Investigating the function of a small secreted protein family in *Physcomitrella patens*

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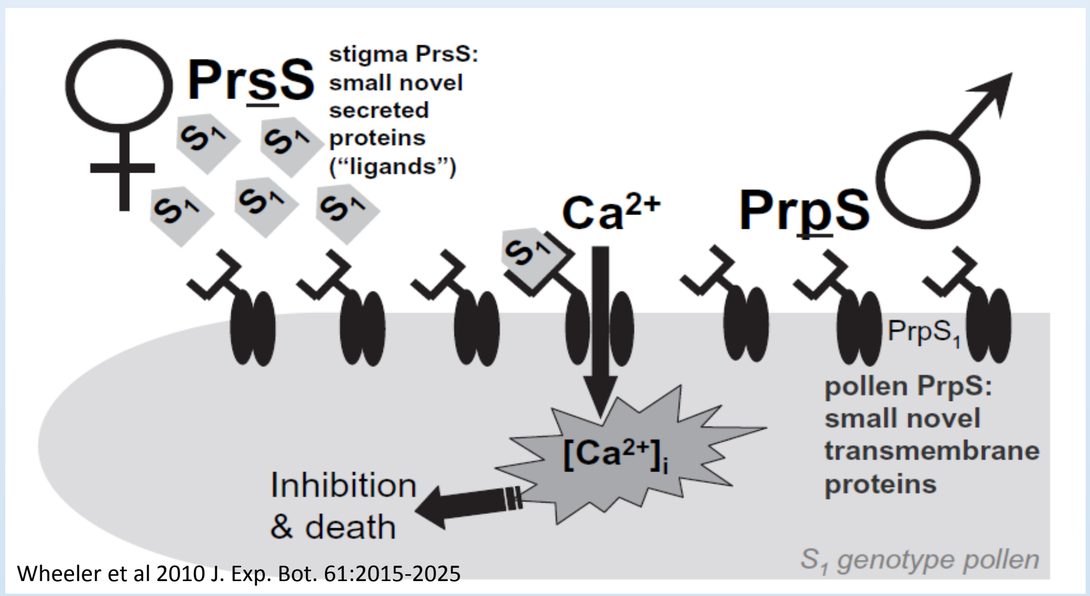
The correct development of multicellular organisms depends upon the perception of signals secreted by cells in order to co-ordinate cell differentiation. The *Physcomitrella patens* genome encodes many components of potential signaling systems, including putative receptor proteins and putative secreted protein ligands, yet at present little characterization of these proteins has been carried out. We are currently attempting to characterize the expression pattern and function of a family of 6 secreted proteins exhibiting homology to PrsS, the ligand that controls self-incompatibility (SI) in *Papaver rhoeas* (field poppy). In poppy, PrsS interacts with a receptor on the surface of pollen tubes, PrpS, causing SI by programmed cell death. Homologues of this protein (SPH – S-Protein Homologues) exist in dicotyledonous plants and bryophytes but not in other plant taxa. We aim to determine spatiotemporal expression differences between these proteins via reporter gene analysis and qPCR of cDNA. In addition we are in the process of creating targeted gene knockouts for all 6 of the (*PhyscoSPH*) genes in *P. patens*. We are also searching for receptors of PhyscoSPHs in *Physcomitrella* using a bioinformatic strategy alongside phage display. In accomplishing this we hope to determine the function of a small novel secreted protein family in *Physcomitrella* and in addition we hope to elucidate the function of SPH proteins in *Arabidopsis*.

