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Overexpression of Full-length Expansin A4 or its N-terminal Domain is Toxic in *Physcomitrium patens*

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ABSTRACT

Plant expansins are a large family of cell-wall anchored proteins involved in cell wall loosening, plant growth and development, and organogenesis. The objective of this project was to identify an expansin gene from the model plant, *Phycomitrium patens*, that is highly expressed in tip-growing protonemata, and use it to localize the sites of active secretion. We identified PpEXPA4 as a gene highly expressed in protonemata and created constructs of PpEXPA4 tagged with either a C-terminal HaloTag or a C-terminal triple fluorescent protein. For our initial study, we expressed the proteins from a strong constitutive promoter. Our hypothesis was that PpEXPA4 would be secreted and accumulate on the cell wall. Instead, we found that high levels of expression of PpEXPA4 were toxic to the cell. To alleviate this and partially disrupt expansin's activity, we removed the C-terminal domain of PpEXPA4 and created tagged constructs containing only the tagged domain 1 of PpEXPA4. Expression of this construct, even at lower concentrations, was also found to be toxic. Although the tagged protein was identified in the cell's interior, none could be identified on the cell wall. Our results suggest that the toxicity of PpEXPA4 at high levels is due to domain 1 of the protein. Future studies should evaluate the localization and toxicity of PpEXPA4 expressed from its endogenous promoter as well as the overexpression of domain 2.

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Lastly, I would like to thank Richard Thyden, who's initial project proposal was unfortunately not achieved, but set the groundworks for this project to begin

CHAPTER 1. INTRODUCTION & LITERATURE REVIEW

1.1 Expansins

Expansins are a large family of cell-wall anchored proteins with a total of four subgroups, alpha-expansins (EXPA), beta-expansins (EXPB), expansin-like A, and expansin-like B. For this project we chose to focus on alpha-expansins, also known as expansin As. These proteins are important in cell wall loosening, plant growth and development, are involved in organogenesis and a number of other processes (Carey & Cosgrove, 2007). They are relatively short proteins, about 250-275 amino acids comprising two domains, with domain one of EXPA being similar to the p12 and barwin genes, and domain two is most similar to the grass group-2 pollen allergens (G2A) gene of *Phleum pretense* (Sampedro & Cosgrove, 2005). As they get anchored in the cell wall, and are relatively small proteins, they may be a vital group of proteins in localizing the sites of active secretion at the cellular level.

The barwin and p12 genes also share introns with domain 1 of expansin, with p12 sharing two intron locations and barwin sharing one intron location. The p12 gene is found in the xylem of blighted citrus trees and is known to participate in a signaling function, but no cell-wall loosening functions have been identified (Sampedro & Cosgrove, 2005). Barwin is part of the pathogenesis-related protein-4 family (PR-4), which are known to express in the event of wounding or pathogen presence in barley plants (Franco et al., 2018). No homologous introns were found between G2A and domain 2 of expansin and their biological function is not known. However, they still share 35-45% identity, suggesting a common ancestor and function (Sampedro & Cosgrove, 2005).

Although it is not known when expansins first appeared in plants, the expansin-like A and B families are traced back to the last ancestor of gymnosperms and angiosperms (Sampedro & Cosgrove, 2005). As time went on, the families continued to grow and diversify, with the last

common ancestor of *Arabidopsis*, poplar, and rice having 12 EXPA genes, and rice having 34 (Sampedro & Cosgrove, 2005).

As *Physcomitrium patens* is a bryophyte, it contains no EXLA or EXLB genes, suggesting these arose after the split of *P. patens* and angiosperms. However, intron patterns of EXPA and EXPB that relate to angiosperm intron patterns support their ancestral relation. Especially the EXPB genes in *P. patens*, which have heavily conserved areas of the binding surfaces of the proteins to angiosperms (Carey & Cosgrove, 2007). The relation between *P. patens* and angiosperms expansin genes is still there, but the superfamily itself is beginning to change as each expansin takes up a possibly different role in the cell.

Although the family continues to evolve, there are still similarities between the subfamilies and between the species. Highly conserved regions of EXPA and EXPB genes between species, shown in **Figure 1**, suggest the functions of the proteins are likely the same in *P. patens* as they are in angiosperms (Sampedro & Cosgrove, 2005). Most of the changes in the gene sequences between the species are in-between the conserved areas (**Figure 2**), having little effect on the protein's function.

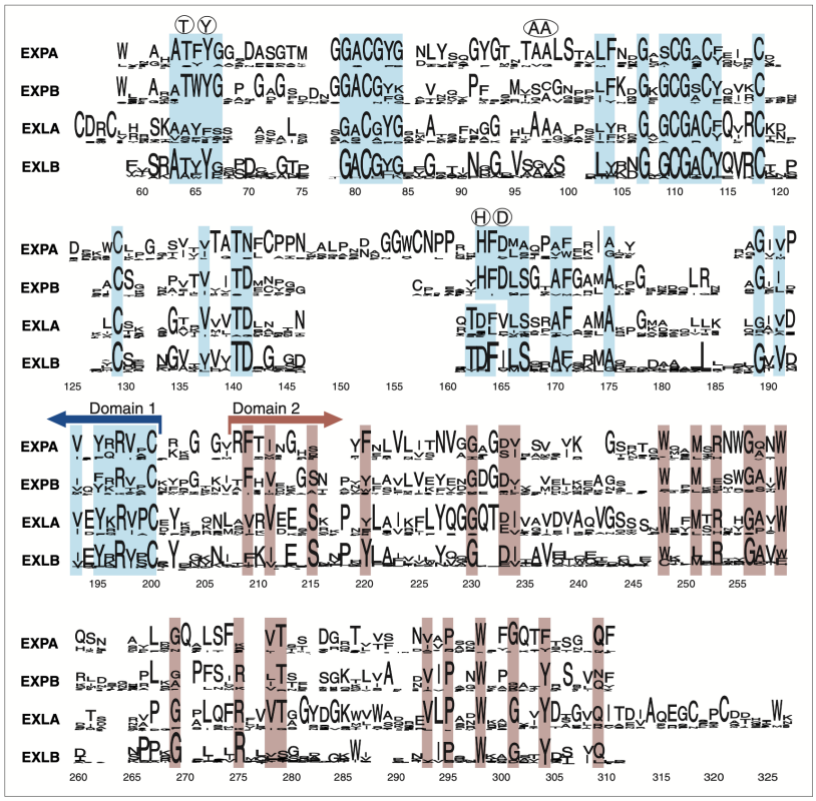


Figure 1. Areas of conservation between EXPA, EXPB, EXLA, and EXLB genes. Domain 1 areas of conservation are highlighted in blue and domain 2 areas of conservation are highlighted in brown. Figure adapted from (Sampedro & Cosgrove, 2005).

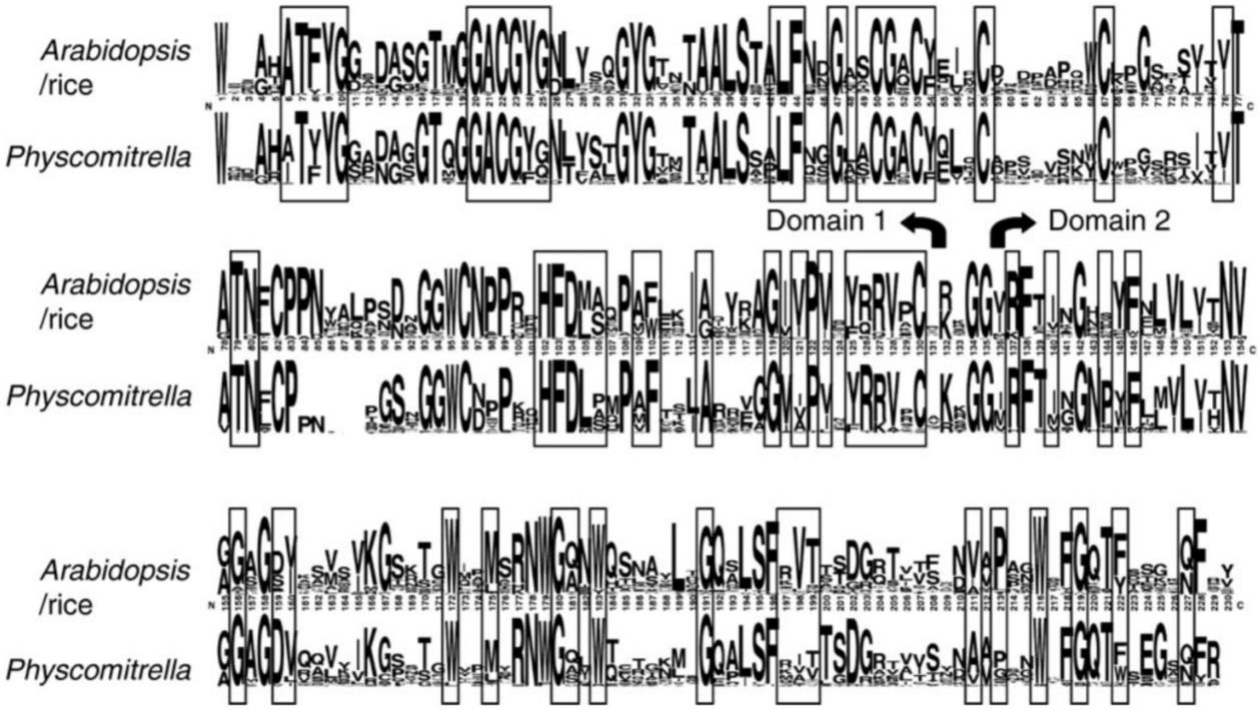


Figure 2. Conserved regions of the amino acid sequences of EXPA genes from *Arabidopsis*/rice and *Physcomitrella patens.* Areas of high conservation are denoted by larger letters and boxes. The two arrows point in the direction of domain 1 and domain 2. Figure adapted from (Carey & Cosgrove, 2007).

If the expansin genes are similar enough between species that their functions are likely the same, which species would be most suitable for this project? Many of the species that have been studied have varying numbers of EXPA and EXPB genes, and some contain EXLA and EXLB genes. Although the function of the EXLA and EXLB genes are not as well understood, the abundance of EXPA and EXPB genes is likely to factor into what species to select for localizing the sites of active secretion.

1.2 *Physcomitrium patens*

Plant-based models have been an emerging alternative as they are scalable and much safer hosts compared to bacterial model systems (Reski, Parsons, & Decker, 2015). Particularly mosses have

been being used in biotechnology due to their low structural complexity and primarily haploid gametophytic growth. Belonging to the bryophyte group, they lack true roots and require a wet environment for growth (Decker & Reski, 2020), and are typically stable over long periods of time compared to other plant cultures (Reski et al., 2015). Mosses can be grown in self-contained systems in pure mineral media including petri dishes, Erlenmeyer flasks, and bioreactors (Reski et al., 2015), making them an easy model organism for this project.

One such moss known as *Physcomitrium patens* (**Figure 3**) has been used in research because of its comparably easier and precise genome-engineering via homologous recombination, ability to grow in large bioreactors up to 500 L, homogeneity of protein products, and stability of products from batch to batch (Reski, 2018). Because of its easy genome-engineering, creating stable lines of moss expressing our protein of interest should be simpler compared to other model organisms. Not only is *P. patens* an ideal target for a project concerning genetic engineering, it also contains a large number expansin proteins in the EXPA subfamily, being the largest out of *A. thaliana, Oryza sativa,* and *Populus trichocarpa* (Carey & Cosgrove, 2007).

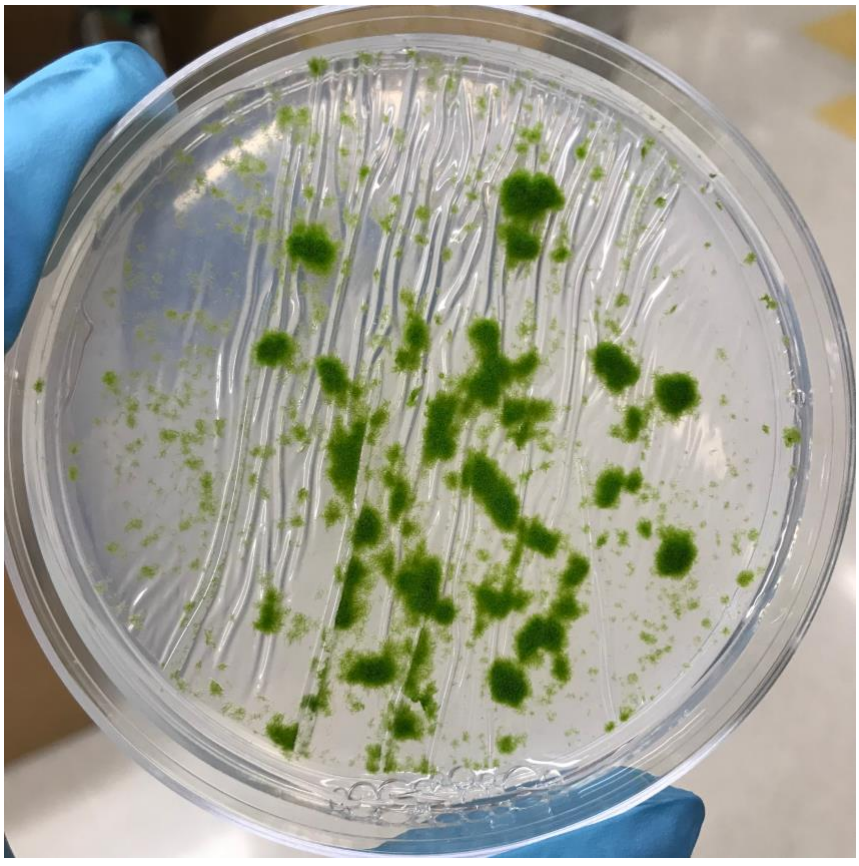


Figure 3. *P. patens* Gransden line grown on cellophane over $PpNH_4$ solid media.

1.3 *Physcomitrium patens* and expansins

For this project, we decided to focus on expansin As, as this subfamily is the largest in *Physcomitrium patens,* consisting of 27 different genes and only 7 EXPB genes (Carey & Cosgrove, 2007). As previously mentioned, Expansin-like A and expansin-like Bs are also absent in *P. patens,* indicating that these genes arose after a divergence of bryophytes from vascular plants (Carey & Cosgrove, 2007). This may also mean that the large subfamily of EXPAs is due to duplications of genes throughout time. The fact that these expansins are still present in *P. patens* suggests that they now serve a new and specific purpose. Expression levels of each EXPA also support this, as the different genes are each expressed in different cell types and at different levels throughout the plant (see Appendix A).

1.4 SNAP tag vs. HaloTag

In order to know whether our experiment is successful, a tag is needed to identify the target protein. We were interested in two tags, the SNAP tag and the HaloTag, each with their own uses and benefits. The SNAP tag was looked into because of its ability to attach to either the N- or C-terminus of a protein and its ability to be detected using fluorescent scanning and SDS-page gels (Cole, 2013). The SNAP tag has also been successfully used in plants before. Iwatate et al (Iwatate et al., 2020) demonstrated the use of the tag in *Arabidopsis thaliana,* using multiple varieties of the tag and comparing how it worked with different binding targets.

