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Physiological and cell biological characterisation of two novel membrane proteins in *Arabidopsis thaliana*

by

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Thesis submitted in fulfillment of the requirements for the Degree of Master of Science (by research)

Institute of Molecular, Cell and Systems Biology
School of Life Sciences
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Abstract

This thesis describes the characterisation of AtPQL4 and AtPQL6, two members of a hitherto uncharacterized gene family of A. thaliana with six members, AtPQL1-6. As their counterparts in other species and kingdoms AtPQL proteins contain seven transmembrane domains and two copies of the so-called ‘PQ-loop’ domain. AtPQL4 and AtPQL6 show high amino acid sequence identity between each other and they are the closest A. thaliana homologues of the mammalian LEC35/MPDU1 protein, which has been shown to be required for all types of Man-P-Dol dependent glycosylation in the ER.

To address the functional homology between the two AtPQL proteins and MPDU1, the sub-cellular localisation of AtPQL4-GFP and AtPQL6-GFP fusion proteins was investigated. Confocal laser scanning microscopy analysis of Nicotiana tabacum leaf cells expressing AtPQL4-GFP or AtPQL6-GFP showed fluorescence patterns typical for ER. ER localisation of AtPQL4 and AtPQL6 was further confirmed by co-expression with the ER marker, YFP-HDEL.

A second set of experiments employed YFP-fusion proteins of AtSYP121, a plasma membrane SNARE protein, and AtTIP2, a tonoplast aquaporin. Confocal microscopy confirmed plasma membrane/tonoplast localisation of the YFP proteins when expressed on their own in tobacco leaf cells. However, both proteins were found to be retained in the ER when co-expressed with AtPQL4-GFP or AtPQL6-GFP fusion proteins. These new findings point to a role of AtPQL4 and AtPQL6 in protein processing in the ER thereby enforcing previous results from microarray experiments indicating ER-stress in AtPQL4 and AtPQL6 mutants.

Finally, a number of AtPQL4 and AtPQL6 knockout and AtPQL6 overexpressor lines were tested under a variety of environmental stresses to investigate the function of the two AtPQLs at whole-plant level. Low sucrose conditions resulted in growth inhibition of mutants compared to wild type plants. Considering previous findings that (a) AtPQL4 and AtPQL6 are localised in the ER (b) ATPQL mutants show differential expression of genes involved in the unfolded protein response (UPR) and (c) over-expression of AtPQL4 and AtPQL6 impacts on the targeting of other proteins, the observed phenotype could be linked to the unfolded protein response and autophagy that occurs during sugar starvation.
In conclusion, it is proposed that *AtPQL4* and *AtPQL6* proteins function in retaining membrane proteins for sufficient time in the ER to allow ER-quality control and related processes to take place. Further experiments to investigate such function are discussed.
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Author’s declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

[Signature]

Maria Papanatsiou, April 2011
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### Abbreviations

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<td>Uridine diphosphate-mannose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGGT</td>
<td>UDP-glucose:glycoprotein glucosyltransferase</td>
<td></td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td></td>
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<tr>
<td>UTR</td>
<td>Untranslated Region</td>
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Chapter 1: General introduction

A novel family of *Arabidopsis thaliana*, named “PQ-loop repeat” (PQL) was characterised by Richard Pattison (PhD thesis, 2008, University of Glasgow). The *AtPQL* gene family consists of six members, *AtPQL1*-6, and is characterised by the presence of the PQ-loop domain. The objective of this project was the functional characterisation of two of the *AtPQL* proteins, *AtPQL4* and *AtPQL6*. In this chapter I will describe what is known for the *AtPQL* family from the work carried out by Pattison (2008). In addition, I will review existing literature on yeast and mammalian PQL proteins that show high sequence identity and/or potential functional similarity with *AtPQL* proteins, especially with *AtPQL4* and *AtPQL6*.

1.1 Structural features of *AtPQL* proteins

Six genes encoding *AtPQL* proteins are present in the *Arabidopsis* genome, *AtPQL1* (At4G20100), *AtPQL2* (At2g41050), *AtPQL3* (At4g36850), *AtPQL4* (At5g59470), *AtPQL5* (At5g40670) and *AtPQL6* (At4g07390). The *AtPQL* proteins have two copies of the defining PQ-loop domain. The PQ-loop domain is a region of 40-60 amino acids including the highly conserved proline-glutamine (PQ) motif. Hydropathy analysis of each *AtPQL* protein showed that they are integral membrane proteins with seven transmembrane domains and a N-terminus facing the non-cytoplasmic side of the membrane. The seven transmembrane domains are a common characteristic of all PQL proteins identified so far (Hardwick and Pelham, 1990; Anand *et al*., 2001; Chung *et al*., 2001; Kalatzis *et al*., 2001). In addition, hydropathy analysis showed that the PQ-loop domain usually spans two transmembrane domains and consists of two hydrophobic regions surrounding a hydrophilic core. The significance of PQ-loop is not yet clear, but it is suggested to constitute a way of forming the transmembrane domains of proteins. All *AtPQL* proteins contain a conserved PQ motif at position 22-23 of the PQ-loop domain. It was found that the PQ motif is close to the transition region of the transmembrane and non-transmembrane domain. It might facilitate the change in direction of the polypeptide chain, as proline has the ability to bind the amino acid chain twice (Betts and Russell, 2003). Nevertheless, mutations in residues within the PQ-loop have suggested an additional role for the PQ-loop (discussed below). The alignment of full-length *AtPQL* amino acid sequences with other known non-plant PQL proteins led to the separation of *AtPQL* proteins into three sub-groups. Figure 1.2 represents a phylogenetic tree showing the
relationship between the *AtPQL* and PQL proteins from other species and the domain organization of each of the proteins. Sub-group 1 contains *AtPQL1*, *AtPQL2* and *AtPQL3*, sub-group 2 contains *AtPQL4* and *AtPQL6* and sub-group 3 is defined by *AtPQL5*. The *AtPQL* genes are found throughout the genome, with *AtPQL2* being on chromosome 2, *AtPQL1*, *AtPQL3* and *AtPQL6* on chromosome 4 and *AtPQL4* and *AtPQL5* on chromosome 5. The *AtPQL* genes also differ in intron-exon organisation, *AtPQL1* contains no introns while its closest homologues *AtPQL2* and *AtPQL3* have 11 and 10 introns, respectively.

![Predicted topology of PQL proteins.](image)

**Figure 1.1:** Predicted topology of PQL proteins.

PQL proteins are integral membrane proteins. They contain seven transmembrane domains and two PQ-loop domains. The PQ-loop domains typically span two transmembrane domains with the conserved PQ situated at the interface between the cytoplasmic loop and the second transmembrane domain.
Figure 1.2: Classification of AtPQL proteins in three different subgroups.

A) Phylogenetic tree shows the relationship between the AtPQL1-6 proteins and PQL proteins characterised in other eukaryotes. The tree was created from full-length sequence alignments of selected PQL proteins. Node numbers indicate bootstrap values. B) The distribution of the domains for each PQL protein and number of amino acids is also shown. Transmembrane domains are shown in red color, while PQ-loop domains are shown blue color (Figure taken from Pattison, 2008).

1.2 AtPQL1, AtPQL2, AtPQL3 and STM1

The sub-group 1 proteins AtPQL1-3 show high sequence identity with a PQL protein, STM1, from the fission yeast *Schizosaccharomyces pombe*. STM1 has been proposed to act as a G-protein coupled receptor (GPCR) and was implicated in the control of cell cycle under nitrogen-deficient conditions. It is suggested that nitrogenous signals may bind to STM1 resulting in its activation through structural changes and therefore facilitating its interaction with heterotrimeric protein Gpa2. Gpa2 is involved in sexual differentiation in response to poor nutritional conditions (Chung *et al.*, 2001). This interaction occurs through the C-terminal region of STM1 that contains a putative Gpa2 binding site of 7-8 residues within the second PQ-loop. Site-directed mutagenesis in the binding site revealed a lysine residue at position 199 to be important for the function of STM1. A Gpa2 binding site is absent in the AtPQL1-3 proteins.

Transcriptional profiling by Pattison (2008) showed that *AtPQL1* and *AtPQL2* have similar expression patterns and were strongly expressed in floral tissues. *AtPQL2* was also found
in rosette leaves and roots, though in much lower amounts. In contrast, \textit{AtPQL3} showed higher mRNA levels than \textit{AtPQL1} and \textit{AtPQL2}, with highest expression in root tissues. Despite the overlapping expression profiles of \textit{AtPQL1} and \textit{AtPQL2}, both genes exhibited temporal differences of expression. \textit{AtPQL1} showed peak expression in developing pollen whereas \textit{AtPQL2} was more strongly expressed in mature pollen. Transient expression of \textit{AtPQL1-GFP} fusion protein in epidermal cells of \textit{Nicotiana tabacum} indicated localisation to the tonoplast. No co-localisation data were obtained for \textit{AtPQL2} and \textit{AtPQL3} proteins.

Pattison (2008) argued that a tonoplast localisation might not exclude a GPCR function of \textit{AtPQL1} as its N-terminus faces the vacuolar lumen where it could interact with the only identified \textit{Arabidopsis} Ga subunit, GPA1. Such interaction could take place at points where the tonoplast and plasma membrane are in close contact. Huang \textit{et al}. (2006) reported interaction of GPA1 with THF1, a plastid localised protein, at points where the plastid membrane is in very close proximity to the plasma membrane. GPA1 is expressed throughout development in almost all tissues, with the exception of mature seeds and mature pollen (Weiss \textit{et al}.., 1993). It is therefore unlikely that \textit{AtPQL2}, which is highly expressed in mature pollen, interacts with GPA1. Single knockout lines of \textit{AtPQL1} and \textit{AtPQL3} and overexpressors of \textit{AtPQL1}, \textit{AtPQL2} and \textit{AtPQL3} did not show a phenotype different from wild type (Col0) when grown on soil or in different nutrient conditions (Pattison, 2008).

### 1.3 \textit{AtPQL5} and cystinosin

Human cystinosin (\textit{CTNS}) is the closest homologue of \textit{AtPQL5} with 29.9\% sequence identity at the amino acid level. CTNS is the only member of the PQL protein for which a function has been established at the molecular level. CTNS is a human H\textsuperscript{+}-driven transporter, mediating the efflux of cystine from lysosomes (Kalatzis \textit{et al}.., 2001). Cystine is the disulfide form of the amino acid cysteine (cys) and is a by-product of lysosomal protein hydrolysis. Cystine is exported into the cytosol, where it is reduced to cys (Gahl \textit{et al}.., 2002). Mutations in \textit{CTNS} result in cystinosis, an inherited lysosomal storage disease. Defective cystine efflux leads to cystine accumulation inside lysosomes where reduction to cys cannot take place (Kalatzis \textit{et al}.., 2004). CTNS is one of the PQL proteins that is affected in its function by mutations in the PQ-loop. 16 different point mutations leading to clinical symptoms have been shown to fall within the two PQ-loops of CTNS (Attard \textit{et al}.., 1999; Kalatzis \textit{et al}.., 2001; Kalatzis \textit{et al}.., 2004). Most of these mutations affected the cys transport activity of CTNS and not its localisation.
Based on expression profiling (Pattison, 2008) AtPQL5 appears to be the most strongly expressed member of the AtPQL family. Expression was apparent in all tissues, with highest transcript levels occurring in open flowers. In addition, AtPQL5 showed diurnal changes in expression, as transcript level was highest in the middle of the day, in both short and long days. This finding might argue against a function of AtPQL5 in cys production, as other genes involved in the cys production are more expressed at the end of the night to provide sufficient cys for sulfur metabolism in the morning (Saito et al., 2004). Similar to what was observed for the subgroup 1 PQL proteins, AtPQL5 was highly expressed in pollen and exhibited strong changes in expression level during pollen development. Sub-cellular localisation experiments suggested that AtPQL5 also localises to the tonoplast (Pattison, 2008). This is in agreement with a similar function to CTNS, which is localized to the lysosome. Lysosomes are small organelles containing a plethora of hydrolytic enzymes and they are responsible for the degradation macromolecules derived from extracellular environment through endocytosis or from the cytosol through autophagy (Sun-Wada et al., 2003). In plants, the vast majority of hydrolytic activity resides inside the vacuoles. However, vacuoles have additional functions such as storage of ions and various metabolites, control of osmotic pressure and detoxification (Marty, 1999). Given the localisation of AtPQL5, a CTNS-like function of AtPQL5 could be possible. In plants, the reduction of cystine to cys is essential, as cys is a key intermediate for sulfur metabolism (Saito et al., 2004). However, AtPQL5 knockout mutants did not show a phenotype when grown in low sulphur conditions (Pattison, 2008). The ers1Δ mutant disrupted in the yeast PQL gene ERS1, is sensitive to hygromycin (Gao et al., 2005). Expression of CTNS in the ers1Δ mutant resulted in increased hygromycin tolerance. However, functional complementation was not observed for any of the AtPQL proteins expressed in ers1Δ, suggesting either a function different from CTNS or a loss of function due to heterologous expression.

1.4 AtPQL4, AtPQL6 and MPDU1

An alignment of AtPQL4 and AtPQL6 full amino acid sequence showed 74% identity between the two proteins. AtPQL4 and AtPQL6 genes displayed similar expression patterns with little variation in transcript level between different tissues (Pattison, 2008). AtPQL4 was more strongly expressed in open flowers, while AtPQL6 showed peak expression in floral buds. In addition, AtPQL4 and AtPQL6 genes showed diurnal changes
in expression. In both short and long days, the transcript levels were increased throughout
the course of the day and reached a maximum at the end of the day. Experiments using
green fluorescent protein (GFP)-tagged *AtPQL4* and *AtPQL6* suggested localisation to the
endoplasmic reticulum (ER), as GFP fluorescence was observed in net-like structures
(Pattison, 2008).

Transcriptional profiling of mutant lines for *AtPQL4* and *AtPQL6* genes as well as an
overexpressor line for *AtPQL6* gene showed differential expression of genes in the mutant
lines compared to wild type. The differentially expressed genes could be classified into
three main groups. The first group includes pathogenesis related (*PR*) genes and genes
involved in cell-wall modification, such as lipid transfer proteins (LTPs), xyloglucan
endotransglycosylase (XET) and glycosylphosphatidylinositol (GPI) anchored proteins. *PR*
genes are induced in response to pathogen infection and encode proteins with various
functions (Van Loon *et al*., 2006). *PR-1* was up-regulated in both knockout and
overexpressor lines for *AtPQL6*. *PR-2* was up-regulated in all knockout lines, while down-
regulated in the *AtPQL6* overexpressor line. Opposite changes in knockout and
overexpressor lines were also observed for several LTPs the cell-wall modification
associated gene *XTR7*. Members of the COBRA family were up-regulated in *AtPQL6*
knockout plants. All 12 members of the COBRA family encode GPI anchored proteins
with a regulatory role in the deposition of cellulose microfibrils thus controlling the
direction of cell wall expansion (Roudier *et al*., 2002). The second group of differentially
expressed genes consists of lipid metabolism and ER stress-related genes. Several genes
involved in lipid metabolism were differentially regulated in *AtPQL4* and *AtPQL6*
mutants. The most interesting observation was the down-regulation in the *AtPQL4*
knockout of a *DPS*-like gene, which encodes for a dehydrodolichol pyrophosphate (dedol-
P-P) synthase that is responsible for the synthesis of dedol-P-P (Cunillera *et al*., 2000).
Dedol-P-P is a precursor molecule of dolichol-pyrophosphate (Dol-P-P), which is the lipid
acceptor of sugars in the synthesis of lipid-linked oligosaccharides (LLO) during N-
glycosylation in the ER. ER stress results from accumulation of unfolded proteins in the
ER and is accompanied by increased expression of a number of genes implicated in the
unfolded protein response (UPR) (Liu and Howell, 2010), such as the binding protein
(*BiP*), the protein disulfide isomerase (*PDI*), calnexin (*CNX*) and calreticulin (*CRT*). *BiP3*,
one of the three *A. thaliana BiP* genes, was up-regulated in both *AtPQL6* knockout and
overexpressing lines. The *UTr1* gene was also up-regulated in the *AtPQL6* overexpressing
line. *UTr1* is a UDP-glucose transporter that supplies glucose to the CNX/CRT cycle for
the making of incorrectly folded proteins (Reyes *et al*., 2009). The third group contains
genes encoding N-glycoproteins and proteins targeted to the secretory pathway. Such proteins were over-represented among the differentially expressed genes in the \textit{AtPQL} knockout lines, and under-represented in the \textit{AtPQL6} overexpressor line.

