A Barley Efflux Transporter Operates in a Na⁺-Dependent Manner, as Revealed by a Multidisciplinary Platform

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Plant growth and survival depend upon the activity of membrane transporters that control the movement and distribution of solutes into, around, and out of plants. Although many plant transporters are known, their intrinsic properties make them difficult to study. In barley (Hordeum vulgare), the root anion-permeable transporter Bot1 plays a key role in tolerance to high soil boron, facilitating the efflux of borate from cells. However, its three-dimensional structure is unavailable and the molecular basis of its permeation function is unknown. Using an integrative platform of computational, biophysical, and biochemical tools as well as molecular biology, electrophysiology, and bioinformatics, we provide insight into the origin of transport function of Bot1. An atomistic model, supported by atomic force microscopy measurements, reveals that the protein folds into 13 transmembrane-spanning and five cytoplasmic α-helices. We predict a trimeric assembly of Bot1 and the presence of a Na⁺ binding site, located in the proximity of a pore that conducts anions. Patch-clamp electrophysiology of Bot1 detects Na⁺-dependent polyvalent anion transport in a Nernstian manner with channel-like characteristics. Using alanine scanning, molecular dynamics simulations, and transport measurements, we show that conductance by Bot1 is abolished by removal of the Na⁺ ion binding site. Our data enhance the understanding of the permeation functions of Bot1.

INTRODUCTION

Plants regulate the acquisition of water and essential nutrients from the soil and their distribution within the plant through membrane transporters. Additionally, plants use membrane transporters as vehicles to dispose of or compartmentalize toxic substances. Limited information is available on the molecular function of most plant transport systems, which are challenging to explore due to their hydrophobic nature. Investigations of the fundamental function of plant transporters have been largely driven by genetics and physiology, but knowledge of molecular function is required if we are to modify the properties of these transporters to enhance uptake or export of specific nutrients (Schroeder et al., 2013; Bouguyon et al., 2015). Targets include improving the nutritional quality of plant products for humans, such as increasing iron and zinc, and exclusion of toxins such as cadmium and arsenic. Modifying nutrient fluxes is also important to protect plants from excessive accumulation of elements such as salt and boron, which become toxic at high concentrations. Determining the molecular function of transporters demands integrative approaches or new platforms. Using such platforms, rapid advances on plant transporters could deliver vital information leading to improved plant survival, yields, and nutritional quality (Schroeder et al., 2013).

In this work, we propose a multipronged approach that represents a synergy of biological and biophysical tools. This platform encompasses computational (molecular modeling combined with molecular dynamics simulations), biophysical (small-angle x-ray scattering and atomic force microscopy) and biochemical (cell-free synthesis combined with nanotechnology and recombinant protein expression) tools as well as molecular biology (site-directed mutagenesis), electrophysiology (oocytes and patched bilayers), and bioinformatics that are used for detailed descriptions of the molecular function of a transporter. As a case study, we examined a barley (Hordeum vulgare) efflux transporter Bot1 from landrace Sahara underlying high soil boron tolerance, for which a three-dimensional structure and molecular basis of permeation...
are unknown. This knowledge can be applied to target alternative sources of variation in landraces and wild relatives (Pallotta et al., 2014) and to generate new variants through editing of promoters and targeted genome editing (Li et al., 2012; Li et al., 2013) or with genetic engineering (Schroeder et al., 2013) to deliver improved crops.

Plant transporters facilitating tetrahydroxy borate anion \([\text{B(OH)}_2]^2\) efflux are classified in the 2.A.31 anion exchanger (AE) family, together with distant orthologs of mammalian and yeast carriers (Saier et al., 2014). Plant members of the AE family play important roles in tolerance to both high and low boron concentrations in soils (Takano et al., 2002; Nakagawa et al., 2007; Sutton et al., 2007; Pallotta et al., 2014). For example, under conditions of limited boron supply to Arabidopsis thaliana, the At-Bor1 transporter is expressed specifically on the inner plasma membrane of pericycle and endodermal cells of the root, exporting boron to the xylem for transfer to shoots (Takano et al., 2010). Similarly, rice pericycle and endodermal cells of the root, exporting boron to the approaching pK2002; Nakagawa et al., 2007; Sutton et al., 2007). In barley roots, and reduce yeast intracellular boron concentrations (Takano et al., 2007) facilitating the shoot accumulation of boron (Nakagawa et al., 2007). In barley, the efflux of borate anions can be reduced by anion channel inhibitors and is pH dependent, where efflux no longer occurs at pH values approaching the pK of boric acid (9.24) (Hayes and Reid, 2004). These observations are consistent with Hv-Bot1 operating as a borate anion-permeable transporter. Two alternative models for efflux of borate anions, based either on Hv-Bot1 working as an anion channel or through an anion exchange mechanism, have been suggested for Hv-Bot1 (Hayes and Reid, 2004). Another member of the AE 2.A.31 family, mammalian NaBC, functions as an electrogenic voltage-regulated and Na+-coupled borate anion transporter (Park et al., 2004), while YNL275w from Saccharomyces cerevisiae, confer tolerance to high boron and reduce yeast intracellular boron concentrations (Takano et al., 2002; Nakagawa et al., 2007; Sutton et al., 2007). In barley roots, efflux of borate anions can be reduced by anion channel inhibitors and is pH dependent, where efflux no longer occurs at pH values approaching the pK of boric acid (9.24) (Hayes and Reid, 2004). Similar oligomerization patterns of Bot1 were observed using cell-based expression in P. pastoris. Mono-, di-, and trimeric forms were readily detectable via immunoblot analysis, with trimeric Bot1 being the most abundant (Figure 1B). We confirmed the membrane localization of Bot1 by confocal imaging of chimeric Hv-Bot1-GFP in P. pastoris and in onion epidermal cells that transiently expressed the 3SS::Hv-Bot1-GFP construct (Supplemental Figure 2). Through SDS-PAGE we observed variation in the mobility of Bot1 forms derived from WG-CFPS and P. pastoris expression, which may be explained by differential solvation of proteins by SDS. This anomaly is not unparalleled (Rath et al., 2009).

**RESULTS**

**Full-Length Mono- to Trimeric Forms of Hv-Bot1 Are Produced through Cell-Free Synthesis and in Pichia pastoris**

Wheat germ cell-free protein synthesis (WG-CFPS) was used to produce full-length barley Bot1 via cotranslational insertion into liposomes in a decoupled bilayer mode (Periasamy et al., 2013). The translation reaction was performed in the presence of a variety of liposomes (Figure 1; Supplemental Figure 1), including a mixture of lipids to mimic the composition of a plant membrane (Magdy et al., 1994). SDS-PAGE indicated that Bot1 was embedded in mono-, di-, and trimeric forms in asolectin liposomes with a yield of 0.16 mg of monomer per milliliter of the reaction mixture (Figure 1A; Supplemental Figure 1). On the other hand, blue native PAGE revealed the presence of only trimeric Bot1 (Figure 1A). We examined WG-CFPS in the presence of mild surfactants Brij58, digitonin, and Tween 80 as well as acetylated surfactant peptides, which led to similar yields of monomeric Bot1 but to lower yields of oligomeric forms (Supplemental Figure 1), while nonacylated surfactant peptides were detrimental to WG-CFPS of Bot1. Given that the incorporation of membrane proteins in liposomes cannot be mediated by a protein-conducting channel (White and von Heijne, 2004), it is evident that the presence of soluble chaperones and native lipids in a wheat germ extract and a close proximity of ribosomes to liposomes maintain protein synthesis and help cotranslational insertion in an integration-competent conformation, without the need for surfactants. The identity of synthesized Bot1 was verified through immunoblot analysis (Figure 1; Supplemental Figure 1) and electrospray ionization mass spectrometry (ESI-MS) showing that peptide ions matched peptide sequences of Bot1 (listed in the Methods section under “Tryptic Mapping of Bot1 by ESI-MS”).

Similar oligomerization patterns of Bot1 were observed using cell-based expression in P. pastoris. Mono-, di-, and trimeric forms were readily detectable via immunoblot analysis, with trimeric Bot1 being the most abundant (Figure 1B). We confirmed the membrane localization of Bot1 by confocal imaging of chimeric Hv-Bot1-GFP in P. pastoris and in onion epidermal cells that transiently expressed the 3SS::Hv-Bot1-GFP construct (Supplemental Figure 2). Through SDS-PAGE we observed variation in the mobility of Bot1 forms derived from WG-CFPS and P. pastoris expression, which may be explained by differential solvation of proteins by SDS. This anomaly is not unparalleled (Rath et al., 2009).

