Plant Major Intrinsic Proteins - natural variation and evolution

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2010

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Printed in Sweden by Media-Tryck AB, Lund, 2010

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P.O. Box 124
SE-221 00 Lund, Sweden
ISBN 978-91-7422-259-3

Abstract

Major Intrinsic Proteins (MIPs, also called Aquaporins, AQPs) are channel forming membrane proteins. Although initially functionally characterized and named after their water channeling property in human red blood cells, it has become increasingly evident that MIPs are present in all types of organisms and transport a variety of small, uncharged molecules besides water. MIPs have a highly conserved structure with a constriction region and electrostatic repulsion filter allowing the combination of high transport rate and selectivity, characteristic of MIPs.

In plants, MIPs form a large and varied protein family, with roughly three times as many isoforms as found in animals. Even though the abundance implies that MIPs have important functions in plants, the roles of individual MIPs have so far only been described for a handful of isoforms. Variations in the filter regions as well as experimental data, suggest differences in substrate specificities and localization for different MIP subfamilies. However actual functions of different isoforms remain largely unknown as traditional knock out/knock down experiments to a large extent have failed to reveal any clear phenotypes.

Using another approach, we used naturally occurring genetic variants (accessions) of the model plant *Arabidopsis thaliana* to see if differences in traits, such as drought tolerance, could be linked to differences in MIP regulation. We also looked at the evolution of the MIP family in plants, to see if this could for example be linked to events such as the emergence of a vascular system or the development of a terrestrial lifestyle. Therefore we identified the whole MIP family in the moss *Physcomitrella patens* and in nine different green algae and investigated how these relate to those of higher plants. An amazing diversity of MIPs was found and surprisingly some of the higher plant subfamilies were present also in these simple plants.

Keywords: MIP, aquaporin, plant, natural variation, evolution, Arabidopsis, Physcomitrella patens, NIP, XIP

List of Publications

This thesis is based upon the following papers, which in the text are referred to by their Roman numerals:

I. **Unexpected complexity of the Aquaporin gene family in the moss Physcomitrella patens.**
Danielson JÅH and Johanson U
BMC Plant Biology, 8:45, 2008

II. **Transcriptional regulation of aquaporins in accessions of Arabidopsis in response to drought stress.**
Alexandersson E, Danielson JÅH, Råde J, Moparthi VK, Fontes M, Kjellbom P and Johanson U
Plant Journal, 61(4):650-60, 2010

III. **Phylogeny of major intrinsic proteins.**
Danielson JÅH and Johanson U
Advances in Experimental Medicine and Biology, 679:19-31, 2010

IV. **Algae MIPs.**
Danielson JÅH, Anderberg H and Johanson U
Manuscript

Additional Publications

Persistent LTP without triggered protein synthesis.
Abbas AK, Dozmorov M, Li R, Huang FS, Hellberg F, Danielson JÅH, Tian Y, Ekström J, Sandberg M, Wigström H.
Neuroscience research, 63(1):59-65, 2009

Fibrinogen gene variation and ischemic stroke.
Jood K, Danielson JÅH, Ladenvall C, Blomstrand C, Jern C.
Journal of thrombosis and haemostasis, 6(6):897-904, 2008

Contribution to the Papers

I. JD took part in designing the study. JD did the acquisition, analysis and interpretation of data and drafting of the manuscript. JD took part in revising the manuscript.

II. JD took part in all the experiments except the GUS experiment. JD took part in analyzing the data and drafting of the manuscript. JD took part in revision of the manuscript

III. JD took part in designing the study. JD did the acquisition, analysis and interpretation of data. JD took part in drafting and revising the manuscript.

IV. JD took part in designing the study. JD did the acquisition of data. JD took part in the analysis. JD took part in the drafting of the manuscript.

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Abbreviations

AQP	Aquaporin
ar	aromatic
bb	backbone
cRNA	copy RNA
DNA	Deoxyribonucleic Acid
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
GIP	GlpF-like Intrinsic Protein
GlpF	Glycerol Uptake Facilitator
HGT	Horizontal Gene Transfer
HIP	Hybrid Intrinsic Protein
MIP	Major Intrinsic Protein
miRNA	micro RNA
NIP	NOD-26 like Intrinsic Protein
NJ	Neighbor Joining
PBM	Peribacteroid Membrane
PIP	Plasma membrane Intrinsic Protein
PM	Plasma Membrane
RNA	Ribonucleic Acid
RNAi	RNA interference
sc	sidechain
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIP	Small and basic Intrinsic Protein
TIP	Tonoplast Intrinsic Protein
TM	Trans Membrane
WUE	Water Use Efficiency
XIP	X Intrinsic Protein
YFP	Yellow Fluorescent Protein

1 Introduction

In a way, life is confinement, as all life as we know it is cellular, existing only in that unit of space, the cell. Membranes, consisting of lipid bilayers, define not only the cell but also many of the organelles within the cell and effectively isolate what is inside from the outside. This makes it possible to create the controlled environment necessary for all the chemical reactions that is life to occur. However, having a lipid bilayer only confines the space, and in order to make it controlled, transports across the membrane must be regulated. In cells, transports over the membranes are maintained by three different kinds of proteins, pumps that use energy to actively transport substances, channels that facilitate the passive diffusion of substances over the membrane and carriers that can either be active or passive transporters.

For a long time it was believed that cellular water transport occurred entirely by passive diffusion through the lipid bilayer, and that differences in permeability between different membranes were depending only on differences in lipid composition. In the 1950s researchers did experiments showing that membranes from certain tissues (e.g. kidney and red blood cells) were not only highly permeable to water, but also that this water transport could be blocked in a reversible way by mercury (Stein and Danielli, 1956). This indicated that proteins were somehow involved in the permeabilities of these membranes, but how this occurred and which proteins were involved would not be revealed until almost 40 years later.

Major Intrinsic Proteins (MIPs, also referred to as aquaporins, AQPs) is a group of channel forming proteins of ancient origin, predating the emergence of the three domains of life (Pao et al., 1991, Park and Saier, 1996, Kozono et al., 2003). Although identified early on as major constituents of cellular membranes, they first gained massive attention in the early 1990s when CHIP28 (a MIP later renamed AQP1) was identified as the long sought water channel protein (Preston et al., 1992). This did not only solve the old ongoing debate on the involvement of proteins in cellular water transport, but also resulted in a burst of MIP related research. It turns out MIPs are not only water channels, but rather they are involved in transport of several other small uncharged molecules (e.g. glycerol, urea, silicic acid, ammonia, hydrogen peroxide) across cellular membranes (which is why the use of MIPs is preferred over the more catchy, but misleading, AQPs in this thesis).

The first plant MIP was identified in 1987 (Fortin et al., 1987) as a membrane protein of root nodules, but it was not until 1993 (Maurel et al., 1993) that a plant MIP was for the first time being experimentally determined to be a water channel. Since then, numerous different plant MIPs have been identified and the transport properties have been experimentally determined for several of them. It turns out that plants contain a very large MIP family with more than 30 isoforms, almost three times as many as found in mammals. The abundance of isoforms suggests that MIPs have important roles in plant life, but largely the functions of the different subfamilies and individual isoforms is still unknown.

It was in this context that the subject of my doctoral project was formulated, to expand on the knowledge of plant MIPs, the diversity of the superfamily, the roles of different subfamilies and isoforms. In my project I used both traditional molecular biology approaches (such as GFP/GUS tagging of proteins, knock out/knock down plants, transcriptional profiling and so on) and more genetics based approaches, looking at differences between naturally occurring variants (accessions) in one species, or at variation between more distantly related species.

2 Plants

Even though water is essential for all living organisms, it plays an extremely large and crucial role in the life of plants. For the process of photosynthesis, plants have to take up carbon dioxide from the air, but in doing so they also lose water, and they lose a lot of water. For every gram of matter produced by photosynthesis, approximately 500 g of water has to be absorbed by the roots, transported throughout the plant and lost to the atmosphere (Taiz and Zeiger, 2006). Water transport in the plant is passive in the way that it is driven by this transpiration, and water uptake and transport throughout the plant is a spontaneous movement of water from regions of higher to lower free energy. This, together with the crucial role of water and the fact that plants are immobile, makes it of utmost importance for plants to be able to regulate water flow in response to environmental conditions.

Water has many different functions in the plant, at a cellular scale it is *the* solvent of the cell and therefore influencing everything from chemical reactions to protein stabilities, but at a macroscopic level it also constitutes the plant long-distance transport system, being both the transporter of mineral nutrients from roots to shoots in the xylem, and the transporter of sugars made in the photosynthesis from shoot to root in the phloem. Water also has a fundamental role in the very first steps of photosynthesis in plants, where water is oxidized to oxygen by the use of light, oxygen that ultimately all organisms depending on aerobic respiration (such as ourselves) depend on for energy conversion.

2.1 The Plant Cell

There are numerous unique features of the plant cell compared to that of other eukaryotes, such as animals. One of the more striking is that the plant cell is enclosed by a cell wall, a rigid cellulosic structure giving strength and toughness but also restricting morphological changes of the mature cell. The cell wall allows the plant cell to build up a positive hydrostatic pressure (called turgor or turgor pressure), pressing the plasma membrane (PM) of the cell to the wall, resulting in among other things further rigidity. This hydrostatic pressure is mainly created by large water filled vacuoles that occupy up to 95 % of the total cell volume of mature plant cells (Taiz and Zeiger, 2006). By actively accumulating solutes in the vacuole, an osmotic gradient is created over the tonoplast (the vacuolar membrane), resulting in water uptake and increased turgor of the cell.

Even though the vacuole occupies a large volume of the plant cell, the remaining part of the cytosol is highly compartmentalized, containing many different organelles consisting of at least one membrane type as seen in Figure 1. Most plant cells are interconnected via plasmodesmata, microscopic tubular extensions of the endoplasmic reticulum (ER) surrounded by PM that traverse the cell walls of adjacent cells.

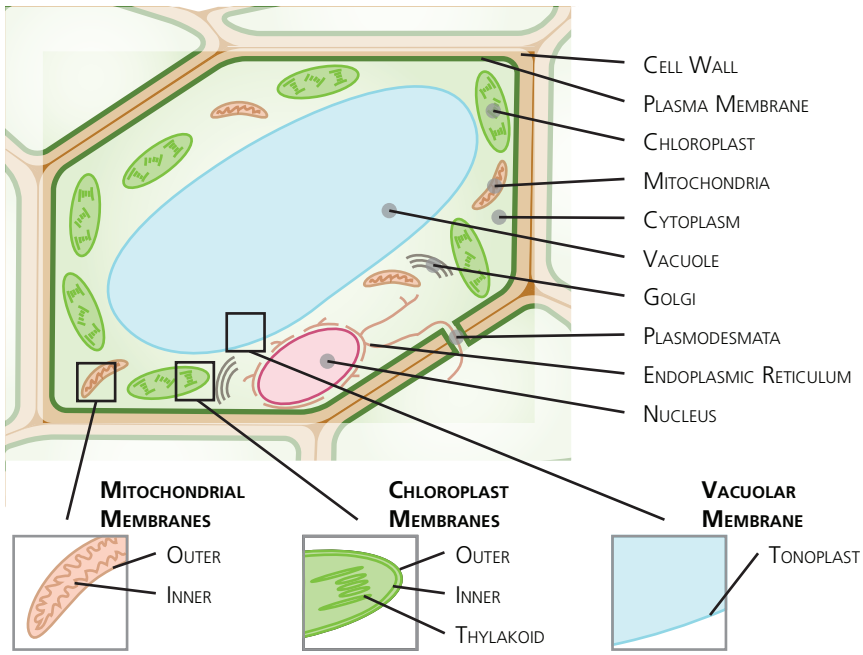


Figure 1. Schematic picture of the plant cell. Top part shows the whole cell with different organelles. Below are enlargements of three different organelles displaying the naming of their membranes.

2.2 Water Transport in Plants

Water can move through the plant via three different pathways: the apoplastic, the symplastic and the transmembrane pathway, illustrated in Figure 2. The apoplast consists of the continuous system of cell walls, intercellular airspaces and lumens of dead cells (e.g. xylem and fibers) found in the plant and in the apoplastic pathway, water moves in the plant without having to cross any plasma membranes. In the symplastic pathway water moves through the symplast, that is the network of cell cytoplasm interconnected by plasmodesmata. In order for the water to enter and exit the cytoplasm it needs to pass the plasma membrane, but when it moves in the symplast, no membranes need to be crossed. In the third, transmembrane, pathway water moves from cell to cell via the cell wall and therefore needs to cross the plasma membrane at least twice for each cell it passes. Most likely the relative importance of the three pathways differs in different tissues and species and although this has not yet been clearly established some general implications can be seen. For example the existence of the casparian strip, a radial band of cell walls impregnated with the hydrophobic substance suberin, in roots prevent water movement in the apoplast and force water to cross the plasma membrane of these cells in order to reach the vascular system of mature roots. This enables plants to regulate water permeability of the root at a local level by changing the permeability of the plasma membrane at the casparian strip, thereby affecting the water uptake of the root system globally.

