# Role of the plasma membrane-associated SEC14-like phosphatidylinositol transfer protein PATELLIN 2 in the dynamic regulation of iron acquisition in *Arabidopsis thaliana*

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*Karolin Montag*
Düsseldorf, 26.11.2019

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# 1. Preface

In the beginning of this work the results will be summarized. Next, the introduction will provide a brief overview of the importance of lipid dynamics, membrane composition and the role of SEC14-like phosphatidylinositol transfer proteins in plant cells. Additionally, the role of IRT1 in iron acquisition will be discussed. At the end of the introduction the aims of this thesis are enumerated. The three manuscripts presented here are building the basis of this thesis. Finally, the results obtained are discussed.

Manuscript 1

**Phylogenetic analysis of plant multi-domain SEC14-Like phosphatidylinositol transfer proteins and structure-function properties of PATELLIN 2**

Karolin Montag*, Jannik Hornbergs, Rumen Ivanov and Petra Bauer

In the first manuscript the evolution of the SEC14-like phosphatidylinositol transfer protein superfamily in the green lineage is evaluated by phylogenetic analysis. Additionally, the *Arabidopsis thaliana* SEC14-GOLD protein PATELLIN 2 (PATL2) is used as an example to demonstrate the functions of its different domains in lipid binding and membrane association.

Manuscript 2

**SEC14L-PITP PATL2 interacts with IRT1 and protects membranes from oxidative damage in Arabidopsis**

Karolin Montag*, Tzvetina Brumbarova, Regina Gratz, Kalina Angrand, Rubek Merina Basgaran, Rumen Ivanov and Petra Bauer

In this manuscript the interaction of PATL2 with IRON-REGULATED TRANSPORTER 1 (IRT1) is evaluated. Furthermore, the role of PATL2 in iron acquisition and its contribution to membrane integrity is analyzed.

Manuscript 3

Review **- Structure and function of SEC14-like phosphatidylinositol transfer proteins with emphasis on SEC14-GOLD domain proteins**

Karolin Montag*, Rumen Ivanov and Petra Bauer

Finally, we collect and review the knowledge of SEC14-like phosphatidylinositol transfer proteins, with special focus on SEC14-GOLD proteins and their regulatory role in complex cellular pathways through lipophilic ligand binding and protein-protein interaction.

## 2. Summary

Although iron is an essential micronutrient for the model plant *Arabidopsis thaliana* (Arabidopsis), overaccumulation of iron negatively affects plant survival. Hence, maintaining a balanced iron level is essential and requires tightly regulation of iron uptake through IRON-REGULATED TRANSPORTER 1 (IRT1), which is the main iron importer in Arabidopsis roots. While it is known that IRT1 activity is regulated on transcriptional level and on protein level through protein-protein interactions and cellular trafficking, the exact mechanism controlling IRT1 activity is not fully discovered till now. In this thesis, the SEC14-GOLD protein PATELLIN 2 (PATL2), which belongs to the SEC14-like phosphatidylinositol transfer protein (SEC14L-PITP) family, is presented as an interaction partner of IRT1.

SEC14L-PITPs contribute to membrane identity and fulfill regulatory functions in membrane trafficking by their ability to sense, bind, transport and exchange lipophilic substances between membranes, such as phosphatidylinositol, phosphoinositides and α-tocopherol. Arabidopsis exhibits several SEC14L-PITPs with a modular domain structure. In particular, SEC14-GOLD proteins are associated with cell division, plant development, and plant stress response. The contribution of their individual domains to protein function is poorly analyzed till now. This study contributes to the understanding of SEC14L-PITP evolution in the green lineage by revealing an increased number and modular complexity of SEC14L-PITPs in higher plants compared to green algae. The increasing number and complexity of SEC14L-PITPs might reflect the high versatility of roles addressed in adaptation to dry land. The first SEC14-GOLD proteins appeared in mosses. Through the example of PATL2 we analyzed the domain functions of SEC14-GOLD proteins regarding lipid binding, membrane association and protein-protein interaction. While the CTN-SEC14 module is essential for general membrane attachment of PATL2, the C-terminal GOLD domain is critical for the plasma membrane association by binding selectively to phosphatidylinositol-4,5 biphosphate. The long unstructured N-terminus of SEC14-GOLD proteins is unique to the plant kingdom and differs in its sequence for each protein. We found that the N-terminal extension in the case of PATL2 is essential for protein-protein interaction with IRT1. Detailed characterization of PATL2 function in Arabidopsis revealed that PATL2 negatively affects iron reductase activity and lipid peroxidation in roots, but positively influences growth of IRT1-complemented *fet3 fet4* yeast. Taken together, these results suggest a role of PATL2 in regulation of iron acquisition on protein level and membrane protection by keeping the iron level in a proper non-toxic but sufficient range. This leads to the conclusion that SEC14-GOLD proteins are not only able to control membrane trafficking, and the phospholipid-signaling pathway, but also actively contribute to membrane integrity and plant stress response by interacting with critical membrane proteins and preventing membrane damage.

## 3. Zusammenfassung

Obwohl Eisen ein essentieller Micronährstoff für *Arabidopsis thaliana* (Arabidopsis) ist , führt eine Anreicherung zum Tod der Pflanze. Die Kontrolle der Aktivität von IRON-REGULATED TRANSPORTER 1 (IRT1), dem wichtigsten Eisenimporter in Wurzeln von Arabidopsis, ist daher essentiell, um einen ausreichenden, aber ungiftigen Eisenvorrat bereitzustellen. Die Aktivität von IRT1 wird sowohl auf der Ebene der Transkription als auch auf Proteinebene reguliert. Auf der Proteinebene wird die IRT1 Aktivität durch Protein-Protein-Interaktionen und Membranbewegungen kontrolliert. In dieser Arbeit wird das SEC14-GOLD Protein PATELLIN 2 (PATL2) als ein IRT1-Interaktionspartner präsentiert. SEC14-GOLD Proteine sind Mitglieder der SEC14-ähnlichen Phosphatidylinositol Transferprotein (SEC14L-PITP) Familie. Diese übernehmen regulatorische Aufgaben in Bezug auf Membranidentität und Membranbewegungen innerhalb der Zelle, da sie die Eigenschaft besitzen lipophile Substanzen zu erkennen, zu transportieren und zwischen Membranen auszutauschen. Höhere Pflanzen, wie Arabidopsis, weisen eine große Anzahl an SEC14L-PITPs auf. Viele dieser SEC14L-PITPs haben außerdem mehrere Domänen. Besonders SEC14-GOLD Proteine werden mit Pflanzenentwicklung, Zellteilung und der Reaktion der Pflanze auf Stress in Zusammenhang gebracht. Die genaue Aufgabe ihrer verschiedenen Domänen ist bis heute nicht genau untersucht. Diese Arbeit soll dazu beitragen die Evolution von SEC14L-PITPs bei Pflanzen zu verstehen. Wir konnten zeigen, dass während der Entwicklung von Landpflanzen die Anzahl und Komplexität von SEC14L-PITPs zugenommen hat. Diese Beobachtung ist vielleicht auf die Anpassungen und die erweiterten Aufgaben im neuen, trockenen Lebensraum zurückzuführen. Die ersten SEC14-GOLD Proteine bei Pflanzen sind bei Moosen gefunden worden. Am Beispiel von PATL2 haben wir die Funktion der einzelnen Domänen analysiert. Dabei wurde ein besonderes Augenmerk auf die Lipid- und Membranbindung des Proteins gelegt. Wir konnten zeigen, dass das CTN-SEC14 Modul wichtig für die generelle Bindung des Proteins an Membranen ist, während die C-Terminale GOLD Domäne wichtig für die Bindung an die Plasmamembran ist, da sie selektiv Phosphatidylinositol-4,5 Biphosphat bindet. Der unstrukturierte N-Terminus von SEC14-GOLD Proteinen ist einzigartig für das Pflanzenreich und unterscheidet sich bei jedem SEC14-GOLD Protein. Der N-Terminus ist essentiell für die Interaktion von PATL2 mit IRT1.

Die Charakterisierung von PATL2 zeigt, dass PATL2 einen negativen Effekt auf die Eisenreductaseaktivität und Lipidperoxidationsrate in Wurzeln von Arabidopsis hat. Allerdings scheint PATL2 die Zellfitness positiv zu beeinflussen. Diese Ergebnisse verdeutlichen, dass PATL2 möglicherweise auf Proteinebene ein Regulator der Eisenaufnahme ist, da es die Zerstörung der Zellmembrane verhindert und auch den Eisengehalt der Zelle kontrolliert. Zusammen mit Daten aus der Literatur bedeutet dies für uns, dass SEC14-GOLD Proteine nicht nur Aufgaben in der Membranbewegung und dem Phospholipidsignalweg übernehmen, sondern auch aktiv zum Membranerhalt und der Reaktion der Pflanze auf Stress beitragen, indem sie mit kritischen Membranproteinen interagieren und Schäden an Membranen verhindern.

# 4. Introduction

## 4.1 Membrane identity is driven by lipid dynamics and composition

Cellular membranes are hydrophobic permeable barriers shielding their enclosed space from the outer environment. Such barriers allow to maintain and to control the intern milieu by selectively regulating information and substance flow. Due to their unique tasks lipid and protein composition varies giving each membrane its specific identity (Mamode Cassim et al., 2019; Nicolson, 2014; Watson, 2015). While the plasma membrane (PM) is important for the cell shape and cell-to-cell signal transduction (Cooper and Hausman, 2000; Luschnig and Vert, 2014), the chloroplast thylakoid membrane uses an electron gradient to generate energy in form of ATP (O'Connor and Adams, 2010). Lipids not only build the membrane backbone, but they also have regulatory functions (Stevenson et al., 2000). Adjustments in lipid composition and minor lipid modification can lead to major changes in crucial cellular pathways (Harayama and Riezman, 2018). During this discussion the major focus will be laid on the lipid dynamics and composition of plant membranes. *Arabidopsis thaliana* (Arabidopsis) membranes are composed of 7 % sphingolipids, 47 % glycerolipids and 46% sterols (Uemura et al., 1995). All these lipids have different functions keeping the cell adaptable. Belonging to the glycerolipids, the head groups of phosphatidylinositol (PI) can be reversibly phosphorylated to seven phosphatidylinositol phosphates (PIPs)- PI(3)P, PI(4)P, PI(5)P, PI(3,4)$P_2$, PI(3,5)$P_2$, PI(4,5)$P_2$ and PI(3,4,5)$P_3$ (Irvine, 2016). While PI(3)P, PI(4)P, PI(5)P, PI(3,5)$P_2$ and PI(4,5)$P_2$ were found in plants, the presence of the other two PIPs was not reliably verified till now (Heilmann, 2016). Although PI and PIPs are minor lipid components in plant membranes with a proportion of less than 1%, they are essential for membrane identity (Gerth et al., 2017; Simon et al., 2014; Simon et al., 2016). They have regulatory functions being relevant for membrane-associated signaling by binding to a great number of interaction partners and thereby influencing multiple processes, such as plant growth, development, cellular polarization and stress response (Heilmann, 2016; Roman-Fernandez et al., 2018). While in animal membranes PI(3)P is localized to membranes of the early endosome, where it is involved in protein degradation, cell signaling and protein recycling (Christoforidis et al., 1999; Jean and Kiger, 2012; Simonsen et al., 1998), in plants, it is found in a decreasing gradient from the pre-vacuolar compartment (PVC), over the late endosomes to the tonoplast, indicating a possible function in vacuolar trafficking (Simon et al., 2014; Vermeer et al., 2006). Additionally, PI(3)P serves as a pool for PI(3)P 5-kinases (type-III PIPkinases) to generate PI(3,5)$P_2$ (Michell et al., 2006), which, for example, is enriched in somatic cells and tobacco pollen tubes during osmotic stress (Meijer, 1999; Zonia and Munnik, 2004). In yeast PI(3,5)$P_2$ accumulates in membranes of late endosomes (Eugster et al., 2004; Friant et al., 2003). PI(4)P can mainly be found at the PM in plants, but

is also present in membranes of endosomal compartments, the Golgi, secretory vesicles and at the cell plate (Gerth et al., 2017; Hammond et al., 2009; Simon et al., 2014; Varnai and Balla, 2006). Among others, the distribution of PI(4)P and other PIPs was determined using biosensors, which consist of specific lipid binding domains (Simon et al., 2014). A difficulty of such sensors is that their overexpression might influence the function and localization of PIPs within a cell (Simon et al., 2014; Varnai and Balla, 2006). To overcome this problem one possible solution is to use promoters with a milder expression pattern than commonly used promoters (Simon et al., 2014). The PI(4)P pool at the PM and the cell plate biophysically affects the surface charge of these membranes (Simon et al., 2016). Thereby, and by its function as a signaling lipid PI(4)P is able to influence localization of several proteins, which are involved in developmental processes, nutrition and plant immunity (Hammond et al., 2012; Simon et al., 2016). Whereas, the PI(4)P pool at the Golgi is involved in protein recycling by regulating protein sorting via membrane trafficking either to the endosome or the PM (Daboussi et al., 2012; Jean and Kiger, 2012; Szentpetery et al., 2010). Moreover, PI(4)P is the source for $PI(4,5)P_2$ generation (Dickson et al., 2014). Both PIP pools are tightly co-regulated and critically important in controlling signal transfer through endoplasmic reticulum (ER)-PM contact sites (Sohn et al., 2018; Stefan et al., 2011). Their levels are changing in response to different environmental stimuli, e.g. cold stress and hyperosmotic stress (Cho et al., 1993; Konig et al., 2007; Ruelland et al., 2002). However, the PI(4)P concentration is up to 30 times higher than the $PI(4,5)P_2$ pool in Arabidopsis seedlings (Munnik, 2014; Munnik et al., 1994). $PI(4,5)P_2$ is localized in microdomains within the PM acting as biological landmarks able to start several cellular pathways, such as endocytosis and exocytosis (Heilmann, 2016; Konig et al., 2007; Martin, 2012; Sun et al., 2013). Furthermore, $PI(4,5)P_2$ at the PM is a substrate of receptor-stimulated phospholipase C, triggering different second messenger molecules, e.g., diacylglycerol (DAG) (Gericke et al., 2013; Katan, 1998). The phospholipid is involved in controlling ion channels as well as in mediating protein-membrane interactions of membrane trafficking or signaling proteins (Balla, 2013; Hammond et al., 2012; Matteis and Godi, 2004; McLaughlin and Murray, 2005; Suh et al., 2006). It was demonstrated that light-induced stomata opening in plant leaves depends on increasing $PI(4,5)P_2$ concentrations inhibiting anion channel activity (Lee et al., 2007). For a long time PI(5)P had not been identified in plants, but it could be shown that PI(5)P contributes in a relevant way to the PIP pool (Meijer et al., 2001). PI(5)P is localized to the cell nucleus and the PM where it is involved as a signaling lipid in stress response (Meijer et al., 2001; Poli et al., 2019; Sarkes and Rameh, 2010). $PI(3,4)P_2$ and $PI(3,4,5)P_3$ cannot be found in plants, but in human cells they mediate protein anchoring at the PM and thereby influence different cellular pathways, e.g., growth-factor stimulation (Cantley, 2002; Karathanassis et al., 2002). As pointed out above, the lipid and protein composition of a

dynamic membrane is characteristic for each membrane and connected to exactly defined biological functions. It is important that membranes have the capability to adapt in consequence to environmental stimuli by changing their surface composition. To ensure the correct signal transduction and stress response of membranes their protein and lipid composition, the membrane rearrangement and the lipid metabolism need to be strictly regulated. A possible option is the usage of regulatory proteins that are able to control the metabolic steps themselves working as coordinators between lipid signaling and metabolism. Proteins with such abilities are phosphatidylinositol transfer proteins (PITPs).

## 4.2 The role of SEC14L-PITPs in plants

### 4.2.1 The SEC14 domain

The conserved cellular membrane trafficking pathway is essential for the distribution of proteins, lipids and other macromolecules (Goring and Di Sansebastiano, 2017; Tsvetanova, 2013). Hence, it needs to be tightly coordinated and regulated. Regulatory proteins controlling vesicle trafficking in response to developmental cues and external stimuli are linkers between lipid sensing, metabolism, and signaling. Thus, they are able to influence membrane composition. PITPs are one crucial group exhibiting such characteristics in regard to PI and PIPs. They can be clustered in two protein families, which have clearly separated biological functions (Wirtz, 1991). The first protein family is defined through its phosphatidylinositol transfer protein and Lipin/Ned1/Smp2 (PITP/LNS2) domain (Cockcroft and Raghu, 2018), whereas the other PITP protein family has a characteristic SEC14/CRAL-TRIO domain (Bankaitis et al., 2010). Defects in both PITP families can lead to developmental defects and server diseases, such as cancer (Cockcroft, 2012; Curwin and McMaster, 2008; Hsuan and Cockcroft, 2001; Nile et al., 2010). During this work it will be focused on the SEC14-like phosphatidylinositol transfer protein (SEC14L-PITP) superfamily. In this chapter a short overview will be provided on the role of SEC14L-PITPs in plants, while they will be discussed in more detail in manuscript 3.

Proteins containing a SEC14 domain can be found in yeast, plants, invertebrates, animals and humans suggesting a conserved evolutionary origin and an essential function (Aravind et al., 1999; Mousley et al., 2007; Saito et al., 2007). Generally, members of the SEC14L-PITP superfamily are defined as regulators of membrane trafficking and the phospholipid-signaling pathway by sensing, binding, transporting and exchanging single lipophilic substances between membranes (Kf de Campos and Schaaf, 2017; Mousley et al., 2007). SEC14L-PITPs are not only able to sense, bind, transport and exchange PI and PIPs between membranes, but they are also able to sense, bind, transport and exchange other lipophilic substances, such as phosphatidylcholine (PC), phosphatidylglycerol (PG), tocopherol and squalene (Chin and Bloch, 1985; Cleves et al., 1991; Kempna et al., 2003). Identified in a

screen for secretory mutants (termed “SEC”) yeast Sec14p (304 AA) was the first characterized SEC14L-PITP and is the model for the SEC14 domain (Bankaitis et al., 1990; Novick et al., 1980). Sec14p is essential for yeast survival and able to either bind PI or PC (Bankaitis et al., 1990; Cleves et al., 1991). The protein is involved in lipid synthesis and turnover as well as in the Golgi secretory process (Bankaitis et al., 1990; Holic et al., 2004). The SEC14 domain is formed by twelve α-helices, six β-strands, eight $3_{10}$-helices, and two distinct domains, which build together the characteristic hydrophobic lipid-binding pocket (Sha et al., 1998). A similar pocket fold was observed in the mammalian proteins CELLULAR RETINAL-BINDING PROTEIN (CRALBP), α-TOCOPHEROL TRANSFER PROTEIN (α-TTP) and TRIO (Crabb et al., 1998; Min et al., 2003). That is why the SEC14 domain is also termed CRAL-TRIO domain (Panagabko et al., 2003). A unique feature of the SEC14 domain, which differentiates it from other lipid-binding domains, is that the lipophilic ligand is bound within the core of the domain (D'Angelo et al., 2006; Min et al., 2003; Schaaf et al., 2006; Stahelin, 2009). Due to that not only the lipid headgroup, but also its fatty acid tail might contribute to binding specificity of molecules to the SEC14 domain. Interestingly, the alpha helical amino (N)-terminus of the SEC14 domain is defined as a separate domain and called CRAL-TRIO-N-terminal extension (CTN). Nevertheless, it is part of the globular SEC14 domain, but absent in some SEC14L-PITPs (Saito et al., 2007). Due to its position the CTN domain is normally able to interact with the headgroup of the bound ligand. It was speculated that the CTN domain might be able to identify the pocket-bound ligand (Saito et al., 2007). Previous experiments indicate that the CTN plays a critical role in membrane binding of SEC14L-PITPs (Skinner et al., 1993).

Crystallization studies of several SEC14L-PITPs revealed two major conformations of the SEC14 domain, an open conformation and a closed conformation, which encloses a bound lipophilic monomer within the SEC14 domain (Schaaf et al., 2008; Sha et al., 1998). These conformational changes might be crucial for biological activity of this domain and classifies SEC14L-PITPs as potential peripheral membrane proteins (Kono et al., 2013; Ryan et al., 2007; Schaaf et al., 2011; Schaaf et al., 2008). It is assumed that the open conformation is the membrane bound version, while the closed status is believed to be the soluble form of the SEC14 domain (Tripathi et al., 2014). Due to the putative conformational changes of the SEC14 domain and on the basis of additional data, a possible lipid-presentation model was developed to explain the mechanism of the SEC14 domain. It is assumed that the SEC14 domain senses membrane-bound PC and presents the domain bound PI to PI(4)P-OH kinases. Next, PI is phosphorylated to PI(4)P and exchange with membrane-bound PC. The presence of PI(4)P at the membrane is then initiating further cellular processes, e.g., vesicle formation (Kf de Campos and Schaaf, 2017).

### 4.2.2 Function of multi-domain SEC14L-PITPs in plants

Simple eukaryotes like yeast exhibit a small number of single-domain SEC14L-PITPs (SEC14-only proteins), while complex organisms do have an increased number of SEC14L-PITPs. Additionally, they exhibit larger multi-domain proteins containing a SEC14 domain (multi-domain SEC14L-PITPs) (Mousley et al., 2007; Saito et al., 2007). The increasing number and complexity of SEC14L-PITPs might demonstrate the high versatility of their regulatory roles within membrane trafficking and lipid metabolisms. The additional modules of multi-domain SEC14L-PITPs are mostly recognizable domains, also identified in other classes of proteins (Anantharaman and Aravind, 2002; Saito et al., 2007).

Arabidopsis has both SEC14-only proteins and multi-domain SEC14L-PITPs (Mousley et al., 2007). One class of multi-domain SEC14L-PITPs is the plant-specific SEC14-nodulin family. SEC14-nodulin proteins contain an N-terminal SEC14 domain and a nodulin domain at their carboxy (C)-terminus. They are involved in polarized membrane trafficking as regulatory proteins (Denance et al., 2014; Ghosh et al., 2015; Huang et al., 2013; Kapranov et al., 2001; Vincent et al., 2005). AtSfh1, a member of the SEC14-nodulin family, regulates the tip directed gradient of $PI(4,5)P_2$ (and PI(4)P) in root hairs, which controls root-hair biogenesis (Bohme et al., 2004; Ghosh et al., 2015; Preuss et al., 2006; Vincent et al., 2005).

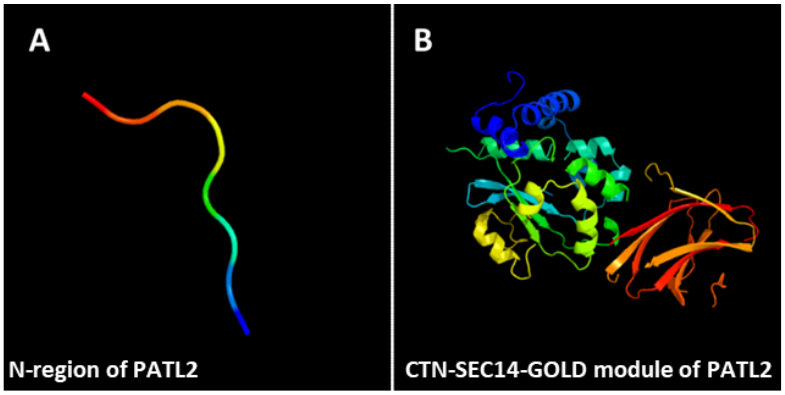


**Figure I. Modeled PATL2 protein structure using Phyre2.**
(A) The N region of PATL2 could not be meaningful predicted, since no known structure could be used as a basis. (B) The CTN-SEC14-GOLD module of PATL2 could be modeled with a 99.9% confidence, demonstrating its conserved structure.

The other class of multi-domain SEC14L-PITPs in Arabidopsis is the SEC14-GOLD protein family, which is also present in humans (Mousley et al., 2007; Saito et al., 2007). All SEC14-GOLD proteins contain an additional golgi dynamics (GOLD) domain at their C-terminus. The GOLD domain seems to be involved in regulating vesicle trafficking and Golgi function by mediating protein-protein and protein-membrane interactions (Anantharaman and Aravind, 2002; Sohda et al., 2001). Arabidopsis has six SEC14-GOLD proteins, named PATELLINs (PATLs) (Fig. I) (Peterman et al., 2004). PATL proteins exhibit a characteristic N-terminal extension (N region) with a unique amino acid composition for each protein. The N regions contain a high amount of charged amino acids and known patterns, e.g. coiled coil motifs or Pxxp motifs (Diella et al., 2008; Liu et al., 2006; Neduva and Russell, 2006; Peterman et al.,

2004). Analyses of *patl* mutants together with localization studies indicate that they might play an important role in the regulation of cell polarity and pattering as well as in plant development (Peterman et al., 2004; Suzuki et al., 2016; Tejos et al., 2017). Since multiple *patl* mutants exhibited a decreased polarization of the auxin transporter PIN-FORMED 1 (PIN1) and defects in early plant development (Tejos et al., 2017). PATL1, PATL2 and PATL3 are peripheral membrane proteins localized to the PM, cell plate and cytosol, dependent on the cell status, and are able to bind PIPs *in vitro* (Peterman et al., 2004; Suzuki et al., 2016; Tejos et al., 2017; Wu et al., 2017). PATL1 is able to bind all PIPs and PI but prefers PI(5)P, PI(3)P and PI(4,5)$P_2$, while PATL2 only binds to PIPs but not to PI (Peterman et al., 2004; Suzuki et al., 2016). PATL3 mainly binds PI(4)P and PI(4,5)$P_2$, which correlates with its observed localization at the PM and the cell plate (Wu et al., 2017). PATLs do not only interact with lipids, but they are also able to interact with proteins. It was demonstrated that PATL1 is able to interact with the $Ca^{2+}$ sensor CALMODULIN-4 (CaM4), and PM $Na^+/H^+$ antiporter SALT OVERLY-SENSITIVE1 (SOS1) suggesting a regulatory role in the response to cold and salt stress (Chu et al., 2018; Zhou et al., 2018). Furthermore, PATL1 and PATL2 were identified in a complex with proteins all having a reported or expected function in intracellular membrane trafficking (Isono et al., 2010). Furthermore, PATL2 is a substrate of MPK4 MAP Kinase and phosphorylation might influence the activity of PATL2 (Suzuki et al., 2016). PATL3 is able to interact with the exocyst component EXO70A1 through its GOLD domain (Wu et al., 2017). Additionally, PATL3 and PATL6 impede alfalfa mosaic virus movement (Peiro et al., 2014). Moreover, the homolog of PATL6 in tomato binds α-tocopherol and contributes to chloroplast function by maintaining its membrane structure (Bermudez et al., 2018). This observation suggests a role of PATLs in membrane protection against reactive oxygen species (ROS) and radicals. This idea is supported by studies on PATL1 linking it to ROS inhibition in response to environmental stress (Zhou et al., 2018).

Taken together, all data indicate that SEC14L-PITPs in general are regulators of several cellular pathways, by controlling membrane trafficking and lipid metabolism. Particularly, members of the PATL family seem to be basic regulatory proteins influencing plant tolerance to altered environmental conditions.

## 4.3 Iron acquisition strategy of Arabidopsis

### 4.3.1 The importance of iron for living organisms

Although iron is the fourth abundant element on earth with a 5% share of total earth´s crust, animals and humans are not able to use soil iron, since it is predominantly present in its oxidized form and bound to minerals (Wedepohl, 1995). But the fact that iron is able to changes its redox stage, from a reduced ferrous to oxidized ferric form and back, makes it an

useful component of different biological pathways. It is an essential part of several proteins and enzymes, e.g. iron-sulfur clusters or heme proteins (Balk and Pilon, 2011; Miller, 2013). That is why iron deficiency leads to severe health problems. One result of insufficient iron supply is the lack of healthy red blood cells, due to a reduced hemoglobin amount, leading to iron-deficiency anemia, which is one of the most common micronutrient deficiencies worldwide (Miller, 2013; Stoltzfus, 2001). Iron-deficiency anemia affects in particular pregnant women and young children, since they generally require more iron for developmental processes then adult men. Pregnant women with iron-deficiency anemia are more likely to have developmental problems of their unborn baby, miscarriage, infant and maternal mortality, birth complications and depressions (Black, 2012; Christian et al., 2015). Furthermore, iron deficiency during growing up can lead to negative effects on children`s growth and development, including cognitive development problems (Bobonis, 2004; Grantham-McGregor and Ani, 2001). Iron-deficiency anemia can be ascribed to limited excess to food providing a sufficient amount of micronutrients. It can be observed that nowadays human diet often consists of energy rich carbohydrates. Plants as autotrophic organisms are able to utilize soil bound iron. Hence, vegetable products are the main iron source in human diet. Therefore, it is important to breed food crops that provide optimized amounts of micronutrients. One problem to overcome is the fact that soil bound iron is poorly utilizable for plants (Guerinot and Yi, 1994; Wedepohl, 1995). That is why the availability of iron can be a limiting factor for plant growth, biomass production and reproduction (Vert et al., 2002), since it is an essential component of the electron transport chains of photosynthesis, respiration and in mitochondria (Abadia, 1992; Balk and Schaedler, 2014; Briat et al., 2015). Iron-deficient plants exhibit characteristic phenotypes with stunted roots, increased number of root hairs and chlorotic leaves, which results in decreased photosynthetic activity due to a reduced number of chloroplast (Jakoby et al., 2004; Römheld and Marschner, 1981; Schmidt, 1999).The other problem to solve is the fact that iron has a catalytic activity that could possibly result in the formation of ROS and radicals via the Haber-Weiss reaction/Fenton reaction, when accumulating in high amounts (Kehrer, 2000; Winterbourn, 1995). This leads to severe damage of DNA, proteins and lipids in plant cells (Connolly et al., 2002; Le et al., 2019; Tripathi et al., 2018). Plants unable to limit iron import or exposed to extremely high levels of bio-available iron display coalesced tissue necrosis (bronzing), blackening and/or flaccidity of the roots (Laan, 1991; Welch and Larue, 1990). Therefore, it is essential for plant survival to keep a sufficient but non-toxic iron pool by regulating iron import. To improve the iron availability for human diet through vegetable products it is critical to exactly understand the molecular mechanisms of iron uptake in plants.

### 4.3.2 Regulation of iron uptake in Arabidopsis

Due to the fact that the concentration of bio-available iron in the soil - particularly in an alkaline and calcareous environment - is limited, higher plants use different approaches to acquire iron from the soil appropriately (Römheld, 1987; Schmidt, 1999). While 'Strategy I' is a reduction-based mechanism, 'Strategy II' is a chelation-based strategy (Römheld and Marschner, 1986). Generally, it can be noticed that grasses are using Strategy II, whereas flowering plants, for instance Arabidopsis, follow Strategy I (Fig. II).

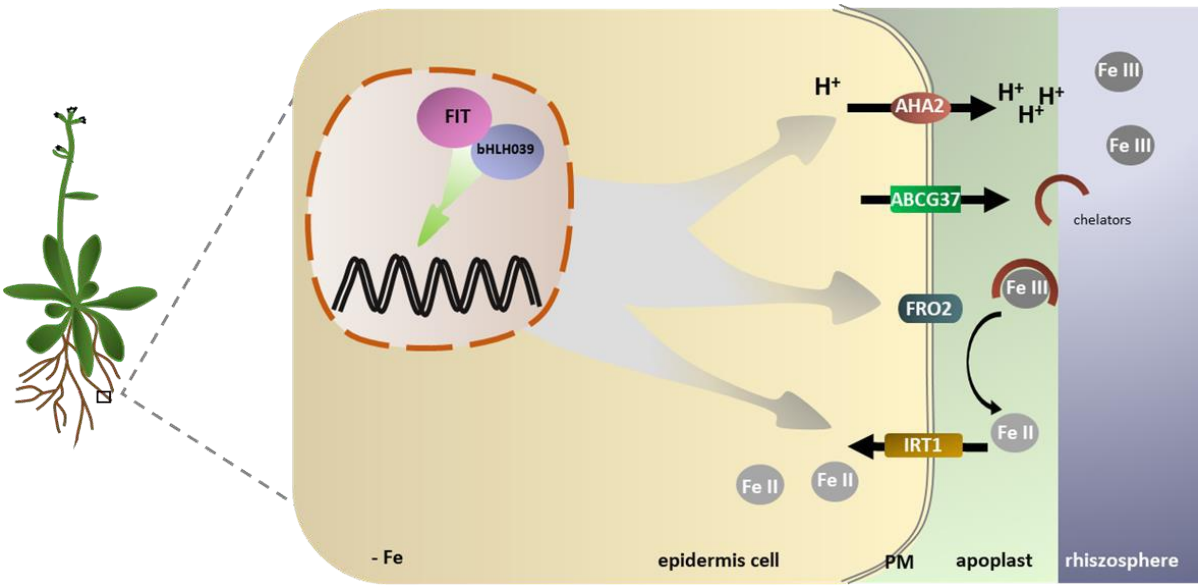


**Figure II. Schematic model of iron deficiency response in epidermis cells of Arabidopsis roots.** Arabidopsis uses a reduction based iron uptake mechanism - 'Strategy I'. Under iron starvation the plant response is induced by the transcription factor FIT dimerizing with other bHLHs. First, the soil pH is lowered by protons transported through AHA2. In parallel chelators are transported into the apoplast by ABCG37. Next, the chelated ferric iron is reduced to ferrous iron by FRO2. Finally, iron is imported in the epidermis cell by IRT1.

In response to iron starvation genes encoding for proteins involved in iron uptake are upregulated by subgroup Ib basic helix-loop-helix (bHLH) transcription factors FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) and bHLH039 in epidermis cells of Arabidopsis roots (Blum et al., 2014; Brumbarova et al., 2015; Naranjo-Arcos et al., 2017). The FIT-dependent iron deficiency response is promoted by its interaction with the calcium dependent and iron-deficiency induced CBL-INTERACTING PROTEIN KINASE 11 (CIPK11), which phosphorylates FIT at Ser272 and contributes to FIT protein activity (Gratz et al., 2019). The upregulation of gene expression is the initial step in response to iron deprivation in Arabidopsis roots and followed by three further steps. First, the soil pH is acidified through pumping protons in the rhizosphere by ARABIDOPSIS H+-ATPASE 2 (AHA2) enhancing the iron solubility (Santi and Schmidt, 2009). This leads to a pool of dissolved ferric iron, which then can be chelated by phenolics exported in the apoplast by the

ABC-family transporter ABCG37. This mechanism provides a constant availability of solubilized iron (Fourcroy et al., 2014; Rodriguez-Celma et al., 2013). Next, the ferric iron is reduced to ferrous iron by the PM localized FERRIC REDUCTASE-OXIDASE 2 (FRO2) (Robinson et al., 1999; Yi and Guerinot, 1996). Finally, the reduced iron is taken up into the epidermis cell by the ZIP-family transporter IRON-REGULATED TRANSPORTER 1 (IRT1), which is located mainly at the PM (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002).

### 4.3.3 Regulation of IRT1 transporter activity on protein level

To maintain a constant but non-toxic iron pool in Arabidopsis it is necessary to highly control iron uptake through IRT1. Interestingly, IRT1 is not only localized to the PM but also to other cellular compartments, such as the early endosome and the *trans*-Golgi network, indicating a role of intracellular trafficking in the regulation of IRT1 activity and iron/metal import (Barberon et al., 2011; Dubeaux et al., 2018; Ivanov et al., 2014). For that reason, it can be concluded that IRT1 activity is not only controlled by transcriptional regulation but also through regulation on protein level.

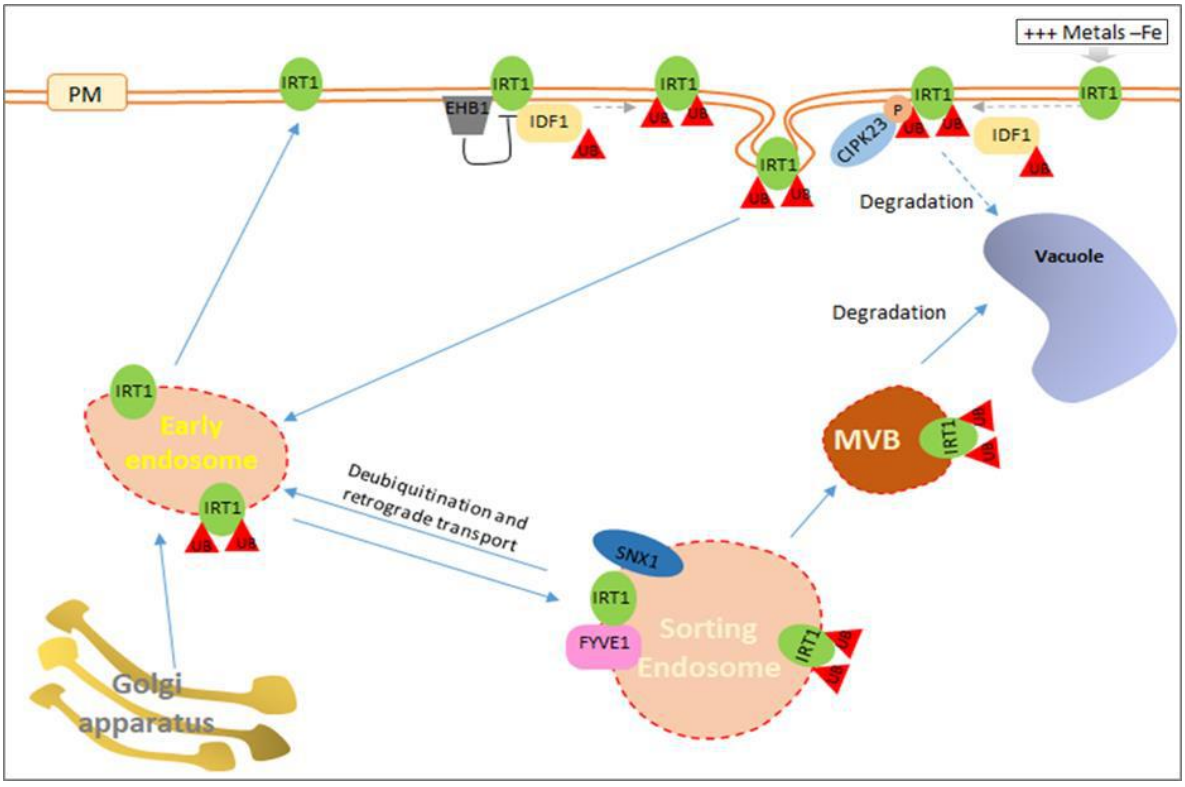


**Figure IV. Regulation of IRT1 activity, stability and intracellular trafficking is dependent on protein-protein interactions.**

Ubiquitination of IRT1 is the signal for endocytosis. At the sorting endosome the fate of IRT1 is decided. The transporter is either sent for degradation to the vacuole, or dependent on FYVE1 and SNX1 sent back to the PM. Presence of non-iron metals results in phosphorylation of IRT1 at the PM and leads to degradation of the protein.

This was confirmed by the observation that constant *IRT1* overexpression leads to accumulation of IRT1 in iron-deficient roots of Arabidopsis, while no transporter protein could be verified during sufficient iron supply indicating post-transcriptional regulation (Connolly et al., 2002). The assumption that IRT1 is regulated on protein level was also corroborated by additional studies, which demonstrated that IRT1 activity is regulated by protein-protein interactions and by intracellular trafficking (Fig.III) (Barberon et al., 2014; Barberon et al., 2011; Dubeaux et al., 2018; Ivanov et al., 2014; Khan et al., 2019; Shin et al., 2013).