**Thickness of Liposomal Bilayers with Cotranslationally Inserted Bot1 Is Locally Perturbed**

Studies of membrane structures using small-angle x-ray scattering (SAXS) techniques are well established (Luzzati and Husson, 1962; Kucerka et al., 2007; Pabst et al., 2010), where changes in the local structure of membranes upon peptide/protein
insertion are detected by scattering approaches and models of electron density profiles are generated (Pabst, 2006). Here, insertions of Bot1 in membrane bilayers of liposomes were analyzed by SAXS under solution conditions, using synchrotron radiation to monitor the local perturbation of the bilayer structure following WG-CFPS. Modeled bilayer electron density profiles derived from SAXS data (Pabst, 2006) for multilamellar 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and unilamellar asolectin liposomes (Figure 2) indicated that significant alteration of the bilayer structure accompanied insertion of Bot1 (Figure 2; Supplemental Table 1). The position of the head group ($\rho_0$), hydrocarbon tail width ($\rho_r$), and amplitude ($\rho_\phi$) were altered for both tested liposomal environments. The average distances between phosphate head groups across both bilayers ($d_{ph}$, taken to be the approximate bilayer thickness) decreased upon insertion of Bot1 in DMPC and asolectin liposomes consistent with membrane embedding. Similar findings were reported for nanodisc-reconstituted bacteriorhodopsin (Kynde et al., 2014). In summary, the SAXS data demonstrate that Bot1 was inserted in the bilayer, as its thickness was locally perturbed.

An in Silico Atomistic Model Reveals Complex Structural Folding of Bot1

3D structural information on plant, mammalian, or yeast carriers of the AE 2.A.31 family is unavailable. To gain insight into the structural framework of the plant members of this family, we constructed in silico an atomistic model of Bot1 ($H. vulgare$, genotype Sahara 3771) consisting of 574 residues, although we acknowledge that computational models have interpretative limitations. Based on the crystal structure of the uracil/H$^+$ symporter (Lu et al., 2011), identified as a distant structural homolog of Bot1 through I-Tasser (Zhang, 2008), we predicted dispositions of secondary structural elements and boundaries of membrane $\alpha$-helices, using a variety of independent tools (cf. Methods), to arrive at the consensus topology for Bot1. The Bot1 protein is predicted to fold into a complex architecture containing an independently folded membrane and cytoplasmic $\alpha$-helices (Figure 3). The membrane-integral part of the Bot1 model resembled the inward-open conformation of the uracil/H$^+$ symporter, which harbors 13 transmembrane $\alpha$-helices and forms a singular pore (Lu et al., 2011). The topological distributions of secondary structures indicate that the N terminus of Bot1 is exposed to the cell exterior, while the C terminus is oriented to the cell interior. Notably, nearly all potential phosphorylation sites (16 out of 18) are located on the intracellular side of the protein or on short intracellular $\alpha$-helices. Seven $\alpha$-helices shape the pore (Figures 3A and 3B, pink), while six additional $\alpha$-helices surround the pore-forming $\alpha$-helices, some of which are kinked (Figures 3A and 3B, green). In addition to the membrane $\alpha$-helices, five short $\alpha$-helices form an independently folded domain we have named “sombrero,” which protrudes from the membrane plane by $-17$ Å into the cell interior (Figures 3A and 3C). MD simulation of Bot1 in the environment of a dioleoylphosphatidyl-choline (DOPC) lipid bilayer (Figure 3C) revealed the presence of a Na$^+$ binding site that co-localizes to the proximity of pore-forming $\alpha$-helices (Figure 3; Supplemental Figure 3). A close view of the Na$^+$ ion binding site indicated that six residues participate in the nonahedral coordination of Na$^+$. These are Asn-63 (carboxyl O and OD1), Thr-68 (carboxyl O and OG1), Glu-70 (OE1 and OE2), Thr-71 (OG1), Gin-113 (OE1), and Gly-117 (carboxyl O). While four Na$^+$ bonding oxygen atoms (Asn-63, OD1; Thr-68, OG1; Glu-70, OE2; Thr-71, OG1) were identified at distances of $-2.2$ to 3.2 Å during the last 34 to
60 ns of MD simulation time, the other five Na+-bonding partners (Asn-63, carbonyl O; Thr-68, carbonyl O; Glu-70, OE1; Gln-113, OE1; Gly-117, carbonyl O) were at distances of 3.2 to 5.6 Å (Supplemental Movies 1 and 2). Surface electrostatic potential calculations predicted a higher density of electronegative charges near the Na+ binding site, while a lower density of charges was observed in the membrane-embedded regions (Supplemental Figure 3).

Given that Bot1 readily forms di- and trimers irrespective of the lipid system via cell-free synthesis (Figure 1A; Supplemental Figures 1A and 1C) and through recombinant expression in P. pastoris (Figures 1A and 1B), we predicted quaternary structures of Bot1 using SymmDock, which uses geometry-based docking and searches for complementary interfaces between neighboring protomers. We obtained rational estimates of spatial dispositions of individual protomers within di- to hexameric assemblies (Figure 4). The predicted trimer conformation stood out as the most compact, with the lowest interface atomic contact configuration (Figures 3E and 4). Based on electrophoretic analyses and docking calculations, we propose that trimeric Bot1 is the predominant conformation in planta or that mono-, di-, and trimeric forms are in a dynamic equilibrium. It is common for transporters to form multimeric complexes, e.g., the structures of functional dimers of chloride channels (Lim et al., 2013; Stölting et al., 2014) and trimers of ammonium transport proteins (Khademi and Stroud, 2006; Wacker et al., 2014) have been described.

**Bot1 Protrudes 1.7 nm above the Bilayer Surface**

Atomic force microscopy (AFM) is considered to be superior to other imaging techniques in that images can be taken at ambient environment. AFM of Bot1 that was cotranslationally inserted in DMPC liposomes revealed distinctive morphological and structural characteristics. The images were compared with those of bilayers of empty DMPC liposomes and DMPC liposomes that underwent WG-CFPS in the absence of Bot1 mRNA (Figure 5). We only observed surface-protruding features in the DMPC liposomes containing Bot1 (Figure 5C). In 66% of these high-resolution scans (≥1 × 1 μm; minimum pixel resolution 512 × 512), we found protruding features on an otherwise flat surface, which we presumed to be Bot1 integrated into the bilayer (Figure 5C, white arrows). The average values of heights and diameters of these features were 1.7 ± 0.6 nm and 19.2 ± 7.5 nm, respectively. By contrast, only 10% of high-resolution scans on the DMPC samples undergoing WG-CFPS in the absence of Bot1 mRNA presented any form of protrusions (Figure 5B), while no protrusions (Figure 5A) were observed in empty DMPC bilayers. It may be expected that Bot1 is inserted in liposomes in either orientation; therefore, we looked for two distinct populations of protruding features through AFM. It is theoretically possible for AFM to detect the Bot1 protein inserted in the opposite orientation (given that the atomistic model predicted these protrusions to be 9 to 10 Å and the vertical detection limit of AFM was calculated to be 0.1 nm), but our measurements did not reveal their presence. Only a single topology feature was observed (Supplemental Figure 4). The height (1.7 nm) of the protrusions in bilayers was consistent with the modeled size of the sombrero component, which was structurally predicted to extend from the membrane by approximately the same distance. These observations suggested that Bot1 was incorporated in DMPC liposomes essentially uniformly with its N terminus directed to their exterior surface (Supplemental Figure 2.

**Figure 2. Global Analysis of Bot1 Liposome Bilayer Profiles Using SAXS.**

DMPC (A) and asolectin (B) liposomes. I(q) represents the scattering intensity, and q is the magnitude of the reciprocal space momentum transfer vector. Fits of modeled bilayer electron density profiles to experimental SAXS data are for empty (open circles) and Bot1-inserted (full circles) liposomes. Insets show the corresponding bilayer electron density profiles of empty (solid lines) and Bot1-inserted (dashed lines) liposomes, where the bilayer electron density, ρ, is plotted on the vertical axis against the distance from the center of the bilayer, z, on the horizontal axis.
Figure 3. An in Silico Atomistic Model of Bot1 and the Architectures of Its Oligomeric Assemblies.

(A) and (B) A cartoon of the Bot1 model viewed along the membrane plane (A) and from the cell interior (B), revealing 13 transmembrane α-helices (pink and green) and five short α-helices (blue). Views in (A) and (B) are related by 90° about the horizontal axis.

(C) The structure of Bot1 embedded in a DOPC bilayer. DOPC surrounding Bot1 are within 8 Å distance. The lipid bilayer thickness is between 28 to 29 Å.

(D) Details of the Na⁺ ion binding site with six residues Asn-63 (carbonyl O, OD1), Thr-68 (carbonyl O, OG1), Glu-70 (OE1 and OE2), Thr-71 (OG1), Gln-113 (OE1), and Gly-117 (carbonyl O).

(E) Predicted dimeric (left) and trimeric (right) assemblies viewed along the membrane plane (top) or from the cell interior (90° rotation of the left panel to the viewer) (bottom). Cartoons of the left-hand side protomers are colored as in (A). Two other protomers are colored in cyan (left) or cyan and yellow (right) with intracellular α-helices in blue. All protomers are depicted with surface representations. Images in (A) to (E) were created in PyMOL.
The values of interface atomic contact energies were rounded to three and four significant digits. Individual protomers in cartoon and surface representations are color-coded. The images were created in PyMOL.

