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Characterization of AtSEC12 and AtSAR1

Proteins Likely Involved in Endoplasmic Reticulum and Golgi Transport

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Transport of cargo proteins from the endoplasmic reticulum (ER) to the *cis*-Golgi network is mediated by protein-coated vesicles. The coat, called COPII coat, consists of proteins that are recruited from the cytosol and interact with integral membrane proteins of the ER. In yeast, both cytosolic proteins (Sec13/31, Sec23/24, and Sar1) and ER-associated proteins (Sec12 and others) have been purified and characterized and it has been possible to demonstrate transport vesicle formation in vitro. *Arabidopsis thaliana* homologs of Sar1 and Sec12 have recently been identified, but little is known about the properties of the proteins or their subcellular distribution. Here we demonstrate that AtSAR1, a 22-kD protein that binds GTP, and AtSEC12, a 43-kD GTP-exchange protein, are both associated with the ER. However, about one-half of the cellular AtSAR1 is present in the cytosol. When AtSAR1 is overexpressed in transgenic plants, the additional protein is also cytosolic. When tissue-culture cells are cold-shocked (12 h at 8°C), AtSAR1 levels appeared to decline and a larger proportion of the total protein was found in the cytosol. Given the known function of AtSAR1 in yeast, we propose that the amount of ER-associated AtSAR1 is an indication of the intensity of the secretory process. Thus, we expect that such a cold shock will adversely affect ER-to-Golgi transport of proteins.

In eukaryotic cells newly synthesized proteins destined for secretion or residence in organelles of the secretory pathway must enter and transit the secretory pathway for proper processing and targeting. This pathway consists of a number of membrane-bound organelles, including the ER, the Golgi apparatus, and vacuoles. The proteins are transported between these organelles in small vesicles (Palade, 1975) that bud from the donor membrane and are targeted to the acceptor membrane, where they fuse (Rothman, 1996). Since proper functioning of the secretory pathway is crucial for normal cell operation, transport between the various compartments must be tightly regulated to ensure that vesicles fuse only with the appropriate membrane. Both biochemical and genetic approaches have led to the identification of numerous components of the molecular machinery that mediate this transport.

In yeast transport of proteins from the ER to Golgi complex is a well-characterized vesicular transport step. Budding of vesicles from the ER involves a number of peripheral membrane proteins (Sar1p, Sec23/24p complex, and Sec13/31p form a complex collectively termed the COPII coat), together with at least one integral ER-membrane protein, Sec12p (Salama et al., 1993; Barlowe et al., 1994; Salama and Schekman, 1995). Functional plant genes with homology to the *SAR1*, *SEC12*, *SEC13*, and *SEC23* genes have been isolated (d'Enfert et al., 1992; Bar-Peled et al., 1995; M. Bar-Peled and N.V. Raikhel, unpublished data). Many vesicle transport steps, including the ER-to-Golgi step, are thought to be regulated by GTP-binding proteins (Ferro-Novick and Novick, 1993). Several cDNAs encoding GTP-binding proteins have been isolated from higher plants (for review, see Verma et al., 1994), and these include members of the RAB/YPT family (Cheon et al., 1993; Bednarek et al., 1994; Park et al., 1994; Loraine et al., 1996), the ARF (Regad et al., 1993), dynamin (Dombrowski and Raikhel, 1995; Gu and Verma, 1996), and the Sec1 family (D. Bassham and N.V. Raikhel, unpublished data). The localization and function of most of the GTP-binding proteins isolated from plants are yet to be determined.

Studies of yeast and mammalian cells have indicated that some GTP-binding proteins play a regulatory role during protein transport (Serafini et al., 1991; Barlowe and Schekman, 1993; Elazar et al., 1994; Søgaard et al., 1994; Jedd et al., 1995). Indeed, in the few cases studied, reducing the levels of GTP-binding protein led to adverse cellular effects. The subcellular location and tissue distribution of these proteins in plant cells may provide clues to their function. Furthermore, in a multicellular organism different cells may have specific secretory activities. In plants there are numerous examples of such specialization: secretion of specific proteins during pollen germination, vacuolar accumulation of storage proteins in seeds, secretion of mucilage in growing root tips, and formation of a new membrane and cell wall during the late stages of cell mitosis (phragmoplast formation). Recently, it was observed that at least two types of vesicle populations can bud from the ER (Aridor et al., 1995; Bednarek et al., 1995), suggesting specific cargo selection. To date it is unknown how the

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Abbreviations: AtSAR1, *Arabidopsis thaliana* secretion-associated RAS super family; BiP, immunoglobulin heavy chain-binding protein; COP, coat protein; GST, glutathione *S*-transferase.

COPII products involved in ER-to-Golgi protein transport are located in plant cells.

In this paper we characterize the properties of the gene products of *AtSEC12* and *AtSAR1*. The cDNAs were isolated based on their ability to complement yeast mutants defective in transport of vesicles from the ER to the Golgi apparatus (d'Enfert et al., 1992). Both proteins are associated with the ER, which is known to be contiguous with the outer nuclear membrane. However, AtSAR1 was also found in the cytosol. The intracellular localization of AtSAR1 is modified by cold-shock treatment, which causes a reduction of AtSAR1 associated with the ER membrane. Based on the analysis of wild-type and transgenic plants, we suggest that the relative distribution of AtSAR1 between the cytosol and membrane may reflect the activity of the secretory pathway in plant tissue.