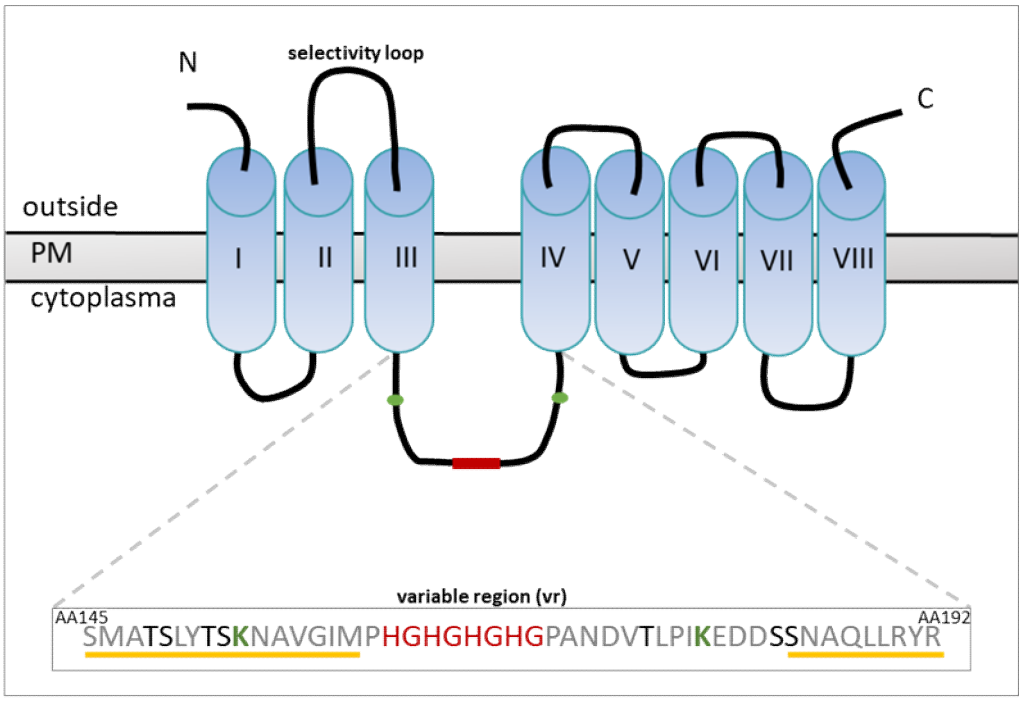


**Figure IV. Structural model of IRT1 focusing on IRT1vr.** Its amino acid (AA) position within IRT1 protein is indicated by numbers. The histidine-rich motif of IRT1vr is labeled in red, while the lysine residues, which are ubiqitinated by IDF1, are marked in green. The putative phosphorylation sites of IRT1vr are highlighted in black. EHB1-target sites are underlined in yellow.

IRT1 is a member of the Zinc-regulated transporter, Iron-regulated transporter-like protein (ZIP) family (Maser et al., 2001). ZIPs are potential integral membrane proteins able to transport different metals and can be found in several organisms, such as bacteria, fungi, plants, insects, vertebrates (Guerinot, 2000; Hall, 2006; Maser et al., 2001). The loss of IRT1 activity results in severe leaf chlorosis and plant lethality, since IRT1 is the main iron importer in Arabidopsis roots (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). The membrane protein is also able to transport other heavy metals such as zinc, copper and manganese, as well as deleterious nickel and cadmium, if present (Rogers et al., 2000; Vert et al., 2002). It is predicted that IRT1 exhibits eight transmembrane domains with a cytosolic variable region (IRT1vr; residues 145–192) spanning between transmembrane

domain three and four (Fig. IV). The cytosolic variable region, spanning between two transmembrane domains, is a characteristic feature of the ZIP family (Eide, 2006; Eng et al., 1998; Guerinot, 2000; Nishida et al., 2008). IRT1vr is known to be essential for the control of protein stability and metal binding (Grossoehme et al., 2006; Guerinot, 2000; Kerkeb et al., 2008; Potocki et al., 2013; Rogers et al., 2000). While Asp144 is important for iron import, demonstrated upon IRT1 heterologous expression in yeast (Rogers et al., 2000), the histidine motif of IRT1vr (residues 162-168) was also identified as a potential metal binding (Grossoehme et al., 2006; Kerkeb et al., 2008).

To prevent an uncontrolled import of iron IRT1 can be negatively regulated through calcium-dependent interaction of IRT1vr with ENHANCED BENDING 1 (EHB1) (Khan et al., 2019). This interaction occurs through the helical regions of IRT1vr, which flank the transmembrane domains three and four (Khan et al., 2019). Additionally, ubiquitination of IRT1vr by E3 ligase IRT1-DEGRADATION FACTOR 1 (IDF1) at Lys154 and Lys179 can start its clarthrin-mediated endocytosis and supports its degradation (Barberon et al., 2014; Barberon et al., 2011; Kerkeb et al., 2008; Shin et al., 2013). *idf1* mutants showed a delayed IRT1 degradation resulting in constant accumulation of IRT1 at the PM (Shin et al., 2013). Stable localization of IRT1 to the PM, due to the exchange of lysines with arginines - unable to be ubiquitinated, causes a metal-toxicity phenotype and plant death underlining the importance of metal uptake control by regulation of IRT1 localization (Barberon et al., 2011; Kerkeb et al., 2008). After internalization the transporter is temporarily stored in the *trans*-Golgi network. The decision of IRT1 degradation or recycling depends on the presence of regulatory proteins such as FREE1/FYVE1 and SORTING NEXIN 1 (SNX1). If they are present, IRT1 is redirected back from the sorting endosome to the PM (Barberon et al., 2014; Ivanov et al., 2014). In the absence of SNX1 IRT1 degradation is increased (Ivanov et al., 2014). This fits to the observation that members of the SORTIN NEXIN (SNX) family are critical regulators of protein trafficking during plant stress response in Arabidopsis (Heucken and Ivanov, 2018). Besides, FREE1/FYVE1 is essential for the rhizosphere-directed polarization of IRT1 at the PM and involved in vacuole biogenesis (Barberon et al., 2014; Kolb et al., 2015). While the pure presence of iron does not influence IRT1 PM localization, its other metal substrates do, maybe to prevent non-iron metal toxicity (Barberon et al., 2014; Dubeaux et al., 2018). It could be demonstrated that the presence of non-iron metal substrates in a massive amount leads to phosphorylation of IRT1vr through protein kinase CIPK23 followed by IDF1 induced internalization and final degradation (Dubeaux et al., 2018).

Due to the toxicity of its substrates it is clear why IRT1 activity needs to be highly controlled in Arabidopsis, but till now very little is known about the active sensing of metal toxicity and its effect on IRT1 regulation and intracellular trafficking. Despite all the gained knowledge on

the post-translational regulation of IRT1, the exact regulatory mechanism is still unknown. Thus, other mechanisms and proteins must undertake regulatory roles controlling IRT1 activity.

## 4.4 Previous work

Prior to this thesis the regulation of IRT1 activity by cellular trafficking and its dependency on several regulatory proteins was demonstrated (Barberon et al., 2011; Dubeaux et al., 2018; Ivanov et al., 2014; Shin et al., 2013). However, still many components of the exact mechanism regulating IRT1 activity, and thereby iron uptake in Arabidopsis roots, are missing. Thus, it is suggested that additional proteins might undertake regulatory functions controlling IRT1 activity and cellular trafficking.

On the basis of the suggestion that IRT1vr is essential for the regulation of protein stability and activity at the PM and thereby controlling iron acquisition, a yeast two-hybrid screen was performed to identify potential regulatory candidates on protein level. For the yeast two-hybrid screen an iron-deficient cDNA library and IRT1vr as bait were used. (For a more detailed description see manuscript 2.) A few promising interaction partners were identified. Next to EHB1 (Khan et al., 2019), PATL2 was identified as an interaction partner of IRT1vr. Furthermore, the protein-protein interaction was confirmed by a targeted yeast two-hybrid assay and BiFC studies in tobacco (R.Ivanov, T. Brumbarova, R.Gratz, K. Angrand, R.M. Basgaran and P.Bauer, unpublished; see manuscript 2).

## 5. Aims of this study

As mentioned above, during previous work PATL2 was identified as a potential interaction partner of IRT1. Due to the function of its SEC14-domain as a lipid transfer domain and the regulatory role of SEC14L-PITPs in lipid metabolism and membrane trafficking, we suggest a possible influence of membrane lipid composition and membrane trafficking on IRT1 activity, maybe regulated by PATL2. Since PATL1, the closet homolog of PATL2, fulfills functions in stress tolerance through interaction with PM proteins, PATL2 could fulfill a similar task by interacting with IRT1. We hypothesize that PATL2 might be a peripheral membrane protein able to sense, exchange, transport or modify PIPs or other lipophilic ligands. Thus, PATL2 could control membrane trafficking. Additionally, PATL2 could be a regulator of iron acquisition in response to iron stress recruited via protein-protein interaction with IRT1.

1. *The first aim was to evaluate the development of the SEC14L-PITP superfamily in the green lineage and to identify the roles of the different PATL2 domains in lipid binding and membrane association.*
   In order to retrace the evolution of SEC14L-PITPs in the green lineage SEC14L-PITP sequences of selected species, representing different stages of evolution, were analyzed to identify the number of proteins and their domain composition. Additionally, functional aspects of SEC14-GOLD proteins were investigated by performing phylogenetic and expression analyses. To understand the function of PATL2 it was essential to identify the lipid-binding and membrane-association behavior of its different domains. Therefore, a set of recombinant proteins, including full-length PATL2 and deletion mutants, was generated and protein-lipid overlay assays and liposome-binding assays were performed. Additionally, cellular localization of PATL2 and its deletion derivatives was determined to study the protein-membrane association *in vivo*.

2. *An additional aim was to verify the interaction between PATL2 and IRT1 and to characterize the physiological role of PATL2 in iron acquisition and IRT1 regulation.*
   As the potential interaction of PATL2 and IRT1 was identified in a yeast two-hybrid experiment, the interaction needed to be confirmed *in planta*. Furthermore, the interactions of PATL2 and its deletion mutants with IRT1vr were tested to identify which domain of PATL2 is involved in the interaction with IRT1vr. For interaction studies in plants bimolecular fluorescence complementation (BiFC) and Co-immunoprecipitation (CoIP) were used. To investigate the physiological effect of *PATL2* on iron uptake and IRT1 regulation, PATL2 loss-of-function mutants and

*PATL2* overexpressing plants were analyzed using different laboratory methods and assays. Furthermore, to understand the interaction and the regulatory effect of PATL2 on IRT1 in more detail a complementation assay was performed in yeast.

3. *The third aim was to elaborate the role of SEC14L-PITPs, especially SEC14-GOLD proteins, in organism stress response and fitness by classifying their identified functions.*

   In order to analyze the role of SEC14L-PITPs in organism fitness and stress response functional aspects of SEC14L-PITPs were investigated by performing an analysis of the domain structure and literature studies. All data were collected and used to reclassify their role in membrane trafficking, stress response and organism fitness.

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# 6. Manuscript 1

## Phylogenetic analysis of plant multi-domain SEC14-like phosphatidylinositol transfer proteins and structure-function properties of PATELLIN 2

# Phylogenetic analysis of plant multi-domain SEC14-like phosphatidylinositol transfer proteins and structure-function properties of PATELLIN 2

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**Keywords**: SEC14, PATELLIN, membrane, GOLD domain, liposome, phosphatidylinositides

**Key message:**
SEC14L-PITPs guide membrane recognition and signaling. An increasingly complex modular structure of SEC14L-PITPs evolved in land plants compared to green algae. SEC14/CRAL-TRIO and GOLD domains govern membrane binding specificity.

**Abstract**

SEC14-like phosphatidylinositol transfer proteins (SEC14L-PITPs) provide cues for membrane identity by exchanging lipophilic substrates, ultimately governing membrane signaling. Flowering plant SEC14L-PITPs often have modular structure and are associated with cell division, development, and stress responses. Yet, structure-function relationships for biochemical-cellular interactions of SEC14L-PITPs are rather enigmatic.

Here, we evaluate the phylogenetic relationships of the SEC14L-PITP superfamily in the green lineage. Compared to green algae, land plants have an extended set of SEC14L-PITPs with increasingly complex modular structure. SEC14-GOLD PITPs, first apparent in a moss, diverged to three functional subgroups, represented by the six PATELLIN (PATL) proteins in Arabidopsis. Based on the example of Arabidopsis PATL2, we dissect the functional domains for *in vitro* phosphatidylinositide, phosphoinositides and liposome binding and plant cell membrane association. While the SEC14 domain with its N-terminal CRAL-TRIO (CTN) domain extension serves general membrane attachment of the protein, the C-terminal GOLD domain directs it to the plasma membrane by recognizing specific phosphoinositides. We discuss that the different domains of SEC14L-PITPs integrate developmental and environmental signals to control SEC14L-PITP-mediated membrane identity, important to initiate dynamic membrane events.

## Abbreviations

CTN, CRAL-TRIO-N-terminal extension; GOLD, GOLGI dynamics; **PATL, PATELLIN; PC,** phosphatidylcholine; **PI,** phosphatidylinositide;

PIP, phosphatidylinositol phosphate; **PITP,** phosphatidylinositol transfer protein; PM, plasma membrane

## Introduction

Biological membranes define two milieus by selectively regulating the exchange of substances and flow of information. Membrane proteins dynamically interact with membrane lipids to monitor and elicit regulatory steps at membranes and in between different membranes, such as during secretion and endocytosis.

One protein family with membrane recognition and signaling properties is the SEC14-like phosphatidylinositol transfer protein (SEC14L-PITP) superfamily. SEC14L-PITPs, first identified in yeast (*Saccharomyces cerevisiae*) by screening for secretory mutants, share their characteristic SEC14 domain, also known as CRAL-TRIO domain [33]. The SEC14 domain of yeast prototype Sec14p starts with three alpha helices, sufficient for stable Golgi membrane association [43, 46]. Not all SEC14 domains contain this alpha-helical region, named CRAL-TRIO-N-terminal extension (CTN) [40]. The SEC14 domain acts as a pocket with lid for binding, transferring and exchanging different types of single lipophilic substrates between membranes, e.g. phosphatidylinositol (PI), phosphatidylinositol phosphates/ phosphoinositides (PIPs) and phosphatidylcholine (PC) [3, 10]. This non-vesicular transport and heterotypic exchange of chemically different substances affect membrane identity, e.g. through exchanging PC against PIPs, or by recruitment of a PI-kinase [25, 42]. Resulting signals affect downstream cellular events, such as lipid kinase efficiency and vesicle formation [4]. Heterotypic exchange can also occur against tocopherol and retinal, altering membrane characteristics [26, 35, 40]. SEC14L-PITPs act in complex physiological processes. Human diseases and defects in plant development, and stress response have been associated with mutations in SEC14L-PITP-encoding genes [11, 53].

How SEC14L-PITP activities are regulated in the cell is a question still under investigation. However, it is discussed that the modular composition and specialization of protein functions of the large SEC14L-PITP families in higher eukaryotes serve to integrate the SEC14L-PITP activities in the different cellular contexts.

While yeast has only six members with only the fundamental SEC14 or CTN-SEC14 domains, higher eukaryotes, e.g. humans or the flowering plant Arabidopsis (*Arabidopsis thaliana*), not only have high numbers of SEC14L-PITPs, but also many of them with modular complex composition. These complex SEC14L-PITPs carry at their C- or N-terminus additional stretches, mostly with recognizable domains, also found in other types of proteins [1, 40].

Arabidopsis has six multi-domain SEC14L-PITPs, named PATELLINs (PATLs), containing an additional Golgi dynamics (GOLD) domain at their C-terminus [37]. GOLD domain proteins are involved in Golgi function and vesicle trafficking, and this domain might play a role in mediating protein-protein interactions with putative cargo proteins as well as mediating

protein-lipid interactions [1, 8]. Arabidopsis PATL3 recruitment to the plasma membrane (PM) is dependent on an interaction with exocyst component EXO70A1 via the GOLD domain [52]. The exocyst complex is involved in cytokinesis and establishment of cell polarity [14, 18]. PATL proteins also exhibit a large, often acidic N-terminal region with variable amino acid composition, a high amount of charged amino acids [e.g. (K)KE(E); (EE)EK repeats], coiled coil and PxxP motifs [13, 31, 37].

Plant mutant analysis and protein localization suggest that PATLs are important for cell division, polarity and patterning [37, 49]. Multiple homozygous gene knock-outs have drastic developmental defects in Arabidopsis. The plants lack proper polarization of the auxin transporter PIN-FORMED1 (PIN1) [49]. PATL1 localizes to the developing cell plate and is able to bind PI and PIPs, preferring PI(5)P, PI(3)P and PI(4,5)$P_2$ on lipid strips [37]. PATL2 binds all PIPs but no PI and localizes to the PM and the developing cell plate during cytokinesis in roots [48, 49]. Furthermore, environmental response phenotypes are reported, indicating that PATL proteins play a role in the proper functioning of organs. PATL1 interacts with the $Ca^{2+}$ sensor Calmodulin-4 (CaM4), and PM $Na^+/H^+$ antiporter SALT OVERLY-SENSITIVE1 (SOS1), and contributes to the plant tolerance to cold and salt stress [9, 54]. PATL1 negatively influences reactive oxygen species formation, linking it to cellular damage protection under stress [54]. PATL3 and PATL6 disturb alfalfa mosaic virus movement by interaction with a plasmodesmata targeting movement protein (AMV MP) [36].

Here, we performed a phylogenetic analysis of the SEC14L-PITP family in the green lineage, to assess whether the complexity of SEC14L-PITPs increased during land plant evolution, as was the case in the lineage leading to animals. Based on the example of Arabidopsis PATL2 protein, we dissect the modular structure of a multi-domain SEC14-GOLD protein to investigate the contribution of individual domains to lipid binding and membrane association.

## Materials and Methods

### Phylogenetic analysis and alignments

SEC14 protein sequences were identified via Phytozome v12.1 (https://phytozome.jgi.doe.gov) and protein BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) using as query yeast Sec14p and the SEC14 domain of AtPATL2, and via InterPro using the CRAL-TRIO domain. Sequences were retrieved from selected species of green algae, a moss, a lycophyte, and of selected flowering plants, and analyzed for the presence of multiple domains. The positions of domains were determined using NCBI Conserved Domain Search NIH (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), PROSITE (https://prosite.expasy.org/) and

InterPro (https://www.ebi.ac.uk/interpro). InterPro domain accession numbers are GOLD-IPR009038, SEC14/CRAL-TRIO-IPR001251, CTN-IPR011074, GDAP2/Macro domain-IPR035793, DDT domain-IPR018501, PHD domain-IPR019787, WHIM1 domain-IPR028942, WHIM2 domain-IRO28941. Nodulin and nodulin-like domains were defined according to Kapranov et al.[23] and Vincent et al. [50].

Phylogenetic trees were generated as described by Ivanov and Bauer [21] using the http://www.phylogeny.fr server.

## Accession numbers

*Arabidopsis thaliana* TAIR10: At4g344580 (AtSfH1); AT2G21540 (AtSfH3); At4G36490 (AtSfH12); At1g72150 (PATL1); At1g22530 (PATL2) ; At1g72160 (PATL3); At1g30690 (PATL4); At4g09160 (PATL5); At3g51670(PATL6); At4g39180; At1g01630; At1g05370; At1g14820; At1g19650; At1g22180; At1g55840; At1g75170; At1g75370; At2g15670; At2g16380; At2g18180; At2g21520; At3g22410; At3g24840; At3g46450; At4g08690; At4g36640; At4g39170; At5G47730; At5g56160; At5g63060;At1G69340; At4g35750; At3g10210; At1g55690

*Chlamydomonas reinhardtii* v5.5: Cre10.g444250; Cre12.g503950; Cre12.g527050.t1.2; Cre10.g448051; Cre02.g141950; Cre03.g166201; Cre02.g147800; Cre02.g101200 ; Cre17.g718100; Cre17.g703200; Cre11.g467563

*Volvox carteri* v2.1: Vocar.0070s0030; Vocar.0004s0276; Vocar.0005s0399; Vocar.0008s0014; Vocar.0036s0128; Vocar.0037s0062

*Chara braunii* [32]: CBR_g84; CBR_g29298 ; CBR_g36387; CBR_g31494; CBR_g38007; CBR_g39624; CBR_g4074

*Marchantia polymorpha* v3.1: Mapoly0098s0038; Mapoly0054s0137; Mapoly0008s0106; Mapoly0027s0072; Mapoly0054s0139; Mapoly0064s0114; Mapoly0091s0075; Mapoly0245s0002; Mapoly0114s0025; Mapoly0030s0043; Mapoly0153s0026

*Selaginella moellendorffii* v1.0: 439610; 271658; 114753; 11806; 168470; 121430; 93038; 92905; 91570; 43741; 65145; 90159; 89782; 91055; 77842; 22919

*Solanum lycopersicum* iTAG2.4: Solyc07g066090; Solyc10g053900; Solyc04g082050; Solyc04g040200; Solyc11g051160; Solyc08g078680; Solyc11g040280; Solyc01g109870; Solyc01g005270; Solyc02g070210; Solyc01g005290; Solyc01g109860; Solyc03g118150; Solyc02g083250; Solyc10g053970; Solyc10g005740; Solyc01g005280; Solyc05g054570; Solyc01g005260; Solyc12g089130; Solyc06g075980; Solyc05g012610; Solyc09g015080 (*Sl*TBP); Solyc11g027880; Solyc09g025230; Solyc04g080690; Solyc06g064940; Solyc09g060090; Solyc11g012790; Solyc03g112640; Solyc07g065700; Solyc04g005490

*Zea mays* PH207 v1.1: Zm00008a000784; Zm00008a007108; Zm00008a021866; Zm00008a034613; Zm00008a000376; Zm00008a008524; Zm00008a018020;

Zm00008a031256; Zm00008a016003; Zm00008a033937; Zm00008a031138; Zm00008a013174; Zm00008a027397; Zm00008a019107; Zm00008a032518; Zm00008a032332; Zm00008a025849; Zm00008a025849; Zm00008a039685; Zm00008a008448; Zm00008a022847; Zm00008a022585; Zm00008a025654; Zm00008a018864; Zm00008a017887; Zm00008a003138; Zm00008a012744; Zm00008a037114; Zm00008a037115; Zm00008a019021; Zm00008a004286; Zm00008a022784; Zm00008a012865; Zm00008a016847; Zm00008a028031; Zm00008a030680; Zm00008a005640; Zm00008a025640; Zm00008a026335; Zm00008a009102; Zm00008a017141; Zm00008a033936; Zm00008a012432; Zm00008a013571; Zm00008a025498; Zm00008a028943; Zm00008a008572; Zm00008a033935; Zm00008a032805; Zm00008a034926; Zm00008a020872; Zm00008a020873; Zm00008a014049; Zm00008a021294

## Visualization of gene expression and gene co-expression analysis

Expression data for Arabidopsis *PATL1, PATL2*, *PATL3*, *PATL4* and *PATL6* genes were obtained using the ePlant Heatmap Viewer module (http://bar.utoronto.ca/eplant/) [51]. Co-expression networks were built using the Network Drawer module via ATTED-II version 9.2 (http://atted.jp/) [34]. The gene ontology (GO) feature of ATTED-II was used for GO term enrichment.

## Generation of recombinant vectors

Full-length Arabidopsis *PATL2* and derived deletion mutants (Table S2) were generated as follows: The *PATL2* coding sequence was amplified from cDNA, obtained from wild-type Col-0 roots, using primers PATL2B1F and PATL2B2stopR (Table S3) and transferred via Gateway cloning into pDONR207 (BP reaction, Life Technologies). This plasmid was used as a template for generating all different *PATL2* deletion mutant coding sequences by PCR (Table S3 for primers), subsequently transferred into pDONR207. Then, *PATL2* and deletion-mutant forms were transferred into pH7WGY2 vector via Gateway cloning (LR reaction, Life Technologies), allowing constitutive expression of N-terminally tagged YFP proteins in plant cells via pCaMV35S [24]. The pDONR plasmids served as templates for generating recombinant pET-52b (+) plasmids (Novagen) with PATL2 and deletion mutant inserts, using restriction-ligation cloning with BamHI and NotI restriction enzymes, allowing Strep-tagged protein expression in bacteria.

## Transient tobacco epidermis transformation and confocal microscopy

*Rhizobium radiobacter* strain C58C1(pTiB6S3ΔT)$^{H}$ containing recombinant pH7WGY2 with PATL2 or PATL2-mutant inserts was used for transforming tobacco (*Nicotiana benthamiana)*

by leaf infiltration, as previously described [19]. Overnight *R. radiobacter* cultures in YEB medium were pelleted and resuspended to an $OD_{600}$ of 0.4 in tobacco infiltration solution (2mM $NaH_2PO_4$, 50mM MES, 0.5% Glucose) supplemented with 100 µM acetosyringone. The suspension was infiltrated into young tobacco leaves. After 24-48h, leaves were used for confocal microscopy. For co-localization studies, the PATL2 vector-containing bacteria were mixed with bacteria containing plant vectors for expression of PM marker AHA1-mRFP [7] and ER marker mOFP-HDEL [5].

Confocal images of fluorescent signals were collected using a LSM780 system (Zeiss). YFP signals were excited at 514 nm, and emission was detected at 520–550 nm. mRFP and mOFP were excited at 561 nm, and emission was detected at 580–630 nm.

## Protein expression and purification with the Strep-tag®/Strep-Tactin® system

BL21 (DE3) pLysS containing pET-52b(+) vectors with recombinant PATL2 or PATL2 deletion mutants were grown in 50 ml LB (100 µg/ml ampicillin, 34 µg/ml chloramphenicol, $OD_{600}$ of 0.08). The temperature was lowered from 37°C to 30°C when the culture reached an $OD_{600}$ of 0.3-0.4. Protein expression was induced 30 min after the temperature shift with 1 mM isopropyl-ß-D-thiogalactopyranosid (IPTG) and cells were harvested three hours later. StrepII-tagged protein of interest was purified using the Strep-tag®/Strep-Tactin® system (IBA Lifesciences) according to the manufacturer's protocol for Strep-Tactin® Macroprep® or via Strep-Tactin®XT Superflow® cartridge using ÄKTA prime plus (GE Healthcare Life Sciences). Protein expression and purification were assessed following standard SDS-polyacrylamide gel electrophoresis, either followed by Coummassie Brilliant Blue staining or by blotting on Amersham™ Protran™ 0.2 µm nitrocellulose membranes (GE Healthcare Life Sciences) and affinity detection via enhanced chemiluminescence (GE Healthcare Life Sciences) of StrepII-tagged protein using Strep-Tactin®-horseradish peroxidase (HRP) conjugate (Iba Lifesciences).

## Protein-lipid overlay assays

Membrane Lipid Strips™ or PIP Strips™ (Echelon Biosciences) were blocked with 3 % (w/v) BSA in TBST (150 mM NaCl, 2.7 mM KCl, 24.7 mM Tris-HCl, 0.05 % v/v Tween-20, pH 7.4) for 2 ½ hours at room temperature in a Petri plate. The protein-lipid overlay incubation was performed with 2 ml Strep-tagged protein (0.5 µg/ml protein in 3 % (w/v) BSA in TBST) at 4°C overnight. The strip was washed five times for eight minutes with TBST and incubated with 1:1000 diluted custom-made α-PATL2-1 IgG antibody, generated against the peptide TKKEETPVAPAPVEC (GenScript), in 3 % (w/v) BSA in TBST for one hour at room temperature. The strip was washed four times for ten minutes with TBST and incubated for one hour with 1:2000 Goat anti-rabbit IgG-HRP-conjugated antibody (Agrisera) in 3 % (w/v)

BSA in TBST. After four washing steps with TBST for 10 min, the protein-lipid binding was detected by enhanced chemiluminescence (GE Healthcare Life Sciences) and quantified.
For signal quantification of lipid dot binding, the Multiplex Band Analysis feature of AlphaView®Software (ProteinSimple) was used. Circular regions of same sizes were selected for each dot, and by using multi-regional background subtraction the corrected signal was quantified as background-corrected sum. Relative signal intensities were calculated by dividing each background corrected signal coming from one lipid drop by the sum of corrected signals from PI, PI(4)P, PI(4,5)$P_2$ and PI(3,4,5)$P_3$ or from PI and all phosphorylated derivatives (Membrane Lipid Strip™ or PIP Strip™, Echelon Biosciences).

## Liposome-binding assays

All lipids used in the assay were purchased from Avanti® Polar Lipids, Inc. The liposome-binding assays were performed as described in Julkowska et al. [22] with the modifications that sonication (10 % amplitude for 1 min, 3 sec. on, 20 sec. off, Digital Sonifier® W-250 D, Branson Ultrasonic Corporation) was used for liposome formation instead of extrusion, and 250 mM sucrose was contained in the extrusion buffer (25 mM Tris-HCl pH 7.5, 1 mM DTT) instead of raffinose. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (PI), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol-4'-phosphate) (PI(4)P) and 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) (PI(4,5)$P_2$) were each dissolved 20:9:1 in chloroform:methanol:water, mixed to a total of 300 nmol with 7% PI, and pelleted by vacuum centrifugation. The resulting pellet was rehydrated in extrusion buffer and sonicated. Liposomes were harvested at 50,000 x *g* for 15 min and suspended in 25 μL binding buffer. 12 nmol of protein in 25 μL ml of binding buffer was added and incubated for one hour at room temperature. Supernatant (s) and pelleted membrane fractions (m) were separated by centrifugation at 210000 x *g* and both analyzed by SDS-PAGE followed by immunoblot using the α-PATL2-1 IgG antibody and detection, as described above.

## Results

### Complexity of the SEC14L-PITP family expanded during land plant evolution

We retraced the evolution of SEC14L-PITPs in the green lineage by evaluating the number of proteins and their domain composition. Sequences were recovered from selected species of green algae as well as plants, representing different evolutionary stages of the green lineage. Unicellular *Chlamydomonas reinhardtii* and multicellular *Volvox carteri*, both belong to *Chlorophyta*, have a low degree of cell differentiation. In the representatives of this ancestral green algae taxon, we identified six and eleven single-domain SEC14 proteins (SEC14-only proteins) (Figs. S1A, S1B). In most of them, a CTN domain is associated with the SEC14 domain. This resembles the organization of the yeast SEC14-PITP family. *Charophyta* have a complex morphology, and are evolutionarily advanced as closest living relatives of land plants. The charophytic algal model *Chara braunii* has six SEC14-only proteins, one of them devoid of the CTN, and one multi-domain SEC14L-PITP (Fig. S1C). The latter, (CBR_g38007), has a N-terminal plant homeodomain (PHD) and a DNA-binding homeobox - Different Transcription factors (DDT) domain along with the WHIM motifs 1 and 2 involved in packing the DDT domain [2]. Such a combination was not identified in any other species investigated here.

Liverworts count as most ancient Bryophytes and first land plants. Eleven SEC14L-PITPs were found in the liverwort *Marchantia polymorpha* (Fig. S1D). Seven were SEC14-only proteins, three with and four without CTN. The remaining three proteins were SEC14-GOLD domain proteins, similar to Arabidopsis PATLs. The lycophyte *Selaginella moellendorffii*, an ancient vascular plant, has 14 SEC14L-PITPs (Fig. S2A). Ten of them are Sec14-only proteins, one without CTN. Three are SEC14-GOLD proteins. We found one new multidomain protein with an N-terminal domain similar to the human Ganglioside-induced differentiation-associated protein 2 (GDAP2) domain. ADP-ribose is a potential substrate for the GDAP2 domain [17, 29]. The function of the protein is unknown, but could be required for developmental transitions occurring only in vascular plants.

In Arabidopsis, we identified two new (At3g10210 and At4g35750) SEC14L-PITPs than previously reported, summing up to 35 proteins [30] (Fig. 1A). 15 of them are SEC14-only proteins, nine with a CTN. 20 proteins are multi-domain proteins, including the six PATLs with an additional GOLD domain, one with an additional GDAP domain and 13 SEC14L-PITPs with a nodulin or nodulin-related domain. In other distantly related flowering plants, such as the eudicot tomato (*Solanum lycopersicum*, *Solanaceae*) and the monocot maize (*Zea mays*, *Poaceae*), 32 and 54 SEC14L-PITPs are present, comprising 11 and 20 multi-domain proteins, again with either GOLD, GDAP, nodulin or nodulin-related domains (Figs S2B, S3). The plant-specific SEC14-nodulin protein family is only present in investigated

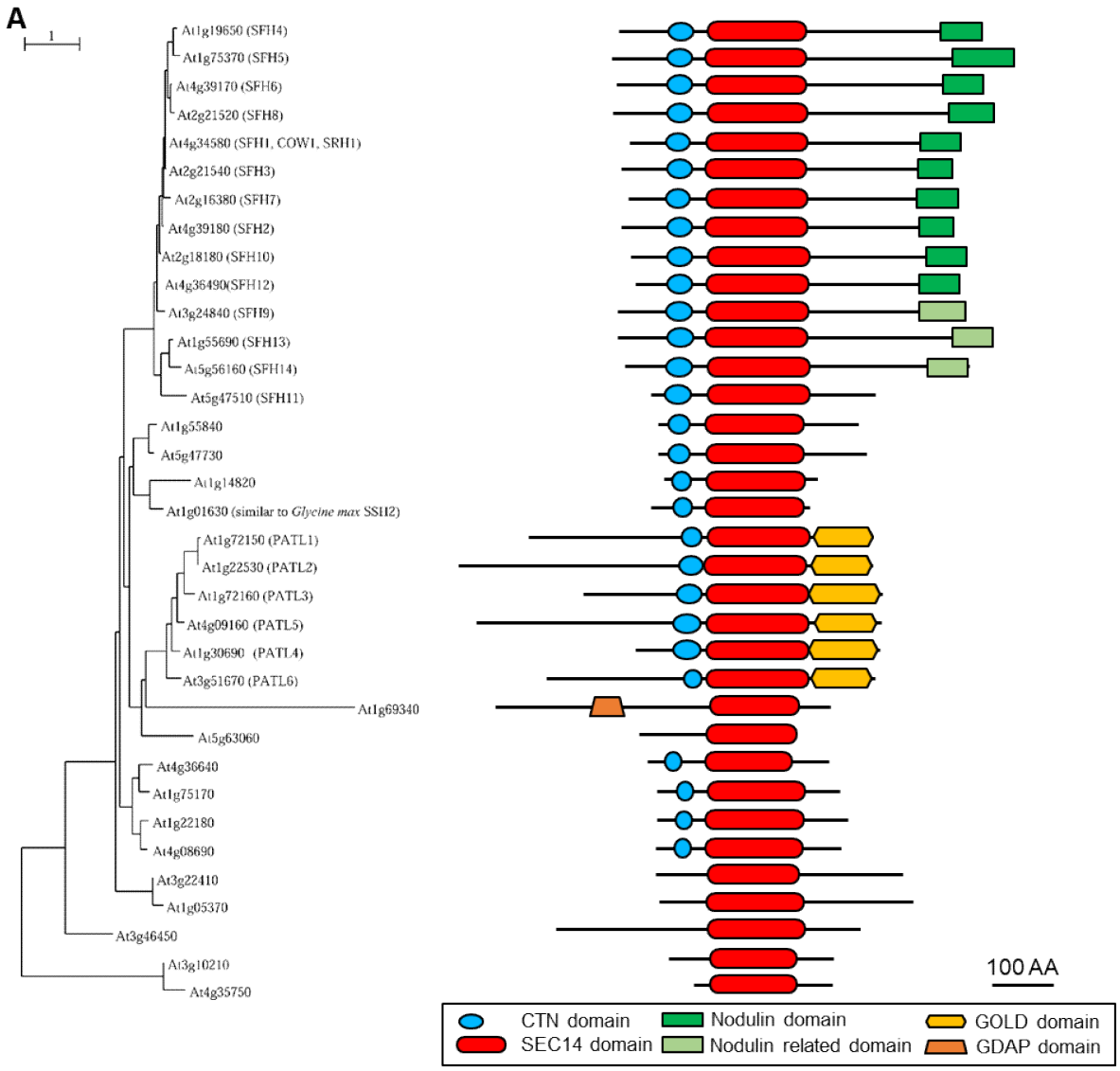
flowering plants [12, 23, 50]. Some of these SEC14-nodulin proteins were shown to function as general regulators in polarizing membrane trafficking [16, 20, 50].

Taken together, the numbers and modular complexities of SEC14L-PITPs increased with the developmental complexity of multicellular eukaryotes in the green lineage. The increasing complexity is likely linked with new functions acquired to coordinate plant development with environmental constraints on dry land.

## Plant SEC14-GOLD (PATL) proteins divide into three clades based on sequence comparisons

Plant multi-domain SEC14L-PITPs group in similar branches of the phylogenetic trees, in contrast to SEC14-only proteins, that are scattered in different branches (Fig. 1A; Figs S1, S2, S3). Three distinct PATL clades were suggested from Arabidopsis-tomato PATL comparisons, namely AtPATL6/SlTBP, AtPATL4 and AtPATL1/2/3/5 [6], and hypothesized to mediate different cellular functions [38]. Phylogenetic analysis with all land plant PATL-protein sequences, identified in this work, either using full length or CTN-SEC14-GOLD (termed here “C”) sequences (Fig. 1B, 1C), confirms the evolution of three clades. The AtPATL1/2/3/5 clade is only present in vascular plants, while the AtPATL6 clade is exclusive to flowering plants. The AtPATL4 clade, closer to AtPATL1/2/3/5 than AtPATL6 clade, is most related to the ancient moss PATLs. The N-terminal regions (termed here “N”), without CTN-SEC14-GOLD domains, have a low degree of sequence similarity (Fig. S4). N regions alone are not sufficient to construct a meaningful phylogenetic tree (Fig. 1D). N stretches are also absent in the human SEC14-GOLD α-tocopherol-associated proteins (TAPs) (Fig. 1D; Fig. S5).

Sequence diversification into three clades suggests that PATLs might fulfill diverse functions within the plant, perhaps associated with canonical roles (via CTN-SEC14-GOLD) and adaptive responses (via N).


