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Plants fighting back: to transport or not to transport, this is a structural question

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Short title: Protein structure-function relationships underpinning soil toxicity tolerance

Key words: abiotic stress, borate transporters, crops, high soil boron, HKT, protein structural modelling, salinity, structural bioinformatics.

Abstract – Membrane transport proteins are fundamental to life; their co-ordinated action controls the movement and distribution of solutes into, around and out of cells for signalling, metabolism, nutrition, stress tolerance and development. Here we outline two transport system case studies that plants use to tolerate soil elemental toxicity, demonstrating how iterative studies of protein structure and function result in unparalleled insights into transport mechanics. Further, we propose that integrative platforms of biological, biochemical and biophysical tools can provide quantitative data on substrate specificity and transport rates, which are important in understanding transporter evolution and their roles in cell biology and whole plant physiology. Such knowledge equips biotechnologists and breeders with the power to deliver improvements in crop yields in sub-optimal soils.

Introduction – A major global limitation on crop yield is the presence of sub-soil constraints, including low water availability, extreme pH or elements that are toxic to plants if they accumulate to high concentrations within cells. Crop traits that result in tolerance to common elemental sub-soil constraints such as aluminium, arsenic, boron, chloride and sodium, are often multiple, but can rely upon the

presence or absence of specific transport processes. These processes are predominantly mediated in cells by membrane-embedded transport proteins that control the exclusion, exudation or compartmentation of elements across cellular membranes to guard against elemental toxicity [1]. In this article, we explore how a quantitative understanding of protein structure is important for understanding transport function and hence the cell biological processes underpinning stress tolerance.

Transport proteins form channels or transporters that facilitate movement of solutes (*e.g.* water, ion, sugar or gas) in charged and uncharged forms, either down or against (electro-)chemical gradients to fulfil a role in nutrition, signalling, metabolism or stress tolerance. Transport proteins also function as transceptors – a composite of 'transporter' and 'receptor' [2] – *e.g.* CHL1/NRT1.1/NPF6.3 fulfils transport and signalling functions in response to external nitrate concentrations *via* different protein parts. Furthermore, recent work on the ferrous iron IRT1 transceptor described how intracellular excess of non-iron metals triggers IRT1 degradation *via* a signalling cascade to avoid root intoxication [**3].

The activity of transporters can be regulated transcriptionally through positive or negative transcriptional regulators [4], post-transcriptionally through oligomerisation, protein-protein and protein-lipid interactions [5], and post-translationally (*e.g.* phosphorylation, N- and O-glycosylation, S-nitrosylation) [6]. The co-ordinated action of multiple transporters in a membrane, and sometimes throughout the plant, is important for combating elemental soil toxicity, which is the molecular basis for numerous classical observations of differential transport processes being evident in different tissue types as gained through isotope flux analysis [7]. Therefore, the location of transporters within the cell or in the plant is essential for dictating their influence on cellular and whole plant function. It has increasingly been demonstrated that specific transport processes occur within certain key cell-types, *e.g.* root exclusion of aluminium (Al^{3+}) [1], shoot sodium (Na^{+}) [8] or borate ($[B(OH)_4]^-$) [*9]. Specific cell-types that confer elemental toxicity tolerance have consequently been coined as 'gatekeeper' cells and rely upon the activity of specific transport proteins [10].

Quantitative data on transport mechanics of plant transporters – Multiple techniques can address the permeation function of transporters at quantitative levels. Commonly, quantitative data acquired *via* flux or electrophysiological investigations of transporters expressed in heterologous systems such as *Xenopus laevis* oocytes, yeast, or HEK cells are compared with transport activity *in vivo,* or with the lack of activity following misexpression *in planta* [8]. Purified proteins can also be reconstituted in

membrane-mimicking environments of artificial liposomal vesicles, tethered bilayers and nanodiscs [*9,11,12]. These defined environments lead to transporter reconstitution in fluid trans-bilayers and if oligomerisation is required for function, transporters may adopt quaternary structures, where lipid-protein interactions and bilayer forces contribute to stability and ultimately function. Precisely because transporters are present in bilayers in highly purified states, this approach allows the definition of their transport mechanics, although at a somewhat reductionist level, and may introduce artefacts due to the lack of co-factors. Similarly, in heterologous systems of native membranes there are complexities in analysis due to the presence of other native transport proteins or in 'knockout' plants the pleiotropic upregulation of other transporters to compensate for the lack of the target protein [13]. Furthermore, quantitative data *per se* can be difficult to compare across studies due to the influence of environmental regulation specific to those experiments. The exception perhaps comes from fully reductionist systems, which leads us to the role that structural studies can play in better understanding transport phenomena – as an ultimate distillation of reductionism.