**Figure 4.** Interface Atomic Contact Energies and Spatial Arrangements of Multimeric Forms of Bot1 Were Calculated Using SymmDock.

<table>
<thead>
<tr>
<th>Multimeric form of Bot1</th>
<th>Interface atomic contact energy (kcal/mol)</th>
<th>Spatial arrangement</th>
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<td>Dimer</td>
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<tr>
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<tr>
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<tr>
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<td>Hexamer</td>
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**Bot1 Is a Channel-Like Transporter with High Affinity for Borate Anions and a Requirement for the Na\(^+\) Ion**

To examine the transport properties of Bot1, we used (1) two-electrode voltage clamp (TEVC) electrophysiology of *Xenopus laevis* oocytes expressing Bot1 (Supplemental Figure 5) and (2) patch-clamp electrophysiology (Steller et al., 2012) of giant asolectin liposomes with cotranslationally inserted Bot1 (Figures 6 and 7).

The ability of Bot1 to mediate electrogenic movement of borate anions was demonstrated by electrophysiology, where TEVC of X. *laevis* oocytes injected with Bot1 capped RNA (cRNA) increased both inward and outward currents in the presence of external boronic acid at three pH values (pH 7.0, 8.0, and 9.0), whereas no boronic acid-inducible currents were detected in water-injected control oocytes (Supplemental Figure 5). The largest current differences between the control oocytes and the Bot1-cRNA-injected oocytes were recorded at pH 7.0, while smaller currents were measured at pH 9.0. The intersection reversal potential (E<sub>rev</sub>) values plotted against borate concentrations for the X. *laevis* Bot1 cRNA-injected oocytes indicated a trend line with \( r = -0.888 \). The individual intersection E<sub>rev</sub> values were 49.9, -12.5, and -59.9 mV at pH 7.0 (0.14 mM borate), 8.0 (1.33 mM), and 9.0 (9.0 mM), respectively (Supplemental Figure 5). Assuming a pH value of 7.69 in the cytosol of *X. laevis* oocytes (Sasaki et al., 1992) and complete equilibrium of boric acid across the plasma membrane, intersection E<sub>rev</sub> values indicated that Bot1 operated as a passive uniporter in a Nernstian manner with respect to borate anions.

Patch-clamped giant liposomes with Bot1 were used to further study transport properties of Bot1 (Figure 6). While no currents were observed when liposomes underwent WG-CFPS in the absence of Bot1 mRNA, we observed channel opening and closing events with liposomes containing Bot1. At least two levels of conductance states were observed that could potentially be attributed to openings of individual protomers (Figure 6A). These data indicated that Bot1 operated as an ion channel between -60 mV and +100 mV (Figure 6A). The currents were completely abolished (Figure 6A) by applying 80 µM 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), a known blocker of anion channels (Liu et al., 1998). Currents measured with NaCl and boric acid (5 mM each on the internal face, pH 7.5) and increasing concentrations of borate ions (0 to 0.85 mM on the external side, pH 7.5), moved E<sub>rev</sub> values toward more negative values (Figure 6B); a good correlation \( r = -0.996 \) was observed from the plot of E<sub>rev</sub> as a function of borate concentrations. This followed the calculated shifts in the Nernst potential for borate anions (0, -8.5, -18.4, and -35.9 mV at 0.085, 0.2125, 0.425, and 0.85 mM borate, respectively), which was consistent with the movement of borate anions via Bot1. The movement of borate anions through Bot1 was also evident from the plot of E<sub>rev</sub> as a function of borate concentrations, where a linear correlation was obtained (Figure 6B, inset). No currents were observed when borate anions were absent or when NaCl (internal face) was replaced with KCl or LiCl on the same bilayer. These observations validate our MD simulations suggesting the existence of a Na<sup>+</sup>-ion binding site within the structural fold of Bot1 (Figure 3; Supplemental Figure 3).

Borate anion transport via Bot1 followed Michaelis-Menten \( (v_0 = V_{\text{max}}[S]/(K_m + [S])) \) kinetics with \( K_m = 104.1 \pm 5.4 \) µM and \( G_{\text{max}} = 15.8 \pm 0.18 \) pS; Figure 6C). Conductance measured in the presence of 25 mM chloride, phosphate, nitrate, and sulfate anions indicated that Bot1 permeated a variety of polyvalent anions (Figure 6D). Conductance values for chloride, phosphate, nitrate, and sulfate anions were 7.8, 7.3, 6.2, and 5.9 pS, respectively. In contrast to borate, with 0.5 to 10 mM of these anions, we could not observe reliable currents. Therefore, under physiological conditions, the predominant ion permeating Bot1 is likely to be monovalent tetrahydroxy borate, in line with the in planta physiological evidence (Sutton et al., 2007).

**The Na<sup>+</sup> Ion Binding Site Located Near the Pore Regulates Borate Permeation**

To examine if Bot1 operates as a Na<sup>+</sup>-dependent channel or a Na<sup>+</sup>-cotransporter, we measured currents in proteoliposomes with 0 to
75 mM concentrations of NaCl or sodium glutamate on the external side in the presence of 0.425 mM borate. This allowed us to investigate the kinetic properties of Bot1 in the absence of other proteins in a bilayer. The data showed that there were either no changes (Supplemental Figure 6, NaCl) or small changes (Figure 7A, sodium glutamate) in $E_{\text{rev}}$ values, suggesting that the Na$^+$ ion was activating currents rather than being transported by Bot1. We also derived the $K_v$ value for Na$^+$ based on conductance values at $-20$ to $+40$ mV with 0 to 75 mM sodium glutamate; $K_v$ was estimated to be $3.93 \pm 0.78$ mM. These data are in good agreement with in silico observations.

We systematically varied each of the six key residues (Asn-63, Thr-68, Glu-70, Thr-71, Gln-113, and Gly-117; Figure 3) of the Na$^+$ ion binding site both individually and in combinations by alanine scanning. We then measured transport function using patch-clamp proteoliposomes (Figures 7B and 7C) and by MD simulations evaluated Na$^+$ residence time, Na$^+$ diffusion coefficients and number of water molecules in the first hydration shells of the Na$^+$ binding site in wild-type and variant Bot1 (Supplemental Table 2 and Supplemental Figures 7 and 8). Transport measurements (using comparable levels of proteins; Figure 1C) demonstrated that while the permeation rates of borate anions in each of the single variants did not markedly deviate from those of wild-type Bot1, the transport rates of double (ATEAQG) and triple (AAEAQQG) mutagenized variants progressively decreased, while another double (NTETAA) mutagenized variant showed unaffected transport rates (Figure 7B; Supplemental Table 2). No transport was recorded for 4-fold (AAAAAQG), 5-fold (AAAAAG and AAEAAQ), and 6-fold (AAAAAA) variants (Figure 7B; Supplemental Table 2 and Supplemental Videos 3 and 4). Growth of S. cerevisiae cells expressing wild-type and variant sequences of Bot1 on solid medium with 20 mM boric acid (Figure 7C) correlated with the decreased transport capacity of the variants in patched bilayers (Figure 7B). MD simulations of wild-type and variant Bot1 revealed that the measured transport rates and formation of transport pores were interrelated with the predicted residence time of Na$^+$ in the ion binding site (12 to 50 ns) and the number of water molecules associated with Na$^+$ in a first hydration shell (Figure 8; Supplemental Table 2 and Supplemental Figures 7 and 8). Additional MD simulations of wild-type Bot1 with K$^+$ in the ion binding site or without an ion (Supplemental Table 3) demonstrated that Na$^+$ was the viable option for permeation (compared with Supplemental Tables 2 and 3). We observed that K$^+$ exhibited a lower diffusion coefficient than Na$^+$, which can be explained by nonspecific interactions of K$^+$ with residues inside the ion binding site. Interestingly, an experimental estimate of the energy associated with the most prevalent state, a K$^+$ monohydrate, is 18 kcal/mol and that of the most prevalent trihydrated Na$^+$ state is 15.78 kcal/mol (Rao et al., 2008) (Supplemental Figure 8), while an ab initio estimate of a hydrated borate cluster is 15.71 kcal/mol (Zhou et al., 2013). Energetically, the exchange of water molecules from hydration shells between ions will favor those with comparable thermodynamic penalties. For example, borate would prefer water exchange with Na$^+$ rather than with K$^+$, and if no hydrated cation cluster is present in the ion binding site, no borate transport occurs. On the other hand, pore dimension lengths are affected by the internal ion environment due to localized ion-residue interactions and hydration states of ions. Importantly, the disposition of pores in the Bot1 model and in the uracil/H$^+$ symporter structure, the latter used as an initial template for simulations, showed similar spatial characteristics. Evaluations of predicted pore dimensions of Bot1 (Chovancova et al., 2012) revealed that in the wild-type and mutagenized variants with preserved transport function, the pores penetrated through the $\alpha$-helical bundle, while only short tunnels were formed in non-transporting variants (Supplemental Tables 2 and 3 and Supplemental Figure 8).

