Exploring Unorthodox Aquaporins

Characterization of NbXIPs & AtNIPs

Ampah-Korsah, Henry

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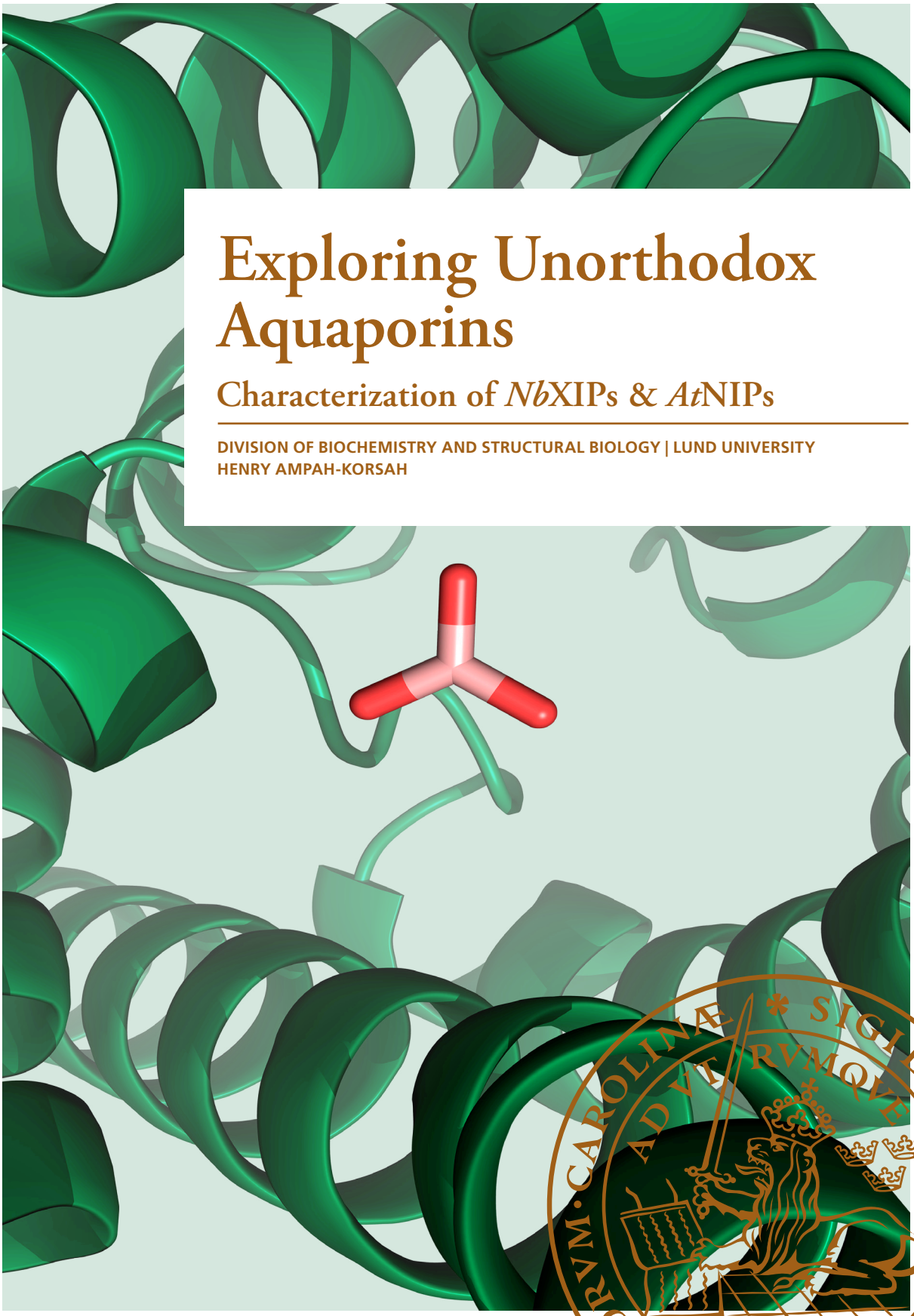

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DIVISION OF BIOCHEMISTRY AND STRUCTURAL BIOLOGY | LUND UNIVERSITY
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LUND
UNIVERSITY

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Abstract

Aquaporins are membrane integral proteins that facilitate the transport of water and/or other small neutral molecules across biological membranes in cells in all forms of life. Among the subfamilies of aquaporins in higher plants, the X Intrinsic Proteins (XIPs) are the most recently discovered subfamily and the least characterized. However, the aromatic/arginine (ar/R) selectivity filter and the proposed substrate specificity of XIPs resemble that of some NIPs aquaporin isoforms. The aim of the studies in this thesis was to functionally and structurally characterize *Nicotiana benthamiana* XIP1;1s and *Arabidopsis thaliana* NIP1;1 and NIP5;1 aquaporins.

By using the *Pichia pastoris* expression system, *Nb*XIP1;1s, *At*NIP1;1 and *At*NIP5;1 were successfully expressed. *Nb*XIP1;1s and *At*NIP5;1 increased the sensitivity of *P. pastoris* cells to boric acid. Furthermore, the N-terminally His-tagged splice-variant *Nb*XIP1;1α appeared more opened to boric acid than the C-terminally His-tagged protein when expressed in *P. pastoris*. In *P. pastoris* spheroplasts, differences in water and glycerol permeability of *Nb*XIP1;1α and *At*NIP5;1 were observed. *At*NIP5;1 was permeable to water and glycerol but *Nb*XIP1:1α was impermeable to both substrates. *Nb*XIP1;1α and *At*NIP5;1 proteins were purified by Ni-NTA affinity chromatography. While the yield obtained for *Nb*XIP1;1α was adequate for both functional and initial crystallization studies, the initial yield for *At*NIP5;1 was only sufficient for functional characterization. The current purification scheme for *At*NIP5;1 needs to be optimized to obtain sufficient amounts of protein for a comprehensive crystallization study.

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Key words

Aquaporins, XIPs, NIPs, phosphorylation, *Pichia pastoris*, overexpression, boric acid, membrane proteins

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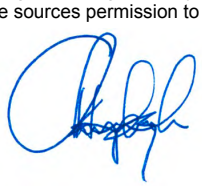
Signature  Date 21-09-2016

Exploring Unorthodox Aquaporins

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Henry Ampah-Korsah

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Division of Biochemistry and Structural Biology

2016

Front cover photo: Exploring unorthodox aquaporins

Back cover photo: “Gye Nyame”. An adinkra symbol from Ghana which means “Except for God” and signifies the Supremacy of God.

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To My Family

“And as ye would that men should do to you,
do ye also to them likewise”

Luke 6:31

Content

List of publications

This thesis is based on the following papers. The papers are included at the end of the thesis.

I. The aquaporin splice variant *Nb*XIP1;1α is permeable to boric acid and is phosphorylated in the N-terminal domain

Ampah-Korsah H, Anderberg HI, Engfors A, Kirscht A, Nordén K, Kjellström S, Kjellbom P & Johanson U

Front. Plant Sci. 7:862.

II. Single amino acid substitutions in the selectivity filter render *Nb*XIP1;1α aquaporin water permeable

Ampah-Korsah H, Sonntag Y, Engfors A, Kirscht A, Kjellbom P & Johanson U

Manuscript submitted.

III. The aquaporin splice variant *Nb*XIP1;1α is regulated by its N-terminal domain

Ampah-Korsah H, Engfors A, Leavy S, Kjellbom P & Johanson U

Manuscript

IV. Purification and characterization of *Arabidopsis thaliana* NIP1;1 & NIP5;1 aquaporins

Ampah-Korsah H, Kirscht A, Engfors A, Leavy S, Kjellbom P & Johanson U

Manuscript

Contribution to papers

I. HA-K participated in the design of the study, constructed the *P. pastoris* clones, performed the expression, boric acid growth toxicity assay, immunoblot assays, isolation of plasma membranes, spheroplast preparation, purification, circular dichroism experiment, reconstitution into lipid vesicles, phosphorylation/dephosphorylation assays and functional studies. HA-K took part in the mass spectrometry experiments and was involved in the analysis of the MS data. HA-K drafted and was involved in the revision of the manuscript.

II. HA-K participated in the design of the study, constructed *Nb*XIP1;1 mutant *P. pastoris* clones, carried out the immunoblot assays, prepared spheroplasts and performed the functional studies. HA-K took part in the analysis of the homology modeling data. HA-K drafted and was involved in the revision of the manuscript.

III. HA-K took part in the design of the study, supervised parts of the work, performed parts of the immunoblot assays and carried out the phosphorylation/dephosphorylation experiments. HA-K drafted and was involved in the revision of the manuscript.

IV. HA-K took part in the design of the study, transformed *P. pastoris* cells with constructs made by AK, performed the expression, immunoblot assays and purification experiments. HA-K drafted and was involved in the revision of the manuscript.

Abstract

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Popular science summary

Water is very important for life. In order to survive every organism needs to control the amount of water in its cells. One of the main ways the cell regulates its water content is through aquaporins. Aquaporins are membrane proteins that allow water and/or small neutral molecules to enter or exit the cell. Aquaporins form a large family and are present in all life forms, however, plants have more aquaporins than other organisms. Aquaporins are also known as Major Intrinsic Proteins (MIPs). Members belonging to the aquaporin family have the same overall three dimensional (3D) structure, however, some aquaporins have different selectivity filters. Orthodox or classical aquaporins are aquaporins that are permeable to water only as the name aquaporin suggests. Unorthodox aquaporins are aquaporins that have selectivity filters that allow other small neutral molecules to pass.

In 2008, new members of the aquaporin family were recognized in the early land plants, i.e. mosses. Since the function of these new aquaporin members was not known, they were called X intrinsic proteins (XIPs), X standing for unknown function. However, the selectivity filter of XIPs resemble that of some previously known plant aquaporins called Nodulin 26-like Intrinsic Proteins (NIPs). Different XIPs have been reported to facilitate the transport of boric acid, hydrogen peroxide, urea, glycerol but not water. However, the physiological function of XIPs is still unknown. Therefore, the purpose of the projects in this thesis was to increase the knowledge about *Nicotiana benthamiana* XIPs (*Nb*XIPs) and *Arabidopsis thaliana* NIPs (*At*NIPs) aquaporins.

The yeast, *Pichia pastoris*, has previously been used to produce and study many proteins including aquaporins. *P. pastoris* was therefore used to produce *Nb*XIP1;1s, *At*NIP1;1 and *At*NIP5;1 aquaporins. Since the production of *Nb*XIP1:1s and *At*NIP5;1 was better than that of *At*NIP1;1 in the yeast, *Nb*XIP1;1s and *At*NIP5;1 were chosen for further studies. Both *Nb*XIP1;1s and *At*NIP5;1 facilitated the transport of boric acid into intact yeast cells which retarded the growth of the cells since boric acid is toxic to yeast. On the contrary, boric acid is beneficial to plants, as plants need boron for proper growth and yield. In *P. pastoris* cells with weakened cell walls, the splice-variant *Nb*XIP1;1α was not permeable to water or glycerol, while *At*NIP5;1 was permeable to both molecules. In order to study *Nb*XIP1;1α and *At*NIP5;1 proteins in a controlled environment away from

interfering effects of other proteins, *Nb*XIP1;1α and *At*NIP5;1 were isolated and purified from the yeast cells. Though the amount of purified *At*NIP5;1 obtained after the initial purification trial was enough for functional studies, it was not enough for crystallization studies aimed at solving the 3D structure of *At*NIP5;1. Therefore, the initial purification protocol for *At*NIP5;1 will be improved to generate large amounts of pure *At*NIP5;1 protein for crystallization studies. A 3D structure of *At*NIP5;1, together with functional studies, will help clarify how the structure of *At*NIP5;1 affects its function. However, the yield of purified *Nb*XIP1;1α obtained after purification was sufficient for both functional and crystallization studies.

The transport capabilities of purified aquaporins are usually studied by inserting aquaporins into lipid vesicles and measuring the shrinking or swelling of the vesicles in response to rapid changes in sugar or salt concentration. A rapid change in boric acid concentration revealed that lipid vesicles with inserted purified *Nb*XIP1;1α protein were two-fold more permeable to boric acid as compared to empty lipid vesicles. An examination of the *Nb*XIP1;1α protein revealed that the purified *Nb*XIP1;1α protein was partially modified with phosphates. Five amino acid residues in the N-terminal part of the protein had phosphate groups attached. Phosphate modification of aquaporins usually controls aquaporins in two ways; by serving as a signal to transport the aquaporin to a specific membrane location in the cell or by causing a structural change in the aquaporin that allows the pore to open. When the N-terminal part of *Nb*XIP1;1α with the phosphate groups was cut from the protein, the protein became more permeable to boric acid in intact yeast cells. This suggested that the N-terminal part of *Nb*XIP1;1α regulates its function, e.g. permeability. In an attempt to understand how the selectivity filter of *Nb*XIP1;1s works, the water-impermeable filter of *Nb*XIP1;1α was exchanged for the water permeable filter of *At*TIP2;1, a close relative of XIPs.