On the other hand, the HaloTag can be used in protein purification, molecular imaging, protein assays, and *in vitro* cellular imaging (England, Luo, & Cai, 2015). The HaloTag can be additionally tagged using fluorescent proteins such as GFP and mCherry, resulting in a much brighter image compared to the SNAP tag while also being less prone to bleaching rapidly (Erdmann et al., 2019).

And although more difficult compared to the SNAP tag, N- and C- terminus versions of the HaloTag can be created to attach to the opposite terminus protein. Based on this information, we decided to use the HaloTag in this project, primarily because of its ability to be internally and externally stained using the HaloTag Ligand TMR and Alexa488.

1.5 Objective of the project

From this background research, we were able to select a gene family from the model plant *Physcomitrium patens* which is anchored in the cell wall. Once a specific gene has been selected, it can be used to localize the sites of active secretion at the cellular level using the HaloTag fluorescent staining, and if necessary, purify the protein for further investigation. To accomplish this, we selected a gene from the EXPA family based on our own research into phylogenetics and expression levels and localization patterns of each EXPA gene known in *P. patens*.

CHAPTER 2 – METHODOLOGY

2.1 Moss plating and cultures

Moss cultures were started from the Gransden cell line. Seven-day old moss was removed from its $PpNH_4$ plate, grinded for 15-20 seconds, and re-plated on 2 – 4 $PpNH_4$ plates. Cellophane was placed over $PpNH_4$ media to enable easier removal of moss from the plate. Moss was incubated in a growth chamber for later use. The growth chamber is set at 25˚C and cycles through 16 hours of light and 8 hours of darkness.

2.2 Phylogenetics, expression levels, & localization

To begin, an extensive look into expansin phylogenetics was performed. Using databases of documented expansin protein sequences from both *P. patens* and *A. thaliana*, sequence alignments were performed using Geneious and a phylogenetic tree was created (**Figure 7**). The phylogenetic tree created contained all known expansin A genes in both species and was compared to the tree created in the Expansin superfamily paper (Carey & Cosgrove, 2007). To maintain consistency in naming, the proteins were sequence matched and renamed in our tree to match the naming used in the Carey & Cosgrove paper.

In order to select an expansin A to work with, the expression levels and localizations of each protein were determined using the Physcomitrella eFP Browser, a database with *P. patens* expression localizations. Each expansin A in the database was recorded and analyzed for possibly selection (Appendix A). The target location for expression was in tip-growing protonemata, specifically the caulonema, where EXPA 4 had the highest expression, and was localized to only that area (**Figure 8**).

The EXPB genes from *P. patens* were also researched and their expression levels and localizations recorded in Appendix B. Each expansin B was also sequence matched to the genes presented in the Carey et al paper and re-named accordingly.

2.3 Vector design and construction

Sequences for the HaloTag N terminus, C terminus, and EXPA 4 were imported into Geneious. A forward and reverse primer were added to the EXPA 4 gene and attB1 and attB5r sites on either side for use in Gateway cloning. The gateway cloning vector pDONR221-P1P5r was used to clone the EXPA 4 gene in virtually and create the pENT construct. This was also performed to generate the HaloTag N terminus tagging construct. For the C-terminus tagging construct, the HaloTag gene was virtually amplified by primers for flanking with attB5 and attB2 sequences, and the PCR product cloned into the vector pDONR221-P5P2. After the constructs were created, the sequences containing the att sites were sent to Genewiz for construction into a plasmid with kanamycin resistance.

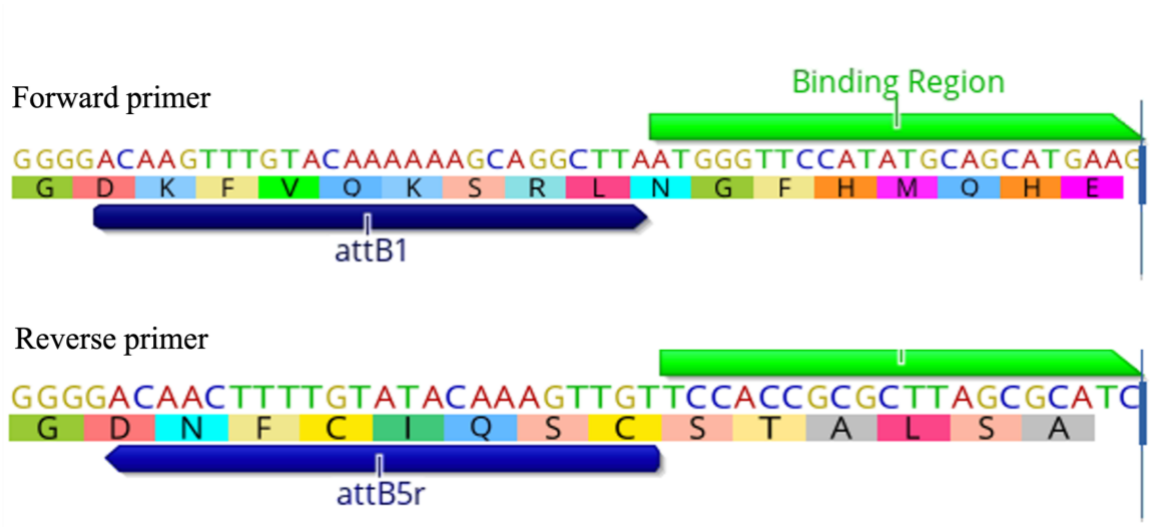


Figure 4. Forward and reverse primers used in the PCR procedure to amplify domain 1 of EXPA4 highlighted in green. The attB1 and attB5r sites were added for insertion into the pDONR221-P1P5r vector after purification of the PCR product.

A subsequent EXPA 4 domain 1 construct was also created. **Figure 4** shows the primers that were created virtually in Geneious which defined the region of domain 1 in EXPA 4. Using the primers, PCR was used to amplify the region (Section 2.14). One amplified, the EXPA4d1 gene with flanking attB1 and attB5r sites was inserted into the pDONR221-P1P5r vector through a BP reaction (**Figure 6**).

2.4 BP reaction of target gene and entry vector

N-terminus proteins were BP cloned into the pDONR221-P1P5r entry vector and C-terminus proteins were BP cloned into the pDONR221-P5P2 entry vector. The entry vector and target gene vector were combined (0.5-1 µL target gene vector, 3.5 µL entry vector) in a tube with 1 µL clonase II enzyme and incubated at room temperature overnight or up to 24 hours. The reaction was stopped by pipetting 0.5 µL of proteinase K to the tube and incubating for 10 minutes at 37˚C. *E. coli* transformation and selection for correct constructs are continued in sections 2.6 – 2.8.

2.5 LR reaction of two vectors

Seventy-five ng of each vector was added to one tube with 75 ng of the entry clone, pTH-Ubi-Gateway. TE buffer was added to 4 mL. The whole mixture was added into a tube containing a clonase enzyme, vortexed briefly, centrifuged for 30 seconds, and then incubated overnight (~16 hours) at 25˚C. Proteinase K (0.5 µL) was added to each tube to stop the reaction, vortexed, and centrifuged. *E. coli* cells were transformed with whole LR mixture. **Figure 5** shows one of the constructs created in this project – pTH-Ubi-EXPA4d1-HaloTag. The remaining constructs can be seen in Appendix C.

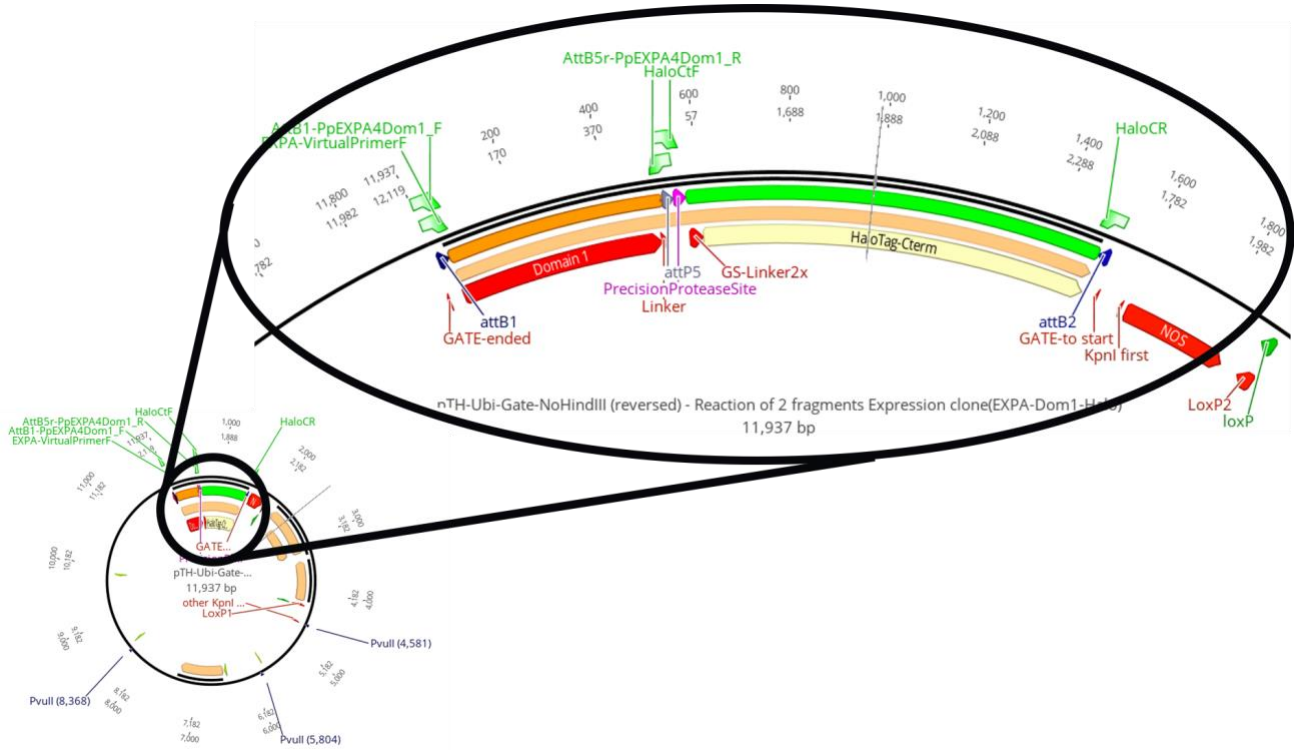


Figure 5. The pTH-Ubi-EXPA4d1-HaloTag construct created virtually using Geneious Gateway reactions. In red is the EXPA4d1 gene and in yellow is the HaloTag gene. A hygromycin resistance gene is also coded in the construct, but not shown. Restriction enzymes were selected using Geneious for restriction enzyme analysis after transforming *E. coli* with the DNA.

2.6 Bacteria transformation

DH5 alpha *E. coli* cells were thawed on ice for 10 minutes and split into tubes, 25 µL each. LR (or BP) reaction mixtures were added to corresponding *E. coli* tubes and sat on ice for 30 minutes. Cells were heat shocked at 42˚C for 30 seconds and then iced for 5 minutes. After, 250 µL LB was added to each tube and then incubated while rotating at 37˚C for one hour. Carbenicillin plates were warmed in the incubator during this time. After one hour, tubes were centrifuged for 1 minute at max speed and 150 µL of supernatant was pipetted out. Pellet was resuspended with remaining LB, plated on warmed plates, and spread with glass beads. Plates were incubated at 37˚C overnight.

After incubating overnight, colonies were selected and put into 2 mL of LB and 2 µL of carbenicillin (1:1000) and incubated at 37˚C while rotating overnight to prepare for DNA miniprep.

2.7 DNA miniprep

DNA from the selected colonies was purified using the Zyppy Plasmid Miniprep Kit procedure:

E. coli cultures previously transformed with constructs from LR reactions were transferred into 1.5 mL tubes and centrifuged for 30 seconds and the supernatant discarded. The pellet was resuspended in 600 µL H_2O and 100 µL 7x lysis buffer was added and tubes were mixed by inversion. After incubating at room temperature for 2 minutes, 350 µL cold neutralization buffer was added and mixed thoroughly. Tubes were centrifuged for four minutes. The supernatant was transferred into Zymo-spin INN columns and centrifuged for 30 seconds. The flowthrough was discarded and 200 µL of Endo wash buffer was added. The tubes were centrifuged for 30 seconds, the flowthrough discarded, and 400 µL Zyppy wash buffer was added. After centrifuging for 1 minute, the columns were transferred to 1.5 mL tubes. Forty µL of Zyppy elution buffer was added and centrifuged for 1.5 minutes. The columns were discarded and the remaining supernatant was saved. Concentrations of each sample prepared were taken using the Nanodrop with the Zyppy elution buffer as the blank.

2.8 Restriction enzyme analysis

Restriction enzymes were selected using the virtual gel function in Geneious. DNA was prepared to be digested by creating a solution with 150 ng of DNA, 0.3 µL of chosen restriction enzyme,

2.0 µL cutsmart buffer or NEB 3.1 buffer, and sterile H_2O to bring the final volume up to 20µL. The mixtures were incubated at 37˚C for 1 hour.

Agarose gels were made using 1x TBE (40 mL for short, 80 mL for long) and SeaKem LE agarose (0.32 g short, 0.64 g long) to have a final concentration of 80% agarose. Safe DNA gel stain was added (4 µL short, 8 µL long) before pouring the gel. Four µL of gel loading dye purple 6x was added to each sample before loading. Purple 1 KB ladder was used as the DNA ladder. Gels were run at 200 V for 20+ minutes depending on length.

2.9 DNA maxiprep

DNA from the selected colonies was purified using the ZymoPURE Plasmid Maxiprep Kit:

E. coli cultures for the maxiprep were prepared using 1mL of starter culture in 150 mL LB and 150 µL carbenicillin. Cultures were incubated at 37˚C overnight while shaking.

Cultures were centrifuged for 10 minutes at 4500x g using the JA-10 insert. The supernatant was discarded and 14 mL of Zymo Pure P1 was added to resuspend the pellet. Fourteen mL of Zymo Pure P2 was added and mixed by inverting. After incubating at room temperature for 2-3 minutes, 14 mL of Zymo Pure P3 was added and mixed by inversion. Zymo Pure syringe filters were set up with 50 mL conical tubes placed underneath. Lysate was poured into filters and allowed to sit for 8 minutes. The lysate was pushed through the filter using a syringe. Fourteen mL of Zymo Pure binding buffer was added to the filtered lysate and mixed by inversion.

The vacuum manifold was set up with Zymo-spin V-P columns. The lysate mixture was poured into the column and the vacuum was turned on until all the lysate had gone through the filter. Five mL of Zymo Pure wash 1 was added and the vacuum turned on. Five mL of Zymo Pure wash 2 was added and the vacuum turned on; this step is repeated once. The columns were placed into

collection tubes, centrifuged for 1 minute, and the supernatant discarded. The columns were transferred to 1.5 mL tubes and 400 µL of Zymo Pure elution buffer was added directly onto each filter. After 2 minutes of room temperature incubation, tubes were centrifuged for 1.5 minutes and the filters discarded.

