibiting a long primary inflorescence and numerous short secondary inflorescences.
(E) Representative flowers from sar1-1 and sar1-1 sar3-1 plants. Bars = 1 mm.
(F) Regular spacing of siliques in a wild-type plant.
(G) Examples of the irregular spacing of siliques that occurs in sar1 or sar3 single mutants (left) and sar1-1 sar3-1 double mutants (right).
(H) Proportion of total siliques that are irregularly spaced in sar1-1, sar3-1, and sar1-1 sar3-1 mutant plants. Measurement from wild-type plants is omitted, because these do not develop any irregularly spaced siliques. Error bars represent SE (n = 7 or greater).
the stability of the AXR3/IAA17 protein (Gray et al., 2001). Seedlings were stained for β-glucuronidase (GUS) expression either directly after a 2-h heat shock or 90 min after return to 22°C. The results shown in Figure 8A indicate that sar1-1 roots exhibit stronger GUS staining than wild-type roots directly after heat shock and after 90 min. To determine the effects of sar1-1 on the stability of AXR3NT-GUS, GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) assay at intervals after the seedlings were returned to 22°C (Figure 8B). Surprisingly, GUS activity levels in sar1-1 seedlings at time 0 were consistently lower than those in the wild type (experiment repeated three times), despite the fact that GUS staining was always stronger in the mutant. For the experiment shown, GUS activity at time 0 was 482.9 ± 69.8 and 101.1 ± 17.9 nM MU/µg protein for Col-0 and sar1-1, respectively. The reason for this discrepancy is unknown. In any case, when GUS activity was expressed relative to time 0, our results indicate that the fusion protein was more stable in sar1-1 than in wild-type plants (Figure 8B).

Because the Aux/IAA proteins are repressors of auxin response, we expected their stability to be lower in the suppressors rather than higher. However, a closer examination of the cellular localization of the AXR3NT-GUS protein may resolve this paradox. Consistent with its role as a transcriptional repressor, the Aux/IAA proteins are localized to the nucleus (Oeller and Theologis, 1995; Gray et al., 2001). In wild-type plants, nuclear localization of AXR3NT-GUS can be clearly observed in Figure 8C. However, in sar1 and sar3 mutants, GUS staining was observed throughout the cell, suggesting that SAR1 and SAR3 are required for either nuclear transport or retention of AXR3NT-GUS (Figures 8D and 8E). This staining pattern is dependent on a relatively short incubation of seedlings with the enzyme substrate. After a longer incubation, we observed a broader distribution of GUS staining in wild-type roots as well (Figure 8E). In axr1(HSAXR3NT-GUS) roots, the staining pattern was also nucleus-localized after a short incubation time (Figure 8G). However, in the sar3 axr1 double mutant, the pattern of GUS staining was similar to that observed in the single sar1 and sar3 mutants (Figure 8H). This finding indicates that the alteration in AXR3NT-GUS expression in sar1 and sar3 is independent of AXR1.

**DISCUSSION**

Despite the importance of nucleocytoplasmic transport for cellular function, almost nothing is known about this process in plants. In this report, we describe the identification and characterization of two putative subunits of the NPC. In animals and yeast, nucleocytoplasmic transport is mediated by the activity of the NPC, carrier proteins termed karyopherins, and the small GTPase Ran (Gorlich and Kutay, 1999). Many NPC subunits are conserved between animals and yeast, indicating that the
Figure 8. The sar1 and sar3 Mutations Affect the Localization of the AXR3NT:GUS Protein.

(A) Seven-day-old HS:AXR3NT:GUS seedlings in a wild-type or sar1 background were heat-shocked at 37°C for 2 h and then placed at 22°C for 90 min. Samples from time 0 or 90 min (90°) were stained with GUS overnight.

(B) Seedlings were treated as described for (A). Protein extracted from seedlings at each time point was tested in a MUG fluorescence assay. The amount of fluorescence produced in each sample was expressed as a percentage of the fluorescence at the end of the heat-shock period. Values reported are means of two determinations. Similar results were obtained in a second, independent experiment.