Full-length alignments of amino acid sequence showed 29.3\% and 30.4\% identity of \textit{AtPQL4} and \textit{AtPQL6} respectively with the mannose-phosphate-dolichol utilisation 1 (\textit{MPDU1}) gene from humans. The three proteins show similar organisation of the seven transmembrane and the PQ-loop domains. LEC35/MPDU1 has been shown to be required for the utilization of dolichol-phosphate (Dol-P) linked sugars in different types of glycosylation processes (Anand \textit{et al}., 2001, see below). Mutations in \textit{MPDU1} and similar genes cause congenital disorders of glycolsylation (Kranz \textit{et al}., 2001; Schenk \textit{et al}., 2001; Koiwa \textit{et al}., 2003).

\subsection*{1.5 Synthesis of Dol-P linked sugars}

Glycosylation is a post-translational modification of proteins that is highly conserved in all eukaryotes. There are five different types of glycosylation, N-glycosylation, O-glycosylation, P-glycosylation, C-mannosylation and GPI anchoring, differing in the sugar-amino acid bond formed, all of which are initiated in the ER and Golgi. The first steps of N-glycosylation, C-mannosylation and GPI in the ER depend on mannose-phosphate-dolichol (Man-P-Dol) and glucose-phosphate-dolichol (Glc-P-Dol) (Spiro \textit{et al}., 2002). In addition, a special type of O-glycosylation, known as O-mannosylation, is dependent on Man-P-Dol. In this chapter I will describe only those types of glycosylation that are dependent on Man-P-Dol.

Dolichols are long-chain polyisoprenoid lipids comprising 15 to 23 isoprenic units depending on the organism. Biosynthesis of dolichols involves formation of geranyl (GPP) and farnesyl (FPP) diphosphates through the mevalonate pathway (Swiezewskaa and Danikiewicz, 2005). Elongation of the polyisoprenoids is catalyzed by \textit{cis}-prenyltransferases that add isopentenyl diphosphates (IPPs) onto FPP. The \textit{lew1} mutant of \textit{A. thaliana} is defective in a gene encoding a \textit{cis}-prenyltransferase. \textit{lew1} plants show a hypersensitive response to tunicamycin and have altered levels of glycosylation (Zhang \textit{et al}., 2008). Newly synthesized Dol-P is available for synthesis of Man-P-Dol and Glc-P-Dol that are required for glycosylation. This process takes place on the cytoplasmic side of the ER and is mediated by Dol-P- mannosyltransferase and Dol-P-glusosyltransferase.
using the nucleotide sugar donors, UDP-Man and UDP-Glc, respectively (Helenius et al., 2002). Both Man-P-Dol and Glc-P-Dol require re-orientation in the ER membrane to be available for glycosylation-associated enzymes residing in ER lumen. Experiments using water-soluble analogues of Man-P-Dol and Glc-P-Dol suggested that translocation into ER sealed vesicles requires the involvement of so-called flippases (Schenk et al., 2001a). However, this remains to be identified.

1.6 Protein glycosylation in the ER

N-glycosylation of proteins is catalysed by the oligosaccharyltransferase (OST) complex in the ER lumen, where the LLO synthesized in the ER membrane is transferred to asparagine (Asn) residues of a strict consensus sequence Asn-X-Ser/Thr, with X being every amino acid except proline (Spiro et al., 2002, Koiwa et al., 2003). Several mutants defective in this process have been identified in plants. For instance, knockout of STT3a but not STT3b results in a salt sensitive phenotype in A. thaliana (Koiwa et al., 2003). The genes are homologues of yeast STT3 subunit of OST enzyme. Although single mutants are viable, double mutants of stt3a/stt3b show a gametophytic lethal phenotype. It was suggested that the two genes are redundant. At least the STT3a is involved in glycosylation, as stt3a mutants display decreased levels of glycosylation of proteins (Koiwa et al., 2003).

A schematic representation of all the steps of the LLO biosynthesis along with the known mutations in A. thaliana and chemical inhibitors affecting this process is displayed in Figure 1.3 from Pattison and Amtmann, 2009. Biosynthesis of LLO requires the sequential attachment of sugar residues to Dol-P and is topologically split across the ER membrane. The first seven reactions take place on the cytoplasmic side of ER, whereas the remaining seven occur in the ER lumen. First, glycosyltransferases catalyse the addition of two N-acetylglucosamine (GlcNAc) and five mannose (Man) residues to Dol-P using the nucleotide sugar donors, UDP-GlcNAc and GDP-Man. Subsequently, the Man5GlcNAc2-P-Dol intermediate must be flipped across the ER membrane for elongation and completion of LLO. Man5GlcNAc2-P-Dol is flipped to the luminal side by an ATP-independent flippase (Rush and Waechter, 1995). The RFT1 protein of Saccharomyces cerevisiae was shown to be required for this translocation process (Helenius et al., 2002). The yeast strain alg11 is deficient in GDP-Man-dependent mannosyltransferase, which is required for the synthesis of Man3GlcNAc2-P-Dol, and shows accumulation of Man3GlcNAc2-P-Dol on the cytoplasmic side of the ER (Cipollo et al., 2001).
Overexpression of RFT1 in alg11 cells caused the accumulation of Man7GlcNAc2-P-Dol in the ER lumen suggesting that RFT1 is a potential candidate for an ER membrane flippase (Helenius et al., 2002). Once the Man5GlcNAc2-P-Dol intermediate is flipped to the luminal side of the ER, a further four mannose and three glucose (Glc) residues are added to make the complete LLO, Glc3Man9GlcNAc2-P-Dol. Sugar donors for these reactions are Man-P-Dol and Glc-P-Dol. The complete oligosaccharide is transferred from dolichol to proteins by the OST complex and further modified as part of the CNX/CRT cycle (see 1.8 section). Additional modifications of the glycan take place in post-ER compartments such as the Golgi apparatus (Strasser et al., 2006).

Although many genes are involved in N-glycosylation, few mutants have been isolated in plants that would allow understanding of the molecular significance of the individual proteins taking part in this process. However, the use of chemical inhibitors such as tunicamycin has given a great insight into the process of N-glycosylation. Tunicamycin is a specific inhibitor of UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-P transferase (GPT), the enzyme that catalyses the first glycosyltransferase reaction during LLO synthesis. Tunicamycin treatment is lethal for all eukaryotes, including plants (Zeng and Elbein, 1995). Sub-lethal doses of tunicamycin induce the expression of ER stress-related genes, particularly those involved in UPR. The first loss-of-function mutant of N-glycosylation identified in A. thaliana was asparagine-linked glycosylation 3-2 (alg3-2). The ALG3 gene encodes for α1-3-mannosyltransferase, the enzyme responsible for the addition of the first Man residue after translocation of the LLO to the ER luminal face. Although alg3-2 mutants show an enhanced UPR, they do not show any physiological phenotype, suggesting that the latter steps in N-glycosylation are not vital (Henquet et al., 2008). Treatment of Cucumis sativus (cucumber) with sub-lethal amounts of tunicamycin resulted in an increased expression of PR proteins, increased levels of salicylic acid and increased systemic acquired resistance (SAR) against the fungal pathogen, Colletotrichum lagenarium (Sticher and Metraux, 2000), pointing to a close relationship between N-glycosylation and defence responses. The effect of AtPQL4 and AtPQL6 mutation on both UPR and defence related genes is therefore in agreement with a possible function in N-glycosylation as suggested by its homology to MPDU1 (see 1.4 section).

O-glycosylation comprises the linkage of glycans to amino acids containing a hydroxyl functional group like serine, threonine, tyrosine, hydroxyproline and hydroxylysine (Spiro et al., 2002). O-glycans are highly variable in structure as each sugar residue has 3 or 4
attachment sites for other sugar residues and they can also adopt different conformational states. In addition, O-glycans can be long polysaccharide chains, namely glycosaminoglycans (GAGs) that consist of multiple disaccharide repeats such as N-acetylgalactosamine (GalNAc) or GlcNAc linked with glucuronic acid (GlcA) or galactose (Gal) residues (Wopereis et al., 2006). Few O-glycans contain Man residues and therefore require Man-P-Dol. This special form of O-glycosylation, named O-mannosylation, involves the attachment of Man residues to either serine or threonine residue of proteins (Spiro et al., 2002). To date few O-glycosylated proteins have been identified. Most of them are derived from S. cerevisiae and the best described being a mammalian skeletal muscle extracellular matrix protein, the α-dystroglycan (Worepeis et al., 2006).

C-mannosylation is another type of protein modification that occurs in the lumenal ER and involves the carbon-carbon linkage of a Man residue to the C-2 of the first tryptophan (Trp) residue of a consensus sequence Trp-x-x-Trp. (Spiro et al., 2002). The Man residue is derived from Man-P-Dol, as lec15 mutants, deficient in Man-P-Dol synthase activity, show a decrease in C-mannosylation (Doucey et al., 1998). Few proteins containing C-mannosylation sites have been identified in mammals, with the most well described being the ribonuclease2 (RNase2) protein (Krieg et al., 1997, Doucey et al., 1998).

GPI anchoring is an additional type of glycosylation that enables tethering of proteins to the plasma membrane and is found in all eukaryotes. The first two steps of GPI synthesis occur at the cytoplasmic side of the ER. They involve the attachment of a GlcNAc residue to phosphatidylinositol (PI) and the subsequent deacetylation and acylation of the inositol ring of PI to produce the GlcN-acyl-PI intermediate. Re-orientation of the GlcN-acyl-PI in the ER membrane facilitates the attachment of additional Man residues derived from Man-P-Dol sugar donors. Further species-specific modifications take place on the luminal side of ER. A transamidase transfers the mature GPI to nascent proteins with a GPI-attachment site at carboxyl terminus (Kinoshita and Inoue, 2000).
Figure 1.3: Lipid linked oligosaccharide biosynthesis in the ER.

N-glycosylation starts at the cytoplasmic side of ER membrane with (a) the attachment of two GlcNAc and five Man residues from nucleotide sugar donors to Dol-P generating the Man$_5$GlcNAc$_2$-P-Dol intermediate. (b) This intermediate is subsequently flipped across ER membrane and further four Man and 3 Glc residues are added to form the Glc$_3$Man$_9$GlcNAc$_2$-P-Dol. The Man and Glc residues are derived from Man-P-Dol and Glc-P-Dol sugar donors. (d) The complete glycan is transferred to protein as they are co-translationally translocated into the ER lumen. *A. thaliana* mutants and chemical inhibitors of individual steps are shown in red color. (Figure is taken from Pattison and Amtmann, 2009)
1.7 LEC35/MPDU1 and Man-P-Dol usage in plants

The MPDU1 protein, also known as LEC35, is implicated in all types of Man-P-Dol dependent glycosylation. The LEC35 gene was isolated from Chinese hamster ovary (CHO) cells through a screen using concanavalin (ConA), which binds high-mannose oligosaccharides (Kornfeld and Kornfeld, 1985), in combination with castanospermine (Cs) and swainsonine (Sw) that inhibit the Golgi-localised N-linked glycan processing enzymes. Cs inhibits glucosidase I and II whereas Sw inhibits mannosidase II (Elbein, 1987; Tulsiani et al., 1982). lec35 mutants showed increased tolerance to both glycosylation inhibitors Cs and Sw suggesting that they could produce complex glycans via an alternative pathway (Lehrmann and Zeng, 1989). The authors suggested that lec35 mutants were defective in LLO composition and therefore by-passed the necessary activity of the three enzymes to produce complex glycans. In addition, high-pressure liquid chromatography (HPLC) analysis of LLOs incubated with GDP-[3H]-Man showed an accumulation of Man$_5$GlcNAc$_2$-P-P-Dol at the expense of the mature LLO, Glc$_3$Man$_3$GlcNAc$_2$-P-P-Dol (Lehrmann and Zheng, 1989). The accumulation of this LLO intermediate was not the consequence of a defective enzyme involved in Man-P-Dol synthesis, as Man-P-Dol synthetase and transferase activity was the same in mutant and wild type CHO-K1 cells (Anand et al., 2001). lec35 mutants were also affected in glucose-dolichol-phosphate (GDP)-dependent LLO synthesis. Lack of glucosylated LLOs was not due to presence of incomplete mannosylation of LLOs, as in lec15 mutants, which are defective in Man-P-Dol synthetase, glucosylation of the Man$_3$GlcNAc$_2$-P-P-Dol intermediate takes place producing Glc$_3$Man$_3$GlcNAc$_2$-P-P-Dol instead of Glc$_3$Man$_3$GlcNAc$_2$-P-P-Dol. Anand et al. (2001) also reported a defect of C-mannosylation of tryptophan in lec35 mutants. lec35 cells expressing the C-mannosylated RNase 2.4 exhibited a 7.4-fold decrease in mannosylation compared to wild type CHO-K1 cells. Moreover, mutations in the LEC35 gene resulted in a block of mannosylation of GPI during GPI synthesis. lec35 mutants accumulate GlcN-acyl-PI that suggests an arrest in elongation of GPI at the first mannosylation step in the ER lumen (Camp et al., 1993). From the combined results, Anand et al. (2001) concluded that MPDU1/LEC35 has a role in utilisation of Man-P-Dol, but its exact mode of function has not yet been identified.

Man-P-Dol and Glc-P-Dol mediated glycosylation is essential for plant viability, as several mutants deficient in such processes resulted in gametic or embryonic lethality. However, the severity of the phenotype depends on the stage and location of glycosylation. As mentioned above, mutations occurring in later stages of N-glycosylation in the ER are not
lethal while those occurring in the first step of this process are. In addition, mutations that affect N-glycosylation in the Golgi are less severe than those occurring in the ER (Strasser et al., 2006). A possible explanation for the lethality associated with the inhibition of N-glycosylation is the saturation of chaperones and folding catalysts in the ER lumen that causes ER dysfunction due to inability of such proteins to respond to ER stress (Vitale and Ceriotti, 2004). Although it was speculated that *At*PQL4 and *At*PQL6 might have a potential role in N-glycosylation in the ER, no gametic and embryonic lethality was observed (Pattison, 2008).

### 1.8 ER Quality Control (ER QC)

Many proteins have to enter the secretory pathway in order to be secreted or targeted to a specific sub-cellular location. Such proteins are termed secretory proteins and can be secreted proteins or proteins residing in the plasma membrane or in the lumen of different organelles such as vacuoles (Sanderfoot and Raikhel, 2003). The secretory pathway, alternatively known as anterograde pathway, begins with the co-translational translocation of a protein into the ER, followed by its flow to the Golgi and subsequent sorting to its final destination (Bassham et al., 2008). The newly synthesized secretory proteins are subject to a quality control mechanism (QC) in the ER to adopt the correct conformation. ER QC involves retention of proteins that are not yet mature in the ER and targeting of misfolded proteins for degradation through the ER-associated degradation (ERAD) pathway (Vitale and Denecke, 2006). ER stress causes accumulation of non-native proteins in the ER that can form protein aggregates due to nonspecific interactions between hydrophobic residues that are exposed in the newly synthesized protein (Vitale and Boston, 2008).

Once the synthesized secretory protein enters the ER lumen, chaperones and other folding helper proteins bind to the nascent polypeptide chain to facilitate correct folding. The first folding helper is the enzyme signal peptidase that removes a N-terminal signal peptide present in the majority of secretory proteins (Vitale and Denecke, 2006). Subsequently, chaperones and co-chaperones bind to hydrophobic regions of proteins to prevent the formation of any aggregates (Liu and Howell, 2010). Several such folding proteins have been identified, with the most well described being the ER luminal BiP. In *A. thaliana* three *BiP* genes have been found, named *BiP1*, *BiP2* and *BiP3*. BiP is responsible for several additional processes in the ER. For instance, it facilitates the movement of the
protein through the translocation pore in association with the Sec61 protein (Vitale and Denecke, 2006). Studies have reported that environmental stresses such as drought, heat and pathogen attack can cause ER stress that in turn results in the enhanced expression of all three AtBiP genes (Wang et al., 2005; Iwata et al., 2008).