The selectivity filter of aquaporins is made up of five amino acid residues that determine which molecules can pass through the pore of the aquaporin. However, a substitution of only one of the selectivity filter residues made the *Nb*XIP1;1α protein water permeable. Interestingly, a substitution of another residue outside the selectivity filter also made *Nb*XIP1;1α water permeable. The water permeable *Nb*XIP1;1α constructs provide the possibility to explore the functional properties of *Nb*XIP1;1s in great detail. *At*NIP5;1 has been shown to be one of the proteins responsible for boric acid transport into plant cells. Since the selectivity filter and the choice of substrates of *Nb*XIP1;1s are similar to that of *At*NIP5;1, a possible physiological function of *Nb*XIP1;1s could be that they facilitate the transport of boric acid into distinct plant cells.

Chapter 1

Aquaporins

Introduction

Water is essential for living organisms since it plays a central role in many physiological processes that sustain life. The basic unit of life, the cell, is composed of approximately 70-80% water. The cell and its organelles have boundaries which are lipid bilayers. These lipid bilayers protect the cell and help with the organization of water and other constituents within the cell and its subcellular compartments. It was correctly hypothesized by scientists that water simply diffuses through the lipid bilayer, however, simple diffusion alone could not sufficiently explain the high water permeability seen in certain cell types such as erythrocytes and renal tubules. It was therefore envisioned that channels must be present in these cells to assist with the water transport. This led to the search for these so-called "water channels".

In the early 1990s, Agre and his co-workers isolated and functionally characterized the first water channel (CHIP28) which they had earlier discovered by "sheer blind luck" in red blood cells and renal tubules [Denker *et al*., 1988; Preston and Agre, 1991; Preston *et al*., 1992]. They later renamed this first water channel as aquaporin 1 (AQP1), meaning a pore for water transport in the lipid bilayer [Agre *et al*., 1993]. This did not only definitively confirm the involvement of proteins in the passive transport of water across biological membranes but also it stirred the interest of many researchers, consequently resulting in a boom in aquaporin related research. Prior to the discovery of AQP1, other proteins, such as the *Escherichia coli* glycerol facilitator (GlpF) [Richey and Lin, 1972] and the major intrinsic protein (MIP) of bovine lens fiber [Gorin *et al*., 1984], from the same family as AQP1 were known although not linked to water transport. As a recognition for his achievement in the discovery of water channels, Peter Agre was awarded the 2003 Nobel Prize in Chemistry, which was also jointly awarded to Roderick MacKinnon for his structural and mechanistic studies on ion channels.

Aquaporins are quite ubiquitous and are present in all biological membranes where the regulation of rapid permeability of water and/or other small uncharged

molecules is required in the cell. Aquaporins are also referred to as Major Intrinsic Proteins (MIPs). Since 1993 when the aquaporin name was coined by Agre *et al*., 1993 till date, over one thousand five hundred aquaporins have been discovered in organisms in all life forms including animals, plants and microorganisms [MIPModDB; Gupta *et al*., 2012]. For example, the aquaporin Z (AqpZ) in *E. coli* [Calamita *et al*., 1995] and aquaporin M (AqpM) [Kozono *et al*., 2003] in the archaeon *Methanothermobacter marburgensis* were recognized in 1995 and 2003, respectively. It is now known that mammals have thirteen aquaporin isoforms (AQP0 – AQP12) which are involved in various physiological functions including the secretion of saliva, control of urine concentration in the kidneys, regulation of water homeostasis in the brain, control of perspiration in the skin and regulation of glycerol concentration in fat metabolism [Krane and Goldstein, 2007]. Notably, more than 30 aquaporin isoforms have been found in individual species of plants [Johanson *et al*., 2001; Chaumont *et al*., 2001], with the X Intrinsic Proteins (XIPs) as the most recently recognized aquaporins [Danielson and Johanson, 2008].

With the discovery of many new members of the aquaporin family which have low to nought water permeability but high permeability to substrates such as glycerol, ammonia, boric acid and silicon, it has become apparent that the name aquaporin is somewhat misleading as it only suggests water transport. In the light of this, many researchers have proposed terms such as aquaglyceroporins and superaquaporins to classify these unusual aquaporins [Kayingo *et al*., 2001; Ishibashi, 2006; Nozaki *et al*., 2008; Rojek *et al*., 2008]. In one of these classifications, aquaporins were categorized into three major subtypes based on their transport abilities as, “classical or orthodox” aquaporins specific for water transport, “aquaglyceroporins” permeable to water and glycerol, and “unorthodox” aquaporins with yet unknown function, poor water permeability but permeable to other small uncharged solutes [Rojek *et al*., 2008].

Structure and function

Jung *et al*., 1994 proposed the 'Hourglass model' of the aquaporin structure. In addition, high resolution structures of aquaporins have revealed that aquaporins exist primarily as homotetramers, with each monomer forming a pore through the membrane [Fu *et al*., 2000; Törnroth-Horsefield *et al*., 2006; Horsefield *et al*., 2008; Kosinska Eriksson *et al*., 2013; Kirscht *et al*., 2016a]. Cartoon representations of the high resolution X-ray crystal structure of *At*TIP2;1 monomer, homotetramer and the homotetramer embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid bilayer are shown in Figure 1A, B and C, respectively.

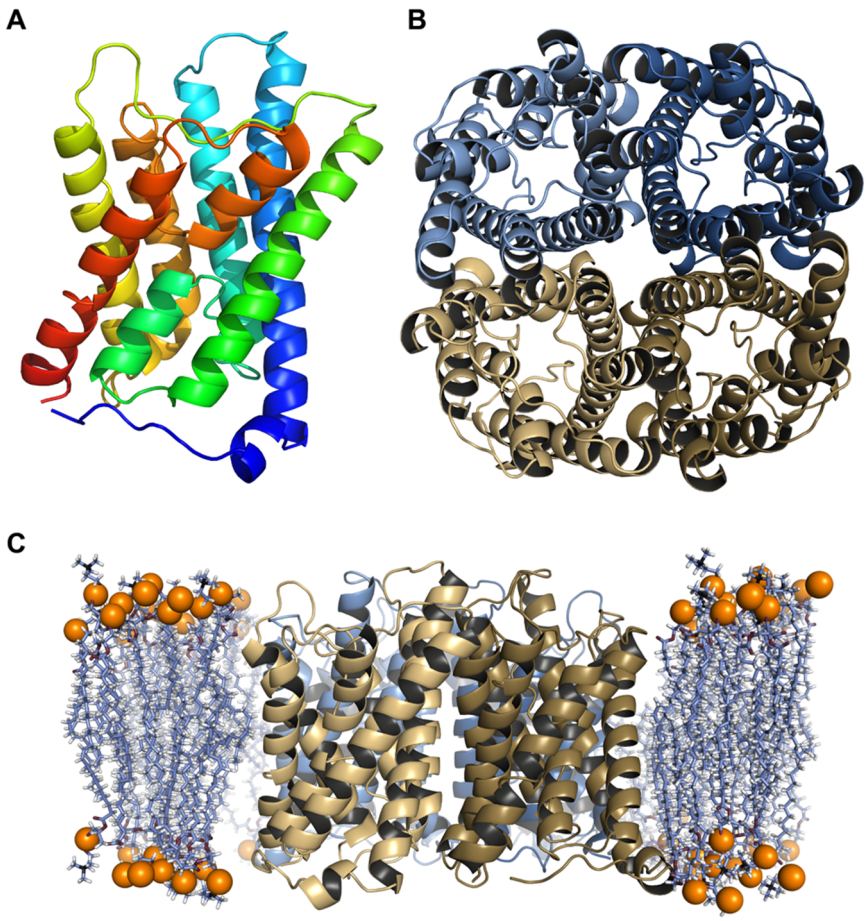

Figure 1. Cartoon representations of *At*TIP2;1 aquaporin adapted from the X-ray crystal structure (PDB ID 5I32) of the protein [Kirscht *et al*., 2016a] **(A)** A side view of the monomer with six membrane spanning helices (coloured from blue to red) showing the two half helices (in green and deep orange) in the middle of the protein. **(B**) Top view of the homotetramer showing the assembly of the four monomers. **(C)** A side view of the homotetramer embedded in a POPC lipid bilayer. The phosphates in the POPC lipids are depicted with orange balls.

The aquaporin monomer consists of six transmembrane α-helices (Helix 1 – Helix 6) connected by five loops (Loop A – Loop E), with both the amino and carboxy termini located in the cytoplasm. Loops B and E contain the asparagine-proline-alanine (NPA) aquaporin motif and form two short α-helices (HB and HE) that fold back into the membrane from opposite sides, forming a seventh pseudo-transmembrane helix [Mitsuoka *et al*., 1999]. A careful look at the primary and secondary structure of aquaporins revealed that the monomer is made up of a tandem repeat, each repeat consisting of two transmembrane helices, a half helix followed by a third transmembrane helix, which may have arisen from a gene duplication event [Pao *et al*., 1991]. The overall fold and topology of aquaporins is highly conserved within the aquaporin family. Substrate specificity of aquaporins is governed by a selectivity filter that is made up of two regions in the pore of aquaporins.

The first region is the conserved NPA region which repels protons from passing through the pore [Murata *et al*., 2000]. The repeated NPA aquaporin motif at the N-termini of the two short α-helices basically form the NPA region. The N-termini of the two short α-helices meet and connect in the middle of the aquaporin, creating a net positive charge that repels protons or any positively charged molecule from passing. The repulsion of protons from passing through the pore of aquaporins is vital in sustaining membrane potential of cells. It has been established by molecular dynamics simulation that water passes through the pore of aquaporins more or less in a single-file hydrogen bonded to each other [Tajkhorshid *et al*., 2002; Chakrabarti *et al*., 2004]. However, at the NPA region the water molecules are reoriented, resulting in a bi-directional orientation of the water molecules in the single-file of water in the pore of aquaporins, as shown in Figure 2.

The second region is the aromatic arginine (ar/R) region which regulates substrate selectivity by providing an aperture and interactions that fit the substrate [de Groot *et al*., 2003]. The recently published high resolution structure of the *Arabidopsis thaliana* TIP2;1 aquaporin isoform revealed that in addition to the four previously recognized residues at specific positions in helix 2, helix 5, loop E and helix E ($H2^P$, $H5^P$, LE^P and HE^P, respectively), a fifth residue at a specific position in the loop C (LC^P) forms part of the ar/R region [de Groot *et al*., 2003; Zhu *et al*., 2004; Kirscht *et al*., 2016a]. The identity of these amino acid residues at the ar/R region restricts which substrate can permeate through the pore. Mutational studies have shown that it is possible to alter the substrate selectivity of aquaporins by instituting point mutations in the ar/R region [hAQP4; Kitchen and Conner, 2005; rAQP1; Beitz *et al*., 2006; hAQP1; Kirscht *et al*., 2016a]. It has been observed that a conserved histidine residue at the $H5^P$ position is characteristic of aquaporins which are water-specific [Sui *et al*., 2001]. By site-directed mutagenesis, Wallace and Roberts, 2005 showed that the residue at the $H2^P$ position is the crucial determinant for both water

and glycerol permeability in TIP and NIP aquaporins. Substitution of the conserved alanine at the $H2^P$ position in the ar/R region of *At*NIP6;1 (NIP II subgroup) for the conserved tryptophan in soybean nodulin 26 (NIP I subgroup) rendered *At*NIP6;1 water permeable in *Xenopus laevis* oocytes [Wallace and Roberts, 2005]. The highly conserved arginine at the HE^P position in the ar/R region has been reported to contribute to the electrostatic repulsion of positively charged molecules [Beitz *et al*., 2006; Kosinska Eriksson *et al*., 2013].