(C) to (H) HS:AXR3NT:GUS seedlings in a wild-type or mutant background were heat-shocked at 37°C for 2 h and then stained with GUS solution for 45 min ([G] to [E], [G], and [H]) or 2 h ([F]). Images show GUS expression in the root tip region, and arrows indicate the positions of nuclei.
function and overall structure of the NPC are likely to be similar in
distantly related eukaryotes (Vasu and Forbes, 2001; Bapteste
et al., 2005). The animal NUP107–120 complex comprises at least
nine subunits and has been shown to be functionally equivalent to
the yeast NUP84 complex (Cronshaw et al., 2002; Lutzmann et al.,
2002; Harel et al., 2003; Bai et al., 2004; Loiodice et al., 2004;
Bapteste et al., 2005). Our own analysis reveals that most sub-
units of the NUP107–120 complex have identifiable homologs in the
Arabidopsis proteome. These include NUP160, NUP133,
NUP107, NUP96, NUP85, NUP43, and two proteins similar to
Sec13 (G. Parry and M. Estelle, unpublished data). The presence of
many putative NUP107–120 proteins in Arabidopsis strongly suggests
that the complex exists in a similar form in plants.

We have shown that the SAR1 and SAR3 proteins are related to
the human nucleoporins NUP160 and NUP96, respectively.
We have demonstrated that SAR1 and SAR3/MOS3 are broadly
expressed and confirm that SAR3/MOS3 localizes to the nuclear
periphery (Zhang and Li, 2005). The sar1 and sar3 mutants have a
similar pleiotropic phenotype with diverse growth defects, con-
firming that the NPC is important for many aspects of plant
development. These results also suggest that SAR1 and SAR3/
MOS3 have related functions in the NPC. Furthermore, sar1 sar3
double mutants exhibit a much more severe phenotype than
either single mutant, suggesting that the loss of both subunits
results in a further decrease in NPC function. Because SAR1 and
SAR3/MOS3 may be part of the NUP107–120 subcomplex, this
phenotype may be caused by a severe defect in the assembly
and/or function of this subcomplex.

The human and yeast homologs of SAR3/MOS3, NUP96 and
NUP145C, are present within equivalent NUP subcomplexes
(Lutzmann et al., 2002; Harel et al., 2003; Walther et al., 2003; Bai
et al., 2004). Most of the investigations regarding the role of
vertebrate NUP96 and yeast NUP145C have focused on the
proteolytic event that releases NUP98 and NUP69 from the
NUP196 polyprotein (or NUP145N and NUP145C from NUP145
in S. cerevisiae). Processing of the polyprotein is achieved by an
autoproteolytic event that is conserved from yeast to vertebrates
(Teixeira et al., 1997, 1999; Fontoura et al., 1999; Rosenblum and
Blobel, 1999; Hodel et al., 2002). Failure to process the
cellular proteins results in severe defects, because the polyprotein is not
localized correctly to the nuclear rim (Fontoura et al., 1999).
SAR3/MOS3 is translated as a single polypeptide that is similar
to vertebrate NUP96. However, examination of the N-terminal
region of SAR3/MOS3 reveals that it has similarity to the
C-terminal region of vertebrate NUP98. This region contains
the peptide motif that is necessary for the autoproteolytic cleav-
age of NUP196. Interestingly, the Arabidopsis genome encodes
two proteins (At1g59660 and At1g10390) that are related to the
other product of autoproteolysis, NUP98. These proteins contain
the functionally important FG repeats in their N-terminal regions
but also contain the proteolytic motif at their C termini. Thus, in
Arabidopsis, NUP96 and NUP98 are synthesized as distinct
peptides that retain the proteolytic motif at their N and C termini,
respectively. The N-terminal region of SAR3/MOS3 shares 45%
identity with the catalytic region of NUP196 and contains the
conserved residues that are necessary for cleavage in verte-
brates (Rosenblum and Blobel, 1999; Hodel et al., 2002). Further
studies are required to determine whether SAR3/MOS3 un-
dergoes processing to release a short N-terminal peptide and
whether this processing is important for function.

Comprehensive analyses of the NUP107–120 complex in S.
cerevisiae, Schizosaccharomyces pombe, Caenorhabditis eleg-
s, and vertebrate cell culture systems have demonstrated
many aspects of its cellular function (Lutzmann et al., 2002; Galy
et al., 2003; Harel et al., 2003; Walther et al., 2003; Bai et al.,
2004; Loiodice et al., 2004). When the function of the NUP107–
120 complex is compromised, it results in a reduction in the
nuclear export of RNA irrespective of the organism of study
(Aitchison et al., 1995; Vasu et al., 2001; Bai et al., 2004; O’Hagan
and Ljungman, 2004). Similarly, we found increased accumula-
tion of mRNA in the nucleus of sar1 sar3 cells relative to wild-type
cells, suggesting that there is a defect in mRNA export in these
plants. It is difficult to assess the precise developmental conse-
quences of nuclear mRNA accumulation, although it is clearly
associated with significant growth defects. Severe defects are also
observed in the los4 mutant that accumulate mRNA in the
nucleus in a similar manner (Gong et al., 2005).