A 1:10 dilution was created for each sample using the Zymo Pure elution buffer and the concentrations of each dilution were taken using the Nanodrop with the Zymo Pure elution buffer as the blank.

2.10 Linearizing DNA

A mixture of 590 µL H_2O, 120 µg DNA, 60 µL NEB 3.1 buffer, and 10 µL SWA1 enzyme was created for each vector being linearized. Mixtures were inverted to mix and incubated at room temperature for 2 – 4 hours. Sixty µL of 3M NaOAc pH 5.2 was added and mixed. Solutions were each split equally into two tubes and 800 µL of absolute ethanol was added. Tubes were incubated at -20˚C for 30 minutes and then centrifuged at max speed for 5 minutes. The supernatant was discarded and 500 µL of 70% ethanol was added. Pellet was not resuspended, but ethanol was instead carefully passed over the pellet to wash. The tubes were centrifuged for another 5 minutes at max speed.

Materials were moved to sterile hood after centrifuging. The supernatant was discarded, pipetting out any remaining liquid. Tubes were tapped down on a kimwipe and left facing the fan of the hood to dry for 20 – 30 minutes. 50 µL sterile TE buffer was added to each tube, but pellet was not responded. The tubes were left for 2 – 4 minutes to rehydrate, then moved to incubate at 37˚C for 10 minutes. Tubes were then moved to room temperature for at least 10 minutes, then moved to 4˚C fridge to store until use.

2.11 Stable & transient moss transformations

Seven-day-old Gransden moss was protoplasted for one hour at room temperature using 2% driselase in 8% mannitol. The solution was filtered into 50 mL tubes and flowthrough was transferred into 15 mL tubes. Solutions were centrifuged for 5 minutes at 700 rpm/250 g and the supernatant discarded. Protoplasts were resuspended in 8% mannitol, inverted to mix. Centrifuged for 5 minutes at 700 rpm/250 g and repeated centrifuging, discarding, and resuspending two more times. Pipetted 10 µL of the protoplast solution onto a cell-counter plate. Protoplasts were counted in four sections of the counter plate and the appropriate amount of MMg to use was calculated:

$$\frac{protoplasts\ counted}{4} \times 10{,}000 = \frac{protoplasts}{mL} \times 10mL = \frac{total\ protoplasts}{1.6 \times 10^6} = mL\ MMg$$

Centrifuged protoplast solution for 5 minutes at 700 rpm/250 g and discarded the supernatant. Pipetted the appropriate volume of MMg medium and resuspend the protoplasts for a final concentration of 1.6 x 10^6 protoplast/mL of MMg. The solutions were allowed to sit for 20 minutes at room temperature.

Six hundred µL of protoplast solution was added to 60 ng DNA and mixed gently (used linearized DNA if performing a stable transformation). Seven hundred µL of PEG 4000/Ca was then added and mixed gently and allowed to sit for 30 minutes at room temperature. The solution was diluted with 3 mL W5 medium and mixed by inversion. Centrifuged for 5 minutes at 700 rpm/250 g to remove PEG and discarded supernatant. Protoplasts were resuspended with 2 mL of PRMT/$CaCl_2$. The resulting solution as split into two plates (PRMB medium with mannitol to avoid osmotic shock) with 1 mL each and spread evenly by the tilting plate. The plates were then taped closed

with micropore tape and labeled accordingly. The moss was incubated for four days in the growth chamber.

Only for stable transformation:

After four days, the moss was transferred onto hygromycin plates for selection and incubated for another 7 days. Colonies that survived were placed onto hygromycin plates for further selection and incubated for another 7 days or longer. Once large enough, the colonies were grinded and plated onto $PpNH_4$ and allowed to grow for further experimentation.

2.12 Internal moss staining and imaging

The moss used was transiently transformed moss 4 days after transformation. In a sterile hood, 14 mL of $PpNH_4$ was added to each plate of moss to be stained. $PpNH_4$ was carefully passed over moss to resuspend the cells and pipetted back up and into a 15 mL tube. Solutions were centrifuged for 5 minutes at 700 rpm/250 g and the supernatant was removed using a plastic pipette until ~1mL remained. The pellet was resuspended, transferred to a 1.5 mL tube, and centrifuged for 5 minutes at 700 rpm/250 g.

One µL of HaloTag Ligand TMR was resuspended in 500 µL of $PpNH_4$ and stored in a dark place for use. The supernatant was removed from the pellets and 80 µL of the HaloTag Ligand TMR solution was added, and the pellet resuspended. The moss was incubated for 45 minutes at room temperature in a dark place. After incubating, the moss was centrifuged for 5 minutes and the supernatant discarded. One mL of $PpNH_4$ was added, and the pellets resuspended. After centrifuging for another 5 minutes and the supernatant discarded, 250 µL of $PpNH_4$ was added, and the pellets resuspended again. The moss was centrifuged for another 5 minutes and the supernatant pipetted out until ~30 µL remained. The pellets were resuspended and 30 µL was

pipetted onto a glass slide. A glass cover was placed over the moss and the edges sealed with wax. The slides were then imaged using a fluorescent microscope.

2.13 External moss staining and imaging

The moss used in this procedure was more developed than the moss used in the internal staining. Moss was selected directly off each plate (~200 µL) using tweezers and put into a 1.5 mL tube. Moss that was less developed was selected. The moss was resuspended in 200 µL of $PpNH_4$. In a tube with 1 µL of HaloAlexa488, 200 µL of PpNH4 was added (5X). Fifty µL of the stock dye was added to each tube (four total) and incubated at room temperature in the dark for 1 hour.

After incubating, the moss was washed with $PpNH_4$ by pipetting out the liquid and adding 1 mL of $PpNH_4$. This was repeated three times. For the last wash, approximately 500 µL was left in the tube and a small amount was pipetted onto a slide for imaging using a fluorescent microscope. As the moss was more developed, the tip of the pipette was cut off to fit.

2.14 PCR of EXPA 4 domain 1

The primers were centrifuged for 3 minutes and then diluted with H_2O, 36 µL of the forward primer to 3.6 nmol and 131 µL of the reverse primer to 13.1 nmol. A 1:10 dilution was created using 2 µL of each stock primer created and 18 µL H_2O. 1µL of each primer was added to 4 µL of EXPA4-L1R5, 0.2 µL polymerase, 4 µL of 5x Phusion HF reaction buffer, 9.4 µL H_2O, and 0.4 µL dNTP (deoxynucleotide solution mix). The mixture was centrifuged briefly for 10 seconds. The reaction was run on the Phusion setting at a 66˚C annealing temperature. A gel was run of the PCR product and the DNA recovered using gel purification (**Figure 6**).

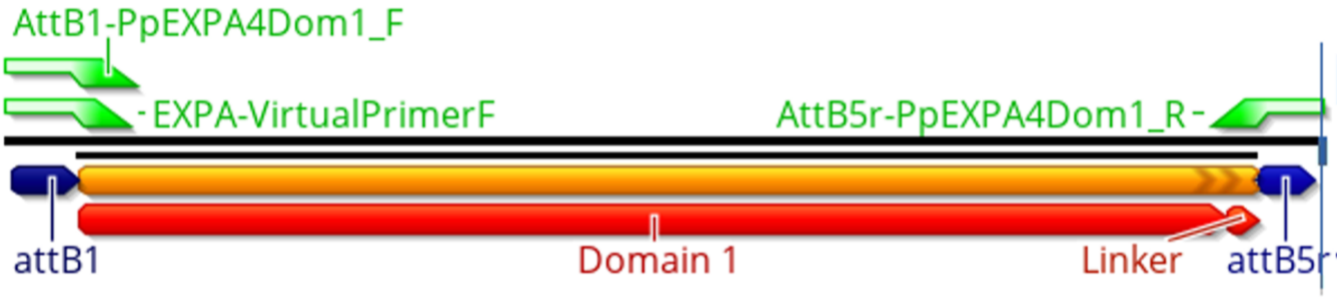


Figure 6. PCR amplified product of domain 1 of EXPA4 from the EXPA4-L1R5 vector. Flanking attB1 and attB5r sites were used in a BP reaction to insert the gene into the pDONR221-P5P2 vector.

2.15 Gel purification

DNA from the PCR reaction was recovered using the Zymoclean gel DNA recovery kit:

The band was cut from the gel under UV light using a razor. The gel was weighed in a 1.5 mL tube and 330 µL of ADB was added (1 g gel = add 1 mL ADB). The mixture was incubated at 45˚C for 10 minutes until dissolved. The solution was transferred to a filter and centrifuged for 1 minute and the flowthrough discarded. Two hundred µL of a DNA wash buffer was added to the filter and centrifuged for 30 seconds. The flowthrough was discarded and another 200 µL of the DNA wash buffer was added and centrifuged for 30 seconds. The filter was transferred to a 1.5 mL tube and 8 µL of DNA elution buffer was added onto the filter and centrifuged for 1 minute. The concentration was taken using the Nanodrop with the DNA elution buffer as the blank.

2.16 Western blot using anti-HaloTag and anti-GFP antibodies

After transforming moss with a construct and resuspending in 1 mL of PRMT/$CaCl_2$, the moss was left in its tube and incubated in the growth chamber for two days. After two days, the solution

was transferred to a 1.5 mL tube and centrifuged for 6 minutes in a cold room centrifuge. The supernatant was pipetted out and the moss was stored in a -80˚C freezer until use during the western blot.

Moss was removed from the freezer and placed on ice to defrost. 100 µL of lysis buffer was added and cells were muddled 10 times while rotating the muddler. The tubes were centrifuged for 10 minutes in the cold room centrifuge. 80 µL of the supernatant was transferred to a 1.5 mL tube and 5 µL DTT and 25 µL SDS was added and immediately transferred to a heat block set to 95˚C. After 7 minutes the tubes were removed.

A gel buffer was prepared using 100 mL 10x MOPS buffer, 900 mL H_2O, and 5 mL 20% SDS. The gel compartment was filled with the buffer and loaded with the samples. 10 µL of the protein ladder and 30 µL of each sample was used. The gel was run for approximately 35 minutes at 200 volts.

The gel was removed from the compartment and cracked open to remove. The teeth and foot were removed, and the gel was resuspended face down in 1x transfer buffer. Two filters and a membrane were hydrated in 1x transfer buffer. The transfer compartment was assembled. In a cold room, the transfer compartment was filled to the “blotting” line with 1x transfer buffer and run overnight at 30 volts and 99mAmps.

After running overnight, the membrane was removed from the compartment and the ladder lines marked with a pen. The membrane was briefly covered with Ponceau Red 0.1% and washed off with water. The membrane was immersed in H_2O and placed on the shake table for approximately 10 minutes.

A TBSt buffer was prepared using 900 mL of H_2O, 100 mL 10x TBS pH 2.5, and 500 µL Tween polysorbate 20. A milk solution was created using 5 g nonfat dry milk and 100 mL of 1x TBSt

buffer previously made. H_2O was poured out from the membrane, 50 mL of milk solution was added, and the membrane was shaken for 1 hour.

The milk was poured out and the membrane was resuspended in 1x TBSt buffer. 1mL of milk was added to 5 µL of the HaloTag antibody (1:1000). This mixture and another 4 mL of milk were added to the membrane and placed on the shake table for at least 1 hour. A longer period allows for better antibody blotting. After blotting, the milk was discarded, and the membrane quickly washed with 50 mL of 1x TBSt. Three 10-minute washes were performed using 50 mL 1x TBSt. A secondary antibody wash was performed using 3.3 µL of an anti-mouse IgG HRP conjugate and 10 mL of milk for one hour. A quick wash was performed using 50 mL 1x TBSt and three 10-minute washes were performed using 50 mL 1x TBSt.

A solution of 10 mL of ECl and 33.3 µL H_2O_2 was created. The membrane was rehydrated in this solution briefly, and then transferred onto the tray for the imager. The blot was imaged using 10 second cumulative intervals and the images were saved. If necessary, the membrane was stored in 1x TBSt in a 4˚C fridge for later use.

2.17 Media procedures

- Liquid LB for miniprep and maxiprep

Liquid LB was created using 150 mL H_2O and 3.75 g LB powder. These were autoclaved before use.

- PpNH4 (liquid and solid)

Solid $PpNH_4$ media was created using 1 L H_2O, one bag $PPNO_3$ moss medium, 0.5 g diammonium tartrate, and 8 g plant agar. This was stirred for 30 minutes and autoclaved. The media could be left to solidify or plated when cooled.

Liquid $PpNH_4$ media was created using 1 L H_2O, one bag $PPNO_3$ moss medium, and 0.5 g diammonium tartrate. This was stirred for 30 minutes and autoclaved.

- PRMB

PRMB media was created using 1 L H_2O, one bag $PPNO_3$ moss medium, 0.5 g diammonium tartrate. This was stirred for 30 minutes and autoclaved. When the media cooled to approximately 45˚C, 10 mL of 1M $CaCl_2$ was added and mixed. The media was plated.

- Hygromycin media

Hygromycin media was created using 1 L H_2O, one bag $PPNO_3$ moss medium, 0.5 g diammonium tartrate. This was stirred for 30 minutes and autoclaved. When the media cooled to approximately 45˚C, 300 µL 50 mg/mL hygromycin was added for a final concentration of 15 µg/mL. The media was plated.

- Carbenicillin media

Carbenicillin plates were created using 1 L LB media and 500 µL carbenicillin for a final concentration of 15 µg/mL. The media was plated.

CHAPTER 3 – RESULTS

3.1 Phylogenetics, expression levels, & localization

To select a suitable expansin for our vector construct, we conducted a phylogenetic analysis. **Figure 7** shows the expansin As from *P. patens* and *A. thaliana*. The three *P. patens* genes grouped at the bottom with the *A. thaliana* genes were analyzed first for their sequence similarity. The expression patterns and levels of the genes were looked up using the Physcomitrella eFP Browser, a database with *P. patens* gene expression levels in different tissues. For this project, tip-growing protonemata, specifically in the caulonema area of the plant, was the desired expression location. The three *P. patens* genes that we looked at did not have the desired expression patterns (expression levels seen in Appendix A). The remaining *P. patens* EXPAs expression patterns and levels were looked up. **Figure 8** shows the expression pattern and levels of EXPA4, highlighted in the phylogenetic tree in green. This gene expresses almost exclusively in the caulonemata, and at a considerably higher level compared to other genes (14692 units compared to others expressing at 1000 to 4000 units), so it was selected for use in the project.

Expansin As from *P. patens* were also matched according to the labeling system used in the article by Carey and Cosgrove (Carey & Cosgrove, 2007) in order to simplify the naming system. Expansins that did not have a match were left unnamed. Expansin Bs were also matched to the Carey and Cosgrove paper and their expression patterns and levels were recorded from the database. The expression levels and patterns were analyzed for a possible second protein of interest (Appendix B), but EXPB genes were not used in this project.