Relatively few crop transporters have been characterised to date at physiological, biochemical and structural levels, and in fact many transporters await to be discovered. It has become apparent from work in plant model organisms such as *Arabidopsis* that transporter selectivity and regulation are difficult to predict based either on sequence alone, or through homology to transporters characterised in other systems. Therefore, despite efforts to sequence multiple crop genomes, reliable functional annotation at present is impossible without the actual transporters first being characterised at multiple levels. Furthermore, the imputation of evolutionary relationships and functional similarities between transporters on sequence alone can be fraught with error.

Natural variation in transporter sequences is a useful tool to examine functional relationships; single amino acid residue variation within a protein can lead to loss of function or change in transport selectivity [14,**15-17]. However, it is impossible to ascertain the mechanism underpinning such functional changes by examining the sequence alone. Furthermore, the same variation in a homologous protein may lead to a different outcome. An example includes the recently solved structure of the *Arabidopsis* two-pore channel [18] that has different gating and selectivity properties compared to its mammalian counterparts. Only by taking into consideration how sequence motifs and residues interact mutually with permeating and non-permeating solutes, and how they are controlled by regulatory factors, the effect on variations could be understood. For this to occur, the atomic structure must be

resolved, and if possible put in the context with its dynamics and conformational states [19]. The power of such approaches is only beginning to emerge; for instance, the molecular basis of several transporters that improve crop tolerance to elemental sub-soil constraints was recently revealed [8] and is being fed into breeding programs [20].

3D structures of plant transporters – Eight hundred and one unique membrane protein structures have been elucidated as of July 2018 (http://blanco.biomol.uci.edu/mpstruc), mostly by X-ray crystallography [21]. Only eight of those are plant transporters: *Spinacia* plasma membrane intrinsic protein (PIP) aquaporin (Protein Data Bank accessions 1Z98) and *Arabidopsis* tonoplast intrinsic protein aquaporin (5I32), *Arabidopsis* nitrate transporter (4OH3), *Oryza* (5CTH) and *Arabidopsis* sweet transporters (5XPD), *Arabidopsis* boron transporter Bor1 (5L25), *Arabidopsis* two-pore channel (5DQQ) and *Arabidopsis* MATE transporter (5Y50). No plant transporter structure has yet been elucidated by other biophysical approaches such as NMR spectroscopy, which is applicable for smaller proteins [22], cryo-electron microscopy [23] or serial crystallography [24]. We expect that these multiple approaches combined with computational techniques, referred to as integrative structural biology with hybrid methods, will have enormous potential to break the structural conundrum of plant transporters.

Technical challenges associated with applying mainstream biophysical approaches to plant transporter structure elucidations are being overcome by rapid advances in cell-based and cell-free synthesis methods (**Figure 1**) [*9,11] coupled with nanotechnology [12]. For X-ray crystallography and cryo-electron microscopy, purified membrane proteins are typically stabilised in controlled settings of micelles, bicelles, swollen lipidic mesophases, lipodiscs, supported lipid bilayer stacks and amphipol belts [25]. These preparations are subjected to crystallisation trials and data collection by *in situ* X-ray screening [26] or cryo-electron microscopy imaging [27]. We have recently seen a revolution in the latter field facilitated by the 2017 Nobel Prize winners in chemistry (Dubochet, Frank, Henderson) [27].

3D protein homology modelling and structural bio-informatics – In the absence of experimental structures, the second-best approach is the generation of 3D models, based on the spatial information of related homologous proteins, using programs such as *e.g.* Modeller [28]. Homology models based on a high or even 'twilight' sequence identity between template and target proteins are enhanced with all-atom molecular dynamics (MD) simulations in lipid environments and in complex with ligands [19]. Importantly, homology models with structural bio-informatics mining allow to satisfy proteomics gaps,

such as the state of post-translational modifications [29], and evolutionary and conservation relationships [30,31].