**DISCUSSION**

Fundamental research of plant membrane transporters using genetics, physiology, and, in isolated instances, biophysics has led to the descriptions of transport mechanisms of a limited number of plant transporters. Consequently, most nutrient sensing mechanisms in plants remain unknown (Schroeder et al., 2013; Munns and Gilliham, 2015). As of November 2015, from 577 unique membrane proteins (http://blanco.biomol.uci.edu/mpstruc/), only three structures of plant transport proteins (an aquaporin from spinach [Spinacia oleracea], a nitrate transporter from Arabidopsis, and a SWEET transporter from rice) are known. Although structural biology (mainly x-ray crystallography) assists
with the precise definition of protein function at the molecular level, determining the structures of membrane proteins remains challenging. Alternative strategies are necessary to deliver a “blue print” approach for investigations of the molecular function of plant transporters, including those playing key roles in photosynthesis or abiotic stress tolerance (Schroeder et al., 2013; Pallotta et al., 2014). This knowledge can be applied to target alternative sources of variation in landraces and wild relatives (Pallotta et al., 2014), for editing of promoters and targeted genome editing (Li et al., 2012; Li et al., 2013), or with genetic engineering (Schroeder et al., 2013) to deliver improved crops.

Using our platform, we unraveled the molecular function of Bot1. We define (1) the transport function of Bot1 using cell-free synthesis combined with nanotechnology, site-directed mutagenesis, and MD simulations; and (2) an in silico atomistic model and its fine structural features, including the presence of a Na⁺ ion binding site that regulates permeation. We also use SAXS to confirm that Bot1 was inserted in the bilayer and AFM to estimate the height of the protrusions in the lipid bilayer.

Our atomistic model of Bot1 can be used to map and predict effects of changes on structure and function of specific residues. For example, we mapped the variant sites L234H and T541M found in the naturally occurring Bot1 allele in barley cultivar Haruna Nijo (Hayes et al., 2015). H234 is positioned on an interhelical loop and neighbors the fully conserved residue Arg-235, which likely fulfills a role in transport function, while Met-541 is located on an intracellular loop in a region of lower conservation (Supplemental Figure 9, asterisks). To validate our computational model, mutagenesis experiments have shown that L234H in Bot1 is critical, while T541M does not affect function (Hayes et al., 2015). Other

Figure 6. Transport Properties of Bot1 Using Patch-Clamped Proteoliposomes.

(A) Membrane potential dependency on unitary current fluctuations of Bot1 (internal side with 5 mM and external with 0.425 mM borate concentrations equivalent to 25 mM boric acid; symmetric 5 mM NaCl at pH 7.5) at holding potentials of between -60 and +100 mV. Single channel recordings with borate were observed. The data below the horizontal dashed line show inhibition of permeation by 80 µM DIDS.

(B) Dependency of conductance on borate (internal side with 5 mM, external with 0 to 0.85 mM concentrations equivalent to 0 to 50 mM boric acid at pH 7.5) at holding potentials of between -100 and +100 mV. Slope values are 0.0086 (r = 0.9961), 0.0118 (r = 0.9979), 0.0134 (r = 0.9592), and 0.0166 (r = 0.9910) for 0.085, 0.2125, 0.425, and 0.85 mM borate concentrations, respectively. Inset: a plot of E_{rev} values as a function of borate concentrations (slope value = -45.8115).

(C) Michaelis-Menten kinetics for borate transport.

(D) Dependency of conductance on symmetric (25 mM) concentrations of chloride, phosphate, nitrate, and sulfate anions, at pH 7.5. The values of shown SE of the mean are based on technical triplicates and in (B) to (D) represent 9, 1, and 8%, respectively.
residue changes found in fully functional Bot1 alleles from the cultivars Tadmor, Alexis, and WI4304 (N108S, K183E, and Y195F) were in noncritical regions of our atomistic model (Supplemental Figure 9).

MD simulations of Bot1 in a lipid environment revealed the presence of the Na⁺ ion binding site trapped among oxygen atoms, which may create a large activation barrier for Na⁺ to escape from this particular conformational state of Bot1 (Figure 3; Supplemental Figure 3). Additional MD simulations using alternative conformations of Bot1 indicated the presence of a second Na⁺ binding site, located in the vicinity of the fifth and sixth membrane α-helices. Although we have not explored the significance of the second Na⁺ binding site, its presence suggests that borate anions may approach the pore-forming region from several directions. Based on our atomistic model, we propose that Na⁺ cations may work as a cofactor and attract borate anions during certain stages of the transport event, creating a possibility for borate to be captured inside the Bot1 pore. Alternatively, Na⁺ may locally neutralize the negatively charged milieu of phospholipids to allow for passage of borate anions through the pore (Supplemental Movies 1 and 2). In good agreement with our in silico observations, we experimentally observed Na⁺ dependency of borate transport with 5 mM minimal concentration. This finding is rather unsurprising, given that co-occurrence of salinity and boron toxicity stresses has been well documented in an agricultural context, although the precise relationship between boron toxicity and salinity in plants is not clear (Grieve and Poss, 2000; Yermiyahu et al., 2008). For investigations of Na⁺ dependency of borate transport, we used proteoliposomes, enabling us to investigate the kinetic properties of Bot1 in the absence of other proteins in a bilayer. We acknowledge that further quantitative work, using, for example, isothermal calorimetry or surface plasmon resonance, will be required to firmly establish the apparent affinity of Na⁺ to Bot1.

Figure 7. Dependency of Conductance of Bot1 on Na⁺ and the Identity of Residues Constituting the Na⁺ Ion Binding Site.
(A) Dependency of conductance on symmetric 0.425 mM borate concentrations equivalent to 25 mM boric acid and 0 to 75 mM sodium glutamate at pH 7.5.
(B) Dependency of conductance on symmetric 0.425 mM borate concentrations equivalent to 25 mM boric acid, and symmetric 5 mM NaCl, at holding potentials of between −100 and +100 mV for the wild-type and double (ATEAQG, NTETAA), triple (AAEAQG), 4-fold (AAAGQG), and 6-fold (AAAAA) mutants of Bot1. Inset shows dependency of conductance on borate for wild-type and single (ATEAQG, NAETQG, NTATQG, NTEAQG, NTETAG, and NTETQA) and double (NTETAA) mutants of Bot1. The values of shown se are based on technical triplicates and in (A) and (B) represent 15% and between 4 and 10%, respectively.
(C) Functional analysis of residues participating in binding of Na⁺ by Bot1. Left and right panels 1 to 5 show growth of S. cerevisiae expressing wild-type and variant Bot1 in the presence (20 mM) or absence (0 mM) of boric acid, respectively. Yeast cells transformed with Bot1 (wild type), an empty plasmid (empty), and variant Bot1 are spotted on each plate in 10-fold serial dilutions from right to left.
residence time, while Asn-63, Gln-113, and Gly-117 may play supporting roles in Na\(^+\) binding, as indicated by the relatively high transport rates of the AAEQG variant (Supplemental Table 2). Alanine scanning suggested a key role of E70 in the Na\(^+\) ion binding site, although additional residues (Thr-68 or Gln-113 and Gly-117) were required to restore full borate transport or a boron tolerance phenotype in S. cerevisiae (Figure 7C; Supplemental Table 2).

Among the mutated forms of Bot1, there was good agreement between predicted Na\(^+\) residence time, Na\(^+\) diffusion coefficients, number of water molecules associated with Na\(^+\) in the first hydration shell, and borate transport rates (Supplemental Table 2). When the Na\(^+\) residence time was more than 12 ns, the mutated forms of Bot1 transported borate anions at rates equivalent to those of wild-type Bot1. All ions must first shed water molecules from their hydration shells to be able to enter protein pores for either transport or ion-protein interactions. The extent of dehydration depends on modeled protein structure, and in the case of Na\(^+\) in Bot1, this was related to the presence of specific residues in the ion binding site. The predicted hydration profile of a Na\(^+\) ion with long residency inside the ion binding site displayed multiple hydration states in the first hydration shell, of which states with two to three water molecules were prominent (Supplemental Figures 7 and 8). Inside the ion binding site, Na\(^+\) interacts with the oxygen atoms of residues to compensate for lost oxygen atoms from water molecules. As a result, distorted octahedron coordination geometry of Na\(^+\) was formed through interactions with both neighboring oxygen atoms and water molecules (Figure 8). Evaluations of predicted partial hydration of Na\(^+\) inside the ion binding site in wild-type and mutagenized Bot1 variants indicated that proteins with high transport rates contained fewer water molecules associated with Na\(^+\) in the first hydration shell than proteins that permeate less effectively (Figure 8; Supplemental Figures 7 and 8). A plausible hypothesis, based on the MD simulation data, is that a specific Na\(^+\) hydration state is required to create an energy barrier for efficient transport of anions by Bot1. However, when the Na\(^+\) hydration shell is more complete, ion exchange becomes less efficient due to a reduced pore space. We further observed that in nontransporting variants (Figure 8B; Supplemental Table 2), the predicted Na\(^+\) ion binding site cross-sectional area was increased by up to 35% compared with wild-type Bot1, allowing for more water molecules in the first hydration shell. This supports a hypothesis that while the ion binding site must be small enough to hold the Na\(^+\) ion inside, an overall larger pore diameter is required for transportation of borate anions. Evaluations of MD simulations of the pore of wild-type Bot1 revealed that it was physically linked to the Na\(^+\) ion binding site and it may be up to 4 Å wide and 25 Å long (Supplemental Table 2 and Supplemental Figure 8), while short pores in double, triple, and 4-, 5-, and 6-fold mutagenized variants failed to penetrate \(\alpha\)-helical bundles and would be ineffective for borate transport. These findings were confirmed through analyses of wild-type Bot1 with K\(^+\) in the ion binding site or in the absence of ion (Supplemental Table 3).