Most of the proteins passing through the secretory pathway are modified by N-glycosylation. N-glycosylation ensures ER QC as the core Glc3Man9GlcNAc2-P-P-Dol oligosaccharide is subject to further modifications inside the ER lumen by entering the calnexin/calreticulin cycle (Pattison and Amtmann, 2009). The CNX/CRT cycle depends on the de-glucosylation and re-glucosylation of the oligosaccharide by enzymes residing in the ER lumen. Figure 1.4 represents the CNX/CRT cycle including all the mutants identified in Arabidopsis to affect this process. Once the oligosaccharide is transferred to the protein, the two terminal glucose residues are removed by glucosidase I and II, respectively. The monoglucosylated oligosaccharide, GlcMan9GlcNAc2, binds to the two lectins CNX and CRT. CNX is an ER membrane protein, while CRT is a luminal ER protein. Both lectins contain N-terminal regions forming β-sandwich domains that interact with the single glucose residue of the oligosaccharide of the proteins (Liu and Howell, 2010). Glucosidase II facilitates the release of the protein from the CNX/CRT cycle by removing the last glucose residue. However, if the protein has not adopted its native form, another ER resident protein, the UDP-glucose:glycoprotein glucosyltransferase (UGGT), will add a further glucose residue to the nonglucosylated oligosaccharide to allow further rounds of CNX/CRT cycle in order to control the folding status of the protein (Liu and Howell, 2010). The cycle is interrupted when the protein is correctly folded and the protein can then be exported to the Golgi (Pattison and Amtmann, 2009).

Mutations affecting proteins involved directly or indirectly in the CNX/CRT cycle have severe effects on plant fitness and phenotypes can range from being completely lethal to no obvious morphological phenotype (Pattison and Amtmann, 2009). For instance, the ER-localised protein, UDP-glucose/galactose transporter (AtUTR) 1 in A. thaliana is responsible for the transport of UDP-glucose into the ER lumen. Mutations in the AtUTR1 gene cause 50% decrease in UDP-glucose uptake suggesting that the protein is essential for the correct functioning of the CNX/CRT cycle (Reyes et al., 2006). In addition, a genetic screen for Arabidopsis mutants insensitive to the surrogate peptide elf18 for the elongation factor (EF)-TU, has identified several genes that provide a link between ER QC and pathogen response, including CRT3, UGGT and the ER retention receptor (ERD2) (Li et al., 2009; Nekrashov et al., 2009).
After transfer of the mature Glc3Man5GlcNAc2 oligosaccharide to protein, the newly produced glycoprotein enters the CNX/CRT cycle. The outermost and following glucose residue are removed by glucosidase I and II. The two lectins, CNX and CRT can bind the monoglucosylated oligosaccharide, promoting correct folding of the protein. After removal of the last glucose residue the protein can be exported to the Golgi. If it is still misfolded, it re-enters the CNX/CRT cycle by addition of a further glucose residue by UGGT (indicated as GT). *A. thaliana* mutants are shown in red color. (Figure is taken from publication by Pattison and Amtmann, 2009)

ER stress induces the UPR which in turn results in the enhanced expression of folding helpers to relieve the stress either by promoting correct folding or by eliminating defective proteins. UPR is not only stimulated by environmental stresses like pathogen attack, but also by normal developmental processes such as seed development that involves accumulation of storage bodies (Pattison and Amtmann, 2009). Studies in yeast have revealed that a key player in UPR is the BiP protein. Under normal conditions BiP is found to be associated with a RNA splicing factor, the inostol-required 1 (Ire1) protein (Liu and Howell, 2010). Upon accumulation of misfolded proteins free BiP decreases causing BiP dissociation from Ire1, which in turn activates a transcription factor that up-regulates expression of ER stress related genes (Travers *et al.*, 2000; Ceriotti and Roberts, 2006). Such genes are not only chaperones and folding helpers, but also genes implicated in the secretory pathway or secretion (Travers *et al.*, 2000). Microarray studies in *A. thaliana* showed a similar set of genes to be up-regulated during UPR, though down-regulation of proteins with signal peptides, especially cell wall proteins, was also evident (Martinez and Chrispeels, 2003). In addition, two ERAD genes, a putative ubiquitin and an AAA-type ATPase, were up-regulated suggesting a link between UPR and ERAD pathway.
As discussed before, N-glycosylation inhibition induces UPR that in turn up-regulates expression of BiP, CNX, CRT and other folding-related proteins (Ceriotti and Roberts, 2006). Many of the chaperones induced by UPR are also implicated in the ERAD pathway. The discrimination between native and misfolded proteins is facilitated by the CNX/CRT cycle. Permanently misfolded proteins remain in the ER lumen much longer than the folding intermediates and therefore the possibility to be trimmed by ER-localised mannosidase increases. This enzyme is responsible for the removal of the last Man residue from the nonglucosylated oligosaccharide. Hence, the re-glucosylation of the Man$_9$GlcNAc$_2$ is less probable than of the Man$_9$GlcNAc$_2$ causing the misfolded protein to be translocated to the cytosol (Ceriotti and Roberts, 2006). However, it is mostly unknown how defective proteins are recognised by folding factors (Liu and Howell, 2010). Studies have suggested that dislocation of these proteins occurs through the Sec61 complex. Translocation to the cytosol is dependent on ubiquitinylation of the defective protein that will allow proteasomal degradation to take place (Ceriotti and Roberts, 2006).

Not all defective proteins follow the ERAD pathway. Degradation of non-native proteins can be achieved in vacuoles through two different mechanisms, the Golgi-mediated pathway and autophagy (Vitale and Boston, 2008). The first mechanism entails the vesicular traffic of the protein through golgi and pre-vacuolar compartment, the subsequent sorting to the vacuole by a Vps 10 receptor and eventually the degradation by vacuolar proteases (Kruse et al., 2006). The alternative mechanism is based on autophagy. ER stress is shown to provoke the assembly of pre-autophagosomal structures (Yorimitsu et al., 2007). Studies in yeast have suggested that the autophagosome requires the early secretory pathway to supply membranes for its formation (Regiori et al., 2004). It has also been reported that ER fragments containing misfolded proteins are selectively recognised and transported to the vacuole within the autophagosome (Rose et al., 2006).
1.9 Objectives and thesis outline

The aim of this project is to give further insight into the function of two AtPQL proteins, AtPQL4 and AtPQL6. This is achieved by investigating the sub-cellular localisation of the two proteins, the effect of overexpression on the localization of other membrane proteins and the phenotypic analysis of mutant lines for both genes under certain environmental conditions. The thesis contains three Results chapters (chapters 3-5). Chapter 2 provides details about the materials and methods used in this project.

Chapter 3 presents the sub-cellular localisation of the AtPQL and AtPQL6 proteins, using GFP-fusion constructs and confocal laser scanning microscopy of tobacco leaf cells. A previous study reported localization to the ER. Here, I confirm this finding by co-expressing the proteins with an ER marker protein. In addition, novel information on the sub-cellular localisation of a third protein from the same family, AtPQL2, is also presented.

Chapter 4 presents new information on the molecular function of AtPQL4 and AtPQL6. Overexpression of the two AtPQL proteins in tobacco leaves is shown to affect the targeting of secretory proteins. This was investigated by co-expressing GFP-AtPQL proteins with a number of different fluorescent-tagged proteins. The implications of this observation for a possible function of AtPQL4 and AtPQL6 in the ER are discussed.

Chapter 5 describes the phenotypic analysis of mutant lines for AtPQL4 and AtPQL6 in a variety of environmental stresses. The observations at whole-plant level are discussed in the context of putative functions of the proteins at the (sub-)cellular level.

A general discussion of all the data presented in the thesis is provided in chapter 6. A possible function of the AtPQL4 and AtPQL6 proteins is proposed and ideas for future experiments are presented.
Chapter 2: Material and Methods

2.1 Materials

2.1.1 Chemicals

Except where otherwise stated, all chemicals used are from Sigma (Poole, UK) and Fischer-Scientific (Southampton, UK). Enzymes for molecular biology were from Invitrogen (Paisley, UK), Promega (Southampton, UK) and New England Biolabs (Hitchin, UK). Kits for DNA extraction were from Fischer-Scientific (Southampton, UK) and kits for gel extraction were from Qiagen (Crawley, UK). Custom primers were purchased from Invitrogen.

2.1.2 Plants

*Arabidopsis thaliana* ecotype Columbia (Col0) was the background for all mutants and transgenic lines. Six different *Arabidopsis thaliana* genotypes were used; wild-type Col0 (WT), *pql4-1*, *pql4-2*, *pql6-1*, *pql4-2/6-1* and *35S::AtPQL6-1*.

2.2 Plant Growth and Treatment

2.2.1 Growth on soil

To grow *A. thaliana* plants on soil, approximately 6 seeds were sown on moist compost (Levington F2, Fisons, Ipswich, UK) inside a standard circular plant pot (4 cm in diameter) and placed in a tray under propagator in darkness at 4°C for two days to allow stratification. The pots subsequently were placed in a controlled growth chamber (Sanyo Fitotron) in either long-day conditions (16 hours light, 8 hours darkness; light intensity 100-120 μmol m⁻² sec⁻¹; temperature approximately 20°C/18°C day/night; relative humidity of 60%/70% day/night) or short-day conditions (9 hours light, 15 hours darkness; light intensity 120-150 μmol m⁻² sec⁻¹; temperature approximately 20°C/18°C day/night; relative humidity of 60%/70% day/night). After one week, plants were transplanted in individual pots and the propagator was removed. Plants were watered every 4th day until senescence. For seed collection, Aracon tubing was placed on top of the rosettes and
watering stopped once the first siliques became discoloured. The plants were allowed to first completely dry out before collecting the seeds.

### 2.2.2 Viral infection

Four seeds per genotype were sown on soil and placed in a controlled growth chamber in short-day conditions as described. After the emergence of the first true leaves, Arabidopsis seedlings were inoculated with CaMV isolate Cabb B-JI. 2 μL of sterile water mixed with a small amount of cellite, used as an abrasive, and 0.1 μg of purified virus were pipetted onto the leaf. The leaf was rubbed using a small glass rod. Control plants were not infected. Three plants from each genotype were pooled to extract DNA at different time points as described below and quantified with qPCR (see below).

### 2.2.3 Growth on agar plates

*A. thaliana* seeds were surfaced sterilised. About 400 seeds were placed in 1.5 mL Eppendorf tube with 1mL 100% ethanol for one minute and mixed by inverting tube several times. The ethanol was discarded and 1 mL of sterilising solution (2.5% sodium hypochlorite and 0.01% Tween 20) was added into the tube and mixed by inverting several times. After incubation for five minutes, the bleach solution was replaced by 1 mL of double distilled water. Washing was repeated five times to ensure complete removal of bleach solution and the seeds kept in water. The tubes were wrapped in aluminium foil and placed in darkness at 4°C for two days to ensure stratification.

Different media were used for physiological experiments. For most of the experiments in this project, a full minimal medium (Control medium) was used (Table 2.1). To prepare the medium, reagents were mixed and diluted with double distilled water to the final concentration. 0.5x Muraskige and Skoog (MS) medium was also used where stated. 2.2 g of MS powder (Sigma, M5519) per litre was dissolved in double distilled water. Media were supplemented with sucrose to a concentration 1% and pH was adjusted to pH 5.6 with either 0.5 M of NaOH or using MES-TRIS buffer. To solidify the media, 1% Agar (Type A, Sigma) was added before autoclaving at 121°C for 20 minutes. Once the autoclaved medium had cooled to about 60°C, it was suspended into square plates (120 x 120 mm, Greiner) using titration pipettes.
Seeds were sown individually onto the solidified media using a sterile cut P200 pipette tip attached to a Gilson pipette. Generally, 6 seeds sown per plate containing 50 mL medium and spaced evenly into horizontal line approximately 15 mm form the top of the plate. The plates were sealed with 3M Micropore Tape (MidMeds Ltd, Nazeing, UK) and placed in controlled growth chamber (24 hours photoperiod; light intensity approximately 110 μmol m$^{-2}$ sec$^{-1}$; temperature 20°C and relative humidity of 60%), except where otherwise stated.

Table 2.1: Final concentration of reagents used to prepare full minimal Control medium.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO$_3$</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td>42.5 μM</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.625 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>2 mM</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>0.16 μM</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>0.38 μM</td>
</tr>
<tr>
<td>MnSO$_4$</td>
<td>1.8 μM</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>45 μM</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$_7$O$_2$$_4$</td>
<td>0.015 μM</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>0.01 μM</td>
</tr>
</tbody>
</table>

2.2.4 Stress treatments

Salt and Osmotic stress

Plants were grown on MS agar plates supplemented with 50 mM NaCl or 50 mM KCl to measure sensitivity to salt and osmotic stress, respectively.
Macronutrient depletion

Plants were grown on agar plates supplemented with varied strength of MS to measure sensitivity of seedlings to macronutrient depletion. 1/3, 1/10, 1/30, 1/100 and 1/300 MS dilutions were used. Four seeds of two different genotypes were sown on agar plate.

Sucrose starvation

Response of plants in varied carbon concentrations was measured by growing plants on MS agar plates supplemented with 0%, 0.5%, 1%, 3% and 4% of sucrose.

2.2.5 Analysis of growth and water content

From nine days of germination and onwards, plates were scanned to allow measurement of the roots using the EasyRhizo software (Armengaud et al., 2000). Fourteen days post germination, shoots and roots were harvested to measure fresh weight and placed in foil at 60°C for three days to measure dry weight.

Water content was estimated according to the following formula:

\[ W_{\text{cont}}(\%) = 100 - (DW/FW \times 100) \]

Statistical analysis was performed using ANOVA test with SOFA Statistics version 0.9.25 (Paton-Simpson & Associates Ltd, Auckland, New Zealand).

2.2.6 Growth on liquid medium

Plants grown in liquid 0.5x MS medium prepared as described above. Sterile seeds were sown directly into either 6-well plates containing liquid medium. The plates were sealed with Micropore tape and placed in control growth chamber (24 hours photoperiod; light intensity approximately 110 \( \mu \text{mol m}^{-2} \text{ sec}^{-1} \); temperature 20°C and relative humidity of 60%).
2.3 DNA and RNA extraction, quantification

For the extraction of either DNA or RNA, plant material was placed into a 2 mL screwcap tube together with a grinding ball and placed directly into liquid nitrogen. Subsequently, the tubes were placed in a pre-cooled rack and the plant material ground using the Qiagen Tissue Lyser for 45-110 seconds depending on the amount of plant material, with a frequency of 28.6 Hz.

2.3.1 DNA extraction

DNA was extracted from *A. thaliana* using the Nucleospin® Plant II kit (Fischer-Scientific) according to the manufacturer’s instructions, with an additional step of digestion with Proteinase K. After lysis step, 8 μL of Proteinase K (20μg/μL) were added into the tubes, followed by thorough vortexing. The tubes were incubated at room temperature for three hours. Proteinase K was inactivated by incubating the samples at 65°C for thirty minutes. DNA was twice eluted from the column in 50 μL of the supplied elution buffer. DNA quantity and purity was determined spectrophotometrically by preparing 1 to 50 dilutions of DNA and measuring the absorbance at 260 nm in an Eppendorf UVette® in the Eppendorf Biophotomoter Plus. Absorbance readings were compared to double distilled water. The purity of DNA was estimated from the A260/A280 ratio.

2.3.2 RNA extraction

To prepare RNAse free water, 1 mL of DEPC (Diethyl pyrocarbonate, Fluka) was added to 1 L of double distilled water and stirred for approximately two hours. This water was subsequently autoclaved and used in all methods involving RNA.

RNA was extracted from *A. thaliana* by adding 1.4 mL of TRIzol solution (0.8M Guanidium thiocynate, 0.4 M Ammonium thiocynate, 0.1M NaAc pH 5.2, 5% Glycerol and 3% Phenol saturated in water pH 4.5) to 100 mg of ground frozen plant material. The samples were mixed by vortexing for one minute and incubated at room temperature for five minutes. This was followed by centrifugation at 8000g for two minutes. The supernatant was transferred into new 2 mL Eppendorf tube and 300 μL of chloroform:isoamylalcohol (24:1) were added. The samples were mixed by vortexing for one minute, incubated at room temperature for two minutes and then centrifuged at 13000g at 4°C for ten minutes. The aqueous phase was carefully transferred into a fresh 1.5 mL
tube followed by adding isopropanol in a volume ratio of 1:1. The samples were mixed by inverting the tubes for several times and left at room temperature for 5 minutes to allow the precipitation of RNA. Subsequently, the samples were centrifuged at 13000g at 4°C for 10 minutes, washed with 1 mL of 70% ethanol and centrifuged at 13000g at 4°C for five minutes. The supernatant was removed and the pellet allowed to dry at air and resuspended in 50 μL of DEPC-treated water. The samples were placed at 42°C to ensure dissolving of dried RNA.

