Highlights

- The plant Na^+/H^+ antiporter SOS1 is critical for salt tolerance.
- SOS1 is a homodimer folded into a transmembrane and a cytosolic domain.
- The regulatory mechanism is based on the concerted rearrangement of these domains
- The SOS1 structure provides a model valuable for biotechnological applications.

Structural insights on the plant Salt-Overly-Sensitive 1 (SOS1) Na⁺/H⁺ antiporter

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Running title: The structure of the plant SOS1 antiporter

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Summary

The *Arabidopsis thaliana* Na⁺/H⁺ antiporter SOS1 is essential to maintain low intracellular levels of toxic Na⁺ under salt stress. Available data show that the plant SOS2 protein kinase and its interacting activator, the SOS3 calcium-binding protein, function together in decoding calcium signals elicited by salt stress and regulating the phosphorylation state and the activity of SOS1.

Molecular genetic studies have shown that the activation implies a domain reorganization of the antiporter cytosolic moiety indicating that there is a clear relationship between function and molecular structure of the antiporter. To provide information on this issue, we have carried out *in vivo* and *in vitro* studies on the oligomerization state of SOS1. In addition, we have performed electron microscopy and single particle reconstruction of negatively stained full length and active SOS1. Our studies show that the protein is a homodimer that contains a membrane domain similar to that found in other antiporters of the family, and an elongated, large and structured cytosolic domain. Both the transmembrane and cytosolic moieties contribute to the dimerization of the antiporter. The close contacts between the transmembrane and the cytosolic domains provide a link between regulation and transport activity of the antiporter.

Introduction

Salinity is a significant environmental factor that adversely affects plant growth and agricultural productivity. The Salt Overly Sensitive (SOS) pathway is responsible for Na^+ homeostasis in plants.¹ SOS1 is a plasma membrane Na^+/H^+ antiporter that is regulated by the SOS2 Ser/Thr protein kinase and two alternative interacting activators, the calcium sensors SOS3/CBL4 and SCaBP8/CBL10.²⁻⁴ An active SOS2-SOS3 complex is responsible for the phosphorylation and subsequent transport activation of SOS1 in response to salt stress. The transporter, in turn, extrudes the toxic Na^+ from the cytoplasm.^{2,3,5} The SOS1 protein is predicted to have an N-terminal transmembrane (TM) region composed of 13 α -helices and a long cytoplasmic tail of around 700 amino acids at the C-terminal end.⁶ The TM portion has sequence similarities with plasma membrane Na^+/H^+ exchangers from animal, bacterial, and fungal cells.^{7,8} On the other hand, the cytosolic moiety is more variable in terms of sequence or size among different species.⁸

Biochemical and molecular genetics studies have shown that the SOS1 cytosolic moiety can be divided functionally into an activation domain, essential for the activity, and an autoinhibitory domain.⁵ The interaction between them maintains the transporter in a resting state. The

phosphorylation of the autoinhibitory domain by the SOS2 kinase activates sodium transport.⁵ The Arabidopsis MAP kinase MPK6 also phosphorylates the C-terminal moiety of SOS1 contributing to the regulation of the antiporter.⁹ In addition, it has been shown that SOS1 interacts with RCD1 and functions in the oxidative stress response.¹⁰ These findings suggest that a variety of protein-protein interactions with the SOS1 cytosolic domain regulate its function. This would imply that SOS1 represents a central hub coordinating different stimuli in response to different stress signals, thus, constituting an important target for biotechnological applications.

SOS1 belongs to the Cation Proton Antiporter (CPA) superfamily of proteins. Structural studies with the bacterial Na^+/H^+ antiporters, NhaA from *Escherichia coli* and NhaP1 from *Methanococcus jannaschii*, have shown that these antiporters are dimers in the membrane and consist of 12 and 13 TM helices, respectively. Their activation is triggered by changes in pH, which suggests structural changes associated with the two transmembrane helices involved in sodium transport.¹¹⁻¹⁶ As in these two antiporters, *Physcomitrella* SOS1 transport is coupled with changes in cytosolic pH.¹⁷ This suggests a common regulatory mechanism where there may be concerted activation by pH and by SOS2-SOS3. There is little three-dimensional structural information on transporters containing long regulatory C-terminal tails, except for the electron microscopy (EM) structure of the full-length HsNHE1. This transporter has a cytosolic domain of around 300 amino acids and has a dimeric structure in which the cytoplasmic region of the dimer forms a single structural domain.¹⁸ On the other hand, despite plant Na^+/H^+ antiporters constitute an important branch within the CPA superfamily with potential biotechnological applications, they have never been characterized from a structural point of view.

