Date of the application 31 January 2018
Scientific field Biological Sciences
FWO Expert Panel Molecular and Cellular Biology (Bio1)
Motivation of panel choice My research proposal aims to determine the near-atomic resolution structure of a plant specific ABC transporter in different functional states. Because my research aims at understanding functioning of a protein at molecular level, I will use a cohort of biophysical and biochemical methods to characterize properties of specific molecules. Therefore, the Bio1 panel of Molecular and Cellular Biology is the most appropriate Expert Panel to evaluate my research proposal.

Title of your PhD dissertation

**English title** Structural and functional characterization of the Arabidopsis thaliana Anaphase Promoting Complex/Cyclosome

**Dutch title** Structurele en functionele karakterisatie van de Arabidopsis thaliana Anaphase Promoting Complex/Cyclosome

Title of your research proposal

**English title** Unveiling the molecular basis of diterpene secretion by structural characterisation of plant specific ABC transporters.

**Dutch title** Onthulling van de moleculaire basis van diterpenessecretie aan de hand van structurele karakterisering van plantspecifieke ABC-transporters.
**Summary in layman's terms**

Plants are chemical factories. They produce specialized metabolites including aromas, flavors and bioactive compounds making them of interest to the various industries. These metabolites play an important role in protection, competition and interaction with their environment. When specialized metabolites accumulate in high amounts, they can be toxic for the plant cells. One way to avoid self-toxicity, is by excreting these metabolites into extracellular spaces located in tiny hairs covering plants (trichomes). This requires the metabolite to move from the site of biosynthesis across the plasma membrane and the cell wall into the extracellular cavities. ABCG transporters have been identified to facilitate this translocation but the exact molecular mechanism in plants remains unknown.

About 60% of all known natural products are terpenoids. They contribute to the scent of eucalyptus, the flavor of cinnamon or the color of tomatoes. To understand how terpenoids are translocated, I will characterize the molecular architecture of the plant specific protein from ABC transporter family, Pleotropic Drug Resistance (PDR), using single-particle cryo-electron microscopy. PDR1 from tobacco plant (lat Nicotiana tabacum) is the archetype of the family that has already been well characterized. By determining the atomic resolution structure of different functional states, I will be able to provide a detailed understanding of diterpenes translocation.

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**RESEARCH**

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<tr>
<th>Main host institution</th>
<th>Vrije Universiteit Brussel</th>
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<tr>
<td>Head of the research unit</td>
<td>Efremov Rouslan ( )</td>
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<td>Additional host institution(s)</td>
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<td>Collaboration</td>
<td>1) Marc Boutry, Institut des Sciences de la Vie, Université catholique de Louvain, Belgium.</td>
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<td>2) Helmut Grubmüller, Theoretical and Computational Biophysics, Max Planck Institute for Biophysical Chemistry, Germany.</td>
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**EXTRA DATA**

**Supervised dissertations**


Manon is currently pursuing a PhD funded by IWT in the Remy Loris Lab.

**Funding applied for elsewhere or already available**

Not applicable
Previous FWO fellowships

In context of my Phd, I have applied and received an Aspirant FWO fellowship (September 2013 - September 2017).

In context of my Postdoctoral research, this will be my first application for a FWO fellowship.

Previous research stays

1) Research stay

- Intensive training at the Eukaryotic Expression Facility of the EMBLE Grenoble (Imre Berger lab) in order to learn how to generate MultiBac baculovirusses (Grenoble, France, November 2014). For this, I applied and received funding by BiostructX.

2) Courses

- EMBO course: The structural characterization of macromolecular complexes at EMBL Grenoble (Grenoble, France) (02/07/2014) - (07/07/2014)

- The 7th Brazil school for single-particle cryo-EM microscopy (Sao Paulo, Brazil) (11/08/2016) – (26/08/2016)

- BioStructX Workshop on Ensemble Refinement of Protein Crytstal structures in Phenix (Lisbon, Portugal) (31/01/2016) – (02/02/0216)

Planned research stays

During the research project I plan to join the group of Computational and Theoretical Biophysics led by Prof. Helmut Grubmüller at Max-Planck Institute of Biophysical Chemistry (Göttingen, Germany). There during a period of three to six months I will learn the method of molecular dynamics simulations, which I will apply to understand the determinants of PDR1 substrate specificity and the substrate translocation mechanism.

Additionally, in course of my project I will perform data collection at several European Biological cryo-EM facilities including NeCEN (Leiden, Netherlands), eBiC (Diamond, UK) and ESRF (Grenoble, France) equipped with the high-end electron cryogenic microscopes.

Scientific awards

I did not receive scientific awards for this research.
Personal statement

At the beginning of my PhD I wanted to know why some plants are able to live much longer than the humans. It was suggested that a key factor is the Anaphase Promoting Complex/Cyclosome (APC/C), a 1.1 MDa multi-subunit E3 ubiquitin ligase that regulates cell cycle division. Driven by interest in understanding how the APC/C functions I chose to determine its atomic structure as a PhD project. Using a combination of structural biology, biophysical techniques and biochemistry, I aimed to understand the molecular mechanism of the plant APC/C.