The sar1-1 and sar3-1 mutations suppress auxin resistance in both
the axr1 and rce1 mutants. This suppression does not
appear to involve a direct effect on the RUB conjugation path-
way, because neither mutation alters the level of RUB-CUL1. An
alternative model is that suppression involves changes in the
transport of auxin response proteins, such as the Aux/IAAs. Our
data suggest that AXR3NT-GUS is stabilized in sar1-1 but that
this change is related to decreased translocation of the fusion
protein into the nucleus rather than to a direct effect on the
degradation machinery. If this model is correct, we expect other
members of the Aux/IAA protein family to be similarly affected.
An overall decrease in the level of these transcriptional repres-
sors in the nucleus could explain the partial suppression of the
phenotype conferred by axr1. However, it is important to note
that we did not observe auxin hypersensitivity in sar1 or sar3
plants, possibly because other regulatory processes compen-
sate for the change in Aux/IAA distribution.

A number of auxin signaling and response genes are targets of
miRNAs. The expression of some AUXIN RESPONSE FACTOR
genomes is modulated by the activity of mi160, and the TIR1/AFB
family of auxin receptors are targets of mi393 (Jones-Rhoades
and Bartel, 2004; Mallory et al., 2005; Wang et al., 2005; Navarro
et al., 2006). Because SAR1 and SAR3/MOS3 are involved in
the nuclear export of RNA, it is possible that the regulation of these
genomes by miRNAs is affected in sar1 and sar3 plants. We
examined the TIR1 transcript level by RT-PCR and found that it
was not altered by either mutation (G. Parry and M. Estelle,
unpublished data). However, it remains possible that the ex-
pression of other miRNA target genes is altered in the mutants.
The effects of mutations in components of the NUP107–120
complex vary in severity depending on the organism. In C. eleg-
s, reduced levels of Ce NUP160 (the SAR1 homolog) result in
nearly 100% embryo lethality, whereas the sar1 mutants are
relatively robust. Although this may reflect differences in the
importance of these proteins to NPC function, it is also possible
that none of the sar1 or sar3 mutations are null. In any case, the
severe phenotype of the sar1 sar3 double mutant suggests that
the NUP107–120 complex, assuming that this complex is pre-
sent in plants, has an important role in NPC function. We have
demonstrated that the sar1 and sar3 mutants are deficient in aspects of protein import and RNA export. Based on the phenotypes observed in sar1, sar3, and double mutant plants, it is likely that these defects affect diverse signaling pathways. In the future, it will be important to determine the functions of SAR1 and SAR3 in the NPC and the precise role of the complex in cellular regulation.

METHODS

Plant Growth Conditions

Plant growth conditions and morphological analyses were as described previously (Lincoln et al., 1990; Cernac et al., 1997). The auxin root elongation assay was performed by growing plants for 4 d on Arabidopsis thaliana solution (ATS) followed by transfer onto fresh ATS with or without auxin. Root growth was measured after an additional 3 d and expressed as a proportion of root growth on ATS medium without auxin. Lateral root number in 10-d-old seedlings was determined by scoring emerged primary roots on a Nikon SMZ1500 microscope. ATS medium consists of 1% sucrose, 5 mM KNO3, 2.5 mM KPO4, 2 mM MgSO4, 2 mM Ca(NO3)2, 50 µM Fe-EDTA, and 1 mL of microinutrients.

Genetic Analyses

A population of γ-mutagenized axr1-3 seeds was prepared as described previously (Ruegger et al., 1997). M2 seedlings were screened at 29°C in constant light at an intensity of 85 μE·m⁻²·s⁻¹. Seedlings with long hypocotyls were selected for further analysis (Gray et al., 1998). The genetic basis for the long-hypocotyl phenotype was determined by backcrossing to axr1-3 plants and analyzing the F1 and F2 generations. To map the SAR3 gene, sar1-3 plants were crossed to Landsberg erecta plants and homozygous sar1-3 plants were identified in the F2 population. By analyzing 680 mutant plants using a variety of CAPS and insertion/deletion PCR-based markers, sar3 was mapped distal to CAPS marker g17311 on chromosome 1 on BAC T21F11 or F23AS. After identification of SAR3, PSI-BLAST analysis revealed that SAR3 is homologous with human NUP196 (accession number AAC96798) and yeast NUP145 (accession number CAA96798).