MATERIALS AND METHODS

Plant Growth

Ten milliliters of *Arabidopsis thaliana* ecotype Columbia cell-suspension line (T87-C33, a generous gift from Dr. Michael Axelos; Axelos et al., 1992) was subcultured weekly into 50 mL of medium (0.32 g L^{-1} Gamborg's B-5 with minimal organics, with the addition of 20 g L^{-1} Suc, and 0.5 mg L^{-1} 2.4-D, buffered to pH 5.7 with KOH) and grown on a rotary shaker at 100 rpm at 22°C under light or dark conditions. Seeds of *A. thaliana* (approximately 15) were sterilized and placed in a 125-mL flask containing 50 mL of germination liquid medium (4.3 g L^{-1} Murashige and Skoog salts mixture [Gibco-BRL], 0.5 g L^{-1} Mes, 10 g Suc, 0.1 g L^{-1} *myo*-inositol, 100 μL L^{-1} of 10 mg mL^{-1} thiamine-HCl, 50 μL L^{-1} of 10 mg mL^{-1} pyridoxine, and 50 μL L^{-1} of 10 mg mL^{-1} nicotinic acid, adjusted to pH 5.7 with KOH). The sterile plants were germinated and grown in the flasks placed on a rotary shaker (50 rpm) in a 22°C incubator under a 12-h fluorescent light/dark cycle. To study tissue-specific protein expression patterns, seeds were germinated either in germination medium-agar plates and grown as described above or in pots and grown in a controlled environment chamber at 22°C under a 12-h day/dark cycle: 120 μmol m^{-2} s^{-1} light intensity, 70% RH. Plant materials (roots, leaves, pollen, stems, and siliques) were collected at different times, as indicated, and analyzed. For the analysis of cold conditions, cell-suspension cultures were placed at 8°C for the periods indicated in "Results." Tunicamycin (Calbiochem) was dissolved in DMSO at 10 mg mL^{-1}. Aliquots from this stock were added directly to the suspension medium to achieve a final concentration of 20 μg mL^{-1}. Controls were treated with DMSO. At the end of each treatment, cells were homogenized and processed.

Protoplast Preparation and Homogenization

Protoplasts were isolated essentially as described by Axelos et al. (1992) with minor modifications. Five milliliters of drained cells from two flasks of 4-d-old Arabidopsis cell-suspension cultures were collected on a 94-μm filter. The cells were incubated with 25 mL of freshly made protoplasting solution (15.4% [w/v] Suc, 0.32% [w/v] Gamborg's B-5 minimal organics, with the addition of 100 mg of caylase 345 L, and 15 mg of pectolyase Y-23) at room temperature for approximately 3 h on a rotary shaker (60 rpm). The treated suspension was adjusted to approximately 40 mL with 15.4% (w/v) Suc and 0.32% (w/v) Gamborg's B-5 minimal organic solution and poured into Babcock centrifuge bottles. Protoplasts were separated from broken cells after centrifugation (1100 rpm in a HNSII clinical centrifuge swinging bucket rotor [IEC, International Equipment Company, Needham Heights, MA] for 10 min at 25°C). The floated protoplast band was collected and transferred to a centrifugation tube containing 20 mL of 0.4 M betaine, 3 mM Mes, and 10 mM $CaCl_2$, pH 5.7. Protoplasts were pelleted after 10 min of centrifugation at 600 rpm (50*g*) at room temperature, washed with 30 mL of 0.4 M betaine, 3 mM Mes, and 10 mM $CaCl_2$, pH 5.7, and pelleted. Protoplasts were resuspended in 4 mL of cold lysis buffer containing 20 mM Hepes-KOH, pH 6.9, 0.4 M Suc, 10 mM potassium acetic acid, 1 mM DTT, and 0.5 mM PMSF. EDTA (1 mM) or $MgCl_2$ (3 mM) was added when indicated. Protoplasts were lysed gently but completely (as confirmed by microscopy) by at least eight passages through a 25⅝-gauge needle.

Total Protein Isolation

Five milliliters of drained cell-suspension culture or plant tissues (up to 1 g) was ground on ice in a mortar with a pestle in the presence of approximately 100 mg of acid-washed glass beads. Total protein was then extracted by further grinding with 4 mL of cold lysis buffer (100 mM Tris-HCl, pH 7.5 [or 50 mM Hepes-KOH, pH 6.9], 0.4 M Suc, 10 mM potassium acetate, and 1 mM EDTA), 8 μL of 1 M DTT, and 20 μL of 0.1 M PMSF. Where indicated, 3 mM $MgCl_2$ was included in the lysis buffer and EDTA was omitted. Samples were placed on a shaker for up to 15 min at 4°C while other samples were extracted. Samples were further lysed by passing the homogenate eight times through a 25⅝-gauge needle, and filtered through three layers of Miracloth (25 μm, Calbiochem) to remove unbroken cells and debris. The homogenate was termed total crude homogenate.

Separation of Intracellular Membranes by Velocity, Size, or Density

Total crude homogenate was centrifuged (1,000*g* for 10 min at 4°C), and the top 3.5 mL of supernatant (termed s1 or postnuclear fraction) was removed from the pellet fraction (termed p1). Total microsomes were prepared by centrifugation of the s1 fraction for 60 min at 150,000*g*, generating a total membrane pellet (termed p150) and a soluble protein fraction (termed s150). Where indicated, the s1 fraction was further centrifuged at 8,000*g* (generating a soluble supernatant termed s8 and a pellet fraction termed p8). The s8 fraction was centrifuged again at 150,000*g* for 1 h (generating a soluble supernatant termed s150 and a pellet fraction termed p150). All pellets (p1, p8, and p150) were rinsed briefly with 1 mL of lysis buffer and further

resuspended in lysis buffer containing 150 mM NaCl, 1% Triton X-100, and 1% sarcosyl. After 15 to 30 min on ice, the solubilized pellets (p1, p8, and p150) were spun (at 150,000*g* and 4°C for 15 min) to remove undissolved matter, and the supernatant was saved for analysis.

For the preparation of nuclei, we used a method described by Morre and Andersson (1994), with slight modifications. Protoplasts were prepared and homogenized in 6 mL of lysis buffer containing $MgCl_2$. The homogenate was centrifuged (at 1,000*g* and 4°C for 10 min), the pellet was washed once with 6 mL of lysis buffer, spun as above, and the pellet containing nuclei and plastid fragments was resuspended in 2 mL of cold lysis buffer and layered on top of 10 mL of a Suc cushion composed of 2 M Suc, 1% (w/v) dextran T500, 20 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$ in an Ultraclear centrifuge tube that fits the SW40Ti rotor (Beckman). After the sample was centrifuged at 50,000*g* and 4°C for 45 min, the top plastid band was removed and the pellet containing nuclei was washed once and resuspended in lysis buffer containing 150 mM NaCl, 1% Triton X-100, and 1% sarcosyl (for immunoblot analysis), or resuspended in 100 μL 10 mM Mes-KOH, pH 5.6, 0.2 M Suc, 3 mM $MgCl_2$, and 20% glycerol, and stored at −20°C.