In silico predictions of the existence of the Na\(^+\) binding site and electrophysiology experiments indicate that there is a tight relationship between Na\(^+\)-dependent gating (Na\(^+\) works as a co-factor regulating hydration states of borate anions approaching
the pore) and borate-dependent conductance in Bot1. In other words, Na+ may function as a local hydration sink/reservoir that allows a dynamic equilibrium exchange of water molecules as borate anions approach the Na+ binding site. Thus, borate may undergo dehydration due to its proximity to Na+ to reduce its ion diameter, enter the binding site and potentially rehydrate to balance thermodynamic hydration penalties of ions (compared with Supplemental File 1). For effective borate transport, its solvation (hydration) and dehydration need to be effective and this is why borate interacts better with Na+ than with K+ (Rao et al., 2008; Zhou et al., 2013). These relationships need to be further explored at all levels, including in plants, to find out if tolerance to borate is modulated by the presence of Na+. Initial experiments using hydroponically grown seedlings suggested that residual intracellular Na+ meets the needs of Bot1 for conductance. Nevertheless, these in planta experiments independently confirmed that Bot1 is not likely to be a Na+/borate cotransporter, as also demonstrated in this work by electrophysiology experiments.

In summary, using a multipronged platform, we unraveled the molecular properties of Bot1 and provide insight into the origin of its transporting function. We defined the transport function of Bot1 using cell-free synthesis combined with nanotechnology, SAXS, site-directed mutagenesis, and MD simulations and revealed an atomistic model and its fine structural features, including the presence of a Na+ ion binding site that regulates permeation. Using an approach combining both in vitro and in silico data, we provide compelling evidence for Bot1 to be annotated as a Na+-dependent, channel-like polyvalent anion-permeable transporter, with high affinity for monovalent tetrahydroxy borate anions. While this knowledge is central to understanding boron transport processes in plants, our approach should be useful for general studies of other transporters.

METHODS

Chemicals and Reagents

The sources of oligonucleotide primers, restriction and DNA modifying enzymes, plasmid extraction kits, EDTA-free Complete Protease Inhibitor Cocktail tablets, and other chemicals were described previously (Periasamy et al., 2013). Benzonase (40 units/mL) was from Invitrogen. Asoslectin lipid from soybean and all other lipids were from Sigma-Aldrich and Avanti Polar Lipids, respectively. Styrene/maleic anhydride copolymer 3:1 (SM) was provided by Michael Overduin and Timothy Knowles (University of Birmingham, Birmingham, UK).

Wheat Germ Cell-Free Protein Synthesis of Bot1

To construct the Bot1 DNA fusion, full-length Bot1 was amplified from cDNA of the barley (Hordeum vulgare) landrace Sahara 3771 (Sutton et al., 2007) and cloned in-frame into the pEU-E01-MCS expression vector (CellFree Sciences) including the N-terminal 6xHis tag followed by the tobacco etch virus (TEV) site (Glu-Asn-Leu-Tyr-Phe-Gln) (Gly) (Phan et al., 2002) to generate the 6xHis-TEV-Hv-Bot1-pEU DNA fusion (Supplemental Table 4).

To prepare unilamellar asoslectin liposomes for cotranslational insertion of Bot1 via WG-CFPS, the lipid was dissolved in chloroform at 10 mM, deposited on the conductive side of an indium tin oxide glass slide, and allowed to dry for 2 to 4 h at ambient temperature. The slide was purged with nitrogen gas continuously for 15 to 30 min to remove residual traces of chloroform. An O-ring was placed around the dried lipid film, into which 200 μL of 100 mM sorbitol was added. Electro-formation of giant unilamellar vesicles proceeded at 37°C using the Vesicle Prep Pro (Nanion Technologies) as described by Nanion Technologies. Preparation of 100- and 400-nm liposomes proceeded in the lipid rehydration buffer (100 mM NaCl and 25 mM HEPES-NaOH, pH 7.5), into which dried lipids were re-suspended to a final concentration of 50 mg/mL. Liposomes were extruded 21 times above the lipid transition temperatures using the Liposofast extruder (Avestin) equipped with 100- or 400-nm pore size polycarbonate filters.

To conduct WG-CFPS of Bot1 in the presence of unilamellar liposomes and surfactants, the synthesis was conducted as described previously (Periasamy et al., 2013), through uncoupled transcription and translation at 25°C for 24 h in a bilayer mode. DMPC, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), and asoslectin lipids were prepared at 50 mg/mL. A mixture of POPC:POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine):POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol)):cholesterol = 6:3:1:0.1 (by weight) was used at 10 mg/mL. For synthesis of Bot1 with surfactants, liposomes were replaced by Brij-58, n-dodecyl-β-D-maltoside, n-decyl-β-D-maltoside, digitonin, Triton X-100, Tween 80, or acetylated (Ac) surfactant peptides Ac-AAAAAAD (Ac-Ala-Ala-Ala-Ala-Ala-Ala-Asp), Ac-IIDD (Ac-Ile-Ile-Ile-Ile-Asp), and Ac-LLKK-NH$_2$ (Ac-Leu-Leu-Leu-Lys-NH$_2$) in the translation reaction as described (Periasamy et al., 2013). Samples from WG-CFPS were evaluated by immuno-dot blot analyses, and the positive samples were analyzed by SDS-PAGE coupled with immunoblot analyses using either a mouse IgG2a isotype anti-6xHis monoclonal antibody (Clontech) or a crude serum raised through immunization of rabbits with the CSVDKDLKSLKDAVLREGDE peptide (residues 419 to 437, positioned between membrane α-helices nine and ten) derived from Bot1. Prior to loading on SDS-PAGE gels, the protein samples were incubated for 30 min at 37°C in SDS-PAGE loading buffer. Immunoblot profiles were developed with the Novex ECL HRP chemiluminescent substrate reagent kit (Invitrogen) according to the manufacturer’s instructions, and the images were scanned (Clix Science Instruments). Blue native PAGE proceeded according to the NativePAGE Bis-Tris Gel protocol (Invitrogen). For analyses of protein synthesis and expression (n = 2), and all transport data (n = 3), we calculated the values of the mean in transport experiments. In transport data we expressed these values as a percentage of standard deviations using GraphPad Prism 6 software.

Liposomes with cotranslationaly incorporated Bot1 were purified using Accudenz density gradient ultracentrifugation as described (Periasamy et al., 2013), except that the solutions did not contain NaCl. Purified Bot1 liposomes were dialyzed for 14 to 18 h at 4°C using 10-KD cutoff Slide-A-Lyser Mini dialysis cups (Thermo Scientific) against 10 mM histidine buffer, adjusted with Bis-Tris propane to pH 7.5, and stored on crushed ice, where they remained stable for up to 5 d.

The diameters of proteoliposomes were determined with a NICOMP 380 particle sizing system operating in a vesicle mode. The data were weighted on ~ 60 liposomes.

Liposome-associated topology of Bot1 in DMPC liposomes was determined by digestion with TEV protease (Phan et al., 2002; Periasamy et al., 2013). Aliquots of nondigested and TEV protease-treated liposomes were detected by immunoblot analyses using a mouse IgG2a isotype anti 6xHis monoclonal antibody (Clontech) and a crude serum raised against the Bot1 peptide specified above. The crude serum with Bot1 antibodies was prepared by immunization in rabbits (Institute for Medical and Veterinary Medicine) using a peptide synthesized by Mimotopes. The immunobLOTS were developed with the Novex ECL HRP chemiluminescent substrate reagent kit (Invitrogen) as described above and scanned.