To obtain purified APC/C, I attended an intensive practical course on Multi-Bac expression system in the Imre Berger lab at EMBL-Grenoble. Upon returning to Brussels, I managed to clone all the 15 individual subunits of APC/C and express the complete plant APC/C in insect cells. To my knowledge, this is the first time such a large multi-subunit plant protein complex has been produced heterologously. Next, I managed to purify and structurally characterize the intact plant APC/C using negative stain electron microscopy. The result of this work is currently submitted in two publications highlighting my ‘hands-on” skills with plant protein complexes.

In parallel, I worked on the project aiming to understand the role of the prokaryotic kinase Doc in persistence. Here, I used a combination of X-ray crystallography, SAXS, ITC and in vitro phosphorylation assays to validate the molecular phosphorylation mechanism of Doc. The results were reported in six publications (two first author) in peer-reviewed journals including high-impact journals: Nat. Chem. Biology and Sci. Advances.

I am applying for a FWO fellowship, to have an opportunity to further broaden my understanding of plant molecular machinery using single-particle cryo-EM. The lack of structural information on the plant specific PDR family hinders our understanding of excretion of specialized metabolites. The project will be conducted in the cryo-EM laboratory of Rouslan Efremov in collaboration with Prof. Marc Bouty from the UCL. This postdoctoral project will allow me to combine my knowledge in structural biology, biophysics and plant molecular biology with new for me methods of cryo-EM, biochemistry of membrane proteins and molecular dynamics simulations.

By June 2018, the top-end single particle cryo-EM facility will be operational at the VUB. The grant will allow me to join a vibrant environment and interact with experienced people from the field. Additionally, the Efremov lab has expertise in biochemistry and single particle cryo-EM of eukaryotic membrane proteins. To further strengthen my understanding of PDR1, I will join the group of Prof. Helmut Grubmüller (MPG, Göttingen) renowned for advanced expertise in MD simulations. There I will learn MD and will apply it to understand specificity of substrate binding to the transporter. This project will strengthen my research skills to allow me in the future pursuing career of an independent researcher.

Career breaks

Not applicable
RESEARCH CONTEXT

How this project fits in the research activities of the research group

The Efremov group is specialized in the structural characterization of nanodisk-embedded membrane proteins using single-particle cryo-EM. This strategy has successfully been used to determine near-atomic resolution structures of important ABC transporters. Together with the establishment of the first cryo-EM facility at the VUB, the Efremov group will provide an ideal working environment for the development of my project.

In return, I will introduce plant proteins into the research portfolio the group. Plant ABC transporters are of great interest to the scientific and industrial community due to their active function of translocation of pharmaceutical and nutraceutical compounds. In addition, my expertise with protein purification, structural determination (x-ray, SAXS, negative stain EM) and biophysical techniques (ITC/SPR) is highly complementary with the research performed in the Efremov group. This will allow us to work together and obtain a complete picture of the PDR1 translocation mechanism.

Currently, time resolved cryo-EM is being developed in the Efremov group. Plant ABC transporters are highly suited for this application since multiple short-lived states are present in the full catalytic cycle. In the future, time resolved cryo-EM can be applied to NtPDR1 to visualize conformational transitions enabling us to provide the first complete catalytic cycle of a plant ABC transporter.

National and international context

ABC transporters are subject of intensive research because of their fundamental role in cellular function and drug evasiveness. Therefore, advances in understanding of the mechanism of these transporters are often published in high-impact journals, including Nature and Science. Interestingly, in spite of common evolutionary origin of ABC transporters, the mechanisms of the transport are different. Therefore, ABC transporters from phylogenetically distant organisms have to be thoroughly characterized to understand their mechanisms and selective transport of organic compounds. Given the recent successes of single-particle cryo-EM in obtaining near-atomic resolution structures of membrane proteins, this cutting edge technique is accelerating the output of high impact research in the membrane protein field.

My research proposal brings together the expertise of two leading Belgian labs in single particle cryo-EM (Efremov, VIB-VUB) and plant transporter physiology (Boutry, UCL). I will collaborate with the Grubmüller laboratory in Max-Planck Institute in Germany where I will perform molecular dynamic simulations to solidify my understanding on how PDR1 translocates its substrate sclareol. Given the lack of any structural information on plant ABC transporters, these results will be highly anticipated in the field since the shed a light on a completely uncharacterized kingdom.

The strategic collaboration of these three labs will accelerate the project immensely. In case of success, my results can be valorized by the Flemish Institute of Biotechnology (VIB), which has world-leading expertise in plant biotechnology. Here, my scientific expertise will be shared with other Flemish and international research groups to achieve new scientific breakthroughs.
In the table below questions are listed on the ethical aspects of your research proposal.

If you mark a ‘yes’ for the question, it follows that

- **For the questions marked with *:** the applicant is legally or on the basis of institutional regulations obliged to ask for an ethical advice at the competent ethics committee of the host institution; please do take into account that even when there is no obligation with regard to the research itself, for the publication of the results a positive advise still can prove to be necessary. If you have answered questions with a * positively, you must submit your proposal to the ethics committee as soon as your application has been approved for funding. Your fellowship can only start when this clearance has been formally given. Only if the advice relates to a work package that is planned for a later stage of the fellowship, it may be submitted just before the start of that part of the research. Please keep in mind that the advisory procedure can take some time and that therefore you should submit your proposal to the ethics committee well in time.

- **For the questions that are not marked:** the applicant and the evaluation panel are invited to reflect on the issue and take, if necessary, the necessary precautionary measures.