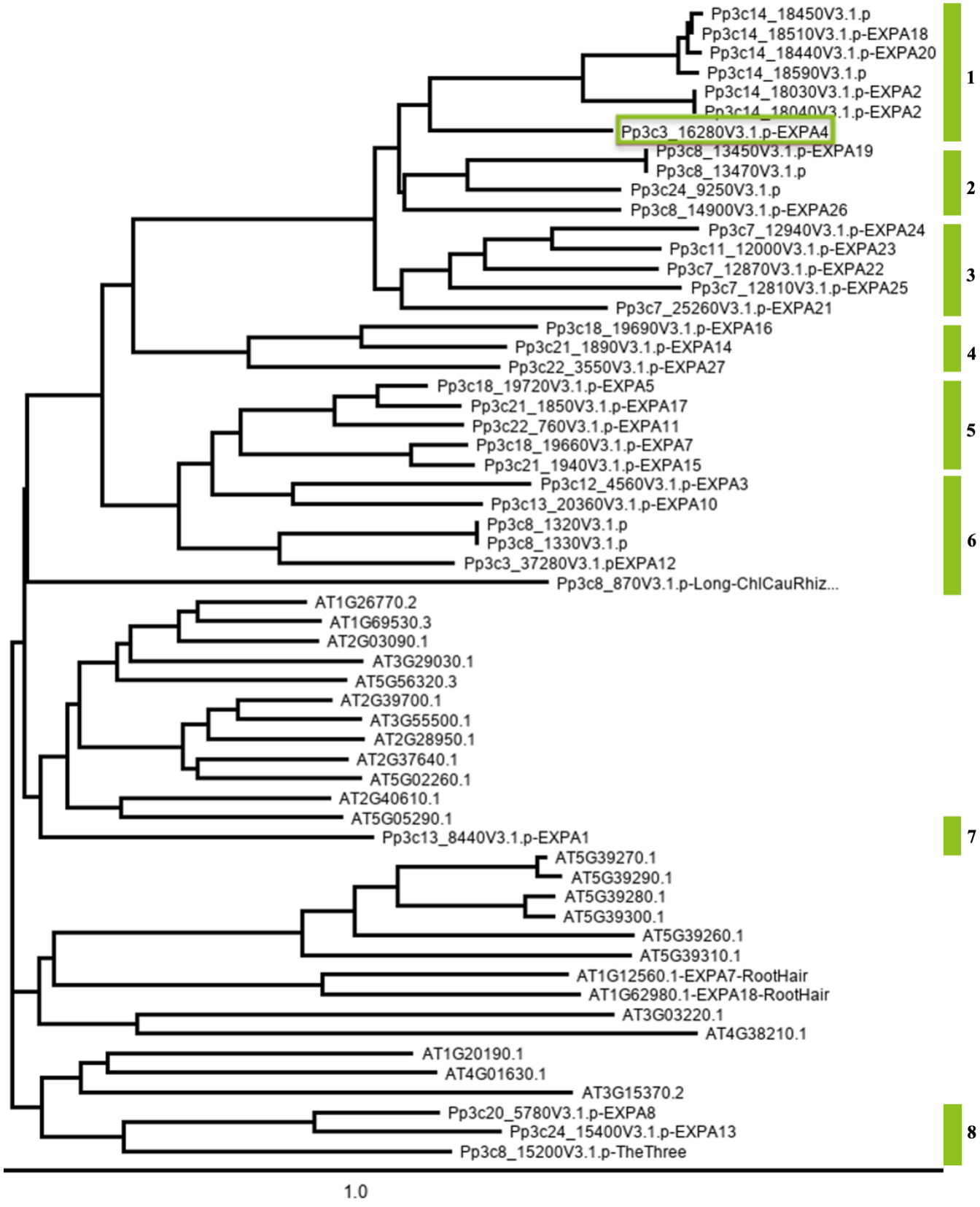


Figure 7. Phylogenetic tree showing EXPAs from *P. patens* and *A. thaliana* created using Geneious. Boxed in green is the gene of interest, EXPA 4 (expression levels shown in **Figure 8**). Clades of sequence similar genes are separated by green on the right (also seen in **Table 2**).

Expression levels and localization patterns of PpEXPA4 can be seen in **Figure 8**. All known *P. patens* expansin A expression and localization patterns were searched and recorded in Appendix A. As PpEXPA4 is highly expressed in the desired area, the caulonemata, and not expressed anywhere else in the plant, it was chosen for this project. Expansin B1 was also found with similar expression levels and localization patterns to this protein and was considered as a second protein of interest (**Figure 9**), but ultimately not used. The remaining expression levels and localizations of EXPB genes can be found in Appendix B.

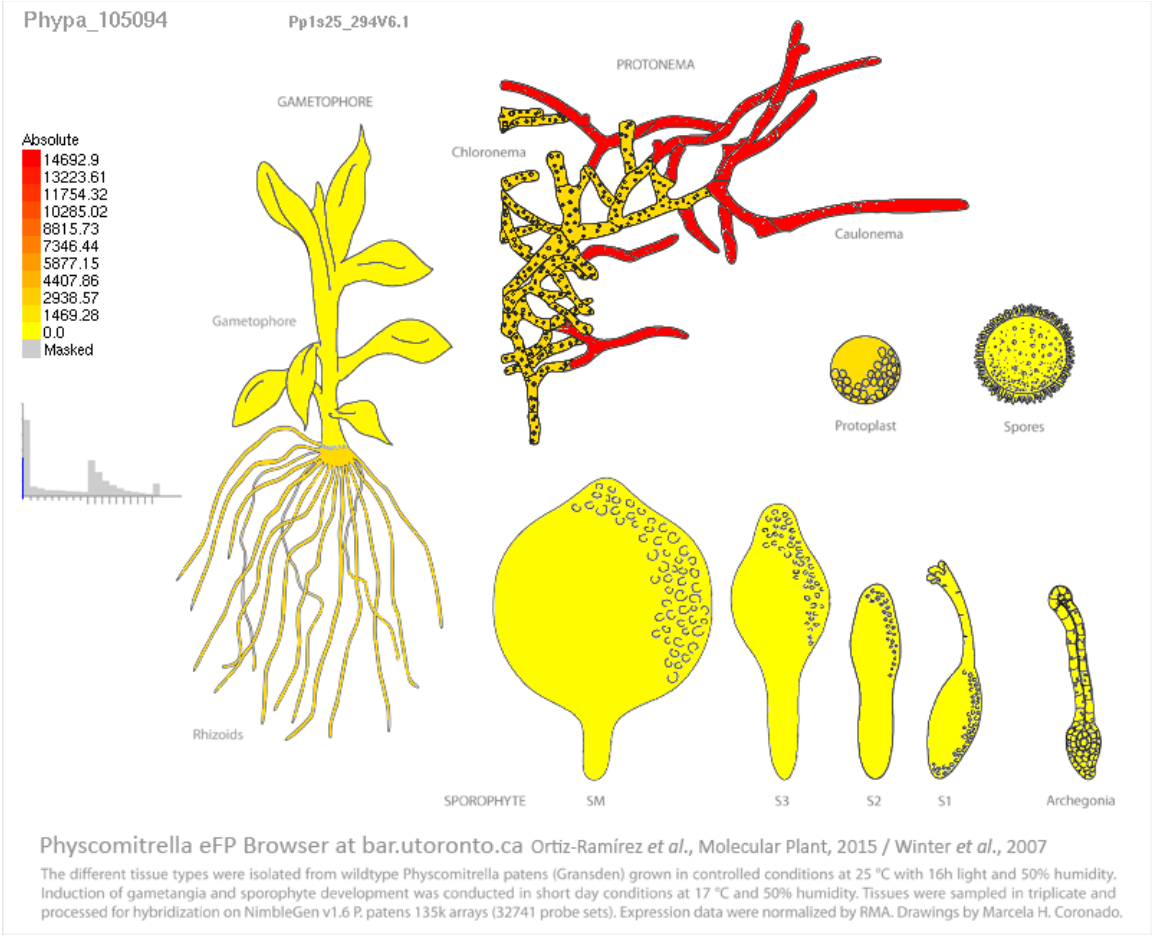


Figure 8. Gene of interest Pp3c3_16280V3.1, also known as EXPA4, expression levels in different parts and stages of growth of *P. patens*. The caulonema area shows the highest level of expression of the protein (~14692 units), while most other areas are close to zero.

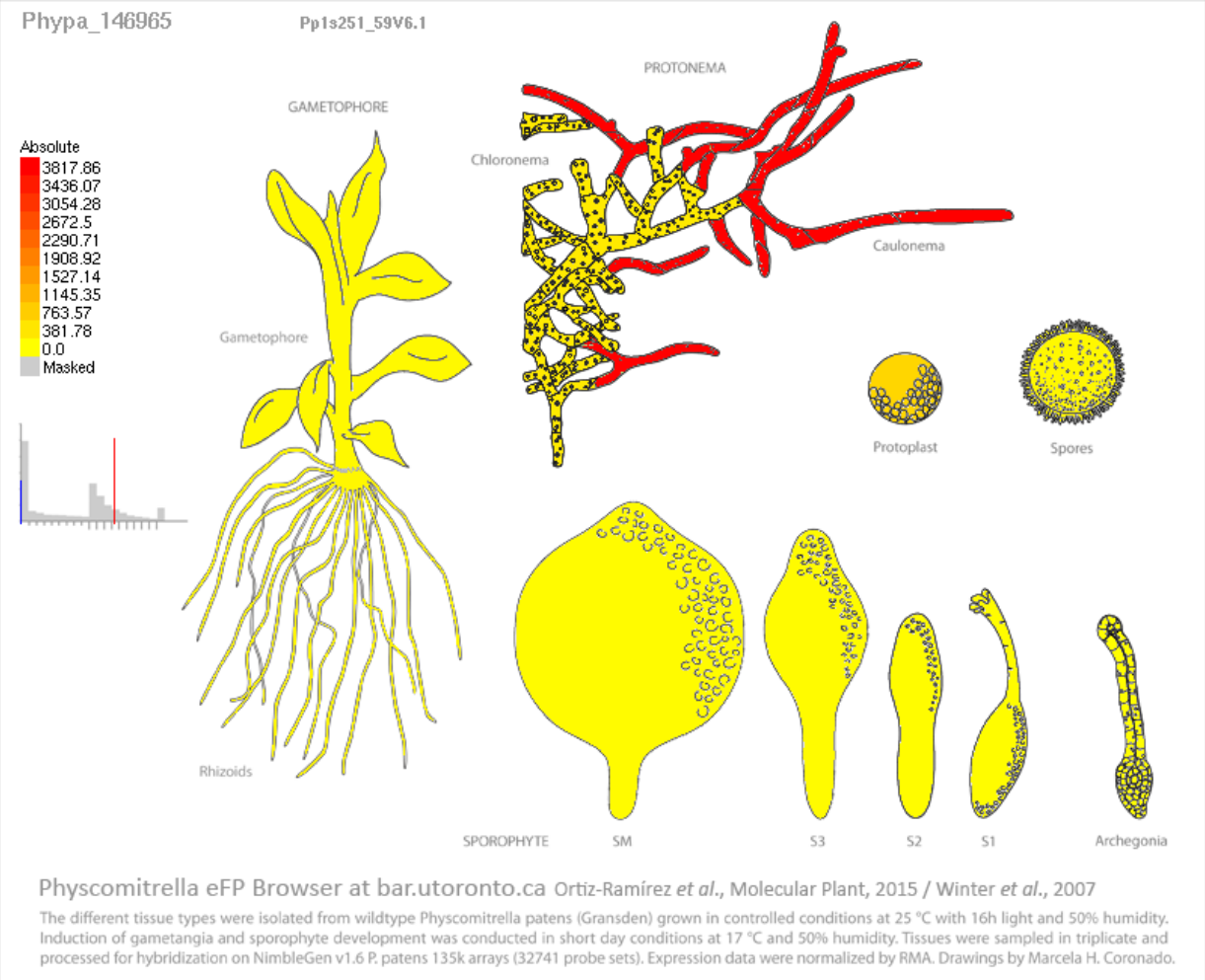


Figure 9. Gene of interest Pp3c33_630V3.1, also known as EXPB1, expression levels in different parts and stages of growth in *P. patens.* The caulonema area shows the highest level of expression of the protein (~3817 units), while most other areas are close to zero.

3.2 Constructs, BP, and LR reactions

The constructs generated in the project are listed in **Table 1**. Multiple gels were run after LR reactions, DNA mini preps, and DNA maxi preps during the creation of the constructs. This section is to highlight the most important gels run that show each construct that was created throughout the project.

Table 1. Constructs created throughout the project. pUC-GW-Kan are the Gateway entry clones created from BP reactions, pTH-Ubi denotes the Gateway vector the genes were cloned into. As this project concerns only *P. patens*, "Pp" was removed from naming throughout experimentation. Plasmid maps of each construct can be seen in Appendix C.

Database Number	Name
323	pUC-GW-Kan-PpEXPA4
324	pUC-GW-Kan-HaloTagC
325	pUC-GW-Kan-HaloTagN
326	pDONR221P1P5r-PpEXPA4d1
327	pTH-Ubi-PpEXPA4-HaloTag
328	pTH-Ubi-PpEXPA4-3mCherry
329	pTH-Ubi-PpEXPA4-3mEGFP
330	pTH-Ubi-3mEGFP-HaloTag
331	pTH-Ubi-HaloTag-3xGFP
332	pTH-Ubi-3mCherry-HaloTag
333	pTH-Ubi-HaloTag-3mCherry
334	pTH-Ubi-CLoG1-HaloTag
335	pTH-Ubi-PpEXPA4d1-HaloTag
336	pTH-Ubi-PpEXPA4d1-3mCherry

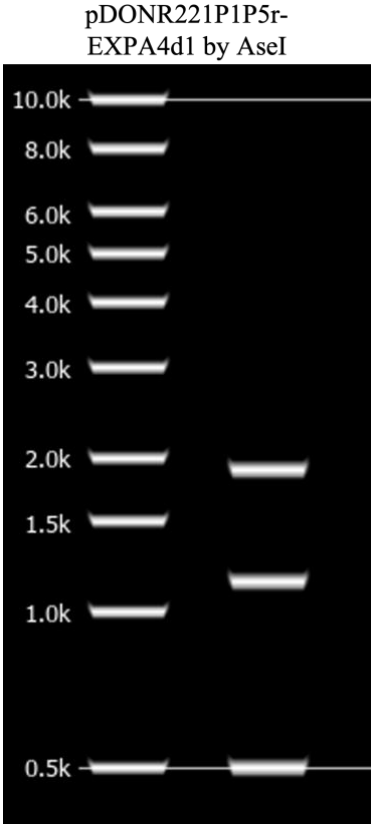


Figure 10. The virtual gel of the expected bands from Geneious of pDONR221P1P5r-EXPA4d1 when digested by AseI.

The image of the gel run after the BP reaction inserting EXPA4d1 into the pDONR221-P1P5r entry clone was unfortunately lost. However, the virtual gel that was used to identify correct bands in the gel can be seen in **Figure 10**. Because BP reactions have a lower success rate compared to LR reactions, six *E. coli* colonies were selected for a DNA miniprep and run through the gel. Of the six chosen, positive colonies were identified and selected for the LR reaction between pDONR221-P1P5r-EXPA4d1 and the two vectors pUC-GW-Kan-HaloTagC and pDONR221-P5P2-3mCherry. These can be seen in the gel in **Figure 11**.

