**Title:** **Extending the type IIS toolkit for subcellular localisation in Marchantia**

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* **Team:**

Connor Tansley (c.tansley@uea.ac.uk, BBSRC DTP PhD student, Miller Lab, Biology Department, UEA)

Connor has been setting up the required facilities to work with *Marchantia* at UEA to investigate calcium signalling in the basal land plant for his PhD. In this project he will carry out the required sequence analysis to find putative localisation domains in comparison to known localisation sequences and clone these sequences into vectors containing fluorescent reporters.

* Dr Susana Sauret-Gueto (ss2359@cam.ac.uk, OpenPlant Research Manager, Prof Haseloff Lab, Plant Sciences Department, University of Cambridge)

Susana is setting up standardised practices for DNA assembly around Loop, compiling a *Marchantia* DNA toolkit and establishing and curating registries to facilitate standardisation and sharing. She has also established and advanced microscopy facility at the OpenPlant in Cambridge, equipped with a series of fluorescent microscopes with different resolution capabilities. She will bring all these resources and expertise into the project.

* Linda Silvestri (ls257@cam.ac.uk, Research Technician, Prof Haseloff Lab, Plant Sciences Department, University of Cambridge)

Linda is responsible for *Marchantia* tissue culture at the Haseloff Lab. She will be teaching Connor the *Agrobacterium*-mediated transformation using 6-12 well plates.

* **Summary:**

Synthetic Biology is an emerging field that employs engineering principles for constructing genetic systems. The relative simplicity of genetic networks in *Marchantia*, combined with the growing set of genetic manipulation, culture, and microscopy techniques, are set to make this primitive plant a major new system for analysis and engineering (1). In order to allow for the systematic engineering of gene expression in relation to cell behaviour and metabolism in *Marchantia*, we need a reliable toolkit of multispectral markers as well as tags for specific subcellular locations.

The Haseloff Lab is setting up standardised practices for DNA assembly, compiling a *Marchantia* DNA toolkit and establishing registries to facilitate standardisation and sharing. The constructs are compatible with the OpenMTA, and thus suitable for open distribution. In the Haseoff Lab, Bernardo Pollak *et al*. developed Loop assembly, a simple and open type IIS assembly system (2). Susana Sauret-Gueto and team have been developing Loop-pCambia, L0 acceptor plasmids designed for Loop, and they are putting together a type IIS DNA tool kit which includes promoters, fluorescent proteins, signal peptides and other tools for synthetic biology, aiming at maximising efficiency and reproducibility of *Marchantia* workflows. Previous studies demonstrated that some examples of localisation sequences found in higher plants also function in *Marchantia* such as localisation sequences for the endoplasmic reticulum (HDEL; 3), the nucleus (SV40; N7; 3; 2) and the plasma membrane (Myr; Lti6b; 2; 3). These sequences represent useful L0 parts and are already available as Loop compatible modules in the *Marchantia* toolkit. Chloroplast transit peptides have also been characterised in *Marchantia* (4), and some of them are also available in the kit.

The aim of this project is to enlarge the *Marchantia* Loop toolkit with protein tags that localise to other important subcellular locations and compartments. This project specifically aims to design and test typeIIS tags for localisation to the tonoplast (vacuole membrane), golgi apparatus, oil bodies, peroxisome and mitochondria. These tools diversify the potential uses to which *Marchantia* can be put by the synthetic biology community, and using the open MTA Loop system, plasmids can be freely shared in between researchers.

* **Proposal:**

This project specifically aims to develop tags in *Marchantia* for localisation to the tonoplast (vacuole membrane), Golgi apparatus, oil bodies, peroxisome and mitochondria.

* **Design of new tags:**

**Tonoplast:**

The tonoplast is an important sink for sequestration of cytoplasmic toxic compounds and plays a role in calcium signalling in response to stress (5). Calcineurin B-Like (CBL) proteins are a family of calcium-binding proteins which contain members that localise to the tonoplast in higher plants. Interestingly, localisation can be proposed based on phylogeny (5). Connor carried out phylogeny of this family for *M. polymorpha* and a single MpCBL was discovered that is likely to localise to the tonoplast. The other two MpCBLs likely localise to the PM, which confirms a previous study (5). MpCBL will be tested for localisation to the tonoplast and, if confirmed, a signal peptide will be derived from it. If not, the AtCBL3 localises to the tonoplast and a N-terminal signal peptide derived from it can be tested in *Marchantia* as well (6; 7; 8; 9). The Miller Lab can also supply a tonoplast localising N-terminal peptide (y-TIP) which has been used in a range of higher plants as a localising module (10).

**Peroxisome**

The peroxisome is an important subcellular localisation site which is involved in jasmonic acid biosynthesis and the production of reactive oxygen species. The peroxisome is also known to take part in the downstream processes of p-glycolate recycling and fatty acid b-oxidation in plants. Therefore, this body is both good at sequestering toxic products from the rest of the cell and is a key organelle for fundamental biology (11). Previous studies in *Marchantia* have used the PST-1 C-terminal tag for localisation of Citrine to the peroxisome for visualisation of cold translocation of the organelle (12). The Miller Lab uses this tag and we will test in *Marchantia* in this study.