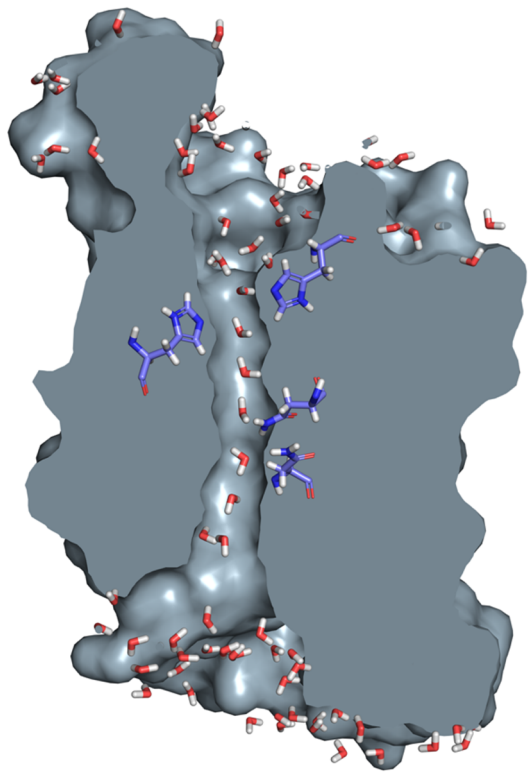

Figure 2. A surface representation of the monomeric structure of *At*TIP2;1 cut open to show the bi-directional orientation of the water molecules in the pore. An energy minimization was done on the water molecules with the protein fixed in NAMD [Philips *et al*., 2005]. The hydrogens of the water molecules in the pore point away from the center of the protein. For simplicity, only the two asparagines in the NPA region (one from each half helix) and the two histidines (at $H2^P$ and LC^P positions) in the ar/R filter are shown. This structure was adapted from the X-ray crystal structure (PDB ID 5I32) of *At*TIP2;1 [Kirscht *et al*., 2016a]. Molecular dynamics simulations were done by Yonathan Sonntag.

Regulation

To adapt to different conditions, living organisms regulate the function of aquaporins in their cells. Regulation of aquaporins already begin at the transcriptional level where depending on the need, different aquaporin isoforms are expressed in certain types of cells. In mammals for instance, water specific aquaporins such as AQP2 and AQP4 are expressed in kidney cells to reabsorb water from urine to avoid dehydration while aquaglyceroporins like AQP7 and AQP9 are expressed in adipose tissues and liver cells, respectively to facilitate the permeation of glycerol in these cells [King *et al*., 2004; Day *et al*., 2014]. Likewise, in plants, different aquaporin isoforms are localized in specific membranes such as the plasma membrane, vacuolar membrane and peribacteroid membrane. Alexandersson *et al*., 2005 showed that under drought stress most aquaporin genes in *A. thaliana* were down-regulated, however, 26 h after re-hydration the transcript levels of all aquaporin genes in the drought stressed plants were up-regulated to the same level as in pre-stress conditions. Also, it has been shown that under boron deficient conditions, the transcript levels of the boric acid permeable *At*NIP5;1 increased in root cells [Takano *et al*., 2006]. Evidence from experimental results indicate that aquaporins, especially NIPs, that are permeable to metalloids such as boron and silicon show a polar localization in cells in which they are expressed, working in harmony with active efflux transporters to support transport of metalloids through plant cells [reviewed in Pommerrenig *et al*., 2015].

Regulation of aquaporins occurs also at the posttranslational level. Phosphorylation of serine or threonine residues modulates aquaporins to alter the water permeability of cells in two ways namely, gating (opening and closing of the pore) and trafficking (targeting to different membranes). It has been reported that phosphorylation enhanced water permeation in *Pv*TIP3;1, *So*PIP2;1, soybean nodulin 26 and *Tg*PIP2;2 [Maurel *et al*., 1995; Johansson *et al*., 1998; Guenther *et al*., 2003; Azad *et al*., 2008]. The high resolution X-ray structures of *So*PIP2;1 in closed and open conformation, revealed that loop D occludes the pore of *So*PIP2;1 from the cytoplasmic side. Phosphorylation of Ser 115 in the cytoplasmic loop B and Ser 274 in the C-terminal region release the loop D, culminating in the opening of the channel [Johansson *et al*., 1998; Törnroth-Horsefield *et al*., 2006]. Likewise, phosphorylation of Ser 262 has been reported to be responsible for the increased water permeability in soybean nodulin 26 [Lee *et al*., 1995; Guether *et al*., 2003]. Molecular dynamic simulations and functional studies have suggested that gating of the yeast aquaporin Aqy1 may be regulated by phosphorylation of Ser 107, whereby phosphorylation of Ser 107 induces the opening of the pore [Fischer *et al*., 2009].

Prak *et al*., 2008 showed that phosphorylation of Ser 283 was necessary to target *At*PIP2;1 to the plasma membrane under resting conditions while salt stress induced

intracellular accumulation of *At*PIP2;1 in transgenic root cells. In animals, it has been reported that the peptide hormone vasopressin induces the trafficking of AQP2 from intracellular vesicles to the apical plasma membrane in kidney cells via a cascade of cAMP dependent events involving AQP2 phosphorylation [Nielsen *et al*., 1995; Brown *et al*., 2003]. AQP1, AQP4, AQP5 and AQP8 have all been suggested to have a regulated trafficking to the plasma membrane [Han and Patil, 2000; Madrid *et al*., 2001; Yang *et al*., 2003; Garcia *et al*., 2001].

Other external conditions such as changes in osmolarity [Johansson *et al*., 1996], pH change [Tournaire-Roux *et al*., 2003; Shelden *et al*., 2009; Leitao *et al*., 2012] and concentration of divalent cations, Ca^{2+} for example, [Nemeth-Cahalan and Hall, 2000; Gerbeau *et al*., 2002; Verdoucq *et al*., 2008] have been reported to modulate water permeability in certain aquaporin isoforms. In addition to Ca^{2+}, Mn^{2+} and Cd^{2+} inhibited *At*PIP2;1 reconstituted into proteoliposomes [Verdoucq *et al*., 2008]. Although mercury has been shown to inhibit many aquaporins, some aquaporins have been reported to be mercury insensitive [Calamita *et al*., 1995]. Mercury increased the water permeability of SoPIP2;1 in proteoliposomes [Frick *et al*., 2013; Kirscht *et al*., 2016b] A conserved histidine in the D loop of plant PIPs has been associated with pH regulation [Tournaire-Roux *et al*., 2003; Törnroth-Horsefield *et al*., 2006]. In *So*PIP2;1, it has been reported that protonation of His-193 in the D loop triggers the closure of the channel [Törnroth-Horsefield *et al*., 2006]. A drop in the cytosolic pH resulted in a decrease in water permeability of *At*PIP2;2 [Tournaire-Roux *et al*., 2003] and VvTIP2;1 [Leitao *et al*., 2012].

Plant AQPs

As previously mentioned, there are more aquaporin isoforms identified in individual species of plants than in species of animals or microorganisms, for example, 35 in *Arabidopsis thaliana* [Johanson *et al*., 2001], 36 in maize [Chaumont *et al*., 2001], 33 in rice [Sakurai *et al*., 2005], 55 in poplar [Gupta and Sankararamakrishnan, 2009] and 71 in cotton [Park *et al*., 2010]. Based on sequence similarity, plant aquaporins have been classified into 7 subfamilies, namely; the Plasma Membrane Intrinsic Proteins (PIPs), the Tonoplast Intrinsic Proteins (TIPs), the Nodulin 26-like Intrinsic Proteins (NIPs), the Small basic Intrinsic Proteins (SIPs), the X Intrinsic Proteins (XIPs), the Glycerol facilitator-like Intrinsic Proteins (GIPs) and the Hybrid Intrinsic Proteins (HIPs) [Danielson and Johanson, 2008]. A schematic tree of plant aquaporins identified in mosses, spikemosses, monocots and dicots is shown in Figure 3. PIPs, TIPs, NIPs, SIPs and XIPs have been found in higher plants while HIPs have been described in mosses and spikemosses (Figure 3). GIPs have only been recognized in mosses.

Though the assigned names of plant aquaporins to some extent correlate with their subcellular localization, results from several studies suggest that some plant aquaporin isoforms are targeted to other membranes than suggested by their names [reviewed in Maurel *et al*., 2008]. The large number of plant aquaporins and their different subcellular localizations highlight the crucial need for plants to tightly regulate water and solute transport across the different membranes in plant cells since plants are stationary and frequently fall victim to extreme changes in their environment. Among aquaporins in higher plants, PIPs can be classified as orthodox aquaporins since they are water-specific. Though not as well characterized as members of the PIP subfamily, members of the SIPs, NIPs and XIPs subfamilies can be classified as unorthodox aquaporins since some of them have unusual aquaporin NPA motifs and/or ar/R regions, hence seem to have evolved specificity for other substrates than water.

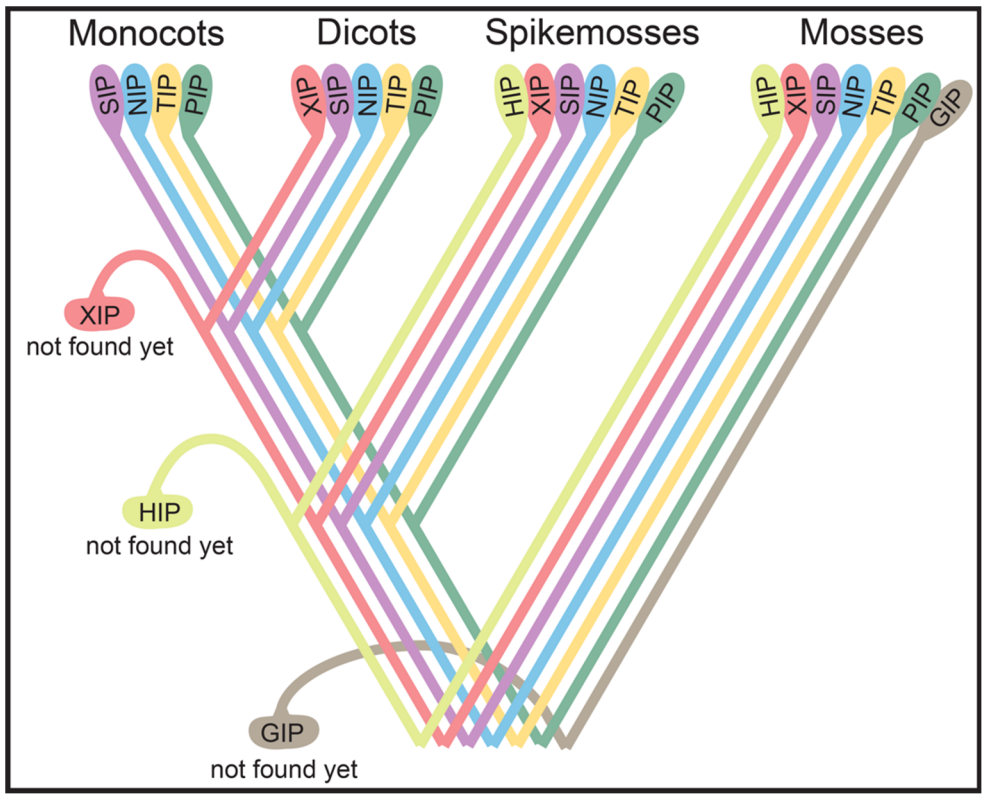

Figure 3. A schematic tree showing the aquaporin subfamilies in mosses, spikemosses, dicots and monocots. GIPs have been identified only in mosses. HIPs have been found in both mosses and spikemosses. XIPs have not been discovered yet in monocots, however, XIPs have been found in mosses, spikemosses and dicots. (The tree was provided by Hanna Anderberg).