The RNA quantity and purity was measured spectrophotometrically in Eppendorf Biophotometer Plus. RNA was 50x diluted in DEPC-treated water and absorbance measured from 220 to 320 nm into an Eppendorf UVette®. RNA concentration was calculated following the formula: (A_{260-230}) \times 40 \times \text{Dilution factor} (\text{ng/μL}). The purity of RNA was estimated from the A_{260}/A_{280} ratio.

2.4 Polymerase Chain Reaction (PCR)

PCR was performed using the Gen Amp PCR System 9700 (Applied Biosystems). Primer sequences are shown in appendix A.

2.4.1 Standard PCR

Standard PCR was carried out using GoTaq DNA polymerase (Promega). Table 2.2 and 2.3 display the composition of the standard PCR mix prepared to a final volume of 25 mL and the cycling conditions. Template was genomic DNA. Aliquots of the mastermix were pipetted into standard thin-wall PCR reaction tubes. Templates were added individually to each PCR tube.

2.4.2 Agarose gel electrophoresis and gel purification

PCR products were resolved by agarose gel electrophoresis. 15 μL of PCR product was loaded on a DNA agarose gel (1.5% agarose, 1x TAE containing 32μL/L ethidium bormide solution). 10 μL of DNA lambda ladder was loaded on each gel. DNA bands were separated at 100mV for 25-35 minutes and visualised under UV light using GelDoc 2000 scanner (Biorad).
PCR products were extracted from gels under UV light and purified using a QiaQuick gel extraction kit (Qiagen) according to manufacturer’s instructions. DNA was eluted in 50μL elution buffer. DNA quantity and purity was determined spectrophotometrically as described above.

Table 2.2: Standard PCR reaction mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Green GoTaq Buffer</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>3</td>
</tr>
<tr>
<td>dNTP Mix (10mM of dATP, dCTP, dGTP and dTTP)</td>
<td>1</td>
</tr>
<tr>
<td>Forward Primer (10mM)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer (10mM)</td>
<td>1</td>
</tr>
<tr>
<td>GoTaq (5U/μL)</td>
<td>0.2</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>11.8</td>
</tr>
<tr>
<td>Template</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2.3: Cycling conditions of standard PCR.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Step</th>
<th>Temperature</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denaturation</td>
<td>95°C</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>Denaturation</td>
<td>95°C</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Primer Annealing</td>
<td>60°C</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Final extension</td>
<td>72°C</td>
<td>5</td>
</tr>
</tbody>
</table>
2.4.3 Quantitative PCR (qPCR)

qPCR was performed in 96-well plates using Mx3000 (Stratagene) real-time PCR system and the Brilliant SYBR Green qPCR kit (Stratagene). Table 2.4 and 2.5 display the composition of the qPCR reaction mix prepared to a final volume of 12.5 mL and cycling conditions. The template was genomic DNA or DNA standards and nucleotide-free water. DNA standards were prepared from gel-extracted PRC products by performing standard PCR using the same primers as for qPCR. Purified PCR products were adjusted to 10 pg/μL and subsequently diluted to produce six different standards ranging from 1 pg/μL to $10^{-5}$ pg/μL.

At the end of the annealing step of every PCR cycle, SYBR green fluorescence was measured and the Ct (the number of cycles required to reach threshold fluorescence) was calculated using the machine software (MX3000). Plotting the Ct value of each dilution against the log initial template quantity created a standard curve. qPCR reactions were made in duplicates. To control and correct any errors occurring in each qPCR, the expression of the gene of interest should be tested relative to a stably expressed gene.

For viral infection experiments, the amount of virus DNA was estimated. The reference gene used was the *Arabidopsis* 18S ribosomal RNA gene. The *Arabidopsis* genome contains a fixed number of copies of the 18S rRNA gene and therefore 18S rRNA provides the most accurate measure of the amount of total DNA. CaMV DNA was quantified by Q-PCR, and for each sample, duplicates of reactions were performed. Table 2.6 displays the primers were used to amplify the CaMV Cabb B-JI gene and the *Arabidopsis* 18S ribosomal RNA gene.
Table 2.4: QPCR reaction mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR green qPCR Master mix</td>
<td>10</td>
</tr>
<tr>
<td>Forward Primer (10mM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Reverse primer (10mM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>4.2</td>
</tr>
<tr>
<td>Template</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.5: Cycling conditions for qPCR.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95°C</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>95°C</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1</td>
</tr>
</tbody>
</table>

Amplification program

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>95°C</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Dissociation program
2.5 Molecular cloning procedures

2.5.1 Transformation of chemically competent *E.coli* cells

Aliquots of chemically competent *E.coli* (strain TOP10™, Invitrogen) cells were thawed on ice for five minutes and then 1 μL of plasmid was added and mixed by stirring for five seconds. The tubes were placed into liquid nitrogen for five minutes. This was followed by placing the tubes in a prewarmed water bath at 42°C for 45 seconds. Luria Bertani (LB) medium was added to each tube that was then incubated at 37°C for one hour by shaking at 200 rpm. Bacteria cells were harvested by centrifugation at 8000g for two minutes. The cells were resuspended into 100 μL of the supernatant and the whole volume was spread on LB agar plates (1% agar) under a flow hood using or autoclaved glass beads or a Drigalski spatula sterilised by ethanol flaming. The agar plates were supplemented with the appropriate antibiotics for selection of positive transformants as shown on Table 2.7. All the plates were incubated overnight at 37°C.

### Table 2.6: Antibiotics used for selection of positive transformants.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Final concentration (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamycin</td>
<td>25</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50</td>
</tr>
<tr>
<td>Spectinamycin</td>
<td>100</td>
</tr>
</tbody>
</table>

2.5.2 Plasmid minipreps

Single colonies picked with a sterile pipette tip and inoculated in 4 mL of LB medium containing the appropriate antibiotics. The cultures were grown overnight at 37°C by shaking at 200 rpm. Once cultures reach saturation they were transferred into 2 mL tubes and centrifuged at 4000g at 4°C fifteen seconds. The cell pellets were resuspended in 400 μL resuspension buffer (50 mM Tris-Cl, 10 mM EDTA, RNase A (10μg/ml), Lysozyme, pH 8 adjust) by vortexing. This was followed by adding 400 μL of lysis buffer (0.2 M NaOH, 1% SDS) and mixing by inverting the tubes for four times. The lysates were resuspend in 400 μL of neutralisation buffer (3M Potassium acetate, pH 5) and centrifuged
at 14000g at 4°C for ten minutes. 1 mL of the supernatant was transferred into fresh 2 mL tube and mixed with 1 mL of chloroform-isoamyl alcohol (24:1) by inverting the tubes for thirty seconds. The suspension was centrifuged at 14000g at 4°C for one minute and 950 μL of the supernatant was transferred into fresh 2 mL tubes. Isopropanol was added in a volume ratio of 1:1, the suspension was mixed and incubated at -20°C for twenty minutes. This was followed by centrifugation at 14000g at 4°C for fifteen minutes. The supernatant was transferred into a new tube containing 500 μL of 70% ethanol and centrifuged at 14000g at 4°C for five minutes. The DNA pellet was allowed to dry at air for ten minutes. The DNA pellet was dissolved in 100 μL of double distilled water by incubating them at 65°C for ten minutes.

### 2.5.3 Restriction digestion reactions

Plasmids were digested with restriction enzymes purchased from NEB or Promega. Table 2.8 shows the composition of the restriction digestion mix in a final volume of 20 μL. The mix was prepared with the appropriate reaction buffers for each enzyme and BSA where required. If necessary, the reaction was performed by using two enzymes into the same tube and choosing a reaction buffer to be compatible with both enzymes. The reactions were incubated at 37°C for approximately 1 hour. The samples were separated by agarose (1.5%) gel electrophoresis as described above.

**Table 2.7: Composition of restriction digestion mix.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer</td>
<td>5.1 μL</td>
</tr>
<tr>
<td>BSA (10μg/μL)</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>0.4 μL</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>11 μL</td>
</tr>
<tr>
<td>Template</td>
<td>3 μL</td>
</tr>
</tbody>
</table>
2.5.4 Transformation of chemically competent *Agrobacterium tumefaciens* cells

Aliquots of chemically competent *A. tumefaciens* (strain GV3101) cells were transformed with binary plasmids using a thermal shock protocol. The cells were left to thaw at room temperature for five minutes and 5 μL of plasmid DNA was added. The cells were incubated first on ice and then on liquid nitrogen for five minutes. The tubes were then heated in a prewarmed water bath at 37°C for five minutes. 1 mL of liquid YEB medium was added into the tubes and the cells were incubated at 28°C for two hours while shaking at 200rpm. The cells were harvested by centrifugation at 8000g for two minutes and were suspended in 100 μL of supernatant. 10%, 30% and 60% of the suspensions were spread on LB or YEB agar plates containing gentamycin and rifampicin in addition the specific antibiotic for selection of the binary plasmid. The plates were sealed with parafilm and incubated at 28°C degrees for two days. Single colonies were picked from the plates with a sterile pipette tip and inoculated in 4 mL liquid LB medium supplemented with the same antibiotics as those used on the plates. The inoculums were incubated at 28°C overnight and used for plasmid extraction, restriction digest analysis, verification of correct transformation of agrobacteria with plasmid DNA, re-inoculation and then production of glycerol stocks, or for direct preparation of glycerol stocks as described below.

2.5.5 Bacterial glycerol stocks

For direct inoculation of transformed agrobacteria, glycerol stocks were produced. 590 mL of 4 mL overnight bacterial cultures were added into 2 mL screwcap tubes containing 900 mL of 40% glycerol under flow hood. The tubes were mixed by inversion and were snap frozen in liquid nitrogen. Stocks were stored at -80°C for long-term use. A sterile filter tip was used to scrape off some frozen bacterial stock for inoculation into liquid LB or YEB medium.

2.6 In vivo subcellular localisation and co-localisation of fusion proteins

The fluorescent fusion protein constructs 35S::*AtPQLs*:GFP used for *in vivo* subcellular localization were created by Dr Richard Pattison (University of Glasgow) using the
Gateway recombination system (Invitrogen). Vectors used for transient *A. tumefaciens* transformations were provided by Dr. Grefen and Prof. Blatt (University of Glasgow).

### 2.6.1 Creation of expression clones

Expression clones were created combining a gateway entry clone with an attR containing destination vector using the gateway LR-clonase II (Invitrogen). The LR-recombination reaction mix was made up to a final volume of 2.5 μL. 1 μL (=150 ng) of destination vector and mixed with 1 μL (=150 ng) of entry clone and 0.5 μL of LR-clonase II. The reaction was incubated at room temperature for one hour. 1 μL of the reaction was used to transform *E. coli* cells and positive transformants were verified by restriction digestion as described before. The destination vectors pH7YWG2 and pH7RWG2 were used to create YFP and RFP fusions respectively. Both vectors contain a cauliflower mosaic virus (CaMV) 35S promoter. The destination vector pUBC-Dest including fluorophores was used to create protein fusion constructs with either GFP or YFP driven by the *A. thaliana ubiquitin-10* gene promotor (Grefen et al., 2010). Table 2.9 displays the expression clones created for this thesis.

Table 2.8: Expression clones created in this thesis using Gateway system.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Entry Clone</th>
<th>Destination vector</th>
<th>Expression clone</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AtPQL4</em></td>
<td><em>pENTR 201 PQL4 NS</em></td>
<td>pH7RWG2</td>
<td>pH7YWG2 35S::<em>AtPQL4</em>-RFP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pUBC-DEST</td>
<td><em>pUB10::AtPQL4</em>-GFP</td>
</tr>
<tr>
<td><em>AtPQL6</em></td>
<td><em>pENTR 201 PQL6 NS</em></td>
<td>pH7RWG2</td>
<td>pH7YWG2 35S::<em>AtPQL6</em>-YFP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pUBC-DEST</td>
<td><em>pUB10::AtPQL6</em>-GFP</td>
</tr>
</tbody>
</table>

### 2.6.2 Transient expression of protein fusion constructs in *Nicotiana tabacum*

*N. tabacum* was used for transient expression of all the constructs using the agroinfiltration method. 4 mL *Agrobacterium* inoculations containing the appropriate construct and antibiotics were grown as described in general methods. Half amount of the cultures were added into 2 mL tubes and centrifuged at 13200g for two minutes. The supernatant was
removed, the other half of the culture was added and the centrifugation step was repeated. The bacteria cells were suspended in 2 mL of transformation buffer (10 mM MgCl2, 100 μM acetosyringone) and centrifuged at 8000g for two minutes. The washing step was repeated three times to remove any residual medium. This was followed by resuspension in 1 mL of transformation buffer to measure the OD$_{600}$. The final OD$_{600}$ was 0.3 and 0.2 for subcellular localisation and co-localisation respectively. The cell suspensions were left at room temperature for approximately one hour. To infiltrate the *N. tabacum* plants, the underside of a young mature leaf was pierced with a fresh razor blade (without cutting through the tissue) and a 1 mL syringe used to inject the cells. The infiltrated areas of the leaf were marked and the plants were returned to the control growth room (16 hours light, 8 hours darkness; light intensity 100-120 μmol m$^{-2}$ sec$^{-1}$; temperature approximately 26°C/18°C day/night; relative humidity of 60%/70% day/night) for two to three days before observation under the confocal microscope.

### 2.6.3 Transient expression of protein fusion constructs in *Arabidopsis*

*A. thaliana* seeds were surfaced sterilised, incubated at 4°C for two days to ensure stratification and then placed in control growth room under continuous light as described before. Seeds were incubated in six-well plates supplemented with 3 mL of 0.5x MS medium (pH 5.7) for three to four days to allow germination. The whole procedure was performed under the flow hood. *Agrobacterium* inoculations were prepared as described in general methods. Bacteria were pelleted by centrifugation at 4000g at 4°C for fifteen minutes and resuspended in 2 mL sterile transformation buffer (0.5x MS medium, 100 μM acetosyringone, 0.003% sylwet-77). This step was repeated twice and bacteria were finally resuspend in 1 mL of transformation buffer to measure the OD$_{600}$. A final OD$_{600}$ of 0.1-0.2 was reached by diluting the bacteria in transformation buffer. The growth medium was removed from the six-well plates using 1 mL filter pipetting tip and replaced with 3 mL of *Agrobacterium* suspension. The plates were resealed and transferred back to the control growth room for two to three days before confocal imaging.

### 2.6.4 Confocal laser scanning microscopy and analysis

Confocal imaging was performed on a Carl Zeiss CLSM-510-META-UV confocal laser-scanning microscope (Carl Zeiss, Inc., [http://www.zeiss.co.uk](http://www.zeiss.co.uk)). 20x lens and 40x immersion oil lenses were used. For visualizing GFP and YFP, an argon ion laser was used. Laser light of 514 nm was used to excite YFP. Reflected light was subsequently
reflected by a secondary NFT490 dichroic mirror and passed through a 530-600 nm bandpass filter to allow collection of fluorescence. For GFP visualization, excitation was at 488 nm and light was reflected by a secondary NFT 545 dichroic mirror. GFP fluorescence was collected as the light passed through a 505 long pass filter. In both cases, emitted light passed through a 635VIS dichroic mirror to allow collection of chloroplast autofluorescence using a META detector of a bandwidth of 621-700 nm.

For co-localisation experiments the settings were slightly altered to minimize potential problems caused by bleed through between the two fluorophores. Sequential acquisition of images in the yellow channel and then green channel was used. YFP fluorescence was collected using the same configuration as that for single expression of fusion proteins. However, for GFP fluorescence detection, excitation occurred at 458 nm and reflected light was collected using a 505-530 bandpass filter.