A
1
At1g19650 (SFH4)
At1g75370 (SFH5)
At4g39170 (SFH6)
At2g21520 (SFH8)
At4g34580 (SFH1, COW1, SRH1)
At2g21540 (SFH3)
At2g16380 (SFH7)
At4g39180 (SFH2)
At2g18180 (SFH10)
At4g36490(SFH12)
At3g24840 (SFH9)
At1g55690 (SFH13)
At5g56160 (SFH14)
At5g47510 (SFH11)
At1g55840
At5g47730
At1g14820
At1g01630 (similar to Glycine max SSH2)
At1g72150 (PATL1)
At1g22530 (PATL2)
At1g72160 (PATL3)
At4g09160 (PATL5)
At1g30690 (PATL4)
At3g51670 (PATL6)
At1g69340
At5g63060
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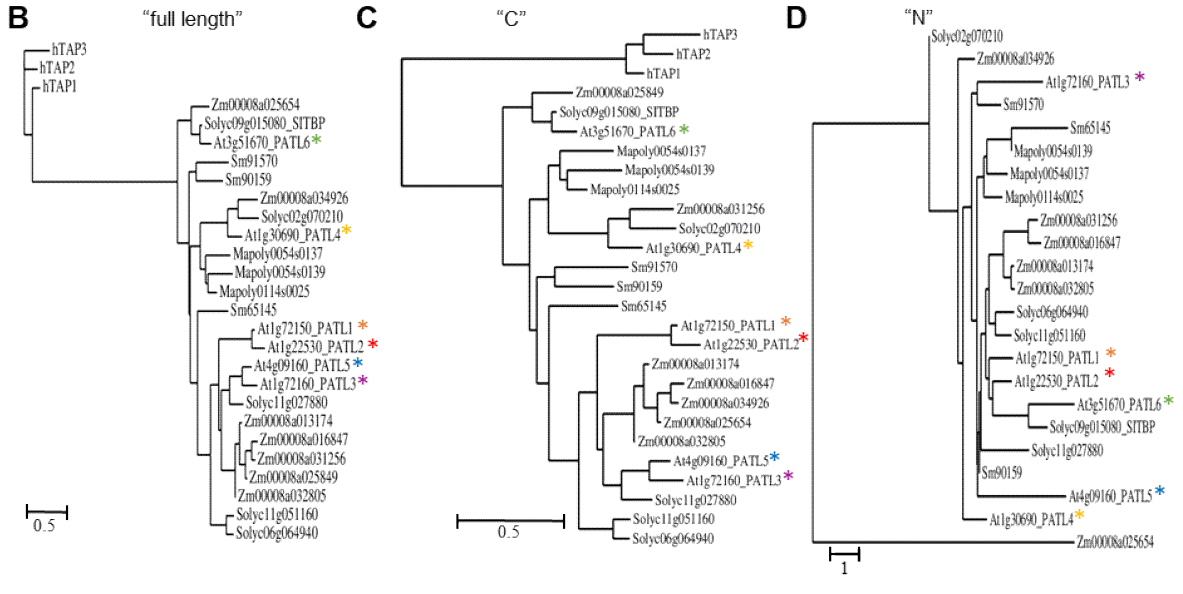
100 AA
CTN domain
SEC14 domain
Nodulin domain
Nodulin related domain
GOLD domain
GDAP domain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Sm90159
At4g09160_PATL5
At1g30690_PATL4
Zm00008a025654
1

**Fig. 1. The flowering plant SEC14L-PITP superfamily is complex, and PATLs form three clades.**
(A) Phylogenetic relationships of *Arabidopsis thaliana* SEC14L-PITPs. Left, phylogenetic tree, generated with full-length amino acid sequences. Right, modular structure of SEC14L-PITPs; different domains, represented in different colors, as indicated. (B-D) Phylogenetic analysis of green lineage SEC14-GOLD proteins, generated with (B) full-length ("full-length"), (C) the CTN-SEC14-GOLD regions ("C"), (D) the variable N-termini ("N"). Three human TAP proteins represented outgroups in B, C. Size bars of phylogenetic trees represent the numbers of substitutions per amino acid position, as indicated.

## Expression analysis of Arabidopsis *PATL* genes confirms functional diversification into three groups

Available transcriptome data for five of the six Arabidopsis *PATL* genes reflect the three different clades (Fig. 2A). Generally, all genes are more highly expressed in stem than root tissues, and regulated under the influence of plant hormones and during development. Particularly *PATL4* and *PATL6* are responsive to abiotic stresses. *PATL1* and *PATL2* are closely co-regulated, which supports the suggestion that they may have arisen from gene duplication events [37]. *PATL4* is associated in a distant way with the *PATL1*, *PATL2*, *PATL3* coexpression cluster (Fig. 2B). Within this coexpression cluster, four genes encode proteins related to actin, kinesin motor proteins and microtubules, suggesting that *PATL1*, *PATL2*, *PATL3* and *PATL4* function might be related to cytoskeleton organization and dynamics (Fig. 2B; Table S1). *PATL6* is part of a different coexpression cluster with an enrichment of auxin-related genes (Fig. 2B; Table S1).

Partially overlapping expression patterns and co-expression indicate functional redundancy within the Arabidopsis PATL family. Despite of that, some specific functions are noted, consistent with the observed sequence diversification of PATL clades.

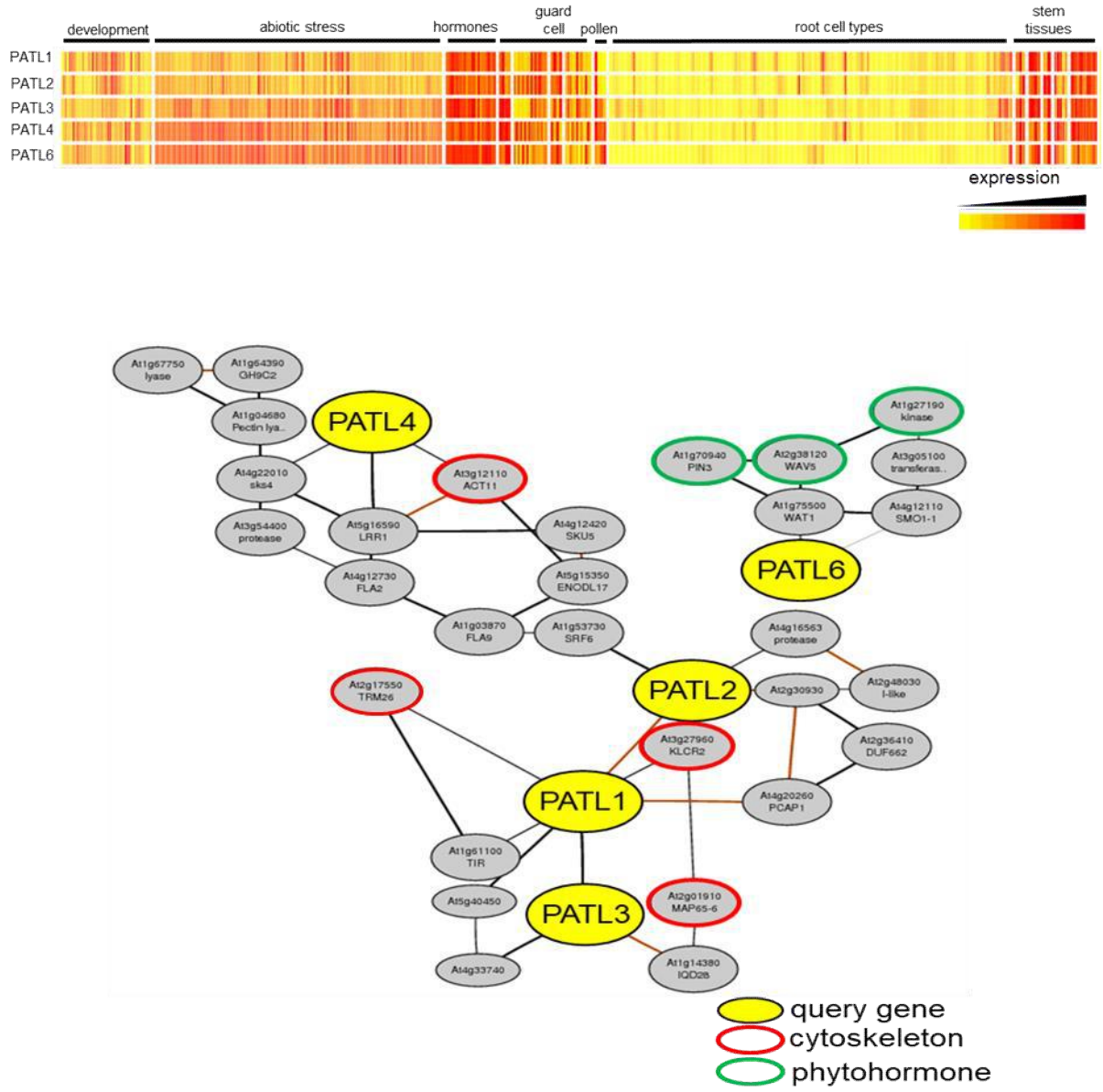
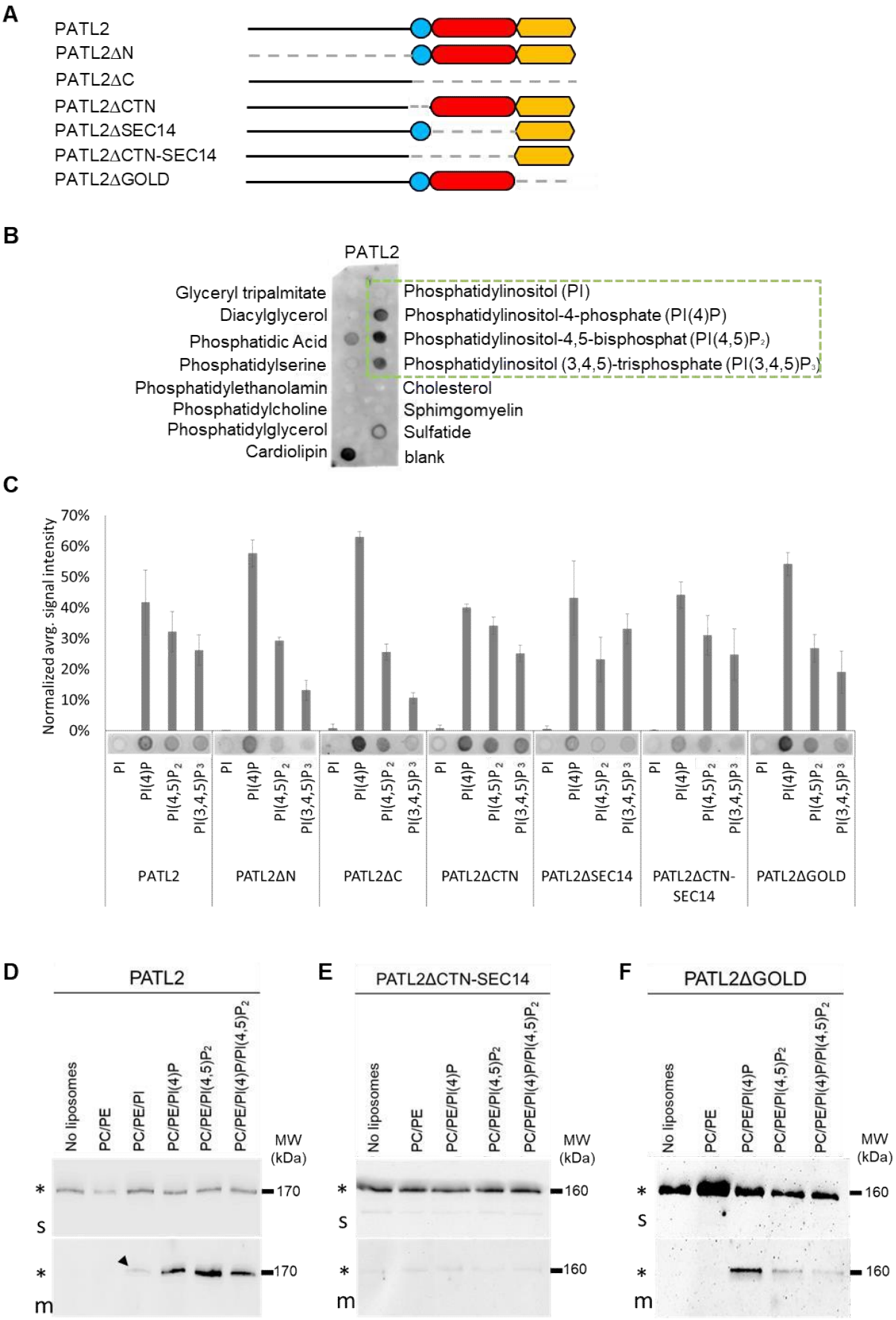



**Fig. 2. Arabidopsis *PATL* genes form three subgroups, based on gene expression profiles.**
(A) Heatmap showing expression patterns of *PATL* genes across development, tissues, environmental and hormone responses. (B) Genome-wide co-expression networks of *PATL* genes with auxin response and cytoskeleton genes. No data are available for *PATL5*. All data are also listed in Table S1.

## The CTN-SEC14 and GOLD domains of PATL2 protein confer different specificities in phosphoinositide binding

The CTN-SEC14 domain is the canonical element of SEC14L-PITPs for lipid transfer functions and this domain associates with the membrane. The GOLD domain is essential for PATL3 recruitment to the PM via EXO70A1 [52]. However, detailed knowledge is lacking about the roles of the different domains of SEC14-GOLD proteins in plants. We used PATL2 from Arabidopsis to dissect the roles of PATL2 domains in membrane attachment *in vitro*.

Deletion mutants lacking domains were generated (Fig. 3A). The PM and cell plate localization of full-length PATL2 and the ability to bind PIPs in protein-lipid overlay assays [48, 49] raised the question whether specific PIPs are recognized by its individual domains. However, all PATL2 deletion versions assayed were able to bind to any type of immobilized PIPs using a lipid strip, including those devoid of CTN, SEC14 and GOLD domains (Figs. 3B, 3C). Cardiolipin, sulfatide and phosphatidic acid were also bound, presumably because of their negative charges, mimicking PIPs (Fig. 3B). Using a specific PIP strip, PATL2 and all deletion proteins bound any type of PIPs. Mostly, however, PI(3)P was preferred, followed by either PI(5)P or PI(3,5)$P_2$ (Figs S6A, S6B). Overlay assays show binding to immobilized phospholipid units, while liposome assays provide information of binding to phospholipids in the context of a curved synthetic membrane. The latter mimics better PIP recognition in a biological membrane environment. To conduct a liposome assay, PI(4)P and PI(4,5)$P_2$ were chosen since they are predominantly present at the PM and the cell plate [44] where PATL2 was found to be localized [48, 49]. Basic liposomes prepared with PC/PE served as controls, and neither PATL2 protein nor any mutant version associated with them in the membrane (m) fractions (Fig. 3D-F). If PI was added to these liposomes, a low amount of PATL2 protein was found in the liposome fraction. Addition of PI(4)P, PI(4,5)$P_2$, or a combination of PI(4)P and PI(4,5)$P_2$ greatly increased the abundance of PATL2 in the membrane fractions (Fig. 3D). Deletion of the CTN-SEC14 domain fully abolished association with any of the liposomes (Fig. 3E) showing that the N and GOLD domains alone are not sufficient for liposome binding. In comparison, deletion of the GOLD domain resulted in reduced binding to PI(4,5)$P_2$, compared to PI(4)P (Fig. 3F). All protein versions were detected in all supernatant (s) fractions, showing that they were recognized by antisera (Fig. 3D-F). Hence, the SEC14 domain is required for association with PIPs in the membrane, while the GOLD domain steers additionally correct PIP membrane targeting of PATL2.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PATL2
PATL2ΔN
PATL2ΔC
PATL2ΔCTN
PATL2ΔSEC14
PATL2ΔCTN-SEC14
PATL2ΔGOLD
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PATL2
Glyceryl tripalmitate
Diacylglycerol
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Phosphatidylserine
Phosphatidylethanolamin
Phosphatidylcholine
Phosphatidylglycerol
Cardiolipin
Phosphatidylinositol (PI)
Phosphatidylinositol-4-phosphate (PI(4)P)
Phosphatidylinositol-4,5-bisphosphat (PI(4,5)P2)
Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3)
Cholesterol
Sphimgomyelin
Sulfatide
blank
C
Normalized avrg. signal intensity
70%
60%
50%
40%
30%
20%
10%
0%
PI
PI(4)P
PI(4,5)P2
PI(3,4,5)P3
PATL2
PATL2ΔN
PATL2ΔC
PATL2ΔCTN
PATL2ΔSEC14
PATL2ΔCTN-SEC14
PATL2ΔGOLD
D
PATL2
No liposomes
PC/PE
PC/PE/PI
PC/PE/PI(4)P
PC/PE/PI(4,5)P2
PC/PE/PI(4)P/PI(4,5)P2
MW (kDa)
170
s
m
E
PATL2ΔCTN-SEC14
160
F
PATL2ΔGOLD

**Fig. 3. Phosphoinositide binding of PATL2 is dependent on CTN-SEC14 and GOLD domains.**
(A) Schematic representation of PATL2 deletion mutants. (B, C) StrepII-PATL2 protein–lipid overlay assay. Green rectangle in (B), phosphoinositide binding signal quantified in (C). (D-F) Liposome-binding assays and immunoblot of StrepII-PATL2 and selected deletion mutants, associated with soluble (s) and membrane (m) fractions. Correct protein bands are indicated by * and hardly visible band is indicated by an arrow, note the altered gel migration behavior of proteins (Figure S6C).

## The GOLD domain of SEC14-GOLD protein PATL2 specifies membrane binding in plant cells

The PATL2-liposome association raised the question of the role of PATL2 domains in a cellular context. When expressed in plant cells, full-length YFP-PATL2 was present mainly at the PM, co-localizing with the PM marker AHA1 (Fig. 4A). Deletion of PATL2-N had no effect on the PM localization (Fig. 4B), indicating that the N region has different significance for the protein. Deletion of the CTN-SEC14 module (PATL2ΔC, PATL2ΔCTN, PATL2ΔSEC14 and PATL2ΔCTN-SEC14 combinations) abolished membrane association, and the proteins were primarily present in the cytosol (Figs. 4C, 4D, 4E, 4F). This is consistent with the observed inability of PATL2ΔCTN-SEC14 to bind to liposomes (this work). A critical role of the CTN domain in the membrane association of SEC14L-PITPs (Fig. 4D) had previously been observed [46]. Deletion of the GOLD domain abolished PM association, and instead, led to localization at the ER membrane, marked by OFP-tagged HDEL (Fig. 4G, 4H). The observation is consistent with the role of the GOLD domain in binding PI(4,5)$P_2$-containing liposomes (this work) and the PM, enriched in PI(4,5)$P_2$ [27].

Taken together, the CTN-SEC14 domain is essential for PATL2 membrane association, however, the GOLD domain carries information for PM targeting. The N region, not involved in membrane targeting, may confer physiological tasks.

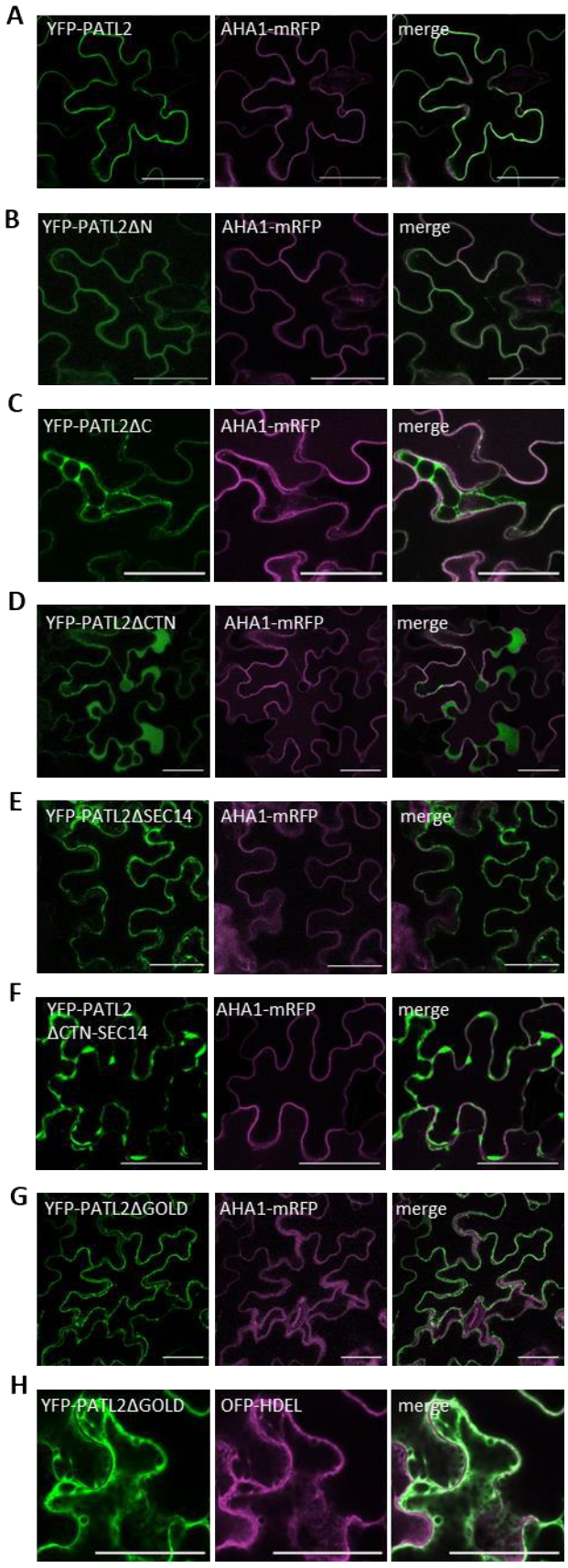


**Fig. 4. Cellular localization of PATL2 is specified by the CTN-SEC14 and GOLD domains.**

(A-H) Co-localization of YFP-PATL2 or its deletion mutants (green) with either PM marker AHA1-mRFP or ER marker HDEL-mOFP (magenta), in tobacco epidermis cells. Size bar 50μm.

## Discussion

In this work, we present a phylogenetic analysis of the SEC14L-PITP superfamily in the green lineage and show that the complexity of SEC14L-PITPs increased during land plant evolution. We dissect the modular domain structure of a SEC14-GOLD protein, Arabidopsis PATL2, and demonstrate that the individual domains of PATL2 contribute differently to lipid binding and membrane association.

The numbers of family members and the modular configurations of SEC14L-PITPs increased during land plant evolution, similar as in the animal lineage. This reflects certainly the high versatility of SEC14L-PITP roles in multicellular organisms, where developmental and environmental specifications of functions become increasingly connected, as highlighted by the case of SEC14-GOLD proteins in Arabidopsis. *Charaphyceae* have some land plant features, related to the function of SEC14-GOLD proteins. Their cell divisions involve formation of a phragmoplast with presence of specific proteins, and a subset of auxin functions seem present, as inferred from the partial presence of auxin signaling and PIN-mediated auxin transport genes in Chara [32]. Thus, it is surprising that SEC14-GOLD proteins are lacking in Chara, since these proteins are involved in cell plate formation and auxin responses in higher plants like Arabidopsis [37, 49]. Perhaps SEC14-GOLD proteins were acquired in land plants to integrate cell division and auxin responses with complex environmental response networks, more important on dry land. The fact that PATL1/PATL2/PATL3 and PATL4 are present in one common co-expression network suggests that they might take a role in regulating cytoskeleton function. This corresponds well to the known involvement of PATL1 and PATL2 in cell division [37, 48], a process dependent on the cytoskeleton. Enrichment of auxin-related genes in PATL6 co-expression analysis is consistent with the function of PATLs in auxin-dependent PIN1 localization [49]. Additionally, analysis of multiple *patl* mutants in Arabidopsis demonstrated a redundant function and critical role of the protein family in polarity and patterning [49]. Taken together, SEC14-GOLD proteins are partly redundant in land plants, while some specific functions of each are noted.

PATL2 is composed of a discrete N region and a C region with the conserved CTN-SEC14 and GOLD domains. We investigated the contributions of individual domains to membrane binding using deletion mutants. All SEC14 proteins share at least the SEC14 domain. Many SEC14 proteins also have the CTN, some even a GOLD domain, and only plant SEC14-GOLD proteins also have an N region. We used only deletions of full domains. Because the respective remaining domains were entities on their own, we are confident that domain deletions did not affect the protein structure in a manner that the functions of individual remaining domains were perturbed.

The C regions with CTN-SEC14-GOLD domains of different SEC14-GOLD proteins do not differ much in their amino acid sequence [37, 38]. PATL2 binds to the PM, and the C region is sufficient for PM-binding. Consistently, the localization of PATL2ΔN did not differ from that of PATL2, while deletion of the C region (PATL2ΔC) results in cytosolic localization of the protein. Deletion of only the CTN, SEC14 or both domains (PATL2ΔCTN, PATL2ΔSEC14, PATL2ΔCTN-SEC14) also results in cytosolic localization of the protein, consistent with the inability of PATL2ΔCTN-SEC14 to bind PI(4)P, PI(4,5)$P_2$ or PI(4)P and PI(4,5)$P_2$-loaded liposomes. The canonical CTN-SEC14 domain might attach to phosphate groups of PIPs through surface-located conserved basic amino acids, two of these are present in PATL2 (R373; K542) (Fig. S5) [26]. The importance of CTN-SEC14 domain is also consistent with literature data. The CTN domain of yeast Sec14p adds membrane-binding capabilities to a predominantly cytosolic rat SEC14L-PITP [46]. Moreover, a point mutation in the SEC14 domain of human p50RhoGAP leads to loss of protein-membrane association [45], similar as the deletion of the CTN-SEC14 domain of human PTP-MG2 [41]. Hence, the conserved function of the CTN-SEC14 domain in membrane-binding also holds true for plant proteins.

The GOLD domain is present in the single-domain protein p24 and in multi-domain proteins. In the latter, the GOLD domain co-occurs with additional domains, having a function in binding membrane lipids and proteins [1, 47]. The GOLD domain of PATL2 adds specificity for PM recognition and for binding to liposomes containing PI(4,5)$P_2$, presumably due to a reported conserved lysine repeat. It may specifically bind PI(4,5)$P_2$, since it aligns well to the PI(4,5)$P_2$-binding motif of two proteins, AP180 and μ2-adaptin, which are involved in clathrin-coated vesicle formation (Fig. S4) [15, 28, 37, 39]. Deletion of the GOLD domain (PATL2ΔGOLD) shifts binding from the PM to the endomembrane system, and from liposomes containing PI(4,5)$P_2$ to liposomes containing only PI(4)P. The above mentioned amino acid similarities among C regions of SEC14-GOLD proteins suggest conserved domain functions for them [1, 15, 28, 37, 39, 41, 45, 47].

The N regions of SEC14-GOLD proteins are unique to the plant kingdom and are not present in human SEC14-GOLD counterparts. Furthermore, they vary in every plant SEC14-GOLD protein. The sequence diversity of the N region suggests rapid evolution and unique function. The N region of PATL2 does not mediate liposome binding, if the CTN-SEC14 domain is lacking (PATL2ΔCTN-SEC14). Also in the cellular context, the N region alone lacking the CTN-SEC14-GOLD module (PATL2ΔC), does not bind membranes. However, the N region confers binding to immobilized phospholipid units, and we explain this with charges and ionic interactions involving the positively-charged [(K)KE(E); (EE)EK] repeats of PATL2-N. Perhaps the N regions of SEC14-GOLD proteins contribute to membrane interactions in cellular contexts by binding to membrane proteins, such as shown in the case of PATL1 binding the salt transporter SOS1 via the N region [54]. Thus, the N regions might confer

specific functions to SEC14-GOLD proteins and be relevant for specific physiological responses.

In the future, it will be important to understand the molecular mechanisms underlying the PATL functions, modulated by their domains and by specific protein-protein and protein-lipid interactions.

## Supplemental information

Supplemental Information includes six figures and three tables.

**Supplemental Figure 1.** Phylogenetic analysis and modular structure of the SEC14L-PITP superfamilies in green algae and liver moss.

**Supplemental Figure 2.** Phylogenetic analysis and modular structure of the SEC14L-PITP superfamilies in a lycophyte and eudicot tomato

**Supplemental Figure 3.** Phylogenetic analysis and modular structure of the SEC14L-PITP superfamily in monocot maize (*Zea mays*).

**Supplemental Figure 4.** Alignment of Arabidopsis PATL protein sequences.

**Supplemental Figure 5.** Alignment of AtPATL2 with human TAP protein sequences.

**Supplemental Figure 6.** Protein–phosphoinositide overlay assay and electrophoretic migration behavior of StrepII-PATL2 and deletion mutants.

**Supplemental Table 1.** List of genes in co-expression modules.

**Supplemental Table 2.** Information on the generation of PATL2 deletion mutants.

**Supplemental Table 3.** List of primers used in this study.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

PB, KM and RI designed experiments. KM and JH performed experiments. KM, JH, RI and PB analyzed data. KM wrote the manuscript. PB and RI revised the manuscript. PB acquired funding. All authors have read and approved this manuscript.

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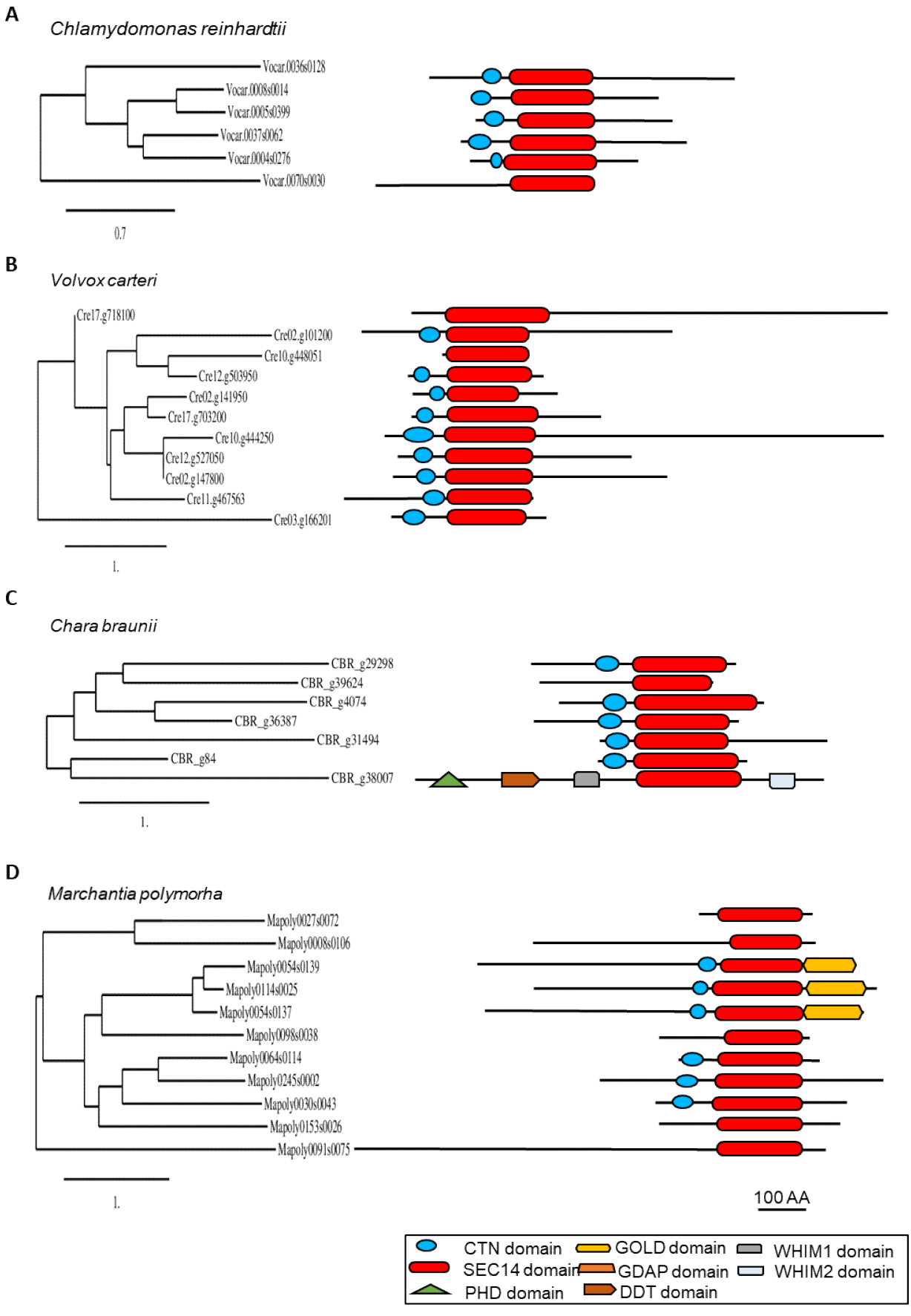
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CBR_g84
CBR_g38007
1.
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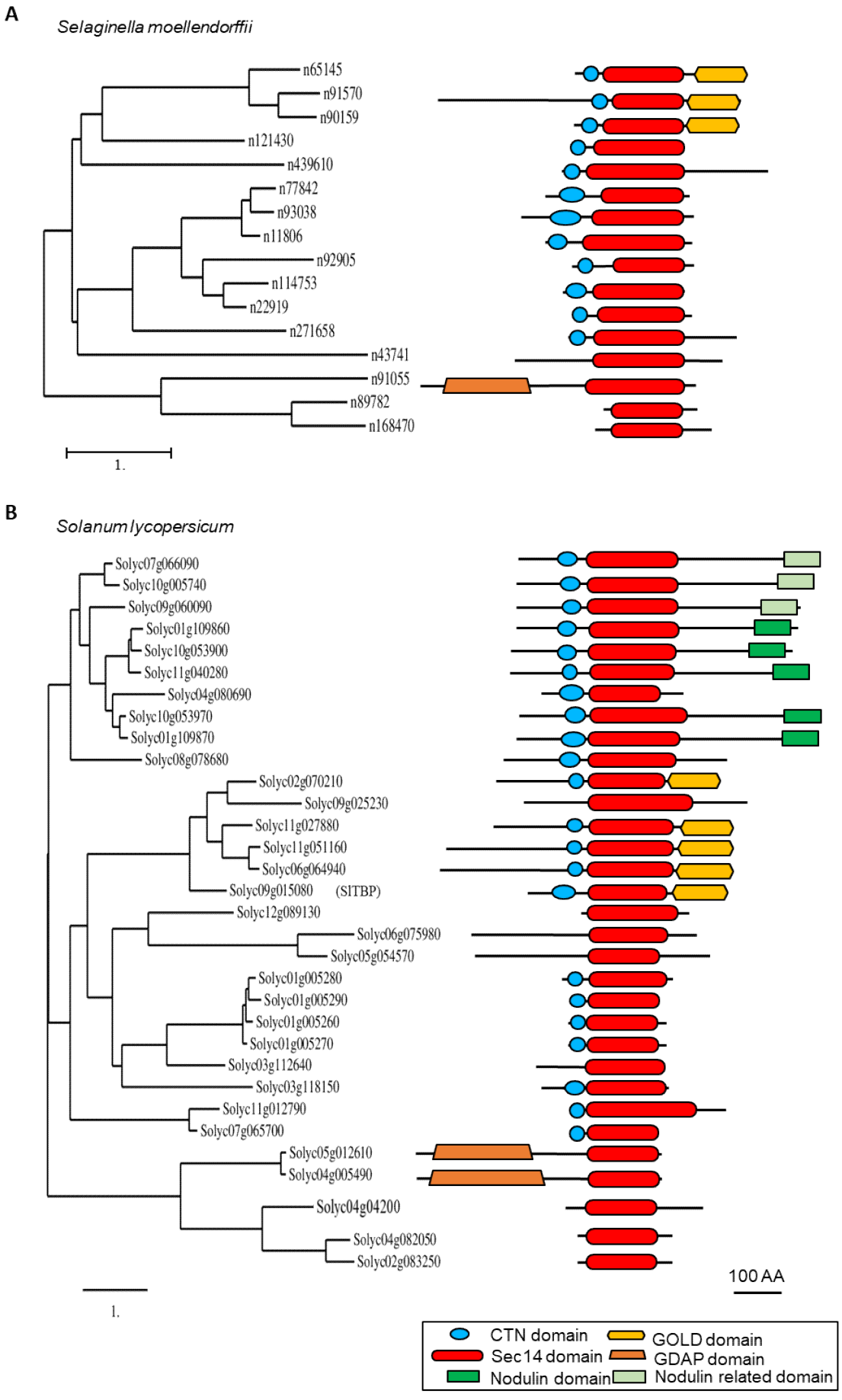
1.
100 AA
CTN domain
GOLD domain
WHIM1 domain
SEC14 domain
GDAP domain
WHIM2 domain
PHD domain
DDT domain

**Supplemental Figure 1. Phylogenetic analysis and modular structure of the SEC14L-PITP superfamilies in green algae and liver moss.**

Left, Phylogenetic trees, generated with full-length amino acid sequences. Right, modular structures of SEC14L-PITPs. (A) *Chlamydomonas reinhardtii*; (B) *Volvox carteri*; (C) *Chara braunii*; (D) *Marchantia polymorha*. Different domains are represented in different colors, as indicated. Primary protein structure and relative length of domains are drawn to scale.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B
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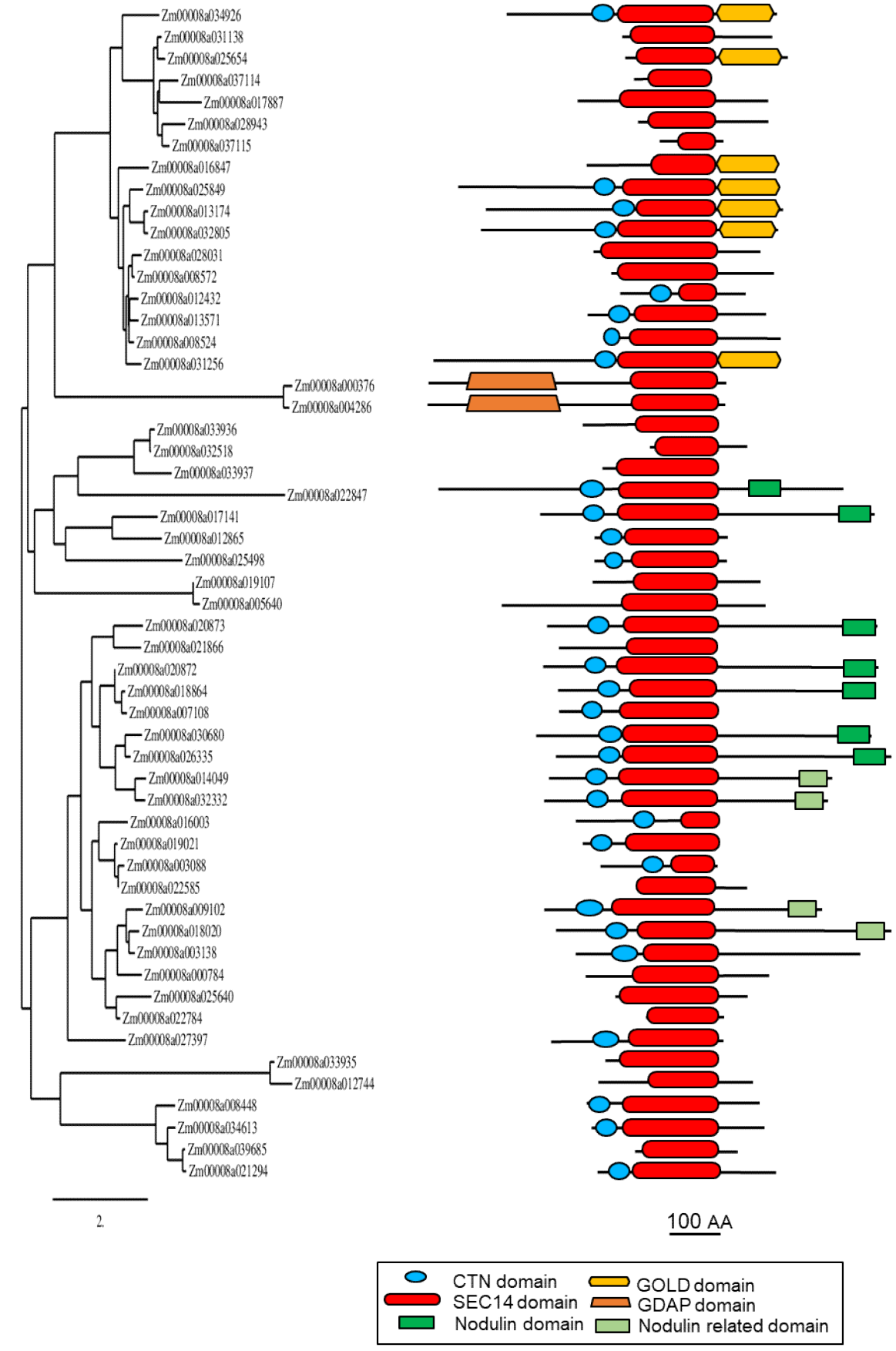
Solyc04g04200
Solyc04g082050
Solyc02g083250
1.
100 AA
CTN domain
Sec14 domain
Nodulin domain
GOLD domain
GDAP domain
Nodulin related domain

**Supplemental Figure 2. Phylogenetic analysis and modular structure of the SEC14L-PITP superfamilies in a lycophyte and eudicot tomato.**

Left, Phylogenetic trees, generated with full-length amino acid sequences. Right, modular structures of SEC14L-PITPs. (A) *Selaginella moellendorffii*; (B) *Solanum lycopersicum.* Different domains are represented in different colors, as indicated. Primary protein structure and relative length of domains are drawn to scale.