For separation of endomembrane organelles based on their densities, and to study ER localization, 1 to 2 mL of packed protoplasts were homogenized in 6 mL of lysis buffer containing 1 mM EDTA or 3 mM $MgCl_2$. After the sample was centrifuged (at 1,000*g* and 4°C for 10 min), the supernatants in each homogenization buffer (s1E or s1M) were collected and applied to linear Suc gradient systems (16–55 or 16–40 or 25–55% [w/v]) or to step Suc gradients. The linear Suc gradients were buffered in 10 mM Hepes-KOH (pH 6.9) and 10 mM potassium acetate, containing either 2 mM EDTA or 5 mM $MgCl_2$, and were prepared in 12-mL Beckman Ultraclear thin tubes that fit the Beckman SW40Ti rotor. The step Suc gradients were made from stock solutions containing 55, 40, 33.5, 26.5, and 16% (w/v) Suc in the above-mentioned buffer containing EDTA or $MgCl_2$ and were prepared by sequential layering of the following stocks into 12-mL Beckman Ultraclear thin tubes: 0.75 mL of 55% Suc solution, three 0.97-mL aliquots of 40% Suc, three 0.77-mL aliquots of 33.5% Suc, two 1-mL aliquots of 26.5% Suc, and two 0.75-mL aliquots of 16% Suc solution. Three milliliters of either s1E or s1M was layered on top of the 9-mL linear or semistep Suc gradient, which was made in the presence of EDTA or $MgCl_2$, respectively. Gradients were centrifuged at 150,000*g* (28,500 rpm) in a Beckman SW40Ti rotor for 2 h at 4°C. Fractions were collected and stored frozen in aliquots until analysis. Aliquots (60 μL) of the fractions were analyzed by immunoblots, and Suc concentrations were determined using a refractometer.

Construction of GST-SEC12 and GST-SAR1 Hybrid Expression Plasmids

The plasmid pMBP56 was constructed to produce an inframe fusion between GST and the entire open reading frame of SAR1 (193 amino acids). An AtSAR1 PCR fragment was generated using Vent DNA polymerase (New England Biolabs), a sense primer having a *Bam*HI recognition sequence 5 bases upstream of the ATG initiation codon of AtSAR1, an antisense primer, and the plasmid pMBP53 containing AtSAR1 cDNA clone as template. The sequence of the resulting PCR-amplified DNA was confirmed, and the *Bam*HI-*Eco*RI fragment was subcloned into pGEX-5X (Pharmacia) generating pMBP56. The plasmid pMBp109 was constructed to produce an inframe fusion between GST and the first 300 amino acids of AtSEC12. It was constructed by ligation of three DNA fragments: the first 120 bp of AtSEC12, which was amplified by PCR and digested with *Bgl*II and *Bam*HI, together with a *Bam*HI-*Hin*dIII fragment of AtSEC12 (nucleotides 120–908) and pGEX-KG (Guan and Dixon, 1991) digested with *Bam*HI and *Hin*dIII. The SEC12 PCR fragment was generated using Vent polymerase, sense primer 3 containing a *Bgl*II recognition sequence 5′ upstream of the initiation ATG codon, antisense primer, and pMBp114 as a template.

Generation and Purification of GST-Fusion Proteins and Preparation of Antibodies

DH5α *Escherichia coli* cells containing pMBP56 or pMBp109 were grown in 100 mL of LB media at 37°C to A_{600} = 0.8 to 1. Protein expression of GST or GST-fusion constructs was induced by adding 0.2 mM isopropylthio-β-galactoside and shifting the cell culture to 28°C. After 3 to 5 h, cells were spun (at 6,000*g* and 4°C for 10 min), resuspended in 10 mL of cold lysis buffer (20 mM Hepes-NaOH, pH 7.5, 1 mM EDTA, and 150 mM NaCl) containing 0.1 mM PMSF, and lysed by passing the culture through a French press (1100 psi). Triton X-100 was added to 1%, and after 20 min at 4°C the homogenate was centrifuged at 12,000*g* for 30 min at 4°C. The clear supernatant (T) was collected and added to 0.5 mL of GSH-Sepharose resin beads (Pharmacia) that had previously been equilibrated with lysis buffer containing 0.5% Triton X-100. After 30 min of incubation at room temperature, the GST-fusion protein bound to the resin was spun (at 500*g* for 2 min) and the effluent was discarded. The resin was washed five more times, each for 5 min, with approximately 15 mL of lysis buffer containing 0.5% Triton X-100. The GST or GST-fusion proteins were eluted from the column using 5 mL of 50 mM Tris-HCl, pH 8.0, and 10 mM GSH essentially as described by Bar-Peled and Raikhel (1996). The fusion protein (approximately 100 μg) was emulsified with Titer-Max (CytRx, Norcross, GA) in a total of 1 mL and injected in rabbits. The rabbits were boosted two more times each with 50 μg of GST-Sec12p or GST-Sar1p emulsified with Titer-Max. Purification of specific antibodies to SEC12 or SAR1 was achieved using a method described by Bar-Peled and Raikhel (1996) and were used at 1:500 dilution. Other antibodies used in this study were anti-AtIMPαp sera at 1:2000 dilution (Hicks et al., 1996) and anti-AtPEP12 at 1:2000 dilution (Conceição et al., 1977). Anti-RD28 and anti-TIP sera at 1:500 dilution and anti-BiP sera at 1:2000 dilution were kindly provided by Maarten Chrispeels (University of California, San Diego).

SDS-PAGE and Immunoblotting

Protein concentrations were determined according to the Bradford assay (Bradford, 1976) using BSA as the standard. Proteins (30 μg/lane) were separated on modified Laemmli-reduced SDS-PAGE (Bar-Peled et al., 1991) and transferred to nitrocellulose membrane (0.45 μm) in 10 mM Tris, 100 mM Gly, 0.05% SDS, and 10% methanol using a semidry apparatus at 2 mA cm^{-2} for 90 min. Blots were stained with Ponceau S (Sigma), incubated with blocking solutions (5% [w/v] powdered milk, TBST [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 0.1% Tween 20]) for 2 to 12 h, reacted with primary antibody at 1:500 dilution, washed, and then reacted with secondary antibody (1:4000) conjugated to alkaline phosphatase. Immune complexes were detected by color assay using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates. The relative density of bands on blots was scanned using a densitometer.