We have performed *in vivo* studies showing that the full-length SOS1 antiporter from the model plant *Arabidopsis thaliana* forms dimers in the membrane. In addition, we describe the expression of SOS1 in *S. cerevisiae* and its purification. Reconstitution of the purified protein sample in proteoliposomes showed that SOS1 was biochemically active. Size exclusion chromatography and dynamic light scattering showed the dimeric nature of purified SOS1. Purified protein was used to determine the 3D structure by electron microscopy and single particle reconstruction techniques. Our analysis further confirms that SOS1 is a dimer that is stabilized by intermolecular contacts involving

both the transmembrane and cytosolic domains. The transmembrane domain displays a remarkable similarity to those found for other Na⁺ transporters of the CPA superfamily such as MjNhaP1 and EcNhaA. The cytosolic moiety displays an elongated and structured shape that provides structural basis to available molecular genetics data.⁵ Additionally, the electron microscopy reconstruction suggests that the cytosolic and transmembrane domains are in contact supporting a regulatory mechanism based on a concerted rearrangement where structural changes in the regulatory cytosolic domain are transmitted to the transmembrane domain.

Results

Expression, purification and oligomeric state of SOS1

The full-length SOS1 antiporter was expressed in *Saccharomyces cerevisiae*, an eukaryotic heterologous system in which SOS1 is fully active.³ The protein had a C-terminal 6x-His tag that allowed its purification from the membrane fraction at pH 8.5 using a Ni²⁺-affinity resin followed by a size exclusion chromatographic (SEC) step. The sample identity was verified by western blot using an antibody raised against SOS1 (Fig. 1a). The protein integrity was also verified by western blot and using an antibody against the C-terminal poly-Histidine tag (Fig. 1a).

The optimization of the purification protocol was crucial to obtain pure and homogeneous sample for structural studies. It was essential to complete the purification in 9 hours and to reduce the detergent contration to 0.05% to obtain a dimeric sample (Supplementary Fig. S1). The size exclusion chromatography profile (Fig. 1b) showed a peak of aggregates (elution volume of 0.94 ml) and a complex peak that could be deconvoluted into 3 peaks (OriginLab, Northampton, MA) with maxima at 1.24 ml (peak 1), 1.46 ml (peak 2) and 1.67 ml (peak 3). Peak 1 and peak 2 contained the full-length SOS1 and migrated above and below the 158 kDa gel filtration standard, respectively (Fig. 1b and 1c), while peak 3 contained degraded protein of low molecular weight (data now shown). The presence of low molecular weight contaminants and/or degradation products was estimated to be less than 15% of the total mass of the protein loaded in the gel. Proteins or fragments of proteins with such molecular weight are hardly detected in electron microscopy images and they are mainly visualized as background noise. We calibrated the gel filtration column using BioRad

standards to estimate the molecular mass of the eluted protein (Fig. 1d). Peak 1 and 2 corresponded to species with apparent molecular mass of 360 kDa and 120 kDa, respectively. The predicted molecular mass of full-length SOS1 is 127 kDa. Considering the fact that the membrane protein is purified with lipids and detergents surrounding the membrane domain, peak 1 could correspond with the dimeric protein and peak 2 could correspond to the monomeric and delipidized protein sample (Fig. 1d).¹⁹⁻²²

The homogeneity of the eluted Ni-NTA protein was also assessed by DLS, which showed that the protein sample was heterogeneous but also that a substantial proportion of sample formed dimers (Fig. 1e). A broad asymmetric size distribution ($Pd=0.78$) was obtained for the eluted Ni-NTA sample, which comprised two different populations (Gaussian distributions) ascribed to the monomeric ($d_{Hmax}=5.6$ nm) and dimeric ($d_{Hmax}=8.4$ nm) species. On the other hand, the experiment with the gel-filtered SOS1 peak 1 sample showed a characteristic narrower distribution ($Pd=0.40$) centered at the corresponding size of the dimeric ensemble, a result that further corroborated the homogeneity and the size of the dimeric SOS1 protein. The weight-average diameter of the dimeric ensemble obtained from the experiments was $d_{Hw}=9.5$ nm, consistent with the particle size visualized by negatively stained EM (see below).