SPH and PrsS proteins

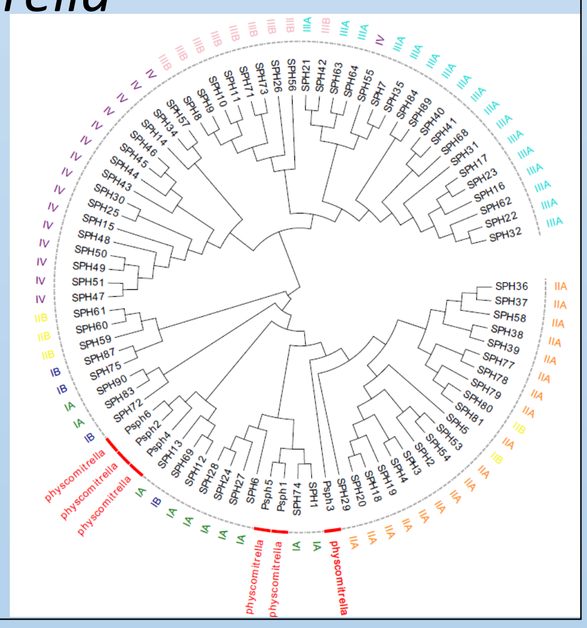


Wheeler et al 2010 J. Exp. Bot. 61:2015-2025

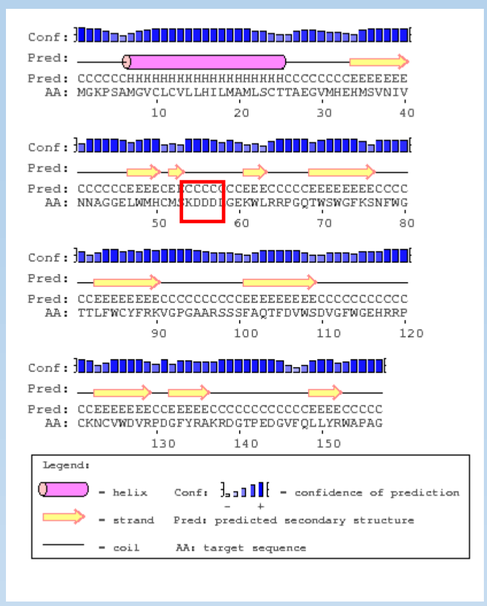
In Poppy SI, polymorphic proteins secreted from the stigma (PrsS) interact with polymorphic receptors on the pollen tube surface (PrpS) in an allele-specific manner (e.g. PrsS1 only interacts with PrpS1) (Wheeler et al 2009). As the genes for both ligand and receptor are completely linked and therefore co-inherited this enables the plant to recognise 'self'. Recognition results in programmed cell death of the pollen tube and hence no self-fertilisation. Since the sequencing of multiple plant genomes we now know there to be homologues of PrsS in other plants (interestingly we have not been able to find homologues of PrpS). These homologous proteins (SPH – **S**-**P**rotein **H**omologues) make up a family of 84 proteins in *Arabidopsis* that exhibit spatiotemporal expression differences (Wheeler et al 2010). As yet the exact function of the SPH proteins in *Arabidopsis* is unknown although mutants of two of them (SPH1 and SPH74) produce plants with a constitutive pathogen response phenotype (Wheeler et al in preparation). The majority of the 84 proteins are expressed in different parts of the flower but the sheer number of genes and the likelihood that many are duplicates makes this family of proteins hard to study in *Arabidopsis*.

SPH proteins in *Physcomitrella*

Phylogeny of all *Arabidopsis* SPH proteins along with the 6 SPH proteins encoded by the *Physcomitrella* genome. The Maximum Likelihood tree was generated using MEGA. Sequences were aligned using MUSCLE with no trimming. Model used for phylogenetic construction was WAG+G. The numerals on the outside relate to SPH subgroups determined on the amino acid composition of hydrophilic loops 2 and 5 (see below). Of the nearest Arabidopsis homologues – SPH1/74 are expressed in immature leaf and stem and SPH12/69 are expressed in pollen.



Primary/secondary structure



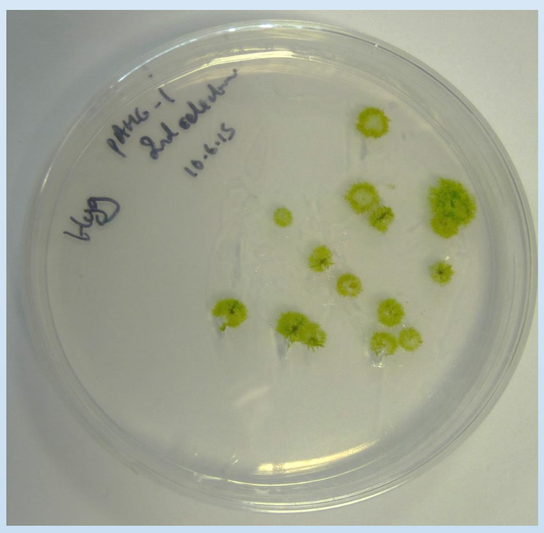
The 6 SPH proteins are heterogeneous but have a conserved secondary structure with 2 completely conserved cysteines which are proximal in the folded protein (Ranjsekar et al in preparation). All SPH proteins including the PrsS proteins share this secondary structural motif. The hydrophilic loop which is known to be critical in the function of PrsS is highlighted in the red box.