Site-Directed Mutagenesis of Bot1

To construct variants for the pEU-E01-MCS expression vector (CellFree Sciences), the QuikChange II.XL site-directed mutagenesis kit (Stratagene) and PfuUltra High Fidelity DNA polymerase (Stratagene) were applied for
mutagenesis (primers in Supplemental Table 5). A 25 μL PCR reaction mixture contained 25 ng DNA template (wild type or mutant), 125 ng of oligonucleotide primers, 25 nM of the deoxy-nucleotide triphosphate mixture, and 1.25 units of the PhUtra High Fidelity DNA polymerase. Cycling parameters for PCR started with a denaturation step at 95°C for 30 s, followed by 18 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 6 min. Then, the PCR mixture was digested with 5 units of DpnI at 37°C for 1 h. Two microliters of the reaction mixture was transformed into XL10-Gold Ultracompetent cells (Stratagene) and plated. Plasmid DNA was isolated using standard methods and inserts were verified by Sanger sequencing.

To construct wild-type and variant Bot1 for expression in Saccharomyces cerevisiae, the protocol and reagents used were as those described above, except that the cycling parameters for PCR commenced with a denaturation step at 95°C for 30 s, followed by 18 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 8 min. DNA of 6-fold variant (N63A/T68A/E70A/A100V) was digested with NotI and XhoI (New England Biolabs) and cloned in-frame into the pPICZ-B expression vector (Invitrogen) to yield the Hv-Bot1-GFP-10xHis-pPICZ-B DNA fusion (Supplemental Table 4). The DNA fusion was linearized and transformed into competent P. pastoris X-33 cells according to the EasySelect Pichia Expression Kit Manual (Invitrogen). Approximately 100 colonies were picked after 3 d of growth on agar plates and used to inoculate 2 mL of liquid BMGY medium (medium composition is specified in the EasySelect manual) in 10-ml conical test tubes. Liquid cultures were grown for 2 d at 25°C, transferred to liquid BMMY medium, and induced for 4 d at 25°C maintaining 1% (v/v) methanol (Nordén et al., 2011). After 4 d, cells were harvested by centrifugation (10 min, 4500g, ambient temperature), pellets were suspended in 10% (v/v) glycine, and 150 μL of cells was lysed in lysis buffer that contained the 90% (v/v) Yeast-Buster reagent (Invitrogen), 1% (v/v) benzonase, 2 mM mercaptoethanol, 10 mM EDTA, 1 mM PMSF, and an EDTA-free complete protease inhibitor cocktail (Roche). The distribution of Bot1 in P. pastoris cellular fractions was evaluated as follows. The mixture was incubated for 20 min at room temperature on a microtiter shaker (Ratek) at 300 rpm and the lysate was collected by centrifugation (10 min, 4500g, ambient temperature) to remove insoluble aggregates. SMA-solubilized Bot1 was mixed with 0.5 mL of the Complete His-Tag Purification Resin (Roche) preequilibrated in 10% (v/v) glycerol, 200 mM NaCl, and 50 mM Tris-HCl, pH 8, and incubated for 2 h at 4°C. The resin with solubilized Bot1 was washed with the equilibration buffer, and Bot1 was released in the elution buffer containing 10% (v/v) glycerol, 200 mM NaCl, 300 mM imidazole, and 50 mM Tris-HCl, pH 8.0, in four elution steps.

Functional Assessment of Wild-Type and Variant Bot1 in S. cerevisiae

Plasmid DNA was transformed in S. cerevisiae (strain INVSc2; Invitrogen) as described (Gietz and Woods, 2002). Transformed cells were spread onto the SC selection medium (0.67% [w/v] bacto-yeast nitrogen base without amino acids, 2% [w/v] glucose, 0.002% [w/v] L-histidine-HCl, and 2% [w/v] bacto-agar) and incubated for 3 d at 37°C. Individual transformants were inoculated into the SC liquid medium with 2% (v/v) glucose and grown at 18 h at 30°C. Cells were centrifuged (10 min, 5000g, ambient temperature), suspended in the SC medium in which glucose was replaced by galactose, and incubated for 18 h at 30°C. To test for boron acid toxicity tolerance, yeast expressing wild-type and variant Bot1 at OD600 = 1.5 were serially diluted 10-fold, 10 μL of cells were spotted on solid 5G galactose medium with or without 20 mM boric acid, and plates were incubated at 30°C for 3 d.

Tryptic Mapping of Bot1 by ESI-MS

The Bot1 protein cotranslationally synthesized in the presence of 400 nm asolectin liposomes was subjected to SDS-PAGE and the band coinciding with the position of Bot1 was cut out from the gel. Tryptic mapping proceeded as described (Periasamy et al., 2013). The ESI-MS analysis revealed ions matching peptide sequences of Bot1 (GenBank accession number AB838562.1): DDWLQLQG, GIVGFFMSPK, MHVEFMDNK, EGDDEGKLAGDFDPR, IQLLVFGASR, VLEGPHASFVESVSSR, FFEPNDSL, and IADEILDELTHR.

Confocal Imaging of Bot1 in P. pastoris

To construct the Bot1 DNA fusion for expression in P. pastoris, amplified Bot1 cDNA was subcloned in-frame into the pPICZ-B expression vector (Invitrogen) through hybridization with GFP cDNA using a GenArt kit (Invitrogen) to yield the Hv-Bot1-TEV-GFP-10xHis-pPICZ-B DNA fusion (Supplemental Table 4). To confirm expression and cellular localization of Bot1, imaging of GFP-tagged Bot1 expressed in P. pastoris was performed using the LSM 5 PASCAL fluorescence microscope (Carl-Zeiss). Cells with an empty pPICZ-B plasmid transformed in P. pastoris were used as a negative control.

Transient Expression of Bot1 in Onion Epidermal Cells

To construct the Bot1 DNA fusion for transient expression, the Bot1 cDNA was cloned into the Gateway entry vector pCR8 (Invitrogen) (Supplemental Table 4) and introduced into the Gateway vector pMDCB83 (Invitrogen) by recombination to yield the 3SS-Hv-Bot1-GFP DNA fusion. The fusion was used to transform onion (Allium cepa) epidermal cells by particle bombardment using a biolistic PDS-1000/He particle delivery system (Bio-Rad). Onion cells were maintained in the dark on Murashige and Skoog medium for 24 h, prior to confocal image analysis. GFP fluorescence was visualized using a 488-nm argon laser, before and after plasmolysis by treatment with 1 M sucrose for 1 min.
Analysis of Insertion of Bot1 in Bilayers of Proteoliposomes by SAXS Using Synchrotron Radiation

The SAXS data of asolectin or DMPC liposomes with and without Bot1 were collected on the Small- and Wide-Angle X-Ray Scattering beamline of the Australian Synchrotron (Melbourne, Australia) using a Pilatus-1M pixel-array detector (Dectris) as described (Kirby et al., 2013). For each data set, 60 frames of a 1-s exposure time were collected from a sample flowing vertically through the x-ray beam at a constant 5 μL/s flow rate in a temperature-controlled 1.5-mm quartz capillary. Solutions of Bot1 inserted in asolectin or DMPC liposomes (extruded through 50- or 100-nm filters) were measured at 23°C in 50 mM Tris–HCl buffer, pH 7.5, containing 300 mM NaCl at ~0.2 to 0.6 mg/mL protein and 5 mg/mL lipid concentrations. The sample-to-detector distance was 1.5 m, covering a range of momentum transfer 0.01 < q < 0.6 Å⁻¹ (q = 4πsinθ/λ, where 2θ is a scattering angle and λ=1.03 Å is the x-ray wavelength). Comparison of successive 1-s frames revealed no detectable radiation damage. Data from the detector were normalized to the transmitted beam intensity, scaled to an absolute intensity using water as an intensity standard, and averaged, and scattering of the buffer solutions was subtracted using the ScatterBrain IDL software package available at the Australian Synchrotron. The SAXS profiles were analyzed using the latest ATSAS package (Petoukhov et al., 2012) and the bilayer electron density profiles that were extracted from SAXS data using a global analysis approach of GAP (Pabst et al., 2000) with asolectin (ULV model) and DMPC (MCT model) liposomal data fits, assuming positionally uncorrelated bilayers and the inclusion of a structure factor, respectively.