*Zea mays*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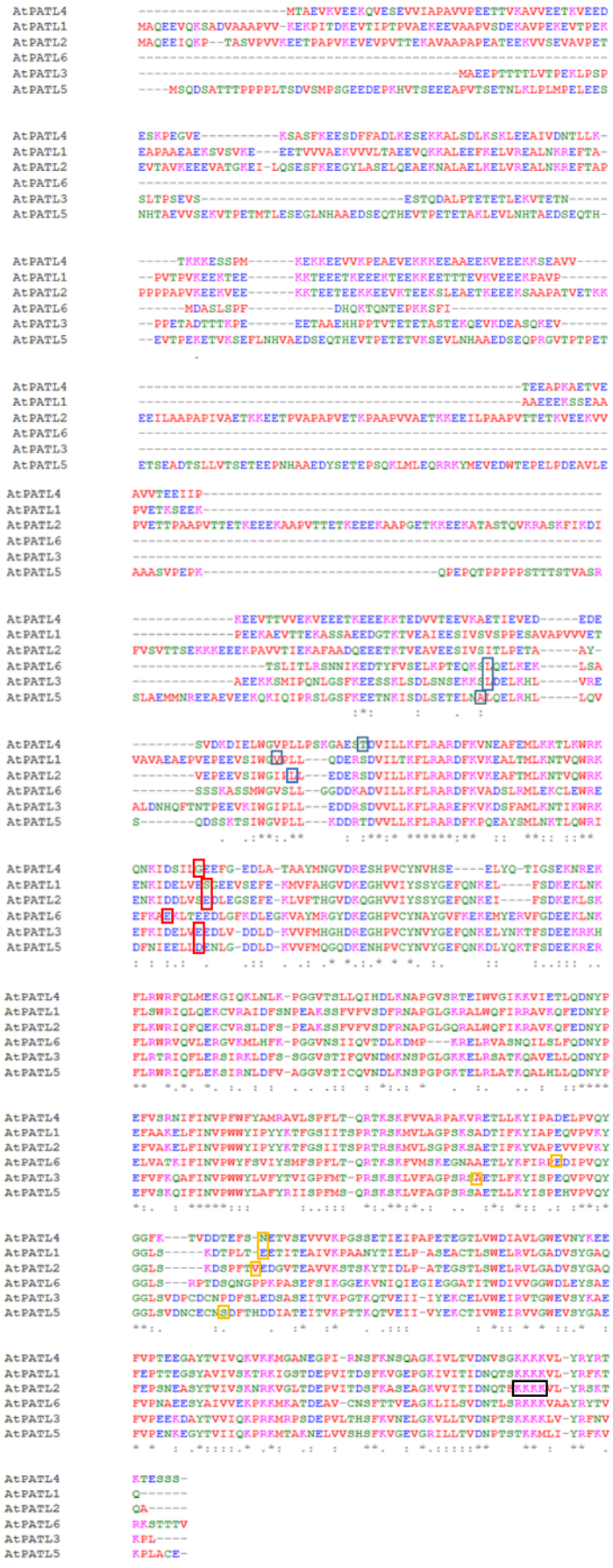
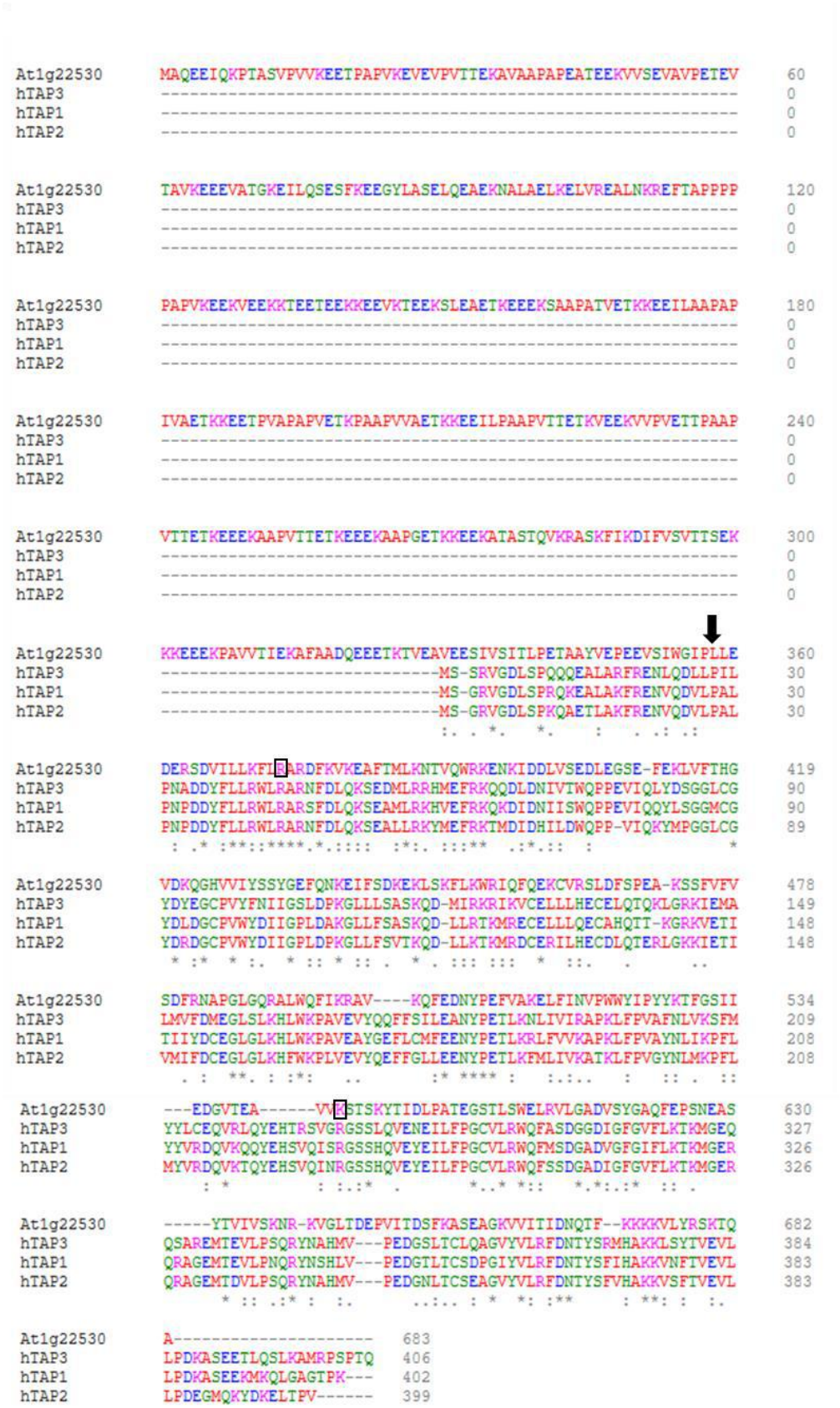
Zm00008a033935
Zm00008a012744
Zm00008a008448
Zm00008a034613
Zm00008a039685
Zm00008a021294
2.
100 AA
CTN domain
SEC14 domain
Nodulin domain
GOLD domain
GDAP domain
Nodulin related domain

**Supplemental Figure 3. Phylogenetic analysis and modular structure of the SEC14L-PITP superfamily in monocot maize (*Zea mays*).**

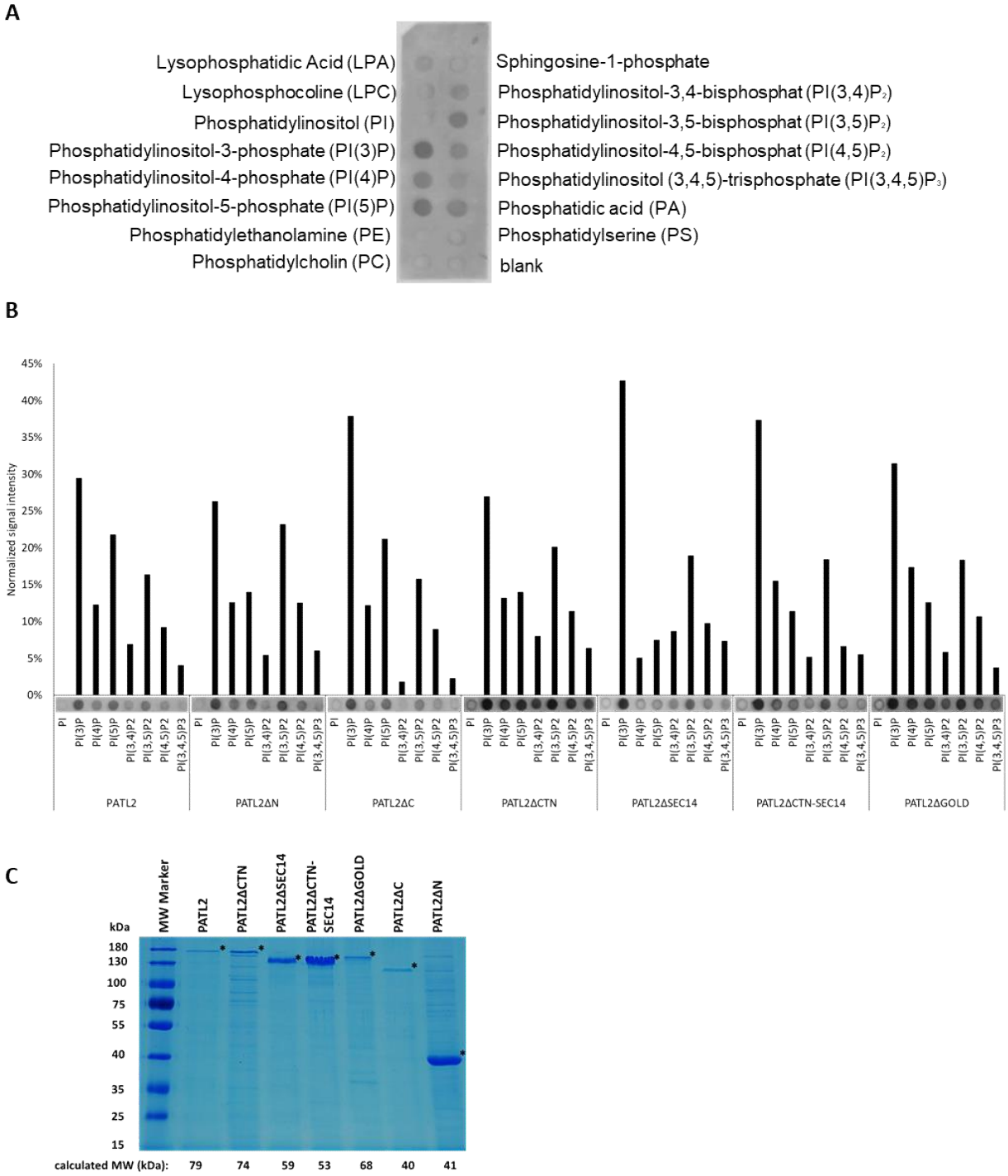
Left, Phylogenetic tree, generated with full-length amino acid sequences. Right, modular structures of SEC14L-PITPs. Different domains are represented in different colors, as indicated. Primary protein structure and relative length of domains are drawn to scale.




**Supplemental Figure 4. Alignment of Arabidopsis PATL protein sequences**. The start of different domains is indicated by a box, in blue, CTN, in red, SEC14, in yellow, GOLD domain. Lysine motif of PATL2 is indicated by a black box. The colors of amino acid letters represent red, hydrophobic; green, polar uncharged; pink, basic; blue, acidic amino acids.




**Supplemental Figure 5. Alignment of AtPATL2 with human TAP protein sequences.** The start of the CTN-SEC14-GOLD region of AtPATL2 is indicated by an arrow. Surface-located conserved amino acids of PATL2 are marked with black boxes. The colors of amino acid letters represent red, hydrophobic; green, polar uncharged; pink, basic; blue, acidic amino acids.




**Supplemental Figure 6. Protein–phosphoinositide overlay assay and electrophoretic migration behavior of StrepII-PATL2 and deletion mutants.**

(A, B) StrepII-PATL2 and deletion mutant protein–phospholipid overlay assay. PIP binding is quantified in (B). (C) Electrophoretic migration behavior of StrepII-PATL2 and deletion mutants, analyzed by SDS-PAGE, followed by Coumassie staining. StrepII-PATL2 and deletion-mutant protein bands are indicated by an asterisk. The theoretical masses of StrepII-PATL2 and deletion mutants are indicated below. In the presence of the N-terminal domain migration of proteins is drastically retarded, presumably due to the acidic character of the N-terminal part, as reported before [37].

**Supplemental Table S1. List of genes in co-expression modules**

| AGI number | Name | description |
|---|---|---|
| The genes in PATL1-4 coexpression module | | |
| At1g72150 | PATL1 | PATELLIN1 |
| At1g22530 | PATL2 | PATELLIN2 |
| At1g72160 | PATL3 | PATELLIN3 |
| At2g17550 | TRM26 | TON1 RECRUITING MOTIF 26 |
| At1g61100 | | disease resistance protein |
| At5g40450 | RBB1 | REGULATOR OF BULB BIOGENESIS1 |
| At4g33740 | | MYB-like transcriptoion factor |
| At3g27960 | KLCR2 | KINESIN LIGHT CHAIN-RELATED 2 |
| At2g01910 | MAP65-6 | Microtubule-associated protein |
| At1g14380 | IQD28 | IQ-DOMAIN 28 |
| At4g16563 | | aspartyl protease |
| At2g30930 | | hypothetical protein |
| At4g20260 | PCAP1 | PLASMA-MEMBRANE ASSOCIATED CATION-BINDING PROTEIN 1 |
| At2g48030 | | DNAse-I-like |
| At2g36410 | DUF662 | transcriptional activator |
| At1g64390 | GH9C2 | GLYCOSYL HYDROLASE 9C2 |
| At1g53730 | SRF6 | STRUBBELIG RECEPTOR FAMILY6 |
| At1g30690 | PATL4 | PATELLIN4 |
| At3g12110 | ACT11 | ACTIN11 |
| At1g03870 | FLA9 | FASCICLIN-LIKE ARABINOOGALACTAN 9 |
| At5g16590 | LRR1 | LEUCINE RICH REPEAT PROTEIN 1 |
| At1g67750 | | Pectate lyase |
| At4g12730 | FLA2 | FASCICLIN-LIKE ARABINOGALACTAN 2 |
| At4g22010 | SKS4 | SKU5 SIMILAR4 |
| At1g04680 | | Pectin lyase-like |
| At3g54400 | | Aspartyl protease |
| At5g15350 | ENODL17 | EARLY NODULIN-LIKE PROTEIN 17 |
| At4g12420 | SKU5 | protein of unknown function involved in directed root tip growth |
| The genes in PATL6 coexpression module | | |
| At1g27190 | BIR3 | BAK1-INTERACTING RECEPTOR-LIKE KINASE 3 |
| At1g70940 | PIN3 | PIN-FORMED 3 |
| At2g38120 | WAV5 (AUX1) | AUXIN RESISTANT 1 |
| At3g05100 | transferase | SAM methyl transferase |
| At1g75500 | WAT1 | WALLS ARE THIN 1 transporter |
| At4g12110 | SMO1-1 | STEROL-4ALPHA-METHYL OXIDASE 1-1 |
| At3g51670 | PATL6 | PATELLIN6 |

**Supplemental Table S2. Information on the generation of PATL2 deletion mutants.**

| PATL2 deletion mutant | AA position | Predicted molecular weight (kDa) | predicted molecular weight stepII-tagged (kDa) | Molecular running weight stepII-tagged (kDa) |
|---|---|---|---|---|
| PATL2 | 1-683 | 76 | 79 | ~ 170 |
| PATL2ΔN | 358-683 | 38 | 41 | ~ 40 |
| PATL2ΔCTN | 1-357; 403-683 | 71 | 74 | ~ 170 |
| PATL2ΔSEC14 | 1-402; 577-684 | 56 | 59 | ~ 160 |
| PATL2ΔCTN-SEC14 | 1-357; 577-685 | 50 | 53 | ~ 160 |
| PATL2ΔGOLD | 1-579 | 65 | 68 | ~ 120 |
| PATL2ΔC | 1-357 | 37 | 40 | ~ 160 |

**Supplemental Table S3. Primers used in this study.**

| Primer | Sequence (5'-3') |
|---|---|
| PATL2B1F_N | GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCTCAAGAAGAGATACAG |
| PATL2B2stopR | GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGCTTGGGTTTTGGACC |
| PATL2ΔGOLDstopB2 | GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAACGGTGAATGGAC |
| PATL2ΔSEC14_F | ATCGACGACCTAGTCTTCACCGTTGAAGAT |
| PATL2ΔSEC14_R | ATCTTCAACGGTGAAGACTAGGTCGTCGAT |
| PATL2ΔCTN-SEC14_F | ATCTGGGGAATCCCATTCACCGTTGAAGAT |
| PATL2ΔCTN-SEC14_R | ATCTTCAACGGTGAATGGGATTCCCCAGAT |
| PATL2ΔCTN_F | CAATCTGGGGAATCCCATCAGAAGATCTTGA |
| PATL2ΔCTN_R | TCAAGATCTTCTGATGGGATTCCCCAGATTG |
| PATL2ΔCstopB2 | GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGGGATTCCCCAGATTGAGAC |
| PATL2ΔNB1_N | GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCTTCTAGAGGACGAAAGATCC |
| PATL2_F_BamHI | AGGAGTGGATCC T ATGGCTCAAGAAGAGATACAG |
| PATL2_R_NotI | AGGAGTGCGGCCGC TTA TGCTTGGGTTTTGGACC |
| PATL2ΔGOLD_R_NotI | AGGAGTGCGGCCGC TTA AACGGTGAATGGACTATC |
| PATL2ΔN_F_BamHI | AGGAGTGGATCC T CTTCTAGAGGACGAAAGATCC |
| PATL2ΔC_R_NotI | AGGAGTGCGGCCGC TTA TGGGATTCCCCAGATTG |

## Authors Contribution to Manuscript 1

**Karolin Montag**

Designed, performed and analyzed following experiments: phylogenetic analyses and alignments; generation of deletion mutants and vectors; transient tobacco transformation and confocal microscopy; protein expression and purification; protein-lipid overlay assays

Designed and analyzed following experiments: liposome-binding assay

K. Montag designed the outline and wrote the manuscript, prepared figures (except of Figure 2) and reviewed / edited the manuscript.

Jannik Hornbergs

Performed following experiments under supervision of K.Montag: protein expression and purification; protein lipid overlay assays; liposome-binding assays

J. Hornbergs contributed to figure preparation of protein-lipid overlay assays and liposome-binding assays.

Rumen Ivanov

Designed, performed and analyzed following experiments: gene expression analysis; co-expression analysis

R. Ivanov contributed to phylogenetic analyses of Arabidopsis (Fig. 1A). R.Ivanov designed the outline of the manuscript, prepared Figure 2, supervised the study and reviewed / edited the manuscript.

Petra Bauer

Designed the outline of the manuscript, supervised the study, provided funding, and reviewed / edited the manuscript.

# 7. Manuscript 2

## SEC14L-PITP PATL2 interacts with IRT1 and protects membranes from oxidative damage in Arabidopsis

# SEC14L-PITP PATL2 interacts with IRT1 and protects membranes from oxidative damage in Arabidopsis

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**Keywords:** iron uptake, SEC14, ROS, lipid peroxidation, IRT1, PATL2, Arabidopsis

**Key massages:**

- Arabidopsis iron import through IRT1 is highly regulated to maintain a sufficient but non-toxic iron pool
- The N region of PATL2 is critical for protein-protein interaction with IRT1
- PATL2 might be a regulator of iron acquisition on protein level
- PATL2 is recruited by IRT1 to the PM and supports membrane integrity

## Abstract

Iron is an essential micronutrient for the model plant *Arabidopsis thaliana.* However, an overaccumulation of iron negatively influences plant survival. Maintaining a sufficient but non-toxic iron pool requires tightly controlled iron import through root iron transporter IRON-REGULATED TRANSPORTER 1 (IRT1). Nevertheless, the complete regulatory mechanism controlling IRT1 activity in roots is not fully discovered till now. We identified the SEC14-GOLD protein PATELLIN 2 (PATL2) as an interactor of the variable region of IRT1 (IRT1vr). Here we examine the interaction of IRT1 with PATL2 and the role of PATL2 in iron acquisition. We found that IRT1 and PATL2 are expressed in the same tissue and are co-localized at the plasma membrane. Furthermore, we could demonstrate that interaction of IRT1vr with PATL2 occurs via the N region of PATL2. PATL2 negatively affects iron reductase activity and lowers the lipid-peroxidation rate. Additionally, the presence of PATL2 had a positive effect on IRT1-complemented *fet3 fet4* cell growth. Taken together, data indicate that PATL2 is a negative regulator of iron acquisition on protein level and prevents iron-caused membrane damage.

## Introduction

Due to its capability to change its redox stage, iron is an essential catalytic component of different biochemical pathways. Although iron is the fourth abundant element on earth it soil bound ferric form is poorly utilizable for plants (Wedepohl, 1995; Yi and Guerinot, 1996). Thus, iron availability can be a limiting factor for plant growth, biomass production and reproduction (Briat et al., 2015). Iron has a catalytic activity which might result in the generation of radicals via the Fenton reaction when accumulating in high levels (Kehrer, 2000; Winterbourn, 1995). This could lead to severe DNA, protein and lipid damage (Connolly and Guerinot, 2002; Le et al., 2019; Tripathi et al., 2018). Hence, it is critical for plant survival to keep a sufficient but non-toxic iron pool by regulating iron homeostasis. To overcome limiting iron availability *Arabidopsis thaliana* (Arabidopsis) uses a reduction-based uptake mechanism – Strategy I, which is upregulated in response to iron starvation by subgroup Ib bHLH transcription factors FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) and bHLH039 (Brumbarova et al., 2015; Colangelo and Guerinot, 2004; Ivanov et al., 2012; Jakoby et al., 2004; Naranjo-Arcos et al., 2017). First, Iron is solubilized and then reduced from ferric to ferrous iron by FERRIC REDUCTASE-OXIDASE 2 (FRO2) (Robinson et al., 1999). Next, the reduced iron is taken up in the epidermis cells of roots by the plasma membrane (PM) located bivalent zinc and iron permease (ZIP) IRON-REGULATED TRANSPORTER 1 (IRT1) (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). A characteristic feature of ZIPs is the cytosolic variable region. The variable region of IRT1 (IRT1vr) is spanning between its transmembrane domains three and four (Eide, 2006; Eng et al., 1998; Guerinot, 2000; Nishida et al., 2008). IRT1vr is critical for the control of metal binding, protein activity and protein stability (Dubeaux et al., 2018; Grossoehme et al., 2006; Guerinot, 2000; Kerkeb et al., 2008; Potocki et al., 2013; Rogers et al., 2000). Furthermore, iron is not the only substrate of IRT1. It can also transport other heavy metals like zinc, manganese, cadmium and cobalt (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000; Vert et al., 2002), but primarily it is critical for iron uptake in plants. Therefore, IRT1 is the starting point to regulate iron import on posttranslational level - not only upon iron deficiency but also due to iron overload. Covalent modifications through protein-protein interactions work hand in hand with cellular trafficking to control IRT1 activity (Barberon et al., 2014; Barberon et al., 2011; Dubeaux et al., 2018; Ivanov et al., 2014; Shin et al., 2013). During iron deficiency IRT1 activity at the PM can be inhibited through the interaction of IRT1vr with ENHANCED BENDING 1 (EHB1) and via internalization, initiated by ubiquitination through the E3 ligase IRT1-DEGRADATION FACTOR 1 (IDF1) (Barberon et al., 2014; Barberon et al.,

2011; Khan et al., 2019; Shin et al., 2013). Recycling of the internalized transporter back to the PM is then dependent on the presents of FREE1/FYVE1 - also being involved in rhizosphere-directed polarization of IRT1 - and SORTING NEXIN 1 (SNX1) (Barberon et al., 2014; Ivanov et al., 2014). Recognizing, via IRT1vr, excess of its non-iron metal substrates in the cytosol results in phosphorylation of IRT1vr by protein kinase CIPK23, followed by IDF1 induced endocytosis and degradation of IRT1 to protect plants from reactive metal accumulation (Dubeaux et al., 2018). However, the complete regulatory mechanism controlling iron import and IRT1 activity in roots is not fully understood till now. That is why additional factors must undertake regulatory functions within iron acquisition. To fill the gabs in the understanding of IRT1 regulation and iron acquisition in Arabidopsis, it was searched for interaction partners of IRT1vr (Khan et al., 2019). PATELLIN 2 (PATL2), a member of the SEC14-like phosphatidylinositol transfer protein (SEC14L-PITP) superfamily, was identified as an IRT1vr interactor.

Members of the SEC14L-PITP superfamily are crucial regulators of membrane trafficking and the phospholipid-signaling pathway by sensing, transporting and exchanging single lipophilic substances between membranes, e.g. phosphatidylinositol (PI), phosphatidylinositol phosphates (PIPs) and phosphatidylcholine (PC), (Bankaitis et al., 1990; Cleves et al., 1991; Kf de Campos and Schaaf, 2017; Mousley et al., 2007). Due to their characteristics SEC14L-PITPs are involved in complex physiological processes within plants taking over regulatory roles in membrane trafficking (Ghosh et al., 2015; Gierczik et al., 2019; Huang et al., 2013; Peiro et al., 2014; Vincent et al., 2005; Zhou et al., 2019). While algae only exhibit a few single-domain SEC14L-PITPs, Arabidopsis has 35 SEC14L-PITPs encoded in its genome (Montag et al., under review). Six of them are multi-domain SEC14-GOLD proteins belonging to the PATELLIN (PATL) family (Peterman et al., 2004). They are characterized through their unique amino (N)-terminal sequence (termed “N region”) followed by a CTN-SEC14 hydrophobic lipid-binding pocket and a carboxy (C)-terminal golgi dynamics (GOLD) domain (Peterman et al., 2004). The GOLD domain is involved in intracellular vesicle trafficking by mediating protein-protein interactions, for example, between AtPATL3 and AtEXO70A1, and protein-membrane interactions, e.g. via binding to $PI(4,5)P_2$ (Anantharaman and Aravind, 2002; Carney and Bowen, 2004; Montag et al., under review; Sohda et al., 2001; Wu et al., 2017). Analyses revealed that PATLs are partly redundant. This was demonstrated through overlapping gene-expression profiles and *patl* mutant analysis (Montag et al., under review; Tejos et al., 2017). PATLs seem to be general regulators involved in plant development and stress response (Chu et al., 2018; Peterman et al., 2004; Tejos et al., 2017; Zhou et al., 2018). PATL1 is working as a regulator of plant tolerance to cold and salt stress by interacting with PM located $Ca^{2+}$ sensor Calmodulin-4 (CaM4) and $Na^+/H^+$ antiporter SALT OVERLY-SENSITIVE1 (SOS1) (Chu et al., 2018; Zhou et

al., 2018). PATL3 is able to interact with EXO70A1, a component of the exocyst complex involve in cell polarity and cytokinesis (Fendrych et al., 2013; He and Guo, 2009; Wu et al., 2017). Additionally, PATL3 and PATL6 interact with a plasmodesmata targeting movement protein interfering with the movement of alfalfa mosaic virus (Peiro et al., 2014). Tomato (*Solanum lycopersicum*) TOCOPEROL BINDING PROTEIN (SITBP), a homolog of Arabidopsis PATL6, is able to bind α-tocopherol (α-TOC) and contributes to maintaining chloroplast membrane structure (Bermudez et al., 2018). The ability of SEC14L-PITPs to bind the antioxidant α-TOC was reported several times (Kempna et al., 2003; Min et al., 2003; Zimmer et al., 2000). PATL2 is a substrate of MPK4 MAP kinase, binds PIPs (and PI) and localizes to the PM and the developing cell plate during cytokinesis (Montag et al., under review; Suzuki et al., 2016; Tejos et al., 2017).

Since the SEC14-GOLD protein PATL2 was identified as an interaction partner of IRT1vr, the aim of this study was to characterize the interaction of IRT1 with PATL2 and to understand the function of PATL2 in iron acquisition. We found that IRT1 and PATL2 are co-localizing at the PM and that the interaction occurs via the N region of PATL2. Additionally, we demonstrated that PATL2 affects iron acquisition and lowers lipid peroxidation upon iron import.

## Results

### Identification of PATL2 as an IRT1vr interactor

In previous studies several IRT1 regulators were identified able to influence iron uptake in Arabidopsis roots, such as IDF1 and CIPK23 (Barberon et al., 2011; Dubeaux et al., 2018; Shin et al., 2013). However, the exact regulatory mechanism controlling iron import through IRT1 in roots cannot be explained totally with this knowledge. Additional factors must play a critical role in the regulation of iron acquisition by controlling IRT1. For the purpose of identifying proteins involved in the regulation of IRT1 a yeast two-hybrid screen was performed. IRT1vr was used as bait and screened against a cDNA library generated from iron-deficient Arabidopsis roots. In this screen EHB1 was identified as an IRT1vr interactor. Later it was shown that EHB1 is a negative regulator of IRT1 (Khan et al., 2019). In the same screen the SEC14L-PITP PATL2 was identified as a putative IRT1vr interactor. The interaction was confirmed by a targeted yeast two-hybrid assay using recloned IRT1vr and full-length PATL2 (Fig. 1A). All tested negative controls showed no growth on the selection medium (Fig. S1A). The already analyzed interaction of AKT1 and CIPK23 served as a positive control (Fig. 1A)(Xu et al., 2006). The outcome illustrated a specific interaction between IRT1vr and PATL2.

## PATL2 interacts with IRT1vr via its N region

PATL2 is composed of different domains, which might be able to interact with IRT1vr, as shown for other PATLs able to interact with PM proteins (Chu et al., 2018; Zhou et al., 2018). To investigate the interaction of PATL2 with IRT1vr further and to identify the domain critical for interaction, we included several deletion mutants of PATL2 in our experiments (Fig. 1B) (Montag et al., under review). The targeted yeast two-hybrid assay showed that mutated PATL2 constructs lacking either the CTN domain (PATL2ΔCTN), or the SEC14 domain (PATL2ΔSEC14) were able to interact with IRT1vr (Fig.1A; Fig. S1A), indicating no function of those protein parts in binding of PATL2 to IRT1vr. However, PATL2ΔN, PATL2ΔCTN-SEC14, PATL2ΔGOLD and PATL2ΔC were unable to interact with IRT1vr in the targeted yeast two-hybrid assay. Due to the fact that neither PATL2ΔN nor PATL2ΔC interacted with IRT1vr in the targeted yeast two-hybrid assay the data were not trustable, since at least one of the constructs should be able to interact with IRT1vr. These results can possibly be traced back to expression or folding problems in the yeast nuclei, comparable to reported problems in a yeast two-hybrid assay for PATL3 (Wu et al., 2017). Therefore, the bimolecular fluorescence complementation (BiFC) method was applied (Grefen and Blatt, 2012) in *N. benthamiana* leaf-epidermis cells as a plant-based interaction system, better mimicking the biological environment. A RFP-signal monitored successful transient transformation of the cells, while an YFP-signal indicated interaction of PATL2, fused to nYFP, with IRT1vr, fused to cYFP (Fig. 1C). A positive BiFC signals was obtained for all PATL2 deletion proteins lacking the CTN domain, SEC14 domain and GOLD domain (Fig. 1D-H). Demonstrating that neither the CTN domain, nor the SEC14 domain, nor the GOLD domain is mediating the interaction of PATL2 with IRT1vr. PATL2 protein with deleted N region was not able to bind to IRT1vr during BiFC analysis (Fig.1I), suggesting that this domain might be critical for PATL2 interaction with IRT1vr. As a negative control EHB1, lacking its CAR signature domain (sig), in combination with IRT1vr was run along the experiment (Fig. S1B) (Khan et al., 2019). Since PATL2 was able to interact with IRT1vr, the next step was to display its ability to interact with the full-length IRT1 protein. Indeed, IRT1-GFP and PATL2-HA were co-immunoprecipitated (Fig. 1J). The obtained result demonstrates that PATL2 is able to interact with full-length IRT1. Summed up, PATL2 is able to interact with IRT1vr and full-length IRT1 *in planta*. The N region is critical for PATL2 interaction with IRT1vr, while the CTN-SEC14 domain does not contribute to the interaction.

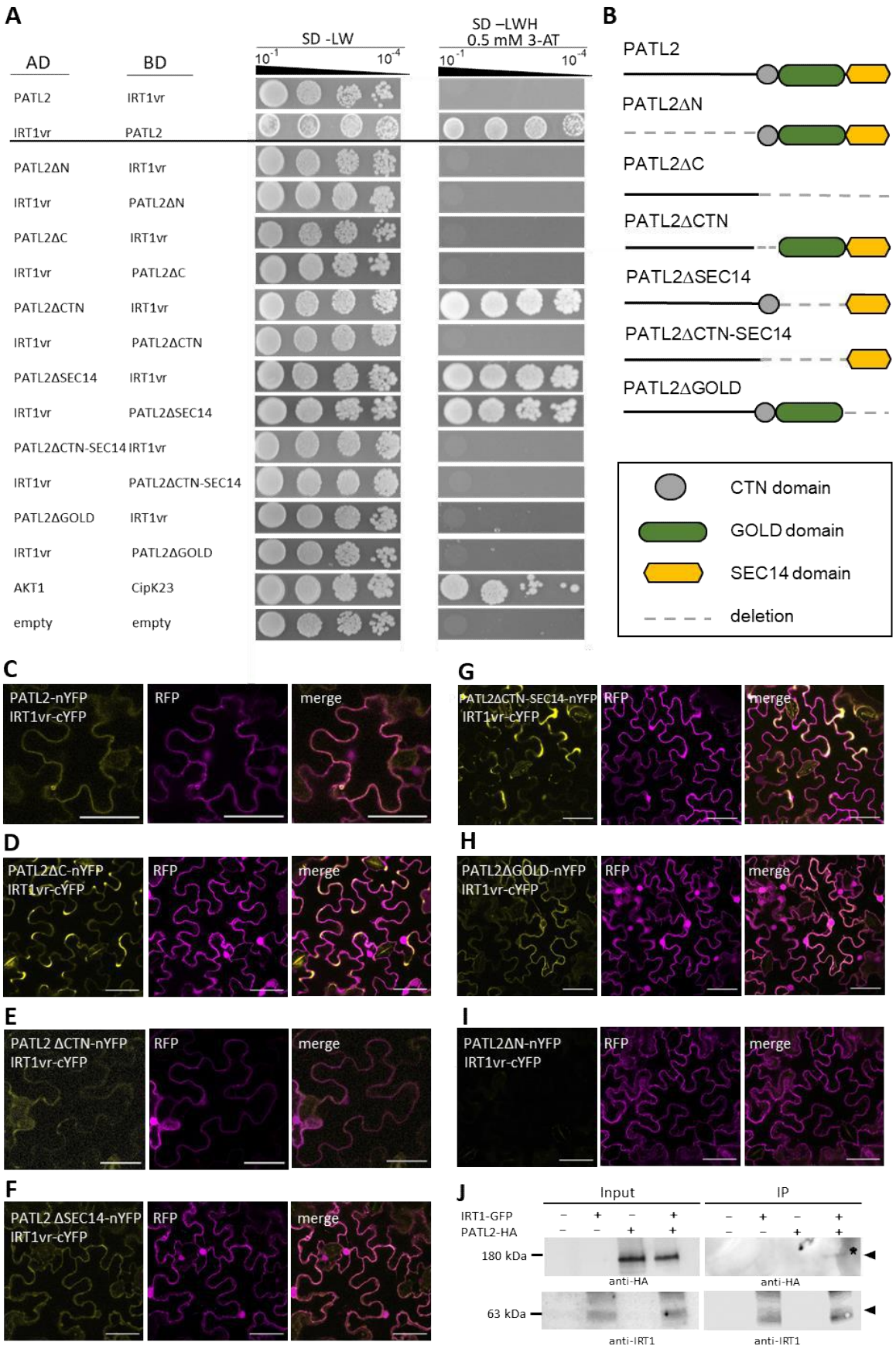
A
AD
BD
SD -LW
SD –LWH
0.5 mM 3-AT
10-1
10-4
PATL2 IRT1vr
IRT1vr PATL2
PATL2ΔN IRT1vr
IRT1vr PATL2ΔN
PATL2ΔC IRT1vr
IRT1vr PATL2ΔC
PATL2ΔCTN IRT1vr
IRT1vr PATL2ΔCTN
PATL2ΔSEC14 IRT1vr
IRT1vr PATL2ΔSEC14
PATL2ΔCTN-SEC14 IRT1vr
IRT1vr PATL2ΔCTN-SEC14
PATL2ΔGOLD IRT1vr
IRT1vr PATL2ΔGOLD
AKT1 CipK23
empty empty
B
PATL2
PATL2ΔN
PATL2ΔC
PATL2ΔCTN
PATL2ΔSEC14
PATL2ΔCTN-SEC14
PATL2ΔGOLD
CTN domain
GOLD domain
SEC14 domain
deletion
C
PATL2-nYFP
IRT1vr-cYFP
RFP
merge
D
PATL2ΔC-nYFP
IRT1vr-cYFP
RFP
merge
E
PATL2 ΔCTN-nYFP
IRT1vr-cYFP
RFP
merge
F
PATL2 ΔSEC14-nYFP
IRT1vr-cYFP
RFP
merge
G
PATL2ΔCTN-SEC14-nYFP
IRT1vr-cYFP
RFP
merge
H
PATL2ΔGOLD-nYFP
IRT1vr-cYFP
RFP
merge
I
PATL2ΔN-nYFP
IRT1vr-cYFP
RFP
merge
J
Input
IP
IRT1-GFP
PATL2-HA
180 kDa
63 kDa
anti-HA
anti-IRT1

**Figure 1. PATL2 interacts with IRT1.**

(A) Targeted yeast two-hybrid assay for verification of interaction between PATL2 and its deletion mutants with IRT1 variable region (vr). Growth on selection media (right) indicates protein-protein interaction. (Controls are shown in Fig. S1A.) (B) Schematic representation of PATL2 deletion mutants. (C) to (I) Targeted BiFC experiment in *N.benthamiana* epidermis cells for verification of the interaction between PATL2 and its deletion mutants with IRT1vr. Showing that PATL2-N is responsible for interaction with IRT1vr. YFP-signal indicates reconstitution of the full-length YFP protein due to protein-protein interaction of tagged proteins. RFP-signal serves as a control for cell transformation. Bars: 50 µM (Control is shown in Fig. S1B.) (J) PATL2 interacts with full -length IRT1 in a immunnoprecipitation (IP) analysis. PATL2-HA and IRT1-GFP or both were expressed in *N. benthamiana* epidermis cells for IP. IP was performed using GFP-Trap_M beads. Samples before (input) and after IP were tested by immunoblot analysis. Specific, but weak, PATL2-HA protein was found in combined IP (highlighted with *).