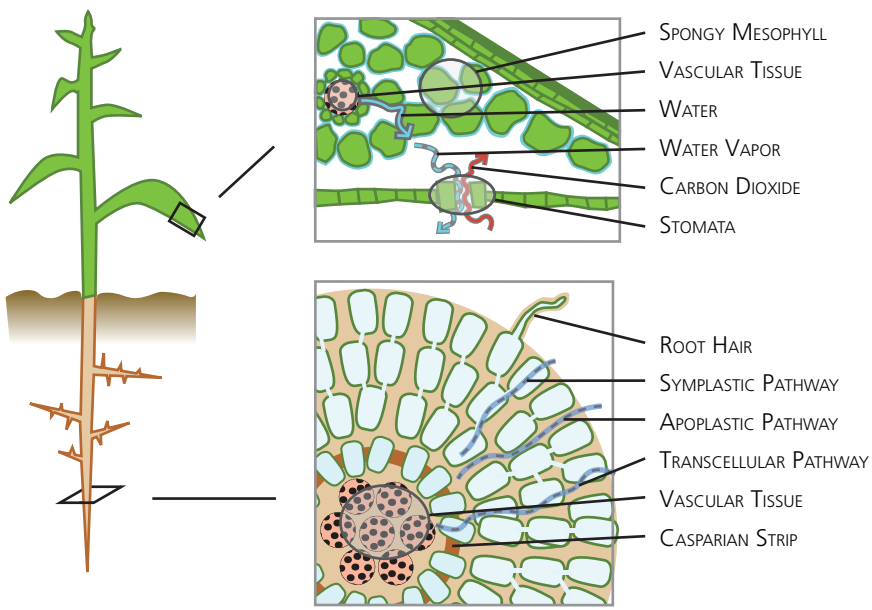


Figure 2. Water Transport Pathways in plant. The Upper picture shows water flow in the leaf, from the xylem to the atmosphere via the stomata and the countercurrent carbon dioxide flow. The lower part shows a cross section of a root with the three water transport pathways and the location of the vascular tissues and casparian strip.

Water flux through the plants can also be regulated at the leaf level, where the aperture of the openings to the atmosphere can be regulated (see Figure 2). These openings, stomata, are composed of two guard cells, forming a pore between them. The guard cells have a special cell wall organization that allows them to change shape depending on the turgor pressure, resulting in stomatal opening/closure. This allows the plant to change the rate of transpiration to environmental cues such as light intensity, temperature, leaf water status and CO_2 concentrations. In a similar way, other cells can also change shape depending on water uptake/loss leading to changes in cell turgor influencing for example cell elongation in newly formed cells or leaf movement. Some of these changes are quite fast, requiring water fluxes across the plasma membrane and the tonoplast membrane that cannot be explained by the permeability of the tonoplast and plasma membrane on their own.

3 Major Intrinsic Proteins

Major Intrinsic Proteins (MIPs) are integral membrane proteins that form channels, permeable to water and other small uncharged molecules, in cell membranes. Unlike membrane transport-proteins that take part in directional, energy demanding, *active* transport of substrates, MIPs form discriminating channels which affect the permeability of the membrane for certain substrates and therefore the rate of *passive* transport of these.

Even though it was shown as early as in the mid 1950s that diffusion of water across the lipid bilayer of cellular membranes was facilitated by some membrane component (Stein and Danielli, 1956), it was still very much the common view that passive diffusion across membranes was the only cellular transport mechanism for water, when in the early 1990s the first water channel protein, Aquaporin-1 (AQP1) was reported (Preston et al., 1992). This work, carried out in Peter Agres group, started out by the identification of an unknown protein in red blood cells in the late 1980s (Smith and Agre, 1991, Denker et al., 1988, Preston and Agre, 1991) and ultimately lead to the publication of the 3.8 Å resolution structure of a water channel MIP in 2000 (Murata et al., 2000). These results changed the view of how water was transported in the cell and the impact of this research lead to the Swedish Royal Academy of Science awarding Peter Agre with a Nobel Prize in chemistry 2003 for "discoveries concerning channels in cell membranes".

This was in fact not the first MIP known, it was not even the first MIP believed to transport something, but it was the first MIP believed and proven to transport water. Prior to this other MIPs had been postulated to transport glycerol in *E. coli* and small molecules in soybean root nodules (Richey and Lin, 1972, Sandal and Marcker, 1988, Baker and Saier, 1990), but it was really the water channeling properties that got the attention and lead to the boom in MIP related research in the early 2000s. Even though the water transporting properties of MIPs was for a long time the main focus of research, lately MIPs have been shown to be permeable to many other substrates and the importance and role of this substrate diversity is a growing research field.

3.1 Phylogeny

In order to evaluate the evolutionary relationship, or phylogeny, of genes or proteins their corresponding DNA or amino acid sequences must be known. Today this is rather easily achieved, but in the days before automated sequencing this was very labor intensive. It is therefore not surprising that, even though MIPs were early on discovered as major constituents of certain membranes, it was not until in 1984 that the first sequence of a MIP, isolated from bovine lens fiber cells, was reported (MIP-26, later renamed to AQP0) (Gorin et al., 1984). This was followed in 1987 by the sequence of another MIP protein isolated from the peribacteroid membrane (PBM) of soybean root nodules (Nod-26, later NIP1;1) (Fortin et al., 1987). At the time it was not realized that these two proteins were related to each other, but in 1988 two groups, independently of each other, found the two sequences to be surprisingly similar (Shiels et al., 1988, Sandal and Marcker, 1988). Sandal and Marcker also concluded that the proteins contained 6 hydrophobic regions large enough to be membrane spanning and suggested that Nod-26 might transport small molecules across the PBM by forming channels. One year later the sequence of *E. coli* glycerol uptake facilitator (GlpF) was released (Muramatsu and Mizuno, 1989) and the following year it was realized that also this, bacterial, protein belonged to the same ancient super family of proteins, the MIP super family (Baker and Saier, 1990).

In 1991 the first phylogenetic analysis of MIPs was presented (Pao et al., 1991), but since only six MIPs were known at the time, the result was rather ambigous. This was however more clear in the analysis of 52 MIPs presented five years later (Park and Saier, 1996). In this, 12 subfamilies could be seen and based on the finding that some prokaryotes had two types of MIPs, it was postulated that all MIPs originate from two ancestral prokaryotic genes, giving rise to the GLP and AQP cluster.

The most thorough phylogenetic analysis up till now was presented in three articles by Zardoya and coworkers between 2001 and 2005 (Zardoya, 2005, Zardoya et al., 2002, Zardoya and Villalba, 2001). They found that MIPs cluster in 8 major groups (GLPs, animal AQPs, bacterial AQPs, PIPs, TIPs, NIPs, SIPs and AQP8s) but also that the relation between these groups could not be resolved.

Since then many more groups have been discovered, but still the phylogeny between these groups remain obscured. It is therefore worth noting that, from a phylogenetic point of view, the commonly used grouping of MIPs into AQPs and GLPs is not correct. For even though the GLPs form a phylogenetically well defined, monophyletic, group, the AQP cluster is in fact only "everything that is not a GLP", and different groups within this cluster might not be more closely related to each other than they are to the GLPs. The present phylogeny of some major MIP subfamilies is presented in Figure 3.

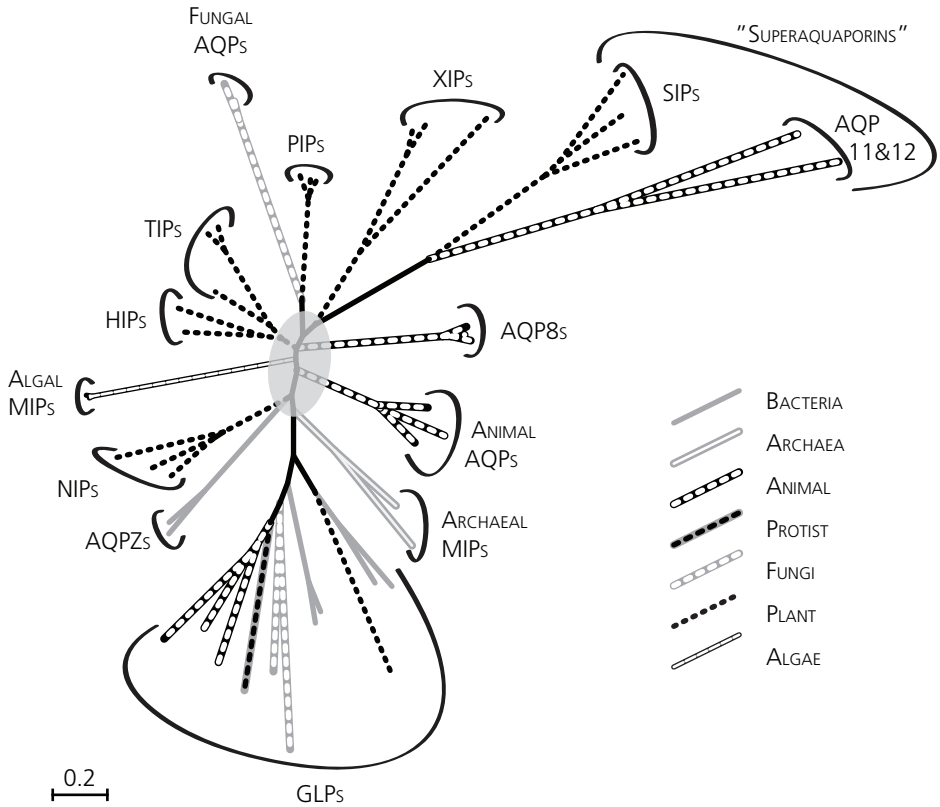


Figure 3. An overview of the phylogeny of the MIP family. A Neighbor Joining (NJ) tree showing the phylogeny of some major MIP subfamilies. Species of MIP sequences are indicated by different line styles and subfamilies are shown by bracketing.The gray shading in the central part of the tree illustrates the uncertainty of the phylogeny of this region indicated by low bootstrap supports in the original analysis.

3.2 Structure

Not only the primary structure (as reflected in the phylogeny), but also the secondary, tertiary and quaternary structure of MIPs are highly conserved. Until now (Nov 1st 2010) high resolution structures of 15 different MIPs have been solved, all sharing the same overall structure with some minor variations.

The MIP structure can be seen in Figure 4 and consists of six transmembrane (TM) helices (H1 to H6) connected by five loops (LA to LE) and is situated in the membrane so that both the amino and the carboxy termini are at the cytoplasmic side. In loop B and E there are two "half-membrane-spanning" helices (HB and HE), which are inserted from opposite sides, making up a seventh transmembrane "broken" helix in the structure.

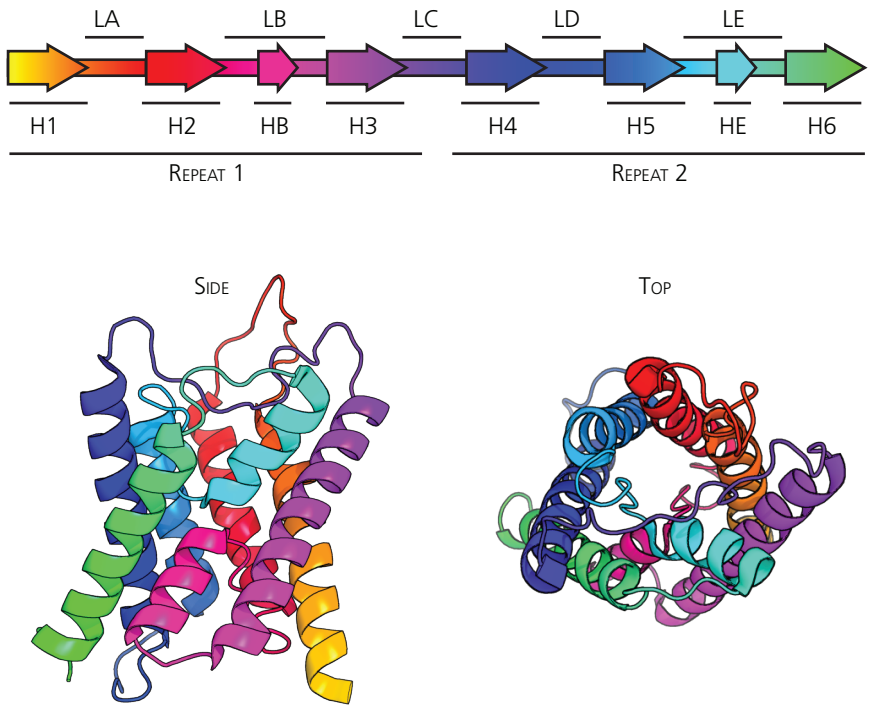


Figure 4. An overview of the MIP Structure. Upper part shows the secondary structure of MIPs, arrows indicate helices. Naming of helices and loops, as well as the positions of the repeats, are indicated. Lower part shows the three-dimensional structure of MIPs from side and top, colored according to the secondary structure cartoon above (The structure is human AQP4, PDBID 3GD8).