AFM Evaluation of Morphological Properties of Bot1 Cotranslationally Synthesized in DMPC Liposomes

All AFM imaging was conducted using a NanoWizard III AFM (JPK Instruments) equipped with a temperature-controlled BioCell stage maintained at 19.5 ± 0.2°C, in 25 mM HEPES-NaOH buffer, pH 7.5, containing 100 mM NaCl. Imaging was performed in contact mode using optimized parameters and MSNL cantilevers (Bruker AFM Probes), whereby the spring constants k, determined by the thermal calibration method, were around 0.17 N/m. This method involves recording the thermal noise spectrum in air before performing a force-distance curve on a hard surface, such as mica, to obtain the cantilever sensitivity. These two values are then used to estimate the spring constant. The force imparted by the tip on the sample during scanning was between 120 and 300 pN. The susceptibility of this technique to cantilever drift resulted in continual adjustment of the scan rate was maintained between 8 and 12 Hz, resulting in a typical velocity of between ~5 to 9 μm/s for small-scale images. All components of the BioCell stage and cantilever mounting apparatus (glass block/spring) were sonicated in ethanol and rinsed in miliQ water prior to use to reduce particles in solution, which could interfere with imaging. The DMPC liposomal fusion with Bot1 proceeded as follows. Mica discs of 9.9 mm in diameter were freshly cleaved and glued to clean glass cover slips of 24 mm in diameter, using a UV curable adhesive (Loctite 358) before being incubated at 27°C until required. Care was taken to ensure that the glue was evenly distributed between the mica and the cover slip and that the glue was fully cured with no air bubbles. Aliquots of proteoliposome stock solution were diluted to 0.5 mg/mL with 25 mM HEPES-NaOH buffer, pH 7.5, in a 1.5-mL centrifuge tube and gently mixed. Before extrusion, both the liposomal solutions and the extruder apparatus were heated to 27°C. A 500-μL volume of the liposomal solution was extruded through a Nucleopore polycarbonate membrane with 100-nm pore size, and 20 μL was spotted onto the freshly cleaved mica and incubated for 5 min at ambient temperature. Prior to imaging, the mica was washed in a constant stream of buffer to avoid dewetting and positioned in the BioCell stage. The stage was mounted on the AFM apparatus and equilibrated for ~15 min before imaging. For analyses of membrane protein heights to construct the frequency histogram, cross sections were drawn across the center point of each protrusion (n = 95) using the JPK Data Processing software (JPK Instruments) and the maximum height values relative to the bilayer surface were recorded. Individual height values were allocated within the predefined bin ranges using Microsoft Excel 2010.

The Bot1 insertion in DMPC liposomes was confirmed by immunoblot analysis. As Bot1 could be distributed in bilayers in two distinct populations, height values of protruding features were recorded by taking cross-sectional parameters through the highest point on the feature, which were indicative of the distance from the membrane surface. The heights recorded to construct the histogram would therefore reflect the real topology of Bot1 in proteoliposomes and describe how Bot1 protruded from the surface of the bilayer imaged by AFM.

TEVC Electrophysiology Recordings of Transport by Bot1 Expressed in Xenopus laevis Oocytes

To construct the Bot1 DNA fusion for TEVC electrophysiology recordings, amplified Bot1 cDNA was cloned into the Gateway entry vector pCR8 (Invitrogen) (Supplemental Table 4) and recombined into pGEMHE:DEST via Gateway LR Clonase II (Invitrogen) to generate the pGEMHE:DEST-Hv-Bot1 DNA fusion. This fusion was linearized with Nhel (New England Biolabs) and used as a template for cRNA transcription, using a mMessage mMACHINE T7 kit (Life Technologies). Oocytes from X. laevis were isolated and maintained as described (Virkki et al., 2006). Oocytes were injected with 46 nL of cRNA (0.7 μg/μL) encoding Bot1 or with RNase-free water using a Nanoinject II automatic nanoinjector (Drummond Scientific). After injection, the oocytes were incubated at 18°C for 2 d in ND96 solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl2, 0.5 mM CaCl2, and 5 mM HEPES-KOH, pH 7.6) containing 2.5 mM of horse serum, 50 μg/mL tetracycline, 5000 units of penicillin, and 5 μg of streptomycin per 50 mL, with the solution changed daily. Experiments were undertaken 2 d after injection of oocytes. TEVC was performed on whole oocytes using an OC-725c amplifier (Warner Instruments) with signals digitized with an Axon 1440A Digidata system. Currents were recorded using pClamp 10.3 software (Axon). Membrane currents were recorded in a control bath solution containing 25 mM N-cyclohexyl-2-aminoethanesulfonic acid and 0.3 mM CaCl2 or in a bath solution containing 25 mM boric acid and 0.3 mM CaCl2. All solutions were adjusted to pH 7.0, 8.0, or 9.0 using 0.5 M Bis-Tris propane. Solution osmolarities were adjusted to 220 Osmol/kg with mannitol. Readings were recorded by clamping the membrane potential at a holding voltage of ~40 mV, while stepwise increments of 20 mV were performed from ~120 to 40 mV for 1.5 s, returning to ~40 mV between each step. Subtracted data (currents in the presence of boric acid less currents in the absence of boric acid for each oocyte; tetraplicates for water-injected and pentaplicates for Bot1 cRNA-injected oocytes) were fitted to the third order polynomial functions. The University of Adelaide Animal Ethics Committee approved the X. laevis oocyte experiments (project number S-2009-044B).

Electrophysiology Recordings of Transport Using Asolectin Patch-Clamped Giant Liposomes with Inserted Bot1

Between 3 and 6 μL of a suspension of giant proteoliposomes, prepared as described above, containing ~5 μg of wild-type or variant Bot1, determined by SDS–PAGE and ImageJ (Schneider et al., 2012), were pipetted onto a microstructured glass chip with an aperture of ~1 μm in diameter. Measurements were performed at ambient temperature with the Port-a-Patch automated patch-clamp system (Nanion Technologies) using borosilicate glass chips. Single-channel events were recorded by adding a solution containing 10 mM histidine, 5 mM NaCl, and 5 mM boric acid, pH 7.5, on the internal side of a glass chip and 0 to 0.85 mM borate concentrations equivalent to 0 to 50 mM boric acid on the external side of the chip. Boric acid was dissolved in the same buffer as that used on the internal side. The osmolarity of all buffers was adjusted to 220 Osmol/kg.
During transport studies, the inhibitor DIDS was used at 80 µM concentration in the presence of 0.425 mM borate concentrations equivalent to 25 mM boric acid. Kinetic parameters $K_m$ and $G_{\text{max}}$ were derived from dependency of conductance on 0.085 to 0.85 mM borate concentrations equivalent to 5 to 50 mM boric acid at pH 7.5. For all other anions (phosphate, chloride, nitrate, and sulfate), symmetric concentrations at 25 mM were used. Dependency of conductance on Bot1 on Na" was measured with symmetric 25 mM boric acid and 0 to 75 mM concentrations of sodium glutamate or NaCl on the external side. Dependence of conductance on borate for variants was measured with symmetric 0.425 mM borate concentrations equivalent to 25 mM boric acid. All solutions were pH-adjusted with Bis-Tris propane unless otherwise indicated. The measurements were recorded using a HEKA amplifier (HEKA Electronics) connected to Ag/AgCl electrodes in the Port-a-Patch system. The signal was filtered using a four-pole low-pass Bessel filter at a frequency of 10 kHz and sampled at 50 kHz, and the signal was acquired using an EPC10 amplifier (HEKA Electronics). The data were analyzed using HEKA software (HEKA Electronics). All measurements were repeated three times, from which the values of $\text{s.e.}$ of the mean were calculated.

**In Silico Construction of a 3D Atomic Model, Oligomeric Assemblies, and MD Simulations**

Predictions of secondary structures of Bot1 performed by PSIPRED (Jones, 1999), MEMSAT3 (Jones et al., 1994), MEMSAT SVM (Nugent and Jones, 2009), MEMPACK (Nugent et al., 2011), SABLE (Wagner et al., 2005), and I-TASSER (Zhang, 2005) were combined. The initial 3D model of a full-length Bot1 protein with truncated loops was obtained from I-TASSER (Zhang, 2008) using the crystal structure of the uracil/H⁺ symporter (PDB accession 3QEF) and the Iterative Threading Assembly Refinement algorithm. Both sequences (Bot1 and uracil/H⁺ symporter) had 18% identity and 30% similarity at the amino acid sequence levels, when aligned using the global pairwise sequence alignment of the Stretcher (EMBOSS) algorithm (Li et al., 2015). Membrane $\alpha$-helices of the initial Bot1 model were manually adjusted using Accelrys Discovery Studio Environment v3.5 (Accelrys Software) to reach agreement with predicted secondary structures. To reconstruct the whole protein in a membrane, a “divide and conquer” method was employed (Cormen et al., 2001). The protein anchored in the DOPC lipid bilayer was divided into region I (membrane $\alpha$-helices) and region II (inter-connecting loops), followed by recombining both regions. MD simulations of the whole system were performed with explicit water, salt, and lipid molecules following minimizations and heating cycles. The end-to-end distances of loops were chosen to fit the corresponding protein gaps, as the dispositions of loops were not known, so the geometries of region I were selected from those at the end of 5 ns MD simulations. To obtain the desired end-to-end distances of the protein structure, two different approaches were employed for short and long loops. For short loops, linear peptide chains were built using the AMBER sequence command, which created topology and coordinate files for AMBER simulations (Case et al., 2012), followed by minimization, heating/equilibration, and MD calculations proceeding typically for 1 to 2 ns at 300K. Out of these MD trajectories, conformations at the desired distances were selected using the gOpenMol visualizer and analyzer (Laaksonen, 1992; Bergman et al., 1997). For long loops, a short MD simulation did not lead to desired conformations. Therefore, each end-to-end distance of a selected conformation was manually adjusted to achieve the desired conformation followed by minimization using Accelrys Discovery Studio Environment v3.5. Since optimization resulted in a deviation from a desired distance, this procedure was repeated until the target distance was reached. In the divide and conquer method, a reassociation procedure, originally used to construct large molecules for quantum mechanics simulations (Lee and Friesner, 1993; Lee et al., 2008), was employed. To reassociate region II with region I, the terminal residues of both regions and several buffer residues were included to overlap terminal residues, such that the atoms of the peptide bonds at the junction of the regions overlapped with high accuracy. Using Accelrys Discovery Studio Environment v3.5, the overlaid atoms and the buffer residues were deleted to reconstruct the complete system without overlapping atoms. The recombined system was partially minimized using Accelrys Discovery Studio Environment v3.5 to avoid clashes of atoms and atypical bond lengths. The modeled protein consisted of 574 residues, where the first 24 N-terminal residues and the last 68 C-terminal residues were not included, as no structural counterparts were identified in databases. Lipids and explicit water and salt molecules were added to produce a system with 102,070 atoms that was minimized by AMBER. The force field algorithms during modeling were ff99SB (Hornak et al., 2006) for protein, lipid11 (Skjevik et al., 2012) for lipid, and TIP3 (Jorgensen et al., 1983) for water molecules. After gradually heating the system to 300K, equilibration MD in duration of 30 ns and production runs of 84 ns were performed, using the SHAKE algorithm with a time step of 2 fs and Langevin dynamics (LD) with the collision frequency of 1.0, a random seed, and surface tension of 10.0 dyne/cm.