PIPs

The Plasma Membrane Intrinsic Proteins (PIPs), as their name suggests, are localized to the plasma membrane of plant cells where they constitute the majority of all proteins in the membrane. In spinach leaf, for example, PIPs form approximately 20% of the total proteins in the plasma membrane [Johansson *et al*. 1996]. In higher plants, PIPs also appear to be the subfamily with the highest number of members, with thirteen PIP isoforms discovered in both *A. thaliana* [Johanson *et al*., 2001] and maize [Chaumont *et al*., 2001]. Though PIPs are divided into two subgroups, PIP1 and PIP2 isoforms, the ar/R selectivity filter is highly conserved in all PIP isoforms [Kammerloher *et al*., 1994; Chaumont *et al*., 2001; Kirscht *et al*., 2016b]. However, results from several studies indicate that PIP2 isoforms are efficient water facilitators as compared to PIP1 isoforms [Kammerloher *et al*., 1994; Johansson *et al*., 1998; Beila *et al*., 1999; Chaumont *et al*., 2000; Moshelion *et al*.,

2002; Fetter *et al*., 2004]. While water is the main substrate for PIPs, it has been reported that carbon dioxide can permeate PIP1 isoforms in tobacco, barley and maize [Uehlein *et al*., 2003; Hanba *et al*., 2004; Otto *et al*., 2010; Heinen *et al*., 2014; Yaneff *et al*., 2015]. Some PIP2 isoforms have been reported to be permeable to hydrogen peroxide [Dynowski *et al*., 2008; Bienert *et al*., 2014]. There are reports suggesting that PIP1 and PIP2 monomers form heterotetramers, which results in the activation and/or trafficking of PIP1 from the endoplasmic reticulum (ER) to the plasma membrane [Fetter *et al*., 2004; Zelazny *et al*., 2007; Otto *et al*., 2010; Yaneff *et al*., 2015]. Finally, as mentioned earlier the three dimensional structure of *So*PIP2;1 in the closed as well as in the open conformation, have shed light on the mechanism of plant aquaporin gating [Törnroth-Horsefield *et al*., 2006].

TIPs

The Tonoplast Intrinsic Proteins (TIPs) are named after the vacuolar membrane (i.e. the tonoplast) in which they are localized. TIPs makes up about 40% of the total proteins in the tonoplast [Higuchi *et al*., 1998] and this may be one of the reasons the tonoplast has a higher water permeability as compared to the plasma membrane [Maurel *et al*. 1997]. Some TIP isoforms have been suggested to be targeted to the plasma membrane, although the tonoplast seems to be the main subcellular localization for TIPs [Alexandersson *et al*., 2004; Gattolin *et al*., 2011]. TIPs are the second largest subfamily of aquaporins in plants with eleven isoforms in maize [Chaumont *et al*., 2001] and ten isoforms identified in both *A. thaliana* [Johanson *et al*., 2001] and rice [Sakurai *et al*., 2005]. There are five subgroups (TIP1-5) of TIPs in higher plants. Some TIP isoforms are permeable to substrates other than water, for instance, glycerol, ammonia, urea, and hydrogen peroxide [Liu *et al*., 2003; Loque *et al*., 2005; Bienert *et al*., 2007; Soto *et al*., 2008; Azad *et al*., 2011; Kirscht *et al*., 2016]. As stated previously, the high resolution X-ray structure of *At*TIP2;1 revealed an additional histidine residue at a specific position in the loop C, which forms part of the residues of the ar/R selectivity region [Kirscht *et al*., 2016a].

SIPs

The Small basic Intrinsic Proteins (SIPs) were first identified in a phylogenetic analysis of the genome of *A. thaliana*. Their given name, SIPs, alludes to the fact that they are smaller than members of the PIPs and NIPs isoforms and contain more basic amino acids than the TIPs [Johanson *et al*., 2001; Johanson and Gustavsson,

2002]. SIPs form two subgroups, SIP1 and SIP2, in higher plants. In comparison with PIPs, members of the SIP subfamily have unusual NPA motifs and ar/R regions. SIPs have been suggested to be expressed in the ER [Ishikawa *et al*., 2005; Maeshima and Ishikawa, 2008] and water permeability has been reported for *At*SIP1;1 and *At*SIP1;2 but not for *At*SIP2;1 [Ishikawa *et al*., 2005]. The physiological function of SIPs is not known and the structure of a member of the SIPs subfamily is yet to be solved.

GIPs

The Glycerol facilitator-like Intrinsic Proteins (GIPs) were named as such due to the fact that they appeared to be closely related to the glycerol facilitator protein (GlpF) in *E. coli* but different as compared to all other aquaporins in plants [Gustavsson *et al*., 2005]. GIPs were first discovered in mosses and were predicted to be localized in the plasma membrane. GIP1;1 was permeable to glycerol but not to water when transiently expressed in *Xenopus laevis* oocytes [Gustavsson *et al*., 2005]. The three dimensional structure of GIPs is not yet available.

HIPs

The Hybrid Intrinsic Proteins (HIPs) were discovered together with the XIPs in the genome of the moss *Physcomitrella patens* [Danielson and Johanson, 2008]. Due to shared characteristics with both PIPs and TIPs, the new subfamily was designated Hybrid Intrinsic Proteins. HIPs seem to be absent in higher plants, however, they have been identified also in the spikemoss *Selaginella moellendorffii* [Anderberg *et al*., 2012]. Functional and structural data are needed to understand the function of HIPs.

NIPs

Soybean nodulin 26, which was discovered in root nodules of the plant, is the archetype of the NIPs subfamily and by which this subfamily was named after [Sandal and Marcker, 1988; Shiels *et al*., 1988; Miao and Verma, 1993]. NIPs have also been found in other model leguminous plants such as *Lotus japonicus* [Guenther and Roberts, 2000] and *Medicago truncatula* [Catalano *et al*., 2004]. The

first NIPs which were characterized from plants other than legumes were *A. thaliana* NIP1;1 and NIP1;2 [Weig *et al*., 1997; Weig and Jakob, 2000].
NIPs form one of the aquaporin subfamilies in plant that are very diverse in terms of their substrate selectivity [Pommerrenig *et al*., 2015]. NIPs have been reported to be permeable not only to water, glycerol, ammonia and urea but also permeable to various metalloids such as boron, silicon, arsenic, antimony and selenium [Rivers *et al*., 1997; Dean *et al*., 1999; Niemietz and Tyerman, 2002; Wallace *et al*., 2005; Ma *et al*., 2006; Takano *et al*., 2006; Bienert *et al*., 2008; Kamiya *et al*., 2009; Kamiya and Fujiwara, 2009; Zhao *et al*., 2010; Hwang *et al*., 2010; Hanaoka *et al*., 2014]. While metalloids such as boron and silicon are essential nutrients for plants, metalloids such as arsenic and antimony are toxic to plants and animals [Takano *et al*., 2006; Ma *et al*., 2006; Pommerrenig *et al*., 2015].

In three independent phylogenetic studies, NIPs were categorized into four subgroups (NIP-1 – NIP-4) [Johanson and Danielson, 2010], six NIP ortholog clusters (NIPCL-I – NIPCL-VI) [Soto *et al*., 2012] or five subgroups (NIP1 – NIP5) [Abascal *et al*., 2014]. Prior to these classifications, *At*NIPs had been primarily classified into 7 phylogenetic groups [Johanson *et al*., 2001; Quigley *et al*., 2002]. In addition to the phylogenetic studies, NIPs have also been classified into three functional groups (NIP-I – NIP-III) based on the amino acids composition of the aromatic arginine (ar/R) selectivity filter [Mitani *et al*., 2008]. *At*NIP1;1, *At*NIP1;2, *At*NIP2;1, *At*NIP3;1, *At*NIP4;1 and *At*NIP4;2 with selectivity filters similar to the filter of soybean nodulin 26 [Wallace and Roberts, 2004] were grouped with the members of the NIP-I functional group which are permeable to water, arsenous acid and antimonous acid. *At*NIP5;1, *At*NIP6;1 and *At*NIP7;1 which have been found to be permeable to boric acid, in addition to the aforementioned substrates of the NIP-I group, were categorized among the members of the NIP-II functional group [Pommerrenig *et al*., 2015]. Members of the NIP-III functional group, which includes OsNIP2;1, are mainly permeable to silicic acid, however, permeability to selenous acid and germanic acid in addition to the abovementioned substrates of the NIP-I and NIP-II groups have also been suggested [Pommerrenig *et al*., 2015]. *Os*NIP2;1 (Lsi1) is the first identified silicon transporter in plant [Ma *et al*., 2006]. Silicon is a vital component of plant cell walls and boosts plants resistance to pest and diseases and helps plants to cope with different stress conditions [Ma *et al*., 2006].

NIPs appear to be mainly localized in the plasma membrane, however, localization to intracellular membranes including the ER membranes have also been suggested [Ma *et al*., 2006; Mizutani *et al*., 2006; Tanaka *et al*., 2008; Giovannetti *et al*., 2012]. The *AtNIP5;1* and *AtNIP6;1* genes were upregulated under conditions of boron limitation and down regulated under high boron conditions [Takano *et al*., 2006; Tanaka *et al*., 2008; Miwa *et al*., 2010]. Interestingly, *Os*NIP2;1 and *At*NIP5;1 have

been proposed to work in concert with the active transporters Lsi2 and *At*Bor1 for the uptake and subsequent distribution of silicon and boron, respectively. In plants, *Os*NIP2;1 and *At*NIP5;1 are localized in the plasma membrane at the distal side of the specific root cells (e.g. endodermis) in which they are expressed while Lsi2 and *At*Bor1 are localized in the plasma membrane at the proximal sides of the same cells [Ma *et al*., 2006; Takano *et al*., 2006; Takano *et al*., 2008, Takano *et al*., 2010]. Guenther *et al*., 2003 showed that phosphorylation of serine 262 enhanced water permeability in soybean nodulin 26. Choi and Roberts, 2007 showed that the *AtNIP2;1* transcript level was upregulated in root cells under oxygen deprived and water logging conditions. In addition, the *At*NIP2;1 protein was permeable to lactic acid when it was expressed in *Xenopus laevis* oocytes. This led to the suggestion that *At*NIP2;1 might play a role in lactic acid fermentation in roots under anaerobic conditions [Choi and Roberts, 2007]. Pommerrenig *et al*., 2015 have proposed that NIPs should be called "metalloido-porins" to highlight the enormous contribution by NIPs to metalloid homeostasis in plants, since NIPs are the only known channels in plants that are essentially responsible for the uptake, translocation or extrusion of several uncharged metalloids. Members of the NIPs subfamily have been studied extensively as reviewed by Wallace *et al*., 2006 and Pommerrenig *et al*., 2015, but so far no three dimensional structure of a member of the NIP subfamily have been deposited in the Protein Data Bank (PDB).

XIPs

The X-Intrinsic Proteins (XIPs) were first discovered in the genome of the moss *Physcomitrella patens* [Danielson and Johanson, 2008]. The 'X' alluded to the lack of information regarding the function and subcellular localization of this previously unrecognized MIP subfamily.

In monocotyledonous plants, XIPs seem to be absent, however, they have been found in most dicotyledonous plants; for instance, grapevine [*Vitis vinifera*; Danielson and Johanson, 2008], poplar [*Populus trichocarpa*; Gupta and Sankararamakrishnan, 2009], tomato [*Solanum lycopersicum*; Sade *et al*., 2009; Reuscher *et al*., 2013], cotton [*Gossypium hirsutum*; Park *et al*., 2010], tobacco [*Nicotiana tabacum*; Bienert et al., 2011; *Nicotiana benthamiana*; Ampah-Korsah *et al*., 2016], bean [*Phaseolus vulgaris*; Ariani and Gepts, 2015], rubber tree [*Hevea brasiliensis*; Zou *et al*., 2015a], castor bean [*Ricinus communis*; Zou *et al*., 2015b], and physic nut [*Jatropha curcas*; Zou *et al*., 2016]. Surprisingly, XIPs are also absent in the genome of the model dicotyledonous plant *A. thaliana* [Danielson and Johanson, 2008]. This explains why XIPs were not recognized earlier since the genome of *A. thaliana* was sequenced before the genome of *P. patens* was known

and have been studied extensively. However, the absence of XIPs in *Arabidopsis* and monocotyledonous plants studied so far, may be due to a functional redundancy that took place during the course of evolution since XIPs share similar substrate selectivity with NIPs though similar substrate specificity do not necessarily imply functional redundancy [Bienert *et al*., 2011]. XIPs have been found also in protozoa [Danielson and Johanson, 2008] and fungi [Gupta and Sankararamakrishnan, 2009].

The most conserved feature or the signature motif of the XIPs subfamily is the NPARC motif showing a conserved cysteine after the second NPA aquaporin motif [Danielson and Johanson, 2008]. This cysteine has been proposed to form a disulphide bond to another conserved cysteine (in the LGGC motif) in loop C of most plant XIPs and some fungi XIPs (see Figure 4). Also, there is a considerable amino acid variation at both the first NPA aquaporin motif and the ar/R selectivity filter of XIPs. The alanine in the first NPA of XIPs is most often replaced by a valine, leucine, isoleucine, serine or cysteine. In a phylogenetic analysis by Lopez *et al*., 2012, XIPs of higher plants were categorized into two distinct clusters (XIP-A and XIP-B), where XIP-A consists of only one subgroup (XIP1) and XIP-B is made up of four subgroups (XIP2, XIP3, XIP4 and XIP5). Additionally, XIPs of land plants have been recently grouped into four subgroups (XIP I-IV) [Venkatesh *et al*., 2015]. *Solanales* XIPs including *N. benthamiana*, *N. tabacum*, *S. lycopersicum* and *Ipomoea nil* XIPs were assigned to the XIP4 group [Lopez *et al*., 2012; Venkatesh *et al*., 2015] which corresponds to the subgroup XIP IV in the recent classification.