## PATL2 expression pattern and subcellular localization overlap with IRT1 expression and subcellular localization

PATLs are characterized as peripheral membrane proteins and previous studies demonstrated that they are localized to the developing cell plate, PM and in the cytosol, depending, among others, on the developmental stage of the cell (Peterman et al., 2004; Tejos et al., 2017). The IRT1 interactor PATL2 N-terminally tagged to YFP co-localized with PM markers in *N.benthamiana* leaf epidermis cells (Fig. 2A) (Montag et al., under review). A PATL2-GFP fusion protein showed localization to the PM, confirmed through treatment of transformed cells with mannitol solution, leading to plasmolysis with green shining PM Hechtian strands (Fig. 2B). These results indicate that tagging PATL2 with fluorescence proteins at its C- or N- terminus does not influence its subcellular localization. Next, we tested the co-localization of YFP-PATL2 and IRT1-mRFP. Subcellular fluorescence signal exhibited a co-localization of the proteins at the PM in *N.benthamiana* leaf cells (Fig. 2C). After a successful selection of homozygous seeds stably expressing PATL2-GFP we could determine signal in 8d old seedling roots. Consistent with previous studies, PATL2-GFP was localized to the PM in the differentiation zone. Additionally, PATL2-GFP was present in the cytosol of cells in the root tip (Fig. 2D) (Tejos et al., 2017). A promoter-GUS study was performed on 8d old seedlings, grown either on iron-sufficient or iron-deficient medium, to pinpoint tissue with PATL2-promoter expression and the influence of iron deficiency on its expression.

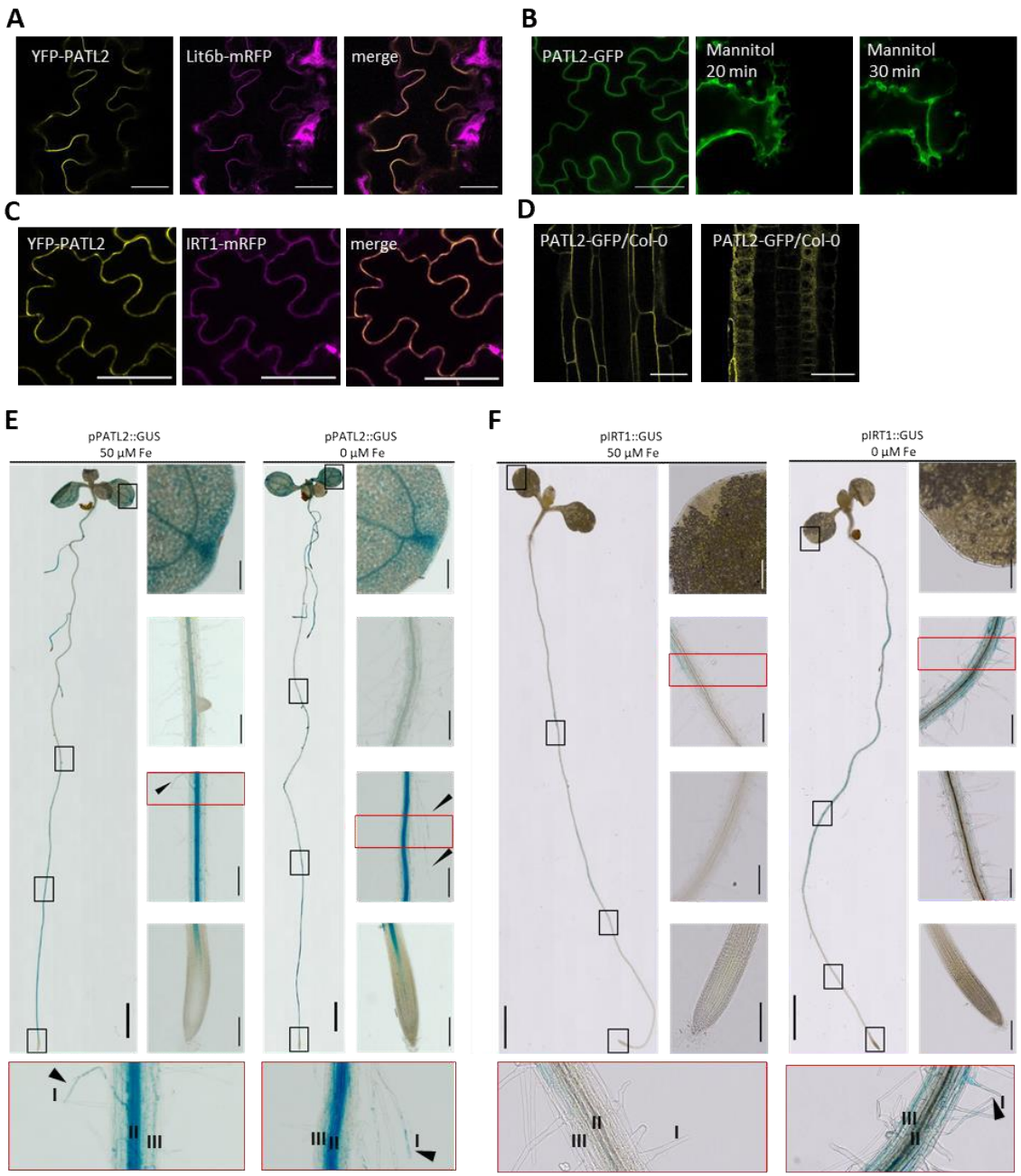


**Figure 2. PATL2 and IRT1 are co-localizing at the plasma membrane (PM).**

(A) Co-localization of YFP-PATL2 with PM marker Lit6b-mRFP in *N.benthamiana* epidermis cells. (B) Localization of PATL-GFP in *N.benthamiana* epidermis cells before and after mannitol plasmolysis. (C) YFP-PATL2 co-localizes with full-length IRT1-mRFP at the PM in *N.benthamiana* epidermis cells. (D) PATL2-GFP localization in differentiation zone and root tip of 8d old Arabidopsis Col-0 seedlings stably expressing PATL2-GFP. (E) and (F) Qualitative analysis of PATL2- and IRT1-promoter activity via GUS-promoter assay in 8d old seedlings grown on either iron-sufficient (50 µM Fe) or iron-deficient (0 µM Fe) Hoagland agar plates. On the left qualitative promoter-GUS activity (blue) on the whole plant. Bars 2000 µm. The squares indicate close-ups on the right, showing a cotyledon, the middle and bottom root zone , as well as the root tip. Bars 200 µm. Red boxes are close-ups, showing promoter-GUS expression in (I) root hairs, (II) the central cylinder and (III) root epidermis. [Arrows indicate promoter GUS activity in root hairs.]

PATL2-promoter activity could be verified in cotyledons, cotyledon vascular tissues , root central cylinder, root hairs and root epidermis cells in seedlings grown on iron-sufficient or iron-deficient medium (Fig. 2E). Additionally, the expression of PATL2-promoter in the root tip expands from the central cylinder under iron-deficient conditions (Fig. 2E). In comparison to that weak IRT1-promoter expression was found in 8d old seedlings grown on iron-sufficient medium, while IRT1-promoter expression is increased in root epidermis cells and root hairs of seedlings grown under iron deficiency (Fig. 2F). Hence, it can be claimed that PATL2 and IRT1 are co-localizing at the PM and are both expressed under iron deficiency in the same tissue.

## PATL2 negatively influences iron acquisition

PATL proteins were shown to contribute to stress tolerance of plants by interacting with PM proteins in response to environmental changes (Chu et al., 2018; Zhou et al., 2018). In order to understand the importance of PATL2 in iron acquisition, we analyzed two independent *patl2* loss-of-function mutants, *patl2-1* and *patl2-2* (Fig. S2A). To study their reaction to iron stress the mutants were exposed to iron-deficient or iron-sufficient conditions. The absence of the *PATL2* transcript was verified using RT-qPCR on cDNA generated from plant roots grown in the 14+3d system (Fig. 3A). The expression level of *PATL2* in response to iron deficiency in wild-type Col-0 (WT) plants showed no changes compared to plants grown under iron sufficient conditions (Fig. 3A). Additionally, roots of *patl2* mutants and WT were examined for expression patterns of iron-deficiency response marker genes *FIT*, *BHLH039*, *FRO2* and *IRT1*, which are all upregulated upon iron starvation. All used maker genes were responding to iron deficiency as expected by being upregulated in WT plants (Figs 3B-E). Both *patl2-1* and *patl2-2* mutants showed comparable upregulation of key iron-deficiency marker genes (Figs 3B-E). Besides of two exceptions, all tested gene expression levels in *patl2-1* and *pat2-2* did not change compared to WT levels (Figs 3B-E). The expression of *FIT* in *patl2-2* was generally increased compared to WT and *patl2-1* (Fig. 3B). Furthermore, *FRO2* gene expression was upregulated in *patl2-1* compared to the other mutant and the WT in response to iron deficiency (Fig. 3D). Taken together, the results showed mostly no changes of gene expression in *patl2* mutants compared to WT. Our next aim was to verify the efficiency of iron-uptake Strategy I in *patl2* mutants. As a marker the activity of FRO2 protein was measured in its role as the main iron reductase in roots. The iron reductase activity was significantly higher in all tested plant lines grown under iron-deficient conditions compared to plants grown on iron-sufficient conditions (Fig. 3F). The absence of PATL2 led to strongly increased iron reductase activity under iron deficiency in roots when compared to WT levels (Fig. 3F).

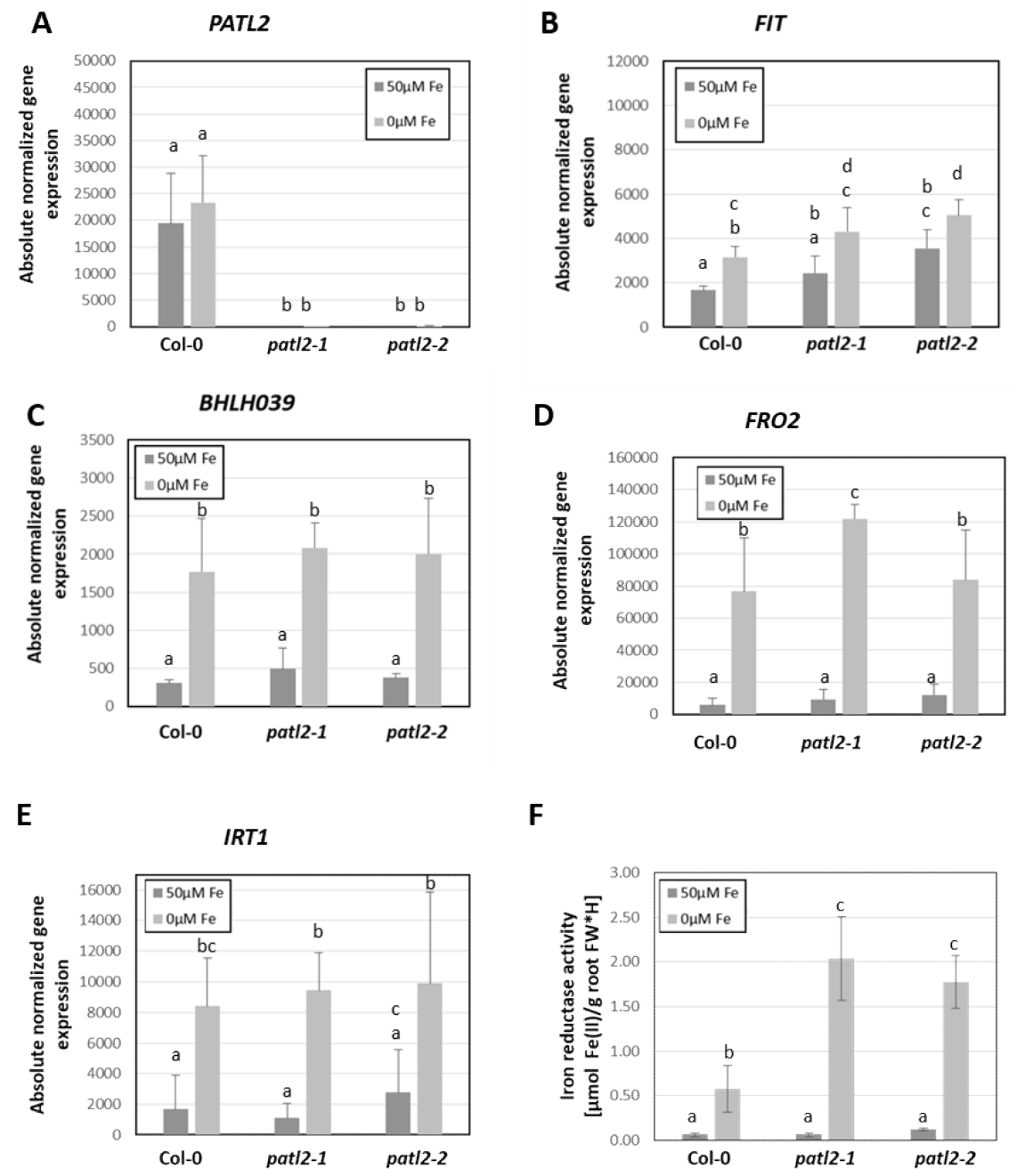


**Figure 3. PATL2 seems to regulate iron acquisition on protein level.**

(A) *PATL2* expression analysis in Col-0 and *patl2* roots. (B) to (E) analysis of iron-deficiency marker gene expressions in Col-0, *patl2-1* and *patl2-2* roots. (F) Iron reductase activity in roots of *patl2* mutants and Col-0. [All plants were grown in the 14+3d system. Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).]

This observation suggests that PATL2 might function as a negative regulator of iron acquisition in Arabidopsis roots. To trace the effect of increased iron reductase activity back to the loss of *PATL2* expression *patl2-1* was complemented with a HA-PATL2 construct. After the selection of homozygous plants overexpressing HA-PATL2 in *patl2-1* background (referred to as "HA-PATL2(1)/*patl2-1*") (Figs S3A, S3C), the root iron reductase activity of this line was tested. The measurement demonstrated the ability of PATL2-HA to complement the observed mutant phenotype, since reductase activity was reduced back to WT level under iron deficiency (Fig. 4A). FRO2 activity was slightly decreased in *patl2-1* and HA-PATL2(1)/*patl2-1* plants compared to WT under sufficient iron supply (Fig. 4A). Overexpression of HA-PATL2 in WT background (referred to as "HA-PATL2(2)/Col-0" and "HA-PATL2(3)/Col-0") (Fig. S3B-C) did not negatively affect iron reductase activity under iron-deficient conditions (Fig. 4B). Due to the reported redundancy within the PATL family in Arabidopsis, we decided to analyze PATL1, the closest homolog of PATL2, in regard to its effect on iron acquisition (Montag et al., under review; Tejos et al., 2017). Therefore a *pat1-1/patl2-2* double mutant and *patl1-1* loss-of-function mutant were analyzed (Fig. S2B) (Peterman et al., 2004). Determining *PATL1* expression pattern using a promoter-GUS assay demonstrated comparable promoter expression of *PATL1* and *PATL2* (Fig. 2E; Fig. S2C). Gene expression analysis of *patl1-1/patl2-2* and *patl1-1* mutants, grown in the 14+3d system, showed that all tested iron deficiency-induced genes were upregulated under iron deficiency (Fig. S2D-G). All genes were expressed at WT level, except of *BHLH039* in *patl1-1* grown under iron-deficient conditions. In this case, *BHLH039* expression was increased compared to iron-sufficient conditions, but not as high as its levels in WT (Fig. S2E). Iron reductase activity of *patl1-1/patl2-2* was comparable to *patl2-2* activity levels and increased compared to WT levels under iron deficiency (Fig. S2H), while FRO2 activity in *patl1-1* was at WT level (Fig. S2I). The outcomes of both experiments indicate no influence of PATL1 on iron acquisition. To analyze on which level the redundancy of the PATL family is regulated we checked gene expression of all family members in roots of *patl2* mutants, *patl1-1/patl2-2* and *patl1-1* grown in the 14+3d system. It can be highlighted that no changes in gene expression due to iron deficiency could be determined (Fig. S4A-F). All *PATL* genes in mutants were expressed at the same level as the tested WT (Fig. S4A-F), except of significant increased gene expression of *PATL1* in *patl2-2* (Fig. S3A). The expression level of *PATL2* was increased in *patl1-1* under iron-deficient conditions compared to WT (Fig. S3B). Since only minor changes could be verified, there is no clear hint that PATLs redundancy is regulated on gene expression levels.

Taken together, gene expression analysis revealed only minor differences in expression levels of iron deficiency-induced genes that would hint to a meaningful phenotype on this level of

regulation. But PATL2 might have a negative regulatory effect on iron acquisition on protein level. Furthermore, it was demonstrated that PATL1 has no effect on iron uptake in Arabidopsis and that the redundancy within the PATL family is not regulated on expression level.

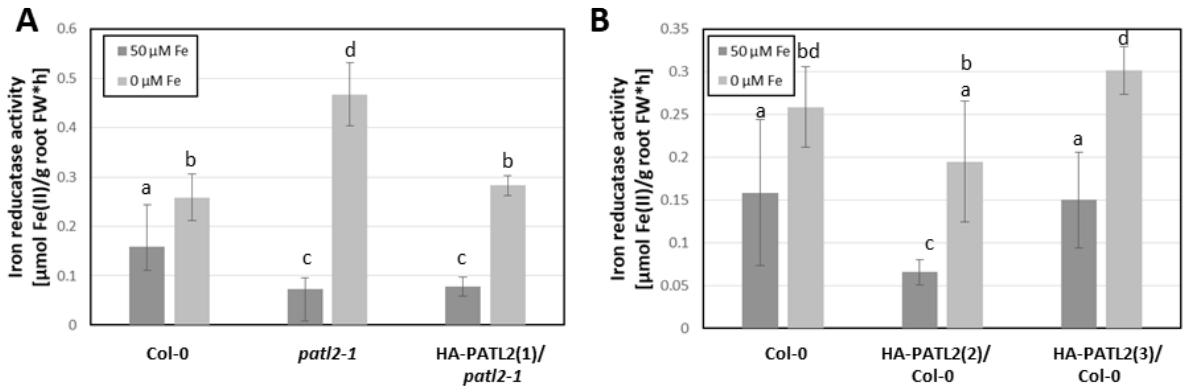


**Figure 4. HA-PATL2 overexpression is able to complement mutant phenotype.**

(A) Iron reductase activity in roots of HA-PATL2(1)/*patl2-1*, which overexpresses *HA-PATL2* in a *patl2-1* background. (B) Iron reductase activity in roots of *PATL2*-overexpression plants in Col-0 background (HA-PATL2(2)/Col-0; HA-PATL2(3)/Col-0). [All plants were grown in the 14+3d system. Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).]

## PATL2 protects membrane from oxidative damage

SEC14-GOLD proteins are able to bind phospholipids and other lipophilic substances like α-TOC (Bermudez et al., 2018; Kempna et al., 2003). α-TOC is known to be important as a lipid-soluble antioxidant neutralizing reactive oxygen species (ROS) at membranes, e.g., preventing the formation of lipid peroxide (Nukala et al., 2018). Having a closer look on the ROS production it can be noticed that ferrous iron is a catalyzer starting the Fenton reaction (Winterbourn, 1995). During one possible Fenton reaction a hydroxyl radical is generated, while in another potential reaction ferrous iron reacts with a lipid peroxide to a lipid alkoxy radical ( Le et al., 2019). Both reactions link iron caused radical formation to unsaturated lipid peroxidation (Minotti and Aust, 1992). To check if lipid-peroxidation rates in *patl2* roots are changed the concentration of malondialdehyde (MDA), a natural by-product of lipid peroxidation, was measured (Zhang and Huang, 2013). In both *patl2* mutants, grown on iron-sufficient medium, the root MDA content was significantly increased compared to WT levels (Fig. 5A). An increase in lipid peroxidation was also verifiable in *patl1-1/patl2-2* and *patl1-1* roots (Fig. S5A). HA-PATL2(1)/*patl2-1* showed MDA concentration on WT level, indicating the complementation of the mutant phenotype (Fig. 5B). For *patl1* mutant it had been demonstrated that the $H_2O_2$ accumulation is increased and that

regulation of ROS formation is interrupted in parts in response to salt stress (Zhou et al., 2018). Salt stress is not the only abiotic stress resulting in changed ROS accumulation in the plant cell, iron acquisition can as well be linked to the ROS-metabolic pathway (Le et al., 2016). That is why we measured the $H_2O_2$ content in roots of *patl2* plants. No significant changes in the $H_2O_2$ accumulation in *patl2-1* and *pat2-2* roots could be detected compared to WT samples (Fig. 5C). Similar results were obtained for the $H_2O_2$ content in *pat1-1patl2-2* and *patl1-1* roots (Fig. S5B). The results suggest a function of PATL2 in membranes protection from lipid peroxidation linking it to ferrous iron uptake via IRT1.

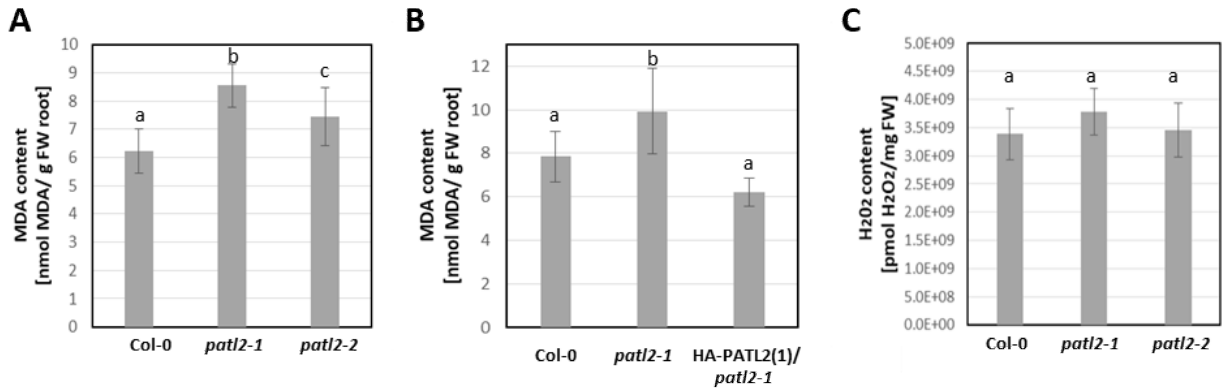


**Figure 5. *patl2* mutants show an increased lipid-peroxidation rate.**

(A) Lipid-peroxidation rate was determined in 17d old Col-0, *patl2-1* and *patl2-2* roots by measuring MDA content. (B) MDA concentration in 17d old HA-PATL2/(*patl2-1*) roots indicating lipid peroxidation. (C) Measured $H_2O_2$ concentration in roots of 17d old Col-0 , *patl2-1* and *patl2-2* plants. [Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).]

## PATL2 positively influences *fet3 fet4* yeast growth during iron acquisition via IRT1

To examine the direct effect of PATL2 on IRT1, its role in iron acquisition, and membrane protection from oxidative damage, the iron uptake mechanism of Arabidopsis roots was reconstructed in a heterologous yeast expression system (Eide et al., 1996; Khan et al., 2019). The heterologous environment was provided by the *fet3 fet4* strain lacking FET3, a multicopper oxidase, and FET4, a bivalent iron transporter. Due to the deletions the *fet3 fet4* strain has a dramatically reduced iron uptake capacity, but is maintaining its iron reductase activity. The presence of low-affinity iron transporters keeps the strain capable to survive (Khan et al., 2019). *fet3 fet4* was complemented in the experiment with full-length IRT1 and either full-length PATL2, PATL2ΔSEC14 (unable to bind to PIPs/membranes) or PATL2ΔN (unable to interact with IRT1vr). As a control a yeast WT strain was transformed along with the *fet3 fet4* strain. Iron

depleted medium was generated by adding BPDS. IRT1 expression alone was able to rescue the growth phenotype of *fet3 fet4* (Eide et al., 1996). The growth of *fet3 fet4* cells co-expressing IRT1 with either PATL2ΔCTN-SEC14 or PATL2ΔN was comparable to yeast growth expressing IRT1 alone (Fig. 6). The expression of full-length PATL2 in combination with IRT1 led to a faster growth under iron-sufficient and iron-depleted conditions (Fig. 6). The expression of PATL2 alone in *fet3 fet4* yeast, without completing the yeast with IRT1, showed no positive effect on their growth (Fig. 6). These results suggest a positive effect of PATL2-IRT1 interaction on *fet3 fet4* growth behavior.

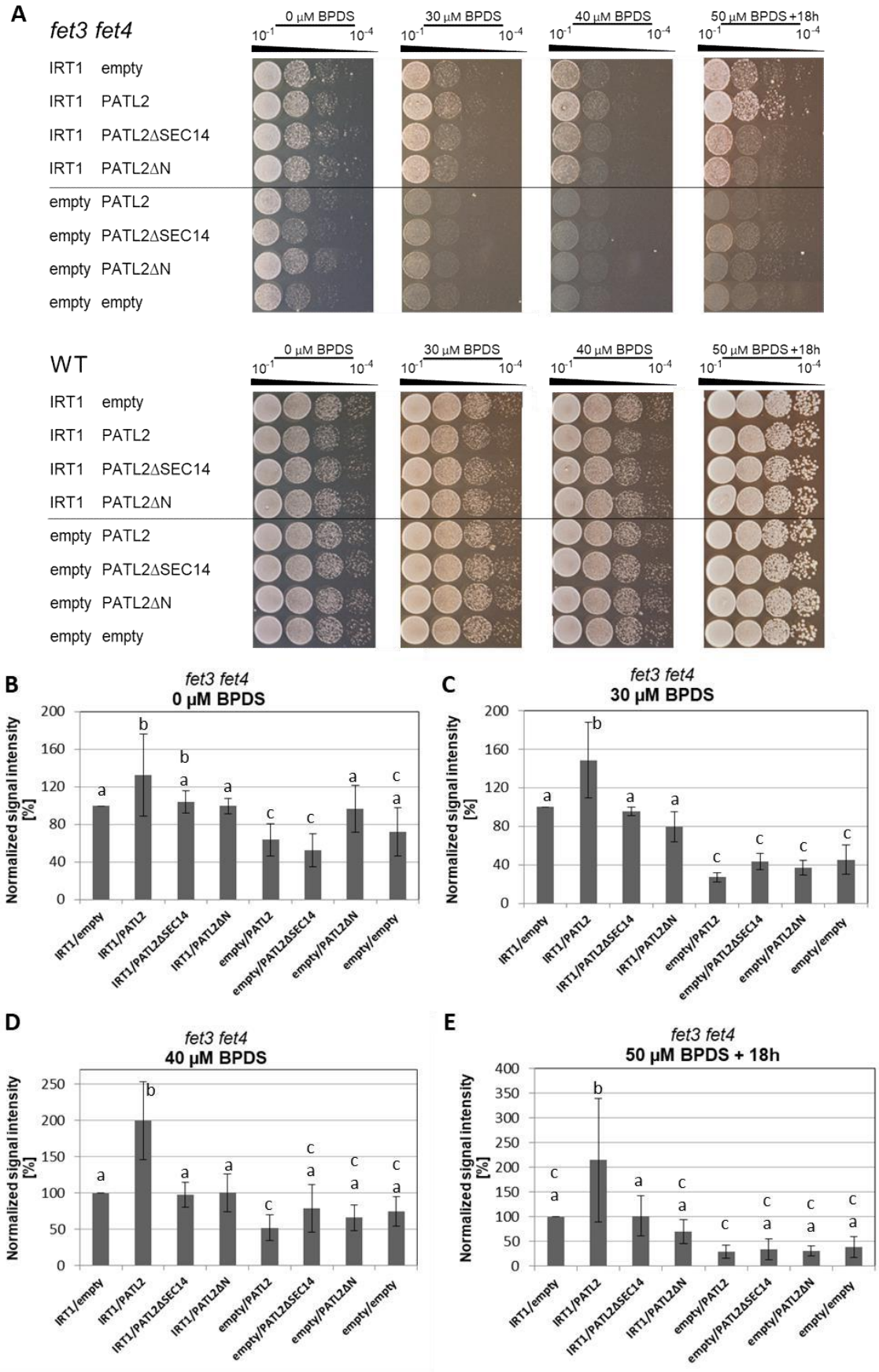
A
fet3 fet4
0 µM BPDS
30 µM BPDS
40 µM BPDS
50 µM BPDS +18h
10-1
10-4
IRT1 empty
IRT1 PATL2
IRT1 PATL2ΔSEC14
IRT1 PATL2ΔN
empty PATL2
empty PATL2ΔSEC14
empty PATL2ΔN
empty empty
WT
B
fet3 fet4
0 µM BPDS
C
fet3 fet4
30 µM BPDS
D
fet3 fet4
40 µM BPDS
E
fet3 fet4
50 µM BPDS + 18h
Normalized signal intensity [%]
IRT1/empty
IRT1/PATL2
IRT1/PATL2ΔSEC14
IRT1/PATL2ΔN
empty/PATL2
empty/PATL2ΔSEC14
empty/PATL2ΔN
empty/empty

**Figure 6. PATL2 positively affects yeast growth during IRT1-mediated iron import.**
(A) Yeast growth of transformed wild-type (WT) and *fet3 fet4* strains on iron-deficient (BPDS) and iron-sufficient (0 µM BPDS - control) medium was tested. Growth of *fet3 fet4* yeast is negatively affected when iron transport is limited. Arabidopsis IRT1 is able to rescue this phenotype. (B) to (E) Quantification of *fet3 fet4* growth when transformed with IRT1 and either PATL2 or its deletion mutants, in relation to *fet3 fet4* transformed with IRT1. [Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).; n=4]

## Discussion

In this work we present the analysis of IRT1 interaction with the SEC14-GOLD protein PATL2 and the significance of PATL2 for iron acquisition. We found that IRT1 and PATL2 are interacting *in planta*, being expressed in the same tissue and co-localizing at the PM. Furthermore, we could demonstrate that interaction occurs via the N region of PATL2. The effect of PATL2 on iron-reductase activity points to the fact that PATL2 regulates iron acquisition on protein level. Additionally, PATL2 prevents membrane damage and contributes positively to *fet3 fet4* growth during iron acquisition via IRT1.

*PATL2 might be a regulator of iron acquisition on protein level*

Next to the performed interaction studies, the ability of PATL2 to interact with IRT1 is supported through overlapping expression patterns under iron deficiency and protein co-localization at the PM. Observed PM localization of PATL2 correlates to its ability to bind PI(4)P and PI(4,5)$P_2$, both enriched at the PM (Montag et al., under review; Simon et al., 2014). PI(4)P at the PM is able to act as a signaling molecule (Hammond et al., 2012). It also serves as the pool for PI(4,5)$P_2$ generation (Dickson et al., 2014), which accumulates at the membrane in microdomains and functions as a signaling lipid, starting several physiological responses, e.g. endocytosis and exocytosis (Heilmann, 2016; Martin, 2012; Sun et al., 2013). The fact that we identified the SEC14-GOLD protein PATL2 as an IRT1 interactor fits to the observation that its closest homolog PATL1 is able to interact with PM proteins, functioning as a regulator in plant tolerance to cold and salt stress (Chu et al., 2018; Zhou et al., 2018). SEC14-GOLD proteins are members of the SEC14L-PITP superfamily, known to be involved in membrane trafficking able to recognize, exchange and transport molecules between membranes, e.g., phospholipid (Kf de Campos and Schaaf, 2017; Mousley et al., 2007). Thereby they function in the phospholipid-

signaling pathway, e.g., by regulating lipid modification (Bankaitis et al., 2010; Kf de Campos and Schaaf, 2017).The interaction of IRT1 with PATL2 suggests a regulatory role of PATL2 in iron stress. PATL2 is a peripheral membrane protein and its localization to the PM and the cytosol, depends, among others, on the developmental stage of the cell or the applied stress. This observation is consistent with previous results (Suzuki et al., 2016; Tejos et al., 2017). While IRT1 gene expression is upregulated in response to iron starvation PATL2 is constitutively expressed, supporting the idea that SEC14-GOLD proteins might be general regulators on plant response to several abiotic stresses (Chu et al., 2018; Zhou et al., 2018). The interaction of PATL2 with IRT1vr via its N region is comparable to data on PATL1 interacting by its N region with salt antiporter SOS1 (Zhou et al., 2018). The CTN-SEC14 lipid-binding pocket is not contributing to protein-protein interaction. This is not surprising since the domain module seems to be critical for membrane association of PATL2 (Montag et al., under review). The N region can only be found in SEC14-GOLD proteins in the plant kingdom and its exact function is not fully characterized yet (Montag et al., under review). Each N region of SEC14-GOLD proteins has a unique amino acid sequence without known domains (Montag et al., under review). Under iron starvation the N region of PATL2 is phosphorylated (Lan et al., 2011), but it is also phosphorylated in response to other environmental influences, e.g., salt stress (Chang et al., 2012; Hsu et al., 2009; Mattei et al., 2016; Tang et al., 2008). This data leads to the suggestion that the N region might undergo posttranslational modifications and that PATL2 might play a regulatory role in response to multiple abiotic stresses maybe through unidentified interactions with additional membrane proteins. The unique sequence of the N region could also explain why PATL1 is not affecting iron uptake although redundancy of PATLs was reported (Tejos et al., 2017). Taken together, the unique sequence of the N region might be defining the specific regulatory role of each PATL (Montag et al., under review). Furthermore, we could demonstrate that the redundancy of PATLs is not regulated on gene expression level. The results also did not hint to an effect of PATL2 on iron stress-response regulation on expression level. The observed increased ferric reductase activity of *patl2* mutants, together with the determined PATL2-IRT1 interaction and the results from gene expression analysis, suggest that PATL2 acts as a regulator of iron acquisition on protein level. The regulatory function could be traced back to a negative effect of PATL2 on a putative FRO2-IRT1 protein complex at the PM to reduce the iron-import rate, by decreasing the reduction of iron in the rhizosphere, which would affect the uptake rate of ferrous iron by IRT1. It is assumed that such a complex is formed to enhance iron uptake rate, comparable to the Fet3pFtr1p complex observed in yeast, as also hypothesized by Khan et al. (2019). The impact of IRT1vr interactors on reductase activity was already described for EHB1, which negatively regulates iron import (Khan et al., 2019).

*PATL2 supports membrane integrity*

While EHB1 seems to be a negative regulator of iron import and thereby, e.g., influences metal storage in seeds (Khan et al., 2019), PATL2 might affect IRT1 import activity locally to prevent stress caused by high accumulation of ferrous iron at the PM. The exact molecular mechanism underlying regulation of IRT1 activity by protein-protein interaction with regard to PATL2 remains unclear. The ability of IRT1 to only transport ferrous iron links it to the ROS-metabolic pathway (Le et al., 2019; Vert et al., 2002). Import of ferrous iron may cause the difficulty of radical formation at the PM initiated by the Fenton reaction and leading to lipid peroxidation (Winterbourn, 1995). Indeed, *patl2* mutants showed enhanced lipid-peroxidation rates in roots indicating radical caused membrane damage. To control the oxidative stress inevitable produced by the presents of ferrous iron one possibility is to tightly control the iron uptake. First, this might happen through PATL2 indirectly influencing ferrous-iron import by IRT1 through negatively regulating iron reductase activity and thereby lowering the availability of ferrous iron. Second, the presents of PATL2, recruited by the interaction with IRT1, seems to prevent lipid peroxidation. Lipid peroxidation, induced by ferrous-iron caused radical formation, can be stopped by antioxidants (Ayala et al., 2014). A possible antioxidant likely to inhibit lipid peroxidation is the lipid-soluble α-TOC (Boonnoy et al., 2018; Liebler and Burr, 1992), which is proven to be bound by some SEC14-GOLD proteins – in animals and plants (Bermudez et al., 2018; Zingg et al., 2008). For that reason, it can be hypothesized that a potential way to stop iron caused lipid peroxidation is the recruitment of α-TOC to damaged membranes by PATL2. Therefore, IRT1-PATL2 interaction might be necessary to identify the possible target site. The ability of PATL2 to bind α-TOC needs to be verified in future. Since PATL1 has no effect on iron reductase activity, but *patl1-1* has an increased lipid-peroxidation rate, it can be suggested that there is a redundancy in the function of membrane protection, but not in response to iron stress. Maybe PATL1 is able to response to other factors leading to radical and ROS formation and thereby influencing membrane integrity, such as salt stress (Mittler, 2002; Zhou et al., 2018). The data generated in the yeast complementation assay showed a positive impact of PATL2 on growth rate of *fet3 fet4* when complemented with IRT1, maybe indicating the contribution of PATL2 to cellular fitness through its ability to protect membranes from oxidative damage. This suggestion is supported by the fact that the positive effect on cell growth cannot be determined in *fet3 fet4* cells expressing either only IRT1 or IRT1 together with PATL2ΔN or PATL2ΔSEC14. PATL2 mutants either lacking the ability to interact with IRT1 or to associate with membranes are not able to fulfill the biological function of PATL2 (Montag et al., under review).

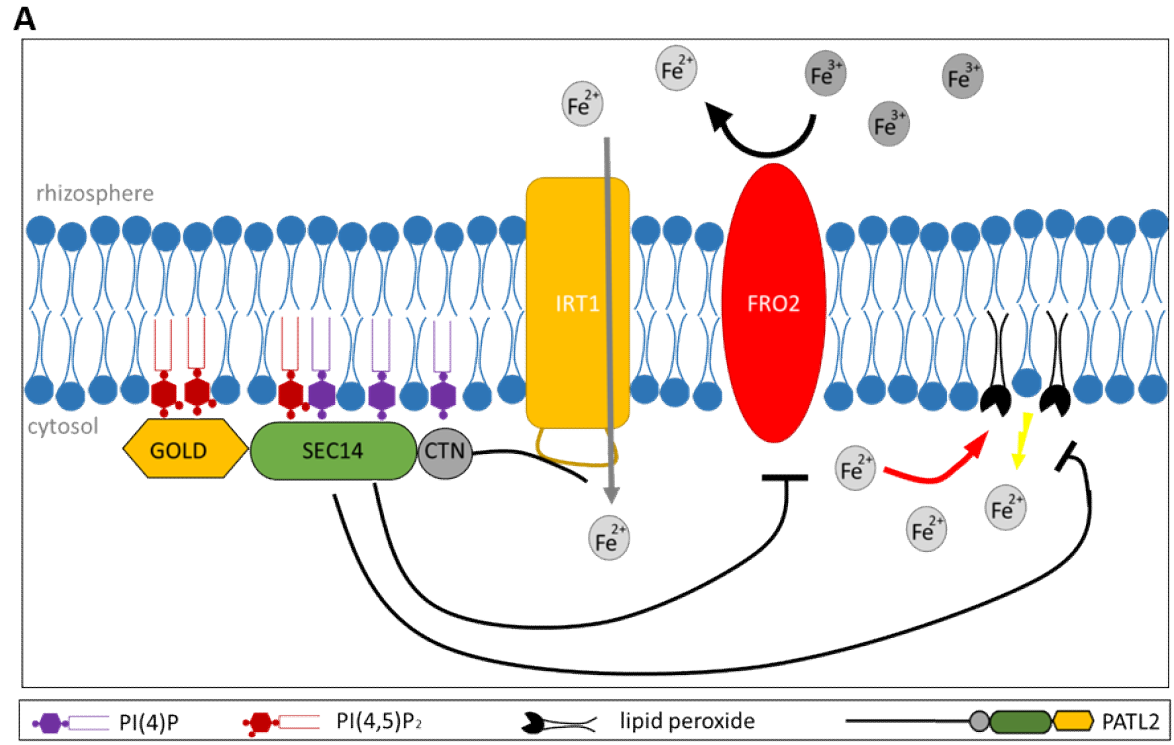


**Figure 7. PATL2 prevents membrane damage and interacts with IRT1.**

(A) Hypothetical mechanism of PATL2 interacting with IRT1. PATL2 binds to the PM membrane via its GOLD domain and the CTN-SEC14 module. Additionally, PATL2 interacts through its N region with the variable region of IRT1. PATL2 is able to negatively regulate the iron uptake machinery and to prevent lipid peroxidation. (B) Chemical reaction liking iron-induced Fenton reaction with lipid peroxidation. Cycle can be stopped through an antioxidant. Here we present the ability of α-TOC to inhibit lipid peroxidation. [LH= = unsaturated lipid; L• = lipid radical; LOO• = lipid peroxyl radical; LOOH = lipid peroxide]

*PATL2 might have a regulatory function on multiple levels in response to iron stress*

Taken together data indicate that PATL2 might act as a regulator of iron acquisition on protein level by undertaking several tasks to keep the iron level of the cell in a proper range to prevent membrane damage (Fig. 7A). As mentioned before, PATL2 seems to function as an inhibitor in the lipid-peroxidation pathway, which is possibly started by the import of ferrous iron (Fig. 7B). Additionally, it negatively influences the iron-uptake machinery on protein level, maybe through inhibiting FRO2 activity and thereby decreasing the ferrous iron pool in the rhizosphere. This might lead to less import of ferrous iron through IRT1 and to less iron accumulating in the cytosol preventing PM damage via lipid peroxidation. For the activity of PATL2 the interaction with PI(4)P and PI(4,5)$P_2$ at the PM as well as the interaction with IRT1vr might be critical to identify its site of action. Since the exact molecular mechanism of PATL2 in response to iron uptake and inhibiting lipid peroxidation is not uncovered right now, this is essential to be discovered in future.