Combined structural and functional studies - The real power of knowing the structure along with quantitative transport data is in predicting and testing key residues that confer substrate specificity, selectivity mechanisms and protein-protein interactions at the molecular level. These predictions improve both the structural model and the understanding of transport function. The first iteration of the model may not be immediately informative for function, so validation experiments are required. We illustrate the power of combining structural modelling and functional assays to enhance the fundamental knowledge of transporters, using two case studies focused on understanding the transport function behind crop tolerance to high soil concentrations of sodium and boron.

HKT transport proteins contribute to dryland salinity tolerance – Shoot salt exclusion is an important trait causing the salinity tolerance of many crops [32]. Plasma membrane-localised High Affinity K^+ Transporters (HKT) [33], associated with the root/shoot vasculature play a key role in minimising the accumulation of Na^+ in aerial tissues [7,32-35]; this interferes with photosynthesis and reproduction, resulting in lower yields (**Figure 2**). The impact of salinity on agriculture is predicted to double by 2050, with up to 40% of irrigated agriculture to be affected. Multiple strategies for improving salt tolerance have been explored, including the role of plant transport proteins [32]. Studies of the structure-function relationships of HKT proteins reveal how natural variation can confer more effective shoot Na^+ exclusion [8,**15,36], which may lead to improved salt tolerance, as delineated below.

Molecular structure and function of plant HKT1;5 transporters (Transporter Classification Data Base: 2.A.38-K^+ transporters, also classified in the KtrB/TrK/TrG/KdpA/HKT superfamily) [*37] – Attempts to crystallise plant HKT proteins have been so far unsuccessful. Instead, high quality homology models using K^+ conducting bacterial members of the Ktr/Trk subfamily [38,*39,40,**41] as structural templates, have been generated [**15,36,42]. This information has directed functional studies that probe the function of key residues through site-directed mutagenesis. For instance, it was shown that TmHKT1;5-A from *Triticum monococcum* and TaHKT1;5-D from *Triticum aestivum* selectively conduct Na^+ ions at different affinities and rates [**15]. Through structural modeling, two of the 27 residue differences between TmHKT1;5-A and TaHKT1;5-D were predicted to confer significant structural changes sufficient to impart functional differences; the predicted mutations were made and

were sufficient to swap the Na^+ transport affinities of the two proteins [**15]. Further, such 3D homology models could be used to interrogate changes in molecular interactions that underpin changes in selectivity. For instance, the mechanism of Na^+ *versus* K^+ exclusion in plant HKT transporters *via* Gly to Ser variation in the 'selectivity filter' prevents K^+ transport [43], but the interactions of these residues with other structural elements and the alterations in molecular interactions of ions within the transporter have yet to be shown.

Boron toxicity tolerance is conferred *via* aquaporins and anion permeable efflux transporters – Boron (B) is a naturally occurring soil metalloid, essential for plant growth. Under adequate B supply, its uptake from the soil to plant root is largely passive. High soil B leads to toxicity that is widespread worldwide [44]. In Australia, a study involving 233 trials over 12 years estimated that B toxicity in tolerant genotypes generated a 14%-16% yield advantage [45].

B toxicity-tolerant crop genotypes accumulate lower concentrations of B than intolerant ones (**Figure 3**). In all plant species, the primary mechanism of B toxicity tolerance is linked to a limited entry of B in the form of boric acid (BA) (a weak Lewis acid with pK_a of 9.24), into the roots, and the disposal of excess BA through leaves *via* hydathode guttation [46].

B toxicity tolerance in barley is thought to be afforded by two genes: (i) *NIP2;1* on chromosome *6H*, encoding an aquaporin that carries neutral BA [47]; and (ii) *Bot1* on chromosome *4H* encoding an efflux transporter [46] that carries an anionic form of BA [*9]. Both genes encode α-helical membrane transporters (**Figure 3**), which reside in the same environment of gatekeeper epidermal root cells.