Control experiments were performed to test for bleed through between the fluorophores. For this purpose, single expression of GFP-tagged proteins were analysed using different confocal microscope configurations. GFP was excited at 458 nm, 488 nm or 514 using a range of light transmission from 1% to 30% and collecting fluorescence using GFP and/or YFP settings. In addition, single expression of YFP-tagged proteins was carried out. Measurements of fluorescence intensity from images (512x512) suggested that false YFP fluorescence was apparent in single expression of GFP-tagged protein as transmission exceeds 20%. However, YFP fluorescence detected in the co-expression experiments was 30.4-fold and 2.81-fold higher than the false YFP fluorescence detected when using 10% and 20% of transmission, respectively. Therefore, the transmission used to visualize GFP and YFP fluorescence in this thesis was from 3% to 10%.

Colocalisation events were evaluated using Zeiss LSM 510 AIM (v3.2) for analyzing line scans and IMAGEJ 1.43u software - particularly the Mander’s/Pearson’s coefficient plugin created by Tony Collins (Wright Cell Imaging Facility, Toronto, Canada) and Wayne Rasband (NIH, USA) – for creating scatter plots based on Pearson’s coefficient (http://www.rsbweb.nih.gov/ij).
Chapter 3: Subcellular localisation of AtPQL2, AtPQL4 and AtPQL6.

3.1 Introduction

In this chapter, I will present data for the subcellular localisation of three proteins of the AtPQL family, AtPQL2, AtPQL4 and AtPQL6. Previous studies have provided evidence for the ER localisation of AtPQL4 and AtPQL6 homologous proteins. However, no subcellular localisation data are available for AtPQL2. The first part presents the subcellular localisation of the three proteins, and the second part presents supporting data showing co-localisation with an organelle marker.

To identify the subcellular localisation of the proteins Agrobacterium-mediated transformation was used. AtPQL proteins fused to green fluorescent protein (GFP) at the C-terminus were transiently expressed in leaf epidermal cells of N. tabacum. Expression was under the control of the strong constitutive 35S promoter (P35S) of cauliflower mosaic virus (CaMV). Figure 3.1 displays a schematic representation of the expression cassette of the plasmids used to express the AtPQL proteins. Analysis of the localisation was carried out using confocal laser microscopy, 72 hours after transformation, when protein expression was expected to be at its maximum.

![Diagram of expression cassette](image)

**Figure 3.1: Expression cassette of plasmid containing the AtPQL2, AtPQL4 and AtPQL6 gene.**

The diagram shows the pGWB5 vector. The Gateway attB recombination sites and plant selectable markers NPTII and HPTII, which confer resistance to hygromycin and kanamycin respectively, are indicated. The position of the coding region relative to the CaMV 35S promoter, sGFP tag and NOS terminator is also shown. (Figure is taken from PhD thesis by Pattison, 2008)

3.2 Results

3.2.1 Subcellular localisation of AtPQL2

Confocal images of tobacco epidermal cells transformed with AtPQL2-GFP fusion protein are presented in Figure 3.2. Frames a-c are projections of image stacks. Frame d is a single plain image and shows the bright field image for frames a-c. GFP fluorescence (shown in
green colour) is visible as a smooth line at the edge of the cell suggesting localisation of AtPQL2 to the tonoplast. However, without a membrane marker it is difficult to distinguish between plasma membrane and tonoplast. One point, where separation of the plasma membrane from tonoplast appears to take place, is indicated by the arrow in frame b. The green spherical structures observed in frame b were chloroplasts, as indicated in frame c showing chloroplast autofluorescence. Autofluorescence mainly depends on the laser intensity used to visualise the fluorophore. In this particular case, high-intensity light was used to visualise AtPQL2-GFP resulting in chloroplast fluorescence contributing to the GFP signal. AtPQL2-GFP fluorescence was detected only in a small number of cells, although a number of various time points (36h, 48h, 72h, 84h) after infiltration were analysed and infiltration of tobacco leaves with Agrobacterium at various densities (OD$_{600}$ nm) was tested.
Figure 3. 2.: AtPQL2 localises to the tonoplast.

Tobacco leaf epidermal cells expressing AtPQL2-GFP fusion protein. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-c are z-stacks of 9 optical sections of 3.50 μm each and show composite fluorescence (a), GFP fluorescence (b) and chloroplast autofluorescence (c). Frame d is the single plane, bright-field image for a-c. GFP fluorescence labels the tonoplast (arrow). Scale bar = 10μm
3.2.2 Sub-cellular localisation of AtPQL4 and AtPQL6.

Confocal images of tobacco epidermal cells expressing AtPQL4-GFP and AtPQL6-GFP fusion proteins are shown in Figures 3.3 and 3.4, respectively. In both figures, frames a-c are single plane images. Frames d-f represents projections of image stacks. The GFP fluorescence signal is shown in green colour in frame e, and frame g is the single plain bright-field image for frames d-f. In contrast to AtPQL2-GFP, the fluorescence signal of AtPQL4-GFP and AtPQL6-GFP was detectable in approximately 50% of the transformed cells. GFP fluorescence was clearly visible in internal compartments forming net-like patterns within the cell (Figure 3.3c and 3.4c). GFP fluorescence also labeled the nuclear envelope that is continuous with the lattice-like network (see arrow in figures 3.3e and 3.4c). Additionally, fluorescence signal was distributed in long fast-moving strands, described as cytoplasmic ER (see arrow in Figures 3.3b and 3.4e). Fluorescence patterns were indicative of typical ER (Fluckiger et al., 2003, Sparkes et al., 2009) and provide evidence for ER localisation of the AtPQL4 and AtPQL6 proteins.

To exclude the possibility of mislocalisation due to overexpression under the control of the strong constitutive $P_{35S}$ promoter, the coding sequences of AtPQL4 and AtPQL6 were also expressed as C-terminal fusions to YFP under the control of the ubiquitin-10 ($P_{UBQ10}$) promoter that facilitates moderate expression of the transgene (Grefen et al., 2010). YFP fluorescence was again detected in typical ER-like patterns (data not shown). In addition, photobleaching experiments showed that both proteins appeared to be highly mobile when imaged during a time series (data not shown).
Figure 3.3: *AtPQL4* localises to the ER.

Tobacco leaf epidermal cells expressing *AtPQL4*-GFP fusion protein. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-c are single plane images from different cells. Frames e-f are z-stacks of 8 optical sections of 3.50 \( \mu \text{m} \) and show composite fluorescence (d), GFP fluorescence (e) and chloroplast autofluorescence (f). Frame g is the single plane, bright-field image for d-f. GFP fluorescence labels the cortical ER (tubules and cisternae), cytoplasmic ER and nuclear envelope (arrows). Scale bar = 10\( \mu \text{m} \)
Figure 3.4: *AtPQL6* localises to the ER.

Tobacco leaf epidermal cells expressing *AtPQL6*-GFP fusion protein. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-c are single plane images from different cells. Frames e-f are z-stacks of 12 optical sections of 3.50 μm and show composite fluorescence (d), GFP fluorescence (e) and chloroplast autofluorescence (f). Frame g is the single plane, bright-field image for d-f. GFP fluorescence labels the cortical ER (tubules and cisternae), cytoplasmic ER and nuclear envelope (arrows). Scale bar = 10μm
3.2.3 Co-localisation of AtPQL4 and AtPQL6 with ER marker

To confirm the ER localisation patterns of AtPQL4 and AtPQL6, the fusion proteins were co-expressed with an ER lumenal marker protein. A construct in which the ER retention signal HDEL was fused to the C-terminus of the yellow fluorescent protein (YFP) was already available in the laboratory. Expression of fusion protein was driven by the strong constitutive $P_{35S}$ promoter. Confocal images of tobacco cells expressing the YFP-HDEL fusion protein are shown in Figure 3.5. Frames a-c are projections of image stacks. Frame d is the single plain bright-field image for frames a-c. YFP fluorescence labelled the typical ER as has been reported in previous studies (Brandizzi et al., 2003; Sparkes et al., 2009).

For co-localisation experiments tobacco leaves were infiltrated with Agrobacterium containing the appropriate constructs at a ratio of 0.2 (OD$_{600}$ nm) (see chapter 2, Molecular cloning procedures section). Figure 3.6 and 3.7 present co-localisation data for AtPQL4-GFP and AtPQL6-GFP with YFP-HDEL, respectively. Frames a-e are projections of image stacks. Frame f is the single plain bright-field image for frames a-e. Both GFP and YFP fluorescence signals produced network-like patterns (Figures 6(b,c) and 7(b,c)). Figures 3.6d and 3.7d show merged images that clearly show co-localisation of the two fluorescence signals and confirmed the ER localisation of the two AtPQL proteins. Quantification of the co-localisation events are shown in Figures 3.6g and 3.7g and was achieved by using fluorescence intensity analysis and Pearson’s correlation analysis. Fluorescence intensity analysis was performed along a line scan (red arrows, Figure 3.6g and 3.7g). Fluorescence intensities were measured in arbitrary units and plotted against the distance from the origin of the line scan. The coefficient produced by the Pearson’s correlation analysis provided evidence for the degree of the co-localisation between the two fluorophores. The value can range from -1 to 1, with 1 indicating complete positive correlation, 0 no correlation and -1 negative correlation (Bolte and Cordelières, 2006). AtPQL4-GFP and YFP-HDEL co-expression resulted in complete co-localisation (Figure 3.6g). The GFP and YFP fluorescence signals displayed the same intensity patterns and completely overlapped along the line scan. Pearson’s correlation analysis also suggested a high degree of co-localisation, with Pearson’s coefficient of 0.948. Similarly, co-expression of AtPQL6-GFP and YFP-HDEL resulted in high co-localisation (Figure 3.7g). Although GFP and YFP fluorescence intensities did differ at some points along the line scan, the two fluorescence signals generally overlapped. A high degree of co-localisation was also evident from the GFP/YFP scatter plot, with a Pearson’s coefficient of 0.920.
Figure 3.5: HDEL labels the ER.

Tobacco leaf epidermal cells expressing YFP-HDEL fusion protein. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-c are z-stacks of 25 optical sections of 1.15 μm and show composite fluorescence (a), YFP fluorescence (b) and chloroplast autofluorescence (c). Frame d is the single plane, bright-field image for a-c. YFP fluorescence is distributed across tubular ER, cisternal ER and nuclear envelope. Scale bar = 10μm
Figure 3.6: Co-localization of AtPQL4 with ER marker.

Tobacco leaf epidermal cells co-expressing AtPQL4-GFP and YFP-HDEL. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-e are z-stacks of 19 optical sections of 1.15 μm. Frames show (a) composite fluorescence, (b) GFP fluorescence, (c) YFP fluorescence, (d) GFP and YFP overlay (yellow color) and (e) chloroplast autofluorescence. Frame f is the single plane, bright-field image for a-e. In frame g fluorescence intensities of GFP (green line) and YFP (red line) are plotted against position on a line scan (red arrow). Fluorescence intensity is shown in arbitrary units. GFP/YFP scatter plot for frame d indicating strong positive correlation between two signals (P = 0.948). GFP and YFP co-localise to cortical ER and nuclear envelope. Scale bar = 10μm
Figure 3.7: Co-localization of *AtPQL6* with ER marker.

Tobacco leaf epidermal cells co-expressing *AtPQL6*-GFP and YFP-HDEL. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-e are z-stacks of 20 optical sections of 1.15 μm. Frames show (a) composite fluorescence, (b) GFP fluorescence, (c) YFP fluorescence, (d) GFP and YFP overlay (yellow color) and (e) chloroplast autofluorescence. Frame f is the single plane, bright-field image for a-e. In frame g fluorescence intensities of GFP (green line) and YFP (red line) are plotted against position on a line scan (red arrow). Fluorescence intensity is shown in arbitrary units. GFP/YFP scatter plot for frame d indicating strong positive correlation between two signals (P = 0.920). GFP and YFP co-localise to cortical ER, cytoplasmic ER and nuclear envelope.

Scale bar = 10 μm
3.3 Discussion

3.3.1 Subcellular localisation of AtPQL2 protein

The subcellular localisation data for AtPQL2-GFP provide the first in vivo evidence for tonoplast localisation. The results are consistent with the previously detected tonoplast localisation of AtPQL1, which is a close homologue of the AtPQL2 (Pattison, 2008). To confirm the tonoplast localisation, co-localisation experiments using tonoplast marker proteins, such as TIP3;1 and TIP2, were carried out. Although the statistical analysis of colocalisation of AtPQL2-GFP with TIP2-YFP suggested a high degree of colocalisation, this result was only obtained once as the detection of AtPQL2-GFP fusion protein was very rare. The absence of fluorescence signal might be the result of the strong constitutive P35S promoter. Overexpression of fusion proteins in combination with the Agrobacterium-mediated transformation might trigger post-transcriptional gene silencing (Johansen and Carrington, 2001; Mishiba et al., 2005). For this reason, experiments using gene silencing suppressors might facilitate the expression of AtPQL2-GFP fusion protein. In addition, expression of fluorescent-tagged AtPQL2 protein under the control of a weak or even better its native promoter could be more effective and might allow co-localisation data with fluorescent-tagged marker proteins. An equivocal tonoplast localisation of AtPQL1 and AtPQL2 argues against previously reported plasma membrane localisation of STM1, a GPCR protein from S. pombe, which is the closest homologue of these two proteins (see chapter 1). In line with its speculated function as a GPCR, STM1 was proposed to be localised in the plasma membrane with its N-terminus facing the extracellular space, where it can bind external ligands (Chung et al., 2001). It will therefore be worthwhile to co-express AtPQL2 with a plasma marker protein to exclude the possibility of plasma membrane localisation.

3.3.2 Subcellular localisation of AtPQL4 and AtPQL6 proteins

Confocal microscopical analysis of AtPQL4-GFP and AtPQL6-GFP proteins showed in typical ER pattern. The term “typical ER” refers to nuclear envelope and cortical ER. The latter describes the interconnected membrane system formed by two distinct domains of ER: the cisternae and the tubules. Tubules can extend and fuse by three-way junctions forming polygonal networks. Loss of the high angle curvature of the polygon results in the formation of flattened membrane sheets, known as cisternae (Sparkes et al., 2009). The cortical ER is subject to dynamic modifications depending on the physiological conditions.
For instance, overexpression of GFP-tagged calnexin resulted in enlargement of cisternae (Runions et al., 2006). In addition, pathogen attacks induced cisternalisation. Takemoto et al. (2003) showed that upon inoculation of A. thaliana cotyledons with oomycete pathogens, more cisternae were formed. This shift to the cisternal form of the ER might be the consequence of increased production of proteins and lipids required for a defence response. Similar ER modifications have been observed under a range of stresses, such as temperature, radiation and water stress (Quader and Zachariadis, 2006). Moreover, the cortical ER network is in close proximity to the plasma membrane and is also continuous with the outer nuclear envelope. It was suggested that the outer nuclear envelope functions as a distinct ER domain that regulates the exchange of proteins between the nucleus and ER (Quader and Zachariadis, 2006). The outer nuclear envelope also controls the flow of membrane lipids required for the quick and proper reassembly of the nuclear envelope during the last stages of cell division (Staehelin, 1997; Quader and Zachariadis, 2006). The AtPQL6-GFP fluorescence signal also stained another distinct domain of the ER, the cytoplasmic ER. The cytoplasmic ER describes the long fast-moving strands that extend the ER membrane to distant cellular locations such as the vacuole (Sparkes et al., 2009).