You find more on the FWO policy and procedure concerning ethical issues and on legal and other documents on the FWO web page dedicated to that topic.

I confirm that none of the issues below apply to my proposal.  

I hereby confirm having taken note that an ethical clearance is needed for the start of my project. I will thus ensure submission of my proposal to the research ethics committee of my host institution.

Please specify which ethics committee(s) deal(s)/will deal with your application.

In case you will submit your proposal to the committee only before the start of work package(s) (WP) that are concerned:

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<th>Number/description of WP(s)</th>
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1. Human Embryos/Foetuses

ETHICS ADVICE RELATED TO THESE QUESTIONS SHOULD ALWAYS BE REQUESTED BEFORE THE START OF THE RESEARCH PROJECT AS A WHOLE AND ALSO REQUIRE AN EXAMINATION BY THE FEDERAL COMMISSION FOR EMBRYOS

- Does your research involve Human Embryonic Stem Cells (hESCs)?*  
  - Will the hESCs be directly derived from embryos within this project?  
  - Are the hESCs previously established cell lines?  

- Does your research involve the use of human embryos?*  

- Does your research involve the use of human foetal tissues / cells?*

2. Humans

- Does your research involve human participants?*  
  - Are they volunteers for social or human sciences research?  
  - Are they persons unable to give informed consent?  
  - Are they vulnerable individuals or groups?  
  - Are they children/minors?  
  - Are they patients?  
  - Are they healthy volunteers for medical studies?  

- Does your research involve physical interventions on the study participants?*  
  - Does it involve invasive techniques?  
  - Does it involve collection of biological samples?

3. Human Cells/Tissues

- Does your research involve human cells or tissues (other than from Human Embryos/Foetuses, i.e. section 1)?*  
  - Are they obtained from commercial sources?  
  - Do they originate from another laboratory/institution/biobank?  
  - Were they produced or collected by you from previous research activities?  
  - Are they produced or collected by you as part of this project?²

4. Personal Data

- Does your research involve personal data collection and/or processing?* (?)  
  - Does it involve the collection and/or processing of sensitive personal data?  
  - Does it involve collecting/processing of genetic information/data?  
  - Does it involve tracking or observation of participants?

- Does your research involve further processing of previously collected personal data (‘secondary use’)?*
5. Animals

Does your research involve research procedures to live non-human vertebrate animals (incl. independently feeding larval forms, foetal forms of mammals in the last trimester of their normal development and cephalopods, and also forms in earlier stages if the experiments have consequences in later stages)?*  
- Are they vertebrates or live cephalopods? N/A  
- Are they non-human primates? (²) N/A  
- Are they genetically modified animals? N/A  
- Are they cloned farm animals? N/A  
- Are they endangered species? N/A

6. International Collaboration

Do you plan to use local resources (e.g. animal and/or human tissue samples, genetic material, live animals, human remains, materials of historical value, endangered fauna or flora samples, etc.)? N/A  
Do you plan to import/export any material from/to other countries? N/A  
- Name of country/ies:

If your research involves low and/or lower middle income countries, are benefits-sharing measures foreseen? N/A  
Could the situation in the country put the individuals taking part in the research at risk? N/A

7. Environment & Health and Safety

Does your research involve the use of elements that may cause harm to the environment, to animals or plants? N/A  
Does your research deal with endangered fauna and/or flora and/or protected areas? N/A  
Does your research involve the use of elements that may cause harm to humans, including research staff? N/A

8. Dual Use

Does your research have the potential for military applications? N/A

9. Misuse

Does your research have the potential for malevolent/criminal/terrorist abuse? N/A

10. Other Ethics Issues

Are there any other ethics issues that should be taken into consideration? Please specify.
For these issues the Belgian commission on privacy protection (Commissie voor de bescherming van de persoonlijke levenssfeer) has to be consulted. You cannot consult the commission directly, but always first contact the research coordination of your host institution.

In this case you already have to submit your proposal to the ethics committee in the application phase.
Unveiling the molecular basis of diterpene secretion by structural characterization of plant specific ABC transporters.

1) State of the art

Plants are chemical factories. They provide building blocks for food, cloths, medicines and regulate the earth’s atmosphere (Keddy 2007; Mora et al. 2011). To survive in their natural environment, plants synthesize a diversity of specialized metabolites such as, but not limited to, volatile organic compounds and defensive molecules. These compounds are self-toxic at high concentration and plants have developed several mechanisms to keep the metabolite concentration level low in sensitive areas (Sirikantaramas et al. 2007). One way to avoid self-toxicity is by excreting these metabolites into extracellular spaces. This requires the metabolite to move from the site of biosynthesis across the plasma membrane and cell wall towards the apoplasm. In plants, membrane-embedded ATP-binding cassette (ABC) exporters that facilitate this transport have been identified (Lefèvre et al. 2015). Currently, only a few eukaryotic ABC transporter structures have been solved limiting our understanding of their molecular mechanism. Recent models determined by single-particle cryo-electron microscopy (EM) are now bridging this gap. They suggest that transport mechanisms differ between transporters. Therefore, the absence of structural information on plant ABC transporters is limiting the understanding of the metabolite transport in plants.