Analysis at the protein level to investigate functional divergence of XIPs in plants revealed that regarding the ar/R selectivity filter residues, the amino acid at the $H2^P$ position showed significant functional divergence in XIPs, with the largest difference between *S. moellendorffii* and *P. patens* XIPs sequences in the XIP I subgroup formed by XIPs of more primitive plants, and XIPs sequences from the other subgroups [Venkatesh *et al*., 2015]. In the XIP I subgroup, a hydrophilic residue (His or Gln) reside at the $H2^P$ position, while in the other XIP subgroups a hydrophobic residue occupies this position [Venkatesh *et al*., 2015]. This may suggest a difference in substrate specificity between XIPs in primitive land plants (mosses and spikemosses) and other XIPs.

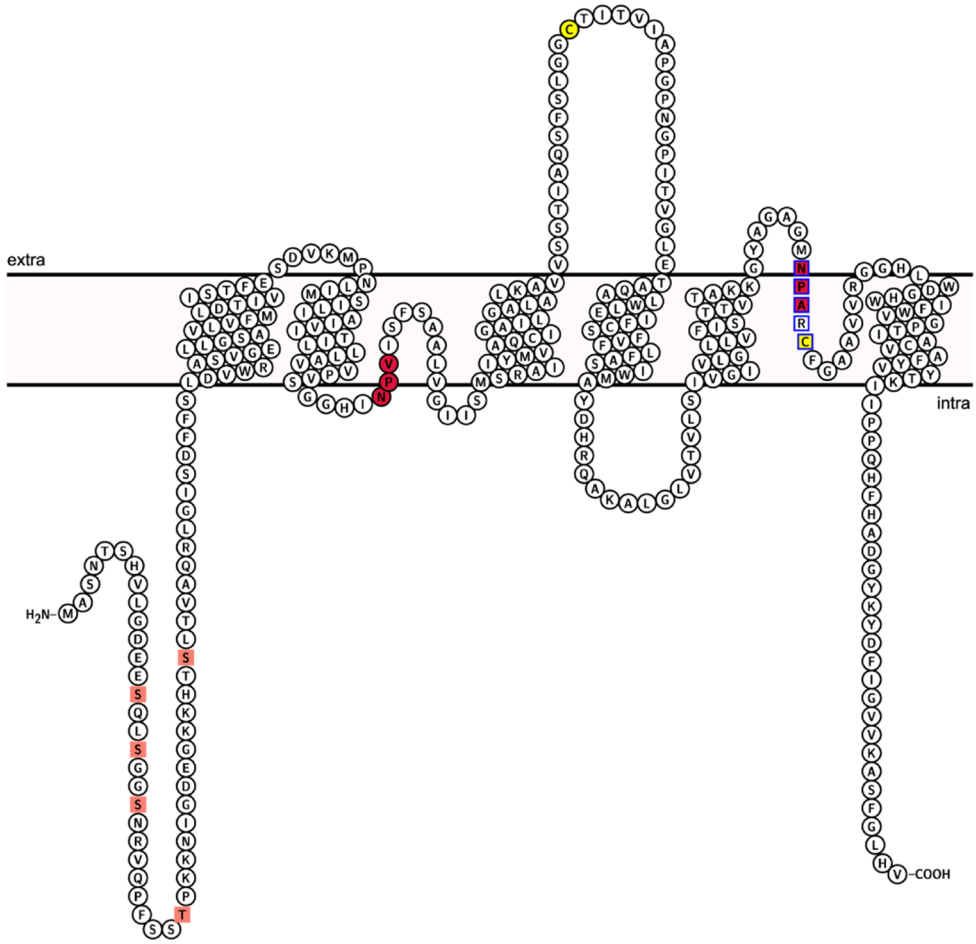

Figure 4. Topology of *Nb*XIP1;1α. The NPA aquaporin motifs are shown in red fill. In *Nb*XIP1;1α, the alanine in the first NPA is exchanged for a valine residue. The NPARC XIP signature motif is shown in blue frame. The conserved cysteines in loop C and loop E are in yellow fill. The identified phosphorylation sites in the N-terminal region of *Nb*XIP1;1α are shown in salmon fill [Ampah-Korsah *et al*., 2016].

Alternative splicing occurs in the pre-mRNAs of some *XIP1* genes in *Solanaceae* and this produces two variants of the transcript and hence results in two slightly different proteins [Bienert *et al*., 2011; Ampah-Korsah *et al*., 2016]. In *N. benthamiana*, two of the three identified *XIP1* genes undergo alternative splicing resulting in five different *Nb*XIP1 proteins (*Nb*XIP1;1α, *Nb*XIP1;1β, *Nb*XIP1;2α, *Nb*XIP1;2β and *Nb*XIP1;3) [Ampah-Korsah *et al*., 2016]. The β-splice-variants are one arginine longer than the α-splice-variants and additionally in the *Nb*XIP1;1s, asparagine 32 in *Nb*XIP1;1β changes to a lysine in *Nb*XIP1;1α. Alternative splicing may alter the function, stability, posttranslational modifications or subcellular location of proteins, however, no difference in substrate specificity or subcellular

location was seen for the *Nt*XIP1;1α and *Nt*XIP1;1β splice variants [Bienert *et al*., 2011].

XIP1:1 proteins have been found to be mainly localized in the plasma membrane when transiently or heterologously expressed in tobacco or yeast cells, respectively [Bienert *et al*., 2011; Ampah-Korsah *et al*., 2016]. However, ER membrane localization has been suggested for the grapevine *Vv*XIP1 aquaporin [Noronha *et al*., 2016]. Five amino acids in the N-terminal region of *Nb*XIP1;1α were found to be phosphorylated [Ampah-Korsah *et al*., 2016]. Some of these phosphorylated sites were proposed to have a role in targeting the *Nb*XIP1;1α protein to the plasma membrane since phosphorylation of aquaporins has been shown to be important for both trafficking of aquaporins to the plasma membrane and for gating the channel. A topology of *Nb*XIP1;1α is shown in Figure 4. When transiently expressed in the tobacco plant, *NtXIP1;1* mRNAs were found to be expressed in all organs of the plant [Bienert *et al*., 2011].

Based on the comparatively more hydrophobic nature of the selectivity filter, XIP1;1s were proposed to be not primarily water transport facilitators [Danielson and Johanson, 2008; Bienert *et al*., 2011]. *Nb*XIP1;1α rendered *P. pastoris* cells more sensitive to boric acid as compared to control cells and increased the permeability of proteoliposomes to boric acid [Ampah-Korsah *et al*., 2016]. Beside boric acid, *Nt*XIP1;1s have been shown to be permeable to glycerol, hydrogen peroxide and urea [Bienert *et al*., 2011]. In addition to boric acid, glycerol and hydrogen peroxide, *Vv*XIP1 has been reported to be permeable to arsenic, copper and nickel [Noronha *et al*., 2016]. With the exception of *Pt*XIP2;1 and *Pt*XIP3;3 which were reported to facilitate the transport of water in *Xenopus laevis* oocytes, no water permeability has been reported for *Nt*XIP1;1s, *Nb*XIP1;1s and *Vv*XIP1, including other *Pt*XIPs [Bienert *et al*., 2011; Lopez *et al*., 2012; Ampah-Korsah *et al*., 2016; Noronha *et al*., 2016].

VvXIP1 transcripts were shown to be down-regulated in cultured grape cells under drought stress, which led to the proposition that the *Vv*XIP1 protein may play a role in osmotic regulation and metal homeostasis [Noronha *et al*., 2016]. So far, there is no structure of a member of the XIPs subfamily. A high resolution three dimensional structure and further functional characterization may reveal the substrate of choice of XIPs and hence the physiological relevance of XIPs in plants that have retained them.

Chapter 2

Methods

In this chapter, the main methods and techniques used to study the *Nb*XIP1;1s and *At*NIPs proteins are described. These methods and techniques are commonly used to study membrane proteins.

Overexpression

It is necessary to study the function of membrane proteins in great detail in order to understand the roles they play in various physiological processes in the cell. Such studies require the use of large amounts of proteins. However, membrane proteins are by default expressed in small quantities in their native membranes. It is therefore laborious to obtain sufficient amounts of membrane proteins from their native membranes for functional and structural studies.

To circumvent this bottleneck in the studies of proteins, several expression systems have been developed for producing large quantities of proteins. Expression of proteins in heterologous systems also makes it possible to carry out mutational studies on the proteins which otherwise will be difficult in the native organism. One of the commonly used heterologous expression systems is *E. coli*. However, a major limitation of the *E. coli* expression system is that, as a prokaryote, it lacks the machinery to correctly fold eukaryotic membrane proteins with the necessary posttranslational modifications required for their function. Also, highly expressed proteins usually end up as inclusion bodies in *E. coli* cells. The *P. pastoris* expression system, as a eukaryote, present a better alternative for producing eukaryotic membrane proteins. In addition to having a strong methanol inducible *alcohol oxidase 1* (*AOX1*) promoter, stable cell lines can be created by insertion of the gene of interest into the *Pichia* genome by homologous recombination and also, *P. pastoris* can grow to very high cell densities [Daly and Hearn, 2005].

To boost our chances for successful high expression in *P. pastoris*, we used the strategy proposed by Nordén *et al*., 2011, i.e. increasing the gene dosage which leads to enhanced recombinant production of aquaporins in *P. pastoris*. By this strategy, the cDNA encoding the protein of interest can be isolated from the native source, amplified and cloned into a plasmid. The resulting plasmid is then sequenced and transformed into *P. pastoris*. Subsequently, selection of *P. pastoris* clones with multiple insertions of the cDNA encoding the protein of interest, as well as a gene encoding antibiotic resistance, is done on plates containing increasing concentrations of the antibiotic zeocin. Finally, a small scale expression screen is carried out to isolate the best *P. pastoris* clone with the highest protein expression. We expressed the *Nb*XIP1;1α wildtype, *Nb*XIP1;1α mutants, *Nb*XIP1;1β, *At*NIP1;1 and *At*NIP5;1 proteins in *P. pastoris*.

The critical steps involved in obtaining multi-copy *P. pastoris* clones, i.e. clones with multiple insertions of the gene of interest, and good expression are highlighted below:

The first critical step is the transformation method or the means by which the linearized pPICZB plasmid with the cloned gene of interest is introduced into the *Pichia* cells. It is paramount that *P. pastoris* cells are transformed via electroporation since electroporation usually results in higher transformation efficiencies than chemical transformation and so it will be easier to isolate multi-copy integrants. Electroporation refers to the process by which electric current is used to create pores in the cell wall and plasma membrane of cells through which the foreign DNA passes. In the preparation of the *P. pastoris* cells for electroporation it is crucial to wash the cells thoroughly with ice-cold sterile deionized water to minimize the concentration of salts in the transformation mixture since high amount of salts cause sparks in the electroporation cuvette which may kill the cells.

The second and most critical step is the optimization of expression levels by selection on increasing concentrations of zeocin. The *Pichia* plasmid pPICZB has the zeocin resistant gene, *Sh ble*, and this gene is by homologous recombination inserted together with the gene of interest into the *Pichia* genome. Multiple incorporation of the gene of interest which is also equivalent to multiple insertion of the *Sh ble* gene concomitantly leads to enhanced resistance to zeocin in the clones. Therefore, *P. pastoris* transformants that thrive on YPD agar plates with high levels of zeocin harbour multiple copies of the *Sh ble* gene and the gene of interest. After screening for high zeocin concentration resistant clones, it is essential to verify the gene dosage by qPCR and also to evaluate the actual protein levels in these multi-copy clones by western blot analysis since increased resistance to zeocin does not automatically result in high protein expression levels.