(i) Cereal multifunctional aquaporins (Transporter Classification Data Base: 1.A.8-Major intrinsic protein superfamily) [*37] – Six major groups of aquaporins are recognised in plants, but only two groups are known to conduct BA, the nodulin-26-like intrinsic proteins (NIPs) [47] and the PIPs [48]. NIPs enable water transport, but also conduct glycerol, H_2O_2, CO_2, BA, and silicic, arsenious and germanic acids [47]. Defined signatures of selectivity filter residues and the width of the pore are proposed to underpin the broad solute specificity of NIPs [**49]. The 3D computational model of barley NIP2;1 predicts an α-helical bundle fold; however, its atomic structure needs to be defined to understand its precise *in planta* roles.

(ii) Cereal anion permeable efflux transporters (Transporter Classification Data Base: 2.A.31-Anion exchanger (AE) family), also classified in the Solute Carrier (SLC) superfamily) [*37] – Anion efflux transporters from barley and wheat are the distant orthologues of mammalian carriers. Studies in *Arabidopsis* imply that anion effluxers respond to BA [50]; however, it is unknown if uniport, anion/anion and/or anion/cation symport account for transport. An *in-silico* 3D atomistic model of the barley efflux transporter Bot1 (**Figure 3**) has been constructed, and mono-, di- and trimeric forms were detected *in vitro* and in *vivo* [*9]. Electrophysiology revealed that Bot1, prepared through co-translational cell-free reconstitution, mediated a Na^+-dependent polyvalent anion transport in a Nernstian manner and had channel-like characteristics. The crystal structure of related *Arabidopsis* Bor1 in a dimeric form [*51] showed a good agreement with the barley Bot1 model [*9]. However, it remains to be established which oligomeric forms are functional *in planta*. Ground-breaking structural work from several laboratories [*51-54] revealed that SLC superfamily transporters operate through the so-called 'elevator' mode of action (**Figure 3**).

Conclusions and future perspectives

Structural biology and transport mechanics data provide the essential quantitative information for understanding function – Through structural knowledge of transport mechanics useful deductions can be made to suggest preferred transporter substrates and their hydration/dehydration states. This knowledge also informs us how substrates interact with essential co-factors during transport, and how competing substrates affect transport. Furthermore, the effects of residue substitutions on permeation events can be predicted by examining variation sites mapped onto atomic structures or 3D models of transporters. These molecular models combined with MD simulations offer the useful structural information (**Figure 4**), as they provide the descriptions of structural elements and motifs that are fundamental to permeation. Based on this information, the accurate annotation of corresponding gene and protein families can be enhanced. Additionally, by untangling the structure-function horizon of plant transporters, we may better understand the significance of natural variation of homologous and divergent genes in an evolutionary context.

Efforts to apply structural and functional information on plant transport proteins in a high-throughput manner should be encouraged, embraced and built upon. Examples of these endeavours include the 'Crop Cure' project (http://ucsdnews.ucsd.edu/feature/crop_cure) to develop biological tools for growing hardier crops, and the allied 'CROPS' project (http://crops.ucsd.edu) focussed on a structural

pipeline of plant membrane transporters. Such efforts are augmented by databases 'Gramene' (http://www.gramene.org), 'International Service for the Acquisition of Agri-biotech Applications' (http://www.isaaa.org) and 'CropTiPS' (http://www.croptips.org), which aims to deliver a comprehensive knowledgebase of membrane transport and signalling systems for various plants.

New frontiers emerge after the convergence of existing multidisciplinary knowledge – Undoubtedly, the prerequisite for further progress in this field is dependent on precise quantitative knowledge of transport permeation function and its regulation, that can be obtained *via* integrative multidisciplinary platforms [*9,20]. One such platform involving biophysical and biochemical tools, and plant and molecular biology, electrophysiology, and chemi- and bio-informatics has been suggested [*9]. Here, *in silico* descriptions of the first-principle MD calculations suggested that the Na^+-dependent barley effluxer Bot1 permits an efficient exclusion of borate anions from plant cells back to soil, possibly through 'quantum tunnelling'. This term refers to kick-starting BA disposal from a plant directly linked to the presence of hydrated Na^+ in a specific location of Bot1; the need for Na^+ may create an energy barrier to drive BA anions efflux [*9]. Notably, co-evolution of boron and Na^+ (salinity) stresses is well-documented in an agricultural context [55], thus it is apparent that there is often a need to combine traits (pyramiding of traits [20]) before improvements in yield can be achieved. Nevertheless, *via* these integrative platforms and through genomics, genetics, metabolomics and transcriptomics associations, essential insights into the origin of permeation will deliver vital information that could pave the way to develop more resilient crops to sustain crop yields under sub-optimal soil conditions.

