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

George Sherrard, Gill Woolard and Mike Wheeler

University of Worcester, Henwick Grove, Worcester WR2 6AJ

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, PrpS1, causing SI by programmed cell death. Homologues of this protein (SPH – Secreted Protein Homologues) exist in dicotyledonous plants and bryophytes but not in other plant taxa. We aim to determine spatiotemporal expression differences between these viruses through 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

In Poppy SI, polymorphic proteins secreted from the stigma (PrsS) interact with polymorphic receptors on the pollen tube surface (PrpS1) 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 inherited this enables the plant to recognise ‘self’. Recognition results in programmed cell death of the pollen tube and hence no self-fertilisation. 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 – Secreted Protein Homologues) 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 SPH47) produce plants with a constitutive pathogen resistance so that we can create double mutants if needed. Physcomitrella patens (Giraudes) was transformed according to the methods of Schaefer et al 1991. Briefly protoplast tissue was digested using Driselase for 30 minutes, washed several times with Manitol before total protoplast number was determined. 15μg linearised pPHG1 or pMLB10 complete with inserts was added to cells at a density of ~1.5 x 10^6/mL. After 7 days continuous light cellophane discs containing transformed protoplasts 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 building up the number of transformants before analysing any phenotypic changes.

Studying PhyscoSPHs (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. 10μg -1.5μg of sequence upstream and downstream of each of the SPH genes has been inserted into pAHG1 or pMLB10.

In order to ensure that any duplicate genes are covered PhyscoSPHs and PhyscoSPH6 sequences were placed into pMLB10 (kanamycin/G418 resistance) while PhyscoSPH1-4 were placed in pAHG1 (hygromycin resistance) so that we can create double mutants if needed.

Studying PhyscoSPHs (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’Donahue 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 protenomal tissue, gametophore, early sporophyte and late sporophyte tissue.

Studying PhyscoSPHs (3) - interacting proteins

Other than the interaction with PrsS 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 PrsS 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 we are attempting to use a phage display approach. A library of phage with 12mer oligopeptides attached to a coat protein is used to screen recombinant PhyscoSPHs. After 3 cycles of phage purification clones are sequenced and examined for stretches of common sequence before the Physcomitrella protein database is searched for candidate proteins.

<table>
<thead>
<tr>
<th>SPH proteins in Physcomitrella</th>
</tr>
</thead>
<tbody>
<tr>
<td>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 – SPH1/14 are expressed in immature leaf and stem and SPH12/69 are expressed in pollen.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary/secondary structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>The 6 SPH proteins are heterogeneous but have a conserved secondary structure with 2 completely conserved cysteines which are proximal in the folded protein (Ranjekar 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.</td>
</tr>
</tbody>
</table>