When looking at the primary and secondary structure of MIPs it is clear that they are built up from two repeats, each consisting of 2 TM helices followed by a half TM helix and then a final TM helix. In an analysis by Pao and coworkers (Pao et al., 1991) the relationship between the repeats was investigated and it was shown that, for all MIPs in the analysis, the first halves were more closely related to each other than to any of the second halves and vice versa, indicating a very ancient origin of the repeats and the gene duplication leading to this MIP structure.

The helices are inserted in the membrane, tilted at a slight angle and in a right-hand fold, giving rise to what is often referred to as the hourglass fold, with two conical vestibules meeting with their tips in the middle of the protein. However, the narrowest region of the pore is actually 8 Å more to the extracellular side, where four amino acids from H2, H5, LE and LE make up the aromatic/arginine (ar/R) filter, a size restriction region largely responsible for the substrate specificity of the MIP. Another region of utmost importance for MIP function is found in the middle of the pore where two highly conserved NPA box motifs are found at the ends of HB and HE. The positions of the ar/R filter and the NPA boxes can be seen in the stereo pictures in Figure 5.

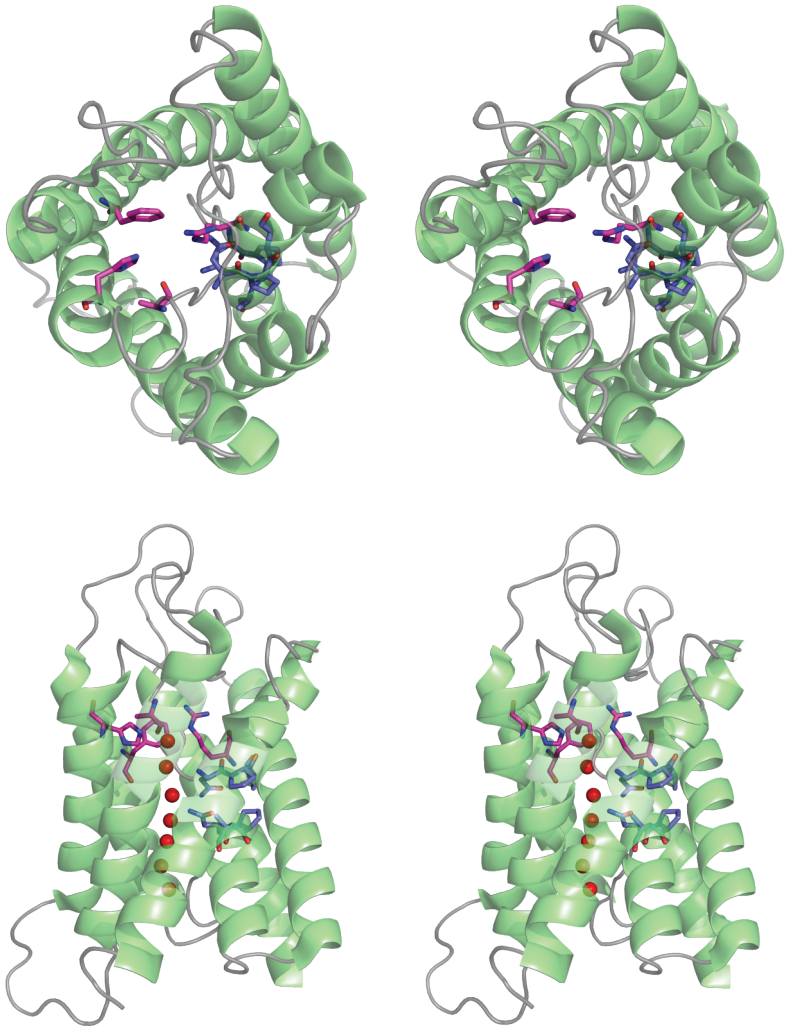


Figure 5. Stereo pictures showing the ar/R filter and NPA motifs. The MIP (HsAQP4, 3GD8) is shown in a green cartoon representation with the ar/R filter and the NPA box motifs shown as sticks in magenta and blue respectively. The upper part shows a top view (from extracellular side) while the lower part shows a side view were parts of helices 4 and 6 are transparent to make the ar/R and NPA regions more visible. The red dots show the positions of waters in the crystal structure.

3.3 Substrate Specificity

Even though some MIPs transport only water, others have physiological roles transporting a whole range of other solutes (reviewed in Carbrey and Agre, 2009). Glycerol and urea were among the first substrates shown for MIPs (such as the aquaglyceroporins AQP 3, 7, 9 and 10), but other substrates include anions such as nitrate (AQP6), ammonia and hydrogen peroxide (AQP8). Some MIPs have been shown to be permeable to the gases carbon dioxide and nitrous oxide, but the physiological relevance for this is still debated. Recently, MIPs have also been shown to be permeable to metalloids (e.g. B, Si, Sb, As) and the physiological importance of this can for example be seen in the interesting case of the protozoan parasites of the genus *Leishmanias*. These parasites cause the disease leishmaniasis, a disease usually treated with antimony and arsenite. It was recently shown that some drug resistant variants have increased arsenite tolerance, achieved by them altering their MIP expression (Lin et al., 2008). Metalloid transport is also of great importance in the context of arsenic contamination of ground water which is a major health problem for hundreds of millions of people around the world, threatened either directly through contaminated drinking water, or indirectly through crops grown in farmlands irrigated with contaminated water.

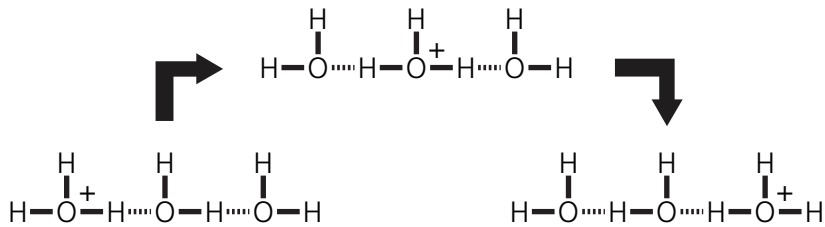


Figure 6. A schematic picture of the Grotthuss mechanism showing how proton movement in water can be achieved by rearrangement of bonds.

Proton pumping across membranes is a fundamental part of energy production in all organisms and it is therefore crucial that transport proteins in these membranes do not disrupt this proton gradient. Proton movement in bulk water is extremely fast, due to the "proton wiring" effect of the Grotthuss mechanism (see Figure 6 and Cukierman, 2000), and for quite some time after the water transport properties of MIPs were first reported (Preston et al., 1992) it was puzzling how the pore could let water through and at the same time block the passage of protons. The answer turned out to have two parts. First, hydrogen bonding between the water molecules is broken when the water, which runs through the pore in a single file, is forced to reorient at the NPA boxes. Secondly, a positive charge at the NPA boxes, created by the dipole moment of the half α-helices, results in an electrostatic repulsion of the positively charged protons, effectively preventing them from passing, see Figure 7.

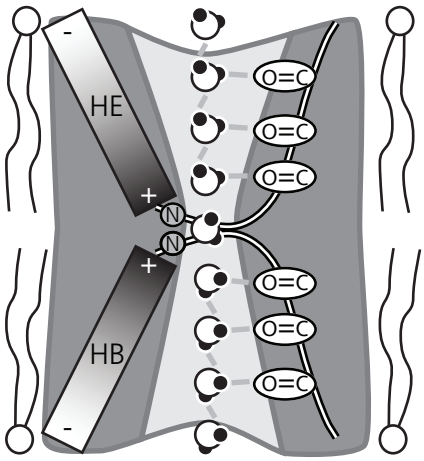


Figure 7. Schematic picture showing the reorientation of water molecules in the pore. On the left side are the half TM helices, showing how their dipole moments result in the positive charge of the electrostatic repulsion filter. Grey lines show how the water molecules hydrogen bond to each other and to the backbone. A reorientation at the NPA boxes breaks this chain of interactions.

As previously mentioned, MIPs are permeable to a wide range of substrates (water, ammonia, urea, glycerol, arsenite, antimony to name a few) and Froger and coworkers early found out that amino acids at five different positions in MIPs seemed to be indicative of substrate specificity for two groups of MIPs, transporting either water or small neutral solutes. These positions, called P1 to P5, where identified by their conservation in amino acid alignments (Froger et al., 1998). However, when high-resolution structures became available it became obvious that the P1 to P5 positions could not interact with any substrate directly and therefore perhaps did not have any functional role, but rather the invariability is just reflecting a closer evolutionary distance of MIPs with the same substrate specificity. In the structures, an intricate hydrogen-bonding network across the pore could be seen and it also became apparent that there was a size constriction region of importance for substrate specificity, the ar/R filter (Sui et al., 2001, Murata et al., 2000, Fu et al., 2000). The ar/R filter consists of four amino acids, encircling the pore of the MIP. Whereas three of the amino acids (H2, H5 and LE2) have their side chains facing the pore, the fourth (LE1) interacts with the substrate through the backbone carbonyl group thereby allowing a greater variation at this position. The effects of the side chains of the other amino acids have been evaluated by point mutations in AQP1, showing that water selectivity can be modulated to allow ammonia, urea and glycerol transport just by changing the ar/R filter (Beitz et al., 2006). But, even though the ar/R filter is important for substrate specificity, it is not solely responsible for it, as was shown in a study where a water permeable PIP was point mutated to mimic ammonia and urea transporting TIPs and NIPs, requiring not only point mutations in the ar/R filter but also at an extra position (Dynowski et al., 2008a). In a recent paper Savage and coworkers (Savage et al., 2010) sequentially engineered the three signature amino acids of glycerol conducting GLPs into the water transporting AqpZ from *Escherichia coli*. Crystal structures and transport rate experiments showed that mutants had a pore size similar to that of wild type AqpZ and that even though water transport was diminished, no glycerol transport could be detected. This suggests that the ar/R filter region is important for water transport

(and possibly other small molecules) whereas transport of larger molecules such as glycerol seem to depend on channel cross-section size. There have also been several simulation studies supporting both the role of the NPA boxes and the importance of the ar/R filter and to various degrees explaining the experimental permeabilities seen (see for example Hub and de Groot, 2008, Phongphanphanee et al., 2010).

Mainly three types of transport assays have been used for experimental determination of MIP permeation; the oocyte system, stopped flow measurements on proteoliposomes and yeast complementation assays. In the oocyte system, eggs from the frog *Xenopus laevis* are injected with cRNA encoding the MIP under investigation. After the eggs have been given time to express the protein (which hopefully localizes to the plasma membrane) they are either transferred to hypo/hyper osmotic media to observe swelling/shrinking, or they are transferred to a media containing labeled substrate to observe uptake of this. The proteoliposome assay is also based on shrinking/swelling, but in this case, purified protein is reconstituted in lipid vesicles (liposomes). Due to the small size of liposomes (and therefore the high surface/volume ratio) the swelling/shrinking is very fast. Measurements therefore have to be made in a stopped flow spectrophotometer, ensuring rapid mixing and fast measurements. Finally, complementation assays in yeast has proven to be useful for identifying a range of substrates, some of which would have been difficult to measure with the other methods. The rationale here is to look at substrates that are either beneficial or detrimental for the yeast and by deleting a naturally occurring transporter, making the yeast less or more responsive to the substrate. Different MIPs are then expressed in the deletion mutant and MIPs transporting the substrate can be identified by identifying clones with restored wild-type sensitivity.