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Conflict of interest

The authors have declared no conflicts of interest.

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The work reveals that the structure of the bacterial membrane KdpFABC complex consists of a channel-like subunit (KdpA) that belongs to the superfamily of potassium transporters, the membrane-associated gating ring KdpB and a pump-like subunit classified in the superfamily of P-type ATPases. This work suggested that the presence of K^+ in the selectivity filter of KdpA leads to a charge transfer through the protein-embedded tunnel to the membrane-associated gating ring of KdpB. This may trigger its phosphorylation by the P-type ATPase leading to movement of an α-helix in KdpA that allows K^+ release to the cytosol. Another component of the KdpFABC complex, periplasmic KdpC may participate in the occlusion of the KdpA selectivity filter. Based on these findings, the authors proposed a novel mechanism for bacterial Ktr transporters that may allow facilitation of the active transport of ions across a biological membrane.

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This work summarises the latest advances on plant aquaporins and explains that the grouping of aquaporins into several subfamilies within the ancient superfamily of major intrinsic proteins, is based on sequence homology and subcellular localisation. Genome-wide identifications of aquaporin genes available from around 15 plant species provide a source of sequence data for molecular studies through structural bioinformatics, three-dimensional modelling and molecular dynamics simulations. These studies have the capacity to reveal novel information, unavailable to X-ray diffraction studies of time- and space-averaged molecules confined in crystal lattices.

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The structure of *Arabidopsis* Bor1 (Solute Carrier 4 transporter) displays a dimeric architecture and two-domain organisation, whereby dimerisation is facilitated by central gate domains. Comparisons with the related human Band 3 transporter in an outward-open state reveal that the core domains of Bor1 have rotated inwards to achieve an occluded state. These data suggested that Solute Carrier 4 (eukaryotic plant transporters such as Bor1), Solute Carrier 26 (prokaryotic fumarate transporters, such as SLC26Dg) and nucleobase-ascorbate uracil transporters UraA and UapA, share the' elevator' transport mechanism, in which alternating access is provided by core domains that permeate substrates across membranes.

Divergence between dimeric *Arabidopsis* Bor1 formed *in crystallo* (under high concentrations of salts and polyethylene glycol), and oligomeric assemblies of *Hordeum* Bot1 formed *in vitro* and *in vivo* [*8] may result from a dynamic equilibrium imposed on Bot1 in lipid environments. Notably, other SLC transporter such as the fumarate transporter crystallised in a monomeric form.

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Legends to figures

Figure 1 Barley membrane efflux transporter Bot1 is produced in functional forms *via* cell-free synthesis.

(a) Schematics of the cell-free synthesis workflow for membrane proteins, based on the wheat-germ extract. Membrane proteins are embedded in defined bilayers of liposomal vesicles through co-translational insertion. Cell-free synthesis systems bypasses the energy constraints required for cell survival and channel resources towards synthesis of a single transcript.

(b) Left image (modified from [*9]): Bot1 is synthesised and incorporated in 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (lanes 1) or asolectin (lanes 2) liposomes. The -mRNA lane contains the reaction lacking mRNA of Bot1. Right image: Yields of 1- to 3-mers. Yields of Bot1 obtained in liposomes show low to medium mg quantities per ml of the cell-free reaction mixture.

Figure 2 Na^+ tolerance is mediated by wheat HKT1,5 transporters.

(a) Wheat plants growing in a green-house. The plants on the right are smaller with fewer 'heads' of grain compared to those grown in salinity-affected soil. Image courtesy of South Australian Research and Development Institute.

(b) 3D model of the TaHKT1;5 transport protein in two orthogonal orientations with Na^+ translocating the permeation trajectory. TaHKT1;5 is embedded in a cell membrane and transports Na^+ through tube-like channels (black mesh), by-passing the selectivity filter (SGGG motif shown in atomic sticks) with Na^+ (purple sphere). Na^+ is likely to enter and exit the translocating permeation trajectory from several locations on both sides of TaHKT1;5, but always by-passes the selectivity filter constriction (modified from [**15]).