Transgenic Plants

To generate plants overexpressing AtSEC12, the full-length *AtSEC12* cDNA was cloned in the sense orientation downstream of the 35S promoter in PGA748 (An et al., 1988) to generate pMBp111. Similarly, *AtSAR1* cDNA was cloned in the sense orientation into pGA748 to generate pMBP54. pMBP54 or pMBp111 were each introduced to *Agrobacterium tumefaciens* strain GV3001 (pMP90). The agrobacteria were subsequently vacuum-infiltrated into 3- to 4-month-old wild-type *A. thaliana* (ecotype Columbia) plants. All transgenic lines were confirmed to generate a ratio 3:1 (Kan^r:Kan^s) on selective plates containing 50 mg L^{-1} kanamycin. Transgenic independent lines homogenic for resistance to Kan were selected. T_3 and T_4 progeny of such independent homogenic lines were used in the experiments described.

RESULTS

Characterization and Tissue Distribution of AtSEC12 and AtSAR1

AtSEC12 cDNA encodes an approximately 43-kD protein with a short hydrophobic core near the carboxy terminus. The cDNA of *AtSAR1* encodes an approximately 22-kD protein that also contains a hydrophobic core. A GST-fusion protein containing the hydrophilic portion of AtSEC12 (amino acids 1–350) or the entire open reading frame of AtSAR1 was expressed in *E. coli*, affinity-purified over a GSH-Sepharose column (Bar-Peled and Raikhel, 1996), and injected into rabbits to raise antibodies. The AtSEC12 antiserum reacted with a 43-kD polypeptide in total protein extract of various wild-type Arabidopsis plant tissues (Fig. 1A), and anti-AtSAR1 reacted with a 22-kD polypeptide (Fig. 1A), as expected from each of the deduced amino acid sequences. Preimmune sera had no reactivity with plant protein extract (data not shown). Hydropathy plot analysis predicts that AtSEC12 is a membrane protein and AtSAR1 is presumably a membrane-associated protein. To find out the membrane association of

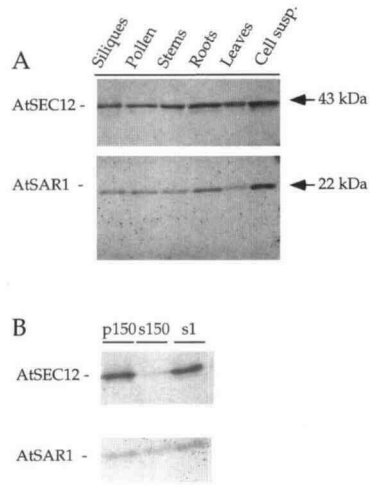

Figure 1. Distribution of AtSEC12 and AtSAR1 in Arabidopsis tissues. A, Total protein was extracted from Arabidopsis green siliques, pollen, or stems of mature plants, from roots or leaves of 2-week-old plants, or from Arabidopsis cell-suspension culture. Equal amounts of proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-AtSAR1 and anti-AtSEC12 antibodies. B, Proteins were extracted from Arabidopsis cell-suspension culture and further fractionated by centrifugation into total protein (s1), total soluble protein (s150), and total membrane fractions (p150). Blots were probed with anti-AtSEC12 or anti-AtSAR1 antibodies.

these proteins, their intracellular distribution was examined by isolating a microsomal fraction at 150,000*g* and comparing immunoblots of total proteins (s1) and soluble (s150) and microsome (p150) fractions from wild-type Arabidopsis plants. The antibody raised against AtSEC12 detected only a membrane-associated protein (Fig. 1B), whereas antibodies raised against AtSAR1 detected a single 22-kD protein band in both cytosolic and membrane fractions (Fig. 1B, bottom).

To determine the tissue distribution of these two proteins, total s1 protein (postnuclear) fractions were isolated from siliques, pollen, stems, roots, and leaf tissues, as well as from cell-suspension cultures (Fig. 1A). AtSEC12 and AtSAR1 were detected at various levels in all tissues examined (Fig. 1A), with the highest levels relative to total protein in cell-suspension cultures. The level of AtSAR1 in 2-week-old leaves appeared to be reduced compared with roots from plants of the same age or when compared with green cell-suspension cultures (Fig. 1A).

Since AtSAR1 is localized in cytosolic and membrane fractions (Fig. 1B), we compared its relative distribution between membrane and soluble fractions in various plant tissues (Fig. 2). Relative to total protein, the amount of AtSAR1 associated with membranes of roots, young leaves, and stems was higher compared with mature leaves (Fig. 2, compare lanes 1, 3, and 7 with lane 5). The AtSEC12 level in mature leaves was lower compared with young leaves (Fig. 2, compare lane 3 with lane 5). However, the ratio between AtSEC12 and the membrane-associated AtSAR1 appeared to be similar in all tissue analyzed (Fig. 2). It is possible that the overall reduction in AtSAR1 and AtSEC12

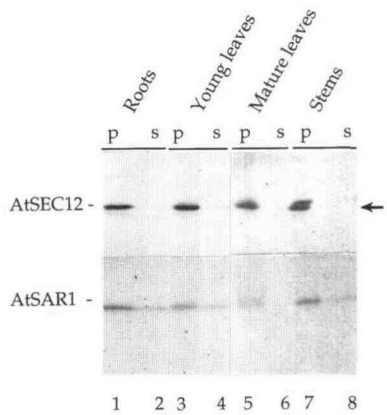

Figure 2. AtSEC12 is a membrane-associated protein, whereas AtSAR1 is a soluble and membrane-associated protein. Total (s1) protein extracted from roots or leaves of 2-week-old plants or from mature leaves of 8-week-old plants or stems was further centrifuged at 150,000*g* to generate total membrane (p150 = p) and total soluble (s150 = s) fractions. Equal amounts of soluble and membrane proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-AtSEC12 and anti-AtSAR1 antibodies. The arrow indicates a lower 42-kD cross-reacting band in stems.

in mature leaves may reflect lower secretory activity compared with young leaves and roots. Consistent with findings reported in this paper, similar observations were made for other proteins (AtPEP12 and AtELP) that are presumably involved in the secretory protein trafficking in Arabidopsis plant cells (Conceição et al., 1977; Ahmed et al., 1997). Often, a lower immunogenic protein band (approximately 42 kD) appeared when blots were probed with antibody raised against AtSEC12. The use of various protease inhibitors did not reduce this cross-reactive band.