Figure 1. Phylogenetic analysis of Arabidopsis ABC protein sequences reveals eight distinct subfamilies of ABC transporters (A-G,J) (Sánchez-Fernández et al. 2001; Verrier et al. 2008). The ABCG subfamily contains a large group of full-size pleotropic drug resistance transporters (PDR) uniquely found in fungi and plants (green). Structural homologues from recently determined structures are shown as representative models of eukaryotic ABC exporters: human ABCA1 (5XJY), bovine MDR1 (6BH), yeast ATM1 (4MYC), mouse MRPI (3G5U), human CFTR (5UAK), human ABCG2 (5NJ3) and human ABCG5/G8 (5DO7). The structural information on plant ABC transporters is lacking.

Plant ABC transporters are highly diverse with more than 120 distinct members organized into eight subfamilies (A-G,J) (Verrier et al. 2008) (Figure 1). They are essential for plant development and are involved in the transport of hormones, lipids, metals, xenobiotics, specialized metabolites, and, more generally, in plant-pathogen interactions (Rea 2007). Due to the importance of ABC transporters in prokaryotic drugs evasiveness and severe human diseases, the general architecture and molecular function of ABC transporter has been extensively characterized (Wilkens 2015). Two nucleotide-binding domains (NBD) are found on the cytosolic
side and co-operate to bind and hydrolyze ATP. These domains couple hydrolysis of two ATP molecules with conformational changes in trans-membrane domains (TMD), which bind and translocate substrates unidirectionally across the lipid bilayer against their electrochemical gradient (Higgins 1992). In eukaryotes, an ABC transporter, consisting of two NDB and two TMD can be encoded into in a single polypeptide (‘full-size’) or be by the oligomerization of two halves forming homo- or hetero dimer (‘half-size’).

About 60% of all known natural products are terpenes. They contribute to the scent of eucalyptus, the flavor of cinnamon or the color of tomatoes. They are classified by the number of isoprene (C5) units. Diterpenes (C20) are composed of four isoprene units and have desirable properties for the food, cosmetic, pharmaceutical, recreational and biotechnological industries. **While their biosynthesis pathways have been well characterized, how diterpenes are transported in the plant remains unclear** (Figure 2). Recently, it was demonstrated that the diterpene scclareol is translocated by the *Nicotiana tabacum* Pleiotropic Drug Resistance transporter 1 (NtPDR1) (Pierman et al. 2017). This full-size ABC subfamily of transporters is uniquely found in plants and fungi (Figure 1). NtPDR1 substrates are known, this transporter has been purified in an active form, its ATPase activity is stimulated by its substrates and preliminary EM analysis reveals good sample quality. Therefore, NtPDR1 is suited for structural characterization to reveal if the transporter uses a conserved or specific translocation mechanism.

**Figure 2.** Architecture of a mature trichome reveals glandular cells at the tip of the stalk. a. In *N. tabacum*, PDR1 located in the plasma membrane of the trichomes facilitates the export of diterpenes (e.g., scclareol) b. By adjusting the substrate specificity of NtPDR1 (red), other metabolic substrates can be transported in trichomes of *N. tabacum*. The structure of a tomato trichome and human ABCG2 is used as models for this figure.

NtPDR1 is expressed in glandular trichomes, tiny hairs at the outside of the leaf, and plays an important role in the defense against pathogens (Crouzet et al. 2005). A key and unique feature of glandular trichomes is their ability to synthesize and secrete large amounts of a metabolites, marking these cells chemical export facilities (Huchelmann et al. 2017). For instance, in some tobacco varieties, the metabolites secreted by the trichomes represent up to 15% of their leaf biomass (Wagner et al. 2004). In recent years, biotechnology explored the possibility of metabolic engineering to increase the number of metabolites produced in these specialized cells. These modification processes will result in plants that are more resistant to pathogens or produce compounds with high industrial or pharmaceutical values. However, many specialized metabolites are toxic at high concentration and their effective over-production can only be achieved if specialized transporters were present on cell membranes (Widhalm et al. 2015; Adebesin et al. 2017). In this respect, understanding of how PDR transporters select and translocate their substrates will enable rational design of transporters capable of facilitating efficient transport of new metabolites.

An example of such a molecule is taxadiene, the precursor of the important anticancer drug Paclitaxel (Taxol®). This tricyclic diterpene is currently being extracted from *Taxus spp.*, heterologous produced in cell cultures, or by total synthesis and semi-synthesis procedures. However, current demand is environmentally unsustainable (Soliman & Tang 2014). To address this issue, taxadiene was heterologous produced in *Nicotiana benthamiana* by ectopic expression of taxadiene synthase in the trichomes (Hasan et al. 2014). While this metabolic engineering strategy was successful, the overall yield is still too low, which is likely due to the lack of a specific
exporter. This problem can be resolved by structure-guided redesigning of NtPDR1 specificity towards exporting taxadiene thus significantly increasing production of this anticancer precursor in Nicotiana spp. To understand the plant-specific transport of diterpenes the following questions need to be answered: what is the structure of NtPDR1? How does NtPDR1 translocate diterpenes across the cellular membrane? Is the PDR1 subfamily operating through a conserved or unique transport mechanism? Can residues important for substrate binding be identified? Is it possible to re-engineer NtPDR1 to alter its substrate specificity?

My proposal aims at answering these questions.

2) Objectives of the research.