Na⁺/H⁺ exchange activity of reconstituted SOS1

To assess that the purified SOS1 protein was biochemically active, Na⁺ transport assays were conducted by reconstituting the protein sample into proteoliposomes. Na⁺/H⁺ proton exchange in response to varying Na₂SO₄ concentrations was monitored by the fluorescent pH sensitive probe pyranine. The rate of exchange increased with increasing substrate concentration following a first order kinetics (Fig. 2a). Sodium gluconate salts produced lower exchange rates than sulfate and ZnSO₄ produced no exchange, indicating that proton exchange was specific to Na⁺ ions (Fig 2b). Control liposomes did not show significant antiport activity. Nonlinear regression fitting to Michaelis-Menten kinetics estimated a K_m of 54 ± 16 mM Na⁺ ($R=0.983$). Sodium gluconate salts produced lower exchange rates than sulfate and ZnSO₄ produced no exchange, indicating that proton exchange was specific to Na⁺ ions.

***In vivo* oligomerization state of SOS1**

To prove the dimerization of SOS1 *in vivo* we performed BiFC assays. The fluorescent complex, which is formed by two non-fluorescent halves of YFP, is restored through the association of interacting proteins fused to these fragments. Epidermal cells cotransformed with SOS1-nYFP and SOS1-cYFP displayed bright yellow fluorescence in the plasma membrane, indicating that these two proteins are capable of forming a dimer *in vivo* (Fig 3a and 3c). Signal was also detected in the endoplasmic reticulum, probably as a consequence of SOS1 overexpression.²³ To demonstrate the specificity of the BiFC analysis, SOS1-cYFP was coexpressed with the known interacting protein YFP-SOS2(CIPK24) or the non related plasma membrane transporter HKT1-nYFP. Yellow fluorescent signal was detected in the first case (Fig 3e) and no fluorescence could be observed in the second one (Fig 3g).

Electron Microscopy and Single Particle Reconstruction of SOS1

Freshly gel filtered and purified dimeric SOS1 protein was observed under the electron microscope for the structural determination. Homogeneous single particles of around 10 nm diameter were observed in the grids prepared with the sample from gel-filtered peak 1. On the other hand, a heterogeneous particle size distribution was observed in those grids prepared with the peak 2 sample and 3D reconstructions failed (Supplementary Fig. S2).

About 12,000 single images were collected from grids prepared with the peak 1 sample. They were reference-free classified and aligned to increase the signal to noise ratio by averaging images corresponding to similar views of the transporter. The reference-free averages revealed that the protein interacts with the support film in different orientations (Fig. 4b and Supplementary Fig. S3A). A clear two-fold symmetry axis was observed in some of the averages of the protein (upper row in Fig. 4b). This finding additionally supports that SOS1 is arranged as a dimer. Some other reference-free averages showed an elongated shape of different length with a main mass connected with a smaller one (lower column in Fig. 4b). These averages could represent side views of the transporter.

The three-dimensional combination of these reference-free averages by the common-line method implemented in EMAN,²⁴ produced a map that served as initial model for 3D Maximum likelihood classification (ML3D).²⁵ ML3D classification yielded two different maps that satisfactory explained all the reference-free averages described before (Fig. 4c and Supplementary Fig. S3B). The maps were calculated with and without imposing 2-fold symmetry and similar results were obtained (results not shown). Although both maps displayed a similar mass organization, the map from class 2 is smaller and has a more compact shape (Fig. 4c).

The SOS1 reconstruction can be divided into two regions. The first one is a globular domain that can be assigned to the transmembrane region and the second is an elongated and structured volume that can be assigned to the cytoplasmic region (Fig. 5). In the class 1 structure the cytoplasmic domain seems to be in a more extended conformation in comparison with the class 2 structure (Fig. 4c). The map representation threshold was chosen to accommodate the mass of the cytoplasmic domain of the dimeric SOS1 in the lower part of the maps. Although the envelope of the maps matches the shape of EcNhaA structure (PDB code 3FI1) (Fig. 5), at this threshold the fitting is slightly loose. This observation is consistent with an undefined belt of detergents and lipids around the transmembrane domains. The resolution of the structures, calculated using a 0.5 cut-off of the FSC, was about 25 Å (Supplementary Fig. S3C).