Distribution of AtSEC12 and AtSAR1 and Their Association with the Nucleus and ER

To investigate biochemical properties and the intracellular distribution of AtSAR1 and AtSEC12, we used actively dividing suspension-cultured cells as the starting material (Fig. 1A). To minimize endomembrane disruption during homogenization, protoplasts were generated from cell-suspension cultures and gently lysed (see "Materials and Methods"). Several methods were used for the subcellular fractionation of AtSEC12 and AtSAR1: velocity sedimentation (differential centrifugation), Suc gradients, and gel-filtration chromatography. Using velocity sedimentation, which separates organelles based on their size, we detected AtSEC12 and AtSAR1 in all crude membrane fractions, including nuclei and plastids (which sediment at low centrifugation force, 1,000*g* = p1), a membrane fraction enriched with ER, vacuoles, and mitochondria (some of which sediment at 8,000*g* = p8), and in the microsomal fraction (150,000 *g* = p150) (Fig. 3A).

In yeast the outer nuclear membrane is contiguous with peripheral ER and carries out ER functions (Preuss et al., 1991; Bednarek et al., 1995; Strambio-de-Castillia et al., 1995). In plants a soluble ER-localized fusion protein (phytohemagglutinin fused to the carboxy-terminal tetrapeptide KDEL) was reported to be found in both the nuclear envelope and ER structure (Herman et al., 1990). Since portions of AtSAR1 and AtSEC12 proteins were detected in the low-speed pellet (Fig. 3A), we analyzed the presence of these proteins in a fraction enriched for intact nuclei. The enriched, intact fraction of nuclei was separated from plastids on dextran/Suc gradients (Fig. 3B), essentially as described previously (Morré and Andersson, 1994). Both AtSEC12 and AtSAR1 were associated with a fraction enriched with isolated nuclei (Fig. 3B) but not with the plastid fraction (data not shown). The nuclear preparation was free of other endomembrane microsomes (Fig. 3B), since it did not react with sera recognizing AtPEP12, which was localized to post-Golgi vesicles, possibly in a post-Golgi compartment (Conceição et al., 1977), with serum to the vacuole membrane protein γ-TIP (Höfte et al., 1992), or with the plasma membrane protein RD28 (Daniels et al., 1994). However, the nuclear preparations were strongly stained with antisera to the AtIMPα (Fig. 3B), a nuclear protein (Hicks et al., 1996).

Velocity sedimentation fractionation is not sufficient to adequately discriminate between the various endomembrane compartments. Therefore, we used a method that separated organelles based on their densities for AtSEC12 and AtSAR1 localization. Postnuclear supernatants (s1) were layered onto Suc gradients; after centrifugation, gra-

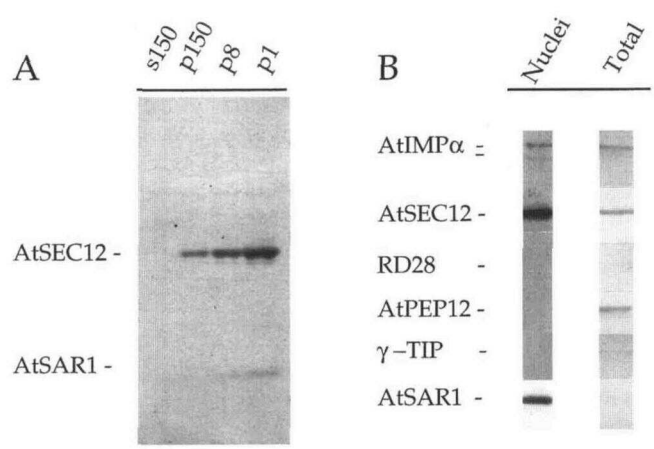

Figure 3. Localization of AtSAR1 and AtSEC12 to nuclei/ER-enriched fraction. A, Total protein extracted from protoplasts of Arabidopsis suspension-cultured cells was centrifuged at low speed, 1,000*g*. The pellet (p1) was saved and the supernatant (s1) was centrifuged again at 8,000*g*. The soluble (s8) fraction was centrifuged again at 150,000*g*, generating soluble (s150) and membrane pellet (p150) fractions. Equal amounts of protein from each of the pellets (p1, p8, and p150) and the supernatant (s150) fractions were separated by SDS-PAGE, blotted, and reacted with anti-AtSEC12 and anti-AtSAR1 antibodies. B, Nuclei, isolated from protoplasts of suspension-cultured cells (total), were enriched over a Suc/dextran gradient. The nuclei were solubilized, and proteins were separated by SDS-PAGE, blotted, and reacted with anti-AtSEC12 and anti-AtSAR1 antibodies. In addition, blots were probed with antibodies to the nuclear marker (54- to 66-kD doublet AtIMPα), the vacuolar marker (γ-TIP), the plasma membrane marker (RD28), and the prevacuolar marker or post-Golgi marker (AtPEP12).

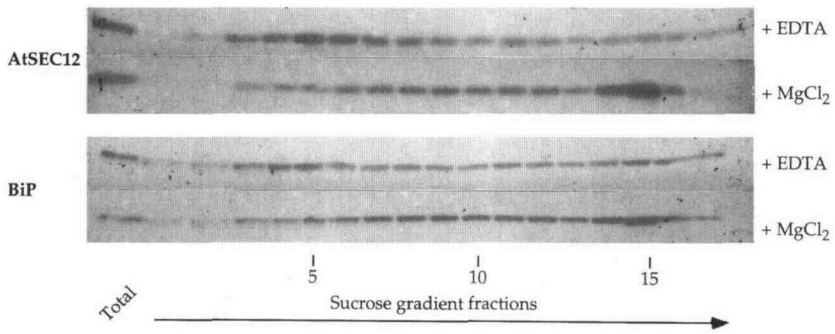

Figure 4. AtSEC12 is an ER-membrane protein. Protoplasts were made from Arabidopsis suspension-cultured cells, resuspended in lysis buffer containing either EDTA (top) or $MgCl_2$ (bottom), and then lysed. The homogenates were centrifuged at 1,000*g* to remove nuclei, and the supernatant (s1) was loaded on a linear 25 to 55% Suc gradient. After samples were centrifuged, fractions were collected from the top to the bottom of the gradients, and aliquots were separated on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-AtSEC12 antibodies and with anti-BiP antibodies. The Suc concentration was approximately 30% in fraction 5 and approximately 45% in fraction 15.