Finally, the method of cultivation of the *P. pastoris* clones is very important for good protein expression. Cell cultivation in a bioreactor, fermenter, is recommended over cell cultivation in flask. This is because conditions such as temperature, pH and dissolved oxygen which affect protein expression can be better regulated in a fermenter than in a flask. Since too much methanol is toxic to the cells and molecular oxygen is needed in the oxidation of methanol in the methylotrophic *P. pastoris* cells, it is crucial that the amount of dissolved oxygen in the culture medium and methanol are tightly controlled, in order to induce the *AOX1* promoter and enhance protein expression. Furthermore, by avoiding anaerobic conditions, high cell densities can be reached by using a bioreactor for cultivation of *P. pastoris* cells. For instance, we routinely obtained cell densities of approximately 300 OD_{600} in a fermenter-based cultivation as compared to 20-25 OD_{600} in a flask-based cultivation.

Membrane protein purification

Membrane preparation and protein solubilization

Membrane proteins, as their name suggests, are synthesized and attached or inserted into the membranes of cells. This presents a challenge in working with membrane proteins since it usually requires the use of detergents to extract these proteins from the membrane.

After obtaining a *P. pastoris* clone with high expression of the protein of interest, the next step is to isolate membranes from the cells. Since *P. pastoris* cells contain thick cell walls, they need to be disrupted to obtain the membranes. *P. pastoris* cells can be lysed by using the Beadbeater™ method which uses glass beads to mechanically break the cells or by using the French press method which uses shear forces caused by pressure variations to break the cells. In this work, the Beadbeater™ method was used to prepare crude membranes from *P. pastoris* cells. With the current protocol, the efficiency of cell lysis using the Beadbeater™ method is approximately 70% (data not shown). To increase the yield of broken cells, the cells can be treated with enzymes to degrade the cell wall before disrupting the cells with the glass beads. It is very important to keep the cells sufficiently cold in the lysis process to minimize protein degradation. Peripheral membrane proteins can be removed by washing the crude membranes with first urea and then sodium hydroxide. However, the sodium hydroxide treatment can be harsh on the protein of interest and so the effect of this wash on the protein should be investigated. For instance, in the purification of the splice-variant *Nb*XIP1;1α, the sodium hydroxide wash was discarded due to high protein losses in this (wash) step. However, it was observed that without the urea wash step it was difficult to solubilize the *Nb*XIP1;1α protein from the crude membranes.

After isolating membranes from the *P. pastoris* cells expressing the protein of interest, the next step is to extract or solubilize the protein from the membranes. Detergents are employed in solubilizing the protein from the membranes. However, several detergents need to be screened to identify the optimal detergent for the protein of interest. The optimal detergent is the one that solubilizes a large fraction of the protein from the membrane at the same time as keeping the protein in its native form. At this stage, the stability of the protein in different detergents, buffers and at different temperatures can be evaluated. However, a common practice is to solubilize and purify proteins at cold temperatures, usually on ice (0°C) or in the cold room (10°C), to minimize protein aggregation.

Purification

The next step after identification of the optimal detergent for the protein, is to purify the protein of interest from other solubilized proteins. The methods commonly used to purify proteins separate them based on their size, charge or binding affinity to specific molecules.

Immobilized Metal-ion Affinity Chromatography (IMAC) was used to purify *Nb*XIP1;1α and *At*NIP5;1 proteins. IMAC utilizes immobilized divalent ions such as Ni^{2+} on a nitrilotriacetic acid matrix coupled to an agarose resin to purify recombinant proteins containing a polyhistidine tag. The imidazole ring in the side chain of histidine binds to the immobilized Ni^{2+} ion. Hence a recombinant protein with a His-tag can be bound to the immobilized Ni^{2+} ions on the matrix. After removing the non-bound proteins by washing the matrix material, the bound protein can be eluted by either decreasing the pH or addition of imidazole. The eluted proteins can be purified further to obtain homogeneous protein samples by using Size-Exclusion Chromatography (SEC). SEC separates proteins according to size with large molecular weight proteins eluting first followed by smaller ones in a decreasing order. If SEC is not needed, it is recommended to desalt the eluted proteins to minimize the amount of imidazole in the eluted proteins since high concentrations of imidazole can interfere with downstream procedures. The purity of the eluted proteins can be evaluated on a Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel stained with silver or Coomassie brilliant blue dye.

The amount of the protein of interest in the eluted fraction can be estimated by western blot and more accurately by measuring the protein concentration by spectrophotometric methods (e.g. Bearden, 1978).

Functional characterization

Growth assay

This method normally uses mutant intact cells lacking the endogenous transporter(s) for the substrate of interest. For instance, if the aim is to test the function of a novel aquaporin protein, then mutant cells deficient in the resident/endogenous aquaporins are usually employed. Hence, by transforming the mutant cells with the gene of the novel aquaporin, aquaporin function will be restored in the mutant cells. Depending on whether the substrate tested for is beneficial or toxic to the transformed cells, the growth of the cells is either improved or retarded, respectively, on plates containing the substrate. Here, we tested for boric acid permeability in *Nb*XIP1;1s and *At*NIP5;1 expressed in the wild-type *P. pastoris* X-33 cells. Even though mutant *P. pastoris* strains deficient in the endogenous Aqy1 protein were not used, *Nb*XIP1;1 and *At*NIP5;1 could still render the wild-type *P. pastoris* cells expressing the individual proteins more sensitive to boric acid than control X-33 cells. The positive results of the growth assay also suggested that at least some fraction of the expressed proteins, *Nb*XIP1;1α and *At*NIP5;1, were correctly folded and localized in the plasma membrane of *P. pastoris* cells.

Stopped-flow spectrometry

Stopped-flow spectrometry is a spectrometric technique used to study the kinetics of fast reactions in solution on millisecond to second timescale. It involves an UV/VIS spectrophotometer coupled with a rapid mixing system. Two or more solutions are mixed rapidly in a chamber and the changes in light signal (either, scattering, absorbance or fluorescence) of the solutions are measured. Stopped-flow measurements assaying for water, glycerol and boric acid permeability were done on *P. pastoris* spheroplasts expressing *Nb*XIP1;1α or *At*NIP5;1 and also on proteoliposomes containing the purified *Nb*XIP1;1α protein.

Yeast spheroplast are usually prepared by treating yeast cells with enzymes such as zymolyase or lyticase that degrade the cell wall. However, spheroplast preparation with zymolyase failed to yield reproducible results. Instead, yeast spheroplasts were prepared by destabilizing the yeast cell wall with β-mercaptoethanol. Disulphide linkages in the cell wall are reduced by β-mercaptoethanol and this results in a destabilization of the yeast cell wall.

Reconstitution of a purified membrane protein into lipid vesicles, liposomes, provides a way to study the function of the membrane protein in a defined system, free from the interfering effects of other proteins. However, the reconstituted membrane protein may require an interacting protein partner to be active. Proteoliposomes may be prepared by first mixing detergent solubilized lipids and detergent micelles containing the purified protein and then removing the detergent from the mixture. The removal of the detergent causes the protein and the lipids to spontaneously associate to form vesicles, proteoliposomes, made up of the lipids and the protein. The detergent can be removed by diluting the detergent/lipid/protein mixture or by dialysis or by the addition of hydrophobic polystyrene beads to adsorb the detergent molecules. Detergents with high critical micelle concentration (CMC) can efficiently be removed by dilution or dialysis, while detergents with low CMC can only effectively be removed by the addition of hydrophobic beads.

In the water permeability assay, yeast spheroplasts or proteoliposomes are subjected to a hyperosmolar solution and the shrinkage upon mixture due to the efflux of water from the spheroplast or proteoliposomes causes an increase in light scattering which is recorded at 90° angle to the incoming light in the stopped-flow apparatus. In the glycerol or boric acid permeability assay, the spheroplasts or proteoliposomes are mixed with hyperosmolar solution containing glycerol or boric acid, respectively. This causes an initital fast shrinkage due to fast water efflux followed by a gradual swelling due to the influx of glycerol or boric acid followed by water. The rate at which water, glycerol or boric acid passes through the aquaporin is depicted by the exponential rate constants of the curves. These rate constants are compared with that of control spheroplasts or liposomes (without protein) treated in the same manner.

The function of an aquaporin can also be tested by transiently expressing the aquaporin in *Xenopus laevis* oocytes and measuring the shrinkage or swelling of the oocytes in hyperosmolar or hypo-osmolar solutions, respectively.

Structural characterization

Mass spectrometry

In protein identification and characterization by mass spectrometry (MS), the protein, in solution or separated on a gel, is reduced and alkylated to protect cysteine residues in the protein. The protein is then digested with a protease, usually trypsin, to create peptides with different masses. The peptides are fractionated by chromatography and fed into the mass spectrometer. In the mass spectrometer, the peptides are ionized and subsequently separated based on their mass-to-charge ratios. This generates experimentally determined peptide mass values which can be used to identify the protein by matching these observed peptide mass values with theoretically calculated peptide masses created by *in silico* digestion of a protein sequence database (e.g. Mascot database). An in-house Mascot database comprising of amino acid sequences of *N. benthamiana* XIP1;1s and *A. thaliana* NIP5;1 and NIP1;1 was used to identify peptides of *Nb*XIP1;1α protein.

In tandem MS (MS/MS), individual peptide ions are isolated and fragmented by collision induced dissociation in the mass spectrometer, resulting in an ensemble of fragment ions. The masses of these fragment ions are recorded and used to obtain the sequence of the peptide. By compiling and evaluating the sequences of the peptide ions fragmented in tandem MS, structural data such as posttranslational modifications of the protein can be obtained. A high sequence coverage is required to fully characterize a protein and its modifications. MS was used to identify phosphorylated amino acid residues of *Nb*XIP1;1α.

Phostag™ method

This method utilizes biotinylated Phos-tag™ zinc(II) complexes (Wako Pure Chemical Industries, Ltd), such as Phos-tag™ BTL-104 and Phos-tag™ BTL-111, to detect phosphorylated proteins on a polyvinylidene difluoride (PVDF) membrane. The Phos-tag™ complex binds to Zn^{2+} ions which in turn binds to phosphates in phosphorylated proteins. Hence, by binding streptavidin-conjugated horseradish peroxidase to the biotin, phosphorylated proteins can be visualized by chemiluminescence just as in immunoblot assays. Phos-tag™ BTL-111 was used to detect phosphorylation in *Nb*XIP1;1α.

Homology modeling

Homology modeling is based on the fact that proteins which share high sequence identity generally tend to have similar overall three dimensional structures. In general, structure is more conserved than sequence. Hence, proteins that share more than 35% sequence identity will typically have the same fold. It is therefore possible to create a homology model of the three dimensional (3D) structure of a protein of interest based on an experimentally determined 3D structure of a closely related protein. Caution should be taken, though, when interpreting results based on a homology model since homology models are not based on experimental data. However, a homology model of a protein can be used to some extent to rationalize experimentally generated results from functional studies of the protein, in order to obtain some insights on how the structure of the protein affects its function.

Basically, a homology model of a protein is generated by aligning the amino acid sequence of the protein to that of a closely related protein with a solved 3D structure. The alignment is then submitted to a server such as SWISS-MODEL or I-TASSER to generate the model. The model obtained from the server is submitted to energy minimization softwares to minimize and validate bond angles in the model. Homology models of the wild-type and mutant *Nb*XIP1;1α proteins were constructed by using the high resolution X-ray crystal structure of *At*TIP2;1 as a template.

Paper summary

Paper I

After the discovery of the XIP subfamily in 2008, work begun in different research groups to characterize isoforms of this new subfamily. Till date, the physiological function and the *in vivo* substrate/s of XIPs are yet to be discerned. It is therefore necessary to characterize XIPs further with respect to selectivity, structure and regulation. In this paper, we described the expression, purification and characterization of *Nb*XIP1;1 splice variants in *P. pastoris*. Furthermore, we demonstrated that the *Nb*XIP1;1α was phosphorylated in its N-terminal region.

In order to generate sufficient amounts of protein for structural and functional characterization of *Nb*XIP1;1 proteins, cDNAs encoding the α and β splice variants of *Nb*XIP1;1 were transformed into *P. pastoris*. A His-tag was placed at either the N-terminus or the C-terminus of the amino acid sequence of *Nb*XIP1;1 for identification by immunoblot assays and for purification by Ni-NTA affinity chromatography. After optimization of the protein expression levels, by screening multi-copy clones selected at different levels of zeocin concentration, both splice variants expressed successfully and at similar protein levels. However, the *Nb*XIP1;1α splice-variant was chosen for further studies since it had a slightly higher expression level than the *Nb*XIP1;1β splice-variant.