**Mitochondria**

The previously mentioned study on cold induced translocation of organelles also included visualisation of the mitochondria using a mitochondrial targeting sequence (MTS) derived from SD3 in *A. thaliana* (12). SD3 is a translocase of inner mitochondrial membrane 21 (TIM21) homologue that localises to the mitochondria (13). This further validates that *A. thaliana* signal peptides may function as signal peptides in *Marchantia*. MTS-SD3 will be tested in the *Marchantia*. In addition, the Miller Lab has the ScCOX4 protein tag originally derived from *Saccharomyces cerevisiae* which has been shown to function in higher plants for mitochondrial localisation (10). Therefore, it is intended to validate this tag in *Marchantia* to broaden the options for mitochondrial localisation.

**Golgi:**

The golgi is important in secretion and post translational modification of proteins. The golgi has a single localisation sequence known to function in *Marchantia* (ST, 14), which will be included in the Loop toolkit. Other golgi localising sequences are known to function in several higher plants and the Miller Lab uses one of these signal peptides, (GmMan1; 10). Therefore, this tag can be tested for function in *Marchantia*. Depending on previous results and timing, the golgi tags could be expanded with sequences derived from *Marchantia* SNARE proteins, which are involved in membrane trafficking between the ER to post golgi transport and are therefore attach to a range of organelles and vesicles including the Golgi (14). *Marchantia* has six SNARE proteins that have already been characterised to localise to the Golgi although the specific localisation sequence is unknown. Their orthologous sequences in *Arabidopsis* also localise to the golgi, so comparison to these sequences should allow detection of the domain required for golgi localisation (15). As a control for the SNARE-derived tags generated, a full-length SNARE gene would be cloned and fused to fluorescent reporters as previously done (14).

**Oil bodies:**

The oil body is a unique structure in *Marchantia* that contain a range of bioactive compounds (16). In the study on MpSNARE proteins, it was found that MpSYP12B localises to the oil bodies. As SNARE proteins localise via the C-terminus it may be possible to determine the localisation sequence. The full-length MpSYP12B will be analysed for consensus to other SNARE proteins to assess the length of the c-terminal domain required for localisation in the oil bodies. As a control for the localisation of the tags generated, the full-length gene will be cloned and fused to fluorescent reporters as previously done. (14)

* **Implementation:**

Due to the relatively low cost of DNA synthesis, ease of *Marchantia* transformation and relative ease of microscopic characterisation, it will be possible for us to synthesise and test multiple localisation tags within the timeframe of the project.

Tags will be introduced by PCR at N- or C-terminal of a fluorescent protein (eGFP, mTurquoise, mVenus are routinely used at the Haseloff Lab) and cloned into L0 acceptor vectors for Loop, or they will be synthesised as a L0 part including the tag fused to a fluorescent protein. Also, when whole genes are used as a tag, they will be synthesised as L0 N- or C- terminal parts (CDS12 or CTAG) in the openMTA acceptor pUAP1 (Table 1). L0 parts with localisation tags and fluorescent reporters will be assembled as L1 constructs, and L1s into L2 constructs by type IIS Loop cloning (Miller; guidance from Haseloff). Haseloff Lab will provide the necessary L0 promoters to assemble L1s, and the L1 resistance cassettes to assemble final L2 constructs.

L2 constructs will be introduced by *Agrobacterium*-mediated transformation into *M. polymorpha* (Haseloff Lab; Connor). Linda will teach the medium throughput sporeling transformations to Connor (modified from 17). She is currently transforming *Marchantia* sporelings in 6 and 12 well plates, with positive transformants screened in 2/3 weeks and next generation gemmae ready in about a month and a half. Positive transformants will be screened based on resistance to antibiotics and fluorescence (Haseloff lab). Subcellular localisation studies will be performed using fluorescence microscopy (Susana, Leica stereo microscope with fluorescence and Leica SP8 confocal microscope).

We expect to do first batch of constructs, transformations and first characterisation in 3 months and a second batch in the following 3 months, together with further characterisation of tags.

* **Benefits and outcomes:**

The benefit of this project will be to contribute to the development of tags for specific subcellular locations in *Marchantia*. All functional L0 tags will be added to the *Marchantia* toolkit collection, which is in the processes of being submitted to Addgene for distribution under Open MTA.

Importantly, a new connection between the Haseloff lab (Cambridge) and the Miller lab (UEA, Norwich) will be formed in the project. Plans for this project involve the sharing of type IIS Loop vectors, parts from the *Marchantia* toolkit, and protocols with the Miller lab for the cloning part. It is a two-way collaboration, as the Miller lab will also share their experience with fluorescent proteins and contribute new protein tags into the collection. The Haseloff lab will also demonstrate the transformation procedure they use for *Marchantia* which is higher throughput than the current method used in the Miller lab.

Susana has set up Benchling at the Haseloff Lab as a collaborative platform, sequence editor and registry for Loop and the *Marchantia* kit, and this will be introduced to the Miller group as an additional benefit of the project.

* **Sponsor for the Research and Cost centre:**

Dr Ben Miller (Code: To be confirmed with the Research Office)

I confirm that I have the full support of the sponsor listed above and that they can be added to the OpenPlant Fund mailing list to receive project updates (to which they can unsubscribe at any time).

* **Budget:**

Additional funding: The Haseloff lab will provide free access to the OpenPlant microscopy Hub, as well as to transformation consumables.

Gene synthesis: Susana negotiated special discount for OpenPlant projects with Genewiz for synthesis into pUAP1 (L0 acceptor plasmid under open MTA).

Funding allocation;

£2850 synthesis and sequencing of constructs

£750- cloning consumables including Sap1, Phusion etc. (Miller Lab)

£400- travel of Miller Lab to Cambridge

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