3.4 MIP Regulation

To only *increase* the permeability of a membrane for a certain substrate might not be very useful for an organism, but to be able to *regulate* the permeability gives the organism the possibility to adapt to different conditions, which is beneficial. It is therefore perhaps not very surprising that MIP mediated permeabilities can be regulated in many different ways.

At a supracellular level, expression of MIPs can be controlled by different transcription factors and mRNA stability can be affected by different miRNAs. This is likely to be part of the explanation to why some species have so many MIP isoforms, but it has also turned out that posttranslational regulation of MIP activity is common, making the regulatory possibilities far more complex than can be achieved simply by a large number of MIP genes.

Posttranslationally, MIPs can either be regulated at a structural level, where alteration in a protein structure mediates a change in permeability of that protein, or at a localization level, where permeabilities of membranes are affected by changes in the subcellular localization of MIPs. MIP gating (opening and closing of the pore) has been shown to occur for both animal, plant and yeast MIPs (Gonen et al., 2004, Harries et al., 2004, Tornroth-Horsefield et al., 2006, Fischer et al., 2009) and to respond to a whole range of cues such as pH, divalent cations and phosphorylation. It has also been suggested that there is a "solute gating" mechanism operating by a cohesion/tension mechanism, in which solute exclusion from the pore leads to negative pressures in the channel resulting in a collapse of the MIP channel (Ye et al., 2004).

Some MIPs have been shown to have a polar localization in the cell. This allows MIPs to work in series with active transporters, coupling passive diffusion to active transport of substrates. MIPs have also been shown to exhibit a dynamic subcellular localization where MIPs translocate from vesicles or the ER to the membrane upon some signal. This has for example been shown for AQP2, where cyclic AMP activates protein kinase A, which in turn phosphorylates AQP2 residing in vesicles, then fusing with the cell membrane (reviewed in Nedvetsky et al., 2009). Finally, MIP abundancy can be regulated by degradation and it has been shown that MIPs can be targets for E3 ubiquitin ligases, suggesting that targeted degradation via the 26S proteasome is an additional regulatory pathway (Lee et al., 2009).

4 Plant MIPs

The diversity and abundance of MIPs found in plants suggests that they have important functions in whole plant water and nutrient status. However, it was not in a search for proteins involved in these functions that MIPs were first identified, but rather their discovery was a consequence of them being major constituents of different membranes (see Figure 8). As previously mentioned, one of the very first MIPs identified was isolated from the peribacteroid membrane of soybean root nodules, but plant MIPs were also identified as major components in plasma membranes and tonoplast (the vacuolar membrane) (e.g. Kjellbom and Larsson, 1984, Johansson et al., 1996). A MIP from tonoplast, Arabidopsis γ-TIP, was the first plant MIP demonstrated to have water-channel activity. This experiment was published just a year after the report on the first water channel from Peter Agres group, using the same technique with the oocyte system (Maurel et al., 1993). Since then, a great number of plant MIPs have been shown to be permeable to water, but also to a whole range of other substrates including glycerol, carbon dioxide, urea, ammonia, different metalloids and hydrogen peroxide.

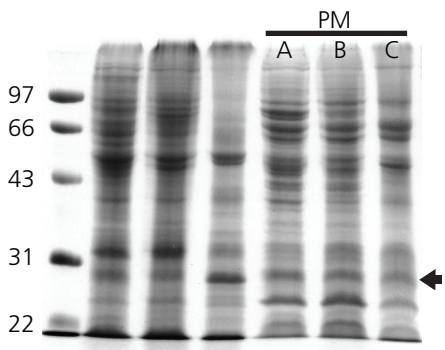


Figure 8. SDS-PAGE gel picture of total PM proteins from poplar. The arrow points at the band identified as a MIP. Poplar PM was isolated from xylem (A), cambium/phloem (B) and leaf (C). (Kindly provided by Katja Bernfur).

Plant MIPs are primarily expressed in two types of tissues. One of these is the vascular tissue, where plant MIPs are expressed in cells in or surrounding the vascular bundle and likely take part in transport to and from the phloem and xylem. The other is in cells where regulated rapid water flows occur, such as in elongating cells, guard cells of the stomata, motor cells controlling leaf movement or in seeds (Maurel et al., 2008).

4.1 The Land Plant MIP Family

Already in one of the first phylogenetic analysis of the 18 MIP sequences known at the time two subfamilies of plant MIPs were identified, the tonoplast intrinsic protein (TIP) and the NOD, later renamed to nodulin-26 like intrinsic protein (NIP), subfamilies (Reizer et al., 1993). During the following years, several more phylogenetic analyses were published and it soon became clear that plants also had a third group of MIPs, the plasma membrane intrinsic protein (PIP) subfamily, and that these three subfamilies were specific for plants (Kammerloher et al., 1994).

Even though it was already in these early analyses obvious that plants contained many different isoforms of MIPs, belonging to several different subfamilies, the complete identification of all MIPs within one species was just not possible until when the first plant genome became available in the year 2000 (The Arabidopsis Genome Initiative, 2000). When this important identification was done in 2001, it did not only became clear that plants had nearly three times as many isoforms as there were in mammals (Arabidopsis has 35 MIPs compared to 13 in humans), but also a fourth "new" subfamily of plant MIPs was discovered, the small and basic intrinsic proteins (SIPs) (Johanson et al., 2001). The largest subfamily was the PIPs (with 13 isoforms), followed by the TIPs and the NIPs (10 and 9 members respectively) and the smallest was the SIPs (with only 3 isoforms). It was also evident that within the subfamilies there were MIPs with higher degrees of similarity, forming subgroups, and when naming the MIPs they were named after MIP subfamily, subgroup number and number within subgroup (i.e. PIP1;1, belongs to the PIP subfamily, to the first subgroup of PIPs and is the first MIP in that subgroup). The identification of all Arabidopsis MIPs and their phylogeny forms a framework for classification of other plant MIPs and its usefulness is made clear by the fact that, since then, several other higher plant species have been shown to contain a comparable number of MIP isoforms (e.g. 33, 36 and 37 MIPs in rice, maize and tomato respectively) with a division into subfamilies and subgroups consistent with that of Arabidopsis (Sakurai et al., 2005, Chaumont et al., 2001, Sade et al., 2009). Recently some higher plants with very large number of MIPs have been reported (55 and 71 for poplar and cotton respectively) (Park et al., 2010, Gupta and Sankararamakrishnan, 2009), however this is also consistent with the Arabidopsis findings, since poplar have gone through a recent whole genome duplication and cotton is polyploid.

Interestingly, even though the total number of MIPs is rather conserved in higher plants, it does not seem possible to find specific pairs of corresponding genes (orthologs) of MIPs in different species. At the same time, all higher plants seem to contain the same subfamilies and to a large extent also the same subfamily groups. Taken together these findings implies that there are certain restrictions for the subfamilies and subfamily groups, but that apart from these constraints, MIPs are rap-

idly evolving. It also suggests that the specific functions of subfamilies and subgroups are conserved in all these plants, hence the conserved MIP family structure. This provoked an interest in us to find out to what extent MIP subfamilies and subgroups would be conserved in a more primitive plant, that is a plant with a more ancient shared ancestry with higher plants thus lacking many of the features of higher plants. Therefore, in **paper I** we identify all MIPs in the moss *Physcomitrella patens*, a plant that diverged from the lineage leading to higher plants around 450 million years ago. We found that the *P. patens* MIP family shows an even greater variation with seven subfamilies, including the four found in Arabidopsis. One of the three subfamilies not found in Arabidopsis was previously identified and found to belong to the GLP group of MIPs and were therefore named GLP like intrinsic protein (GIP) (Gustavsson et al., 2005). The remaining two subfamilies had not been reported previously. One of them showed similarities with both the PIP and the TIP subfamilies and was therefore named hybrid intrinsic protein (HIP) subfamily, whereas the other did not show similarity to any known MIP family and to emphasize this lack of information it was named the X intrinsic protein (XIP) subfamily. After extensive searches it appears to be that the GIP and HIP subfamilies are absent in higher plants, but members of the XIP subfamily was found also in several higher plants (such as tomato, tobacco and poplar) although the subfamily seems to have been lost in monocots and some eudicots, such as Arabidopsis.

The present phylogeny of land plant MIPs is shown in Figure 9. Note that not only the subfamilies, but even some of the subgroups are conserved in mosses. As can be seen, the NIP subfamily is the most diverse of the three larger subfamilies (PIPs, TIPs and NIPs) and with the more relaxed subgroup criteria, first used in maize and rice, three subgroups of NIPs are formed (Chaumont et al., 2001, Sakurai et al., 2005). The extraordinary homogeneity of the PIP1 and PIP2 subgroups also stand out. As of yet it is not clear why these subgroups show this conservation, but one possibility is that they are subject to some extra constraints, making the neutral evolutionary space more confined than for other MIPs. One such constraint might be interactions with other proteins, for example it could be an effect of PIP heterotetramerization. However, this ability of PIP1s and PIP2s to form tetramers that include both isoforms is still debated and the physiological function of this is largely unknown, but this will be discussed more in detail in the next subchapter.

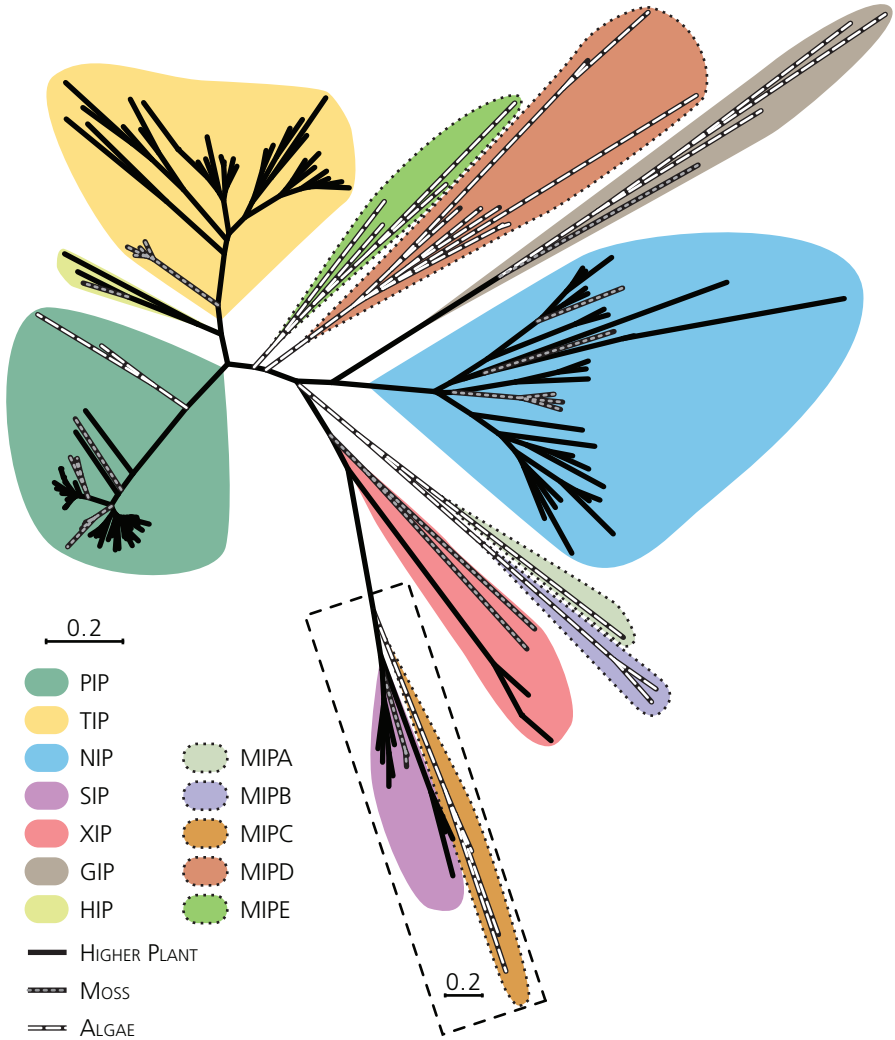


Figure 9. The phylogeny of plant MIPs. A NJ tree constructed using the complete set of MIPs from Arabidopsis, tomato, *P. patens*, and the nine algal species described in **paper IV**. Origin of different MIPs are indicated by line type and subfamilies are indicated by colored background. The algal specific subfamilies are indicated by a dotted outline of the colored area. The SIP/MIPC part is shown at a different scale in order to fit in the same picture. This is indicated by the dashed box and the smaller size of the scale bar.