The constructs created from the LR reaction were pTH-Ubi-EXPA4d1-HaloTag, pTH-Ubi-EXPA4d1-3mCherry, and a control cytosolic protein pTH-Ubi-CLoG1-HaloTag. These were run in a gel shown in **Figure 11**. pTH-Ubi-EXPA4d1-HaloTag and pTH-Ubi-EXPA4d1-3mCherry were cut with the restriction enzyme PvuII, and pTH-Ubi-CLoG1-HaloTag with KpnI. The virtual gels used to predict the band placements of each construct from Geneious are shown above the gel that was run. As LR reactions have a higher success rate compared to BP reactions, all the colonies that were selected showed the correct band placements.

a.

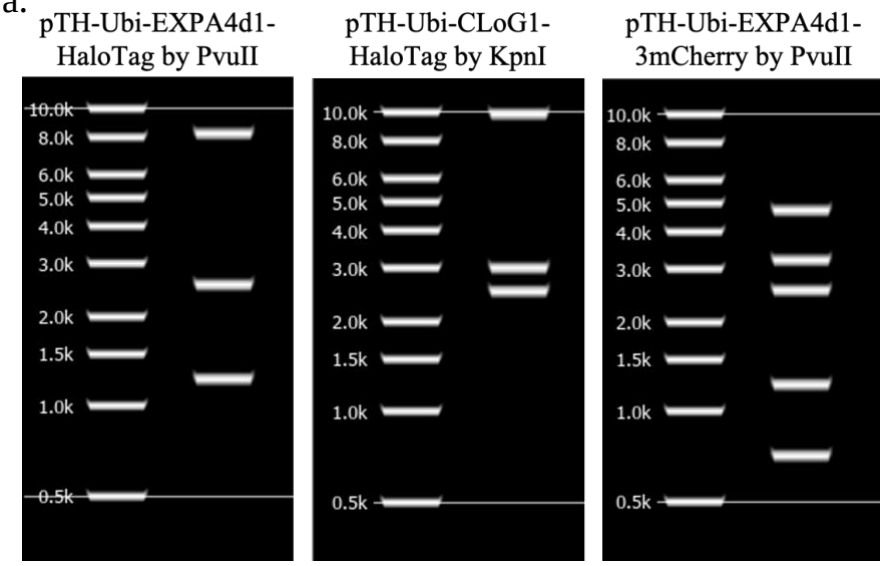
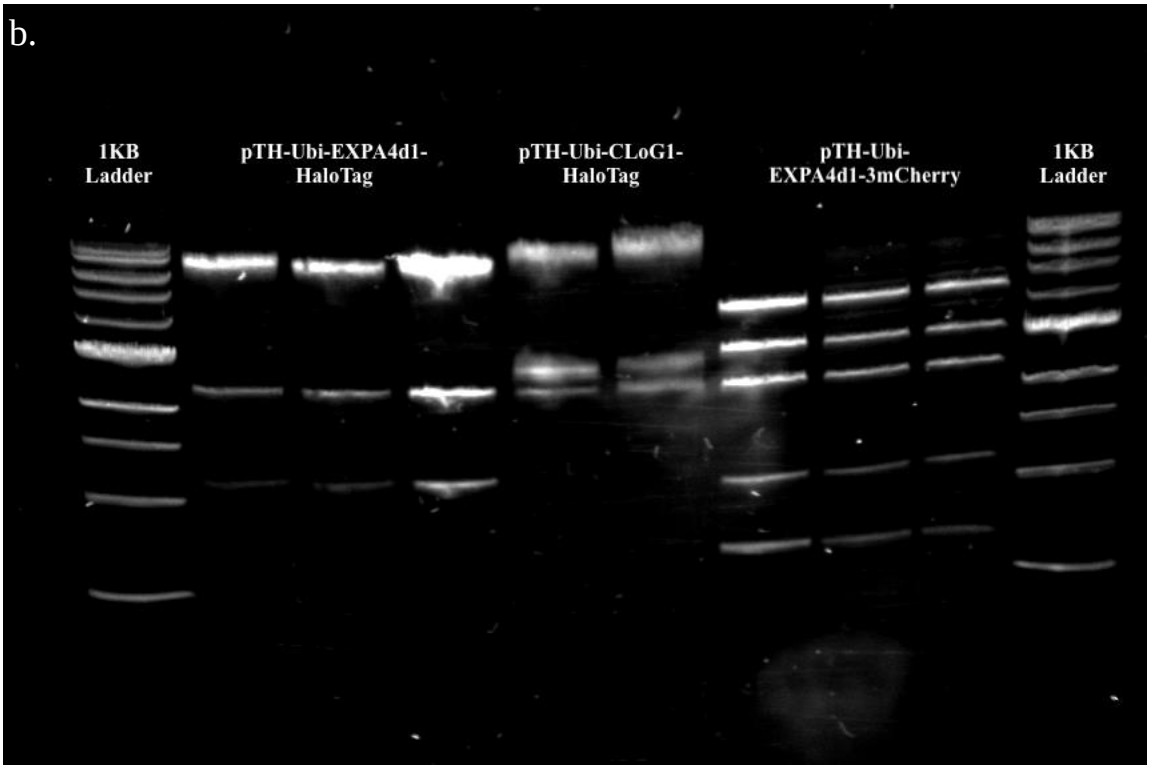


Figure 11. (a) Virtual gels of the expected bands from Geneious. (b) The gel run after LR reactions and *E. coli* transformation of the constructs pTH-Ubi-EXPA4d1-HaloTag, pTH-Ubi-CLoG1-HaloTag, and pTH-Ubi-EXPA4d1-3mCherry. There are only two lanes of pTH-Ubi-CLoG1-HaloTag as the third colony selected did not yield enough DNA.

b.



The other constructs created throughout this project can be seen in **Figure 12**. This gel was run after LR reactions creating the constructs pTH-Ubi-EXPA4-3mCherry, pTH-Ubi-EXPA4-HaloTag, pTH-Ubi-EXPA4-3xmEGFP, pTH-Ubi-3xmEGFP-HaloTag, pTH-Ubi-HaloTag-3xmEGFP, pTH-Ubi-3mCherry-HaloTag, and pTH-Ubi-HaloTag3mCherry. They were all cut by the restriction enzyme PvuII and accurately cut DNA, producing the correct fragments, is starred in red above the lane.

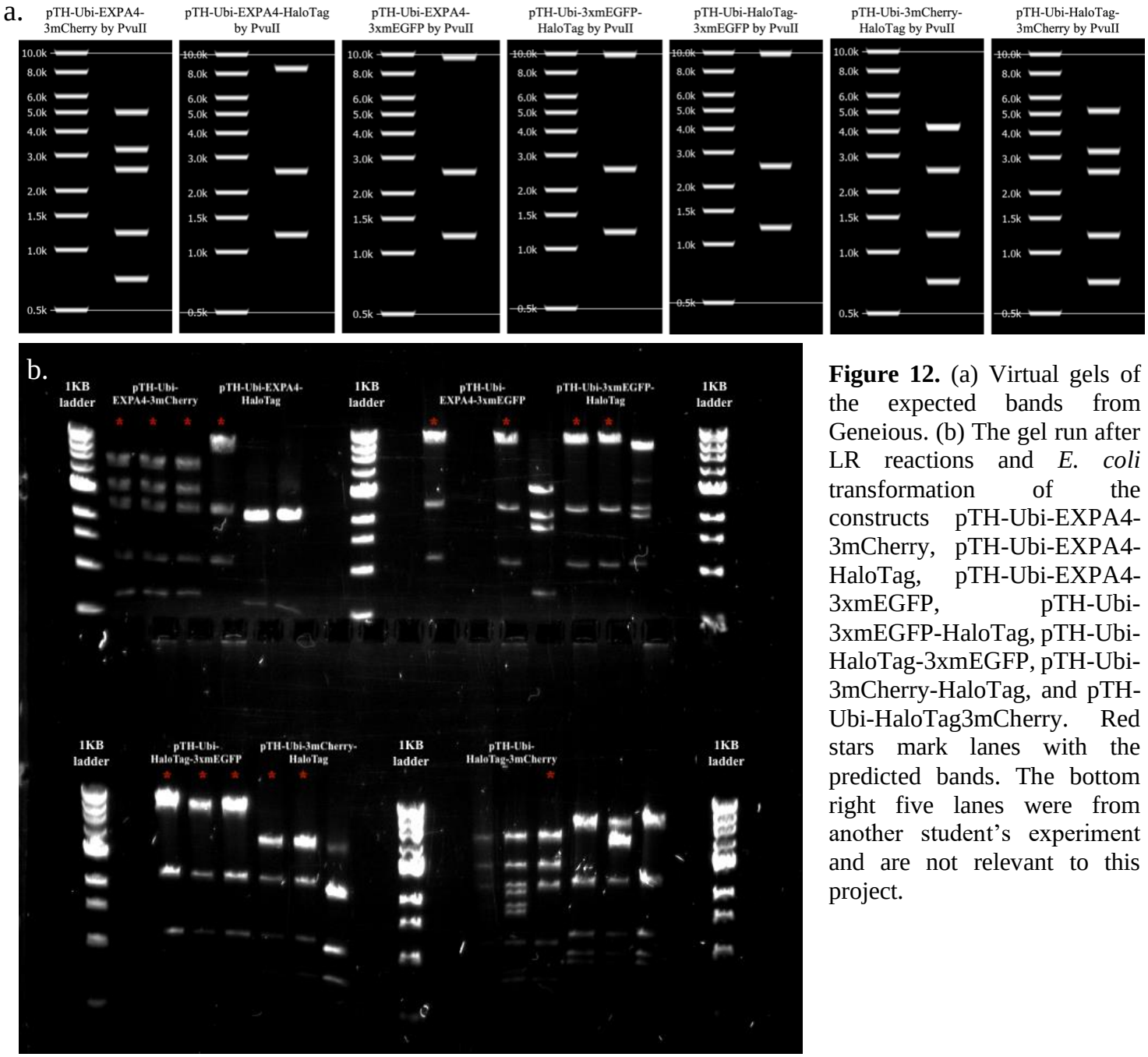


Figure 12. (a) Virtual gels of the expected bands from Geneious. (b) The gel run after LR reactions and *E. coli* transformation of the constructs pTH-Ubi-EXPA4-3mCherry, pTH-Ubi-EXPA4-HaloTag, pTH-Ubi-EXPA4-3xmEGFP, pTH-Ubi-3xmEGFP-HaloTag, pTH-Ubi-HaloTag-3xmEGFP, pTH-Ubi-3mCherry-HaloTag, and pTH-Ubi-HaloTag3mCherry. Red stars mark lanes with the predicted bands. The bottom right five lanes were from another student's experiment and are not relevant to this project.

3.3 Moss transformations: Rate of survival and growth

Moss that was transformed with pTH-Ubi-EXPA4-HaloTag, both linearized and circular, had a lower rate of transformation compared to control moss transformed with pTH-Ubi-3xmEGFP, pTH-Ubi-3xmEGFP-HaloTag, and pTH-Ubi-CLoG1-HaloTag. After the first transformation using pTH-Ubi-EXPA4-HaloTag at 60µg of DNA per 600µL protoplasts, lower concentrations of DNA were tested (15µg and 30µg DNA) for toxicity. Two controls, 30µg pTH-Ubi-3xmEGFP vector and a combination of 30µg pTH-Ubi-EXPA4-HaloTag and 30µg pTH-Ubi3xmEGFP vector were also transformed. Out of these transformations, the 15µg EXPA4-HaloTag vector concentration yielded 4 small colonies of moss that survived. Although these results were better compared to the original 60µg concentration of DNA, the rate of transformation was still significantly lower compared to controls. The colonies that survived hygromycin selection were individually selected and transferred to a separate hygromycin plate for further selection and growth. These selected stable plants with the EXPA4-HaloTag mosses were later used in a Western blot and internal and external staining.

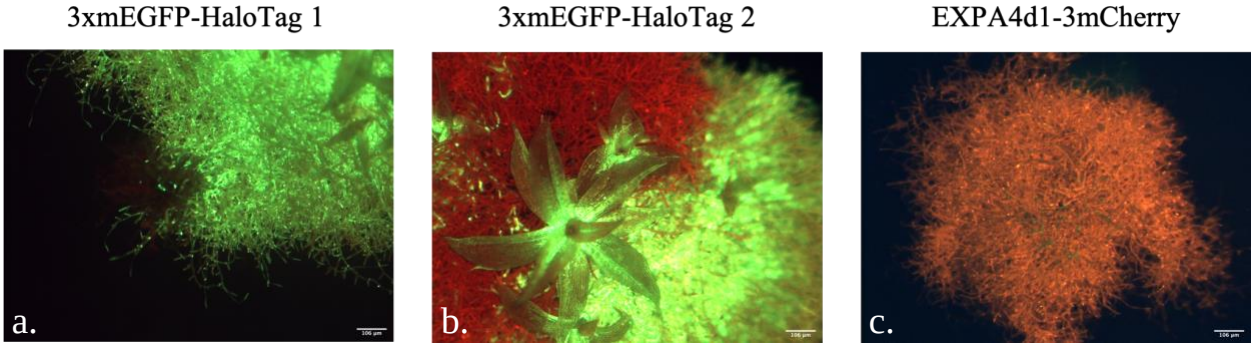


Figure 13. Images taken under a fluorescent microscope at 20x of pTH-Ubi-3xmEGFP-HaloTag and pTH-Ubi-EXPA4d1-3mCherry stably transformed moss. (a-b) pTH-Ubi-3xmEGFP-HaloTag moss was imaged under a GFP and chloroplast filter and (c) pTH-Ubi-EXPA4d1-3mCherry moss was imaged under a mCherry filter. GFP fluorescence appears green, chlorophyll appears red, mCherry fluorescence appears orange.

Due to the significantly lower rate of transformation, a PpEXPA4 domain 1 construct was created using PCR (Section 2.14). Moss transformed with either the pTH-Ubi-EXPA4d1-HaloTag or pTH-Ubi-EXPA4d1-3mCherry construct showed a slightly better rate of survival, but still seemed to be toxic compared to controls. **Figure 13** shows moss stably transformed with pTH-Ubi-3xmEGFP-HaloTag and pTH-Ubi-EXPA4d1-3mCherry. Moss transformed with the pTH-Ubi-EXPA4d1-3mCherry construct (**Figure 13c**) grew at a much slower rate compared to the control, whereas moss transformed with pTH-Ubi-3xmEGFP-HaloTag was developing much more (**Figure 13b**).

3.4 External HaloAlexa488 staining

Most of the external staining results of pTH-Ubi-EXPA4d1-HaloTag transformed cells were negative; however, one cell possibly stained with the Alexa488 external HaloTag stain was found (**Figure 14**). The negative control, pTH-Ubi-3mCherry, showed very little background in the HaloTag channel due to most of the cells surviving the transformation. Further images of external staining of pTH-Ubi-EXPA4-HaloTag stable moss can be seen in Appendix D Figures 24-25.