Figure 3 Mechanistic model of BA toxicity tolerance in barley, involving NIP2;1 and Bot1.

(a) BA toxicity symptoms in the leaf of the BA-intolerant Clipper barley cultivar. Image courtesy of Dr Mahmood Hassan, CSIRO, Australia.

(b) The magnitude and direction of fluxes of BA into and out of the roots of Sahara (BA-tolerant) and Clipper (BA-intolerant) cultivars when excessive BA is present outside of the root (modified from [44]).

(c) Left panel: The barley NIP2;1 computational model with glycerol, water and BA in the pore (cylindrical cyan α-helices and loops in magenta; glycerol in sticks in atomic colours, water molecules in red spheres, BA in black atomic sticks. Right panel: The barley Bot1 computational model with cyan membrane-embedded and grey intracellular α-helices, and loops in magenta (modified from [47,*9]).

(d) A scheme of the elevator transport mechanism that is believed to be common to SLC superfamily transporters and is likely to occur in barley Bot1. The three stages of the mechanism include inward facing (binding the substrate), occluded state (movement of the substrate through the pore) and outward facing (releasing the substrate) (modified from [*51,53,54]).

Figure 4 3D protein homology modelling and structural bio-informatics combined with definition of transport mechanics lead to the definition of transport function. We suggest that the following iterative structure-function testing loop is used to construct the 3D homology model from the protein sequence; the best-scoring model is enhanced by all-atom MD simulations. The significance of the loop to deconvolute the transporter structure-function relationship is highlighted in black thick arrows. In each step, a single or a variety of computational tools are implied, accessible from web site portals or useable *via* standalone packages. The list of these tools is by no means exhaustive.

Highlights:

- The fundamental complexity underpinning plant transport function needs to be comprehensively understood at quantitative levels.
- Structures of plant transporters must be put to context with molecular dynamics and conformational states.
- Combining protein structural modelling and transport data of plant transporters contributes to the understanding of their mechanics at molecular levels.
- New frontiers emerge after the convergence of existing and the novel multidisciplinary knowledge of plant transporters.
- Comprehensive characterisation of plant transporters will deliver vital information paving the way to develop more resilient crops for precision agriculture and to sustain crop yields under sub-optimal soil conditions.