The ER localisation of AtPQL4 and AtPQL6 supports the notion that they might have a similar function as their closest characterised homolog MPDU1. The human MPDU1 is considered to be a chaperone required for utilisation of Man/Glc-P-Dol as sugar donors in glycosylation (Anand et al., 2001). Although experimental data confirming ER-localisation of MPDU1 have not been published, ER localisation is assumed, as Man/Glc-P-Dol is synthesised and utilised in the ER. ER localised fluorescence of both GFP-tagged AtPQL proteins was additionally confirmed by co-localisation with YFP-HDEL fusion protein. The tetrapeptide Lys-Asp-Glu-Leu (KDEL) in mammals and His-Asp-Glu-Leu (HDEL) in the yeast S. cerevisiae have been shown to facilitate retention of proteins in the ER (Pelham, 1990). Reticuloplasmins, which are soluble proteins that function and reside in the luminal ER, carry the tetrapeptide H/KDEL at the C-terminus (Pagny et al., 1999, Frigerio et al., 2001). Reticuloplasmins escaping from ER are retrieved back to it by the action of a receptor that recognises the H/KDEL signal. In mammals, the receptor is distributed along the intermediate compartment between ER and Golgi apparatus and the early Golgi stacks (Griffith et al., 1994). The retrieval of reticuloplasmins from post-ER compartments has been suggested, as proteins residing in the ER were found to have undergone N-glycan modifications that occur in such compartments. For instance, addition of the KDEL signal to the lysosomal glycoprotein, cathepsin D, caused the protein to localise to the ER, but it also carried the N-acetylglucosamine-1-phosphate glycan that is
formed in the cis-Golgi compartment (Pelham, 1990). However, the distance over which ER retrieval occurs and the role of the HDEL/KDEL receptor in plants is under debate (Pagny et al., 2000; Frigerio et al., 2001). The results from the co-localisation experiments with YFP-HDEL provided evidence for the ER localisation of \textit{AtPQL4} and \textit{AtPQL6} proteins. However, the observed degree of co-localisation was surprising. The \textit{AtPQL} proteins are membrane proteins and would be expected to integrate into the ER membrane, whereas YFP-HDEL is a soluble protein residing in the ER lumen. Furthermore, while localisation of \textit{AtPQL4-GFP} and \textit{AtPQL6-GFP} to typical ER is plausible, YFP-HDEL was expected to be constricted to discrete parts of the ER network.

It would be useful to perform co-expression of the \textit{AtPQL4} and \textit{AtPQL6} proteins with a marker protein that resides in the ER membrane. Such a marker could be the ER membrane-bound protein calnexin, a lectin-like protein promoting the correct folding of glycoproteins (Helenius, 1994). Moreover, beside the nuclear envelope that serves as a distinct ER domain (as explained above), there are several other functional domains within the ER network (Sparkes et al., 2009). For instance, ER exit sites (ERES) are defined as sites where anterograde protein transport from ER to Golgi takes place. Several proteins are involved in this process, such as the Sar1p guanosine triphosphatase (GTPase), Sec12 and the coat protein (COP)II components Sec24/Sec23 and Sec13/Sec31 (Hawes et al., 2008). Such proteins can be used as markers for ERES. Co-expression of \textit{AtPQL4} and \textit{AtPQL6} proteins with proteins that reside and function in discrete subdomains of the ER may provide further insights into the specific localisation and function of the two \textit{AtPQL} proteins. Finally, it would be reasonable to investigate the localisation of the \textit{AtPQL} proteins in \textit{A. thaliana} to exclude any possibility of mislocalisation due to heterologous expression. Unfortunately, infiltration of Arabidopsis seedlings with fluorescent-tagged \textit{AtPQL} proteins was not successful. Despite using a range of \textit{Agrobacterium} densities and observation by confocal laser scanning microscopy at different time points, the fluorescence signal to noise ratio in the seedlings was very low making subcellular localisation very difficult.

In conclusion, the results presented in this chapter provide new information on the subcellular localisation of the three \textit{AtPQL} proteins. They suggest localisation of \textit{AtPQL2} to the tonoplast implying functional similarity with its homolog, \textit{AtPQL1}. In addition, they confirm localisation of \textit{AtPQL4} and \textit{AtPQL6} to the ER, supporting an involvement of these proteins in glycosylation and/or other ER.
Chapter 4: Effect of overexpressing AtPQL4 and AtPQL6 on the intracellular targeting of other proteins.

4.1 Introduction

The previous chapter presented evidence for the subcellular localisation of AtPQL4 and AtPQL6 proteins to the ER obtained by the co-localisation of either AtPQL protein with an ER marker protein. However, these experiments did not exclude the possibility of residual localisation to other membranes. To assess other subcellular locations, the plasma membrane protein AtSYP121, the tonoplast proteins AtTIP2 (δ-TIP) and AtTIP3;1 (α-TIP) and the glycoproteins EFR and FLS2 were obtained as fusion proteins with GFP or YFP. Transient Agrobacterium-mediated transformation of tobacco through leaf infiltration was used. Epidermal cells were analysed by using confocal laser scanning microscopy, 72 hours post transformation.

4.2 Results

4.2.1 Co-localisation with membrane marker proteins

AtSYP121 is a member of the Soluble N-ethylmaleimide-Sensitive Fusion Protein Attachment Protein Receptor (SNARE) superfamily in Arabidopsis. AtSyp121 resides at the plasma membrane where it functions in positioning of the Shaker channel KAT1 and in non-host resistance against barley powdery mildew fungi (Bassham & Blatt, 2008). In the plasma membrane of root cells AtSyp121 forms a complex with KC1 and AKT1 (Honsbein et al., 2009). Confocal images of tobacco leaf epidermal cells transformed with SYP121-YFP fusion protein are presented in Figure 4.1. Frames a-c are projections of image stacks. Frame d is the single plain, bright-field image for frames a-c. YFP fluorescence (shown in red colour, frame b) was visible at the periphery of the cell. Fluorescent-tagged SYP121 protein was previously shown to localise to the plasma membrane in transient expression experiments (Uemura et al., 2004; Honsbein et al., 2009; Kato et al., 2010). High intensity laser light was required for detection of the YFP fluorescence signal, since expression under the control of PUBQ10 promoter was weak. Consequently, strong chloroplast autofluorescence is also seen (frame c).
Confocal images of tobacco epidermal cells co-expressing *At*PQL4-GFP or *At*PQL6-GFP with SYP121-YFP are shown in Figure 4.2 and 4.3, respectively. The frames a-e are projections of image stacks and frame f is the corresponding bright-field image. GFP fluorescence (shown in green colour) was localised at the ER, as expected (Figure 4.2b and 4.3b). Surprisingly, when co-expressed with *At*PQL4-GFP or *At*PQL6-GFP, YFP fluorescence (shown in red colour), deriving from SYP121-YFP, also labelled the ER network and the nuclear envelope (Figure 4.2c and 4.3c). Frame d displays an overlay of the two fluorescence signals, and regions where the two signals co-localise appear in yellow colour.

Fluorescence intensity analysis and Pearson’s correlation analysis confirmed the co-localisation. In Figure 4.2, the GFP/YFP scatter plot indicates a good correlation of GFP and YFP fluorescence signals, with a Pearson’s coefficient of 0.702. In addition, Figure 4.2g shows a high degree of co-localisation of the two fusion proteins at the nuclear envelope. Figure 4.2h also shows that the two fluorescence signals mostly overlapped. However, there were points along the line scan where they appear to be discrete. This might be due to the strong chloroplast autofluorescence signal that is shown in Figure 4.2c. Similar quantitative results were gained for the co-localisation experiments of *At*PQL6-GFP with SYP121-YFP (Figure 4.3g). As evident from the fluorescent intensity graph, the two fluorescence signals appeared to overlap along the greatest part of the line scan. Similarly, the GFP/YFP scatter plot showed a positive correlation of the two fluorescence signals, with a Pearson’s coefficient of 0.720.

EFR-GFP and FLS2-GFP fusion proteins (provided by Zipfel, JIC) had previously been shown to localise to the plasma membrane when expressed in *Arabidopsis* roots. Unfortunately, it was not possible to express these proteins in tobacco. Despite several modifications of the protocol, very few cells expressed the fusion proteins and GFP fluorescence was very low.
Figure 4.1: SYP121-YFP labels the plasma membrane.

Tobacco leaf epidermal cells expressing SYP121-YFP fusion protein. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-c are z-stacks of 15 optical sections of 1.46 μm. Frames show (a) composite fluorescence, (b) YFP fluorescence and (c) chloroplast autofluorescence. Frame d is the single plane, bright-field image for a-c. GFP fluorescence labels the plasma membrane.

Scale bar = 10μm
Figure 4.2: SYP121-YFP co-expressed with *At*PQL4-GFP localises to the ER.

Tobacco leaf epidermal cells co-expressing *At*PQL4-GFP and SYP121-YFP. Cells transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-e are z-stacks of 20 optical sections of 1.46 μm. Frames show (a) composite fluorescence, (b) GFP fluorescence, (c) YFP fluorescence, (d) GFP and YFP overlay (yellow color) and (e) chloroplast autofluorescence. Frame f is the single plane, bright-field image for a-e. In frame g and h fluorescence intensities of GFP (green line) and YFP (red line) are plotted against position on a line scan (red arrows). Fluorescence intensity is shown in arbitrary units. GFP/YFP scatter plot for frame d indicating positive correlation between two signals (P = 0.702). GFP and YFP co-localise to cortical ER, cytoplasmic ER and nuclear envelope. Scale bar = 10μm
Figure 4.3: SYP121-YFP co-expressed with *At*PQL6-GFP localises to the ER.

Tobacco leaf epidermal cells co-expressing *At*PQL6-GFP and SYP121-YFP. Cells transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-e are z-stacks of 26 optical sections of 1.46 μm. Frames show (a) composite fluorescence, (b) GFP fluorescence, (c) YFP fluorescence, (d) GFP and YFP overlay (yellow color) and (e) chloroplast autofluorescence. Frame f is the single plane, bright-field image for a-e. In frame g fluorescence intensities of GFP (green line) and YFP (red line) are plotted against position on a line scan (red arrow). Fluorescence intensity is shown in arbitrary units. GFP/YFP scatter plot for frame d indicating positive correlation between two signals (P = 0.720). GFP and YFP co-localises to cortical ER and nuclear envelope. Scale bar = 10μm
4.2.2 Co-localisation with tonoplast marker proteins

Co-localisation of either \textit{AtPQL4} or \textit{AtPQL6} protein with SYP121 resulted in the ER retention of the latter. The question arises whether this phenomenon is specific for SYP121. Therefore, I performed colocalisation experiments expressing the two \textit{AtPQL} proteins with the tonoplast marker proteins, TIP2 and TIP3;1. Tonoplast intrinsic proteins (TIPs) function as aquaporins to regulate the transport of water and other molecules across the tonoplast (Jauh \textit{et al.}, 1999; Gattolin \textit{et al.}, 2009). YFP fusion constructs for these vacuolar markers were already available in the lab. Figure 4.4 show tobacco leaf epidermal cells overexpressing TIP2-YFP. Frames a-c are projections of image stacks and frame d represents the brightfield for a-c. In agreement with previous studies (Hunter \textit{et al.}, 2007), YFP fluorescence (shown in red colour) was found at the tonoplast (frame b). The YFP fluorescence also appeared in small spherical structures (see arrow), known as bulbs, which are a typical feature of tonoplast labeling. Little is known about the function of these structures, but a role as reservoir of membranes to facilitate vacuole expansion and/or as distinct vacuolar subregion, where hydrolytic activities occur, has been proposed (Saito \textit{et al.}, 2002; Hunter \textit{et al.}, 2007).

Tobacco cells co-expressing TIP2-YFP with either \textit{AtPQL4}-GFP or \textit{AtPQL6}-GFP are presented in Figures 4.5 and 4.6, respectively. Frames a-e are projections of image stacks. Frame f is the brightfield image for frames a-e. As shown before, \textit{AtPQL4} and \textit{AtPQL6} proteins are localised in ER. YFP fluorescence is also seen in a lattice-like network and the nuclear envelope suggesting ER localization for TIP2 when co-expressed with \textit{AtPQL4} and \textit{AtPQL6} (Figures 4.5c and 4.6c). Co-localisation (appearing in yellow color) of the fusion proteins is more obvious in the merged images (Figures 4.5d and 4.6d). Figure 4.5g shows the fluorescence intensity graph, in which the fluorescence signals derived from TIP2-YFP and PQL4-GFP follow the same pattern. The overall higher intensity of YFP fluorescence is due to the higher-intensity laser light required to excite TIP2-YFP. In addition, the GFP/YFP scatter plot indicates a positive correlation of \textit{AtPQL4}-GFP and TIP2-YFP, with a Pearson’s coefficient of 0.755. The fluorescence intensity graph for the \textit{AtPQL6}-GFP and TIP2-YFP co-localisation event was more complex, but considerable overlap along the line scan of the two fluorescence signals was detected (Figure 4.6g). Positive correlation of GFP and YFP fluorescence signals is also evident in the GFP/YFP scatter plot, with a Pearson’s coefficient of 0.850.
Colocalisation experiments of the two GFP-tagged *At*PQL proteins were also performed with TIP3;1-YFP (data not shown) and similar results were obtained, as TIP2. Thus, co-expression with PQL4 or PQL6 leads to ER retention of both tonoplast intrinsic proteins.

**Figure 4.4: TIP2-YFP labels tonoplast.**

Tobacco leaf epidermal cells expressing TIP2-YFP fusion protein. Cells were transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-c are z-stacks of 30 optical sections of 1.46 μm and show (a) composite fluorescence, (b) YFP fluorescence and (c) chloroplast autofluorescence. Frame d is the single plane, brightfield image for a-c. YFP fluorescence labels the tonoplast.

Scale bar = 10 μm
Figure 4.5: TIP2-YFP co-expressed with AtPQL4-GFP localises to the ER.

Tobacco leaf epidermal cells co-expressing AtPQL4-GFP and TIP2-YFP. Cells transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-e are z-stacks of 20 optical sections of 1.46 μm. Frames show (a) composite fluorescence, (b) GFP fluorescence, (c) YFP fluorescence, (d) GFP and YFP overlay (yellow color) and (e) chloroplast autofluorescence. Frame f is the single plane, brightfield image for a-e. In frame g fluorescence intensities of GFP (green line) and YFP (red line) are plotted against position on a line scan (red arrows). Fluorescence intensity is shown in arbitrary units. GFP/YFP scatter plot for frame d indicating positive correlation between two signals (P = 0.745). GFP and YFP colocalises to cortical ER, cytoplasmic ER and nuclear envelope. Scale bar = 10μm
Figure 4.6: TIP2-YFP co-expressed with *AtPQL6*-GFP localises to the ER.

Tobacco leaf epidermal cells co-expressing *AtPQL6*-GFP and TIP2-YFP. Cells transformed by agroinfiltration and analyzed by confocal microscopy 72 hours post transformation. Frames a-e are z-stacks of 22 optical sections of 1.46 µm. Frames show (a) composite fluorescence, (b) GFP fluorescence, (c) YFP fluorescence, (d) GFP and YFP overlay (yellow color) and (e) chloroplast autofluorescence. Frame f is the single plane, brightfield image for a-e. In frame g fluorescence intensities of GFP (green line) and YFP (red line) are plotted against position on a line scan (red arrows). Fluorescence intensity is shown in arbitrary units. GFP/YFP scatter plot for frame d indicating positive correlation between two signals (P = 0.850). GFP and YFP colocalises to cortical ER, cytoplasmic ER and nuclear envelope. Scale bar = 10 µm
4.3 Discussion

4.3.1 Co-localisation of AtPQL4 and AtPQL6 proteins with SYP121

SNARES are thought to have an important role in membrane trafficking facilitating the flow of proteins and other molecules across the endomembrane system. The SNARE SYP121 in *Arabidopsis* has been shown to be involved in K⁺ channel trafficking. Studies using the dominant negative cytosolic Sp2 fragment showed that traffic of KAT1 to the plasma membrane is facilitated by SYP121 (Sutter *et al*., 2006b). Additionally, SYP121 promotes gating of other K⁺ channels, such as the K⁺ inward-rectifying channel AKT1. Honsbein *et al*.* (2009) demonstrated that SYP121 directly and selectively interacts with KC1 to enable assembly of SYP121 with AKT1 forming a tripartite SNARE-K⁺ complex at the plasma membrane. Root hair protoplasts of *Arabidopsis syp121, akt1* and *kc1* mutants showed a decrease in K⁺ inward current and net K⁺ uptake. These results imply a role of SYP121 in plant K⁺ nutrition. Moreover, SYP121 forms a tetrameric complex with VAMP722 and SNAP33 to promote secretion of antifungal compounds at the site of penetration of powdery mildew fungi (Kwon *et al*., 2003). *Arabidopsis syp121* mutants have been shown to be more sensitive to the barley powdery mildew demonstrating a specific role of SYP121 in non-host resistance (Bassham and Blatt, 2008).