But the idea might also serve as a model for general PATL function. We hypothesize that, PATL function is activated at the site of action only under stress conditions when several requirements are fulfilled. First, specific PIPs need to be present as signaling molecules at the membrane. Second, a membrane associated protein, which is able to interact with a PATL, is required. Only this combination might then lead to a further regulatory effect of the PATL (Heilmann, 2016). The unique N region mediating protein-protein interactions and the substrate affinity of the SEC14 lipid-binding pocket might define the abiotic stresses targeted by different PATLs.

## Supplemental Data:

**Supplemental Figure 1.** Interaction studies controls.

**Supplemental Figure 2.** Analysis of *patl2*, *patl1-1* and *patl1-1/patl2-2* T-DNA mutants.

**Supplemental Figure 3.** *HA-PATL2*-overexpressing plants.

**Supplemental Figure 4.** Redundancy of PATLs is not regulated at gene expression level

**Supplemental Figure 5.** ROS accumulation in patl1-1/patl2-2 and patl1-1.

**Supplemental Table 1.** Primer List

## Methods

## Generation of recombinant vectors

Full-length Arabidopsis PATL2 and deletion mutants were generated, as descript in Montag et al., (under review). The coding sequences (CDS) of PATL2, IRT1 and IRT1vr were amplified

from cDNA of iron deficient Col-0 roots using primer-pairs PATL2B1F/PATL2B2stopR, I1B1/FLI1B2 and I1LB1 /I1LB2 and subcloned into into pDONR207 via Gateway cloning technology (BP reaction, Life Technologies). Next, PATL2 was transferred into the vectors pH7WGY2 and pMDC83 (Curtis and Grossniklaus, 2003) via Gateway cloning (LR reaction, Life Technologies), allowing to determine the subcellular localization of PATL2 by expression of N-terminally tagged YFP and C-terminal tagged GFP proteins in plant cells. IRT1 localization was determined using pJNC1:IRT1 vector (Ivanov et al., 2014). Additionally, PATL2 was cloned into pAUL1 to express N-terminally tagged HA protein. IRT1vr, PATL2 and its deletion mutants were cloned into pGBKT7 and pACT2 via Gateway-LR reaction to determine protein-protein interaction using yeast two-hybrid system. PATL2 and its deletion mutants were transferred into pAG425GPD-ccdB-HA (Susan Lindquist, Addgene plasmid # 14250) and IRT1 into pAG426GPD-ccdB-eYFP (Susan Lindquist, Addgene plasmid # 14228) for *fet3 fet4* -complementation assay. The 2in1 pBiFCt-2in1-CC vector was used to verify protein-protein interactions in transient transformed *N. benthamiana* epidermis cells. Therefor primers with attB3 and attB2 Gateway cloning sites were used to amplify PATL2 and its deletion mutants and primers with attB1 and attB4 Gateway cloning sites were used to amplify IRT1vr. Next, PCR products of PATL2 and its deletion mutant were cloned via Gateway-BP reaction into pDONR221-B3B2 and IRT1vr PCR product into pDONR221-B4B1. Combinations of IRT1 and PATL2 or its fragments were inserted into vector pBiFCt-2in1-CC vector via Gateway LR reaction (Grefen and Blatt, 2012). All used primers are listed in Supplemental Table 1.

## Yeast two-hybrid assay

To identify interaction partners of IRT1 the variable region of IRT1 was used as bait in a yeast two-hybrid screen against a cDNA-expression library prepared from iron-deficient Arabidopsis roots, as described in Kahn et al. (2019). For verification of identified PATL2-IRT1vr interaction an independent targeted yeast two-hybrid assay was performed. pGBKT and pACT2 vectors containing IRT1vr, PATL2 and its mutants were simultaneously introduced into yeast strain AH109 and spotted on a double-selective SD medium lacking tryptophan (selects for pGBKT7) and leucine (selects for pACT2), pH 5.8. Grown colonies were checked by PCR and re-spotted onto SD medium lacking tryptophan, leucine and histidine, and supplemented with 0.5 mM 3-amino-1,2,4-triazole (3-AT), pH 5.8. Cell growth on SD media lacking tryptophan, leucine and histidine and supplemented with 0.5 mM 3-AT indicates protein-protein interaction. The yeast growth was pictured after incubated for 4 days at 30°C.

## Transient tobacco leaf transformation

To transform *Nicotiana benthamiana* (tobacco) leaves *Rhizobium radiobacter* strain C58C1(pTiB6S3ΔT)$^{H}$ (Wu et al., 2014) was used containing pH7WGY2 and pMDC83, with PATL2 or deletion mutants (Hotzer et al., 2012; Khan et al., 2019). *R. radiobacter* cultures were grown in YEB medium overnight. Next, they were pelleted and resuspended to an OD600 of 0.4 in tobacco infiltration solution (2mM NaH2PO4, 50mM MES, 0.5% Glucose) and supplemented with 100 µM acetosyringone. The suspension was infiltrated via a syringe into young tobacco leaves. Infiltrated leaves were used for confocal microscopy or co-immunoprecipitation after 48h. For co-localization studies, the PATL2 vector-containing bacteria were mixed with bacteria containing vectors able to express the PM marker Litb6-mRFP (Caesar et al., 2011) or IRT1-mRFP (Khan et al., 2019).

## Protein co-immunoprecipitation and SDS-PAGE

*N. benthamiana* leaves were transformed as described above with combinations of IRT1-GFP and PATL2-HA expressing vectors (cloning method see above). The plant material was ground under liquid nitrogen and directly resuspended in IP buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% (w/v) Triton-x-100, 1x CIP protease inhibitor, Roche). Remaining cell components were removed by centrifugation for 10 minutes at maximum speed (4°C).Next, GFP-Trap_M beads (ChromoTek) were washed in IP Buffer and resuspended to a final volume of 25 µl/reaction. 25 µl GFP-Trap_M beads were added to the supernatant and incubated rotating for 2 hours at 21°C. Beads were collected by a magnet and washed with 1 ml IP buffer three times. Protein elution was performed using 50 µl SDG buffer (62 mM Tris-HCl, pH 8.6, 2.5% (w/v) SDS, 2% (w/v) DTT and 10% (v/v) glycerol) at room temperature. Samples were taken from the original cleaned extract “Input” and after protein elution “IP”.

Both fractions were separated on a 10% SDS-polyacrylamide gel electrophoresis followed by blotting on Amersham™ Protran™ 0.2 µm nitrocellulose membranes (GE Healthcare Life Sciences). PATL2-HA and IRT1-GFP were detected via immunoblot. First, 5%- milk powder in TBST (150 mM NaCl, 2.7 mM KCl, 24.7 mM Tris-HCl, 0.05 % v/v Tween-20, pH 7.4) was used to block the membrane for 15 min at room temperature. Followed by incubated for 1h with first antibody in 2,5%- milk powder. Next, the membrane was washed with TBST and incubated with secondary antibody for 1h in 2,5%- milk powder. After washing with TBST signals were detected by enhanced chemiluminescence (GE Healthcare Life Sciences). Antibody dilutions used: 1: 5000 anti-IRT1 (AS11 1780; Agrisera); 1:5000 goat anti-rabbit IgG horseradish peroxidase (AS09 602; Agrisera); 1:5000 rat monoclonal anti-HA horseradish peroxidase conjugated (3F10

671; Roche); 1:1000 anti-HA high affinity (11867423001; Sigma-Aldrich); 1:1000 anti-HA-peroxidase (12013819001; Roche).

## Protein extraction

Protein of 17d old roots was purified by grinding the frozen plant material. 2µL 2xSDG (124 mM Tris-HCL, pH 6,8; 5% SDS; 4% dithiothreitol, 20% Glycerol; 0.002% bromophenol blue; 1x CIP protease inhibitor, Roche) per 1 mg plant material was used to extract the protein. Next, the sample was centrifuged and the supernatant was heated at 95°C for 5 min.

## Bimolecular fluorescence complementation (BiFC) and confocal microscopy

BiFC experiment was performed as described in Grefen and Blatt (2012). Final pBiCF vectors were introduced into *N. benthamiana* through transient tobacco leaf transformation, as descript above. The RFP-signal is an indicator for a successful transformation event. YFP-signal indicates protein-protein interaction of proteins tagged to YFP forms (nYFP and cYFP), since YFP fragments can reconstitute due to spatial proximity. Confocal images of fluorescent signals were collected using a LSM780 system (Zeiss). YFP-signals were excited at 514 nm, and emission was detected at 520–550 nm. mRFP was excited at 561 nm, and emission was detected at 580–630 nm. GFP-signals were excited at 488 nm, and emission was detected at 510–540 nm. Cell plasmolysis was performed applying 1M mannitol.

## Plant material and growth conditions

Mutant plants *patl2-1* (SALK_009882), *patl2-2* (SALK_086866) and *patl1-1* (Salk_080204) all with *Arabidopsis thaliana* Columbia-0 (Col-0) ecotype background were used in this study. Mutant lines were ordered from Arabidopsis Biological Resource Center and genotyped. *patl1-1/patl2-2* double knock-out mutant was generated via crossing and genotyped by T. Kaye Peterman. Absence of a full-length transcript of *patl2* and *patl1* was verified by RT-qPCR on *PATL2* and *PATL1*. For the generation of the HA-PATL2 and PATL2-GFP lines pAUL1_PATL2 and pMDC83_PATL2 were transformed into *R.radiobacter* C58C1(pTiB6S3ΔT)$^{H}$ strain. Transformation of Col-0 and *patl2* mutants was performed using floral dipping (Clough and Bent, 1998). Dipped plants were selected for positive transformation and homozygous T4 generations were used in this work. PATL1- and PATL2-promoter::GUS lines were also generated by floral dipping. The pIRT1::GUS line was obtained from Vert et al. (2002). Arabidopsis seeds were sterilized for 8 min using sterilization solution containing 6 % NaCl and 0.1 % Triton-X and grown upright on Hoagland (HL) agar plates (Brumbarova and Ivanov, 2016). The HL medium

contained either sufficient 50 µM FeNaEDTA (50 µM Fe) iron or no FeNaEDTA (0 µM Fe) mimicking iron deficient conditions. The plants were first grown on 50 µM Fe medium for 14 days and then transferred either to 50 µM Fe plates or 0 µM Fe plates for three additional days (referred to as "14+3d system"), or plants were directly grown on 50 µM Fe or 0 µM Fe plates for 8 days. To verify the MDA and $H_2O_2$ content in roots plants were directly grown on 50 µM Fe HL agar plates for 17 days. The used *N. benthamiana* plants were grown on soil for two weeks. For seed multiplication plants were grown on soil. Plants were grown at room temperature in a 16h-light/8h-dark rhythm in climate chambers (CLF PlantClimatics).

## Beta-glucuronidase (GUS) assay

To obtain gene expression patterns seedlings were grown on HL plates for 8 days and the promoter::GUS activity was analyzed. Therefore, seedlings were incubating in GUS staining solution (50 mM sodium phosphate, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% Triton X-100, and 2 mM GUS substrate 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid) (Jefferson et al., 1987). Chlorophyll of cotyledons was removed by incubating seedlings in 70% ethanol. Pictures of seedlings were taken using Zeiss Axio Imager M2. Three independent lines were analyzed for each pPATL1::GUS and pPATL2::GUS construct. A representative picture is presented in this work.

## Gene expression analysis by reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was isolated using the peqGOLD Plant RNA Kit (PeqLab) from roots grown in the 14+3d system. For cDNA preparation Oligo dT primer and RevertAid first-strand synthesis kit (Thermo Scientific) were used. RT-qPCR was performed as described in Abdalla & Bauer (2016). The DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Scientific) were used for sample preparation and RT-qPCR was performed in a C100 Touch PCR cycler equipped with the CFX96 Real-Time System (BioRad). Obtained data were analyzed using CFX Manager software (BioRad). Standard curves were generated by mass standard dilution series for quantification of the samples. Gene expression of samples was normalized to the expression of EF1Bα. Additionally, its unspliced form was checked for genomic DNA contamination of the sample. Per experiment three independent sets of plants were used and each replicate contained roots of 12 plants. The experiment was performed twice.

## Iron reductase measurement

The root iron reductase activity was measured by the method described in Let et al. (2016). Plants were grown in the 14+3d growth system. The assay was repeated several times.

### $H_2O_2$ measurement

The $H_2O_2$ content of plant roots grown for 17d on iron-sufficient HL medium was measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific) following the protocol by Brumbarova et al., (2016). The $H_2O_2$ concentration was determined using the fluorescence. The experiment was repeated twice.

### MDA assay

To determine the lipid-peroxidation rate the malondialdehyde (MDA) concentration was measured as described in Zhang et al. (2013). 50 mg of roots grown 17days on iron-sufficient HL media were frozen and grinded. Next 0.5 ml of 0.1 % TCA added. After homogenization and centrifugation for 10 min at maximum speed 350 µl supernatant was taken and added to 1,650 ml TBA 0.5 % in TCA 20 % and incubated for 25 minutes at 95 °C. After cooling down samples were centrifuge 5 min at maximum speed (4 °C) and absorption at 532 nm and 600 nm was measured. The malondialdehyde (MDA) concentration was calculated using its extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$. The experiment was at least repeated twice.

### *fet3 fet4* complementation assay

For yeast complementation assay was IRT1 cloned in pAG426GPD and PATL2 and its deletion mutants were cloned in pAG245GPD and transformed in yeast stains INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52, ThermoFischer Scientific) , serving as the wild-type control, and DEY1453 (MATa/MATa ade2/+ canl/canl his3/his3 leu2/leu2 trpl/trpl ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2), here referred to as *fet3 fet4* (Eide et al., 1996). Positive transformation events were select by growing on SD-U-L media (CloneTech) and controlled via yeast colony PCR. Positive colonies were plated on agar plates containing YPD medium either supplemented with different concentrations of bathophenanthrolinedisulfonic acid (BPDS, iron-deficient conditions) or not (control). Yeast growth was observed 24h or 42h after plating. The identical treated INVSc1 strain served as a control to verify potential adverse effects to yeast growth by transformed constructs in the absence of iron uptake problems. INVSc1 yeast cells were grown with pH 5.8, while *fet3 fet4* needed growth condition with pH 5.5. For quantification of the *fet3 fet4* yeast growth the signal was measured using ImageJ Fiji. The growth of *fet3 fet4* yeasts transformed with only IRT1 were

set as a reference to 100%, the growth of the other transformed yeast were calculated in relation to that (n = 4).

## Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc test, in this case Fisher`s LSD using OriginPro 9.OG software. Statistical significance between data was set at $p < 0.05$. Statistical significance is indicated by different letters.

## Accession numbers

PATL2, At1g22530; PATL1, At1g72150; PATL3, At1g72160; PATL4, At1g30690; PATL5, At4g09160; PATL6, At3g51670; FIT, At2g28160; FRO2, At1g01580; IRT1, At4g19690; bHLH039, At3g56980

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## Author contributions

PB, KM and RI designed experiments. KM, RI and TB performed experiment. RG, RMB and KA assisted during experiments. KM, RI, TB and PB analyzed data. KM wrote the manuscript. PB and RI revised the manuscript. PB acquired funding.

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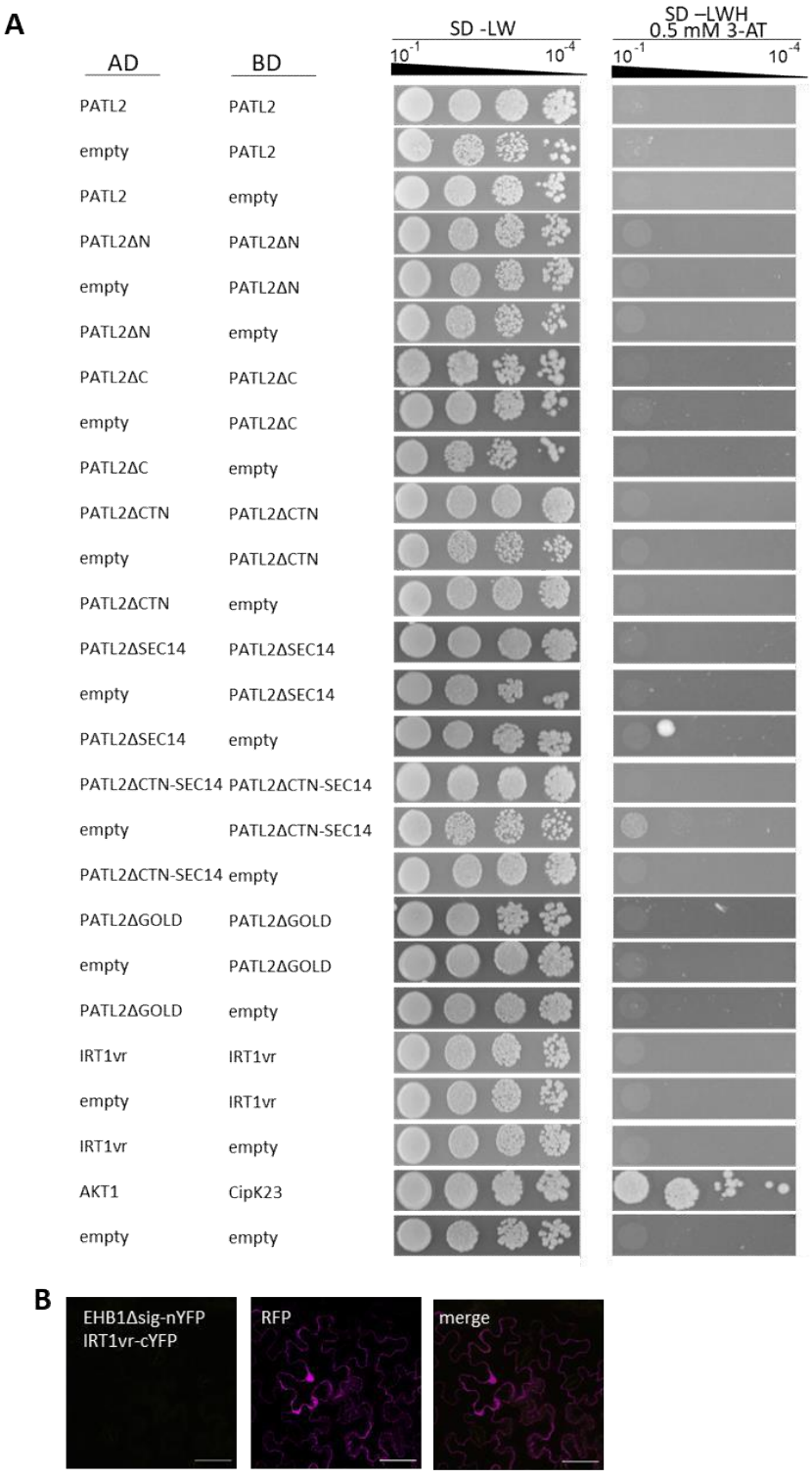
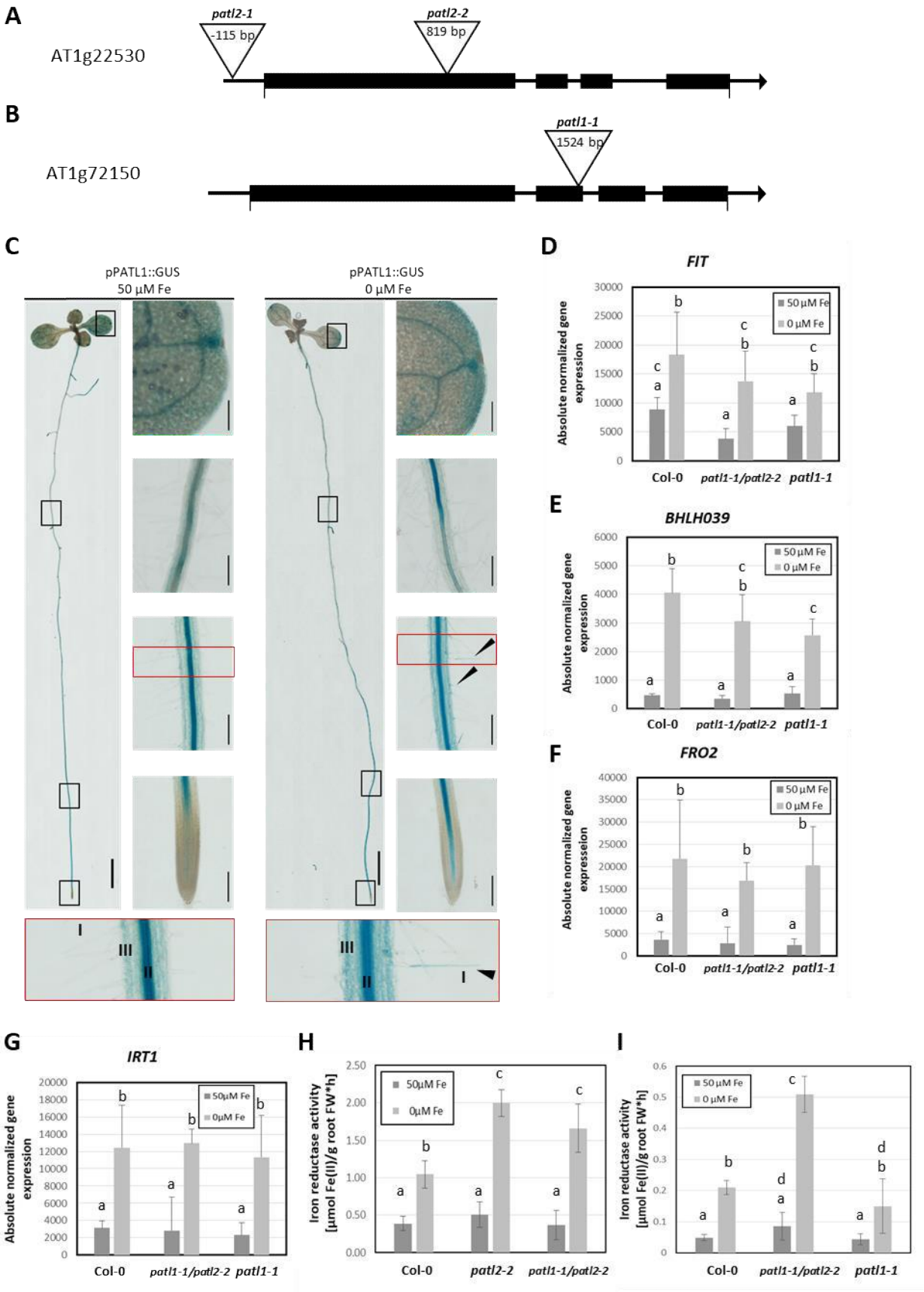


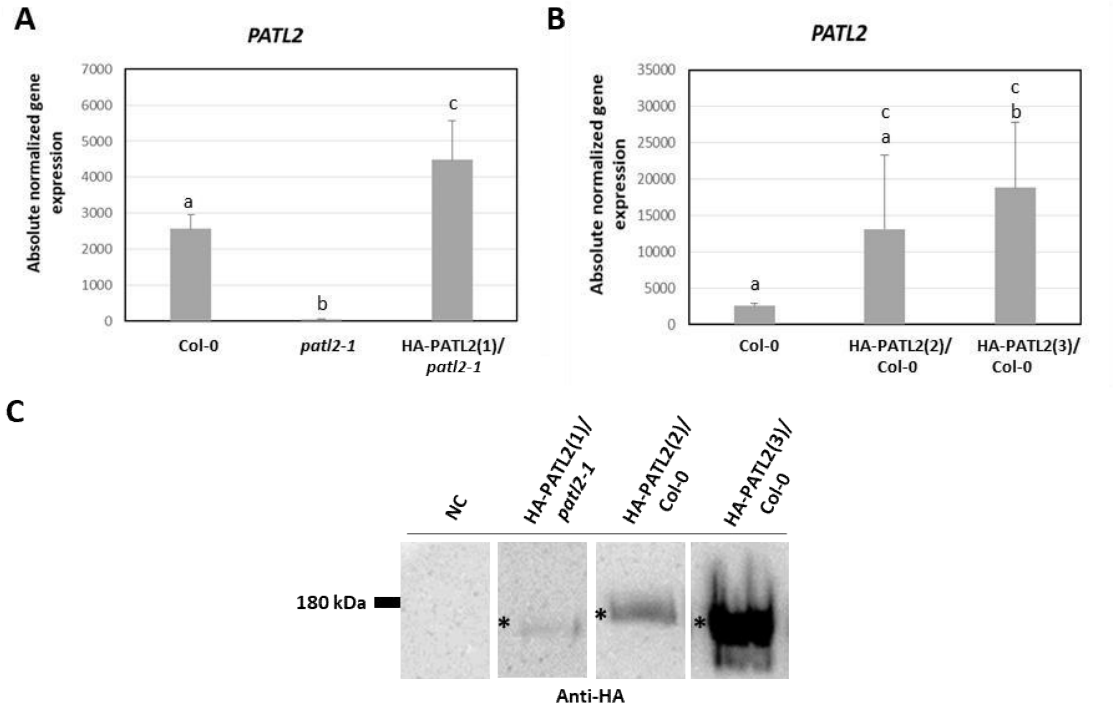

**Supplemental Figure 1. Interaction studies controls.**

(A) Controls of targeted yeast two-hybrid assay to verify the interaction of PATL2 and its deletion mutants with IRT1 variable region (vr). Growth on selection media (right) indicates protein-protein interaction. (B) Negative control of targeted BiFC experiment in *N.benthamiana* epidermis cells. EHB1Δsig is know to not interact with IRT1vr (Kahn et al., 2019). Construct was contemporaneous infiltrated and treated the same like samples in Fig. 1C – 1I. Bar: 50 µM


A
patl2-1
-115 bp
patl2-2
819 bp
AT1g22530
B
patl1-1
1524 bp
AT1g72150
C
pPATL1::GUS
50 µM Fe
pPATL1::GUS
0 µM Fe
I
III
II
III
II
I
D
FIT
Absolute normalized gene expression
50 µM Fe
0 µM Fe
Col-0
patl1-1/patl2-2
patl1-1
E
BHLH039
Absolute normalized gene expression
50 µM Fe
0 µM Fe
Col-0
patl1-1/patl2-2
patl1-1
F
FRO2
Absolute normalized gene expresselon
50 µM Fe
0 µM Fe
Col-0
patl1-1/patl2-2
patl1-1
G
IRT1
Absolute normalized gene expression
50µM Fe
0µM Fe
Col-0
patl1-1/patl2-2
patl1-1
H
Iron reductase activity [µmol Fe(II)/g root FW*h]
50µM Fe
0µM Fe
Col-0
patl2-2
patl1-1/patl2-2
I
Iron reductase activity [µmol Fe(II)/g root FW*h]
50 µM Fe
0 µM Fe
Col-0
patl1-1/patl2-2
patl1-1

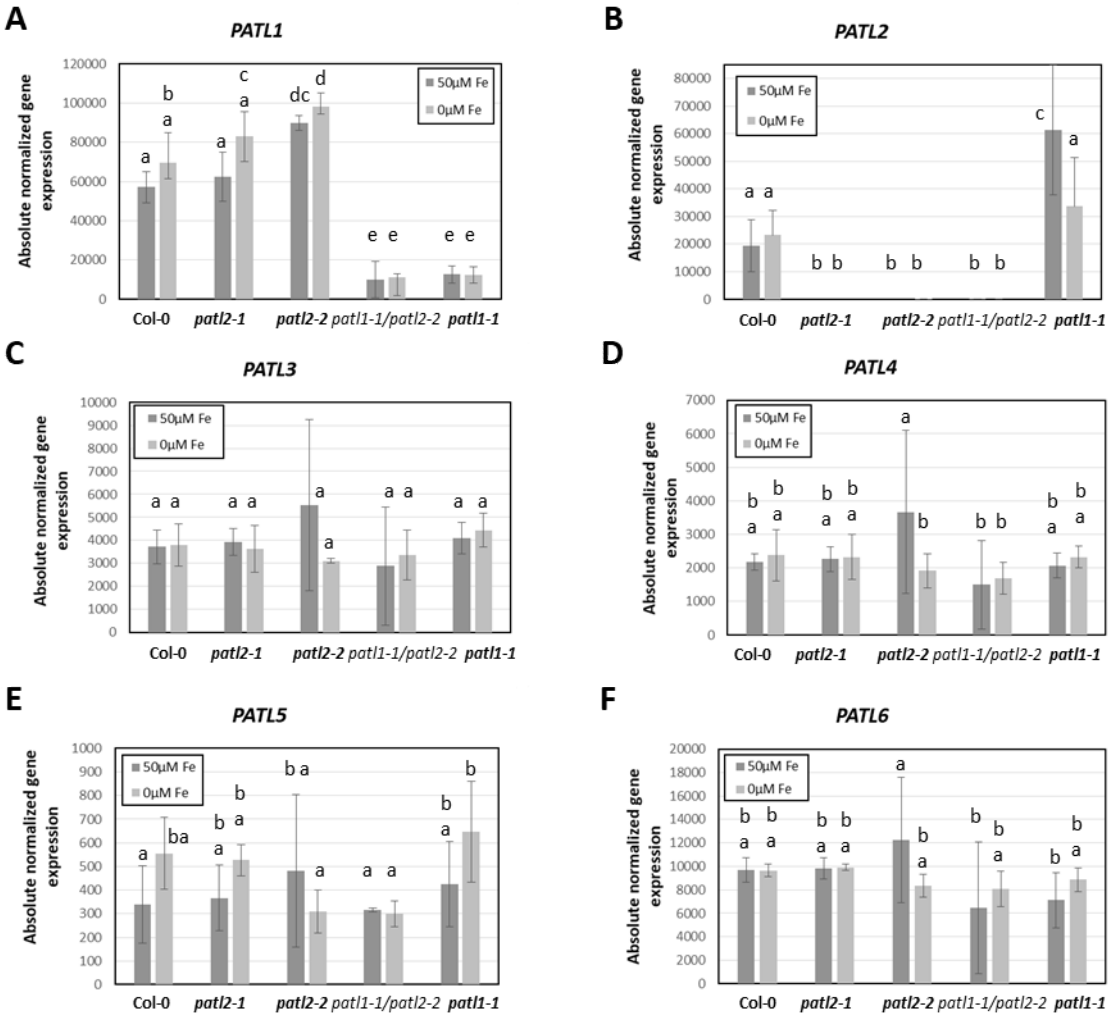
**Supplemental Figure 2. Analysis of *patl2*, *patl1-1* and *patl1-1/patl2-2* T-DNA mutants.**

(A) and (B) schematic representation of *PATL2* and *PATL1* genes. Boxes indicate introns and triangles represent position of T-DNA insertion. (C) Qualitative analysis of *PATL1*-promoter activity via GUS-promoter assay in 8d old seedlings grown on either iron sufficient (50 µM Fe) or deficient (0 µM Fe) Hoagland agar plates. On the left, qualitative promoter GUS activity (blue) on the whole plant. Bars 2000 µm. The squares indicate close-ups on the right, showing a cotyledon, the middle and bottom root zone, as well as the root tip. Bars 200 µm. Red boxes are close-ups, showing promoter-GUS expression in (I) root hairs, (II) the central cylinder and (III) root epidermis. [Arrows are indicating promoter-GUS activity in root hairs.] (D) to (G) analysis of iron-deficiency marker gene expressions in Col-0, *patl1-1/patl2-2* and *patl1-1* roots. (H) to (I) Iron reductase activity of mutant roots and Col-0 roots. [All plants in (D) to (I) were grown in the 14+3d system. Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).]



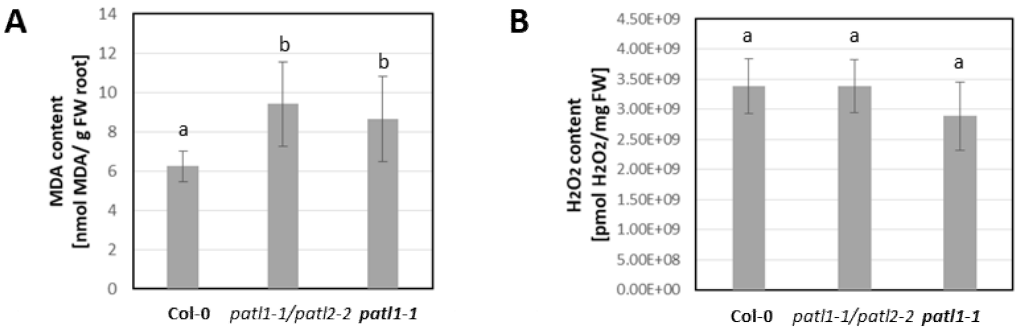

**Supplemental Figure 3. *HA-PATL2*-overexpressing plants.**

(A) *PATL2* expression analysis in roots of *HA-PATL2* expressing plants in *patl2-1* mutant background (HA-PATL2(1)/*patl2-1*). (B) *PATL2* expression in *HA-PATL2* expressing plants with Col-0 background (HA-PATL2(2)/Col-0; HA-PATL2(3)/Col-0). [Plants in (A) and (B) were grown in the 14+3d system. Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).] (C) Analysis of HA-PATL2 protein in roots of 17d old *HA-PATL2* overexpression plants on SDS-PAGE (highlighted with *).




**Supplemental Figure 4. Redundancy of PATLs is not regulated at gene expression level.**

(A) To (F) Expression analysis of *PATLs* in *patl2*, *patl1-1* and *patl1-1/patl2-2* mutants grown in the 14+3d system. [Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).]




**Supplemental Figure 5. ROS accumulation in *patl1-1/patl2-2* and *patl1-1*.**

(A) MDA concentration in roots of 17d old plants indicating lipid-peroxidation rate. (B) Measured $H_2O_2$ concentration in roots of 17d old plants. [Error bars represent calculated standard deviation. Different letters indicate statistically significant differences ($p < 0.05$).]