The goal of my project is to unravel the molecular translocation mechanism of diterpenes in plants. I will structurally characterize NtPDR1 in complex with the substrate sclareol using single-particle cryo-EM. Currently, NtPDR1 is the only well-behaving and well-characterized transporter from this unique subfamily of plant ABCG transporters. Solving the structure is crucial to understanding how diterpene translocation is facilitated. Unveiling how substrates are selected by PDR1 is also of great interest for biotechnological applications. By re-engineering substrate specificity, PDR1 transporters could be used to translocate important pharmaceutical compounds produced in Nicotiana tabaccum expression systems.

To achieve this, the project is divided into four work packages:

**Work package 1:** Structural characterization of NtPDR1 using single-particle cryo-EM.

The primary goal of my project is to solve the molecular architecture of NtPDR1 at near-atomic resolution using single-particle cryo-EM. First, I aim at solving the structure of the transporter in its apo state. Subsequently, I will determine the structure of NtPDR1 in different nucleotide-bound states and in complex with its substrate sclareol. Additionally, other known substrates cembrene and capsidol will be utilized. Together, these intermediate conformations will provide the structural basis for understanding the NtPDR1-mediated diterpene translocation mechanism.

**Work package 2:** Identify substrate selectivity residues using molecular dynamic simulations.

I will use the structures of NtPDR1 in complex with substrates for molecular dynamics (MD) simulations that will be performed in collaboration with group of Prof. Helmut Grubmüller at (MPI, Germany). The goal of the MD simulations to validate the binding site of the substrates and characterize contributions of residues to the binding energy of the substrate. These results will provide insights into how PDR1 selects specific substrates and which residues define the substrate specificity.

**Work package 3:** Validate the binding pocket and translocation mechanism using mutagenesis combined with biochemical assays.

Once important for binding residues have been identified, the translocation mechanism will be validated. In collaboration with Prof. Marc Boutry (UCL, Belgium), I will express and purify mutants of PDR1 transporters mutated at the residues important for substrate binding. After that effect of mutations will be evaluated using substrate binding and translocation assays.

**Work package 4:** Exploring the substrate specificity of NtPDR1 in situ.

At the end of my project I will begin an exploratory work package where based on all the gained data I will aim at redesigning NtPDR1 specificity towards different type of metabolites in situ. Initially, I will focus on the tricyclic diterpene taxadiene as a new target for NtPDR1. Using MD simulations I will identify mutants that alter transporter specificity for binding and translocation of cancer drug precursor taxadiene.
3) Approaches and Methodology

Work package 1: Structural characterization of NtPDR1 using single-particle cryo-EM.

Protein expression and purification
A major bottleneck in structural biology of membrane proteins is the soluble expression and functional purification, often associated with cell toxicity, requirement for chaperones, membrane crowding and stability (Seddon et al. 2004). During my PhD, optimizing the expression and purification of the plant Anaphase Promoting Complex/Cyclosome (APC/C) provided me with valuable expertise on how to optimize these important steps. Using an expression host capable of dealing with these requirements can successfully lead to higher sample quantity and quality necessary for structural characterization. In case of the sclareol exporter, cleavable His-tagged NtPDR1 was stably expressed in N. tabacum BY-2 suspension cells by our collaborator Marc Boutry. Active monomers can be solubilized in detergent dodecyl maltoside (DDM) and purified using Ni-NTA affinity chromatography followed by a size exclusion (Pierman et al. 2017). Reconstitution into asolecin liposomes and addition of the substrates increases the basal ATPase activity of NtPDR1 and initial negative stain electron microscopy experiments revealed the presence of a monodisperse sample (Figure 3). The availability of an active and monodisperse sample reduces the risk associated with this work package. Therefore, at the start of this project, I will grow NtPDR1-expressing BY-2 cells in a bioreactor, solubilize NtPDR1 from microsomal fractions and purify it from this homologous expression system as previously described (Pierman et al. 2017).

Figure 3. Purification and initial negative stain characterization of the Nicotiana tabacum PDR1 transporter purified in detergents. a. Coomassie blue stained SDS-PAGE of the Ni-NTA purification revealed a single protein band after several chromatography steps. b. Gel-filtrated NtPDR1 analyzed by negative stain electron microscopy revealed a monodisperse sample. c. Representative 2D class averages indicate particle projections characteristic of ABC transporter (Pierman et al., 2017).

Sample preparation for single particle cryo-EM
The determination of high-resolution structures of ABC transporters from human and bacteria has been successfully achieved with single-particle cryo-EM, indicating that this goal of work package 1 is feasible. (MsbA – 4.2 Å; ABCA1 – 4.1 Å; ABCG2 - 3.8 Å; CFTR - 3.7 Å and MRP1 - 3.5 Å) (Mi et al. 2017; Qian et al. 2017; Taylor et al. 2017; Zhang & Chen 2016; Johnson & Chen 2017). Although membrane proteins are often purified and structurally characterized in the presence of detergents, recent biochemical data revealed that ABC transporters have higher basal ATPase activity when reconstituted into an environment that approaches physiological conditions (Ritchie et al. 2009; Zoghbi et al. 2016; Mi et al. 2017). An established lipid bilayer mimetic are lipid nanodics, nanometer scale discoidal structures that contain a phospholipid bilayer and are soluble and stable. Recent EM analysis of bacterial MsbA (Mi et al. 2017) and human ABCG2 (Taylor et al. 2017) reconstituted into lipid nanodiscs revealed that cryo-EM reconstitution at near atomic resolution is feasible.
To ensure that the structural studies of NtPDR1 are performed on native conformations of the transporter, I will reconstitute NtPDR1 into nanodics. This expertise in the reconstituting membrane protein into lipid bilayer is available in the Efremov lab, which has major success with reconstituting the muscular isoform of the rabbit ryanodine receptor (RyR1) in the nanodiscs for single particle cryo-EM (Efremov et al. 2015; Efremov et al. 2017) (Figure 4).