The sar1-1 gene had been mapped previously between markers nga280 and nga248 on chromosome 1 (Cernac et al., 1997). Using an additional population of 647 F2 sar1-1 plants, we mapped the gene to a 180-kb region on BACs T9L6, T1609, and F10C21. After the identification of SAR3, PSI-BLAST analysis of genes within this interval indicated that A1tg33410 is related to human NUP160 (accession number NP_056046). SAR3 is related to human NUP196 (accession number AAL56659) and yeast NUP145 (accession number CAA96798). The sar1-1 gene had been mapped previously between markers nga280 and nga248 on chromosome 1 (Cernac et al., 1997).

Protein and RNA Expression

Seedlings were grown for 10 d on agar plates and then transferred to liquid ATS for 1 h with or without 20 mM 2,4-D. RNA was extracted using the Qiagen Plant RNeasy kit, and the RT reaction was conducted on 1 µg of RNA using SuperscriptIII RT (Invitrogen). Subsequent PCRs were conducted using 24 cycles (for IAA1 reaction) or 28 cycles in an Eppendorf mastercycler. We used the following primers in RT reactions: SAR1AF, 5′-ATGGGAGGAATCTGCAGAA-3′; SAR1AR, 5′-TGATATCATCAGCGTCCT-3′; SAR1B, 5′-GCGTGAAGCTATCTGAT-3′; SAR1BR, 5′-GGTCCTTGATCAGGAA-3′; SAR1CF, 5′-AAGGTTGTCACA- GGTTGCT-3′; SAR1CR, 5′-ATCAATGCAAGTCTCCTCC-3′; SAR3AF, 5′-CCGCTTGCTTTGCTGAGTA-3′; SAR3AR, 5′-CTTGACATCTCGAT- CACGTC-3′; SAR3BR, 5′-TGATGAAGACGTAGA-3′; SAR3CF, 5′-CTAAGCTGAGACCGCCGCG-3′; IAA1F, 5′-GGCTCTCTGAAAAC- ACAATCGTT-3′; IAA1R, 5′-CAATAATGATCACATAGGCACGTAGA-3′; IAA5F, 5′-GCGCAATTGAGATTATAATGCTTGACGCT-3′; IAA5R, 5′-TGATAC- CATTCACTTTCCCATACGTAC-3′; ACTINF, 5′-GGGTAGGGC- TGTGATATTCC-3′; ACTINR, 5′-TCTGTAACCGATTCTGGGAC-3′.

For cullin protein gel blots, protein was extracted from 10 mg of floral tissue in extraction buffer (125 mM Tris, pH 8.8, 1% SDS, 10% glycerol, and 50 mM Na2S2O3). For analysis of GFP expression, protein was extracted from 9-d-old seedlings in extraction buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Tween, and a Complete mini protease inhibitor tablet [1 tablet/10 mL; Roche]). GFP (30 µg) or cullin (50 µg) total protein was subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Bio-Rad). The blot was probed with rabbit anti-GFP or rabbit anti-CUL1 antibody (del Pozo and Estelle, 1999) and was visualized using the ECL system (Amersham).

Analysis of GUS Expression

Transgenic HS:AXR3NT:GUS seedlings were grown for 5 to 7 d on ATS medium and then transferred to liquid ATS for a 2-h heat shock at 37°C. Seedlings were then either left at 22°C for 90 min or immediately stained in X-Gluc staining solution for between 45 min and overnight [X-Gluc staining solution consists of 0.05 M Na phosphate buffer, pH 7.2, 10 mM KF, 10 mM KFe(CN)6, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide]. Roots were cleared using a decreasing ethanol series, and root tips were mounted in 50% glycerol. Images were obtained on a Nikon E800 microscope.

For quantitative MUG assays, transgenic HS:AXR3NT:GUS seedlings were heat-shocked as described above. After heat shock, 10 to 15 seedlings were held at 22°C for appropriate times and then frozen in liquid N2. Protein was then extracted in MUG extraction buffer (50 mM Na phosphate buffer, pH 7.0, 0.1% Sarkosyl, 0.1% Triton X-100, 10 mg β-mercaptoethanol, and 10 mM EDTA), and protein concentrations were determined by Bradford assay. The MUG assay was conducted using 50 µg of protein incubated for 16 h at room temperature in MUG reaction buffer (extraction buffer + 2 mM MUG), and the reaction was stopped in 10 X 0.2 M NaCO3. Fluorescence was measured using a TKO 100 fluorometer (Hoefer Scientific), calculated as nanomolar MU per microgram of protein and represented as a percentage of the expression at the end of the heat shock. Experiments were performed in duplicate and the values averaged.