The stereoelectrochemical quality of the Bot1 model was performed using PROCHECK (Laskowski et al., 1993). Evaluation indicated that 99.6% of residues were located in the most favored, additionally allowed and generously allowed regions, when excluding Gly and Pro residues. Two residues, Ser-392 and Leu-120 (0.4%), were located in disallowed regions. Respective overall G-factors, determined by PROCHECK, which estimate overall stereoelectrochemical parameters, were $-0.31$ and $0.17$ for Bot1 and 3QEF, which was used as a template during the initial stages of modeling. The z-score values deduced from ProSa2003 (Sippl, 1993), reflecting combined statistical potential energy, were $-5.39$ and $-8.92$ for Bot1 and 3QEF, respectively.

Electrostatic surface potential was calculated using the Adaptive Poisson-Boltzmann Solver (Baker et al., 2001) with solvent contributions (dielectric constants for solvent and protein components were 78 and 2, respectively) and mapped onto the molecular surfaces generated with a probe radius of 1.4 Å. Molecular graphics was created with PyMOL (http://www.pymol.org).

To obtain estimates of dispositions of protoners within the quaternary structures, we built di- to hexameric assemblies of Bot1 starting from a relaxed monomer model, using the SymmDock tool (http://bioinfo3d.cs.tau.ac.il/SymmDock) that uses rigid and flexible docking, and searches for complementary geometric interfaces between neighboring protoners. The search algorithm first calculates the protoner surface followed by filtering and scoring of matched surfaces. For each oligomeric assembly, a total of 100 models was generated and scored. Selected configurations were based on scores, together with the lowest atomic contact energies and retained ion binding site-forming $\alpha$-helices that were vertically aligned. Docked dimeric and trimeric assemblies were subjected to MD relaxation with implicit and explicit water molecules lacking lipids.

**In Silico Construction of 3D Atomic Models and MD Simulations of Variants**

The macromolecule module of Accelrys Discovery Studio Environment v4.0 was used to perform MD simulations of Bot1 variants. In silico mutational variants of wild-type Bot1 included single substitutions of N63A, T68A, E70A, T71A, Q113A, and G117A that define the Na⁺ ion binding site. We also performed MD simulations of double, triple, and 4-, 5-, and 6-fold (into Ala) variants (Supplemental Table S). Bump functions were used to relax steric clashes of atoms or nonstandard bond lengths. The initial structure of the wild-type Bot1 and lipid/salt/water assembly was obtained after 50 ns of MD simulations. For each variant, this initial structure was used as a reference, from which a new mutagenized structure was isolated. This ensured that initial configurations of lipid/salt/water in all variants remained identical for all MD simulations. During MD simulations, a 574-residue protein was embedded in an aqueous DOPC lipid bilayer, and the
Pore Dimension Calculations

The dimensions of pores in wild-type and variants of Bot1 were calculated by Caver3.0 (Chovancova et al., 2012) using an ensemble of 50 snapshots deduced from each trajectory. Identified pores resulted from a series of associated spheres and sphere radii and corresponded to the closest distances to protein atoms. The narrowest region of a pore is defined as a bottleneck. The data obtained corresponded to the pores with the highest priority score, which was estimated by averaging a sum of pore throughputs over all snapshots, while pore throughputs were averaged over individual clusters. The throughput of a pore was measured as an exponential of cost function, which controls the balance of width and length throughputs over all snapshots, while pore throughputs were averaged over individual clusters. An ensemble of 50 snapshots corresponded to consecutive snapshots that represented a part of a trajectory, before Na+ diffused from the ion binding site of the wild type and variants.

Supplemental Data

**Supplemental Data 1.** SDS-PAGE profiles of Bot1 WG-CFPS reactions in the presence of liposomes and surfactants.

**Supplemental Data 2.** Subcellular localization of chimeric Hv-Bot1-GFP in P. pastoris and after transient expression in onion cells, evaluated by confocal imaging.

**Supplemental Data 3.** Properties and conservation of Bot1.

**Supplemental Data 4.** Schematic representation of a bilayer with incorporated Bot1 observable by AFM and topology of Bot1 in proteoliposomes.

**Supplemental Figure 1.** Inward borate anion currents mediated by Bot1 expressed in X. laevis oocytes.

**Supplemental Figure 2.** Dependency of conductance of Bot1 on Na+.

**Supplemental Figure 3.** Partial hydration profiles of Na+ inside the binding site in wild-type and single, double, triple, and 4-, 5-, and 6-fold mutagenized variants of Bot1.

**Supplemental Figure 4.** Characteristics of wild-type and the Na+ ion binding site-mutagenized AAAAAA variant of Bot1.

**Supplemental Figure 5.** Multiple sequence alignment of Bot1 with other closely related putative plant transporters belonging to the AE2. A.31 family.

**Supplemental Table 1.** Bilayer phospholipid head-group distributions determined from a global fit of the SAXS data using ULV and MCT (asolectin) and MCT (DMPC) models.

**Supplemental Table 2.** Characteristics (with Na+) of the wild-type and ion binding site-mutagenized variants of Bot1, using patched bilayers and MD simulations.

**Supplemental Table 3.** Characteristics (with K+ or without ion) of wild-type Bot1 using patched bilayers and MD simulations.

**Supplemental Table 4.** Primers used for cloning of Bot1.

**Supplemental Table 5.** Primers used to construct the Na+ ion binding site variants of Bot1 for heterologous expression through WG-CFPS and in S. cerevisiae.

**Supplemental Figure 1.** A side view of the atomistic model of Bot1 with the Na+ ion trapped by six residues N63, T68, E70, T71, Q113, and G117.

**Supplemental Movie 1.** A side view of the atomistic model of Bot1 with the Na+ ion trapped by six residues N63, T68, E70, T71, Q113, and G117.

**Supplemental Movie 2.** A top view of the atomistic model of Bot1 with the Na+ ion trapped by six residues N63, T68, E70, T71, Q113, and G117.

**Supplemental Movie 3.** A side view of the atomistic model of the Bot1 variant AAAAAAA with the Na+ ion trapped by six residues N63A, T68A, E70A, T71A, Q113A, and G117A at the beginning of the MD simulation.

**Supplemental Movie 4.** A side view of the atomistic model of the Bot1 variant AAEAAA with the Na+ ion trapped by six residues N63A, T68A, E70A, T71A, Q113A, and G117A.

**Supplemental Movie Legends.**

ACKNOWLEDGMENTS

We thank Michael Overduin and Timothy Knowles (University of Birmingham, UK) for providing styrene/maleic anhydride, Gwenda Mayo and Jingwen Tiong (University of Adelaide) for assistance with imaging, Leanne Kelly and Tony Bacic (University of Melbourne, Australia) for ESI-MS analysis, Peter Kolesik (Bionomics, Australia) for discussions, and Peter Langridge (University of Adelaide) and anonymous reviewers for constructive suggestions on the manuscript. This research was supported by Australian Research Council Discovery Project DP12010900 and by grants from the Grains Research and Development Corporation, the South Australian Government, and the Waite Research Institute. Y.N. and J.R. were supported by Australian Postgraduate Award scholarships from the University of Adelaide, the Australian Centre for Plant Functional Genomics, and the Grains Research and Development Corporation. Small-angle scattering data collection was undertaken on the Small- and Wide-Angle X-Ray Scattering beamline at the Australian Synchrotron.
AUTHOR CONTRIBUTIONS


Received July 14, 2015; revised November 20, 2015; accepted December 11, 2015; published December 15, 2015.

REFERENCES