Discussion

The *Arabidopsis thaliana* plasma membrane Na⁺/H⁺ antiporter SOS1 is essential for plant salt tolerance.¹ SOS1 belongs to the CPA family of Na⁺ transporters. Structural studies of some homologous proteins, HsNHE1, EcNhaA and MjNhaP1, have shown that the members of this family consist of 12 to 13 transmembrane helices arranged as dimers.¹¹⁻¹⁶ These studies showed that transport regulation depends on subtle rearrangement of some transmembrane helices in response to pH. On the other hand, SOS1 transport regulation depends on a large cytosolic multidomain C-terminal moiety of 700 amino acids.⁵ Thus, the knowledge of full-length SOS1 structure is fundamental and provides opportunities to understand, at the molecular level, the mechanism that regulates the activity of this critical protein for biotechnological applications.

The *S. cerevisiae* expression system was used to overexpress a C-terminal His-tagged version of SOS1 protein. Membrane protein solubilization requires high concentration of detergent that should be carefully reduced during the purification procedure to preclude the delipidification and consequent destabilization of the native protein.^{20-22,26} Thus, a careful optimization of the solubilization and purification protocol it is necessary to produce pure, homogeneous and active protein sample for structural studies. To purify SOS1, this included speeding up the process to 9 hours, the adjustment of the optimal detergent and glycerol concentrations, buffer ionic strength, pH and working temperature.

The purified SOS1 antiporter directly catalyzed Na^+/H^+ exchange when it was reconstituted into artificial liposomes. The apparent K_m for Na^+ was relatively high (ca. 54 mM), but this is probably because SOS1 remains in a state with basal activity unless activated by SOS2 phosphorylation⁵

The preliminary biophysical characterization of the SOS1 sample by EM, DLS and SEC showed that the purified protein consisted of a mixture of monomeric and dimeric SOS1 protein species. *In planta* studies verified that SOS1 dimerizes at the plasma membrane. Thus, we conclude that the functionally relevant protein is a dimer.

SOS1 protein was analyzed by single particle electron microscopy to determine the molecular structure of the antiporter. The three-dimensional reconstruction at 25Å resolution displays a defined conformation. The most obvious structural features are the dimeric nature of the protein and the segmentation between the TM and cytosolic domains. (Fig. 5). The comparison of the EM reconstruction at low and high density thresholds showed that the central area of the reconstruction subdivides whereas a continuous density is observed at the TM and cytosolic domains. This clearly indicates that SOS1 dimer is stabilized by intermolecular contacts involving both domains (Fig. 5).

The atomic coordinates of the homologous EcNhaA structure (PDB code 3FI1)¹⁴ were fitted into the EM density map of SOS1 (Fig. 5a and 5b). The quality of the fit allowed us to unambiguously orientate this atomic structure into the dimeric TM domain of SOS1 and to identify the boundaries of this region at the upper part of the complex (as depicted in Fig. 5). However,

sequence analysis indicates that the SOS1 structure would be closer to that of MjNhaP1 than to that of EcNhaA ([Supplementary Fig. S4](#)). The main structural differences between these two transporters are found in the pattern of the dimerization interface interactions. While EcNhaA transporter shows a small β -sheet connecting both protomers,¹⁴ MjNhaP1 involves an extra N-terminal helix and tight interprotomer helix-helix contacts.¹⁶ Sequence analysis indicates that the SOS1 does not present the small β -sheet but contains the extra N-terminal helix. This suggests that dimerization of SOS1 would display a similar pattern to that found in MjNhaP1. The fact that the SOS1 electron microscopy reconstruction weakens at higher thresholds at the position of the NhaA central β -sheet supports this analysis ([Fig. 5c](#)).