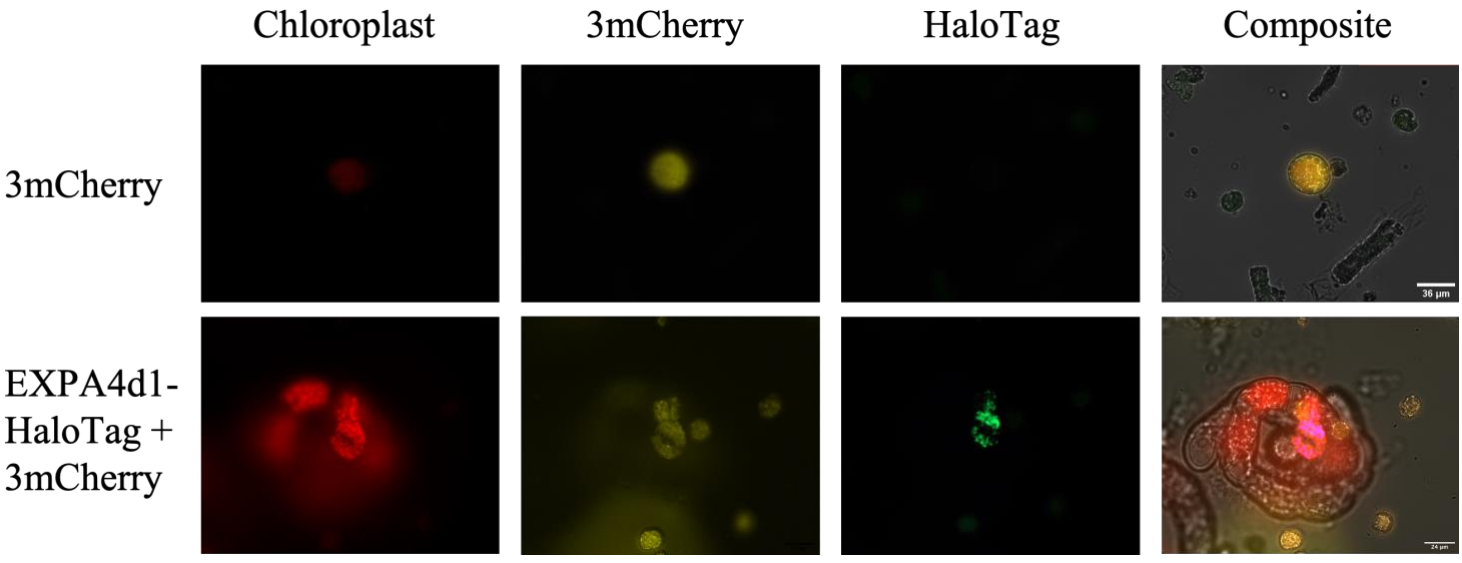


Figure 14. External staining using the Alexa488 HaloTag stain on transiently transformed moss seven days after transformation. The chloroplast filter shows live cells in red, the 3mCherry filter shows 3mCherry expressing cells and dead cells, the HaloTag filter shows cells tagged with the Alexa488 HaloTag stain, the composite shows all filters, including the bright field.

3.5 Internal HaloTag Ligand TMR staining

Internal staining was more successful than external staining. Using the HaloTag Ligand TMR, moss transiently transformed with pTH-Ubi-3xmEGFP (negative control), pTH-Ubi-3xmEGFP-HaloTag (positive control), pTH-Ubi-EXPA4d1-HaloTag, and pTH-Ubi-EXPA4d1-HaloTag + pTH-Ubi-3xmEGFP were stained four days after transformation (**Figure 15**).

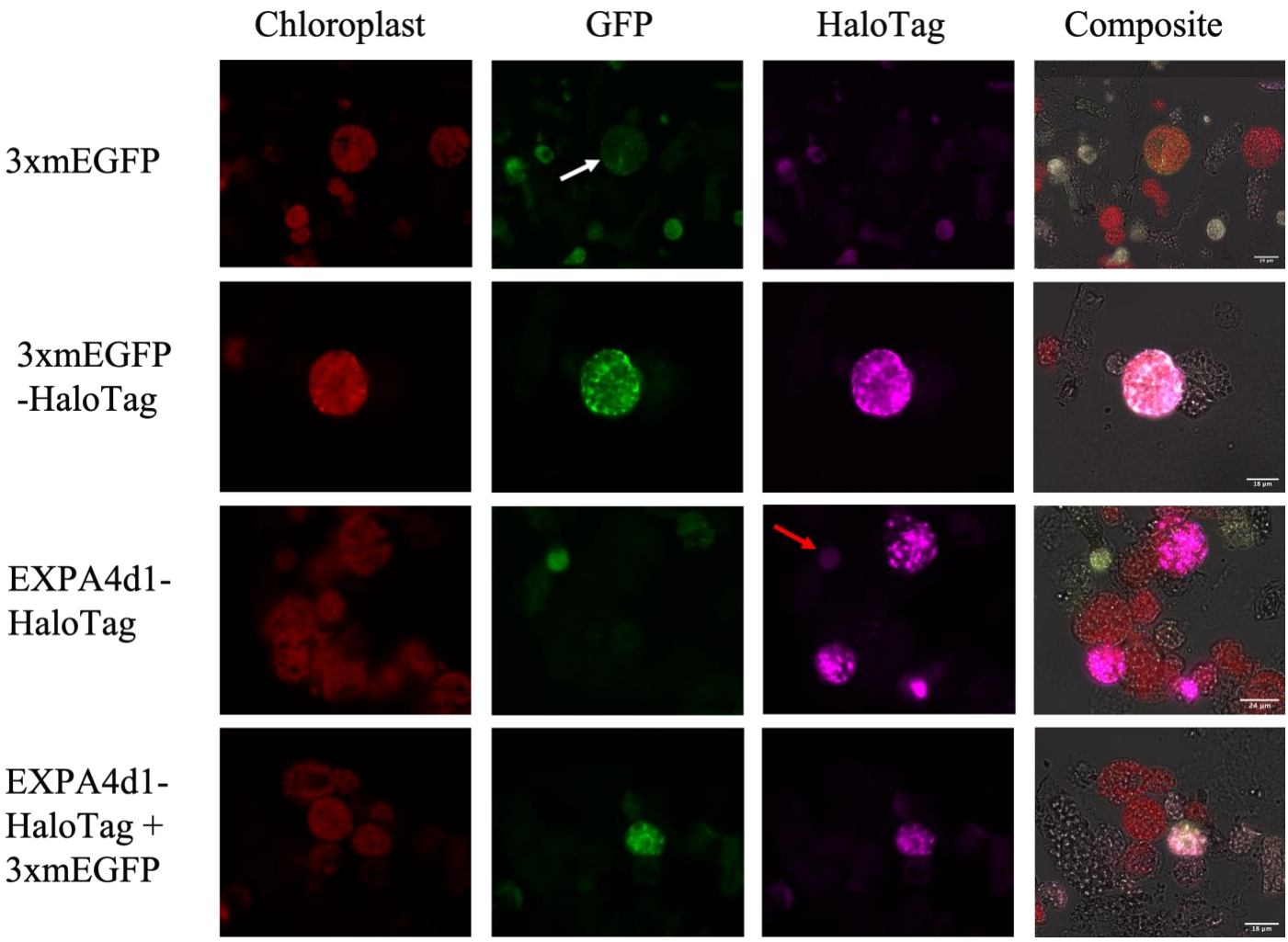


Figure 15. Internal staining using HaloTag Ligand TMR of transiently transformed moss. The chloroplast filter shows live cells in red, the GFP filter shows 3xmEGFP expressing cells and dead cells, the HaloTag filter shows cells tagged with the HaloTag Ligand TMR, the composite shows all filters, including the bright field. The white arrow points to a cell transformed with pTH-Ubi-3xmEGFP; the red arrow points to background in the HaloTag channel from a dead cell.

A white arrow in the negative control 3xmEGFP moss points to a successfully transformed cell. The surrounding cells fluorescing in the GFP and HaloTag channels are cells that have died, as they do not appear in the chloroplast channel. Using this, we determined the background levels of fluorescence that could be expected in the other samples (>7000 fluorescence units). The positive control fluoresces in both the GFP channel and HaloTag channel, suggesting it was successfully transformed and stained. The protein appears to be localized uniformly throughout the cell excluding the chloroplasts. The number of positives found in the pTH-Ubi-EXPA4d1-HaloTag transformations was less than the pTH-Ubi-3xmEGFP-HaloTag transformation, but much more than the external staining of pTH-Ubi-EXPA4d1-HaloTag (**Figure 14**). Three positively transformed and stained cells were found in close proximity. The red arrow in the HaloTag channel points to background from a dead cell. The spotted pattern of the pTH-Ubi-EXPA4d1-HaloTag transformed cells, compared to the uniform localization in the pTH-Ubi-3xmEGFP-HaloTag control, suggest that the protein complex is localized in the endomembrane system of the cell and may not be getting delivered to the cell wall. Lastly, moss transformed with pTH-Ubi-EXPA4d1-HaloTag and pTH-Ubi-3xmEGFP was stained to discern the different localization of the 3xmEGFP and EXPA4d1-HaloTag proteins. Although there is some bleeding over of fluorescence between the GFP and HaloTag channels, it still appears that 3xmEGFP is uniformly spread throughout the cell as seen in the 3xmEGFP control, and that the EXPA4d1-HaloTag protein is localized to the endomembrane system, the same as in the pTH-Ubi-EXPA4d1-HaloTag transformed moss.

3.6 Western blot with anti-HaloTag antibody

Western blots were performed using an anti-HaloTag antibody at a 1:1000 dilution for 1+ hours. No DNA added moss and pTH-Ubi-3xmEGFP transformed moss were used as negative controls, showing very little background in the high exposure image (**Figure 16**). Moss transformed with pTH-Ubi-3xmEGFP-HaloTag was used as a positive control. A band at 117 kDa shows the successfully tagged protein. The other bands in the lane are likely due to degradation of the protein. In the pTH-Ubi-EXPA4d1-HaloTag lane, there is a band at ~45 kDa, although we were expecting this protein to be at 53 kDa. The pTH-Ubi-EXPA4-HaloTag lane shows similar results, with a band appearing at ~50 kDa, while we were expecting 64 kDa. There are a number of different possibilities for why this occured, which will be discussed in Section 4.3.

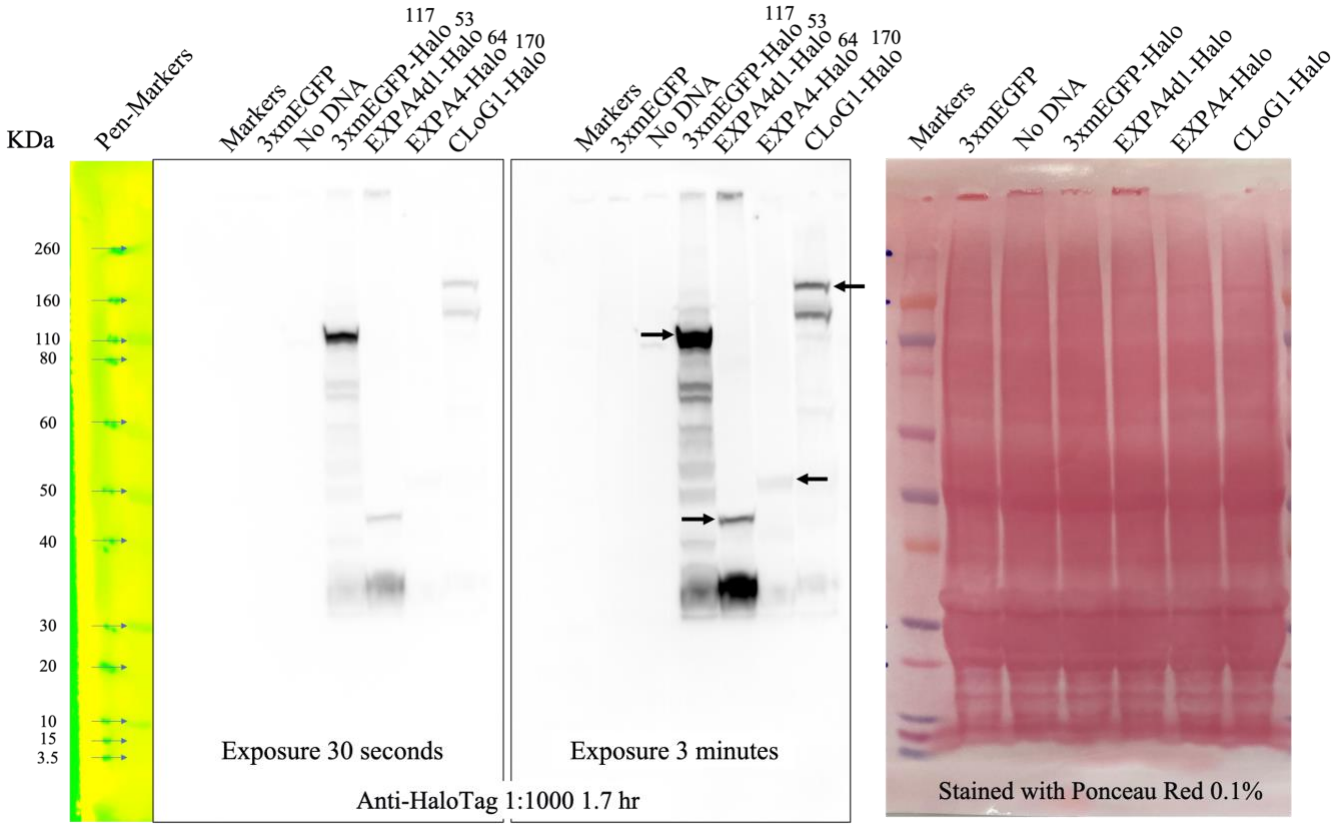


Figure 16. Western blot of proteins from moss transformed with pTH-Ubi-3xmEGFP, no DNA, pTH-Ubi-3xmEGFP-HaloTag, pTH-Ubi-EXPA4d1-HaloTag, pTH-Ubi-EXPA4-HaloTag, pTH-Ubi-CloG1-HaloTag using an anti-HaloTag antibody at a 1:1000 dilution. The image taken after staining with Ponceau red 0.1% shows equal amounts of protein in each lane. Likely bands for our target proteins are labeled with arrows.

The CLoG1-HaloTag protein band appears to be at the correct location, approximately 170 kDa, with a second band slightly under it. Similar to the pTH-Ubi-3xmEGFP-HaloTag control, this is most likely due to degradation of the protein. The bands seen at the bottom of the 3xmEGFP-HaloTag & EXPA4d1-HaloTag, and that are slightly visible under the EXPA4-HaloTag & CLoG1-HaloTag lanes are most likely the HaloTag protein after being cut from its N-terminus protein, as the size of HaloTag is 35 kDa.

Another Western blot was performed using extracts from moss that was stably transformed with pTH-Ubi-EXPA4-HaloTag towards the beginning of the project (**Figure 17**). This moss was grown and selected over a period of approximately 4.5 months after the initial transformation. A control pTH-Ubi-3xmEGFP-HaloTag was used, with a band appearing at approximately 117 kDa, similar to the results of the previous Western (**Figure 16**). What was not expected was every construct selected for expansion and the Western blot had positive results. Each lane of pTH-Ubi-EXPA4-HaloTag moss has a band at approximately 50 kDa. Although the expected size is 64 kDa, this remains consistent with our previous results (**Figure 16**). Each lane also contains a band at approximately 30 kDa, which is most likely the HaloTag protein after being cut from EXPA4. Although these results were not expected, it suggests that these mosses have been expressing EXPA4-HaloTag since the initial transformation 4.5 months before the Western was performed.