Studying *PhyscoSPH*s (1) - knockout strategy

We are in the process of knocking all 6 *SPH* genes out using a homologous recombination approach. The approach utilises deletion plasmids with hygromycin resistance for selection of transformed moss. 1kb - 1.5kb of sequence upstream and downstream of each of the *SPH* genes has been inserted into pAHG1 or pMBL10.

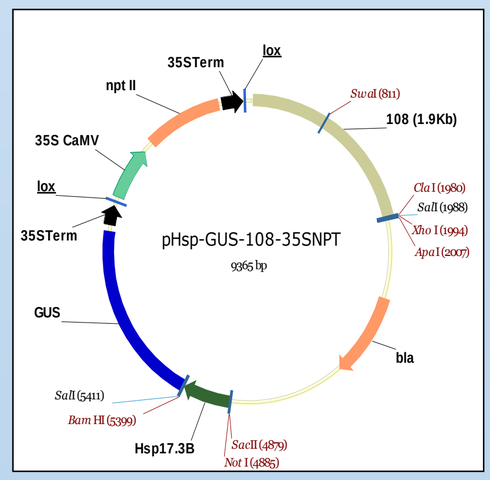
In order to ensure that any duplicate genes are covered *PhyscoSPH5* and *PhyscoSPH6* sequences were placed into pMBL10 (kanamycin/G418 resistance) while *PhyscoSPH1-4* were placed in pAHG1 (hygromycin resistance) so that we can create double mutants if needed.

Physcomitrella patens (Gransden) was transformed according to the methods of Schaefer et al 1991. Briefly protonemal tissue was digested using Driselase for 30 minutes, washed several times with Mannitol before total protoplast number was determined. 15µg linearised pAHG1 or pMBL10 complete with inserts was added to cells at a density of ~1.5 x 10^6ml^{-1}. After 7 days continuous light cellophane discs containing transformed protoplast were transferred to selective media (hygromycin or G418). Two weeks later discs were transferred to non-selective media and incubated for 2 more weeks before transfer back to selective media to select for stable transformants. Currently we are bulking up the number of transformants before analysing any phenotypic changes.



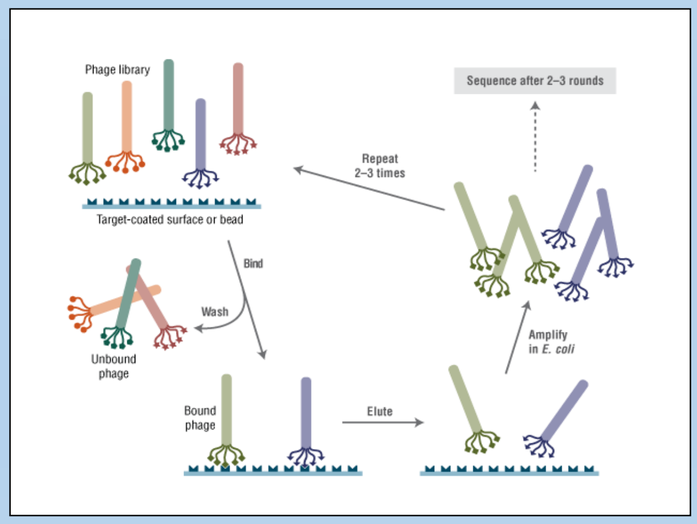
Studying *PhyscoSPH*s (2) - expression analysis

At present we do not know whether the 6 *PhyscoSPH* genes display tight spatiotemporal regulation which may give clues to their function. There is some indication that there are differences in expression during sporophyte development and maturation (O' Donaghue et al 2013). In order to understand the expression pattern of these genes we are utilising a GUS-promoter strategy and also using qPCR on protonemal tissue, gametophore, early sporophyte and late sporophyte tissue.



Studying *PhyscoSPH*s (3) - interacting proteins

Other than the interaction with PrpS in poppy SI (Wheeler et al 2010) we have no indication of what the receptors are for secreted SPH proteins in either *Arabidopsis* or *Physcomitrella*. Homology searching of PrpS has failed to find any homologues and it is likely that this represents an 'orphan gene'. In order to find candidate receptors for PhyscoSPHs and hopefully homologues in angiosperms we are attempting to identify interacting proteins using a phage display approach. A library of phage with 12mer oligopeptides attached to a coat protein is used to screen recombinant PhyscoSPH proteins. After 3 cycles remaining purified clones are sequenced and examined for stretches of common sequence before the *Physcomitrella* protein database is searched for candidate proteins.



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