The two classes of SOS1 reconstructions accommodate quite satisfactorily to the shape of the EcNhaA transporter structure, suggesting that the main changes between structures of class 1 and class 2 take place in the cytoplasmic domain of SOS1 ([Supplementary Movie S1](#)). The cytosolic domain of SOS1 has been proposed to act as a central hub coordinating the interaction of different proteins to orchestrate cell response to stress.⁵ Both EM reconstruction of SOS1 show that the cytosolic moiety forms an elongated structure that extends about 50 Å away from the TM domain. In this sense, the overall shape and size of the cytosolic moiety is extended enough to serve as a docking site for the regulatory SOS2⁵ and MPK6⁹ kinases and also to other proteins such as RCD1¹⁰ thus, merging different stress signals. However, the volume of this region in the class 1 reconstruction is consistent with the size of the SOS1 cytoplasmic domain while the class 2 reconstruction displays smaller size. This suggests that class 2 would correspond to a SOS1 conformer that contains either a partially proteolyzed, unstructured or disordered domain. It is not unusual that flexible regions are smeared after EM refinement and therefore not resolved in EM maps.

Bioinformatic analysis based on secondary structure and globularity predictions and sequence conservation suggests that SOS1 C-terminal moiety consists of three distinct domains ([Fig. 5c](#)).⁶ The first one ranges from residues 526 to 740 and it is almost identical to the cytosolic domain of AtNHX8, a SOS1 homologue involved in lithium transport in Arabidopsis.²⁷ The second domain displays homology to cyclic nucleotide binding domains (CNBD). The third domain is linked to the

second one by a disordered region and it is predicted to include residues 998 to the C-term. Due to the low resolution of our 3D structure it is not possible to assign precisely the position of the predicted domains onto the 3D EM envelope. However, the cytoplasmic moiety forms two different regions at higher density thresholds (Fig 5c). The reconstruction appears to be subdivided in the vicinity of the TM moiety whereas the distal region displays a continuous density (Fig. 5c), suggesting that the latter contributes mainly in the dimerization of the transporter. Thus, we suggest that this region, the distal one, may correspond to the cyclic nucleotide binding domain that has been reported to be involved in the oligomerization of other transporters^{28,29} and the area at the vicinity of the TM domain may constitute the first domain (Fig. 5c). The third domain would be positioned where the differences between the class 1 and class 2 EM models are larger (Fig. 4c, 5c and Supplementary Movie S1), since biochemical data show that this domain undergoes a rearrangement upon protein activation and consequently, it should be flexible and could be partially disordered.⁵

Our work provides a structural framework to the previous molecular genetic studies showing that SOS1 cytosolic moiety can be divided functionally into an activation and an inhibitory moieties.⁵ Such studies suggested a model for SOS1 regulation in which the interaction between these two moieties allosterically regulates the activity of the antiporter. Our structural model provides the basis for such transfer of information since SOS1 is actually structured into subdomains connecting distal regions of the cytosolic domain to the TM domain (Fig. 5a and 5c). The dimeric structure of the transporter provides a stable, large and globular scaffold to serve as a docking platform to integrate the signals coming from different pathways and regulate Na⁺ transport. The detailed mechanisms for such processes remain to be investigated by means of higher resolution structural techniques.

Materials and Methods

Protein expression and purification

A cDNA encoding a C-terminal 6xHistidine tagged version of SOS1 was cloned into the yeast expression plasmid pYPGE15.³ This construct was transformed into the yeast strain AXT3K

(*Δena1::HIS3::ena4*, *Δnha1::LEU2*, *Δnhx1::KanMX4*) for protein expression. Thirty mL of an over-night culture were inoculated into 3 L of YPD and incubated in a shaker at 30°C for 24h.

An optimized purification protocol was developed for SOS1 with all steps performed at 4 °C and the purification completed in 9 hours. Yeast cells (26 g) expressing SOS1-His were resuspended in 26 ml buffer G (50 mM phosphate buffer, 300 mM NaCl, 20% glycerol, 2 mM β-mercaptoethanol (BME), pH 8.5). Yeast cells were disrupted in a bead-beater homogenizer (BioSpec Products) using 0.5 mm glass beads. Crude extract was separated from beads and debris by centrifugation at 1,800 x g. The final cell lysate was centrifuged at 256,630 x g for 50 min and 4 °C. The resulting insoluble membranous fraction (where SOS1-His was present) was incubated for 40 min on a roller with buffer G containing 0.5% N-dodecyl-β-D-maltoside (DDM) (Anatrace) for protein solubilization. Afterwards, a final centrifugation (15 min at 30,600 x g) was carried out to get a clear supernatant.