dient fractions were collected and analyzed by immunoblotting assay. Under normal conditions (Fig. 4, panels +EDTA) AtSEC12 can be found throughout the linear gradient and peaked into fractions 5 and 6 corresponding to approximately 30% Suc. The only positive control available to us as an ER marker in plants is the soluble ER protein BiP, as confirmed by western analysis and immunolocalization, which yield a distribution similar to AtSEC12 on the gradient (Fig. 4, +EDTA panels). It was observed in many systems, including plants (Lord, 1987), that fractionation on Suc gradients containing Mg led to a shift of some ER-associated proteins, which presumably maintains interaction between ribosomes and ER. Indeed, when protein homogenates were prepared in the presence of Mg and subsequently loaded on a Suc gradient containing Mg, a different banding pattern was observed (Fig. 4, +$MgCl_2$ panels). In blots containing Suc gradient fractions prepared with Mg, AtSEC12 peaked at fraction 15 (approximately 45% Suc) and the relative amounts in fractions 5 and 6 were reduced (Fig. 4, compare +EDTA panels with +$MgCl_2$ panel, lanes 5 and 15). As expected, Mg shifted BiP localization on the Suc gradient from fraction 5 (Fig. 4, +EDTA panels) to fraction 15 (Fig. 4, +$MgCl_2$ panels). The fact that AtSEC12 and BiP share similar properties on Suc gradients strongly suggested that AtSEC12 is an ER-membrane protein. We applied postnuclear homogenates to both continuous and discontinuous Suc gradients and found that both AtSEC12 and AtSAR1 co-migrated (data not shown).

AtSEC12 Is an Integral Membrane Protein; AtSAR1 Is a Membrane-Associated and Cytosolic Protein

The subcellular fractionation studies demonstrated that both AtSEC12 and AtSAR1 are associated with the ER. To determine whether AtSEC12 is an integral membrane protein, various conditions and treatments to extract AtSEC12 from the microsomal pellet were examined. Equivalent amounts of the microsomal protein pellet (p150) were resuspended in lysis buffer alone (Fig. 5A, lane 2), in lysis buffer containing nonionic detergent Triton X-100 at 0.1 and 1% (Fig. 5A, lanes 3 and 4) or 2 M urea (Fig. 5A, lane 5), in 0.1 M Na_2CO_3 (Fig. 5A, lane 6), in lysis buffer supplemented with NaCl at 0.5 and 1 M (Fig. 5A, lanes 7 and 8), and in lysis buffer containing ionic detergent sarcosyl at 0.1 and 1% (Fig. 5A, lanes 9 and 10). After incubation on ice for 30 min, the mixtures were centrifuged at 150,000*g*. The content of AtSEC12 in the resulting supernatants was determined by immunoblotting assays after SDS-PAGE. Treatments known to be effective in stripping proteins peripherally associated with membranes (urea, salt, and alkaline conditions) (Fujiki et al., 1982) were not effective in stripping AtSEC12 from the membranes (Fig. 5A). Only nonionic and ionic detergents (Fig. 5A, lanes 3 and 4 and 9 and 10, respectively) were sufficient to extract AtSEC12 from the membrane.

Membrane association of AtSAR1 was analyzed in experiments similar to those performed for AtSEC12 using antibodies against AtSAR1; also, additional concentrations of salt and urea were added to the lysis buffer (Fig. 5B). Although salt treatments (up to 1 M) and low levels of urea (up to 2 M) did not release all of the AtSAR1 from the membranes, alkaline treatment and detergent did elute AtSAR1 from the membrane (Fig. 5B, lane 7). Thus, AtSEC12 behaved like an intrinsic membrane protein, whereas microsome-associated AtSAR1 behaved as a peripheral but tightly associated membrane protein.

Distribution of AtSEC12 and AtSAR1 in Transgenic Plants

Our results show that AtSAR1 is distributed between the cytosol and membrane fractions. In yeast only the membrane-bound, activated Sar1p recruits and initiates the assembly of the COPII coat structure, resulting in the formation of an ER-derived vesicle (Barlowe et al., 1994). Thus, we examined wild-type plants and transgenic plants overexpressing AtSAR1 and AtSEC12 (Fig. 6). The level of AtSEC12 in a total protein extract appeared to increase in independent homozygous plants expressing *AtSEC12* cDNA through the regulation of the 35S promoter (Fig. 6A), and an increase in AtSAR1 was observed in transgenic lines overexpressing AtSAR1 (Fig. 6B). In leaves of wild-type plants the cytosolic amounts of AtSAR1 were similar