Co-expression of SYP121 with AtPQL4 or AtPQL6 resulted in its retention in the ER. Several reports in the literature have demonstrated occasional localisation of SYP121 to intracellular compartments beside the plasma membrane. Kato *et al*.* (2010) reported that in stable transformed *Arabidopsis* seedlings grown under dark conditions, the GFP-SYP121 fusion protein localised to the vacuole. Also, studies using the GFP-tagged tobacco homolog of SYP121, NtSyr1-GFP, showed localisation of the fusion protein in ER and other small internal compartments (Di Sansebastaino *et al*., 2006). With time progressing, these observations changed, and in later stages of expression the fusion protein was detected exclusively in the plasma membrane. However, the mislocalisation of SYP121-GFP reported here was evident at all time points tested (36h, 48h, 72h and 84h post transformation). To exclude that mislocalisation of SYP121 was due to heterologous expression, the *Arabidopsis* lines 35S:AtPQL4-1 and 35S:AtPQL6-1, overexpressing AtPQL4 and AtPQL6 proteins, respectively, were transformed with P*UBQ10*-driven SYP121-YFP. Unfortunately, *Arabidopsis* infiltration posed many problems hindering detection of fluorescence (as discussed in chapter 3). To exclude the possibility that
overexpression of any ER resident proteins would result in the accumulation of SYP121 in the ER. SYP121-GFP was co-expressed with HDEL-YFP in tobacco leaf epidermal cells. As expected, localisation of SYP121 and HDEL in the plasma membrane and in the ER network, respectively (data not shown) could be clearly distinguished.

4.3.2 Co-localisation of AtPQL4 and AtPQL6 proteins with TIP2 and TIP3;1

The AtPQL-mediated ER retention was not specific for SYP121. The same phenomenon was observed when AtPQL4-GFP and AtPQL6-GFP proteins were co-expressed with TIP2-YFP and TIP3;1-YFP fusion proteins. TIPs represent a distinct group of membrane intrinsic proteins (MIP) that are highly abundant in plant vacuoles, where they mediate the transport of water, proteins, gases and small uncharged molecules across the tonoplast (Gattolin et al., 2009). Studies have shown that different TIPs are associated with distinct types of vacuoles. For instance, TIP1 (γ-TIP) is related with lytic-type vacuoles, TIP3;1 with protein storage vacuoles containing seed-type storage proteins and TIP2 with vacuoles that store proteins synthesized in response to environmental and developmental cues (Jauh et al., 1998, Hunter et al., 2007). However, when co-expressed in the same cell they generally co-localise in the central vacuole (Gattolin et al., 2009).

Both TIP2 and TIP3;1 proteins showed fluorescence in typical net-like ER structures and nuclear envelope upon co-expression with the AtPQL proteins. Recently, Gattolin et al. (2010) demonstrated that TIP3;1 shows dual localisation to plasma membrane and tonoplast during seed maturation and germination. In addition to tonoplast and plasma membrane, ER-like structures were detected, but only during the first 24 hours suggesting that accumulation in the ER reflects the passage of the protein through this compartment. The authors speculated that the dual localisation of TIP3;1 might compensate for the low number of PIPs residing in the plasma membrane of seeds. As already pointed out, the ER retention of TIP proteins in cells overexpressing AtPQL4 or AtPQL6 was observed at various time points excluding the assumption that it was due to transient overload of ER.

The observation reported in this chapter indicates that both AtPQL4 and AtPQL6 determine the time of residence of plasma membrane and tonoplast proteins in the ER. Such a role is in accordance with the results from microarray experiments, which showed differential expression of typical ER-stress marker genes (e.g BiP) in AtPQL4 and AtPQL6 mutants (Pattison, 2008). To confirm a function of AtPQL4 and AtPQL6 in ER retention,
localisation of SYP121 and TIP proteins should now be monitored and quantified in Arabidopsis lines overexpressing AtPQL4 and AtPQL6.

4.3.3 Future experiments

One question arising from the co-localisation data is whether the overexpression of the AtPQL4 and AtPQL6 proteins causes an alteration in morphology of the internal compartments hindering the transport of proteins to their final destination? For instance, the decision when a protein enters the secretory pathway or is transported to the vacuole is taken in the Golgi (Hanton and Brandizzi, 2006). Any morphological abnormality of the Golgi might inhibit protein transfer resulting in a build up of proteins in the ER. Co-expression of the two AtPQL proteins with a typical Golgi marker protein, such as ST-YFP, should be carried out to address the effects of AtPQL4 and AtPQL6 overexpression on Golgi morphology and more specifically in the anterograde ER-to-Golgi traffic. In addition, one can imagine that overexpression of AtPQL4 and AtPQL6 may rescue mutants defective in the retrograde pathway, since ER retention will reduce the transport of proteins to the Golgi. For example, it would be worthwhile to overexpress both AtPQL proteins in yeast erd1Δ and erd2Δ mutants defective in ER retention of proteins and monitor survival (Hardwick et al., 1990; Semenza et al., 1990). Chatre et al. (2005) reported that overexpression of the v-SNARE proteins, Sec22 and Memb11 in tobacco resulted in ER localisation of the Golgi marker proteins, ERD2 and ST and in ER retention of soluble cargo (sec-YFP) that was previously shown to be secreted to the apoplast. When co-expression of AtPQL4-GFP or AtPQL6-GFP with sec-YFP was attempted during this project, both fluorescence signals were detected in ER-like structures; however, sec-YFP alone was also found in the ER suggesting that the fusion construct contained an ER retention signal. Regeneration of the sec-YFP fusion protein without the ER retention signal is required before the experiments can be repeated.

Previous microarray experiments showed an upregulation of defence-related genes in AtPQL4 and AtPQL6 mutants (Pattison, 2008). Furthermore, AtPQL4 and AtPQL6 showed increased transcript levels in virus-infected plants. Since the human homolog MPDU1 is involved in ER glycosylation processes, it would be interesting to test whether overexpressing AtPQL4 and AtPQL6 proteins affects the targeting of FLS2 and EFR proteins. FLS2 and EFR are glycosylated plasma membrane proteins that specifically recognise pathogen effectors and elicit plant innate immune responses (Zipfel et al., 2006). Bacterial flagellin, ef-Tu or their peptide surrogates fls22 and elf18 are recognised by
FLS2 and EFR, respectively. Both proteins require transit through the ER to mature before reaching their final destination at the plasma membrane. Mutations in components involved in protein maturation resulted in less accumulation of EFR protein during the plant innate immune response (Zipfel et al., 2006; Li et al., 2009; Nekrasov et al., 2009). For instance, \textit{carleticulin3} (\textit{crt3}) mutants showed insensitivity to \textit{elf18}, which inhibits seedling growth in wild type plants (Li et al., 2009). However, initial attempts during the course of this project to express EFR-GFP and FLS2-GFP fusion proteins in tobacco leaves did not yield any fluorescence signal, despite the infiltration at various ratios (OD$_{600}$ nm) of \textit{Agrobacterium} and observation at several time points after infiltration.

Analysis of EFR and FLS2 localisation in \textit{AtPQL4} and \textit{AtPQL6} \textit{Arabidopsis} mutants, and growth assays of \textit{AtPQL4} and \textit{AtPQL6} mutants with \textit{fls22} and \textit{elf18} are currently underway. Since \textit{AtPQL4} and \textit{AtPQL6} have the same effect on ER retention of proteins when overexpressed, it will be of interest to see if \textit{AtPQL4} and \textit{AtPQL6} function by forming a complex. Use of bimolecular fluorescent complex formation (BiFC) technique will reveal any such interaction between the two \textit{AtPQL} proteins.

In summary, this chapter provided new information on the function of \textit{AtPQL4} and \textit{AtPQL6} proteins. Both \textit{AtPQL} proteins caused retention of the plasma membrane protein SYP121 and the tonoplast proteins TIP2 and TIP3;1 in the ER. From these and previous results, one can speculate that the two \textit{AtPQL} proteins might act as “chaperones” for proteins in the ER during glycosylation, folding and quality control.
5.1 Introduction

In this chapter I will describe further experiments to characterize the function of \textit{AtPQL4} and \textit{AtPQL6} proteins. To assess the function of the two \textit{AtPQL} proteins \textit{in planta}, a variety of \textit{A. thaliana} mutant lines and one overexpressor line were used. The homozygous knockout lines had been isolated from T-DNA insertion lines obtained from the SALK institute (Pattison, 2008). Two knockout mutant lines were used for \textit{AtPQL4}. The first, \textit{pql4-1}, has an insertion in the second exon, 224 bp 5’ of the stop codon, whereas the second, \textit{pql4-2}, has an insertion in the first exon, 109 bp 3’ of the start codon. Neither of the mutant lines produces any mRNA for \textit{AtPQL4} (Pattison, 2008). One knockdown mutant line was used for \textit{AtPQL6}, \textit{pql6-1}, having an insertion in the fifth intron. A small amount of \textit{AtPQL6} mRNA is still produced by this line (Pattison, 2008). The schematic diagram in Figure 1 represents the insertion site for each \textit{A. thaliana} line. A double mutant line, \textit{pql4-2/6-1}, and an overexpressor line for \textit{AtPQL6}, \textit{35S::AtPQL6-1}, were also used.

Previous studies have found no obvious phenotype for the single knockout lines or the overexpressor line under a variety of stresses such as salt, heat and drought (Pattison, 2008). However, this might have been the result of functional redundancy between \textit{AtPQL4} and \textit{AtPQL6}. Thus, I was interested in investigating the response of the double mutant in some of these stresses. Also, a number of additional experiments were carried out to search for physiological phenotypes. The rationale behind each experiment is described below.

5.1.1 Sensitivity to macronutrient depletion and salt stress

Depletion of macronutrients causes disturbance of ion homeostasis and depletion of essential metabolites, thus impacting on plant growth and development. Macronutrients such as nitrogen (N), phosphorus (P), sulphur (S) and magnesium (Mg) are essential for build-up of dry matter and energy, while potassium (K) and calcium (Ca) secure ion homeostasis by regulating ion transport and maintenance of water status (Amtmann and Blatt, 2008). Salinity causes ionic and osmotic stress with a detrimental effect on the fitness of the plant (Parida and Das, 2005). Because overexrepression of \textit{AtPQL4} and
AtPQL6 in tobacco leaves impacts on the targeting of plasma membrane proteins and tonoplast proteins (see chapter 4), one can speculate that the mutants are defective in ion and water transport and therefore show altered salt tolerance and/or sensitivity to macronutrient starvation.

5.1.2 Sensitivity to sugar starvation

Plant growth strongly depends on photosynthetic carbon fixation, and can be further enhanced by external sugar supply. Sugars are the principle substrates for energy generation via respiration and for biosynthesis of macromolecules such as proteins, lipids, DNA and RNA. Upon carbon starvation, cell viability is maintained by differential regulation of genes involved in transport facilitation, transcription, defence, metabolism and protein synthesis (Contento et al., 2004). Carbon starvation induces degradative pathways, such as autophagy to maintain cellular activities and compensate for the lack of carbohydrates (Rolland et al., 2006). Given the many implications of sucrose in plant fitness and its link to autophagy, it was of interest to investigate the growth of mutant lines in a range of sucrose concentrations.

Figure 5.1: T-DNA insertion sites of SALK mutant lines used.

The diagram shows the AtPQL4 and AtPQL6 genes and the T-DNA insertions sites for pql4-1, pql4-2 and pql6-1 mutant lines (Figure taken from Pattison, 2008).
5.2 Results

5.2.1 Sensitivity to macronutrient starvation

To investigate differences in sensitivity to nutrient deficiency between the mutants and wild type, four seeds of each line were sown directly on agar plates supplemented with 1/3, 1/10, 1/30, 1/100 or 1/300 MS. Figure 5.2 shows representative images of these plants 9 days after germination. Dilution of the growth media clearly inhibited growth but no noticeable differences were seen between wild type and mutants.

Figure 5.3 shows the quantitative data for shoot and root fresh weight of the six genotypes used. The fresh weight was measured 14 days after germination. The shoot and root fresh weight of all lines decreased as the nutrient concentration decreased (Figure 5.3A and B). The shoot and root fresh weight of the double knockout mutant and overexpressor line was generally lower than of the wild type and the single knockout mutant lines, but this difference was not statistically significant. In higher concentrations, more variation between mutants and wild type was observed. Figures 5.3C and D presents the response to nutrient depletion within each line relative to the sufficient medium (1/3 MS). There is no obvious change in sensitivity among all the genotypes. To analyze further the sensitivity of the mutants in each condition, data were plotted relative to the wild type profile in each condition (Figures 5.3 E and F). Again, no significant difference was detected. The total root size (i.e. length of both main root and lateral roots) was measured 9 days after germination using EasyRhizo software (Armengaud et al., 2009). In all six genotypes the total root size decreased as the nutrient concentration decreased (data not shown). The mutant lines and overexpressor line did not show any significant difference in the sensitivity of root growth to nutrient deficiency compared to the wild type plants.
Figure 5.2: Effects of macronutrient starvation on *A. thaliana* (Col0) wild type, *AtPQL4* and *AtPQL6* mutants and *AtPQL6* overexpressor line.

Four seedlings each, of wild type or indicated *AtPQL* line, grown on agar plates (120 x 120) containing media of various MS strength. Plants were grown under continuous light with intensity of approximately 120 μmol m⁻² sec⁻¹. Photographs were taken 9 days after germination.
Figure 5.3: Quantification of sensitivity of *A. thaliana* (Col0) wild type, *AtPQL4* and *AtPQL6* mutants and *AtPQL6* overexpressor line to nutrient depletion.

Shoots and roots were harvested and weighed 14 days after germination. A), B) Bars represent the average fresh weight of shoots and roots, respectively, of each line in each condition. Error bars represent ± standard error (n=12 plants). C), D) Bars represent the average relative fresh weight of shoots and roots, respectively, of each line compared to sufficient medium in each condition. E), F) Bars represent the average relative fresh weight of shoots and roots, respectively, of each line compared to wild type within each condition. Error bars represent ± standard error (n=3 replicates of 4 plants).
5.2.2 Sensitivity to salt and osmotic stress

To investigate the sensitivity to salt tolerance, six seeds of each mutant or wild type were sown on plates containing control medium and control medium supplemented with either 50 mM KCl or 50 mM NaCl. Figure 5.4 shows examples of scanned images taken from these plants, 9 days after germination. Inhibition of growth was observed on media supplemented with 50 mM KCl and 50 mM NaCl, with the latter exhibiting a stronger effect on growth. No obvious changes were seen between wild type and mutants.

The fresh weight of shoot and roots was measured 14 days after germination. Figure 5.4A and B show the average fresh weight of shoots and roots in each condition, respectively. The pql4-1 knockout mutant did not show sensitivity on growth to salt and osmotic stress, while the growth of pql4-2 knockout mutant was not strongly affected on 50mM KCl supplemented media. No significant difference was detected between the mutants and wild type. The growth response of the six lines compared to the control medium is shown in Figures 5.4C and D. The two AtPQL4 knockout mutants were less sensitive to osmotic stress and the pql4-1 was also less sensitive to salt stress. The response to salt and osmotic stress of each line within each condition relative to the wild type profile is presented in Figures 5.4E and F. No significant differences in the sensitivity to salt and osmotic stress were seen between the knockout and overexpressor lines comparing to wild type. Table 5.1 presents the percentage of water content of shoots and roots of each line within each condition. No differences in water content were observed between the mutant and wild type plants.
Figure 5.4: Quantification of sensitivity of *A. thaliana* (Col0) wild type, *AtPQL4* and *AtPQL6* mutants and *AtPQL6* overexpressor line to salt and osmotic stress.

Shoots and roots were harvested and weighed 14 days after germination. A), B) Bars represent the average fresh weight of shoots and roots, respectively, of each line in each condition. Error bars represent ± standard error (n=12). C), D) Bars represent the average relative fresh weight of shoots and roots, respectively, of each line compared to control medium in each condition. E), F) Bars represent the average relative fresh weight of shoots and roots, respectively, of each line compared to wild type within each condition. Error bars represent ± standard error (n=2 replicates of 6 plants).

Table 5.1: Percentage of water content of shoots and roots of the wild type and *Arabidopsis* lines.

<table>
<thead>
<tr>
<th>Percentage of Water Content (%)</th>
<th>Shoots</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt</td>
<td>pql 4-1</td>
</tr>
<tr>
<td>Control Medium</td>
<td>98.9</td>
<td>95</td>
</tr>
<tr>
<td>+50 mM KCl</td>
<td>98.5</td>
<td>99.8</td>
</tr>
<tr>
<td>+50 mM NaCl</td>
<td>97.7</td>
<td>96.6</td>
</tr>
</tbody>
</table>
5.2.3 Sensitivity to sucrose starvation

The sensitivity of growth of the six lines to sucrose was determined by growing three seedlings on 0.5 MS media supplemented with 0 %, 0.5 %, 1 %, 3 % and 4 % of sucrose. Figure 5.5 shows example images of the six different genotypes taken 9 days after germination. No noticeable differences were detected between different concentrations of sucrose, and the mutant and wild type plants.