SOS1-His purification consisted of two chromatography steps, namely Ni-affinity chromatography followed by gel filtration. 250 μl Ni-NTA beads (Qiagen) were incubated with solubilized SOS1-His for 45 min. Beads were subsequently washed with buffer G plus 0.15% DDM and 20 mM imidazole. SOS1-His was eluted with buffer H (50 mM phosphate buffer pH 8.5, 300 mM NaCl, 10% glycerol, 0.05% DDM) plus 250 mM imidazole. Fractions of 250 μl were collected. Protein purity was analyzed by SDS-PAGE. Western blot analysis of the samples confirmed the identity and integrity of the purified full-length protein (Fig. 1a). The eluted SOS1-His protein sample was injected into a Superdex 200 PC3.2/30 gel filtration column (GE Healthcare), previously equilibrated with buffer H, for an extra polishing step (Fig. 1b and 1c). Deconvolution of the complex gel filtration trace was performed using the Origin software (version 6.0). The trace was fitted to three or four peaks described by Lorentzian or Gaussian curves using least square analysis. All the fittings produced similar results. Thus, the simplest model (minimum number of peaks) with the best correlation factor was chosen to describe the experimental data. The column was calibrated with molecular weight standards from BioRad.

Production of antisera and western blotting

Polyclonal antibodies were prepared against the peptide RSFRKEHRGMSWPE, which is present in the carboxy-terminal region (amino acid sequence 1006-1015) of Arabidopsis SOS1. Young adult rabbits were immunized initially with 200 µg of the purified peptide in complete Freund's adjuvant. At 2-week intervals, the rabbits were given intramuscular booster injections of 100 µg of the peptide in incomplete Freund's adjuvant. The antisera were collected 7 days after the fifth booster injection.

Protein samples were separated on an 8% polyacrylamide SDS-PAGE gel and then electroblotted onto a Hybond-P PVDF membrane (GE Healthcare). The membrane was blocked for 1 hr with 2% Blotting-Grade Blocker (Bio-Rad) in Phosphate Buffered Saline (PBS) (Bio-Rad). Then the membrane was incubated overnight at 4°C with anti-SOS1 antiserum (1:5,000 dilution) or with anti-polyHistidine monoclonal antibody (1:1,000 dilution, Clone HIS-1, Sigma) in PBS-2% blocker. Secondary antibodies (Anti-Rabbit IgG (whole molecule)-Peroxidase antibody produced in goat and Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in rabbit, Sigma) were used diluted 1:5,000 in PBS-2% blocker for 1 hr at room temperature. The membrane was washed three times in PBS and incubated in SuperSignal West Pico Chemoluminescent Substrate (Thermo Scientific) for 5 min, and then exposed to x-ray film (GE Healthcare) for 1 min.

Electron microscopy and 3D reconstructions

Visualization of proteins as isolated particles is easily achievable by means of electron microscopy (EM) and negative staining of the samples.³⁰ Thus, after image processing, it is currently possible to give accurate results about the shape and size of biological complexes, in close agreement with other biophysical methodologies as dynamic light scattering.³¹ Purified SOS1 was analyzed with a transmission electron microscope after being adsorbed to glow-discharged carbon coated grids and stained with 2% uranyl formate. Grids were observed using a JEOL JEM-2100 transmission electron microscope operated at 200 kV and a nominal magnification of 20,000. Digital images were recorded under low dose conditions using an Orius Gatan CCD camera at a final pixel size of 3.3 Å. The contrast transfer function of the microscope for each micrograph was estimated using CTFFIND3³² and corrected using Bsoft.³³ A total of 12,785 single images of SOS1 were manually

extracted using EMAN.²⁴ 2D reference-free classification and averaging were performed using maximum-likelihood methods²⁵ implemented in XMIPP package.³⁴ Some averages representing projections of the complex from different points of view were selected to build up initial models by common line methods³⁵ using StartAny algorithm.²⁴ As some of the 2D averages displayed clear 2-fold symmetry, common line reconstructions were performed with and without symmetry producing similar results. The heterogeneity exhibited by the conformations of the SOS1 complex was analyzed using 3D maximum likelihood (ML3D) classification.³⁶ The initial map obtained by common line method was low-pass filtered and two seeds were generated by the addition of gaussian noise. Classes 1 and 2 (47% and 53% of the population) yielded maps with a dimension compatible with the mass of SOS1. Independent refinement of images of classes 1 and 2 starting from different initial maps yielded similar results indicating low model bias in the 3D reconstruction. The handedness of reconstruction was defined after fitting the structure of dimeric EcNhaA homologous transporter.¹⁴ The final volumes and fitting were visualized using UCSF Chimera³⁷ and Pymol³⁸ and deposited in the 3D EM database under accession code EMD-2181 and EMD-2182 (<http://www.ebi.ac.uk/pdbe/emdb/>).