Currently, no plant ABC transporter has been reconstituted into nanodics and information on the most optimal lipid composition is lacking. To find conditions for optimal reconstitution, I will screen various membrane scaffold protein (MSP), try different lipid compositions such as soybean lecithin and mixes of synthetic lipids, finally I will optimize NtPDR1:MSP:lipids ratio. The goal is to obtain homogeneous nanodiscs containing reconstituted NtPDR1 with high enzymatic activity. The success of reconstitution will be assessed by ATP hydrolysis assays and negative stain EM. From negative stain and cryo-EM images acquired on a 120 kV microscope, 2D class averages and low-resolution reconstructions (10 - 15 Å) will be calculated. They will provide insights into the most optimal conditions of reconstitution. During my PhD, I have already gained expertise in negative stain EM while screening plant APC/C protein samples.

Single particle cryo-EM structure of the nucleotide free, substrate-bound and activated state
Once NtPDR1 has been successfully reconstituted into lipid nanodiscs, high-resolution single particle cryo-EM reconstruction experiments will be performed to obtain near atomic resolution reconstruction (lower than 4 Å). Initially, plunging condition of cryo-EM samples will be screened aiming at optimal ice thickness and homogeneous distribution of particles. To achieve this, various grid types including holy carbon and graphene oxide grids will be tested, as well as ranges of blotting times and blotting papers. Initial screening on a 120 kV JEOL JEM-1400 microscope equipped with high-sensitivity TVIPS F416 camera and cryo-holder will be followed by data collection on a 300kV cryoARM300 JEOL microscope equipped with Fiend Emission Gun (FEG), phase, plate, energy filter, cryogenic stage and K2 (or K3) direct electron detector that will be fully operational by June 2018 in the host lab. In addition, if necessary microscope time will be available at other European EM facilities including NeCEN (Leiden, Netherlands), eBIC (Diamond, UK) and ESRF (Grenoble, France) ensuring feasibility of the project. The data processing will be performed in cryo-EM processing packages like RELION and SPHIRE (Fernandez-Leiro & Scheres 2017) using available in house GPU station and VUB computer cluster Hydra with several hundred computing cores.

Firstly, the nucleotide free conformation of NtPDR1 will be determined, representing the resting state of the transporter, which is usually the best conformationally defined state of the ABC transporter. While optimization and data processing of the first high-resolution structure will be time-consuming and challenging, once established, the workflow can be easily adapted for determining structures of other NtPDR1 conformations. Secondly, the structure of NtPDR1 with its native substrates sclareol will be determined aiming to reveal the molecular details of the binding pocket. Here, other known substrates cembreme and capsidiol can be used to provide complementary information on substrate specificity.
Next, my goal will be to determine the structures of the different states of the substrate translocation cycle (Figure 5). To mimic the transition state after ATP hydrolysis but before γ-phosphate release, the cryo-EM structure of NtPDR1 in complex with Mg\(^{2+}\)-ADP-vanadate will be determined. This orthovanadate substrate locks NtPDR1 in an inactive conformation as shown by recent ATPase activity assays (Pierman et al. 2017). Finally, the structure of NtPDR1 in complex with ADP will be determined representing the final state of the catalytic cycle. Together, these structures will provide a basis for understanding diterpene translocation by NtPDR1 (Figure 5).

![Figure 5](image.png)

At the end of this work package I expect to obtain detailed structural understanding of NtPDR1’s structure, substrate binding and conformational transitions. However due to limited resolution of cryo-EM (typically to around 4 Å for these proteins) the details of substrate binding need to be thoroughly validated, which is achieved through WP2 and WP3.

**Work package 2:** Identify substrate selectivity residues using molecular dynamic simulations.

At near atomic resolution (3-4 Å) the cryo-EM density will visualize protein backbone and most of the side chain densities permitting building of the atomic model of NtPDR1. Such resolution however, leaves conformations of the side chains, and details of protein-substrate interactions ambiguous. Neither tightly-bound solvent molecules contributing to the substrate binding can be resolved at this resolution. To overcome this limitation and to identify contribution of individual residues to substrate-binding energy and specificity, molecular dynamic (MD) simulations of the complete NtPDR1 with and without substrates will be performed.

Modern high-performance computing allows MD simulations on large systems, including complete ribosomes and membrane proteins imbedded into lipid environment (Hospital et al. 2015). To perform these calculations, I will join the group of Computational and Theoretical Biophysics of Prof. Helmut Grubmüller at Max-Planck Institute for Biophysical Chemistry in Göttingen, Germany. Here, I will learn how to perform MD simulations and initiate this computational research. The department of Prof. Grubmüller is renowned for expertise in the state of the art MD simulations on the large molecular complexes including ABC transporters.