Figure 5.6 presents quantitative data for shoot and root fresh weight of the mutants and wild type. The fresh weight of shoots and roots was measured 14 days after germination. Inhibition of growth on medium without sucrose was detected for all mutants, except *pql4-1* (Figure 5.6A and B). In the other conditions, no significant change in growth was observed. Plants grown on media supplemented with 1% sucrose confirmed the growth pattern of the lines measured on sufficient medium in macronutrient starvation experiment, which contained 1% sucrose. The response to sucrose starvation in each line was therefore analysed relative to the 1% sucrose medium and is presented in Figure 5.6C and 5.6D. Again all the mutants, except *pql-4-1*, showed a higher sensitivity to low sucrose condition. Because differences vary among the six lines, sensitivity to sucrose starvation was investigated for each line relative to wild type within each condition (Figure 5.6E and 5.6F). The knockout and overexpressor lines showed stronger dependence on sucrose than wild type.
Figure 5.5: Effects of sucrose starvation on *A. thaliana* (Col0) wild type, *AtPQL4* and *AtPQL6* mutants and *AtPQL6* overexpressor line.

Three seedlings each, of wild type or indicated *AtPQL* line, grown on agar plates (120 mm X 120 mm) containing 0.5 MS medium supplemented with different concentrations of sucrose (0 %, 0.5 %, 1 %, 3 % and 4 %). Plants were grown under continuous light with an intensity of approximately 120 μmol m⁻² sec⁻¹. Photographs were taken 9 days after germination.
Figure 5.6: Quantification of sensitivity of *A. thaliana* (Col0) wild type, *AtPQL4* and *AtPQL6* mutants and *AtPQL6* overexpression line to sucrose starvation.

Shoots and roots were harvested and weighed 14 days after germination. A), B) Bars represent the average fresh weight of shoots and roots, respectively, of each line in each condition. C), D) Bars represent the average relative fresh weight of shoots and roots, respectively, of each line compared to control medium in each condition. E), F) Bars represent the average relative fresh weight of shoots and roots, respectively, of each line compared to wild type within each condition. Error bars represent ± standard error (n=3 replicates of 3 plants).
5.3 Discussion

In this study several mutant lines for \textit{AtPQL4} and \textit{AtPQL6} genes were used to facilitate functional characterization of the two proteins. If \textit{AtPQL4} and \textit{AtPQL6} are involved in Man/Glc-P-Dol dependent glycosylation, one could expect that mutations in these two genes will lead to gamete and embryo lethal phenotypes, as this type of glycosylation is essential for gametic transmission through pollen. As discussed in chapter 1, several mutants of genes with a role in Man/Glc-P-Dol synthesis showed embryo lethal phenotypes. Isolation of homozygous lines for \textit{AtPQL4} and \textit{AtPQL6} genes suggested that none of the mutations are gamete and embryo lethal. However, Pattison (2008) reported a small reduction in the gametic transmission of both \textit{pql4-1} and \textit{pql4-2}, which was consistent with observations for mutants affected in Man/Glc-P-Dol glycosylation (see chapter 1). Given the overlap in sub-cellular localisation of \textit{AtPQL4} and \textit{AtPQL6}, it is likely that the lack of phenotype is the result of functional redundancy between the two proteins. However, \textit{pql4-2/6-1} double mutants were also viable. In addition, \textit{pql6-1} is not a null mutation, as small amount of transcript is still produced that might explain the lack of a strong phenotype for both the \textit{pql6-1} and \textit{pql4-2/6-1} mutants.

No obvious phenotype was observed at the whole plant level, when all mutants were tested in different condition with the exception of sucrose starvation. All mutant lines, except \textit{pql4-1}, showed a significant reduction in growth on media without sucrose, as determined by shoot and root fresh weight measurements. Sucrose depletion leads to arrest in growth as protein, lipid and amino acid synthesis are reduced. Sugar catabolism is substituted by protein and lipid catabolism allowing cellular homeostasis to be maintained. Two degradative pathways, the selective ubiquitin proteasome-depedent pathway and the non-selective vacuolar autophagy, mediate protein breakdown (Yu, 1999; Roland \textit{et al}., 2006). Rose \textit{et al} (2006) reported the up-regulation of autophagy genes (\textit{AtATG}) when 4 day-old \textit{A. thaliana} suspension-cultured cells were transferred to medium with no sucrose. Several studies have suggested an association between autophagy and ER function (Ishihara \textit{et al}., 2001; Reggiori \textit{et al}., 2004). For instance, ER stress caused by addition of dithiothreitol (DTT) and tunicamycin facilitated the formation of pre-autophagosomal structure (PAS) in \textit{atg11Δ} cells defective in assembly of PAS. As discussed in chapter 1, ER stress stimulates UPR to relieve the stress by inducing genes involved in protein folding and ERAD pathway. Additionally, it is suggested that autophagy functions as a backup to the ERAD pathway if the latter is saturated by degradative substrates (Yorimitsu \textit{et al}., 2007). It is known that proteins involved in folding processes are also implicated in preparing
misfolded proteins for degradation (Liu and Howell, 2010). Hence, one can speculate that the phenotype observed in sucrose-free medium might be the result of impaired flow of degradative proteins to autophagosomes inhibiting protein catabolism. This might be also consistent with the putative function of \textit{AtPQL4} and \textit{AtPQL6} proteins as folding helpers that was suggested by the co-localisation experiments (see chapter 4). Although several conditions reported to cause UPR were tested, it would be useful to investigate the effects of tunicamycin on the mutants as the former blocks the N-glycosylation process and triggers UPR (see chapter 1).

In conclusion, phenotypic analysis of mutants did not yield a great insight into the function of \textit{AtPQL4} and \textit{AtPQL6} proteins. However, the arrested growth of the mutants compared to wild type on sucrose-free media could possibly implicate a function for the two proteins in autophagy-related processes, which should be further investigated in the future.
Chapter 6: General Discussion

6.1 Introduction

The main objective of this thesis was the functional characterisation of two novel proteins, **AtPQL4** and **AtPQL6** from *AtPQL* family from *A. thaliana*. This was achieved by following three different experimental approaches: 1) the subcellular localisation of the two *AtPQL* proteins and functional characterisation 2) at a molecular cellular level and 3) at whole-plant level. Discussion of each of these approaches is made in chapters 3, 4 and 5, respectively. This chapter presents the overall information gained on *AtPQL4* and *AtPQL6* as well as suggests future experiments to answer questions that arose concerning the function of the two *AtPQL* family, with a focus being on *AtPQL4* and *AtPQL6*. The *AtPQL1*, *AtPQL2* and *AtPQL3* showed high identity in amino acid sequence with the fission yeast plasma membrane protein STM1 (Pattison, 2008), which functions as a G-protein coupled receptor (Chung *et al*., 2001). Human MPDU1 is involved in Man-P-Dol dependent glycosylation processes, such as N-glycosylation (Anand *et al*., 2001; Kranz *et al*., 2001, Schenk *et al*., 2001) and shows high sequence identity with the *AtPQL4* and *AtPQL6* (Pattison, 2008). Overexpression of *AtPQL4* and *AtPQL6* proteins resulted in retention of membrane proteins in the ER. ERS1, a PQL protein from *S. cerevisiae*, was identified as a suppressor of the secretion of ER resident protein in *erd1Δ* mutants. Human CTNS is the closest homologue of *AtPQL5* (Pattison, 2008) and is involved in H⁺-driven cystine transport from lysosomes to cytosol (Attard *et al*., 1999; Kalatzis *et al*., 2001). The hygromycin sensitive phenotype of *ers1Δ* yeast mutants was reversed by expression of CTNS (Gao *et al*., 2005).

The novel function of *AtPQL4* and *AtPQL6* in retaining plasma membrane and tonoplast proteins in the ER, discovered in this project, suggests that the previously observed phenotypes of MPDU1, ERS1 and CTNS are in fact different sides of the same “coin”. For example, one could argue that the role of MPDU1 for N-glycosylation, the molecular basis of which has not yet been identified, is in fact linked to retaining proteins for a sufficient time in the ER membrane. This could concern either proteins to be glycosylated or the enzymes that act in different steps of N-glycosylation, or subsets of these in ER membrane associated functional complexes. A role of LEC35/MPDU1 as “chaperone” has indeed been proposed on the basis of the combined experimental data for this protein (Mark Lehrman, personal communication).
Figure 6.1: Information on the function of all the members of AtPQL family.

The diagram shows all the members of AtPQL family and their closest homologues. The sub-cellular localisation of each PQL protein is indicated. The proposed function is indicated with red color. Question marks indicate lack of information on the function of AtPQL proteins. 

AtPQL1, 2 and 3 are localised to the tonoplast and show a high sequence identity with plasma membrane protein STM1, a putative G-protein coupled receptor. AtPQL4 and AtPQL6 are localised in the ER and show high sequence identity with the human protein MPDU1 that is involved in N-glycosylation. Overexpression of AtPQL4 and AtPQL6 resulted to ER retention of plasma membrane and tonoplast proteins. The tonoplast-localised protein ERS1 of S.cerevisiae was isolated as suppressor of erd1A yeast mutants defective in ER retention of proteins. Tonoplast CTNS protein is a proton-coupled cystine transporter and is the closest homologue of AtPQL5, which localises to the tonoplast. CTNS complemented the hygromycin sensitive phenotype of ers1A.
6.2 Is there a functional homology with LEC35/MPDU1?

AtPQL4 and AtPQL6 are the closest homologues of the mammalian PQL protein LEC35/MPDU1. LEC35/MPDU1 is involved in all Man-P-Dol dependent glycosylation processes, such as N-glycosylation and GPI anchoring (Anand et al., 2001). The ER localisation of the two AtPQL proteins is in agreement with a putative role in N-glycosylation. The differential regulation of ER stress-related genes in AtPQL4 and AtPQL6 mutant and overexpressor lines additionally argues in favor of N-glycosylation (Pattison, 2008).

A possible way to address the functional homology of AtPQL4 and AtPQL6 with LEC35/MPDU1 is to express the two AtPQL proteins in lec35 CHO cells and check for complementation. lec35 mutants showed less sensitivity to a combination of glycosylation inhibitors than the wild type cells (Lehrmann and Zeng, 1989). HPLC analysis of lec35 mutants showed an accumulation of Man$_3$GlcNAc$_2$-P-P-Dol instead of the mature LLO, Glc$_3$Man$_9$GlcNAc$_2$-P-P-Dol (Lehrmann and Zheng, 1989). Use of fluorophore-assisted carbohydrate electrophoresis (FACE) analysis will allow visualisation of LLOs from AtPQL4 and AtPQL6 mutants. FACE technique involves the cleavage of LLOs from dolichol with mild acid and the resolution of the removed LLOs on polyacrylamide gel according to size (Gao and Lehrman, 2002). If the two AtPQL proteins are functional homologous to LEC35/MPDU1, an accumulation of the intermediate oligosaccharide is expected. Mutants defective in LLO synthesis showed a hypersensitive phenotype upon treatment with tunicamycin, which inhibits the first step of lipid-linked oligosaccharide (LLO) synthesis (see 1.6 section, Zhang et al., 2008). Phenotypic analysis of AtPQL4 and AtPQL6 mutants and overexpressors treated with tunicamycin should be carried out to check for increased sensitivity.

6.3 Do AtPQL4 and AtPQL6 have a “chaperone” role?

As shown in chapter 4, overexpression of AtPQL4-GFP and AtPQL6 fusions resulted in retention of plasma membrane and tonoplast proteins in the ER. These data indicate a potential role of AtPQL4 and AtPQL6 proteins in retaining proteins in the ER for sufficient time to allow ER quality control, including N-glycosylation, to take place. Several questions arose from these results and potential approaches to address such questions are discussed in chapter 4 (see 4.3.3 section). For instance, investigation of an altered
morphology of the ER resulting from overexpressing \textit{AtPQL4} and \textit{AtPQL6} should be undertaken, as it may cause disturbance of the ER exit sites (ERES) and prevent ER export of proteins. Complementation of yeast \textit{erd1}Δ, \textit{erd2}Δ and \textit{ers1}Δ mutants defective in ER retention with \textit{AtPQL4} and \textit{AtPQL6} might also confirm a function as “chaperones”.

Although, Pattison (2008) did not observe complementation of the \textit{ers1}Δ mutant hygromycin sensitive phenotype by any member of the \textit{AtPQL} family, it will be useful to repeat the same experiments, as he reported a lack of hygromycin phenotype of the \textit{ers1}Δ mutants. Complementation of \textit{ers1}Δ by \textit{AtPQL4} and/or \textit{AtPQL6} would argue in favor of an involvement in ER retention machinery, as \textit{ers1}Δ was isolated in a screen for suppression of \textit{erd1}Δ mutants defective in retention of ER lumenal proteins (Gao et al., 2005). If the chaperone function is valid, then a new mechanism of ER retention independent of the HDEL/KDEL signal might occur in plants. Such information could be valuable for the production of plant-made pharmaceuticals without having the plant-specific post-translational modifications that are immunogenic to humans (Gomord et al., 2010).

6.4 Are \textit{AtPQL4} and \textit{AtPQL6} essential for plant fitness?

A further indication of a “chaperone” role of \textit{AtPQL4} and \textit{AtPQL6} proteins derived from the observed reduced growth of \textit{AtPQL4} and \textit{AtPQL6} mutants in low sucrose. Carbon starvation is known to trigger degradative pathways to ensure protein catabolism and therefore cellular homeostasis by inducing unfolded protein response (UPR) (Reggiori et al., 2004; Rose et al., 2006). It is known that folding factors are also implicated in UPR to ensure ER quality control (Ceriotti and Roberts, 2006). It will be thus interesting to try a variety of stresses, such as tunicamycin and DTT that induce UPR in \textit{AtPQL4} and \textit{AtPQL6} mutants and check for hypersensitive phenotype.

One could expect that mutations on proteins having a role in protein folding, N-glycosylation and ER quality control processes will have a detrimental effect on plant fitness. The lack of embryo or gametic lethal phenotype might be the result of redundancy between \textit{AtPQL4} and \textit{AtPQL6}. Epistatic complementation of the mutants might also explain the lack of a strong phenotype. Furthermore, \textit{AtPQL6} mutant is a knock-down rather than knock-out mutant and thus produces a small level of transcript. Additionally, no information on the amount of protein produced from either \textit{AtPQL4} or \textit{AtPQL6} gene is available, at this stage. For this reason, it could be worthwhile to use the moss \textit{Physcomitrella patens} as another system to establish the function of PQL proteins because
it is an easy and feasible system for targeted ‘knockout’ of genes by homologous recombination.
Appendix A

Table A-1: Primer sequences of AtPQL4 and AtPQL6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AGI</th>
<th>Primers</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPQL4</td>
<td>At5g59470</td>
<td>Forward Primer CTAGGAATCGACTTGAGCTG</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse Primer GAAGCTTAACCGTCATTGAA</td>
<td></td>
</tr>
<tr>
<td>AtPQL6</td>
<td>At4g07390</td>
<td>Forward Primer GCATAAAGGTCTTCCCCCTTTT</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse Primer GATTAATCTGTCCAGCAAGC</td>
<td></td>
</tr>
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</table>

Table A-2: Other primer sequences.

<table>
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<th>Gene</th>
<th>Primers</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMV B-JI</td>
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<tr>
<td></td>
<td>Reverse Primer AAC TTA CCG TAT GCT AGA TTA CCT</td>
<td></td>
</tr>
<tr>
<td>18S RNA</td>
<td>Forward Primer CGT GAT CGA TGA ATG CTA CC</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer GGG GTT TGT TGC ACG TAT TA</td>
<td></td>
</tr>
</tbody>
</table>

Appendix B

CD is provided with supplementary images for Chapter 4

- Co-localisation of AtPQL4-GFP with SYP121-YFP
- Co-localisation of AtPQL6-GFP with SYP121-YFP
- Co-localisation of AtPQL4-GFP with SYP121-YFP
- Co-localisation of AtPQL6-GFP with SYP121-YFP
List of References


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