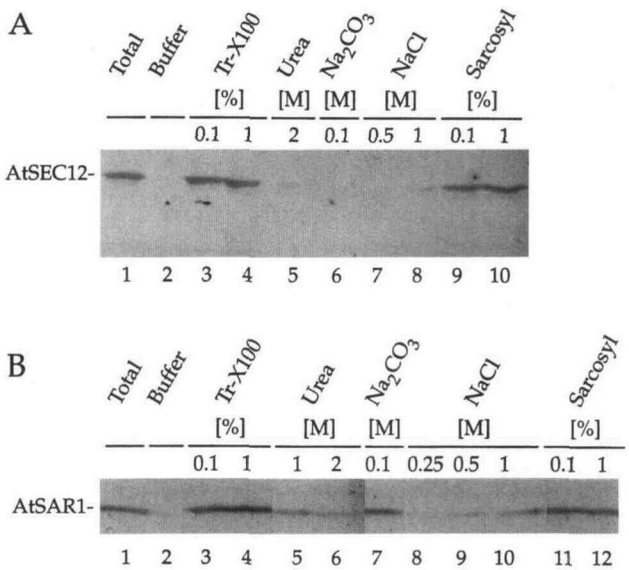

Figure 5. AtSEC12 is an integral ER-membrane protein and AtSAR1 is an ER-peripheral protein. Microsomal fractions were resuspended in various buffers for 30 min on ice and centrifuged at 150,000*g*, and supernatants were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-AtSEC12 (A) or anti-AtSAR1 (B) antibodies. A, Microsomes were resuspended in lysis buffer containing 150 mM NaCl, 1% sarcosyl, and 1% Triton X-100 (Total, lane 1); lysis buffer alone (lane 2); or buffer containing either 0.1 or 1% Triton X-100 (Tr-X100, lanes 3 and 4), 2 M urea (lane 5), 0.5 or 1 M NaCl (lanes 7 and 8), or 0.1 or 1% sarcosyl (lanes 9 and 10). Microsomes were also resuspended in 0.1 M sodium carbonate (lane 6). B, Microsomes were resuspended in lysis buffer containing 150 mM NaCl, 1% sarcosyl, and 1% Triton X-100 (Total, lane 1); lysis buffer alone (lane 2); or buffer containing 0.1 or 1% Triton X-100 (Tr-X100, lanes 3 and 4); 1 or 2 M urea (lanes 5 and 6); 0.25, 0.5, or 1 M NaCl (lanes 8, 9, and 10); or 0.1 or 1% sarcosyl (lanes 11 and 12). Microsomes were also resuspended in 0.1 M sodium carbonate (lane 7).

to the amounts found in the membrane fraction (Fig. 6C, compare lane 1 with 2), and in transgenic plants overexpressing AtSAR1 the distribution of AtSAR1 appeared to be shifted. The majority of AtSAR1 was found in the cytosolic fraction (Fig. 6C, compare lanes 3 and 5 with lane 1). This suggests that the cell could not cope with more AtSAR1 in an active form (membrane associated), thus keeping it cytosolic, in an inactive form. In an attempt to modify AtSAR1 localization, we produced transgenic plants overexpressing full-length AtSEC12. However, no differences in AtSAR1 distribution were observed (Fig. 6C, compare lanes 7 and 8 with lanes 5 and 6).

Cold Alters AtSAR1 Localization

Cold treatment adversely affects transport of proteins along the secretory pathway; transport is slowed or arrested under low temperatures (Saraste and Kuismanen, 1984). Export of proteins from the ER is arrested at low temperature and such treatment is used during in vitro studies of vesicle formation from the ER (Rexach and Schekman, 1991). We have previously reported that in Arabidopsis cold treatment results in elevated levels of *AtSAR1* mRNA compared with control (Bar-Peled et al., 1995). To investigate the effect of cold at the protein level, we placed cell-suspension cultures at 8°C for up to 5 d and measured, by immunoblotting, the relative distribution of AtSAR1 between the cytosol and the membrane fractions. The levels of AtSAR1 in the cytosol and membrane appeared to be similar in control cell-suspension cultures (Fig. 7, lanes 1 and 2); however, a 12-h cold treatment seemed to lead to an overall decrease in AtSAR1 levels in both soluble and membrane fractions (Fig. 7, compare lanes 3 and 4 with lanes 1 and 2), while not affecting AtSEC12 levels. Furthermore, the distribution of AtSAR1 was altered, with relatively more AtSAR1 found in the soluble fraction compared with the membrane fraction (Fig. 7, compare lane 3 with 4). Cell suspensions treated for 3 or 5 d in the cold showed a more pronounced alteration (Fig. 7, lanes 7 and 8). As a control, cell-suspension cultures had been treated with tunicamycin, a drug affecting *N*-linked protein glycosylation in the ER. Previously, we reported that application of tuni-

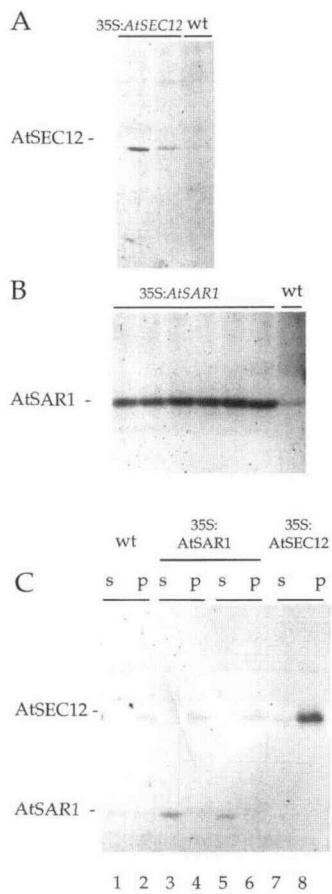

Figure 6. Distribution of AtSAR1 and AtSEC12 in transgenic plants. Immunoblot assays of total protein fractions (s1) from 3-week-old leaves of wild-type (wt) Arabidopsis plants or transgenic homozygous independent lines overexpressing AtSAR1 (A) or AtSEC12 (B). C, Equal amounts of total soluble (s = s150) or membrane (P = p150) proteins from wild-type plants (lanes 1 and 2), two independent transgenic plants overexpressing AtSAR1 (lanes 3–6), or transgenic plants overexpressing AtSEC12 (lanes 7 and 8) were analyzed by immunoblot assays.

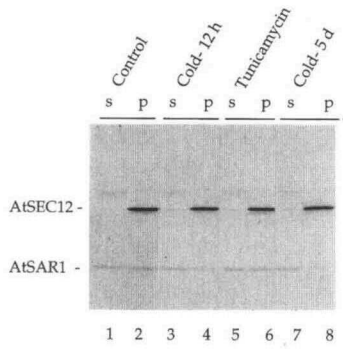

Figure 7. Cold shock shifts AtSAR1 to the cytosolic fraction. Arabidopsis cell-suspension cultures were grown under normal conditions (control, lanes 1 and 2) or were placed at 8°C for either 12 h (lanes 3 and 4) or 5 d (lanes 7 and 8) or were treated with tunicamycin (lanes 5 and 6). Soluble (s = s150) or membrane (P = p150) proteins were isolated and equal amounts were separated on SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted with anti-AtSAR1 and anti-AtSEC12 sera.

camycin affects the transcript levels of several secretory pathway genes (Bar-Peled et al., 1995); however, tunicamycin did not appear to affect the distribution of AtSAR1 between cytosol and microsome when compared with control (Fig. 7, compare lane 5 with 6).