*Nt*XIP1;1 isoforms, which share high sequence similarity with *Nb*XIP1;1s, have previously been shown to be permeable to boric acid. A boric acid growth toxicity assay was therefore employed to ascertain whether *Nb*XIP1;1α was also permeable to boric acid. *P. pastoris* cells expressing the *Nb*XIP1;1α protein were similarly sensitive to boric acid as compared to the cells expressing the control boric acid permeable *At*NIP5;1 protein. This indicated that *Nb*XIP1;1α, just like *At*NIP5;1, facilitated the permeation of boric acid into *P. pastoris* cells. It also suggested that the heterologously expressed *Nb*XIP1;1α was not only targeted to the plasma membrane but also correctly folded and functional. It was observed that the cells expressing the N-terminally His-tagged *Nb*XIP1;1α protein were more sensitive to boric acid than the cells expressing the C-terminally His-tagged *Nb*XIP1;1α protein. This suggested that the N-terminally His-tagged *Nb*XIP1;1α protein was somewhat

more open, which might have implications when discussing a putative gating mechanism involving phosphorylation of the protein. However, the difference observed in the sensitivity of the cells to boric acid could be due to a difference in the expression levels of the *Nb*XIP1;1α constructs in the plasma membrane as the N-terminally His-tagged *Nb*XIP1;1α protein had a higher expression level than the C-terminally His-tagged *Nb*XIP1;1α protein.

Stopped-flow spectrometry was used to investigate whether *Nb*XIP1;1α was permeable to water and glycerol in *P. pastoris* spheroplasts. *Nb*XIP1;1α did not facilitate the permeation of water in *P. pastoris* spheroplasts. This was not surprising based on the comparably more hydrophobic nature of the XIP1;1 ar/R selectivity filter residues as compared to the amino acids comprising the ar/R region of the orthodox aquaporins. Thus, XIP1;1s were proposed not to be water permeable and water permeability has actually not been seen in other XIP1;1 isoforms. The control *At*NIP5;1 aquaporin had approximately two-fold increase in water permeability. However, the interpretation of these water permeability results might be complicated by the fact that the sizes of the different spheroplasts were not verified.

*Nb*XIP1;1α was not permeable to glycerol in *P. pastoris* spheroplasts, however, the control *At*NIP5;1 displayed a four-fold higher glycerol permeability in *P. pastoris* spheroplasts than the spheroplasts transformed with the empty pPICZB plasmid. This was in contrast to the glycerol permeability seen in *Nt*XIP1;1 isoforms when they were transiently expressed in *Xenopus laevis* oocytes [Bienert *et al*., 2011]. Whether this difference in the observation of glycerol permeability is due to the choice of the different experimental systems or due to a difference in the substrate specificity of *Nb*XIP1;1 and *Nt*XIP1;1 isoforms, remains unclear. The recombinant N-terminally His-tagged *Nb*XIP1;1α protein was found not only in intracellular membranes (including the ER) but also in the plasma membrane fraction when sucrose gradient fractions of cell homogenates of *P. pastoris* cells expressing *Nb*XIP1;1α were analysed by western blot. This, however, indicated that the lack of water and glycerol permeability in the *P. pastoris* spheroplasts transformed with the *Nb*XIP1;1α gene was neither due to a lack of expression nor to a disturbance in the trafficking of the protein to the plasma membrane in the *Pichia* cells.

N-nonyl-β-D-glucopyranoside (NG) detergent was used to solubilize urea washed stripped membranes of *P. pastoris* cells expressing the *Nb*XIP1;1α protein. Utilizing the His-tag at the N-terminus, the *Nb*XIP1;1α protein was purified from the solubilized proteins by Ni-NTA affinity chromatography. The eluate was highly enriched in *Nb*XIP1;1α protein and with a yield of 0.15 mg protein per gram of yeast cells obtained routinely, it was sufficient for substrate specificity studies by reconstituting *Nb*XIP1;1α into proteoliposomes. After verifying with circular dichroism that the purified *Nb*XIP1;1α protein was correctly folded and intact, it

was reconstituted into *E. coli* lipids vesicles supplemented with 20% cholesterol to reduce the diffusion of undissociated boric acid through the bilayer of the proteoliposomes.

In a stopped-flow spectrometric analysis, *Nb*XIP1;1α increased the permeability of the proteoliposomes to boric acid two-fold as compared to the empty liposomes. A higher boric acid permeability was expected in the proteoliposomes since a seemingly high boric acid permeation by *Nb*XIP1;1α was seen in *P. pastoris* cells. This suggested that the *Nb*XIP1;1α channel was probably not fully opened in the proteoliposomes, which could be as a result of an unknown gating mechanism occluding the channel, such that only a subfraction of the *Nb*XIP1;1α protein in the proteoliposomes had open channels.

Phosphorylation has previously been shown to regulate the function of other plant aquaporins by gating. In the light of that, the phosphorylation status of the purified *Nb*XIP1;1α protein was investigated to ascertain whether the difference in boric acid permeability of *Nb*XIP1;1α in *P. pastoris* cells as compared to proteoliposomes could be due to a difference in the phosphorylation state of the *Nb*XIP1;1α protein. NetPhosYeast, an *in silico* phosphorylation prediction tool, revealed six putative phosphorylation sites in the N-terminal region of *Nb*XIP1;1α. Probing for phosphorylation with the phosphate binding tag, Phostag™ BTL-111, confirmed that the purified *Nb*XIP1;1α protein was phosphorylated. However, dephosphorylation of *Nb*XIP1;1α by alkaline phosphatase had no appreciable effect on the functionality of the protein with regards to boric acid permeability in proteoliposomes.

Mass spectrometric analysis identified five phosphorylation sites, four serines and one threonine, all in the N-terminal region of *Nb*XIP1;1α. Four of the identified phosphorylation sites coincided with the *in silico* prediction. It was proposed that some of these identified phosphorylation sites may have a role in targeting *Nb*XIP1;1α to the plasma membrane, since phosphorylation of aquaporins has been associated with both gating as well as trafficking of aquaporins to the plasma membrane. The MS data also showed that the purified *Nb*XIP1;1α protein was only partially phosphorylated as identical peptides were identified with and without phosphate groups bound. Inferring from the boric acid growth assay, the stopped-flow spectrometric analysis on the boric acid permeability of proteoliposomes, the dephosphorylation of the purified *Nb*XIP1;1α by alkaline phosphatase and the mass spectrometric analysis, it was speculated that the dephophorylated *Nb*XIP1;1α represented the closed conformation of the protein and that the N-terminal region may need to be fully phosphorylated to open the channel.

In conclusion, *Nb*XIP1;1s were successfully expressed in *P. pastoris*. *Nb*XIP1;1α was found to be functional and permeable to boric acid in *P. pastoris* cells, however no water permeability was seen in *Nb*XIP1;1α. It was also shown that the N-terminal region of *Nb*XIP1;1α was phosphorylated, suggesting that *Nb*XIP1;1α may be gated by phosphorylation.

Paper II

In this paper, we attempted to introduce water permeability in *Nb*XIP1;1α by exchanging its ar/R selectivity filter for the water permeable ar/R selectivity filter of *At*TIP2;1. If successful, this will not only help to us to carry out mutational studies on *Nb*XIP1;1α as water permeability in *Nb*XIP1;1α will be easy to quantify in *P. pastoris* spheroplasts by stopped-flow spectrometry but also, it will help us gain a better understanding of how the selectivity may be tweaked, which may also hold true for other aquaporin isoforms.

After multiple sequence alignment with aquaporins with solved structures, two alternative alignments of residues in the *Nb*XIP1;1α ar/R selectivity filter were obtained. That is, depending on how the helix 5 was aligned, the selectivity filter of *Nb*XIP1;1α could be comprised of either isoleucine, cysteine, valine, alanine and arginine (IC<u>V</u>AR) or isoleucine, cysteine, threonine, alanine and arginine (IC<u>T</u>AR; putative amino acid residue at the helix 5 position ($H5^P$) in the filter has been underlined). Hence, two putative selectivity filters of *Nb*XIP1;1α (I102, C175, V242/T246, A257, R263) were engineered to mimic the *At*TIP2;1 ar/R selectivity filter (H63, H131, I185, G194, R200), to allow for water permeability in *Nb*XIP1;1α.

Considering the conservation of the residues at the loop E (LE^P) and helix E (HE^P) positions and the uncertainty of the loop C alignment, only the residues at the helix two and helix five positions in the filter were considered. In order to sterically accommodate the histidine at the helix two position ($H2^P$), an additional L79G substitution was designed. Hence, two main *Nb*XIP1;1α mutants were constructed, i.e., *Nb*XIP1;1αL79G/I102H/<u>V242I</u> and *Nb*XIP1;1αL79G/I102H/<u>T246I</u>. To exclude the possibility of the long loop C and loop D occluding the channel, *Nb*XIP1;1α mutants with truncations in the loop C and/or loop D were also designed. This set of *Nb*XIP1;1α mutants were successfully expressed in *P. pastoris*, albeit to different levels.

In a stopped-flow spectrometric analysis measuring water permeability in *P. pastoris* spheroplasts, there was no appreciable difference in water permeability between the spheroplasts transformed with the empty pPICZB plasmid and the spheroplasts expressing the wild-type *Nb*XIP1;1α protein, which indicated that the wild-type *Nb*XIP1;1α protein was poorly or not at all permeable to water. After compensating for the difference in the expression levels by relating the rates for water permeability to the individual protein amounts, only the *Nb*XIP1;1αL79G/I102H/<u>V242I</u> triple mutant had a mean specific rate for water

permeability which was significantly different from that of the spheroplasts expressing the wild-type *Nb*XIP1;1α protein.
Truncating the loop C and/or loop D in the mutants did not significantly improve the relative specific rates, as the spheroplasts expressing the mutants with the truncations showed large experimental variation with mean rates not significantly different from that of the wild-type NbXIP1;1α protein or the NbXIP1;1αL79G/I102H/T246I mutant without a deletion. At this stage, it was not clear why the *Nb*XIP1;1αL79G/I102H/T246I mutant could not facilitate the permeation of water in the spheroplasts. Depending on how helix 5 is aligned, the amino acid residue at the $H5^P$ position in the ar/R region could be either T246 or V242. A possible explanation for the lack of water permeability in the *Nb*XIP1;1αL79G/I102H/T246I mutant could be that T246, not V242, resides at the $H5^P$ position in the ar/R region and substituting it for an Ile, in combination with the L79G/I102H substitution, does not favour water permeability.

To investigate whether all three amino acid substitutions in the *Nb*XIP1;1αL79G/I102H/V242I mutant are needed to induce water permeability in *Nb*XIP1;1α, six additional *Nb*XIP1;1α mutants were constructed. Also, to help us rationalize the functional properties of the wild-type and mutant *Nb*XIP1;1α proteins, homology models using the high resolution X-ray crystal structure of *At*TIP2;1 as template were made. All the *Nb*XIP1;1α mutants in this second set were successfully expressed, although to different levels, in *P. pastoris*. As it was done for the first set of *Nb*XIP1;1α mutants, after compensating for the difference in the expression levels by relating the rates of water permeability to the individual protein amounts, all the mutants except the *Nb*XIP1;1αL79G/V242I mutant had lower specific rates that differed significantly from that of the *Nb*XIP1;1αL79G/I102H/V242I triple mutant. However, with the exception of the spheroplast expressing *Nb*XIP1;1αV242I and *Nb*XIP1;1αL79G/I102H, all the mutants had relative specific rates that were significantly higher than that of the spheroplasts expressing the the wild-type *Nb*XIP1;1α protein. This meant that each of the single amino acid substitutions L79G and I102H were individually sufficient to induce water permeability in the *Nb*XIP1;1α protein.

The homology models indicated that T246 is the residue at the $H5^P$ position as it was energetically favourable to place T246 instead of V242 at this position. The models of the wild-type *Nb*XIP1;1α and the mutants revealed a novel orientation of the arginine (R263) in the ar/R selectivity filter, where R263 forms a salt bridge with aspartate (D80) in helix 1. In the *At*TIP2;1 crystal structure, the corresponding arginine in the selectivity filter makes a hydrogen bond to the histidine residue at the $H2^P$ position. However, due to the formation of the salt bridge between R263 and D80, R263 could not make a hydrogen bond to the histidine introduced at the $H2^P$ position in the *Nb*XIP1;1α mutants. Even though according to the model, the

introduced histidine in the *Nb*XIP1;1αL79G/I102H/T246I adopted a TIP-like orientation, it was not enough to orient the arginine to a position similar to that found in the *At*TIP2;1 structure. This suggested to us that even if we have been successful in making *Nb*XIP1;1α water permeable, we have not succeeded in replicating the orientation of the amino acid residues of a TIP-like selectivity filter and therefore could not regulate water permeability in a more expected manner.