**Supplemental Table 1.** Primer list

| Primer | Sequence (5'-3') |
|---|---|
| PATL2B1n | GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCTCAAGAAGAGATACAG |
| AtIRT1c-RT-For | AAGCTTTGATTCACGGTTGG |
| AtIRT1c-RT-Rev | TTAGGTCCCATGAACTCCG |
| bHLH39-RT-3' | GGTGGCTGCTTAACGTAACAT |
| bHLH39-RT-5' | GACGGTTTCTCGAAGCTTG |
| ccdb_F | AAACGCCATTAACCTGATGT |
| Efc-RT-For | TATGGGATCAAGAAACTCACAAT |
| Efc-RT-Rev | CTGGATGTACTCGTTGTTAGGC |
| EFg-RT-For | TCCGAACAATACCAGAACTACG |
| Efg-RT-Rev | CCGGGACATATGGAGGTAAG |
| FIT-RT-For | CCCTGTTTCATAGACGAGAAC |
| FIT-RT-Rev | ATCCTTCATACGCCCTCTCC |
| FRO2c-RT-For | CTTGGTCATCTCCGTGAGC |
| FRO2c-RT-Rev | AAGATGTTGGAGATGGACGG |
| I1LB1 (n) | GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCCATGGCCACGAGCCTATA |
| I1LB1 (c) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTCCATGGCCACGAGCCTATA |
| I1LB2 (stop) | GGGGACCACTTTGTACAAGAAAGCTGGGTTTATCGGTATCGCAAGAGCTGTG |
| I1LB2 ns | GGGGACCACTTTGTACAAGAAAGCTGGGTTTCGGTATCGCAAGAGCTGTG |
| I1LB3 (c ) | GGGGACAACTTTGTATAATAAAGTTGTATCCATGGCCACGAGCCTATA |
| I1LB3 (n) | GGGGACAACTTTGTATAATAAAGTTGATGTCCATGGCCACGAGCCTATA |
| I1LB4 (stop) | GGGGACAACTTTGTATAGAAAAGTTGGGTTTATCGGTATCGCAAGAGCTGTG |
| I1LB4 ns | GGGGACAACTTTGTATAGAAAAGTTGGGTGTCGGTATCGCAAGAGCTGTG |
| LB- SALK T-DNA check | GCGTGGACCGCTTGCTGCAACT |

| | |
|---|---|
| **M13_r** | **CAGGAAACAGCTATGAC** |
| **PAT2seqR1297 (rev)** | **CGTACGAGCTATAGATCACGAC** |
| **PATL1B1F** | **GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCTCAAGAGGAAGTACAG** |
| **PATL1B2nsR** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTTGAGTTTTGAACCTGTAGAGC** |
| **PATL1B2nsR** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTTGAGTTTTGAACCTGTAGAGC** |
| **PATL1B2stopR** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTATTGAGTTTTGAACCTGTAG** |
| **PATL1-RT-F** | **GCAACCCTGAAGCGAAGTCT** |
| **PATL1-RT-R** | **AGCGCGTCTGATGAATTGCC** |
| **PATL1-ST-F** | **TCGTGGTAGAGACTGTCGCC** |
| **PATL1-ST-R** | **CCTTCGGTGGTTGGCTCAAA** |
| **PATL2_g1 (for)** | **GCAAGAGTTAGAAGTGAATCGGAG** |
| **PATL2_g2 (rev)** | **TGTATCTCTTCTTGAGCCAT** |
| **PATL2_g2 (rev)** | **TGTATCTCTTCTTGAGCCAT** |
| **PATL2_g3 (for)** | **AGGAAGAGAAAGTTGAGGAG** |
| **PATL2_g3 (for)** | **AGGAAGAGAAAGTTGAGGAG** |
| **PATL2_g3 (for)** | **AGGAAGAGAAAGTTGAGGAG** |
| **PATL2_g6_R** | **AGGCCCTCTTGACCTGAG** |
| **PATL2_g6_R** | **AGGCCCTCTTGACCTGAG** |
| **PATL2ΔCnsB2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTGGGATTCCCCAGATTGAGAC** |
| **PATL2ΔCstopB2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGGGATTCCCCAGATTGAGAC** |
| **PATL2ΔCTN_F** | **CAATCTGGGGAATCCCATCAGAAGATCTTGA** |
| **PATL2ΔCTN_R** | **TCAAGATCTTCTGATGGGATTCCCCAGATTG** |
| **PATL2ΔCTN-SEC14_F** | **ATCTGGGGAATCCCATTCACCGTTGAAGAT** |
| **PATL2ΔCTN-SEC14_R** | **ATCTTCAACGGTGAATGGGATTCCCCAGAT** |
| **PATL2ΔGOLDstopB2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAACGGTGAATGGAC** |
| **PATL2ΔNB1_N** | **GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCTTCTAGAGGACGAAAGATCC** |
| **PATL2ΔNstartB1c** | **GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCTTCTAGAGGACGAAAGATCC** |
| **PATL2ΔNstartB3c** | **GGGGACAACTTTGTATAATAAAGTTGATGCTTCTAGAGGACGAAAGATCC** |
| **PATL2ΔSEC14_F** | **ATCGACGACCTAGTCTTCACCGTTGAAGAT** |
| **PATL2ΔSEC14_R** | **ATCTTCAACGGTGAAGACTAGGTCGTCGAT** |
| **PATL2B1c** | **GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTCAAGAAGAGATACAG** |
| **PATL2B2nsR** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTGCTTGGGTTTTGGACCTGTAG** |
| **PATL2B2stopR** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGCTTGGGTTTTGGACC** |
| **PATL2B3c** | **GGGGACAACTTTGTATAATAAAGTTGATGGCTCAAGAAGAGATACAG** |
| **PATL2B3n** | **GGGGACAACTTTGTATAATAAAGTTGTAATGGCTCAAGAAGAGATACAG** |
| **PATL2-CnsB2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTGGGATTCCCCAGATTGAGAC** |
| **PATL2-CstopB2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGGGATTCCCCAGATTGAGAC** |
| **PATL2-GOLDnsB2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTGGGATTCCCCAGATTGAGAC** |
| **PATL2-NB3c** | **GGGGACAACTTTGTATAATAAAGTTGATGCTTCTAGAGGACGAAAGATCC** |
| **PATL2-NB3n** | **GGGGACAACTTTGTATAATAAAGTTGTACTTCTAGAGGACGAAAGATCC** |
| **PATL2-RT-F** | **ACCGTTGAAGCAGTCGAAGA** |
| **PATL2-RT-R** | **GAGGAGGATCACGTCGGATCT** |

| | |
|---|---|
| **PATL3 RT FOR** | **AAGATGCCCTACCGACAGAG** |
| **PATL3 RT REV** | **GCGGTTTCTTCTTCTGGCTT** |
| **PATL3 ST FOR** | **GGCTGAAGAACCTACTACTACC** |
| **PATL3 ST REV** | **CGTCTTCAAGTAATGGAATCCC** |
| **PATL4 RT FOR** | **CAGGTGGAGTCAGAGGTTGT** |
| **PATL4 RT REV** | **CCTCAGGCTTGCTCTCATCT** |
| **PATL4 ST FOR** | **GACTGCTGAAGTTAAGGTTGA** |
| **PATL4 ST REV** | **GCTTCTTCGGTAACAACAGCC** |
| **PATL5 RT FOR** | **TCAGAACTCAAACCCACGGA** |
| **PATL5 RT FOR** | **TGCCTTCCGGTGAAGAAGAT** |
| **PATL5 RT REV** | **GGCGTCACCTTCTCTGAAAC** |
| **PATL5 RT REV** | **GTCAGCTTTGTCGTCTCCAC** |
| **PATL5 ST FOR** | **CATTGTCTCCATTCGATCACC** |
| **PATL5 ST FOR** | **CCGCCGCCACCTTTAACC** |
| **PATL5 ST REV** | **CTCTTTAAACACACCATAAGC** |
| **PATL5 ST REV** | **CTTCTGTCTCGGAAGTTACAAGC** |
| **T7_F** | **TAATACGACTCACTATAGGG** |
| **YS3-pACT2_R** | **GAGATGGTGCACGATGCAC** |
| **YS5-pACT2_F** | **AATACCACTACAATGGAT** |
| **I1B1** | **GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCTTCAAATTCAGCACTT** |
| **FLI1B2** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTAGCCCATTTGGCGATAATCG** |
| **FLI1B2Stop** | **GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAGCCCATTTGGCGATAATCG** |

## Authors Contribution to Manuscript 2

**Karolin Montag**

Designed, performed and analyzed following experiments: targeted yeast two-hybrid assay; transient tobacco transformation; BiFC; microscopy; GUS-assays; Genotyping of T-DNA mutants; gene expression analysis via RT-qPCR; iron reductase assays; MDA assays; $H_2O_2$ assays; *fet3 fet4* complementation assay. K.Montag generated deletion mutants, HA-PATL2 expressing plants, PATL2-GFP expressing plants and most recombinant vectors.

K. Montag designed the outline and wrote the manuscript, prepared all figures, performed statistical analyses and reviewed / edited the manuscript

Tzvetina Brumbarova, Regina Gratz, Kalina Angrand, Rubek Merina Basgaran

T.B, R.G., K.A. and R.M.B. contributed to identification of protein-protein interaction by performing the yeast two-hybrid screen. Additionally, they verified the interaction of full-length PATL2 with IRT1vr by BiFC and yeast two-hybrid.

Rumen Ivanov

Designed, performed and analyzed following experiments: yeast two-hybrid screen; Co-immunoprecipitation. R. Ivanov contributed to microscopy.

R. Ivanov designed the outline of the manuscript, supervised the study and reviewed / edited the manuscript.

Petra Bauer

Designed the outline of the manuscript, supervised the study, provided funding, and reviewed / edited the manuscript.

# 8. Manuscript 3

Review

## Structure and function of SEC14-like phosphatidylinositol transfer proteins with emphasis on SEC14-GOLD domain proteins

Review

# Structure and function of SEC14-like phosphatidylinositol transfer proteins with emphasis on SEC14-GOLD domain proteins

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**Keywords:** SEC14 domain, SEC14-GOLD proteins, PATELLIN, membrane trafficking, PITPs

**Key message:**

- SEC14L-PITPs are able to not only bind phospholipids, but also other lipophilic ligands.
- The presence of multi-domain SEC14L-PITPs in higher eukaryotes indicates an increasing variety of functions.
- SEC14-GOLD proteins contribute to the prevention of ROS and radical caused cellular damage.
- SEC14-GOLD proteins function as cellular regulators via protein-protein and protein-lipid interaction to control the response to altered external stimuli.

## Abstract

Intracellular membrane trafficking is a fundamental mechanism to maintain and regulate the molecule transport within a cell and is therefore strongly linked to phospholipid-signaling pathways. Due to their ability to bind phospholipids and interact with cargo proteins, members of the SEC14-like phosphatidylinositol transfer protein (SEC14L-PITP) superfamily are essential key players regulating membrane trafficking.

Our aim is to collect and present in a systematic way the knowledge on different SEC14L-PITPs, with a special focus on the multi-domain SEC14L-PITPs of the SEC14-GOLD class. We review the knowledge on their contributions to the regulation of membrane trafficking and to organism fitness. For this, we concentrate on the structure of SEC14L-PITPs, their ability to not only bind phospholipids but also other lipophilic ligands, and their ability to regulate complex cellular responses through interacting with additional proteins.

## 1. Introduction

Cells are surrounded by membranes, which function as hydrophobic permeable barriers regulating the exchange of molecules and the flow of information. Within the cell, membranes have different compositions resulting in their specific identity and allowing them to fulfill specific tasks (Heilmann, 2016; Mamode Cassim et al., 2019; Watson, 2015). The plasma membrane (PM), for example, is involved in cell-to-cell communication or cell shape changes (Cooper, 2000; Luschnig and Vert, 2014). The thylakoid membrane is essential for photosynthesis and uses an electron gradient to generate ATP (O'Connor and Adams, 2010). In addition to building the basic membrane backbone, lipids may have regulatory roles (Stevenson et al., 2000). Minor changes in lipid composition and structure can result in major modifications to essential cellular processes (Harayama and Riezman, 2018). Especially the phospholipid composition of a membrane has significant effects on the regulation of cellular and tissue functions (Heilmann, 2016). A crucial group of regulatory phospholipids are the phosphorylated derivatives of phosphatidylinositol (PI) - the phosphoinositides (PIPs) PI(3)P, PI(4)P, PI(5)P, PI(3,4)$P_2$, PI(3,5)$P_2$, PI(4,5)$P_2$ and PI(3,4,5)$P_3$ (Irvine, 2016). PI and PIPs provide cues to membrane identity, although they make up less than 1% of membrane lipids (Gerth et al., 2017; Simon et al., 2014; Simon et al., 2016). They are key players controlling growth, development and polarization as well as influencing multiple processes through binding to a great number of interaction partners in adaptation to, e.g., environmental changes (Heilmann, 2016; Roman-Fernandez et al., 2018).

Lipids can be distributed in the cell through vesicle-independent trafficking, which includes spontaneous lipid transfer, flip-flop exchange within bilayers, lateral diffusion or single-lipid transfer by proteins (Lev, 2010). However, trafficking of proteins, lipids and other metabolites between cell compartments is mainly achieved by the highly regulated and coordinated conserved membrane-trafficking mechanism, in which macromolecules are transported within membrane vesicles (Goring and Di Sansebastiano, 2017; Tokarev et al., 2013). Generally, single-lipid transfer and membrane trafficking are controlled by regulatory proteins in response to developmental cues and external stimuli. These proteins are able to link lipid recognition, metabolism and signaling. Phosphatidylinositol transfer proteins (PITPs) exhibit such characteristics. PITPs can be clustered in two independent protein families with distinct separated biological functions (Wirtz, 1991).

The first group is simply named the phosphatidylinositol transfer protein (PITP)-superfamily, defined through its phosphatidylinositol transfer protein and Lipin/Ned1/Smp2 (PITP/LNS2) domain (InterPro accession number (IPR): IPR031315). This domain is thought to promote the

exchange of phospholipids at the membrane contact sites of the endoplasmic reticulum (ER) and the PM by non-vesicular lipid transport (Cockcroft and Raghu, 2018). Proteins containing a PITP/LNS2 domain can be found in mammals, invertebrates and plants (Cockcroft, 2012; Cockcroft and Raghu, 2018). Defects in PITP proteins can lead to neurodegenerative diseases (Cockcroft, 2012; Hsuan and Cockcroft, 2001). In *Arabidopsis thaliana* (Arabidopsis) the PITP/LNS2 domain can be found in two phosphatidate-phosphohydrolase proteins involved in galactolipid synthesis and necessary to maintain membrane structure by lipid remolding due to phosphate starvation (Nakamura et al., 2009; Yoshitake et al., 2017).

The second PITP-superfamily is defined through its SEC14 domain (IPR001251) and therefore named the SEC14-like phosphatidylinositol transfer protein (SEC14L-PITP)-superfamily. SEC14L-PITPs are able to recognize, bind, exchange and transfer small lipophilic molecules between membranes by non-vesicular transport (Bankaitis et al., 1990; Cleves et al., 1991). Additionally, they are involved in regulation of membrane trafficking within a cell (Bankaitis et al., 2010). Yeast Sec14p (304 AA) is the prototype for the SEC14 domain ([37-279AA] 12x α -helices, 6x β-strands, 8x $3_{10}$-helices; 2x distinct domains) (Sha et al., 1998) and was initially identified in a screen for secretory mutants (termed “SEC”) (Novick et al., 1980). Proteins with a SEC14 domain are found in yeast, plants, invertebrates, and mammals, suggesting a conserved and essential role (Aravind and Iyer, 2012; Ren et al., 2011). The SEC14 domain forms a characteristic hydrophobic phospholipid-binding pocket at its carboxy (C)-terminus (Sha et al., 1998). An identical phospholipid binding pocket was observed in several mammalian proteins, including the CELLULAR RETINAL-BINDING PROTEIN (CRALBP), TRIO and α-TOCOPHEROL-TRANSFER PROTEIN (α-TTP) (Crabb et al., 1998; Min et al., 2003). That is why the domain is also called CRAL-TRIO domain (Panagabko et al., 2003). A unique feature of the SEC14 domain is that the lipophilic ligand is bound and enclosed as a whole molecule in the hydrophobic lipid-binding pocket (Min et al., 2003; Schaaf et al., 2006), while other lipid-binding domains, like FYVE or PH, only bind the lipid headgroup (Stahelin, 2009). The alpha helical amino (N)-terminus of Sep14p is defined as CRAL-TRIO-N-terminal extension (CTN) (IPR011074) and cannot be identified in all SEC14L-PITPs (Saito et al., 2007a). The ability to open and close the SEC14 lipid-binding pocket by structural changes seems to be essential for domain activity and the biological function of this domain (Kono et al., 2013; Ryan et al., 2007; Schaaf et al., 2011; Schaaf et al., 2008). The open status is believed to be the membrane attached structure, while the closed conformation - binding a substrate - is understood as the cytosolic version of the SEC14 domain (Tripathi et al., 2014). This fits the observation that the CTN-SEC14 module is crucial for membrane association of SEC14L-PITPs, since loss of the

module leads to the accumulation of the protein in the cytosol (Montag et al., under review; Saito et al., 2007a; Saito et al., 2007b; Sirokmany et al., 2006; Skinner et al., 1993; Sun et al., 2006). To clarify the role of SEC14L-PITPs in membrane trafficking the lipid-presentation model was developed. The model is based on results indicating that the SEC14 domain is essential for promoting membrane trafficking by supporting PI(4)P-OH kinases activity (Strahl and Thorner, 2007). It is believed that the SEC14 domain recognizes membrane-bound phosphatidylcholine (PC) and presents the domain bound PI to the PI(4)P-OH kinases for phosphorylation. Next, the SEC14 domain promotes the exchange of PC with PI(4)P at the membrane. The presence of PI(4)P at the membrane is then initiating vesicle formation (Fig. 1)(Kf de Campos and Schaaf, 2017).

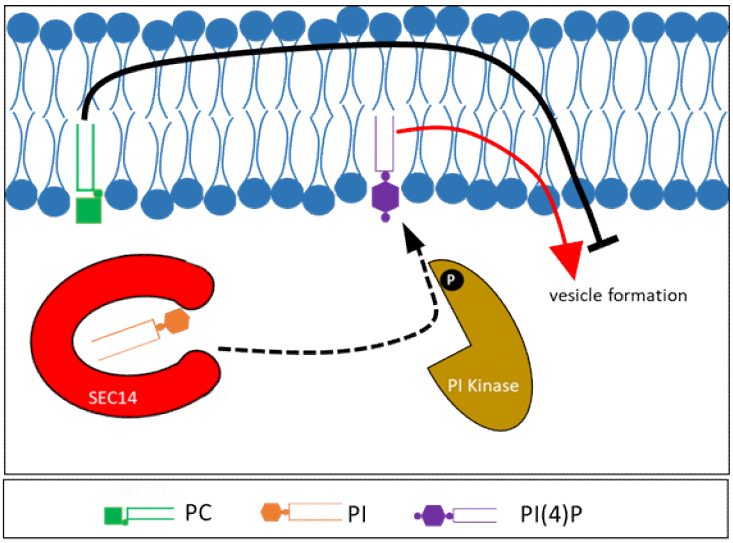


**Figure 1. Lipid presentation model of SEC14L-PITPs.**
On the basis of the crystal structure of Sfh1, binding either PI or PC, de Campos & Schaaf (2017) developed the lipid presentation model of SEC14L-PITPs. The SEC14L-PITP at a targeting membrane senses PC and mediates the heterotypic exchange of PI with PC. Within the exchange the headgroup of PI is presented to a PI-Kinase and phosphorylated to PI(4)P.

It is remarkable that simple organisms mainly exhibit single-domain SEC14L-PITPs (SEC14-only proteins), whereas the SEC14 domain in more complex organisms is often part of larger multi-domain proteins (multi-domain SEC14L-PITPs). While the functions of several SEC14-only proteins are studied well, only a few multi-domain SEC14L-PITPs are characterized. At the same time, their abundance in higher eukaryotes highlights their importance. This raises several fundamental questions. Firstly, in which pathways are they integrated? Secondly, what are the

functions of the different domains within these proteins? Thirdly, which protein-protein and protein-ligand interactions are relevant for this purpose?

Herein, we review the knowledge about SEC14L-PITPs and focus on structure and function of multi-domain SEC14-PITPs. We highlight the subgroup of SEC14-GOLD proteins and analyze the relevance of subdomains for fundamental phospholipid, membrane and protein binding.

## 2. Roles of SEC14-only proteins

Similar to Sec14p and its homologous in yeast (Bankaitis et al., 1990; Cleves et al., 1991; Griac, 2007; Schnabl et al., 2003), SEC14-only proteins can also be identified in higher eukaryotes (Table 1; Fig. 2) (Montag et al., under review; Saito et al., 2007a). While all SEC14-only proteins in yeast are well characterized by demonstrating their roles in different aspects of the phospholipid metabolism, like organization of the actin cytoskeleton, activation of phospholipase D or prevention of saturated fatty-acid accumulation (Desfougeres et al., 2008; Li et al., 2000; Yakir-Tamang and Gerst, 2009), only few human SEC14-only proteins have been studied till now. In spite of this, the studies of human SEC14-only proteins have increased the knowledge about the functions of SEC14L-PITPs and their several essential roles within the organism.

For example, human CRALBP is able to transport 11-cis retinaldehyde, the photosensitive component of rhodopsin, in its SEC14 lipid-binding pocket (Crabb et al., 1998; Fishman et al., 2004). This feature makes CRALBP essential for photoreceptor function. Mutations in its SEC14 domain lead to neurodegenerative diseases affecting the eyesight by photoreceptor involution (Burstedt et al., 2001; Eichers et al., 2002; Maw et al., 1997).

Another important human SEC14-only protein is α-TTP found to be most abundant in liver cells, where it is involved in vitamin E secretion, especially as α-tocopherol (α-TOC), but it is also expressed in mammalian uterine and placental cells during embryogenesis (Arita et al., 1997; Miller et al., 2012; Sato et al., 1993). Vitamin E is known to be an important antioxidant neutralizing reactive oxygen species (ROS) and radicals (Nukala et al., 2018). RRR-α-TOC is believed to be the biologically most active form because of its high binding affinity to the SEC14 domain of α-TTP (Traber et al., 1994). Among other reasons, this could be due to the side chains of tocotrienols (also vitamin E components), which have a lower binding affinity to the SEC14 ligand-binding pocket of α-TTP (Compadre et al., 2014). While it has been shown that α-TTP is important for secretion of α-TOC from liver cells and thereby regulating the vitamin E levels in the organism, it is not required for uptake and intracellular vitamin E distribution (Irias-Mata et al., 2018).

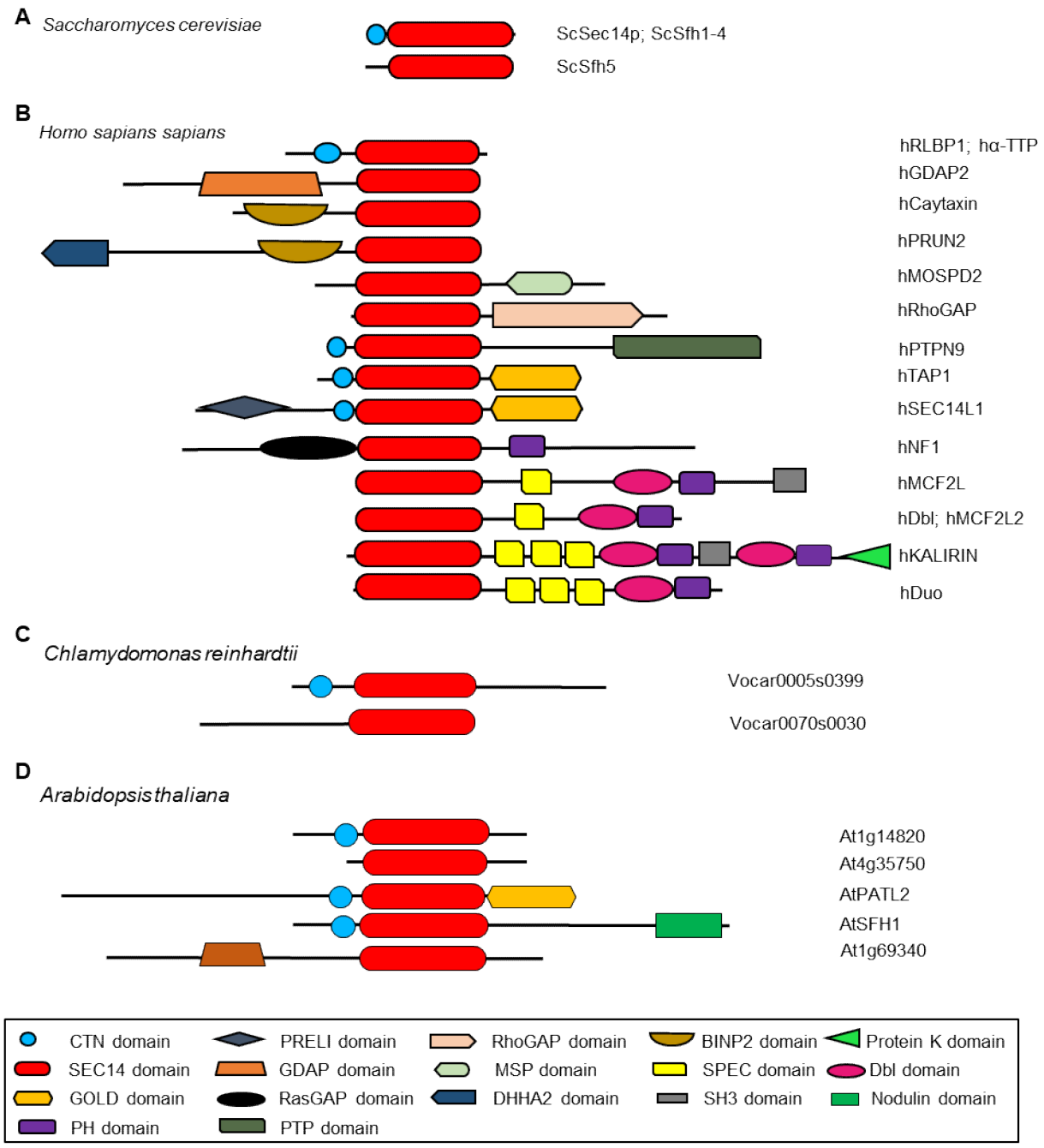


**Figure 2. Schematic structure of chosen SEC14L-PITPs in different species.**
(A) All SEC14L-PITPs in *Saccharomyces cerevisiae*. (B) Representative extract of human SEC14L-PITPs (homologues of proteins can also be found in other metazoan e.g. *Mus musculus*, *Drosophila melanogaster*, *Danio rerio*, *Caenorhabditis elegans*, *Mustela putorius furo* and *pan*). (C) Structure of SEC14L-PITPs in *Chlamydomonas reinhardtii* as representative of green algae. (B) *Arabidopsis thaliana* SEC14L-PITPs and their domain structure as representatives of flowering plants. [Phylogenetic analysis, domain identification and alignments were performed as descript in Montag et al., under review.]

**Table 1.** Selected SEC14-only proteins and their putative function. [phosphatidylinositol (PI) ; phosphoinositides (PIPs) ; phosphatidylcholine (PC); α-tocopherol (α-TOC), phosphatidylethanolamin (PEtN)]

| Protein | Organism | protein-ligand interaction (*in vivo* and *in vitro*) | Protein-protein interaction | cellular role | physiological effect | References |
|---|---|---|---|---|---|---|
| Sec14p | *S.cerevisiae* | PI; PC | Pld1p (phospholipase D) | involved in lipid synthesis, turnover and the GOLGI secretory process ; regulation of PI(4)P levels; negative regulator of phospholipase D | essential for yeast growth | Bankaitis et al., 1990; Cleves et al., 1991; Sreenvivas et al., 1998; Holic et al., 2004 |
| Sfh1p | *S.cerevisiae* | PEtN | unknown | unknown | unknown | Li et al., 2000; Schaaf et al., 2006 |
| Sfh2p | *S.cerevisiae* | PI | Pld1p (phospholipase D) ? | regulates fatty acid synthesis; might regulate phospholipase D | inactivation results in premature growth arrest | Li et al., 2000;Schnabl et al., 2003; Routt et al., 2005, Griac et al.,2007; Desfougeres et al., 2008 |
| Sfh3p | *S.cerevisiae* | PI | unknown | potential role in sterol biosynthesis | mutation leads to changes in sterol composition | van den Hazel et al., 1999; Li et al., 2000; Schnabl et al., 2003; Griac et al., 2007 |
| Sfh4p | *S.cerevisiae* | PI | Pld1p (phospholipase D) ? | might be a part of a membrane-trafficking complex involved in cellular PSer transport; might regulate phospholipase D | unknown | Wu et al., 2000; Li et al., 2000; Griac et al., 2007 |
| Sfh5p | *S.cerevisiae* | PI | unknown | putative role in actin cytoskeleton formation and post-Golgi trafficking | unknown | Li et al., 2000; Briza et al., 2002; Routt et al., 2005; Griac et al., 2007 |
| CRALBP | human | 11-*cis*-retinaldehyde/ 11-*cis*-retinal; 11-*cis*-retinol; 9-*cis* retinal; α-Toc | unknown | unknown | mutation results in neurodegenerative diseases affecting the eyesight | Maw et al., 1997; Crabb et al., 1998;Burstedt et al.; 1999 Eichers et al.,2002; Fishman et al., 2004; Kong et al., 2006; Zhao et al., 2008; |
| α-TTP | human | α-TOC and other vitamin E derivatives; PI; PI(3,4)P2; PI(4,5)P2; PI(3,4,5)P3; 9-cis-retinal | unknown | Vitamin E secretion | mutation results in ataxia, with vitamin E deficiency, and neurodegenerative diseases resulting in, e.g., retinitis pigmentosa | Sato et al., 1993; Traber., 1994; Gotoda et al., 1995; Ouahchi et al., 1995; Arita et al.; 1997; Yokota et al., 1997; Panagabko et al., 2003; Miller et al.,2012; Kono et al., 2013 |

Important for the biological function and localization of α-TTP is its ability to not only bind α-TOC (in its lipid-biding pocket) but also PIPs (at the entrance of the lipid-binding pocket) (Chung et al., 2016; Kono et al., 2013). PIP binding mediates the release of α-TOC at membranes by inducing the conformational change of the SEC14 binding pocket from closed to open (Kono et al., 2013; Meier et al., 2003). Mutations in α-TTP lead to the neurodegenerative disease AVED (ataxia, with Vitamin E deficiency) caused by dramatic vitamin E deficiency, which results in creeping but continuous disturbance of muscle activity (Min et al., 2003; Ouahchi et al., 1995).
These two examples of human SEC14-only proteins show their critical roles in binding and transporting additional lipophilic ligands besides PI, PIPs and PC. Generally, the presence of SEC14-only proteins in simple and complex eukaryotes demonstrates the importance of regulating lipophilic-substance transport within a cell.

## 3. Multi-domain SEC14L-PITPs

However, it can be recognized that the number and modular complexity of SEC14L-PITPs increase in multicellular eukaryotes (Fig. 2). In previous studies, it was shown that through additional domains the functions of SEC14L-PITPs were enlarged (Table 2). Multi-domain SEC14L-PITPs are not only regulators of lipophilic-substance transport but also have the ability to function, e.g. as proteins with enzymatic functions, guanine exchange factors (GEFs) or GTPase-activating proteins (GAPs).
For example, the human multi-domain SEC14L-PITP TYROSINE-PROTEIN PHOSPHATASE NON-RECEPTOR TYPE 9 (PTPN9) has an additional protein phosphatase catalytic (PTP) domain (IPR000242) and functions as a tyrosine phosphatase (Denu and Dixon, 1998). The CTN-SEC14 module of PTPN9 is responsible for protein localization to the outer surface of secretory vesicles binding either phosphatidylserine (PSer) or PI(3,4,5)$P_3$ (and additional other PIPs), which might depend on the specific found biological condition (Huynh et al., 2003; Kruger et al., 2002; Krugmann et al., 2002; Saito et al., 2007a; Saito et al., 2007b; Zhao et al., 2003). Additionally, PTPN9 is a negative regulator of VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 (VEGFR2) by dephosphorylating it at position Tyr1175 (Hao et al., 2012). It is hypothesized that PTPN9 inactivity leads to defects of secretory vesicle formation in the brain and leucocytes with dramatic negative effects on neuronal tube formation, the immune system and bone development (Huynh et al., 2004; Huynh et al., 2003; Kruger et al., 2002; Saito et al., 2007a; Wang et al., 2005).

**Table 2.** Selected multi-domain SEC14L-PITPs and their putative function. [phosphatidylinositol (PI) ; phosphoinositides (PIPs) ; phosphatidylserine (PSer)]

| Protein | Organism | additional Domains | protein-ligand interaction (*in vivo* and *in vitro*) | protein-protein interaction | cellular role | physiological effect | References |
|---|---|---|---|---|---|---|---|
| GDAP2 | Human/ *A.thaliana* | GDAP domain | unknown | unknown | unknown | unknown | Montag et al., unp. |
| Caytaxin | human | BINP2 domain | unknown | unknown | unknown | mutation results in cerebellar cayman ataxia | Bomar et al., 2003 |
| PRUN2 | human | BINP2 domain; DHHA2 domain | unknown | unknown | unknown | prostate cancer suppressor | Salameh et al., 2015 |
| MOSPD2 | human | MSP domain | unknown | unknown | unknown | regulates monocyte migration | Mendel et al., 2017 |
| RhoGAP | human | RhoGAP domain | PI(4,5)$P_2$; PI(3,4,5)$P_3$ | transferrin receptor ? | proto-oncogene; overexpression inhibits transferrin uptake; might effect receptor-mediated endocytosis | plays a role in several types of cancer | Qin et al., 2003; Sirokmany et al., 2006 |
| PTPN9 | human | PTP domain | PI(3,5)$P_2$; PI(3,4,5)$P_3$; PSer | VEGFR2 | plays a role in secretory vesicle formation in brain and leucocytes | mutation results in defects in brain and neuronal tube formation, the immune system, bone development | Krugmann et al.,2002; Kruger et al., 2002; Huynh et al., 2003;Zhao et al., 2003; Huynh et al., 2004; Wang et al.,2005; Saito et al., 2007a; Saito et al., 2007b; Hao et al., 2012; |
| PTPX1 | *Xenopus laevis* | PTP domain; PSer biosynthetic enzyme domain | PI; PSer | unknown | unknown | unknown | Del Vecchio and Tonks, 1994; Saito et al., 2007a |
| NF1 | human | RasGAP domain; PH domain | unknown | unknown | unknown | mutation results in increased cancer risk; Recklinghausen disease | Rasmussen and Friedmann, 2000; D'Angelo et al., 2006; Yap et al., 2014; Rad and Tee 2016; Peltonen et al., 2017 |
| MCF2L | human | SPEC domain; Dbl domain; PH domain; SH3 domain | all PIPs | unknown | unknown | plays a role in several types of cancer | Ueda et al. 2004; Kostenko et al. 2005 |
| MCF2L2 | human | SPEC domain; Dbl domain; PH domain | unknown | unknown | unknown | unknown | |
| Dbl | human | SPEC domain; Dbl domain; PH domain | PI(3)P; PI(4)P; PI(5)P; PI(3,5)$P_2$ | unknown | plays a role in cell differentiation and transformation | developmental disorders, neoplastic transformation, plays a role in several types of cancer | Komai et al., 2002; Sahai & Marshall, 2002; Ueda et al., 2004; Vanni et al., 2015 |
| KALIRIN | human | SPEC domain; Dbl domain; PH domain; SH3 domain; Protein K domain | unknown | unknown | unknown | plays a role in structure and function of synapses | Bateman and Van Vactor, 2001; Herring and Nicoll, 2016 |
| Duo | human | SPEC domain; Dbl domain; PH domain | unknown | unknown | unknown | unknown | |
| Sfh1 | *A.thaliana* | Nodulin domain | PI(4)P; PI(4,5)$P_2$ | unknown | plays role in polarizing membrane trafficking | essential for root hair development | Bohme et al., 2004; Vincent et al., 2005; Preuss et al., 2006 Huang et al., 2013; Ghosh et al., 2015 |

The membrane targeting function of the (CTN)-SEC14 domain and its role in intracellular vesicle trafficking could also be recognized in human multi-domain SEC14L-PITPs functioning as GEFs and GAPs and thereby regulating the Ras/Raf-signaling pathway (Sirokmany et al., 2006; Sun et al., 2006; Ueda et al., 2004). For example, human SEC14L-PITPs with a GAP function are KALIRIN, Dou or RhoGAP, while MCF2 or MCF2L are functioning as GEFs. Mutations in all these proteins are linked to neurodegenerative diseases or cancer (Table 2).

Defects in human NEUROFIBROMIN 1 (NF1), a putative negative regulator of the Ras-signaling pathway, are disease-associated, especially when occurring in the double domain structure of SEC14-PH (Rad and Tee, 2016). The Pleckstrin homology (PH) domain (IPR001849) has a phospholipid binding specificity for $PI(4,5)P_2$ and seems to be involved in protein recruitment to membranes (Hyvonen et al., 1995; Lemmon, 2007; Lemmon and Ferguson, 1998). Patients with an *NF1* mutation are developing the Recklinghausen disease/ Watson syndrome and have a significantly increased cancer risk (D'Angelo et al., 2006; Peltonen et al., 2017; Rasmussen and Friedman, 2000; Yap et al., 2014).

Another example of SEC14L-PITPs playing an important role in human health is the prostate cancer suppressor PROTEIN PRUNE-LIKE PROTEIN 2 (PRUNE2) containing the additional BINP2 domain and DHHA2 domain (IPR004097) at its N-terminus (Salameh et al., 2015).

The human multi-domain SEC14L-PITP GANGLIOSIDE-INDUCED DIFFERENTIATION-ASSOCIATED PROTEIN 2 (GDAP2) contains a GDAP macro domain (IPR035793) at its N-terminus, which possibly binds ADP-ribose, and is localized to the lysosomal membrane (Hassa et al., 2006; Martzen et al., 1999). Its exact function is unknown, but recently homologs were identified in plants, suggesting a conserved function (Montag et al., under review).

A plant-specific subfamily of multi-domain SEC14L-PITPs are SEC14-nodulin proteins exhibiting an additional C-terminal Nodulin domain (Figure 3B) (Denance et al., 2014; Kapranov et al., 2001; Montag et al., under review). SEC14-nodulin proteins were demonstrated to be basic regulators in polarizing membrane trafficking (Ghosh et al., 2015; Huang et al., 2013; Vincent et al., 2005). The best studied member of this protein family is AtSfh1, which is involved in root hair biogenesis by controlling the tip directed gradient of $PI(4,5)P_2$ (and PI(4)P) (Bohme et al., 2004; Ghosh et al., 2015; Preuss et al., 2006; Vincent et al., 2005). Essential for this is the C-terminal lysine motif of the Nodulin domain, which has a high $PI(4,5)P_2$ binding affinity (Ghosh et al., 2015).

Taken together, the presence of multi-domain SEC14L-PITPs indicates the higher versatility of functions addressed in complex organisms as essential regulators within lipid signaling, membrane trafficking and regulation. They play a role in organism development by controlling

lipid signaling and membrane trafficking. Defects in multi-domain SEC14L-PITPs mainly result in developmental disorders, neurodegenerative diseases and an increased cancer risk.

## 4. SEC14-GOLD proteins

Till now the most studied family of multi-domain SEC14L-PITPs is the SEC14-GOLD family (Table 3). They can be found in insects and vertebrates. In the green lineage, SEC14-GOLD proteins were identified in briophytes *Marchantia polymorha* and all following vascular-plant species (Montag et al., under review). All SEC14-GOLD proteins contain a Golgi dynamics (GOLD) domain (IPR009038) at their C-terminus. It has been shown that the GOLD domain functions in membrane trafficking along the secretory pathway by mediating diverse protein-protein and protein-membrane interactions (Anantharaman and Aravind, 2002; Carney and Bowen, 2004; Pastor-Cantizano et al., 2018; Pastor-Cantizano et al., 2016; Sohda et al., 2001). The GOLD domain is either present in single-domain proteins or co-occurs with other domains, which are all involved in lipid binding (Anantharaman and Aravind, 2002; McPhail et al., 2017; Pastor-Cantizano et al., 2016).

### 4.1 Human SEC14-GOLD proteins

The analyses of SEC14-GOLD proteins revealed two subgroups of this family in humans. Next to the two defining domains, SEC14-LIKE 1 (SEC14L1) and SEC14-LIKE 5 (SEC14L5) have an additional N-terminal PRELI/MSF1 domain (Fig. 3A; Table 3) (IPR009038/ IPR006797) (Figure 3A)(Anantharaman and Aravind, 2002). Homologous proteins can also be identified in other higher eukaryotes like Zebrafish*, Drosophila melanogaster*, *Mus musculus*, and *Caenorhabditis elegans,* but not in any of the checked plant and yeast species. It is assumed that the PERLI/MSF1 domain could function in protein association to membranes as well as in transferring lipids (Anantharaman and Aravind, 2002; Yu et al., 2015), due to its involvement in mitochondrial protein sorting and phosphatidylethanolamine metabolism (Hall et al., 2011; Nakai et al., 1993).

hSEC14L1 is able to associate with two transporters, the VESICULAR ACETYLCHOLIN TRANSPORTER (VACht) and the CHOLINE TRANSPORTER 1 (CHT1), on synaptic vesicles (Ribeiro et al., 2007). This indicates that hSEC14L1 may play a fundamental role in intracellular vesicles trafficking (Ribeiro et al., 2007). Additionally, it has a negative regulatory function on RETINOIC ACID-INDUCIBLE GENE I (RIG-I), important for antiviral immunity response. RIG-I

interaction with other proteins is inhibited through its interactions with hSEC14L1, via its PRELI domain and SEC14 domain (Li et al., 2013).

hSEC14L5 was characterized as a potential target for post-traumatic stress disorder (PTSD) found in a study trying to identify molecular and genetic key players in this disease (Chitrala et al., 2016).