4.2 Subfamilies

The original division of plant MIPs into subfamilies was a strict phylogenetic classification and as such only based on sequence similarity. However, as more and more experimental data has become available it seems like this classification also largely reflect subcellular localization and substrate specificity, making the subfamily concept much more useful. A lot of the research on plant MIPs have focused on the PIPs, TIPs and NIPs and still very little is known about the SIP, GIP, XIP and HIP subfamilies. In the following paragraphs, I will try to give an overview of what is known for each of these seven subfamilies.

4.2.1 PIPs

The plasma membrane intrinsic proteins (PIPs) were first identified as major constituents of plant cell plasma membrane, hence the subfamily name. As previously mentioned, PIPs form two highly conserved subgroups, PIP1s and PIP2s (see Figure 10) and it seems that each subgroup has its own unique features. Ultrastructural studies have shown that some PIP2s have a polar localization in root epidermal cells (Hachez et al., 2006) whereas some PIP1s in mesophyll cells seem to be localized to structures called plasmalemmasomas, where the plasma membrane folds inwardly to create a pouch-like structure (Robinson et al., 1996). PIP1s have also been localized to the chloroplast inner membrane by immunogold localization (Uehlein et al., 2008) and shown to have a high degree of retention in the ER (Zelazny et al., 2007).

Whereas PIP2s seem to be highly efficient water transporters, increasing the water permeability 10-20 fold, PIP1s are almost impermeable to water (Moshelion et al., 2002, Fetter et al., 2004). Carbon dioxide have been suggested as an alternative substrate for both PIP1s (Uehlein et al., 2003, Uehlein et al., 2008) and PIP2s (Hanba et al., 2004), and although there have been both transport assay experiments, showing that PIPs do transport CO_2, and experiments showing that PIP1 overexpression *in planta* leads to increased biomass (Sade et al., 2010, Uehlein et al., 2003), the physiological relevance for this transport is still debated. Finally, PIPs have also been suggested both to transport and to be regulated by hydrogen peroxide (Dynowski et al., 2008b, Boursiac et al., 2008a, Henzler et

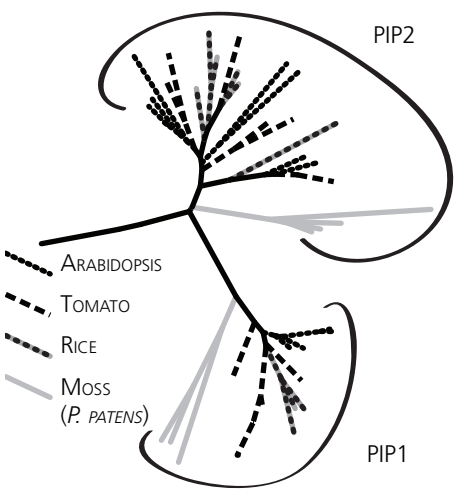


Figure 10. Subtree of the PIPs, showing the conservation of PIP1s and PIP2s. Line types represent species origin of PIPs.

al., 2004, Boursiac et al., 2008b). The function of hydrogen peroxide transport and regulation of PIPs still have to be investigated more in detail, but could potentially be very interesting as hydrogen peroxide is known to work as a signaling molecule in for example pathogen response and thermo tolerance in plants.

That PIPs are gated, i.e. able to change between open and closed conformations, was suggested early on but exactly how this was achieved remained unclear until the structures of open and closed *So*PIP2;1 was solved in 2006 (Tornroth-Horsefield et al., 2006). From these structures it became clear that it is the D-loop that blocks the pore in the closed state and that the stabilities of the two different states can be affected by many different signals such as pH, Ca^{2+} levels and different kinds of kinases. This is illustrated in Figure 11.

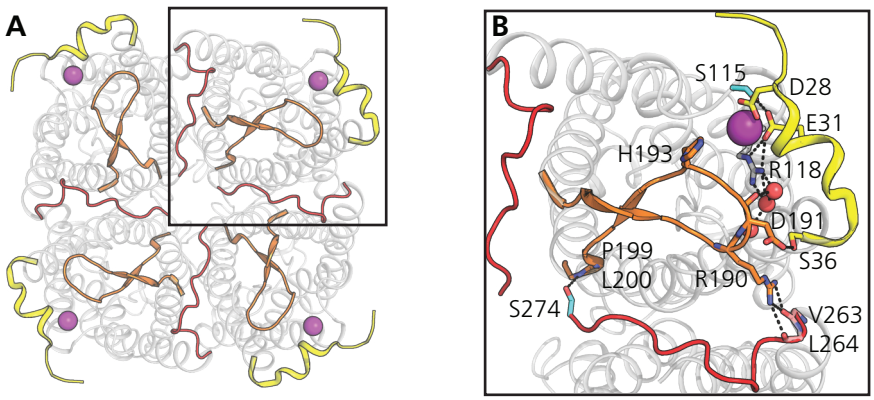


Figure 11. Picture showing the gating of *So*PIP2;1. Picture A shows an overview of a tetramer of closed SoPIP2;1s, while B shows an enlargement of the enclosed area of picture A. The D-loop (obstructing the pore) is colored orange, the proposed position of Ca^{2+} is purple while the N- and C-termini are colored yellow and red respectively. Gating can be affected by phosphorylation of S115 and S274 (colored cyan in picture B), pH sensing (through protonation of H193) and via Ca^{2+} levels. All these will affect the intricate hydrogen bonding network shown by dashed lines in B. In short the C termini and D-loop of two adjacent monomers interacts through the sidechain (sc) of S274 to the backbone (bb) of P199 and L200 and through the sc R190 and bb V263 and bb L264. The N termini and D loop (in the same monomer) interacts though sc D191 to sc S36 and through the bb of R190 and bb D191 via three water molecules to sc R118 and sc E31. Also in the same monomer, sc S115 interacts with sc E31 (Tornroth-Horsefield et al., 2006, Khandelia et al., 2009).

On a slower timescale the permeability of membranes can be affected by changing the localization of PIPs. It has been shown that there is a diacidic motif present in some PIP2s that is important for trafficking (Zelazny et al., 2009) and that in some PIPs one of the residues in the motif can be methylated, possibly altering the trafficking (Santoni et al., 2006). Still, other PIPs lack the diacidic motif entirely but locates properly to the PM anyway (Zelazny et al., 2009). It has also been shown that export

of *At*PIP2;1 from the ER is depending on phosphorylation of the C-termini, and that this phosphorylation is changed upon salt stress, resulting in *At*PIP2;1 being localized to internal structures (Prak et al., 2008). Changes in PIP mediated permeation is also likely to occur via targeted degradation, as it has recently been shown that *At*PIP2;1 can be targeted by an E3 ubiquitin ligase, and subsequent degradation by the 26S proteasome (Lee et al., 2009).

Interactions between PIP1s and PIP2s have been an intensive research area the last couple of years. In 2004 it was shown that, whereas PIP1 expression hardly leads to any increased water permeability, coexpression of PIP1s and PIP2s dramatically increase water permeability, suggesting some form of interaction between the isoforms (Fetter et al., 2004). Since then experiments using various PIP1/PIP2 isoforms have given comparable results, but also shown that coexpression not only affect water transport but also pH sensitivity and shutdown efficiency of the gating, as well as CO_2 transport (Alleva et al., 2010, Bellati et al., 2010, Otto et al., 2010). Although it was unclear in the beginning how the interactions occurred, an increasing amount of experimental data using techniques such as FRET, split-YFP, size exclusion chromatography and gel-electrophoresis now strongly suggest that the isoforms can interact in heterotetramers containing both PIP1 and PIP2 monomers (Otto et al., 2010, Zelazny et al., 2007). The changes in permeabilities found can partially be explained by the finding that coexpression leads to an improved PIP1 PM localization (as PIP1s otherwise show a strong retention in the ER) (Zelazny et al., 2007) but it could also be that physical interactions between the isoforms change the behavior of the proteins, so that for example composition of the tetramer could influence the transporting properties of the monomers. One possibility is that monomers in the tetramer can affect the gating of each other, by stabilizing/destabilizing the D-loop in the open form, something that could potentially change gating properties such as pH sensitivity for the whole heterotetramer. To conclude, PIP mediated permeation can be regulated in response to many cues and on several different levels, some of which are summarized if Figure 12.

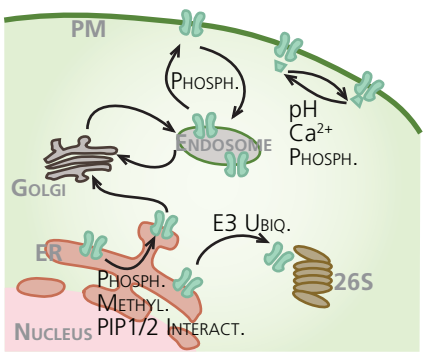


Figure 12. Overview of proposed PIP regulation mechanisms discussed in the chapter.

4.2.2 TIPs

Just as with PIPs, TIPs (Tonoplast Intrinsic Proteins) were named after their main localization which in this case is the vacuolar membrane, the tonoplast. This membrane is extremely permeable to water, with osmotic water permeabilities up to 100 fold higher than those of plasma membrane (Maurel et al., 1997). TIPs are responsible for the high permeability and they can make up as much as 40 percent of the total tonoplast proteins (Higuchi et al., 1998).

Several different kinds of vacuoles exists in plants and in some early localization studies it was found that two types of vacuoles, the protein storage vacuoles of seeds and the large central vacuole of elongating cells, contained different subgroups of TIPs (TIP3 and TIP1 respectively) (Johnson et al., 1989, Ludevid et al., 1992). The concept of multiple vacuolar compartments, originating from different targeting machineries, and residing in one cell has been a source for controversy for the last 15 years or so. In the late 1990s, Jauh and coworkers did some localization studies using confocal microscopy and immunofluorescence labeling with antibodies raised against the three different subgroups of TIPs known at the time (TIP1-3) to see if TIPs could be used as markers for the different vacuolar types. They found multiple types of vacuoles that were differentially labeled and argued that plant cells had the ability to generate at least three different types of vacuoles (Jauh et al., 1999). However, the subject of co-existing, functionally diverse vacuoles have recently been questioned since two independent localization studies, using immunogold and YFP-fusion proteins respectively, both failed to discriminate between vacuoles as all TIP isoforms labeled the same compartment (Hunter et al., 2007, Olbrich et al., 2007). The general conclusion drawn from this is that the expression of TIP isoforms is mostly controlled by developmental cues and that as a consequence of this, some TIPs are associated with special types of vacuoles existing at a specific developmental stage. The multivacuole hypothesis might still be valid for a number of cases, but not universally (Frigerio et al., 2008). Just recently it was reported that some TIPs localize to the plasma membrane, specifically in the seed maturation and the early seed germination stages (Gattolin et al., 2010a). The physiological function of TIPs in the PM is unclear, but they might be involved in imbibition (the rapid initial uptake of water) of dry seeds.

A detailed expression mapping of all Arabidopsis TIPs expressed in root tissue showed that TIPs were mainly localized in mature tissue as well as in the elongation zone, but not in the root meristem (Gattolin et al., 2010b, Gattolin et al., 2009). The two exceptions to this was *At*TIP1;2 and *At*TIP2;1 which were present in the root caps and in lateral root primordia respectively.

Apart from transporting water, TIPs have also been shown to be permeable to glycerol, urea, ammonia and hydrogen peroxide (reviewed in Wudick et al., 2009). The physiological relevance for non-water transport in TIPs remains unclear. A previously reported cell-death phenotype of an Arabidopsis TIP1;1 RNAi line (Ma et al., 2004) has been reevaluated as due to an off-target silencing effect, since two studies on knock-out and knock-down *At*TIP1;1 plants failed to reproduce any severe phenotype (Schussler et al., 2008, Beebo et al., 2009). However, both *At*TIP1;1 and *At*TIP1;2 have been found to be permeable to hydrogen peroxide (Bienert et al., 2007) and double knock-outs of these TIPs showed minor signs of increased oxidative stress under high light conditions, indicating that they might have roles during plant stress (Schussler et al., 2008). *At*TIP1;1 knockout plants were also recently shown to have a reduced root growth when grown in glycerol containing media (Beebo et al., 2009) which is puzzling since *A*tTIP1;1 was found not to transport glycerol in oocyte experiments (Maurel et al., 1993).