Dynamic Light Scattering

Dynamic Light Scattering (DLS) measurements were performed on purified SOS1 from the Ni-NTA eluted protein and from the peak eluted from the gel filtration column using the Zetasizer Nano-ZS apparatus (Malvern Instruments, Worces-tershire, UK). About 14 µl of the solutions were transferred to clean dust-free microcells. The experiments were done after an equilibration time of 5 min at a temperature of $T = 12 \pm 0.02$ °C. DLS technique measures the time-dependent fluctuation in the intensity of scattered light that occurs because of the motion of the particles. The analysis of these fluctuations enables the determination of the translational diffusion coefficients, D , of particles, which can be transformed to a size distribution. The hydrodynamic diameter, d_H , of the particles was calculated using the Stokes–Einstein equation:

$$d_H = \frac{k_B T}{3\pi\eta D} \quad (1)$$

In this equation, k_B is the Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity, $\eta=1.596$ cP. A regularization fitting scheme has been used to find the distribution of particle sizes. In our case, the autocorrelation function of the scattered intensity has been analyzed by means of inverse Laplace transform by Thikonov analysis using the program SEDFIT (from <http://www.AnalyticalUltracentrifugation.com>), in order to obtain the size distribution by intensity.³⁹

Measurement of Na^+/H^+ exchange in vitro

His-tagged SOS1 protein was purified from yeast plasma membrane by Ni^{2+} affinity chromatography as described above. The purified protein was reconstituted in artificial proteoliposomes in the presence of the pH indicator pyranine and $(\text{NH}_4)_2\text{SO}_4$, following the method described by Venema *et al* (2002).⁴⁰ An internal acidification of vesicles was imposed by diluting the proteoliposomes in reconstitution buffer without $(\text{NH}_4)_2\text{SO}_4$. To measure Na^+/H^+ exchange, Na_2SO_4 was added to the incubation buffer and proton efflux coupled to Na^+ influx was monitored by changes in pyranine fluorescence.⁴⁰ Control liposomes did not show significant antiport activity⁴⁰

Generation of Bimolecular Fluorescence Complementation (BiFC) constructs

For BiFC assays the full length cDNA of *SOS1* was transferred to the pSPYCE(M) and pSPYNE173 plasmids⁴¹ using the XbaI and KpnI sites to create C-terminal translational fusions of SOS1 to the N-terminal and C-terminal moieties of YFP, respectively. The cDNA encoding the *Arabidopsis thaliana* HKT1 transporter was cloned into the pSPYNE 173 using the XhoI- KpnI sites. The resulting construct was used as negative control for the interaction with SOS1. The cDNA of SOS2 cloned into pSPYNE(R)173⁴¹ was used to assess the interaction with SOS1. All the YFP fusions were expressed under the control of the 35S promoter. Accuzyme polymerase (Bioline, Randolph, MA) was used for all the PCR amplifications. The PCR products were verified by sequencing.

Agrobacterium-mediated transient expression in *Nicotiana benthamiana*

Tobacco plants were grown in a growth chamber at 23°C with a 16-hour light and 8-hour

dark regime. BiFC plasmid constructs were mobilized into *Agrobacterium tumefaciens* strain GV3101 by electroporation followed by selection on YEP plates containing the appropriate antibiotics. For transient expression, *Agrobacterium* transformants containing the BiFC constructs and the p19 silencing plasmid⁴¹ were grown at OD₆₀₀ of 0.5 and of 0.6 respectively, and co-infiltrated in 5-6-week-old *N. benthamiana* leaves.

Confocal imaging

Agrobacterium-infected cells of the lower epidermis of *N. benthamiana* leaves were analyzed 48-72 hours after inoculation. Confocal imaging was performed using an inverted Olympus FluoView1000 laser microscope with a 60x, N.A. 1.20 water-immersion lens. YFP was excited with a 515 nm wavelength of an argon line and the emission was collected using a 530-630 nm band pass filter.

