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

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Synopsis:

Synthetic Biology is a new field where engineering principles are being applied to biological systems for automation, enterprise and technology development. *Marchantia* is a plant system being developed for synthetic biology with growing sets of tools for genetic manipulation, culture, microscopy techniques (1). A big part of synthetic biology has been the establishment of modular parts and the ability to share these parts freely. Type IIS restriction cloning is integral for scientists to easily share compatible genetic parts. Loop cloning is a form of type IIS restriction cloning and the parts are shareable under openMTA for use in academic and business contexts (2). The compatibility of the Type IIS genetic parts allows these to be treated as 'standard parts' that consist of characterised modules with known functions in particular organisms, e.g. 'constitutive' promoters and terminators. This project focused on expanding the tags that can be added to the coding sequence of a gene to confer a specific subcellular localisation in *Marchantia*. Previously tags had been made to localise proteins to the plasma membrane (Myr; 2; Lit6b; 3), nucleus (SV40; 3; N7; 2) and endoplasmic reticulum (HDEL; 3). This project expanded the localisation tags to other subcellular sites such as the tonoplast, endoplasmic reticulum, Golgi apparatus, peroxisome, mitochondria and the oil bodies.

DNA synthesis and Type IIS cloning has been used to produce new standard genetic parts for protein localisation in *Marchantia*:

Initially a literature review was undertaken to find a broad range of subcellular localisation tags for a range of locations. The Haseloff lab were particularly interested in MpSYP12B (Table 1; 4) as it was previously reported to be localised to the oil bodies of *Marchantia* which produce a wide range of bioactive compounds (5). As MpSYP12B is a SNARE protein, localisation is usually conferred by the C-terminus of the protein but firstly we needed to confirm MpSYP12B is localised to the oil bodies specifically.

The Miller Lab were interested in the tonoplast localising tags due to its importance in calcium signalling. Two of the potential tags were Calcineurin B-Like (CBL) proteins including *Arabidopsis thaliana* CBL3 and the closest homologue CBL in *Marchantia* (Table 1; 6; 7; 8; 9). Both of these tags were synthesised as full length genes. The Miller lab also had another tonoplast tag (y-TIP) which has previously been used in a range of higher plants and therefore this was subcloned for use in the Loop system (Table 1; 10).

The peroxisome, Golgi apparatus and mitochondria were also targeted for tagging as important cell compartments. The Miller Lab already had some tags for some of these sites that work in higher plants (ScCOX4, PST1) which were subcloned and added to the Loop toolkit (Table 1; 11). It is unknown whether these tags confer the same localisation in *Marchantia* but some of these tags come from organisms such as *Saccharomyces cerevisiae* (ScCOX4) and work in higher plants (12). Therefore it would be expected that the tags work in basal land plants as well. To account for the risk that the tags do not localise as expected, a multiple tags for subcellular sites were included such as GmMan1 and the CBLs (Table 1).

The eight subcellular localisation tags were either cloned or synthesised into pUAP4 Loop plasmid (Table 1). Each tag was then fused with a fluorescent marker (mVenus/mTurquoise), a constitutive promoter (p35S) and terminator (NosT-35ST) to form eight level 1 parts.

Lines are being generated for each of the 8 tags using hygromycin to select for positive transformants:

The Haseloff Lab cloned level two constructs for each of the level 1 parts. Initially each level 1 part will be cloned with a hygromycin resistance cassette (*hph*; 13) and spacers. After, some of the tags will be cloned into level 2 constructs with more than one tag. After the initial transformation of spores by *Agrobacterium*-mediated transformation plants will likely be chimeric so gemmae (asexual propagules derived from a single cell) can be taken to form a non-chimeric G1 generation plant.

Localisation of the tagged fluorescent proteins will be assessed by microscopy:

Once the lines have been taken to a non-chimeric G1 generation, the plants will be checked for localisation of the fluorescent markers using microscopy (Leica stereo microscope with fluorescence and Leica SP8 confocal microscope). This characterisation work is currently on-going and is being led by the Haseloff lab in Cambridge.

The OpenPlant project has facilitated a new collaboration:

As part of the collaboration between the lab groups, members of the Miller Lab visited the Haseloff Lab (24/07/19) to learn the sporeling medium-throughput transformation of *Marchantia* and far-red light induced sexual structure formation in *Marchantia*. In return, the Miller Lab shared its protocol for carrying out cryopreservation of gemmae. Alongside this, the Miller lab shared genetic material including for the tags PST1, ScCOX4, Y-TIP. The Haseloff lab also shared material including the loop cloning vectors and CAM1, CAM2 and Tak2 accessions of *Marchantia polymorpha.*

Project outcomes:

The localisation tags have been added to the *Marchantia* type IIS toolkit and will be shared under openMTA. They will also be added to AddGene after localisation has been confirmed. The project has also allowed exchange of techniques and materials between researchers in the Miller and Haseloff lab (including loop cloning and cryopreservation of *Marchantia*). This work forms part of an ongoing interest in both labs to develop shared tools for *Marchantia* and the collaboration will be continued beyond the end of this project.

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Table 1. The level 0 tags produced in the OpenMTA pUAP4 plasmid with subcellular localisation, source and termini the tag was bound to.

Tag	ID	Subcellular location	Synthesised GW or cloned (CT, UEA)	C or N-terminus	L1 done (SSG, UC/ CT, UEA)	L2 done (SSG, UC)	Screening by fluorescence microscopy (SSG, UC)
MpSYP12B	HMO_p0_o946	Oil Body	Synthesised	C	L1_161-CK4, p5-35Sx2:OBS_MpSYP12B_linker-mVenus	L2_230-CsA, p5-35S:HygR, p5-35Sx2:OBS_MpSYP12B_lk_mVenus	Selection of T0 plants done. G1 plants growing. Screening by fluorescent microscopy ongoing
ST	HMO_p0_o944	Golgi Body	Synthesised	C	L1_157-Ck4, p5-35Sx2:GLS-ST-mVenus	L2_226-CsA failed. Needs repeating	
GmMan1	HMO_p0_o950	Golgi Body	Synthesised	C	L1_158-CK3, p5-35Sx2:GLS-GmMan1-mTurq2	L2_227-CsA failed. Needs repeating	
AtCBL3	HMO_p0_o947	Tonoplast	Synthesised	C	L1_162-CK4, p5-35Sx2:TLS-AtCBL3-linker-mVenus	L2_231-CsA, p5-35S:HygR, p5-35Sx2:TLS-AtCBL3-mVenus	same
MpCBL	HMO_p0_o955	Tonoplast	Synthesised	C	L1_160 but wrong, not in frame, because L0 is wrong. Needs repeating		
Y-TIP	HMO_p0_o953	Tonoplast	Cloned from MoClo	C	L1_163-CK3, p5-35Sx2:TLS_Y-TIP-_linker-mTurq	L2_232-CsA, p5-35S:HygR2, p5-35Sx2:TLS_Y-TIP-lk--mTurq	same
ScCOX4	HMO_p0_o952	Mitochondria	Cloned from MoClo	C	L1_159-Ck4, p5-35Sx2:MTS-ScCOX4-mVenus	L2_228-CsA, p5-35S:HygR, p5-35Sx2:MTS-ScCOX4-mVenus	same
PST1	HMO_p0_o954	Peroxisome	Cloned from MoClo	N	L1_164-CK4, p5-35Sx2:eGFP-CTAG_PST1	L2_233-CsA, p5-35S:HygR p5-35Sx2:mVenus-CTAG_PST1	same