Even though lack of TIP isoforms seems to have only minor effects on plants, overexpression of TIPs have been shown to result in increased cell surface and volume (Reisen et al., 2003), as well as increased cell division and cell elongation rates (Okubo-Kurihara et al., 2009). Several studies have shown that overexpression results in plants with increased growth of both vegetative and reproductive tissues (Lin et al., 2007, Peng et al., 2007, Sade et al., 2009). Peng and coworkers also showed that transgenic Arabidopsis plants, overexpressing a ginseng TIP, had an increased tolerance to drought and salt stress. During salt stress, the shoots of the overexpressing plants actually accumulated more sodium than the controls, maybe indicating an increased possibility of intracellular storage due to larger vacuoles and cells (Peng et al., 2007). An increased tolerance to drought and salt stress was also seen in the study by Sade and coworkers, who could not only see an increased biomass, but also an increased yield, of tomato overexpressing *Sl*TIP2;2. They also found that transpiration rates of the TIP-overexpressing tomato plants was increased and that it did not decrease as much during stress as in the wild type, suggesting that maybe TIP overexpression could be a key to convert isohydric plants (drought avoidant, with a tight stomata control) to anisohydric plants (drought tolerant, with less strict stomata control) (Sade et al., 2009). Recently, TIP overexpression was also shown to result in an increased resistance to boron, with overexpressors showing accelerated development as well as increased silique production compared to wild type under high boron stress (Pang et al., 2010). Increased boron content of overexpressors indicates that it might be the same mechanism as is responsible for the increased sodium tolerance.

4.2.3 NIPs

The first plant MIP discovered was NOD26 or Nodulin-26, named after the tissue in which it was discovered, root nodules of soybean. As it later on became apparent that there were many plant MIPs related to NOD26 although not localized to nodules, these MIPs were named NOD-26 like intrinsic proteins (NIPs) (Heymann and Engel, 1999). NIPs have mainly been localized to the plasma membrane (Choi and Roberts, 2007, Ma et al., 2006, Takano et al., 2006, Tanaka et al., 2008, Mitani et al., 2009), but one study has also reported ER localization (Mizutani et al., 2006).

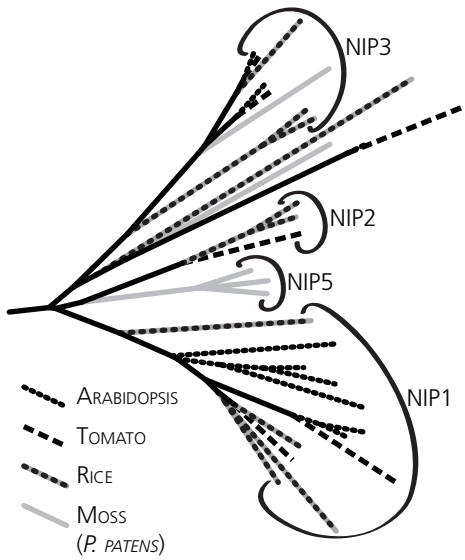


Figure 13. Subtree of the NIPs showing the three major groups of higher plants and the NIP5 group of *P. patens*. Line styles indicate species of origin for MIP sequences.

As seen in Figure 13, NIPs form a very diverse subfamily of MIPs, so diverse in fact that the more stringent subgrouping criteria used for all Arabidopsis MIPs is only useful when comparing this subfamily in closely related species. Therefore when comparing plant species the less rigorous subgroup criteria used in the description of maize and rice NIPs is preferred, resulting in NIPs belonging to three major subgroups (NIP1-3). NIP1s show moderate water permeability, but are also able to transport for example formamide and glycerol, whereas NIP3s showed no water transport but were still permeable to formamide and glycerol as well as larger uncharged solutes such as urea (Wallace and Roberts, 2005, Wallace et al., 2002). The third major subgroup, NIP2, is missing in Arabidopsis and was first recognized as a subgroup when the MIPs in rice and maize were phylogenetically characterized. In 2006 a NIP2 was identified as being responsible for low silicon sensitivity of a rice mutant isolated in a genetic screen (Ma et al., 2006). Not only did this identify a possible physiological role for NIP2s, but it also established metalloids as a whole new group of substrates for MIP transport in general, and specifically NIP2 transport. The identified NIP2 (*Os*NIP2;1 also called Lsi1) is localized in the endo- and exodermal cells, at the location of the suberized casparian strips, in the rice root. Interestingly the subcellular localization is polar, with *Os*NIP2;1 only expressed in the PM facing away from the root center, and it turns out it works in series with an active transporter (Lsi2) localized in the PM facing the other side, this is illustrated in Figure 14. A similar arrangement is found in the xylem parenchyma cells of the shoots, where *Os*NIP2;2 is located on the side facing the vessel (Yamaji et al., 2008). NIP2s in both barley and maize has been found to have a similar location pattern and probably share the same

function *in planta* (Mitani et al., 2009, Chiba et al., 2009). NIP2 overexpressing rice have been shown to have increased silicon content and increased yields, but unfortunately also higher arsenite levels, probably resulting from arsenite and silicon having very similar structures and using the same transport routes in the plant (Ma et al., 2008). Arsenite transport has also been shown to involve both a NIP1 and a NIP3 in Arabidopsis (Kamiya et al., 2009, Isayenkov and Maathuis, 2008), indicating that this substrate is not restricted to the NIP2 subgroup. Two NIP3s in Arabidopsis (*At*NIP5;1 and *At*NIP6;1) has been identified to take part in transport of another metalloid, boron, possibly in a way similar to that of the silicon transporting NIP2s in rice (Takano et al., 2006, Tanaka et al., 2008). *At*NIP2;1, a NIP1, was shown to be upregulated under water logging and to be permeable to lactic acid, leading to the suggestion that it might have a role during lactic acid fermentation in oxygen deprived roots (Choi and Roberts, 2007).

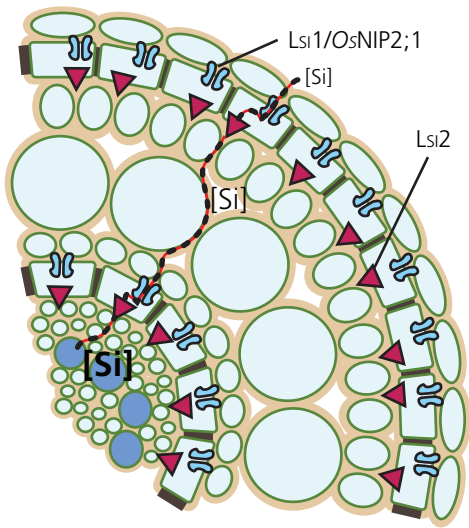


Figure 14. A schematic picture showing how the passive transport through *Os*NIP2;1/Lsi1 works in series with the active transporter Lsi2 in root uptake of silicon (red/black dotted line). Both *Os*NIP2;1 and Lsi2 are expressed in root cells localized to the casparian strips but show opposite polar localization. This arrangement makes it possible for the plant to transport Si against the concentration gradient (as indicated by the sizes of [Si] in the picture).

4.2.4 SIPs

The small and basic intrinsic proteins, SIPs, were originally discovered when the Arabidopsis genome was screened for MIPs and named after their characteristics as being smaller than PIPs and NIPs and more basic than TIPs (Johanson et al., 2001, Johanson and Gustavsson, 2002). Still very little is known about these MIPs, they are very divergent with unusual NPA boxes and ar/R filters, form two subgroups in higher plants and tend to cluster together with the mammalian AQP11 and AQP12 in phylogenetic analyses (as seen in Figure 3). They seem to be localized to the rough ER and some are permeable to water, but so far no phenotype for knock-out plants have been described (Ishikawa et al., 2005, Maeshima and Ishikawa, 2007, Katsuhara et al., 2008).

4.2.5 GIPs

In 2005 a plant MIP was identified in mosses and turned out to be different from all other known plant MIPs in that it belongs to the GLP cluster of MIPs (see Figure 15 and Chapter 3.1). The subfamily was named after this grouping to the GlpF-like intrinsic protein (GIP) subfamily. GIPs have so far only been identified in two species of moss, are predicted to be localized to the plasma membrane and seem to be transporting glycerol (Gustavsson et al., 2005)

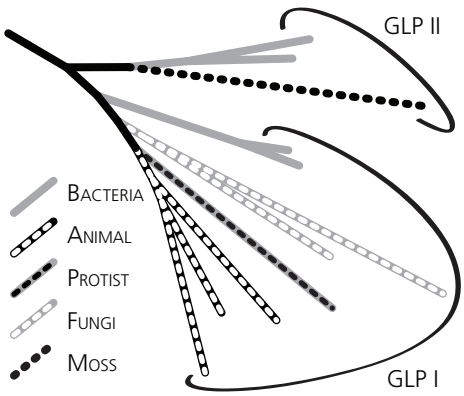


Figure 15. Phylogeny of the GLP subfamily. Line styles show species of MIP sequences and brackets indicate the GLPI and GLPII groups (with GLPII containing the *P. patens* GIP).

4.2.6 HIPs

Another group of plant MIPs that was found upon identifying all MIPs in the moss *P. patens* (**paper I**) are the HIPs. These MIPs had characteristics of both PIPs and TIPs and were therefore named hybrid intrinsic proteins (HIPs). HIPs are not restricted to mosses, but seem to be limited to non-seed plants as they were also found in the spikemoss *Selaginella moellendorffii* but not in any higher plants. To date no HIPs have been experimentally characterized, but they have been predicted to be localized in the tonoplast (**paper I**).

4.2.7 XIPs

The X intrinsic proteins (XIPs) were also identified when characterizing the MIP family of *P. patens*, but contrary to the HIPs, XIPs were also found in several higher plants (**paper I**). An attempt to determine the expression pattern of XIPs by examining the tissues of origin of the cDNA libraries where the different XIP ESTs was made, but no conclusions could be drawn since XIP transcripts were isolated from too many different sources (**paper I**). Analysis of microarray data for poplar also suggests that XIPs are abundantly expressed but do not show any tissue-specific expression (Gupta and Sankararamakrishnan, 2009). Very recently a more detailed study of XIP expression in cotton revealed that the XIP isoform studied seem to be highly expressed in mature leafs and to some extent also in other parts of the shoot but not at any detectable level in the roots (Park et al., 2010). So far no experimental data of XIP permeability is available, but an evaluation of the ar/R filter shows a hydrophobic filter suggesting that XIPs are not mainly water channels but rather transporting other substrates (**paper I**).

4.3 Evolution

The MIP family is an ancient gene family with its origin believed to date back some 2.5 to 3 billion years (Pao et al., 1991, Park and Saier, 1996). The ancient origin is reflected by the fact that MIPs are present in all three domains of life, Bacteria, Archaea and Eukarya. Even though MIPs are present in all kinds of organisms, they are particularly abundant in plants. The abundancy likely reflects that MIPs have important functions in plants and results in that many MIPs probably have overlapping functions. This might explain why traditional loss of function studies, using knock-out/knock-down techniques, largely have been unsuccessful in identifying physiological roles for MIPs. Another approach is to try and deduce the function of proteins by comparing these in different species, in order to see if the evolution of the protein isoforms and families can be linked to the emergence or disappearance of some trait. This can be done either by trying to identify the same specific gene in some evolutionary related species of interest, or in a more "comparative genomics" kind of approach, where whole gene families are identified in genomes of sequenced species and subsequently compared. Using the first approach one is free to chose whatever species one want, but is limited in that conclusions only can be drawn from positive results, since a negative result does not necessarily indicate that the species is missing the gene, only that it cannot be detected. In a genome-sequenced species on the other hand, it is possible to draw that kind of conclusions, making the "comparative genomics" approach more powerful. However, the number of species with a sequenced genome, although rapidly increasing, is still very small.

In order to understand the evolution of the plant MIPs it is necessary to relate it to the evolution of plants. Therefore follows a brief review of plant evolution, which together with Figure 16 hopefully will be useful for understanding the rest of this subchapter. Plants (archaeplastida or "plantae *sensu lato*") are descendants from a unicellular eukaryote that acquired a photosynthetic cyanobacterium as an endosymbiont (the origin of the chloroplast) and they are one of the five or so kingdoms of eukaryotes. Plants contain three groups, a small group of microscopic freshwater algae called glaucophytes, rhodophytes (red algae) that are primarily marine algae including for example the reef-building coralline algae, and viridiplantae (green plants) comprising the chlorophytes and streptophytes. The chlorophyte group of green plants contains the vast majority of all freshwater and marine algae, and contains both uni- and multicellular species. The streptophytes contain the uni- and multicellular charophycean freshwater algae (charophytes), and the multicellular land plants (embryophytes). Land plants are further divided into non-vascular plants (bryophytes) and vascular plants (tracheophytes), and vascular plants into non-seed plants (e.g. ferns and lycophytes) and seed plants. Seed plants are divided into non-flowering (gymnosperms) and flowering (angiosperms), where the flowering plants contain the two groups of monocots and eudicots (having one and two cotyledons

respectively). Many of the main events in plant evolution are reflected in the naming of the groups, but some others are indicated in Figure 16 and include for example multicellularity and apical growth (occurred already in the charophyte-"land plant" ancestor), alternation of generations involving a diploid phase (first land plants) and three dimensional patterning (Bowman et al., 2007).