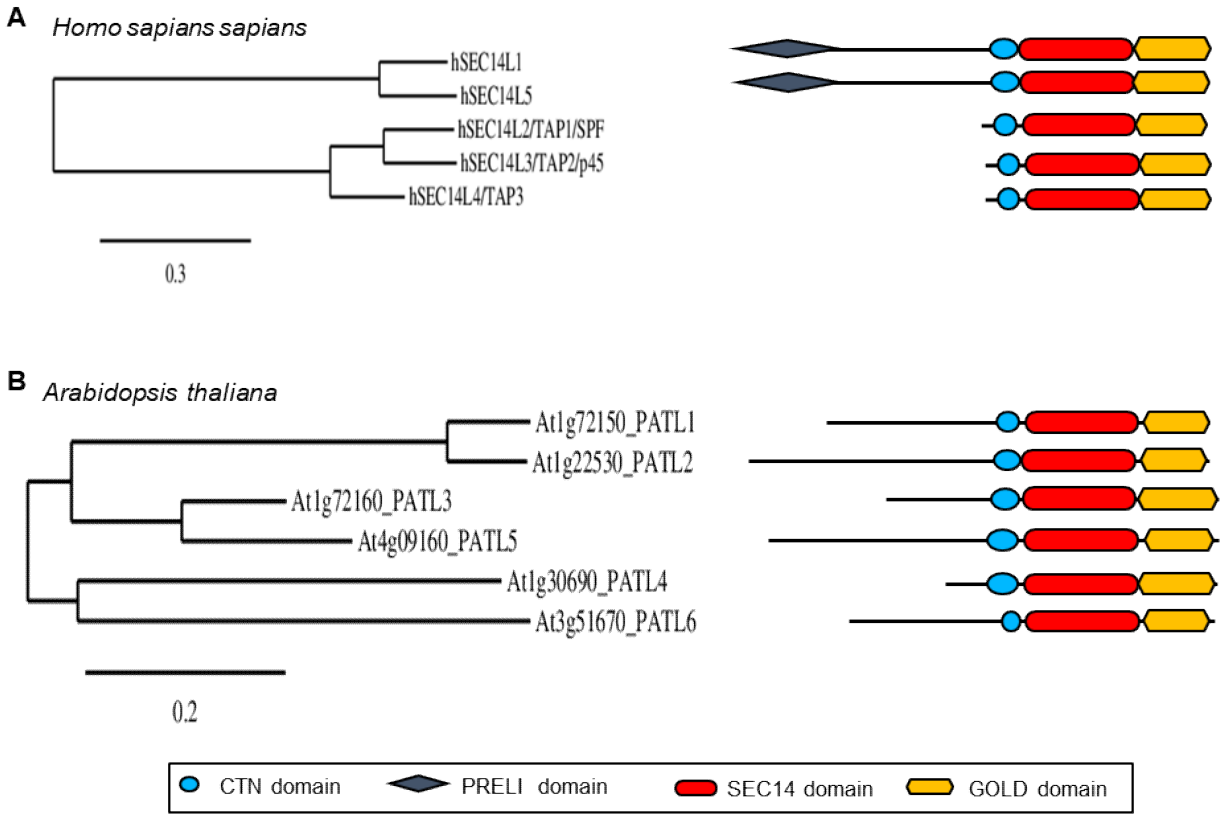


**Figure 3. SEC14-GOLD proteins in humans and Arabidopsis.**
(A) Phylogenetic tree of human SEC14-GOLD proteins and their modular architecture. (B) Phylogenetic tree of PATL proteins in Arabidopsis and their modular structure. [Phylogenetic analysis, domain identification and alignments were performed as descript in Montag et al., under review]

Human TOCOPHEROL-ASSOCIATED PROTEINs (TAPs), TAP1 (SPF/SEC14L2), TAP2 (p45/SEC14L3) and TAP3 (SFP2/SEC14L4), have a SEC14-GOLD domain combination with no additional N-terminal extensions (termed “N region”) (Figure 3A). They have the ability to bind α-TOC and its derivatives, squalene, phosphatidylglycerol (PG), PC, PI and PIPs (Chin and Bloch, 1985; Kempna et al., 2003; Stocker and Baumann, 2003). Recombinant expressed TAP proteins have a Rab-like small GTPase activity (Gong et al., 2017; Habermehl et al., 2005). Furthermore, the ability to bind α-TOC and its derivatives indicates a role of human TAPs in

preventing lipid peroxidation. This is supported by the observation that phosphorylated hTAP1 is able to stimulate cellular cholesterol biosynthesis, since protecting low density lipoproteins (LDL) from oxidation may inhibit cholesterol uptake (Neuzil et al., 1997; Shibata et al., 2001; Stocker and Baumann, 2003). Another hint to that assumption is that the presence of hTAP1 is able to increase vitamin E-mediated membrane protection from lipid peroxidation, which positively influences RNA replication of the hepatitis C virus (HCV) in cell cultures (Li et al., 2018; Saeed et al., 2015). Additionally, hTAP1 and its functional orthologue *Cgr-1* in *C. elegans* are playing a conserved role in the Ras/Raf pathway by being regulators of the Raf-signal activation and thereby suppressing its oncogenic capacity (Johnson and Kornfeld, 2010). Here again its ability to bind α-TOC positively influences health by regulating the uptake of α-TOC into cancer cells to stop cell growth and amplification. But hTAP1 is not only involved in tumor suppression by mediating α-TOC uptake and lipid protection, it also contributes to the regulation of PI(3)P Kinase γ (PI3Kγ) activity, either by blocking its subunit interaction or starting its activity, which then leads to VASCULAR ENDOSOMAL FACTOR (VEGF) expression (Ni et al., 2005; Wang et al., 2009; Zingg et al., 2015; Zingg et al., 2017; Zingg et al., 2014). Another fact linking hTAP1 to carcinogenesis is the observation that it is highly expressed in breast and prostate tissue, but downregulated in prostate and breast cancer cell lines, as well as in human breasts with invasive breast carcinomas (Ni et al., 2005; Wang et al., 2009).

In zebrafish TAP2 is crucial for the hydrolysis of $PI(4,5)P_2$ by phospholipase C (Gong et al., 2017). Rat (*Rattus norvegicus*) p45, a hTAP2 homolog, especially binds $PI(3,4,5)P_3$ *in vitro* and localizes with it in secretory vesicles, the cytoplasm and the extracellular space (Merkulova et al., 2005). Deletion of the SEC14 domain leads to inhibited secretion into the extracellular space, indicating that the SEC14 domain is essential for secretion (Merkulova et al., 2005).

The expression of rat SPF2, a homolog of hTAP3, is mainly observed in skin and respiratory tissue (Kempna et al., 2003; Merkulova et al., 1999). Recombinant SPF2 is able to stimulate the monooxygenase but not as efficient as TAP1 (Mokashi et al., 2004). Its activity is thereby stronger dependent on regulation by protein kinase A phosphorylation, guanine nucleotides and α-TOC, than TAP1 (Mokashi et al., 2004). An alternative splicing pattern was obtained for human TAP3 (Kempna et al., 2003). Due to this, reduced levels of biologically active hTAP3 could increase the risk of disease outbreak associated with the secretory capability of tissues/cells (Kempna et al., 2010; Zingg et al., 2008), underlining possible roles of TAPs as tumor suppressors.

The data on animal SEC14-GOLD proteins demonstrates its role in intracellular vesicle trafficking by interaction with PIPs. Additionally, it shows their function as negative regulators via

protein-protein interactions and demonstrates their oncogenic role. Furthermore, the data highlights their possible function as tumor suppressers, e.g. by mediating vitamin E transport and by preventing cellular damage by ROS and radicals.

## 4.2 Plant SEC14-GOLD proteins

In Arabidopsis and other plants SEC14-GOLD proteins are called PATELLINs (PATLs), named after 'patella', the Latin word for small plate, referring to AtPATL1 localization at the developing cell plate (Peterman et al., 2004). Analysis of the SEC14L-PITP superfamily in Arabidopsis revealed six PATL proteins, with a CTN-SEC14 and GOLD domain but no other N-terminal domains (Fig. 3B) (Peterman et al., 2004). In contrast to human TAPs PATLs display a variable N region of unknown structure, however different small motifs (coiled coil-; pxxp motifs) can be found (Diella et al., 2008; Montag et al., under review; Neduva and Russell, 2006; Peterman et al., 2004). The N regions of PATLs vary in its amino-acid sequence and are unique for each protein (Montag et al., under review). AtPATLs, except of AtPATL6, show an overall acidic N region due to repeats of glutamate (E) (Peterman et al., 2004). But they also show a pattern of lysines (K) surrounding the Es in AtPATL1, AtPATL2 and AtPATL4 (Montag et al., under review). Independently of the N regions, clustering of plant SEC14-GOLD proteins result in the formation of three clades with subgroup-specific amino acid substitutions in the GOLD domain, which may define different functional categories (Bermudez et al., 2018; Montag et al., under review; Peterman et al., 2006). Expression analyses of AtPATLs uncovered overlapping and clade specific clusters, and together with studies on multiple knock-out plants this indicates partial redundancy within the family (Montag et al., under review; Tejos et al., 2017). Multiple *patl* mutants demonstrated the essential role of PATLs in Arabidopsis patterning and polarity by revealing faults in auxin response due to decreased polarization of the auxin transporter PIN-FORMED 1 (PIN1) and defects in early plant development (Tejos et al., 2017). The role of PATLs during plant development can be confirmed by the observation that AtPATL1 expression is increased in developing leaves and vascular tissues and by its cellular localization to the PM and the cell plate during cell division (Montag et al., unp.; Peterman et al., 2004). Comparable distinct localization patterns, partly overlapping, could be visualized for all other AtPATLs. They were found to be peripheral membrane proteins localizing to at the PM, the cell plate and/or within the cytosol. AtPATLs are expressed in leaf-epidermis cells, vascular tissues, during embryogenesis, during development of lateral-root primordia and during differentiation of the root apical meristem (Montag et al., unp.; Suzuki et al., 2016; Tejos et al., 2017; Wu et al., 2017).

**Table 3.** Human and plant SEC14-GOLD proteins and their putative function. [phosphatidylinositol (PI) ; phosphoinositides (PIPs) ; phosphatidylserine (PSer); phosphatidylcholine (PC); α-tocopherol (α-TOC); phosphatidylglycerol (PG); phosphatidic acid (PA)]

| Protein | Organism | additional Domains | protein-ligand interaction (*in vivo* and *in vitro*) | protein-protein interaction | cellular role | physiological effect | References |
|---|---|---|---|---|---|---|---|
| SEC14L1 | human | PRELI domain | unknown | RIG-I; VACht; CHT1 | intracellular vesicle trafficking and synaptic vesicle formation; inhibits protein-protein interactions | invoveld in antiviral immuno response | Ribeiro et al., 2007; Li et al., 2013 |
| SEC14L5 | human | PRELI domain | unknown | unknown | unknown | plays a role in post traumatic stress disorder | Chitrala et al., 2016 |
| TAP1 | human | no | PI; PIPs; PC; PG; α-TOC and other vitamin E derivatives; squalene | PI3Ky | inhibits protein-protein interactions; plays a role in Vitamin E mediated membrane protection, Ras/Raf pathway and phospholipid signaling pathways | cancer suppressor ; plays a role in cholesterol synthesis and atherosclerosis | Neuzil et al., 1997; Kempna et al., 2003; Ni et al., 2005; Wang et al., 2009; Johnson and Korfeld, 2010; Saeed et al., 2015; Zingg et al., 2015; Li et al., 2018 |
| TAP2 | human/rat | no | PI;PIPs (especially PI(3,4,5)$P_3$ in rat) ; PC; PG; α-Toc and other vitamin E derivatives; squalene | PI3Ky | is involved in the regulator of phospholipase D; plays a role in secretion and phospholipid-signaling pathways | | Merkulova et al., 1999; Kempna et al., 2003; Mokashi et al., 2004; Gong et al., 2017 |
| TAP3 | human/rat | no | PI; PIPs ; PC; PG; α-Toc and other vitamin E derivatives; squalene | PI3Ky | stimulates monooxygenase; plays a role in phospholipid- signaling and secretion pathways | putative tumor suppressor | Kempna et al., 2003; Kempna et al., 2010 |
| PATL1 | *A.thaliana* | no | PI; PI(3)P; PI(4)P; PI(4,5)$P_2$; PI(3,5)$P_2$ | CaM4; SOS1; AMSH3; EXO70A1 | plays a role in membrane trafficking; regulator of CaM4 and SOS1 | plays a role in plant tolerance to abiotic stress and plant development | Peterman et al., 2004; Isono et al., 2010; Tejos et al., 2017; Chu et al., 2018; Zhou et al., 2018 |
| PATL2 | *A.thaliana* | no | all PIPs (due to physical interaction), PI(4)P; PI(4,5)$P_2$; cardiolipin; PA; sulfatide | IRT1; AMSH3; MPK4; EXO70A1 | prevents membrane damage; plays a role in iron acquisition and membrane trafficking | plays a role in plant tolerance to abiotic stress and plant development | Suzuki et al., 2016; Tejos et al., 2017; Wu et al., 2017; Montag et al., under review; Montag et al., unp |
| PATL3 | *A.thaliana* | no | PI(4)P; PI(4,5)$P_2$ | EXO70A1; AMV MP | unknown | plays a role in plant development; inhibits alfalfa mosaic virus infection | Peiro et al., 2014; Tejos et al., 2017; Wu et al., 2017 |
| PATL4 | *A.thaliana* | no | unknown | EXO70A1 | unknown | plays a role in plant development | Tejos et al., 2017; Wu et al., 2017 |
| PATL5 | *A.thaliana* | no | unknown | unknown | unknown | plays a role in plant development | Tejos et al., 2017 |
| PATL6 | *A.thaliana* | no | unknown | EXO70A1; AMV MP | unknown | plays a role in plant development; inhibits alfalfa mosaic virus infection | Peiro et al., 2014; Tejos et al., 2017; Wu et al., 2017 |
| SlTBP | *Solanum lycoperisicum* | no | α-Toc | unknown | plays a role in vitamin E transfer between plastids and ER, prevents membrane damage | plays a role in maintaining chloroplast structure | Bermudez et al., 2018 |

Protein localization during development and differentiation links them closely to membrane trafficking supported through the observation that AtPATL1, AtPATL2 and AtPATL3 bind PIPs (Peterman et al., 2004; Suzuki et al., 2016; Wu et al., 2017). Although AtPATL1 preferentially binds PI(5)P, PI(3)P and PI(4,5)$P_2$ and AtPATL3 mainly binds PI(4)P and PI(4,5)$P_2$, both still have the ability to associate with all other PIPs (Peterman et al., 2004; Wu et al., 2017). It was demonstrated that all domains of AtPATL2 contribute to PIP association, which can be traced back to physical interactions (Montag et al., under review). The CTN-SEC14 module of AtPATL2 governs the membrane association of the protein, while the GOLD domain specifies PM localization by recognizing PI(4,5)$P_2$ maybe through its lysine motif (Montag et al., under review). Another hint linking AtPATLs to membrane trafficking is the observation that AtPATL1 and AtPATL2 are able to interact with PM proteins (Chu et al., 2018; Montag et al., unp.; Zhou et al., 2018). Through its GOLD domain AtPATL1 interacts with CALMODULIN-4 (CaM4), a multifunctional sensor for $Ca^{2+}$, and via its N region it interacts with SALT OVERLY-SENSITIVE 1 (SOS1), a Na+/H+ antiporter localized at the PM (Chu et al., 2018; Zhou et al., 2018). Its closest homologue AtPATL2 interacts through its N region with IRON REGULATED TRANSPORTER 1 (IRT1) (Montag et al., unp.). As regulators of this PM proteins AtPATL1 and AtPATL2 contribute to stress tolerance by affecting plant response to cold, salt and iron stress. In addition both seem to be involved in preventing damage caused by ROS and radicals (Montag et al., unp.; Zhou et al., 2018). Furthermore, AtPATL1 and AtPATL2 co-immunoprecipitate with AMSH-LIKE UBIQUITIN THIOESTERASE 3 (AMSH3), a deubiquitinating enzyme required for intracellular trafficking and vacuole biogenesis in Arabidopsis, next to other proteins with reported or expected function in intracellular trafficking processes (Isono et al., 2010).

AtPATL2 is phosphorylated by MPK4 MAP Kinase at position S536 within the SEC14 domain, which might be important for the release of PATL2 from the membrane (Suzuki et al., 2016). Additionally, phosphorylation of the SEC14 domain of AtPATL2 could be determined after short-term cytokinin treatment and sugar stress (Cerny et al., 2011; Niittyla et al., 2007). Further, phosphorylation of the N region was identified at position S77, under Fe deficiency (Lan et al., 2011), and S79, during salt stress, in response to oligogalacturonides, and brassinosteroid signaling (Chang et al., 2012; Hsu et al., 2009; Mattei et al., 2016; Tang et al., 2008). This indicates that AtPATL2 might undergo dynamic post-translational regulation in response to plant stress. AtPATL3 recruitment to the PM membrane depends on interaction of its GOLD domain with EXO70A1, a subunit of the exocyst complex participating in intracellular vesicle transport (Fendrych et al., 2013; He and Guo, 2009; Wu et al., 2017). Interestingly, all other AtPATLs,

except of AtPATL5, are able to interact with EXO70A1 (Wu et al., 2017). Moreover, AtPATL3 and AtPATL6 inhibit stem infection spread of the alfalfa mosaic virus by interfering with virus movement through interaction with a PLASMODESMATA TARGETING MOVEMENT PROTEIN (AMV MP) and thereby preventing subcellular targeting (Peiro et al., 2014). Tomato TOCOPHEROL BINDING PROTEIN (SlTBP) is a homologue of AtPATL6 and clusters together with other plant PATLs due to a plastid-targeting signal (Bermudez et al., 2018). The *SlTBP* gene is mainly expressed in photosynthetic active tissues and the protein is localized to plastids. Its ability to bind α-TOC makes it a key player in controlling vitamin E movement between plastids and the ER, which affects lipid metabolism within these organelle (Bermudez et al., 2018). Additionally, SlTBP is involved in maintaining chloroplast membrane structure, affecting its lipid profile (Bermudez et al., 2018). Interestingly, AtPATL1, AtPATL2 and AtPATL5 were identified as putative cargo receptors in proteomic studies searching for components of the chloroplast vesicle transport pathway. The proteins were identified localizing to the chloroplast envelope (Ferro et al., 2010; Khan et al., 2013; Kleffmann et al., 2004).

Taken together, PI/PIP and α-TOC binding, membrane localization, phosphorylation, increased expression during stress responses, and interaction with membrane and trafficking proteins could indicate that PATLs are basic regulators adapting the cell/organism to altered environmental influences and in response to external stimuli. As regulatory proteins they could be involved in membrane trafficking, e.g., by initiating vesicle formation. Furthermore, they might play a role in protecting the cell from ROS and radical damage, by offering α-TOC as an antioxidant or through regulating the activity of membrane proteins.

## 5. Conclusion

In conclusion, the SEC14 domain, as a part of essential regulatory proteins, is capable to recognize, bind, transport and exchange single lipophilic molecules between membranes. This ability leads to further cellular and physiological effects. Unlike other lipid binding domains the SEC14 domain not only binds phospholipids, but also other lipophilic substances, e.g., α-TOC.
This characteristic and the presence of SEC14L-PITPs in higher eukaryotes indicates a conserved function and highlights the need of controlling lipid signaling and membrane trafficking. Especially, the presence of additional domains in SEC14L-PITPs of higher eukaryotes might indicate the increasing variety of functions addressed by multi-domain SEC14L-PITPs. We hypothesize that these additional domains could be involved in the sensing of the lipid environment at the membrane or in the recruitment of other proteins. This putative

function results in a more specific response of the lipid-signaling pathway to environmental changes.

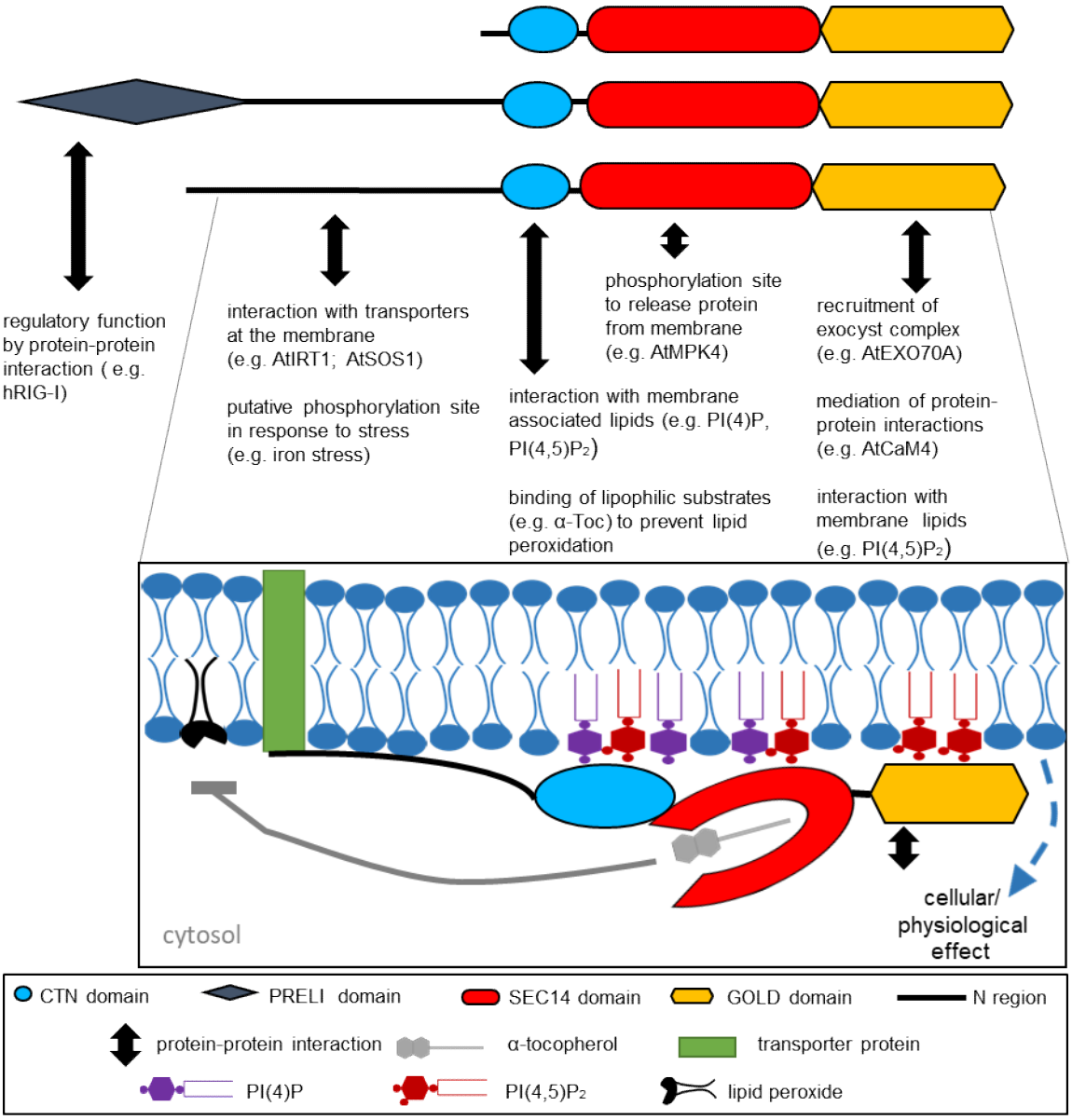


**Figure 4. Summary and model of SEC14-GOLD domain function.**
While the N region is essential for interaction with membrane-bound proteins and a putative phosphorylation site in response to stress, the SEC14 domain is critical for the membrane association of the protein and for the transport of α-tocopherol to prevent lipid peroxidation. The GOLD domain is able to bind membrane lipids, which function as biological landmarks, and recruits proteins to the site of action to start further cellular pathways. The PRELI domain might have a function as a regulatory domain.

The idea is supported by the findings that multi-domain SEC14L-PITPs are able to influence cell division, vesicle formation, lipid signaling and organism development, which directly affects the organism fitness/health. The SEC14 domain is thereby of special interest, since mutations in the SEC14 domain results in defects in development and in the plant-stress response and tolerance, as well as in neurodegenerative diseases and an increased cancer risk in humans.
SEC14-GOLD proteins seem to have an essential regulatory function contributing to organism fitness (Fig. 4). Of special interest is their ability to function as regulators via protein-protein interactions. They might recognize the membrane environment via lipid-protein and protein-protein interaction. Thereby, they are able to sense external stimuli and can initiate the cellular response. In addition, SEC14-GOLD proteins are able to regulate protein activity via protein-protein interaction, e.g., by negatively influencing membrane-protein activity in Arabidopsis to prevent uncontrolled uptake of substances. Last but not least they are able to bind and release vitamin E to protect the cell against ROS and radical caused damage.
Not all complex tasks of SEC14L-PITPs are understood right now and many interesting questions remain to be answered. For example, are SEC14L-PITPs regulated by post-transcriptional regulation? What are the effects of AtPATLs phosphorylation? What is the function of the plant-specific unstructured N region of PATLs? How do SEC14L-PITPs and SEC14-GOLD proteins influence protein activity and metabolic reactions? In which pathways do they play a role?
We believe that identifying the function of SEC14L-PITPs and SEC14-GOLD proteins on cellular levels can help to understand the adaptation of regulatory pathways to environmental changes. One major issue to overcome is definitely the redundancies within the protein family.

## Author contributions

KM searched for the reference collection and wrote the article. KM identified the sequences and illustrated the figures. All authors contributed to discussion. PB and RI revised the manuscript.

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## Authors Contribution to Manuscript 3

**Karolin Montag**

K.Montag searched for the reference collection, designed the outline and wrote the article. K.M. identified the sequences and illustrated the figures.

Rumen Ivanov

R.Ivanov designed the outline of the manuscript, supervised the study and reviewed / edited the manuscript.

Petra Bauer

Designed the outline of the manuscript, supervised the study, provided funding, and reviewed / edited the manuscript.

## 8. Concluding remarks

The results presented in this thesis show that PATL proteins might be essential basic regulators involved in adapting the cell/organism to altered environmental conditions by regulating stress tolerance on several levels in Arabidopsis. Additionally, they play a role in cellular ROS and radical damage protection (Li et al., 2018; Saeed et al., 2015; Zhou et al., 2018).

The increasing number and modular complexity of SEC14L-PITPs in the green lineage does correlate with the increasing organism complexity and is comparable to SEC14L-PITP evolution in the animal lineage (Saito et al., 2007a). The expanding number and complexity of SEC14L-PITPs is likely linked with new functions required to adapt to dry land. Additionally, it might reflect the high versatility of tasks addressed in multicellular organisms, where developmental and environmental specifications of functions become increasingly connected, as highlighted by the case of SEC14-GOLD proteins in Arabidopsis. SEC14-GOLD proteins were possibly required to integrate cell division and auxin response in adaptation to external stimuli during land plant evolution (Peterman et al., 2004; Tejos et al., 2017). Although phylogenetic and expression analyses showed sequence diversification of PATLs into three clades suggesting functional specificity, a partial redundancy of functions is assumed due to overlapping expression patterns and co-expression data (Tejos et al., 2017). The individual domains of PATL2 contribute differently to the diverse functions of PATL2. We found that the CTN-SEC14 module is critical for general membrane association of the protein, confirmed by additional literature data demonstrating that the CTN-SEC14 module maintained its specific function during evolution (Saito et al., 2007b; Sirokmany et al., 2006; Skinner et al., 1993). However, the C-terminal GOLD domain adds clear specificity for PM binding of PATL2, possibly through a conserved lysine motif known to selectively recognize $PI(4,5)P_2$ (Anantharaman and Aravind, 2002; Ford et al., 2001; Mao et al., 2001; Rohde et al., 2002). Previous work indicated that the GOLD domain might play a double role able to bind membrane lipids and to interact with additional proteins (Anantharaman and Aravind, 2002; Sohda et al., 2001). For example, the GOLD domain of PATL3 is critical for its interaction with EXO70A1 (Wu et al., 2017). It is hypothesized that after membrane association of PATL2 the GOLD domain might recruit other proteins to the site of action, starting further cellular pathways in response to iron stress, e.g., vesicle formation for IRT1 internalization. Such speculations need to be experimentally addressed in future. The N region of PATLs is unique to plants and varies in amino acid composition for every checked protein, indicating a specific function and rapid evolution. Since the N region of PATL2 is essential for interaction with IRT1vr, we assume that the N region of PATLs generally contributes to plant stress response by interacting with relevant membrane proteins to identify the site of action. This interaction then enables further actions. Studies on PATL1-N,

critical for protein-protein interaction with PM transporter SOS1, underline this observation (Zhou et al., 2018). Since the CTN-SEC14-GOLD terminus of PATL proteins show a high identity/similarity in sequence (Peterman et al., 2004), it is now suggested that the specific function of each PATL can be traced back to its unique N region. Of course, the ligand-binding specificity of the SEC14 domain might also contribute to specific protein function. Furthermore, the length of the N region might indicate that PATLs are able to interact with more than one membrane protein, which might be critical for stress response in plants. This underlines the potential function of PATLs as basic regulatory proteins in response to plant stress.

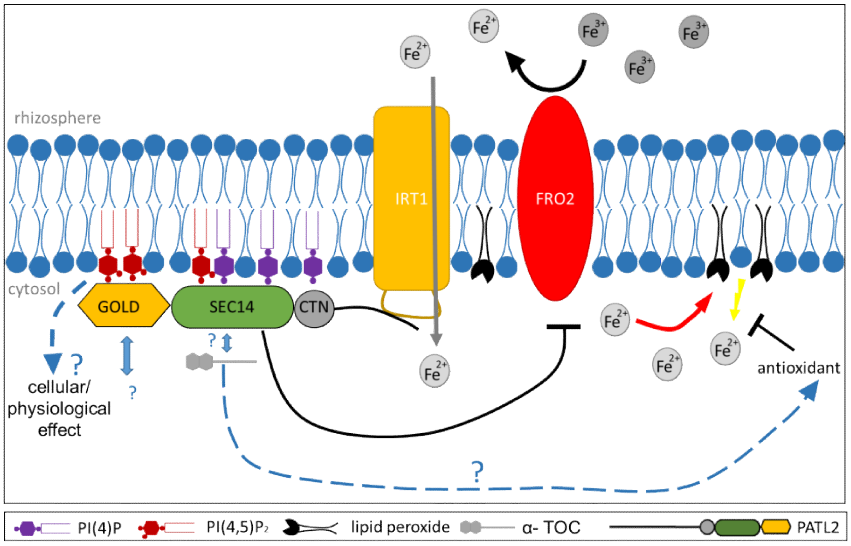


**Figure V. Model of PATL2 interacting with IRT1.**
PATL2 binds to the PM membrane via its GOLD domain and the CTN-SEC14 module. Additionally, its N region interacts with the variable region of IRT1. PATL2 is able to control the iron uptake machinery by negatively regulating FRO2 activity and prevents lipid peroxidation. Still many open questions need to be answered in future. How does PATL2 prevent membrane damages? Does PATL2 bind α –Toc? Does the GOLD domain recruit to additional proteins? Which cellular and physiological effects are regulated by PATL2 at the PM membrane?

For that reason, the interaction of PATL2 with IRT1 might demonstrate a regulatory role of PATL2 in response to iron stress on protein level. This suggestion is supported by the localization of PATL2 to the PM and the cytosol, depending, among others, on the developmental status of the cell or the presence of abiotic stress (Suzuki et al., 2016; Tejos et al., 2017). Our data showed that the function of PATL2 in the regulation of iron import cannot only be traced back to the direct inhibition of iron acquisition machinery through protein-protein interaction. Additionally, the presence of PATL2 prevents lipid peroxidation initiated by local accumulation of IRT1-imported ferrous iron through the Fenton reaction. PATL2 could be involved in the adaptation to iron stress through the sensing of the membrane environment, followed by the initiation of cellular effects to prevent further membrane damage. One possible way, supported by the data in this thesis, is that PATL2 negatively regulates FRO2 activity. This then results in a decreased pool of ferrous iron in

the rhizosphere and might reduce the uptake of reactive ferrous iron by IRT1. Finally, that leads to less accumulation of iron in the cytosol preventing lipid peroxidation. Furthermore, PATL2 might recruit antioxidants to the target site. An antioxidant shown to be bound by the SEC14 domain of SEC14-GOLD proteins is α-TOC (Bermudez et al., 2018; Boonnoy et al., 2018; Liebler and Burr, 1992; Zingg et al., 2008). Further cellular responses could also be mediated by additional proteins recruited through the GOLD domain of PATL2, as described above. Critical for PATL2 to identify the site of action might be the interaction with IRT1. To get a more detailed understanding of PATL2 function there are several perspectives to work on in future. For example, it would be essential to answer the question if PATL2 is able to bind and transport α-TOC. Additionally, it would be important to address the question if the GOLD domain of PATL2 recruits additional proteins to the PM and thereby starts further cellular pathways. Furthermore, the identification of additional PATL2-N interactors would increase the knowledge of PATL2 function.

Generally, identifying the function of SEC14-GOLD proteins and observing their importance on cellular level can help to understand the adaptation of regulatory pathways to altered environmental conditions and external stimuli.

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**Danke**

## 10. Abbreviations

List of the most important abbreviations used in this thesis.

| | |
|---|---|
| % | percent |
| (w/v) | weight per volume |
| °C | degree Celsius |
| µg | microgram |
| µm | micrometer |
| µM | micro molar |
| 3-AT | 3-amino-1,2,4-triazole |
| AA | amino acid |
| AHA | ARABIDOPSIS H+-ATPASE |
| AMSH3 | AMSH-LIKE UBIQUITIN THIOESTERASE 3 |
| AMV MP | plasmodesmata targeting movement protein |
| Arabidopsis | *Arabidopsis thaliana* |
| At | *Arabidopsis thaliana* |
| ATP | adenosine triphosphate |
| AVED | ataxia, with Vitamin E deficiency |
| bHLH | basic helix-loop-helix |
| BiFC | bimolecular fluorescence complementation |
| BPDS | bathophenanthrolinedisulfonic acid |
| BSA | bovine serum albumin |
| CaM4 | CALMODULIN-4 |
| cDNA | complementary DNA |
| CDS | coding sequences |
| CHT1 | CHOLINE TRANSPORTER 1 |
| CIPK | CBL-INTERACTING PROTEIN KINASE |
| CoIP | Co-immunoprecipitation |
| Col-0 | Columbia-0 |
| CRALBP | CELLULAR RETINAL-BINDING PROTEIN |
| CRAL-TRIO domain | CRALBP and TRIO protein domain |
| C-terminus | carboxy -terminus |
| CTN | CRAL-TRIO-N-terminal extension |
| DAG | diacylglycerol |
| DNA | deoxyribonucleic acid |
| DDT domain | DNA-binding homeobox - Different Transcription factors domain |
| DTT | dithiothreitol |
| E | glutamate |
| EDTA | ethylenediaminetetraacetic acid |
| Eflα | Elongation factor 1 α |
| EHB1 | ENHANCED BENDING 1 |
| ER | endoplasmic reticulum |
| Fe | iron |
| FeNa | ferric sodium |
| FeNa-EDTA | ferric sodium EDTA |
| Fig. | Figure |

| | |
|---|---|
| FIT | FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR |
| FRO2 | FERRIC REDUCTASE-OXIDASE |
| g | gram |
| GAP | GTPase-activating protein |
| GDAP domain | Ganglioside-induced differentiation-associated protein domain |
| GDAP2 | Ganglioside-induced differentiation-associated protein 2 |
| gDNA | genomic DNA |
| GEF | guanine exchange factor |
| GFP | green fluorescent protein |
| GO | gene ontology |
| GOLD domain | Golgi dynamics domain |
| GUS | ß-glucuronidase |
| h | hour |
| $H_2O_2$ | hydrogen peroxide |
| HA | hemagglutinin |
| HCl | nuriatic acid |
| HCV | hepatitis C virus |
| HHU | Heinrich Heine Universität Düsseldorf |
| HL medium | Haogland medium |
| HRP | horseradish peroxidase |
| IDF1 | IRT1-DEGRADATION FACTORE 1 |
| IP | immunoprecipitation |
| IPR | InterPro accession number |
| IPTG | isopropyl-6-D-thiogalactopyranosid |
| IRT1 | IRON-REGULATED TRANSPORTER1 |
| IRT1vr | variable region of IRT1 |
| KCl | potassium chloride |
| kDa | kilodalton |
| l | liter |
| lys, K | lysine |
| M | molar |
| m | membrane fraction |
| MDA | malondialdehyde |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| mg | milligam |
| min | minute |
| ml | milliliter |
| mM | milli molar |
| multi-domain SEC14L-PITPs | larger multi-domain proteins containing a SEC14 domain |
| NaCl | sodium chloride |
| $NaH_2PO_4$ | sodium dihydrogen phosphate |
| NF1 | NEUROFIBROMIN 1 |
| nm | nanometer |
| nmol | nanomol |
| N-terminus / N region | amino-terminus |
| OFP | orange fluorescent protein |
| PATL | PATELLIN |

| | |
|---|---|
| PC | phosphatidylcholine |
| PCR | polymerase chain reaction |
| PEtN | phosphatidylethanolamine |
| PG | phosphatidylglycerol |
| pg | picogram |
| PH domain | Pleckstrin homology domain |
| PHD domain | homeodomain |
| PI | phosphatidylinositol |
| PI(3)P | phosphatidylinositol- 3 phosphate |
| $PI(3,4)P_2$ | phosphatidylinositol-3, 4 bisphosphate |
| $PI(3,4,5)P_3$ | phosphatidylinositol-3, 4, 5 trisphosphate |
| $PI(3,5)P_2$ | phosphatidylinositol-3, 5 bisphosphate |
| PI(4)P | phosphatidylinositol- 4 phosphate |
| $PI(4,5)P_2$ | phosphatidylinositol-4, 5 bisphosphate |
| PI(5)P | phosphatidylinositol- 5 phosphate |
| PI3Kγ | PI(3)P Kinase γ |
| PIN1 | PIN-FORMED 1 |
| PIPs | phosphatidylinositol phosphates/phosphoinositides |
| PITP | phosphatidylinositol transfer proteins |
| PITP/LNS2 domain | phosphatidylinositol transfer protein and Lipin/Ned1/Smp2 domain |
| PM | plasma membrane |
| PRUNE2 | PROTEIN PRUNE-LIKE PROTEIN 2 |
| PSer | phosphatidylserine |
| PTP domain | protein phosphatase catalytic domain |
| PTPN9 | TYROSINE-PROTEIN PHOSPHATASE NON-RECEPTOR TYPE 9 |
| PTSD | post-traumatic stress disorder |
| PVC | pre-vacuolar compartment |
| rat | *Rattus norvegicus* |
| RFP | red fluorescent protein |
| RIG-I | RETINOIC ACID-INDUCIBLE GENE I |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| rpm | rounds per minute |
| RT | room temperature |
| RT-qPCR | reverse transcription-quantitative real time PCR |
| s | supernatant fraction |
| SD medium | synthetic defined medium |
| SDS | sodium dodecylsulfate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEC | secretory mutant |
| sec /s | second |
| SEC14L | SEC14-LIKE |
| SEC14L-PITP | SEC14-like phosphatidylinositol transfer protein |
| SEC14-only proteins | single-domain SEC14L-PITPs |
| Ser | serine |
| Sfh | Sec14p homolog |
| SITBP | *Solanum lycopersicum* TOCOPEROL BINDING PROTEIN |

| | |
|---|---|
| SNX | SORTING NEXIN |
| SOS1 | SALT OVERLY-SENSITIVE1 |
| TAP | TOCOPHEROL-ASSOCIATED PROTEIN |
| TBA | thiobarbituric acid |
| TBST | Tris-buffered saline with Tween20 |
| TCA | trichloroacetic acid |
| T-DNA | transfer DNA |
| tobacco | Nicotiana benthamiana |
| Tyr | tyrosine |
| VACht | VESICULAR ACETYLCHOLIN TRANSPORTER |
| VEGF | VASCULAR ENDOSOMAL FACTOR |
| VEGFR2 | VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 |
| WT | wild type |
| YEB medium | yeast extract beef medium |
| YFP | yellow fluorescent protein |
| ZIP | Zinc-regulated transporter, Iron regulated transporter-like protein |
| α-TOC | α-tocopherol |
| α-TTP | α-TOCOPHEROL TRANSFER PROTEIN |