Accession numbers

The final volumes and fitting were deposited in the 3D EM database under accession code EMD-2181 and EMD-2182 (<http://www.ebi.ac.uk/pdbe/emdb/>).

FOOTNOTES

Abbreviations used:

SOS, Salt Overly Sensitive; CPA, Cation Proton Antiporter; TM, transmembrane; DDM, N-dodecyl- β -D-maltoside; EM, electron microscopy; SEC, size exclusion chromatography; DLS, dynamic light scattering; CNBD, cyclic nucleotide binding domain.

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FIGURE CAPTIONS

Fig. 1. SOS1 purification and biophysical characterization of the oligomeric state. (a) SDS-PAGE analysis of the Ni-affinity purified SOS1-His protein. Left, Coomassie blue stained. Right, western blot analysis using an anti-polyhistidine monoclonal and an anti-SOS1 polyclonal antibodies. Molecular weight markers (Mw) are indicated on the left (b) The size exclusion chromatogram of SOS1. The deconvolution of the gel filtration complex peak (black) is shown in grey. Molecular weight markers are indicated as vertical arrows. (c) Silver stained SDS-PAGE analysis of the gel filtration peaks 1 and 2. (d) Gel filtration column calibration curve. $K_{av} = (V_e - V_o) / (V_T - V_o)$, where V_e is the elution volume, V_o and V_T stand for the exclusion and the total volume of the column, respectively. (e) Mass percent size distributions of the samples studied obtained from DLS experiments: (blue squares) Eluted Ni-NTA sample and (red circles) Gel-filtered SOS1 peak 1 sample. The deconvolution of the mass percent size distribution of the eluted Ni-NTA sample is shown as broken lines.

Fig. 2. Na^+/H^+ exchange by SOS1 in proteoliposomes. His-tagged SOS1 was purified and reconstituted in artificial proteoliposomes in the presence of the fluorescent pH indicator pyranine to monitor proton fluxes. (a) Rates of Na^+/H^+ exchange were measured at increasing concentrations of Na_2SO_4 . The experiment was performed twice with independent protein preparations and similar results were obtained. (b) H^+ exchange is dependent of the monovalent cation. Representative transport traces using different salt substrates, 50 mM Na_2SO_4 (black line); 100 mM Na-gluconate (dashed line); 50 mM ZnSO_4 (dotted line). Arrow 1 indicates addition of salt (assay start, proton efflux) and arrow 2 indicates the addition of NH_4Cl (assay end, proton gradient dissipated). Fluorescence is given in arbitrary units.

Fig. 3. Dimerization of SOS1 in planta. Confocal fluorescence (a, c, e, g) and brightfield (b,d,f, h) images of *Nicotiana benthamiana* epidermal leaf cells (scale bars = 10 μ m) expressing the indicated plasmid combinations after 3 days of agroinfiltration. PM, plasma membrane, YN, N-terminal half of YFP; YC, C-terminal half of YFP. All YFP fusions were C-terminal, except SOS2 which had YFP fused to the N-terminal.

Fig. 4. 3D electron microscopy of dimeric SOS1. (a) Representative electron microscopy field of negatively stained peak 1 dimeric SOS1. Representative particles are boxed. (b) ML2D reference-free averages of SOS1. Averages with a clear 2-fold symmetry axis have been placed in the upper row. Side view averages have been placed in the lower row. (c) 3D maps from ML3D classification and refinement observed from different points of view.

Fig. 5. Molecular architecture of SOS1. (a) 3D structure of SOS1 is shown as a grey transparent density. The homodimeric fitted atomic structure of EcNhaA is displayed as a ribbon (PDB ID code 3FII). (b) A zoom of the TM domain in two different views. (c) Domain organization of SOS1. Top: the yellow box indicates the transmembrane (TM) domain; the blue box represents the cytosolic domain of SOS1 that shares high sequence homology with AtNHX8; the green and red boxes indicate the CNBD and autoinhibitory domains, respectively. Functional domains (activation and autoinhibitory, AH) described in Quintero et al.⁵ are also indicated. Bottom: the putative locations of the domains constituting the cytosolic moiety of SOS1 are overlaid on the electron microscopy 3D reconstruction displayed at higher threshold, the central β -sheet involved in the dimerization of EcNhaA is highlighted with a red circle.

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