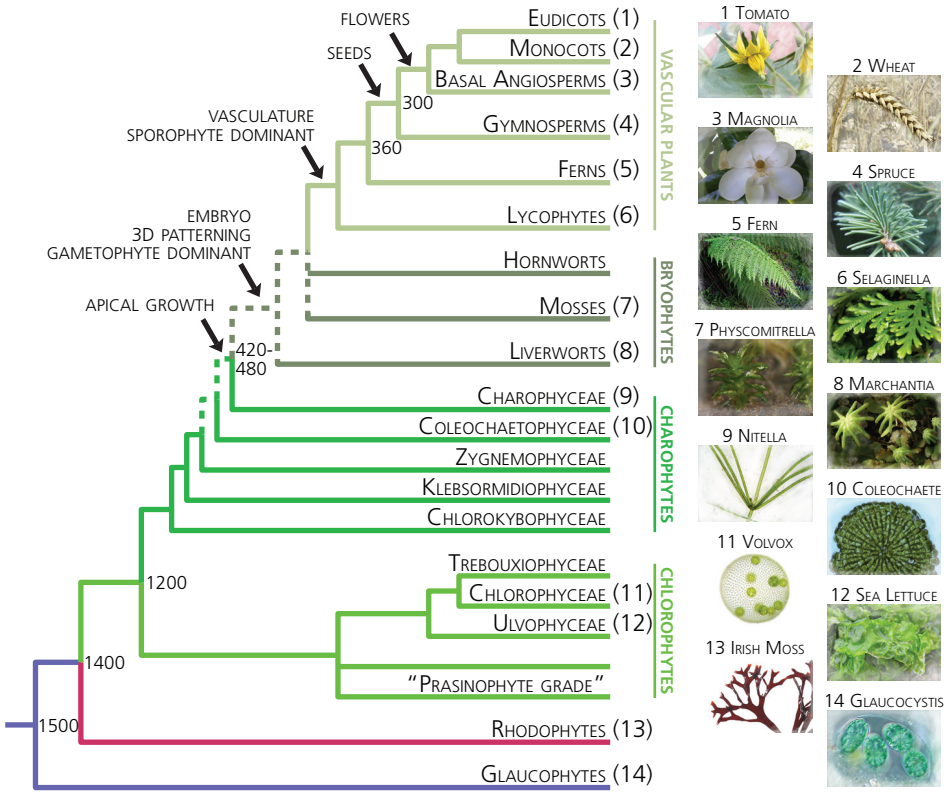


Figure 16. An overview of the evolution of the plant kingdom, adapted from Bowman et al., 2007. The numbers in the nodes of the tree are rough estimates of divergence dates in million years ago. Four different subsets of green plants are indicated by different shades of green and pictures show some examples from different groups. Arrows indicate evolutionary events of special interest. All photos are part of the public domain.

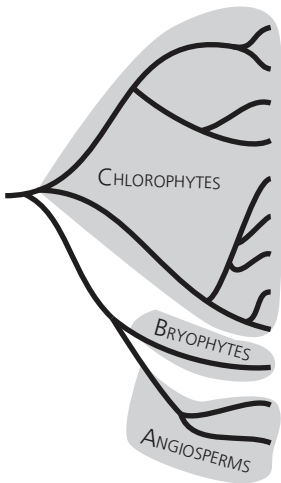
The first two plant genomes that were sequenced were those of Arabidopsis (an eudicot) and of rice (a monocot). In both these genomes, the whole MIP superfamily was identified (Johanson et al., 2001, Sakurai et al., 2005), and when comparing them they seem very similar. But one thing that stands out is that rice seems to have a subgroup of NIPs absent in Arabidopsis (NIP2s). It turns out that this subgroup is also present in other monocots, and later it was identified as being involved in silicon transport in rice (Ma et al., 2006). Silicon is generally not believed to have

any important function in plant cells, and therefore to be non-essential for plants. However, silicon is beneficial for some plants and for example in monocots silicon is deposited extracellularly to give an increased rigidity to the plant and prevent lodging. Most eudicots on the other hand, do not use silicon in this way, which is in agreement with them not having a NIP2 subgroup. Interestingly some eudicots in the cucumber/squash family, for which it is well known that silicon is beneficial, turn out to also have the NIP2 subgroup, even though they are eudicots. This is indicating that the NIP2 subgroup is likely to have a more ancient origin than monocots/dicots and possibly that silicon played an important role in rigidity of early vascular land plants.

In 2007 the genome of the moss *Physcomitrella patens* was released, and upon identification and comparison of the moss MIP superfamily to those of higher plants, they turned out to be surprisingly similar (**paper I**). All the subfamilies and even some of the subgroups found in higher plants were present already in moss, and although the total number of isoforms was lower (23 compared to 35 for *P. patens* and *A. thaliana* respectively) the diversity was greater, with seven subfamilies present in the moss. This was unexpected, as mosses are relatively "simple" plants, lacking many of the features believed to make transport in higher plants more complex, such as root system, vascular tissues, leaf-stem morphology, lignified cell walls and so on. Still it was clear that some features were much more conserved than others, such as the extremely conserved PIP subfamily, in which both the PIP1 and PIP2 subgroups were present in *P. patens*. In the TIP subfamily on the other hand, no subgroup was conserved in mosses and higher plants, even though all subgroups were conserved between monocots and eudicots. This suggests that most of the TIP subgroups of higher plants have higher-plant-specific functions, which is compatible with results from some reports, saying that TIPs are for example involved in seed specific processes (Gattolin et al., 2010a). Likewise, some NIP subgroups seem to be specific for higher plants (NIP2s), while some are found in mosses and higher plants (NIP3s), and yet others are moss specific. While some of the expansion/diversification seen in the subfamilies of higher plants is probably reflecting higher-plant-specific functions, it could also be that some of it is the result of a compensatory effect where subgroups in MIP subfamilies of higher plants have replaced the functions of moss specific subfamilies.

The complete MIP family has been identified in nine algal species, all belonging to the chlorophyte group (**paper IV**). Even though each species only contained a limited number of MIP isoforms (between one and five, on average 2.4) the algal MIPs were very diverse, with a total of seven different subfamilies (see Figure 17). Of these, only two (PIPs and GIPs) were recognized in land plants and one subfamily (MIPC) shows similarities to the "superaquaporin" group consisting of AQP11, AQP12 and SIPs (Morishita et al., 2004). Summarized, the presence of several different subfamilies

might not be very surprising, as several of these algae are very distantly related and the subfamilies might therefore just reflect the phylogeny of the species. But the presence of MIP subfamilies shared with land plants is surprising. Especially since these subfamilies only existed in one to two species of algae and none of these species contained all of these subfamilies (PIP, GIP and MIPC). What makes the results difficult to interpret is that none of the algal species is more closely related to land plants than any other (since they are all belonging to the same sister group of land plants). To explain the shared subfamilies by a shared evolutionary origin would therefore require a complex pattern of gene loss in the algae, and a more likely explanation might therefore be horizontal gene transfer (HGT). A possibility is that this HGT was virus mediated, since an algal virus has been found to contain a GIP type of MIP, closely related to the GIPs found in both algae and *P. patens* (Gazzarrini et al., 2006).



	MIP A	MIP B	MIP C	MIP D	MIP E	GIP	PIP	TIP	NIP	SIP	XIP	HIP	Tot. MIPs	Tot. Subfam.
C. rheinhardtii	-	-	-	**2**	-	-	-	-	-	-	-	-	**2**	**1**
V. carteri	-	-	-	**3**	-	-	-	-	-	-	-	-	**3**	**1**
C. C-169	**1**	-	-	**2**	-	**1**	**2**	-	-	-	-	-	**6**	**4**
C. NC64A	-	-	-	**1**	**3**	**1**	-	-	-	-	-	-	**4**	**3**
O. RCC809	-	**1**	-	-	**1**	-	-	-	-	-	-	-	**2**	**2**
O. lucimarinus	-	**1**	-	-	-	-	-	-	-	-	-	-	**1**	**1**
O. tauri	-	-	**1**	-	-	-	-	-	-	-	-	-	**1**	**1**
M. pusilla	-	-	**1**	-	-	-	-	-	-	-	-	-	**1**	**1**
M. RCC299	-	-	**1**	-	-	-	-	-	-	-	-	-	**1**	**1**
P. patens	-	-	-	-	-	**1**	**8**	**4**	**5**	**2**	**2**	**1**	**23**	**7**
A. thaliana	-	-	-	-	-	-	**13**	**10**	**9**	**3**	-	-	**35**	**4**
O. sativa	-	-	-	-	-	-	**11**	**10**	**10**	**2**	-	-	**33**	**4**

Figure 17. A table showing the size and diversity of the identified MIP families in nine chlorophyte algae, the moss *P. patens* and the higher plants Arabidopsis and Rice. The schematic tree shows how the species are related to each other (but not in scale) and numbers in table are number of isoforms except for last column which shows number of subfamilies.

Horizontal gene transfer might be an explanation for the diversity of plant MIPs and it was suggested already in 2002 that the NIP subfamily originated from a HGT of bacterial AqpZ (Zardoya et al., 2002). However, a later study found no support for this (Zardoya, 2005), but a HGT origin of NIPs cannot be rejected. In **paper III** we looked closer at the phylogeny of NIPs, and successfully identified several bacterial NIP homologs. These bacteria do not correspond to any taxonomic subgroup

and the sequences do not appear to form a stable monophyletic group. Still there is a relatively high support for this, among bacteria widely distributed, MIP subfamily sharing an ancestry with the NIP family (see Figure 18 and **paper III**). Another study has found XIP homologs in fungi (Gupta and Sankararamakrishnan, 2009), possibly indicating an origin involving HGT also for this plant MIP subfamily (see Figure 18).

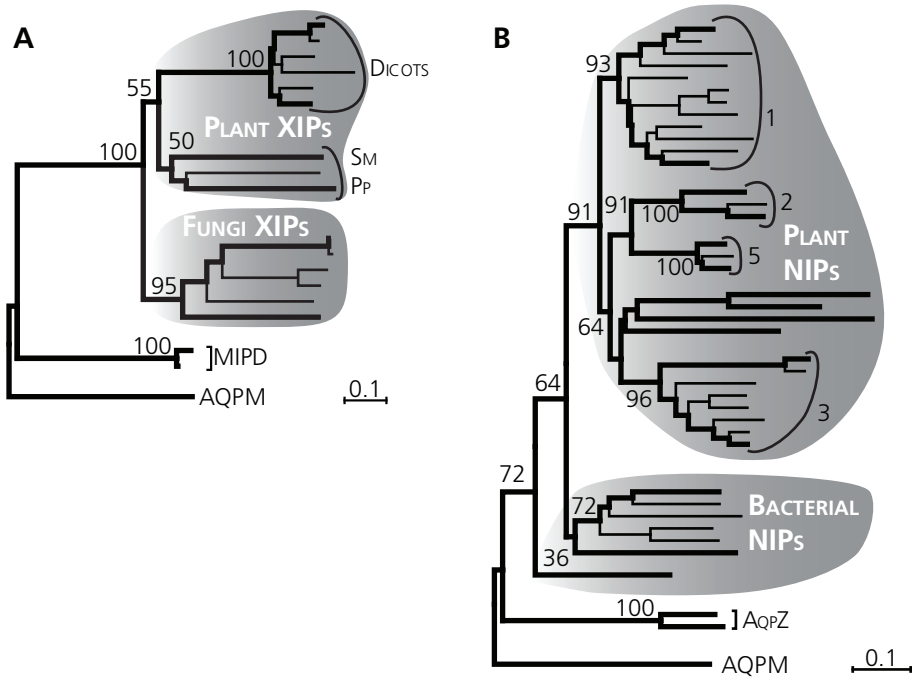


Figure 18. Trees showing possible shared ancestry of plant and non-plant MIPs. Tree A is a NJ tree of plant XIPs and fungi XIPs. MIPD was found to be the subfamily closest to plant XIPs and is included as an outgroup. Sm and Pp is indicating the group of XIPs from *Selaginella moellendorffii* and *Physcomitrella patens*. The tree is rooted with the Achaean AQPM. Tree B is a NJ tree of plant NIPs and bacterial NIPs. AqpZ was found to be the subfamily closest to NIPs in previous studies and was therefore included as an outgroup. The tree was rooted with AQPM. Bootstrap support from 1000 replicates are showed at the nodes of major groups.