DNA Constructs

The PRE/SAR3 cDNA in pBluescript was a kind gift from Jose Martinez-Zapater. To generate the 3SS:SAR3-GFP construct, the SAR3 cDNA was amplified and cloned into pENTR/D-TOPO using standard Gateway protocols (Invitrogen) (primers N96entrF [5′-CACCAGACGTTCTTCATTTCAACCC-3′] and N96entrNSR [5′-AGCTGAGATTTGGCCGCTCTC-3′]). SAR3 was then moved by LR clonase reaction into destination vector pVR-GFP/Ct (a kind gift from Vincent Rubio). Constructs were electroporated into Agrobacterium tumefaciens strain GV3101, and plant transformation was performed by floral dip (Clough and Bent, 1998). Transformants were selected on ATS plates with 200 mg/mL gentamycin (pVR-SAR-GFP). GFP expression was visualized on a Leica TCS SP confocal microscope using the green laser (500 to 550 nm) and viewed through the fluorescein isothiocyanate filter. Cell walls were visualized by staining seedlings with 1 µg/mL propidium iodide in water for 1 min and then washed in water for 5 min.

Whole-Mount in Situ Localization of mRNA

The procedure for in situ localization of mRNA was modified from Gong et al. (2005). Small leaves (<5 mm) were taken from wild-type and sar1-1 mutant plants, and floral transformation was performed by floral dip. Seedlings were grown for 5 to 7 d on ATS medium and then transferred to liquid ATS for a 2-h heat shock at 37°C. Seedlings were then either left at 22°C for 90 min or immediately stained in X-Gluc staining solution for between 45 min and overnight [X-Gluc staining solution consists of 0.05 M Na phosphate buffer, pH 7.2, 10 mM KF, 10 mM KFe(CN)6, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide]. Roots were cleared using a decreasing ethanol series, and root tips were mounted in 50% glycerol. Images were obtained on a Nikon E800 microscope.

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The following materials are available in the online version of this article. NUP133, At2g05120; NUP107, At3g14120; NUP85, At4g32910.1; (synthesized by Gibco BRL) and incubated overnight at 50

the GenBank/EMBL data libraries under accession numbers At1g33410 sections of 0.7

3

580 nm, and images were taken using fluorescein isothiocyanate and

before addition of 1 m

buffer:heptane (fixation buffer consists of 120 mM NaCl, 7 mM Na2HPO4, 2.7 mM KCl, 40 mM EGTA, 0.1% Tween 20, 10% DMSO, and 5% formaldehyde [FA]). Samples were washed for 5 min twice in methanol and three times in 100% ethanol before incubation for 30 min in 1:1 ethanol:xylene. Samples were then washed for 5 min twice with ethanol, once with methanol, and once with methanol:fixation buffer (–FA) and postfixed in fixation buffer (+FA) for 30 min. After fixation, leaves were rinsed for 5 min twice with fixation buffer (–FA) and then once in 1% blocking reagent in fixation buffer (–FA) (Roche 1 093 657). Samples were then blocked for 1 h at 50°C in 2 mL of 1% blocking buffer before addition of 1 μg of 50-mer oligo(dT) with a 5′ fluorescein tag (synthesized by Gibco BRL) and incubated overnight at 50°C. Samples were mounted in water and viewed using a Leica TCS SP confocal microscope. Equivalent laser intensity was used for each set of wild-type and sar3 sar3 samples. Samples were excited at 450 to 500 nm and 530 to 580 nm, and images were taken using fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate filters. Samples were viewed with a ×63 objective, and images were generated by collection of 9 to 12 optical sections of 0.7 μm.

Accession Numbers

Sequence data for the main genes discussed in this article can be found in the GenBank/EMBL data libraries under accession numbers At1g33410 (SAR1) and At1g10860 (SAR3). The putative Arabidopsis homologs of known vertebrate nucleoporins are listed in the data libraries as follows: NUP133, At2g05120; NUP107, At3g14120; NUP85, At4g32910.1; NUP43, At4g30840; Sec13, At2g30050 and At3g01340.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The sar3 Mutation Suppresses the Resistance of axr1 to Methyl Jasmonate.

Supplemental Figure 2. sar rce1 Seedlings Have a Different Pheno-type Than rce1 Seedlings.

Supplemental Figure 3. The SAR3 Protein Is Similar to Yeast NUP145.

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