DISCUSSION

We have characterized the properties of two plant proteins, AtSEC12 and AtSAR1, which are thought to be involved in the formation of ER-to-Golgi transport vesicles. In yeast and mammalian cells, Sar1p is partially cytosolic and partially membrane-bound (Nakano and Muramatsu, 1989; d'Enfert et al., 1991a; Nishikawa and Nakano, 1991; Kuge et al., 1994; Oka and Nakano, 1994). Cytosolic Sar1p is exchanged to GTP form by the ER protein Sec12p, and becomes membrane-bound, activated Sar1p. Subsequently, Sar1p initiates the assembly of the COPII coat structure, composed of the Sec23/24p and Sec13/31p complexes, catalyzing the formation of an ER-derived vesicle (Barlowe et al., 1994). Sar1p hydrolyzes GTP in a reaction stimulated by Sec23p (Yoshihisa et al., 1993), presumably leading to the dissociation of inactive cytosolic Sar1p-GDP from the coated vesicle. COPII (Sec23/24p and Sec13/31p coat structure) initiates budding of the transit vesicle from the ER. We demonstrated that in plants both proteins, AtSEC12 and AtSAR1, co-localized and were found to be associated with the ER, as judged by subcellular fractionation of microsomes on Suc gradients containing EDTA or Mg. Thus, genetic (d'Enfert et al., 1992) and biochemical (this paper) approaches point to a stronger role for AtSEC12 and AtSAR1 in ER to Golgi transport in plant cells. Analysis of enriched, intact nuclear fractions demonstrated that AtSAR1 and AtSEC12 are highly enriched in isolated plant nuclei. This observation is not surprising since the outer nuclear membrane, which is a functional subdomain of the yeast ER (Preuss et al., 1991; Bednarek et al., 1995; Strambio-de-Castillia et al., 1995), consists of the yeast Sec12p. Furthermore, in plants an ER-soluble fusion-protein marker is localized both to the outer nuclei membrane and to the lumen of the ER (Herman et al., 1990). To the best of our knowledge, AtSEC12 is the first plant ER-membrane protein that has been characterized biochemically and immunologically and thus could serve as a marker for the ER.

Although the size of the plant SEC12 is only 43 kD, compared with the 70-kD yeast protein, it is still able to complement the yeast mutant (d'Enfert et al., 1991a). This can be explained by the following similarity between the yeast and the SEC12 proteins. Like the yeast counterpart, AtSEC12 was found to be an integral ER-membrane protein with a single potential hydrophobic membrane-spanning domain, and its NH_2 terminus probably faces the cytosol. The cytosolic portion of the yeast Sec12p functions in exchanging the inactive GDP-bound Sar1p into the active GTP-bound Sar1 form (Barlowe and Schekman, 1993), and this portion has a high amino acid similarity to the plant protein. Furthermore, it has been shown that overexpressing the cytosolic portion of Sec12p in yeast cells results in the recruitment of Sar1p to the membrane (d'Enfert et al., 1991a). Thus, it is likely that neither glycosylation nor the luminal extension have an effect on Sec12p function (d'Enfert et al., 1991a), since the plant protein is able to complement the yeast mutant.

Hydropathy analysis predicted that AtSEC12 would be likely to have one transmembrane domain between amino acids 300 and 334. We thus further studied the membrane topology of AtSEC12 by immunoblot assays of microsomal preparations treated with trypsin in the presence or absence of 0.1% Triton X-100. Like the yeast counterpart, AtSEC12 was found to be a protease-resistant protein. Only at a high concentration of proteases (trypsin or proteinase-K) was AtSEC12 digested. Unfortunately, a high concentration of protease degrades vesicles derived from Arabidopsis cell-suspension cultures. Thus, we could not ascertain AtSEC12 membrane topology using the protease assay.

To date, homologs to the yeast SEC12 protein have been identified only in plant cells (d'Enfert et al., 1992); however, functional homologs to the yeast SAR1 were identified in mammalian (Kuge et al., 1994) and plant cells (d'Enfert et al., 1992; Davies, 1994). The plant AtSAR1 protein, like that of yeast, is approximately 22 kD and shares all of the characteristics of the SAR family, which is the conserved GTP-binding and C-terminal domains. The C terminus of members of the YPT1 and RAB protein family of small GTP-binding proteins is modified by the addition of a lipid, such as a farnesyl analog, which mediates membrane attachment (Ferro-Novick and Novick, 1993). Similarly, the small GTP-binding protein family, ARF, shares an NH_2-terminal consensus sequence for lipid myristylation (Ferro-Novick and Novick, 1993). In contrast, the mechanism that triggers the attachment of Sar1p to the membrane is still unclear. Neither the yeast nor the plant Sar1p homolog has a carboxy-terminal Cys or any site for a lipid modification that could explain its strong affinity for membranes (Nishikawa and Nakano, 1991; d'Enfert et al., 1992).

In yeast attachment to membranes of Sar1p requires activation of the protein from a GDP form to a GTP form. The membrane attachment of the yeast Sar1p is stimulated by elevated levels of Sec12p (d'Enfert et al., 1991a, 1991b), and Sec12p can stimulate dissociation of GDP from Sar1p, leading to Sar1p association with membranes (Barlowe et al., 1993). We could not detect more AtSAR1 associated with membranes in transgenic plants overexpressing AtSEC12. It is possible, therefore, that in plants interaction between AtSAR1 and AtSEC12, which leads to recruitment of AtSAR1 to the membrane, is mediated by other regulatory proteins that may be missing in yeast cells. We postulate that in plant cells a regulatory mechanism may exist that could monitor secretory activity. Such regulation will prevent attachment of AtSAR1 to the ER membrane if it is not required by the system. This could explain why a large portion of the AtSAR1 protein in transgenic plants overexpressing *AtSAR1* is in a cytosolic form. Further support for the existence of a regulatory mechanism monitoring the recruitment of AtSAR1 to the membrane was obtained with the cold-treatment experiments. Under cold-shock conditions, the amount of the GTP-exchange protein, AtSEC12, on the ER membrane is not altered; however, the levels of AtSAR1 associated with the membrane appeared to be significantly reduced compared with the control. The distribution and amounts of AtSAR1 between the cytosol and membranes in plants is also dependent on the type of tissue and on environmental stimuli that may arrest (pause or slow) budding, such as cold shock. It is obvious that more studies should be performed to understand the effect of cold on vesicle trafficking in plants.

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