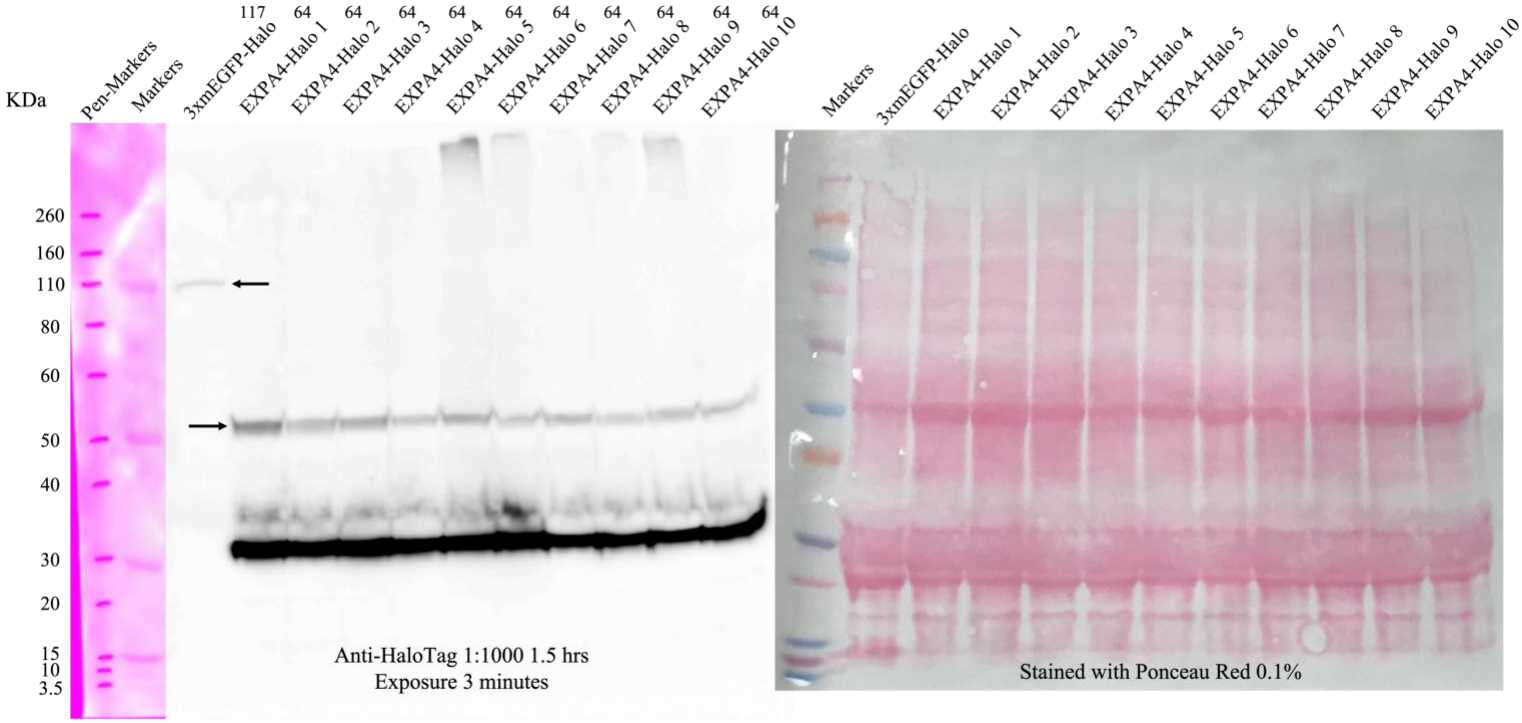


Figure 17. Western blot using anti-HaloTag antibody (1:1000) of moss stably transformed with pTH-Ubi-EXPA4-HaloTag. Moss transformed with pTH-Ubi-3xmEGFP-HaloTag was used as a positive control. The image taken after staining with Ponceau red 0.1% shows equal amounts of protein in each lane. Likely bands for our target proteins are labeled with arrows.

CHAPTER 4 – DISCUSSION

4.1 Relation between phylogenetics, expression, & localization

Our own phylogenetic tree shares similarities to the tree produced in the Carey & Cosgrove (2007) paper on the expansin superfamily, further validating their results. Although, our unmatched EXPA genes that do not appear in their paper would need to be further investigated to validate our results.

The genes in the first clade in Table 2 (Appendix A) all express most highly in caulonema, and some in the protoplast and rhizoids. PpEXPA4, which is next closely related to these EXPA genes also expresses in the caulonema, but at a much higher level. Most of these genes are on chromosome 14, suggesting they evolved from the same ancestral gene by duplication, and eventually developed their own specific expression levels and localizations. The genes in the second clade are most expressed in the chloronemata and protoplast, with the exception of Pp3c24_925, which only expresses at a level of 52 units, compared to 500-8000 of the other genes in this clade. The genes in the third clade do not share significant expression levels or patterns with each other. PpEXPA22 and PpEXPA25 share sequence similarity and both express in the chloronemata, but at extremely different levels (2200 and 50 units respectively). This may suggest that they have the same function, and that PpEXPA25 is expressed less than PpEXPA22 because the cell has no need for both expressing at a high level at once.

The genes in the fourth clade share localization similarities in the archegonia, with the exception of PpEXPA16, which only expresses in the rhizoids at a high level. Although the two other genes which express in the archegonia share this similarity, they have very different expression levels (1220 vs 107 units). These genes are both on different chromosomes, so they are most likely not recent duplicates of the other, and do not share the same function. The genes in the fifth clade are most expressed in the developmental stages of the plant's life (the sporophyte stages and

archegonia). Some share similarities between chromosomes, so it is likely that these are duplicated genes. However, unlike in the third clade, these all express at significantly high levels (6000-16000 units). Although they have overlapping localizations, their expression levels do not complement one another, suggesting that they each have a separate function. The genes in the sixth clade do not appear to share much similarity in localization. Two of the genes, Pp3c8_133 and Pp3c3_3728-PpEXPA12, share similar localization in the developmental stages of the plant (specifically SM), but other than this one similarity, this clade does not appear to overlap in localization and expression, and likely not function.

There are also a number of genes that share sequence similarity with EXPA genes from *A. thaliana* (**Figure 7**), notably PpEXPA1, PpEXPA8, PpEXPA13, and Pp3c8_1520 in clades 7 and 8. These genes share similarity in expression localization in the archegonia and other developmental stages of the moss, and all except PpEXPA1 express at relatively high levels, suggesting different functions. Because of their sequence similarity to the *A. thaliana* EXPA genes, they most likely shared common ancestry before the two species diverged.

4.2 Survival and growth of *P. patens* transformed with EXPA4 constructs

Based on both the stable and transient moss transformations that were done multiple times throughout the project, we can say with confidence that the overexpression of both EXPA4-HaloTag and EXPA4d1-HaloTag are toxic to the cell. Compared to controls, moss transformed with either EXPA4 construct showed a significantly decreased rate of transformation and cells that were transformed and survived grew at a much slower rate on the same media and time frame. However, when moss was stably transformed with pTH-Ubi-EXPA4-HaloTag, a small number of cells were able to survive hygromycin selection over a course of 4.5 months and develop into

larger plants. It is possible the cell expresses the protein but does not utilize its function. For example, the protein may be targeted to the vacuole for degradation (**Figure 27**).

Overexpression of proteins in plants has been known to have possible harmful effects on cell vitality and survivability. In a study done in 2016, overexpression of the PBZ1 gene from rice in *A. thaliana*, resulted in a harmful effect on seed germination, but when the expression was controlled, it had no significant effect (Huang et al., 2016). When the protein of interest has a structure-altering function, such as PpEXPA4 which alters the cell wall by loosening, it is likely that overexpression of the protein will be harmful to the cell. In a study done in 2016, it was found that overexpression of cucumber expansin in *E. coli*, transient tobacco leaves, and *A. thaliana* seeds was unsuccessful, likely because the negative effects of the overexpression of the protein resulted in the cells to repress expression (Yactayo-Chang, 2016).

4.3 Internal & external staining and Western blots

Because only one possible positive external staining of the pTH-Ubi-EXPA4d1-HaloTag transformed moss (Figure 14) was found, and none found in pTH-Ubi-EXPA4-HaloTag transformed moss (Appendix D Figures 24-25), we believe that the protein was not delivered to the cell wall. It is possible that the toxicity of the protein may be due to the attached HaloTag, which could impede the protein's initial folding or function in the cell wall. Our western blot results (Figure 16, 17, & Appendix D Figure 23) suggest that the first 10 kDa of the protein (in both full-length and domain 1 constructs) may instead be cleaved inside the cell, causing it to lose its signal peptide and therefore its ability to be delivered to the cell wall.

Our Western blot results may also suggest that the full protein is still present and migrated in a strange manner compared to control proteins. When considering protein folding and the charge of

the protein, it is possible for the protein to have migrated farther than expected. If the protein is more compact, it may move easier through the gel, or if the protein has a more positive charge, it will migrate faster compared to more neutral or negative proteins. The EXPA4-HaloTag protein has an isoelectric point of 10.08, meaning it is more basic. As the SDS added to the samples before loading the Western binds strongly to the positive hydrophobic residues of proteins, changing the overall charge significantly, the protein may have migrated farther than expected – in this case, by 10 kDa. Whether this can cause a protein to migrate that much farther or not, we do not know. It is still just as likely possible that the first 10 kDa were simply cut off of the protein.

Our internal staining results support the possibility that the signal peptide is lost (Figure 15). The spotty pattern of the stained pTH-Ubi-EXPA4d1-HaloTag cells suggest that the protein is localized to the endomembrane system (ER, Golgi apparatus, & endosomes). Combined with our negative external staining and western blot results, the possibility that the protein is not getting delivered to the cell wall due to degradation appears likely.

Moss that was transformed with pTH-Ubi-EXPA4-HaloTag, selected on hygromycin, and grown over a period of 4.5 months (used in **Figure 17**) was also internally and externally stained (Appendix D **Figures 24-27**). These results further affirm that the protein is not getting delivered to the cell wall, as our external staining attempts were met with negative results again. Internal staining results (Appendix D **Figures 26-27**) suggest that the protein is localized to the vacuole of the cell, unlike the results seen in **Figure 15**, which suggested the protein is localized to the endomembrane system. Fluorescence from the HaloTag Ligand TRM inside the cell is localized throughout except for the chloroplasts, and in one instance the nucleus of the cell was also surrounded by the stain. These results further suggest that EXPA4-HaloTag is toxic to the cell and is not sent to the cell wall.

CHAPTER 5 – CONCLUSION AND RECOMMENDATIONS

Based on the differences between the internal staining of pTH-Ubi-EXPA4-HaloTag and pTH-Ubi-EXPA4d1-HaloTag transformed moss, it is likely that the full-length EXPA4 construct is more toxic to the cell, as it is sent to the vacuole, whereas the domain 1 construct is less toxic, as it appears to be localized in the ER, Golgi, and possibly endosomes. Western blot results also support this, with pTH-Ubi-EXPA4-HaloTag moss exhibiting a fainter band compared to pTH-Ubi-EXPA4d1-HaloTag moss (**Figure 16**).

With the results gained throughout this project, other approaches may be taken in order to complete the original objective of localizing the sites of active secretion at the cellular level. One such approach may be to create an EXPA4 domain 2 – HaloTag construct and test its expression and localization using the methods used in this project. This gene would need to be altered to contain the necessary signal peptide to send the protein to the cell wall, but it is possible that domain 2 of the gene could be less toxic and still retain some cell wall functionality. Other cell wall proteins may be less toxic when overexpressed in *P. patens* and could instead be used to localize the sites of active secretion. One such protein is extensin, a common plant cell wall protein (Srivastava, 2002).

It should also be considered that the attached N-terminus HaloTag protein may be inhibiting or altering the expansin's function, as suggested from our Western blot results. Other tags, such as the SNAP tag previously discussed, could be tested as tagging proteins in order to complete the original objective of this project. As the main goal of this project was to localize the sites of active secretion at the cellular level in *P. patens* using the overexpression of EXPA4-HaloTag, it is unfortunate that we were not able to do so. However, with these results and these possible new approaches provided, the project can be continued the original objective may be accomplished.

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Databases used:

Expansin expression levels and localizations: http://bar.utoronto.ca/efp_physcomitrella/cgi-bin/efpWeb.cgi

Plant genomic database: https://phytozome.jgi.doe.gov/pz/portal.html#

APPENDICIES

Appendix A – Expression levels and localization of EXPA

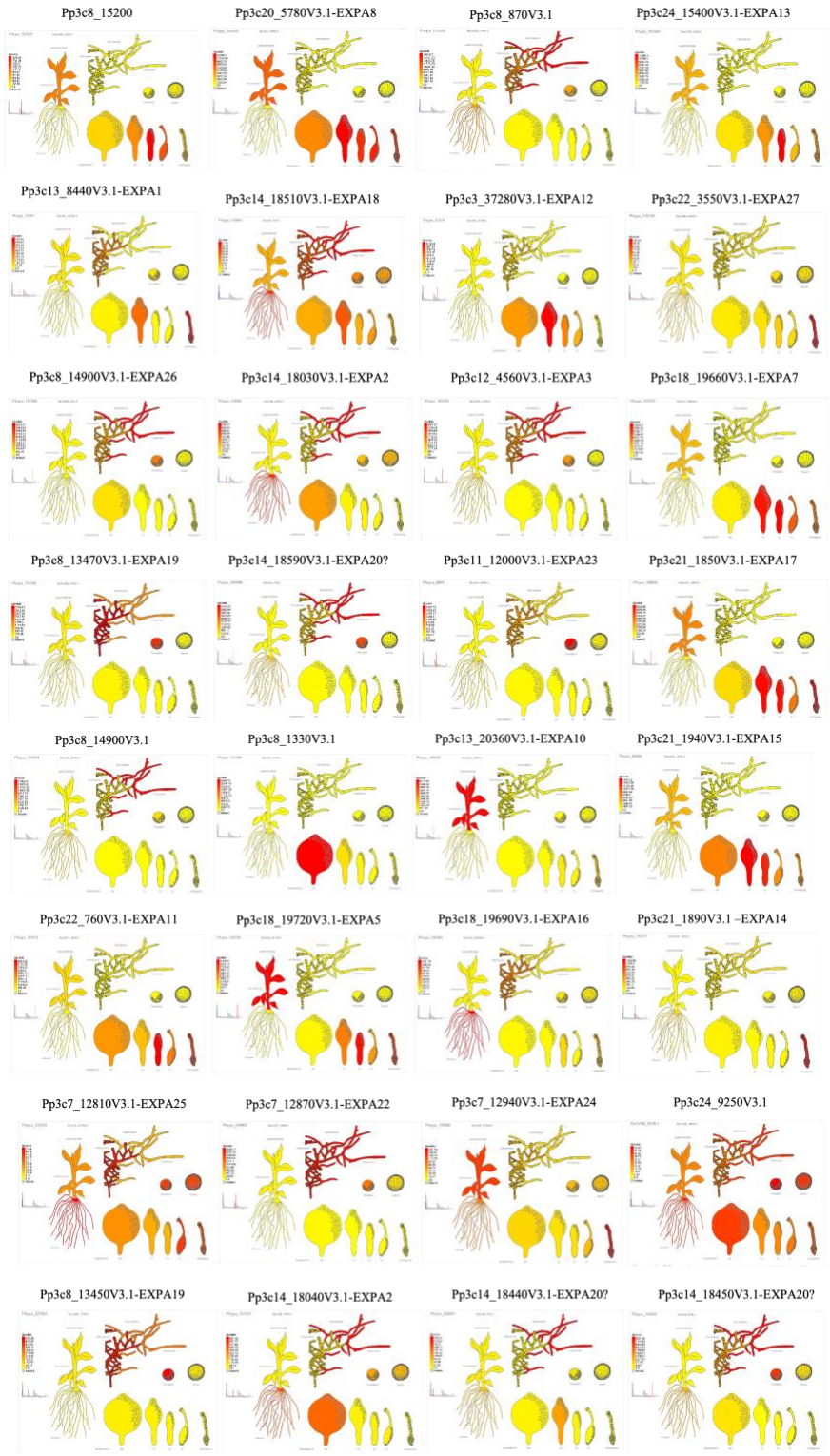


Figure 1. EXPA expression levels and localization patterns gathered using the Physcomitrella eFP database.