Initially, I will use the cryo-EM structure of apo NtPDR1 determined in WP1. This models will be reconstituted into a lipid bilayer modeled in aqueous environment and the stability checked by performing MD simulations up to tens of microseconds. Subsequently, I will perform simulations in presence of the substrates scclareol and cembrene. In case of the Polyglycoprotein transporter (P-gp), MD simulations identified specific residues required for substrate binding highlighting the feasibility of this approach (Barreto-Ojeda et al. 2017). Calculations will be performed using one of the established MD packages such as GROMACS. The appropriate
computational resources for these simulations will also be available: we will use Flemish Supercomputer Cluster (VSC) and GPU nodes to perform the calculations. From the simulated trajectories, I will calculate the affinity of the substrates (Liang et al. 2009). Using these calculations, I will identify residues critical for the substrate binding and predict effect of their mutations in silico. These mutations will be experimentally verified in WP3. Together, WP1 and WP2 will initiate steps for the first time towards a better understanding of how substrates access the translocation cavity in plant ABC transporters.

Once different functional states of NtPDR1 have been determined by cryo-EM, a more challenging work package could be pursued. Using coarse-grained (CG) MD simulations, transitions between the intermediates of the translocation cycle of NtPDR1 could be calculated. Together, this data would reveal molecular details and energetics of the translocation mechanism.

**Work package 3: Validate the binding pocket using mutagenesis and biochemical assays.**

To validate the binding residues of NtPDR1 proposed in WP1 and WP2, mutagenesis studies will be performed. Using sequence and ligation independent cloning (SLIC) strategies, key residues will be individually mutated to alanine residues. In a joint effort with Marc Boutry (UCL, Belgium), I will clone and express different mutated NtPDR1 transporters in *N. tabacum* BY-2 cell using Agrobacterium tumefaciens-mediated transformation. Once purified, I will reconstitute these transporters into asolectin liposomes. The result of the reconstitution will be evaluated by flotation assays using sucrose gradients. If successful, the effect of the mutations on substrate interaction will be via characterized using two biochemical assays:

1) **Substrate binding assays:** ABC transporters are known to hydrolyze ATP. Substrate binding increases this basal activity. Using in vitro ATPase activity assays, reduction of Km can be measured and used to validate the importance of the residue on substrate binding. Here the change in ATPase activity will be measured.

2) **Transport assays:** the uptake of radioactive labeled substrate can be monitored in vitro using liposomes or intact BY2 cells. For this, labeled sclareol is available in the group of Marc Boutry. After incubation with sclareol, radioactivity will be determined by liquid scintillation counting. Key residues influencing substrate translocation will have a decreased $^3$H values.

At the end of this work package, I will understand which key residues determined in WP1 and WP2 are required for substrate binding and specificity.

**Work package 4: Exploring the substrate specificity of NtPDR1 in situ.**

Once key binding residues have been validated in WP3, I will explore the possibility of mutating the substrate-binding pocket to redesign NtPDR1 specificity towards different type of metabolites in situ. In case of the NtPDR subfamily, is has been established that NtPDR1 interacts with other isoprene based molecules such as capsidiol (Pierman et al. 2017). This sesquiterpene adopts a slightly different conformation and binds PDR1 with similar affinity indicating the plasticity of the binding pocket (Figure 2b). Therefore, we believe that NtPDR1 is a feasible target for our strategy.

Taxadiene is the first committed intermediate in the synthesis of the chemotherapeutic Taxol. Since it is metabolized inside the plant cell, a putative transporter is likely not present. Nevertheless, metabolic engineering of the taxadiene synthesis pathway in *N. tabacum* requires the presence of a designated transporter to avoid self-toxicity. Since taxadiene is a tricyclic diterpene, it shows structural similarities with the dicyclic substrate sclareol and capsidiol (Figure 2b). Changing residues in close proximity to the substrate-binding cavity or at regions identified as selectivity filters can facilitate the binding of taxadiene. To do this, I will mutate the key residues identified in WP3 and perform MD simulations with and without taxadiene. The model of NtPDR1 established for MD simulations in WP2 can be reused to perform these experiments. This
way, I will identify residues required for binding the tricyclic diterpene taxadiene in silico. From this, binding efficiency can be calculated and the most optimal changes identified. If the computational efforts are successful, mutant transporters will be generated and biochemical assays will be performed to test specificity of NtPDR1 towards taxadiene.

This package has high-risk and will very likely go beyond the time period allocated for the postdoctoral fellowship. But at least I would like to start these exploratory efforts in performing engineering on the transporter that could potentially revolutionize the field of plant biotechnology.

**Outlook: Unraveling the catalytic cycle of NtPDR1 using time-resolved cryo-EM**

Even though multiple structural intermediates have been solved for ABC transporters, details on the full catalytic cycle are still unanswered (Wilkens 2015). This can be solved however, if the complete translocation cycle could be resolved using structural methods. One potential opportunity to obtain this information is to use time-resolved single particle cryo-EM that can freeze-trap intermediate states with millisecond time resolution (Frank 2017). Time-resolved cryo-EM is one of the research direction of Efremov lab. They are developing methodologies that should enable practical realization of time-resolved cryo-EM. In future this methodology can be applied to NtPDR1 to visualize conformational transitions and the change of populations of the conformations during the catalytic cycle of the ABC transporter.