Cell-free synthesis of the barley Bot1 efflux transporter in functional forms

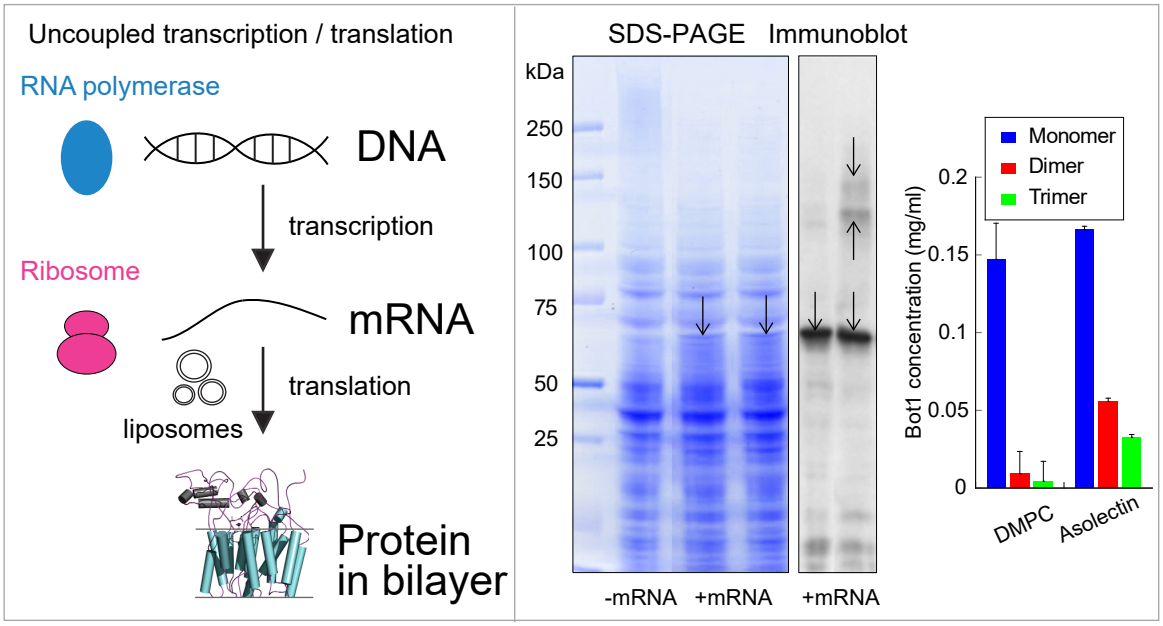


Figure 1

Na^+ tolerance is mediated by wheat HKT1,5 transport proteins

(a) Control and salt-affected wheat plants

(b) 3D model of wheat HKT1;5 transporter

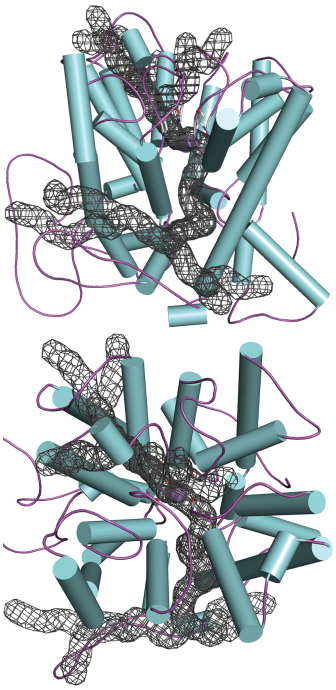
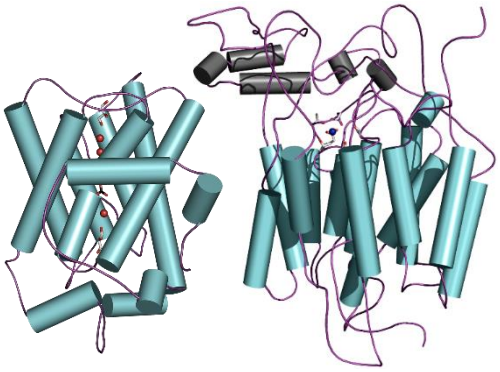


Figure 2

Mechanistic model of BA toxicity tolerance in barley, involving NIP2;1 and Bot1

(a) BA toxicity symptoms in a barley leaf

(c) 3D Protein molecular models



NIP2;1 Bot1

(b) Magnitude and direction of BA fluxes in and out of the roots of tolerant and intolerant barley