The results obtained after estimating the radius of the pore in the models of the wild-type and mutant *Nb*XIP1;1α with the HOLE program suggested that, the pore in the models of the wild-type *Nb*XIP1;1α and the *Nb*XIP1;1αL79G/V242I mutant was not restricted. However, the pore in the model of the *Nb*XIP1;1αL79G/I102H/T246I seemed the narrowest followed by that in the *Nb*XIP1;1αI102H and *Nb*XIP1;1αI102H/V24I models.

Inferring from the results obtained from the functional studies and the homology modelling, it was not clear why the wild-type *Nb*XIP1;1α protein was not permeable to water since the pore in the model appears to be unrestricted and sufficiently wide to allow water permeation. It was therefore assumed that the requisite hydrogen bond network necessary for water permeability in the *Nb*XIP1;1α protein was interrupted by the presence of the hydrophobic residues (Ile 102 and Ala 257 at $H2^P$ and LE^P, respectively) in the *Nb*XIP1;1α selectivity filter. It can also be inferred from the results that V242 does not form part of the *Nb*XIP1;1α selectivity filter as according to the models it is located deep in the pore and also the V242I substitution did not influence water permeability in the single or double mutants. Based on the models, it was not clear why the V242I substitution seemed to be essential for water permeability in the *Nb*XIP1;1αL79G/I102H/V242I triple mutant since the *Nb*XIP1;1αL79G/I102H double mutant had a lower relative specific rate which did not differ significantly from that of the wild-type protein. However, most of the results obtained in the functional studies could be explained from the homology models based on the hydrophobicity and size of the ar/R selectivity filter.

In conclusion, the single amino acid substitutions L79G or I102H were enough to render *Nb*XIP1;1α water permeable. Our results indicate that T246 is the residue at the $H5^P$ position in the ar/R selectivity filter of *Nb*XIP1;1α and that the pore of the protein is likely not to be blocked when the *Nb*XIP1;1α protein is heterologously expressed in *P. pastoris*. The water permeable *Nb*XIP1;1α mutants provide a new tool to study the functional properties of the protein. Furthermore, our models suggest that a salt bridge between an acidic residue residing in the position corresponding to aspartate (D80) directs the orientation of the arginine in the selectivity filter and provides a novel approach for adjusting the selectivity of aquaporins.

Paper III

The X-Intrinsic Proteins (XIPs) are the most recently characterized subfamily among the seven aquaporin subfamilies. However, the three dimensional structure of XIPs has not been solved yet. Since the structure of XIPs will help us understand the function of members of this new aquaporin subfamily, it is important that studies with the aim of solving the structure of a member of this subfamily are initiated. Our initial attempts to crystallize the full length *Nb*XIP1;1α protein to solve the structure of *Nb*XIP1;1α by X-ray crystallography have proved futile.

In this paper, in an effort to increase our chances for successful crystallization of the *Nb*XIP1;1α protein, we set out to engineer *Nb*XIP1;1α constructs differing in the length of their N-terminal region and with good expression in *P. pastoris*. *Nb*XIP1;1α has not only been previously reported to be partially phosphorylated in *P. pastoris* but also phosphorylated at five amino acid residues in its N-terminal region, so truncating the N-terminal region of *Nb*XIP1;1α will yield a homogeneous protein population with regards to phosphorylation for crystallization purposes. However, a series of N-terminal truncations were constructed since removing the whole N-terminal region with the associated phosphorylation sites may affect the expression and subcellular localization of *Nb*XIP1;1α in *P. pastoris*.

All the *Nb*XIP1;1α mutants were successfully expressed in *P. pastoris*, though to different levels with the *Nb*XIP1;1αΔ1-12 mutant having the highest protein expression. It was observed that removing the first 36 amino acid residues in the N-terminal region did not have any adverse effect on the expression of *Nb*XIP1;1α in *P. pastoris*. However, the expression of the protein was drastically affected when the first 44 or 53 amino acid residues in the N-terminal region were truncated, as the *Nb*XIP1;1αΔ1-44 and *Nb*XIP1;1αΔ1-53 mutants had low expression. Further studies are needed to investigate whether the low expression seen in the *Nb*XIP1;1αΔ1-44 and *Nb*XIP1;1αΔ1-53 mutants is due to the effect of the truncation per se or due to the effect of removing the phosphorylation sites in the N-terminal region of the protein. The *Nb*XIP1;1αΔ1-36 mutant was chosen as the ideal candidate for crystallization studies as it has only one of the five reported phosphorylation sites in the N-terminal region and it had a sufficiently high expression level in *P. pastoris*.

All the mutants were localized to the plasma membrane of *P. pastoris* and found to be functional, since they rendered *P. pastoris* cells more sensitive to boric acid as compared to *P. pastoris* cells transformed with the empty pPICZB plasmid. The *Nb*XIP1;1αΔ1-53 mutant, which has most of its N-terminal region with the accompanying phosphorylation sites truncated, rendered *P. pastoris* cells more

sensitive to boric acid as compared to any of the *Nb*XIP1;1α mutants, the full length *Nb*XIP1;1α, and also compared to the boric acid transporter *At*NIP5;1 used as control, though it was expressed in low amounts in *P. pastoris*. This might suggest that the N-terminal domain with its associated phosphorylation sites regulates the function of *Nb*XIP1;1α. However, further studies are needed to examine whether phosphorylation regulates the function of *Nb*XIP1;1α by gating or trafficking or both.

It was also shown that the purified *Nb*XIP1;1α protein was phosphorylated and could be dephosphorylated by alkaline phosphatase. In addition, protein kinase C could rephosphorylate the purified and dephosphorylated *Nb*XIP1;1α protein. This indicated that since the phosphorylation sites in *Nb*XIP1;1α were accessible to kinases in *P. pastoris*, to alkaline phosphatase and to protein kinase C, they would most probably be accessible to kinases in plants. However, further investigations are needed to ascertain whether *Nb*XIP1;1α is phosphorylated in plant cells and the effect of phosphorylation on the function of *Nb*XIP1;1α in plants.

In conclusion, we have shown that the expression of *Nb*XIP1;1α in *P. pastoris* was drastically affected when the N-terminal region of the protein was truncated beyond the first 36 amino acid residues. Our results indicate that the N-terminal region of *Nb*XIP1;1α regulates its function. Of this set of *Nb*XIP1;1α mutants with N-terminal truncations, the *Nb*XIP1;1αΔ1-36 mutant was found to be the preferred candidate for crystallization studies.

Paper IV

In this paper, we set out to overexpress and purify *At*NIP1;1 and *At*NIP5;1 aquaporins in the methylotrophic yeast *P. pastoris*, with the ultimate aim of solving the three dimensional structure of a member of the NIP subfamily of aquaporins by X-ray crystallography. This is important because in addition to functional studies the structure of a NIP channel will help us gain an in-depth knowledge of the mechanism by which various solutes permeate NIP channels. Since such studies require large amounts of pure protein, the *P. pastoris* expression system was employed because it has previously been used for the production of sufficient amounts of other aquaporins.

The cDNAs encoding *At*NIP1;1 and *At*NIP5;1 were transformed into *P. pastoris* X-33 cells. In order to optimize the expression of these proteins in the X-33 cells, we selected for *P. pastoris* clones with multiple insertions of the *At*NIP1;1 or *At*NIP5;1 gene on increasing levels of zeocin concentration, a strategy which has previously been reported by Nordén *et al*., 2011 to enhance the expression of recombinant aquaporins in *P. pastoris*. While this strategy boosted the expression of *At*NIP5;1 in *P. pastoris* cells, it did not enhance the expression of *At*NIP1;1 sufficiently. It was therefore suggested that in addition to increasing the gene dosage, the codon composition and A/T nucleic acid content of the *AtNIP1;1* gene could be optimized for expression in *P. pastoris*. Codon optimization has previously been used to enhance the expression of the *Plasmodium falciparum* aquaporin in *P. pastoris* [Hedfalk *et al*., 2008]. There is no guarantee that this will work for *At*NIP1;1, however, combining these two strategies would increase the chances of enhancing the heterologous expression of *At*NIP1;1 in *P. pastoris*. *At*NIP5;1 was chosen for further studies since *At*NIP5;1 expression in *P. pastoris* was better than *At*NIP1;1 expression and a *P. pastoris* clone with good expression of *At*NIP5;1 would serve as a good starting material for *At*NIP5;1 purification.

The recombinant full length *At*NIP5;1 protein increased the sensitivity of *P. pastoris* cells to boric acid which indicated that the *At*NIP5;1 protein was correctly folded, functional and localized in the plasma membrane of the X-33 cells.

After preparing crude membranes from *P. pastoris* cells expressing *At*NIP5;1 and stripping the peripheral membrane proteins by washing the membranes with urea, the capacity to solubilize the *At*NIP5;1 protein from the stripped membranes by four different detergents was tested. N-decyl-β-D-maltopyranoside (DM) detergent solubilized more *At*NIP5;1 protein than the other three detergents. However, n-dodecyl-α-D-maltopyranoside (DDM) was chosen for large scale solubilization and purification since the *At*NIP5;1 protein seemed more stable in DDM than in DM.

As an identification and a purification strategy, a His-tag had been at the N-terminus of the *At*NIP5;1 protein sequence. Subsequently, Ni-NTA affinity chromatography was used to purify the *At*NIP5;1 protein, utilizing the His-tag. With the aim of performing substrate specificity studies by reconstituting the purified *At*NIP5;1 protein into *E. coli* lipid vesicles in a stopped-flow spectrometric analysis, the DDM detergent used to solubilize the *At*NIP5;1 protein was exchanged for DM in the purification procedure. This is because it would be difficult to prepare proteoliposomes with DDM, due to its low critical micelle concentration (0.17 mM).

Even though, the elution fraction was highly enriched in *At*NIP5;1 protein, a lower thick band below the protein band corresponding to the monomeric *At*NIP5;1 protein was seen, when the elution fraction was loaded onto a SDS-PAGE gel. This seemingly contaminating protein band could also be a truncated form of *At*NIP5;1 lacking the His-tag and co-purified with the protein or an interacting protein partner of *At*NIP5;1 in *P. pastoris*. However, the identity of this lower thick band is yet to be determined by mass spectrometry. To improve upon the purity and the initial yield of 0.04 mg protein per gram of yeast cells obtained, screening of alternative detergents, additional purification by ion-exchange chromatography and gel filtration are approaches likely to succeed.

In conclusion, *At*NIP1;1 and *At*NIP5;1 were successfully expressed in *P. pastoris*, albeit, to different extent with the expression of *At*NIP5;1 being more efficient than the expression of *At*NIP1;1 in *P. pastoris*. The recombinant full-length *At*NIP5;1 protein was functional as it rendered X-33 cells more sensitive to boric acid. The initial purification yield of *At*NIP5;1 protein was enough for functional studies. However, for a full scale crystallization screen, the current purification protocol needs to be optimized to obtain sufficient amounts of monodispersed purified *At*NIP5;1 protein.

Future perspectives

The findings in this thesis have broadened our knowledge of XIPs and NIPs, and have also raised some important questions that need to be addressed in the future in order to fully understand how XIPs and NIPs are regulated and most importantly, how the structure affects the function of these proteins. The immediate question to address is whether XIPs are phosphorylated in plants and what effect does phosphorylation have on XIP function in plants. Secondly, it will be interesting to explore the effect alternative splicing has on XIPs and its physiological relevance in *N. benthamiana*. Answers to these intriguing questions will require the combined efforts of studies using overexpression strategies in plants, mass spectrometry, mutagenesis, functional assays and transcriptomics.

Furthermore, with the availability of water permeable *Nb*XIP1;1α mutant proteins, it will be easier now to investigate the functional properties of *Nb*XIP1;1α by mutagenesis and stopped-flow spectrometry.

With each new solved structure of a member of the aquaporin family, we discern a bit more of how aquaporins function at the molecular level and how they are regulated at the protein level. It is therefore interesting to think about what the structures of SIPs, XIPs and NIPs might reveal, since these aquaporin isoforms are different from other aquaporin isoforms in terms of their substrate specificity.

From a health perspective it is of uttermost relevance to understand the mechanism of metalloid transport into plants by NIPs and XIPs. The mechanism of water transport in aquaporins has partly been elucidated but little is known (little structural information) about how metalloids such as boron, silicon, arsenic and antimony permeate certain aquaporin channels. Three dimensional structures of NIP and XIP proteins may shed light on this important matter.

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