4.4 Physiological Role

Despite a relatively intensive research on plant MIPs in the recent past, the physiological roles of the different plant MIP subfamilies and isoforms are to a large extent still unknown. The physiological functions that have been ascribed some plant MIPs are largely associated with non-water specific permeabilities of these MIP isoforms, such as the involvement of different NIPs in transport of the metalloids silicon and arsenite. However recent research have also identified other potential roles for MIPs. For example Secchi and Zwieniecki identified PIP1s as being important for xylem embolism repair in poplar (Secchi and Zwieniecki, 2010).

There have also been many reports on the general involvement of PIPs in plant water relations, but unfortunately these tend to be unspecific, looking at for instance root hydraulic conductivity or transpiration and to what degree it is blockable by mercury (which blocks some, but not all, MIPs, and also is likely to affect many other proteins). Recently a study was presented that showed a reduction in hydraulic conductivity in *At*PIP1;2 knock-out plants, showing that this specific isoform most likely is a key component in whole plant hydraulics (Postaire et al., 2010). However, the success of using knock-out plants in this research might be specifically linked to *At*PIP1;2 since it is one of the most highly expressed MIPs in Arabidopsis (Alexandersson et al., 2005). For other lowly expressed MIPs knock-out or knock-down experiments have been unable to reveal any phenotypes suggesting a physiological role.

Another alternative is to use naturally occurring genetic variants of a species (accessions) to see if differences in traits can be linked to MIP family differences. In **paper II** we compared the expressional regulation of MIPs during drought in five different accessions with known differences in water use efficiency but could only identify some minor differences. Very recently a study was published that, using a similar approach, looked at MIP responses to drought stress in two poplar accessions known to be drought avoidant (isohydric) and drought tolerant (anisohydric) (Almeida-Rodriguez et al., 2010), identifying some differences possibly linked to hydraulic and stomatal responses.

5 Conclusions and Future Perspectives

Around the turn of the millennium, life science experienced something close to a paradigm shift, as techniques producing large amounts of data (e.g. microarray based RNA detection, automated capillary electrophoresis based DNA sequencing, mass spectrometry based protein detection) made it possible to conduct more large scale high-throughput studies (often referred to as "omics" based, e.g. transcriptomics, proteomics, metabolomics and so on). Although people in general were thrilled about the promising new technologies there were also concerns raised over that they would result in for example a more descriptive kind of research, perhaps failing in asking the relevant biological questions. Now, a decade or so later, well on our way in the "omics"-era the use of these buzzwords, as well as the concerns raised over these techniques, have steadily declined. The techniques have partially shifted the focus of biological research from acquisition to analysis of data and from the reductionism of the traditional hypothesis-driven research to the holism of model building systems biology. Overall the fears have turned out to be unjustified and the massive amounts of publicly available data has turned out to be a valuable resource for the research community at large. In fact, this thesis would never have been possible without the "omics" revolution, as the research in this thesis to a large part is based on analysis of such freely available data.

The numerousness and diversity of the plant MIP family has been known for quite some time and is likely reflecting important roles in several physiological functions in the plant. However, the exact functions of different subfamilies and specific isoforms of MIPs have been difficult to assess and are therefore to a large extent still unknown. We looked at gene expression of water transporting PIPs during drought stress in five different genetic isolates (accessions) of *A. thaliana*. Even though the accessions differed in water use efficiency (WUE, biomass production per water used) we found the overall pattern of PIP expression to be similar in all of them, indicating that differences in PIP expression is not likely to be the cause for differences in WUE. By analyzing publicly available microarray data we found out that the PIPs most down-regulated during drought tend to covary also in other conditions, indicating that they might be part of the same transcriptional network.

By identifying the MIP family in the moss *P. patens* as well as in nine species of chlorophyte algae we have formed a framework for looking at the evolution of this family

in the green branch of life. With the data available we speculate that MIP subfamilies were numerous already in the first land plants and that some of these originate from horizontal gene transfers from other species, possibly chlorophyte algae, fungi and prokaryotes. In prokaryotes, around 80% of all genes show signs of such horizontal gene transfer indicating that this has been extremely common (Dagan et al., 2008). As a result of this it has been suggested that the common use of a tree to illustrate evolution might be better replaced by a web, the web of life. The involvement of horizontal gene transfers in the evolution of the plant MIP family is still only speculations, but it will be interesting to see how the MIP families of other plant species (especially charales) will fit into this as their sequences will become available.

And yes, the phrase in the last paragraph should be "as they become available" and not "if …" because as of today it is a question of "when" and not "if" the genome of a species will be sequenced. The number of nucleotide sequences deposited in GenBank has grown exponentially (see Figure 19) as the costs for sequencing a human genome has plummeted over the last couple of years, from about $10 million in 2007, $1million in 2008, $48 000 in 2009 to only $4 400 in 2010 (Drmanac et al., 2010). The price drop is due to introductions of new technologies for massive parallel sequencing (mostly based on pyrosequencing techniques and referred to as second-generation sequencing). Improvements in these techniques together with recently introduced third generation sequencing techniques (based on single-molecule detection, enabling faster and longer reads) make it likely that the "one thousand dollar genome" goal will not only be reached but surpassed in the near future (Munroe and Harris, 2010). Even though these techniques might not rock the life science community as much as the introduction of the "omics" techniques once did, they are likely to cause a lot of changes to the way life science research is done. One way would be that people are no longer restricted to the genomic sequences available from sequencing consortia, but can simply sequence their organism of interest. Another would be that researchers will no longer be restricted to working with model-species when working with gene expression, but rather can focus directly on the most biologically interesting species.

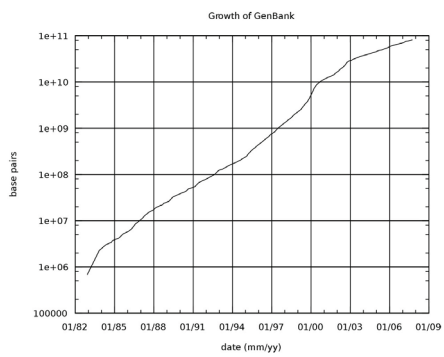


Figure 19. Graph showing the number of nucleotides deposited in GenBank over time. At the moment the database contains more than 1.17×10^{11} bases in sequences belonging to more than 250 000 organisms, with the amount of sequences doubling about every 18 months.

Populärvetenskaplig sammanfattning

Livets minsta beståndsdel, cellen, omges av ett membran som avgränsar den från omvärlden. För att hålla den inre miljön kontrollerad måste cellen kunna styra transporten av olika ämnen över membranet, vilket görs med hjälp av olika proteiner. Dessa proteiner kan antingen vara energikrävande pumpar och transportörer som aktivt förflyttar substanser över membranet, eller de kan vara passiva transportörer och kanaler som påverkar diffusionen av ämnen över membranet.

Ett exempel på det senare är de kanalbildande proteinerna MIPar (Major Intrinsic Proteins). Dessa proteiner identifierades tidigt på grund av att de var så rikligt förekommande i vissa membran (därav namnet) men först senare lyckades man visa deras funktion. En av de första MIParna vars funktion påvisades var en vattentransporterande kanal, varför detta protein och ibland hela gruppen av proteiner även kallas akvaporiner (AQPs). Detta var en mycket stor upptäckt, då det länge varit omdiskuterat huruvida vatten bara var beroende av passiv diffusion genom cellmembranet, eller om det behövdes proteiner för transporten. Upptäckten skulle leda till ett ökat intresse kring MIPar och resulterade även i att Peter Agre, som ledde den grupp av forskare som gjorde de första upptäckterna kring vattentransport i MIPar, tilldelades nobelpriset i kemi 2003.

Sedan proteinfamiljen upptäcktes i slutet av 1980-talet har intensiva studier bedrivits i syfte att kartlägga och karaktärisera olika varianter (isoformer) av MIPar och det har visat sig att MIPar finns i de allra flesta levande organismer och att de inte enbart transporterar vatten, utan även en stor mängd andra ämnen, såsom ammoniak, glycerol, arsenik och urea. Dock kvarstår många frågetecken kring de olika isoformernas lokalisering, specifika funktion, struktur och reglering.

I växter bildar MIPar en stor och omfångsrik familj, med nästan tre gånger så många isoformer som det finns i däggdjur (35 respektive 13). Anledningen till denna omfångsrikedom är i mångt och mycket fortfarande oklar, men det har spekulerats i att den har en grund i växters speciella behov av reglering av transport i allmänhet och vattentransport i synnerhet. Detta behov har sin grund i att växter, till skillnad från djur, inte kan förflytta sig utan måste anpassa sig till förändringar i den miljö de lever i. Det ligger därför nära till hands att tänka sig att en ökad möjlighet att reglera t.ex. vattenflöden under perioder av torka, skulle vara viktigt för växter. Tyvärr har traditionella metoder där man slår ut enskilda MIP-gener (och därmed proteinerna) till stor del misslyckats med att identifiera funktioner hos enskilda isoformer, troligtvis på grund av överlappande funktion hos isoformerna.

Ett annat sätt att få ökad förståelse och kunskap kring MIPar i växter är genom att studera MIP-familjen i olika växter och jämföra dessa. Till exempel har vi i **Paper II** använt oss av den biologiska variationen inom samma art (s.k. ekotypvariation) för att studera huruvida variationen i effektiviteten av vattenanvändande kan förklaras av skillnader i reglering av MIP-gener. I **Paper I** och **IV** har vi istället valt att titta på MIP-familjen i mossa respektive alger för att se hur familjen har utvecklats i växter. Vi har visat att redan mossa, som är en av de mest primitiva landväxterna, har en MIP-familj som är mycket varierad och relativt stor (om än något mindre än den i högre växter). Vidare har de nio arter av alger vi undersökt förvisso endast ett fåtal MIPar per art, men variationen är även här oväntat stor. Intressant nog hittade vi även grupper av MIPar i alger som också återfinns i mossa. Dessa återfanns endast i ett fåtal algarter och detta, tillsammans med det faktum att alla de undersökta algerna är lika avlägset släkt med mossa, tyder på att dessa ”delade” MIP-varianter kan ha överförts med så kallad horisontell genöverföring och alltså inte är nedärvda från en gemensam ursprunglig organism. Att även andra grupper av växt MIPar skulle ha ett ursprung från horisontell genöverföring stöds även av att MIP-gener som visar släktskap med vissa växt-MIPar har identifierats från såväl bakterier (**Paper III**) och svampar.

Det finns redan nu resultat som tyder på att ett modifierat uttryck av MIPar kan påverka såväl växters tålighet och produktion under torkstress, som växters resistens mot arsenik och bor. En ökad kunskap om växt-MIPar kommer på sikt att leda till förståelse kring hur dessa proteiner påverkar transportprocesser i växter. Kunskap som till exempel kan vara avgörande för att på ett effektivt och hållbart sätt kunna möta det behov av en ökad matproduktion som världen ställs inför till följd av en stigande befolkningsmängd.

Acknowledgements

I would like to express my sincere gratitude to all the people who in various ways contributed to the making of this work. Especially I would like to thank:

My supervisor Urban Johanson for the support and advice provided throughout my PhD. Your genuine interest in and enthusiasm for science and plant biology have been inspiring and I am sure I will miss those talks we used to have.

My co-supervisor Per Kjellbom for sharing his great knowledge on Aquaporins and biochemistry in general and for making us feel like a group.

The rest of the Aquaporin group: Kristina Nordén, Maria Fellert, Andreas Kirscht, Sabeen Survery, Hanna Anderberg, Anna Assarsson, Anders Barfod, Lavanya Moparthi, Michael Rützler and Jan Kvassman.

Erik Alexandersson, former group member who helped me during my first struggling time.

Folke Tjerneld and Lisa Thelin for all those music-nerd-talks, sharing records and giving concert tips.

Sinan for being my friend, not only at work.

The innebandy and badminton crew for all the fun.

All the other people at CMPS, making it a nice place to work.

The old friends from Göteborg. I miss you all, and the city, but not the weather.

My brother and sister. I know I can always count on you even though I guess you will always consider me your kid brother.

Mom, for always being supportive and believing in me.

Dad, how I wish you were here.

Finally I would like to thank Helena, for everything.

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