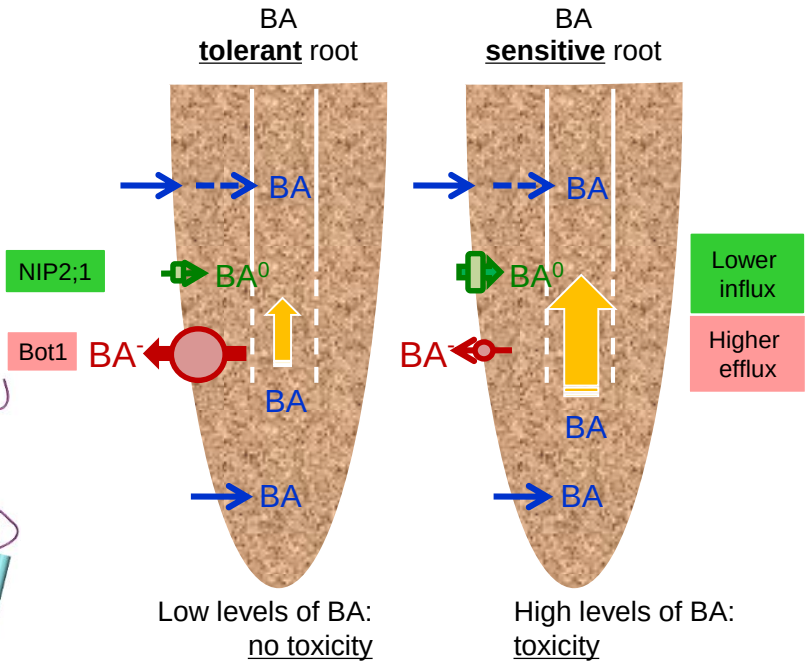


Figure 3

The iterative structure-function testing loop: from sequence to 3D model

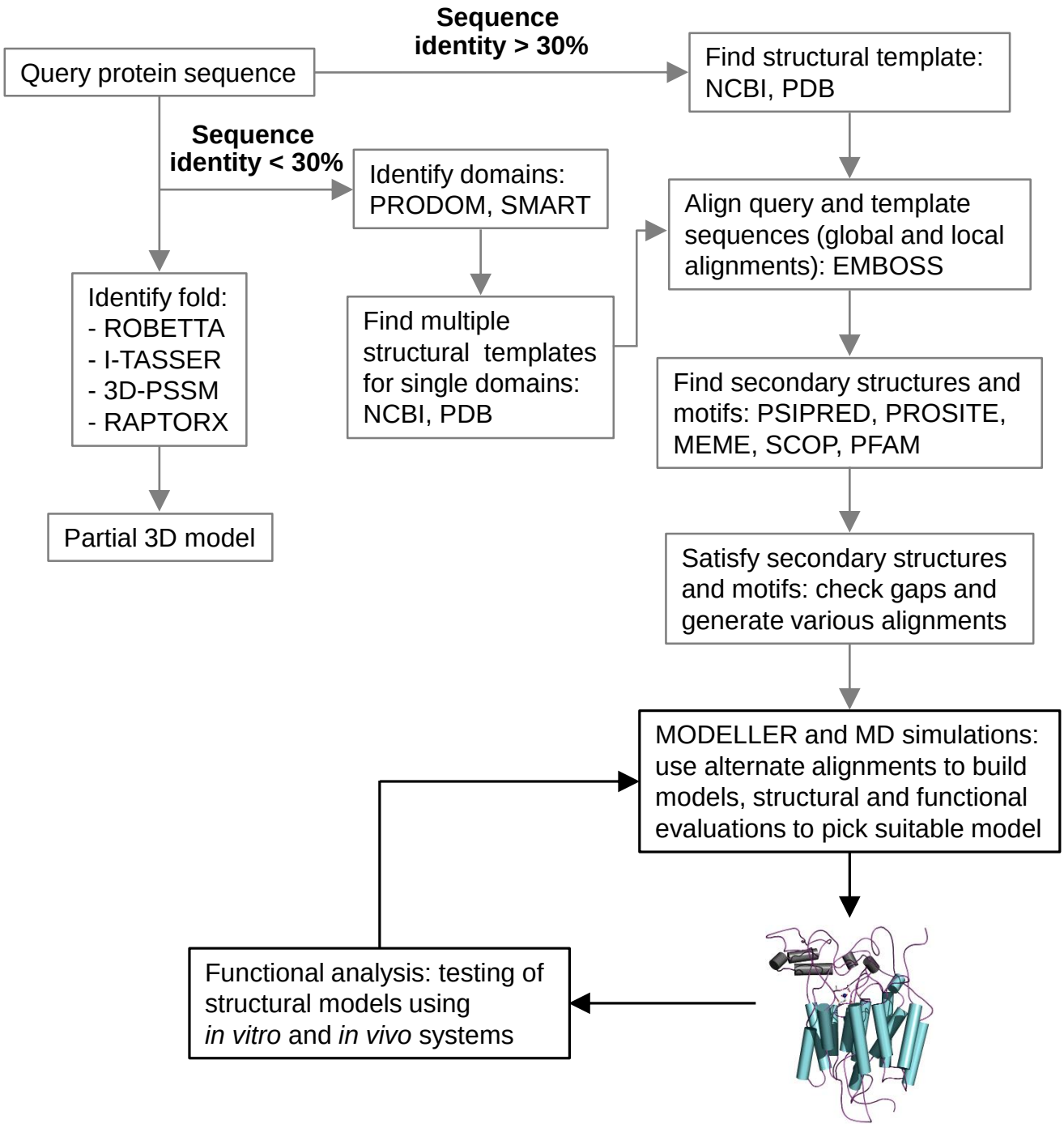


Figure 4