4) Work plan

**Table 1.** Gantt chart of the proposed workflow of the NtPDR1 project.

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<th>Milestone 2</th>
<th>Milestone 3</th>
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<td><strong>Objectives</strong></td>
<td><strong>Year 1</strong></td>
<td><strong>Year 2</strong></td>
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<td>Optimization of NtPDR1 reconstruction into nanodisks and preparing cryo-grids</td>
<td>WP1</td>
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<td>Determine high resolution structure of apo NtPDR1</td>
<td>WP1</td>
<td>WP1</td>
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<td>Determine high resolution structure of NtPDR1-scareol</td>
<td>WP1</td>
<td>WP1</td>
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<tr>
<td>Determine high resolution model of NtPDR1-ADP/vanadate</td>
<td></td>
<td>WP1</td>
</tr>
<tr>
<td>To identify key residues required for substrate modulation by molecular dynamic simulations</td>
<td>WP2</td>
<td>WP2</td>
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<tr>
<td>To validate the binding packet using mutagenesis and biochemical assays</td>
<td>WP3</td>
<td>WP3</td>
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<tr>
<td>Exploring the substrate specificity of NtPDR1 in situ</td>
<td></td>
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5) References


Barreto-Ojeda, E. et al., 2017. Lipid-Uptake Pathways and Lipid-Protein interactions in P-glycoprotein Revealed by Coarse-Grained Molecular Dynamics Simulations.

Crouzet, J. et al., 2005. Organization and function of the plant pleiotropic drug resistance ABC transporter


6) How to communicate the research to a wider public

The topic of the proposed project covers a very general question related to the transport of specialized metabolites in plants and will be attractive to the general audience. The high-resolution structures of important biomolecules are often published in journals targeting wide audience, like Nature and Science.

If the results are appealing to a wider audience they will be communicated by the VIB communication department, which prepares carefully drafted press releases.

7) Full bibliographic details of the 5 most important papers


Beste onderzoeker,

Gelieve een kopie van uw Master diploma op te laden in uw persoonlijk E Loket.

Met vriendelijke groeten,

Anja
Van BIO@fwo.be     Aan stdegiet@vub.ac.be
Datum 26 March 2018 01:14     Gebruiker fwo\vdh     UIT
Betreft 12Q6419N Additional referees Bio1
Dear researcher,

In your application you have proposed ten external referees (at least at postdoctoral level) appointed at a university, research institution or research entity of another type of organization.

Unfortunately insufficient referees have responded to the FWO-invitation. Therefore I would like to ask you to give another five additional foreign referees by March 28, 2018 at the latest.

Not eligible as referee are:

- members of the Board of Trustees of the FWO;
- members of an FWO-Expert Panel;
- persons appointed to a Belgian university, research institute or any other organization; or, in the case of calls for proposals in the framework of bilateral or lead agency agreements, persons appointed to similar institutions or organizations in the country where the foreign project partner is professionally active;
- persons with a professional appointment to a foreign institute where the applicant(s) had been enrolled as a student or professional after January 1st of the year n-3 (n=year of application);
- any co-authors with the applicants of a publication that was submitted or published after January 1st of the year n-3 (n=year of application);
'Co-authorship' is to be understood as follows:
- co-authorship of a monography of which the applicant is co-author as well;
- co-autorship of an article or another type of contribution to a collection (book, journal issue, report, congress proceedings, abstract,...) of which the applicant is co-author as well;
Editors are not regarded as co-authors insofar as they have not also acted as what is understood under 'co-author' as described above. Co-editors of the applicant are not accepted as an external referee.
- partners of the applicant(s) in a research cooperation, whether formalised in a research project or not, that has been applied for or has been running after January 1st of the year n-3 (n=year of application. In this context, the following shall in any case qualify as research cooperation (non-exhaustive list):
  • Cooperation under a research fellowship, granted by the FWO;
  • Cooperation under a research project, whether relating to a specific subject or not or under an international cooperation project, granted by the FWO;
  • Cooperation under the Odysseus programme or the Big Science programme, granted by the FWO;
  • Cooperation under a Scientific Research Network, granted by the FWO;
  • Cooperation under programmes similar to those mentioned above, granted by organisations other than the FWO;
  • Joint research work not formalised in a cooperation structure as defined above;
  • Research carried out in the research areas and/or with research facilities provided by the applicant to the referee or vice versa;
  • ...
Thank you for your cooperation.

Kind regards.
Anja
Geachte onderzoeker,

In het kader van het samenwerkingsprotocol tussen VITO en FWO, worden er jaarlijks twee VITO-beurzen postdoc toegekend.

Omdat uw onderzoek in aanmerking komt voor een dergelijk mandaat, vraag ik vóór maandagochtend 2 juli 2018 uw toestemming te geven om uw dossier door te sturen aan VITO.


Met vriendelijke groeten.

FWO Administratie
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<thead>
<tr>
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PAST AND CURRENT STUDIES

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DISCIPLINES

From molecule to cell
Structural biology
Plant biotechnology

CAREER

Overview of positions NOT related to FWO and connected to a receiving university/organization
SCIENTIFIC PUBLICATIONS

(A1.1) Articles (a) included in Web of Science’s (WoS) Science Citation Index Expanded, Social Science Citation Index and/or Arts and Humanities Citation Index, whose document type is labelled as “Article”, “Review”, “Letter”, “Note” and/or “Proceedings Paper” or (b) in journals included in the Journal Citation Reports (JCR) of Web of Science. In chronological order, starting with the most recent item, with full bibliographic description and with the JCR two-year Impact Factor in the year of publication (or the most recent one).


Overview of FWO-related positions

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