Table 1. Expression levels and localization of EXPA genes listed in the order they appear on the phylogenetic tree (Figure 7). Not all genes are present, as some were not in the Physcomitrella eFP database. Genes are separated into clades by their placement on the phylogenetic tree.

Clade	Gene name	Location of max expression (red)	Location of lesser expression (orange)	Max expression level (units)
1	**Pp3c14_1845**	Caulonema, Protoplast	Chloronema	8591.04
	Pp3c14_1851-EXPA18	Caulonema, Chloronema, Rhizoids, S3	Protoplast, S1, Gametophore	31.83
	Pp3c14_1844-EXPA20	Caulonema	S3	270.25
	Pp3c14_1859	Caulonema, Protoplast	n/a	4315.55
	Pp3c14_1803-EXPA2	Caulonema, Rhizoids	SM	758.27
	Pp3c14_1804-EXPA2	Caulonema	SM, Rhizoids	647.48
	Pp3c3_1628-EXPA4	Caulonema	n/a	14692.9
2	**Pp3c8_1345-EXPA19**	Chloronema, Protoplast	Caulonema	547.39
	Pp3c8_1347	Protoplast, Chloronema	Caulonema	2796.61
	Pp3c24_925	Protoplast, Spores, SM, Caulonema, Rhizoids	Gametophore, S3, S2, S1, Archegonia, Chloronema	52.53
	Pp3c8_1490-EXPA26	Caulonema	Protoplast, Chloronema	8233.37
3	**Pp3c7_1294-EXPA24**	Archegonia, Gametophore	Rhizoids	161.79
	Pp3c11_1200-EXPA23	Protoplast	Chloronema	1837.72
	Pp3c7_1287-EXPA22	Chloronema, Caulonema	Protoplast	2286.17
	Pp3c7_1281-EXPA25	Chloronema, Protoplast, Spores, Rhizoids, S1, Archegonia	SM, Gametophore	51.96
	Pp3c7_2526-EXPA21	-	-	-
4	**Pp3c18_1969-EXPA16**	Rhizoids	Chloronema	1151.774
	Pp3c21_189-EXPA14	Archegonia	n/a	1220.56
	Pp3c22_355-EXPA27	Archegonia	n/a	107.04
5	**Pp3c18_1972-EXPA5**	Gametophore, S2, Archegonia	S3, S1	9163.04
	Pp3c21_185-EXPA17	S3, S2, Archegonia	Gametophore, S1	6328.96
	Pp3c22_76-EXPA11	S2, Archegonia	SM, S1	9060.29
	Pp3c18_1966-EXPA7	S3, S2, S1	Archegonia	10578.4
	Pp3c21_194-EXPA15	S3, S2	SM, S1, Archegonia	16473.2
	Pp3c12_456-EXPA3	Caulonema	Chloronema, Protoplast	6887.07
	Pp3c13_2036-EXPA10	Gametophore	n/a	6115.96

6	**Pp3c8_132**	-	-	-
	Pp3c8_133	SM	n/a	22667.5
	Pp3c3_3728-EXPA12	S3	SM, S2, S1	4430.68
	Pp3c8_87	Caulonema	Chloronema	16819.7
7	**Pp3c18_844-EXPA1**	Archegonia	S3, Chloronema	732
8	**Pp3c20_578-EXPA8**	S3, S2, S1, Archegonia	SM, Gametophore	12258
	Pp3c24_1540-EXPA13	S2	S3, S1, Archegonia, Gametophore	11996
	Pp3c8_1520	S2, S1	S3, Gametophore	1979

Table 2. Matching Carey gene names to database names of EXPA genes.

Carey gene name	**Matching gene**
EXPA1	Pp3c13_8440V3.1.p-TheOne
EXPA2	Pp3c14_18030V3.1.0 & Pp3c14_18040V3.1.p
EXPA3	Pp3c12_4560V3.1.p
EXPA4	Pp3c3_16280V3.1.p-Caulo-14693
EXPA5	Pp3c18_19720V3
EXPA6	No match found
EXPA7	Pp3c18_19660V3.1.p
EXPA8	Pp3c20_5780V3.1.p-TheThree
EXPA9	No match found
EXPA10	Pp3c13_20360V3.1.p
EXPA11	Pp3c22_760V3.1.p
EXPA12	Pp3c3_37280V3.1.p
EXPA13	Pp3c24_15400V3.1.p-TheThree
EXPA14	Pp3c21_1890V3.1.p
EXPA15	Pp3c21_1940V3.1.p
EXPA16	Pp3c18_19690V3.1.p
EXPA17	Pp3c21_1850V3.1.p-S3-6328 (beginning ~18 missing)
EXPA18	Pp3c14_18510V3.1.p (not 100% match)
EXPA19	Pp3c8_13450V3.1.p & Pp3c8_13470V3.1.p
EXPA20	Pp3c14_1844, 1845, 1859 (somewhat similar)
EXPA21	Pp3c7_25260V3.1.p
EXPA22	Pp3c7_12870V3.1.p
EXPA23	Pp3c11_12000V3.1.p
EXPA24	Pp3c7_12940V3.1.p
EXPA25	Pp3c7_12810V3.1.p (beginning ~12 missing)
EXPA26	Pp3c8_14900V3.1.p
EXPA27	Pp3c22_3550V3.1.p

Appendix B – Expression levels and localization of EXPB

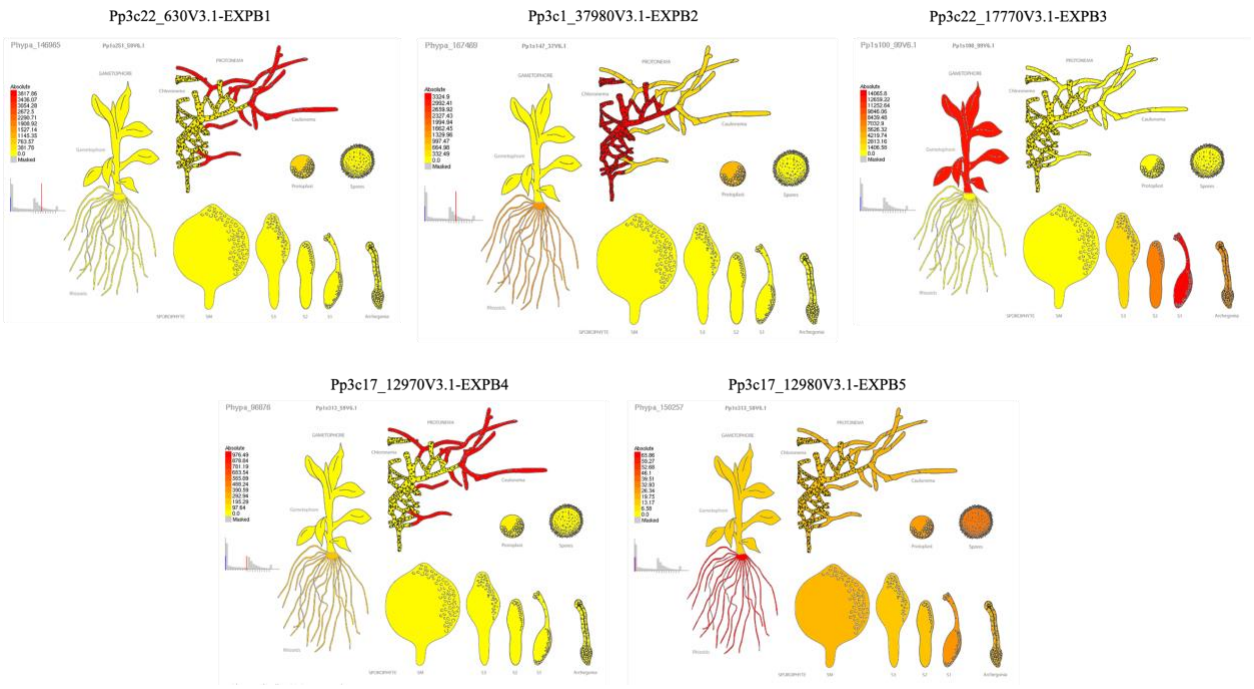
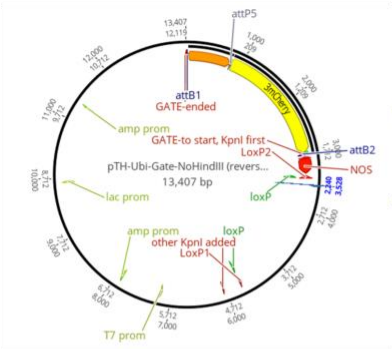


Figure 19. EXPB expression levels and localization patterns gathered using the Physcomitrella eFP database.

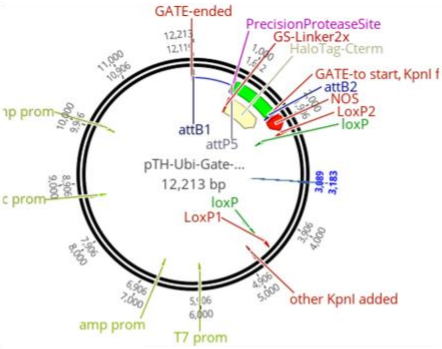
Table 3. Matching Carey gene names to database names of EXPB genes.

Carey gene	Matching gene
EXPB 1	Pp3c22_630V3.1.p
EXPB 2	Pp3c1_37980V3.1.p
EXPB 3	Pp3c22_17770V3.1.p
EXPB 4	Pp3c17_12970V3.1.p
EXPB 5	Pp3c17_12980V3.1.p
EXPB 6	Pp3c2_3390V3.1.p (~15 bp off)

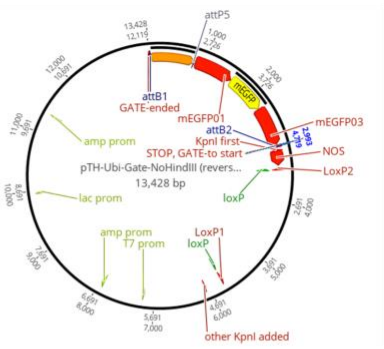
Appendix C – Constructs



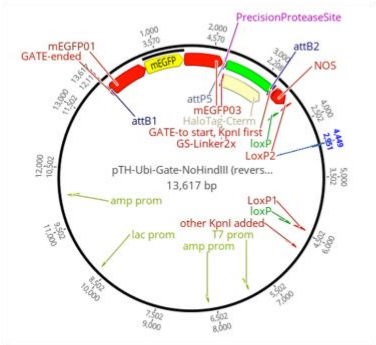
pTH-Ubi-EXPA4-3mCherry



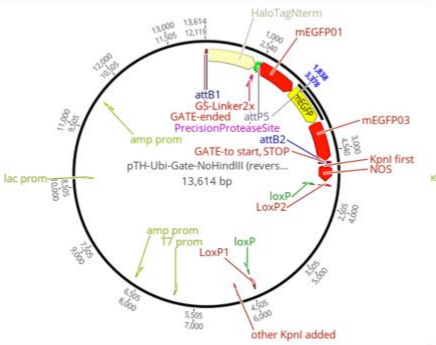
pTH-Ubi-EXPA4-HaloTag



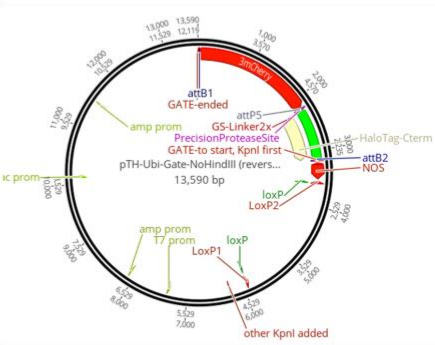
pTH-Ubi-EXPA4-3xmEGFP



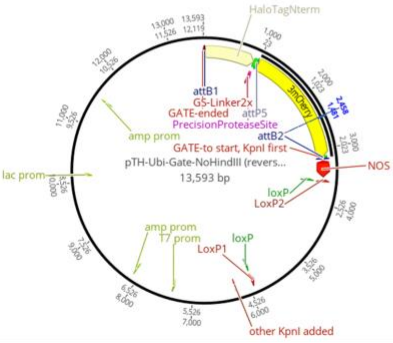
pTH-Ubi-3xmEGFP-HaloTag



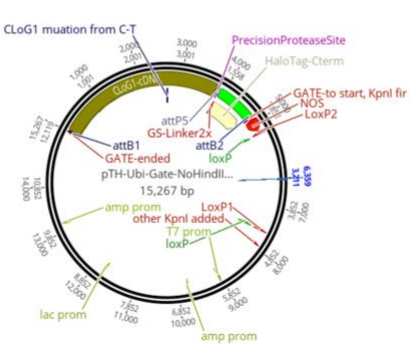
pTH-Ubi-HaloTag-3xmEGFP



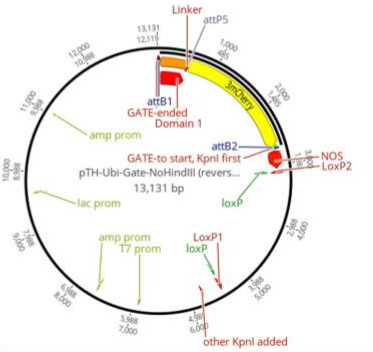
pTH-Ubi-3mCherry-HaloTag



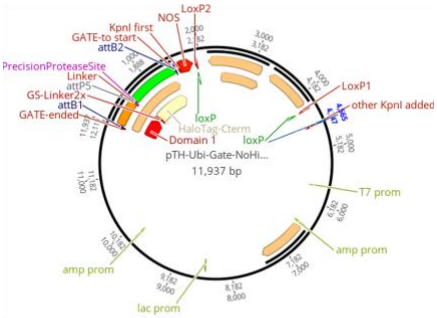
pTH-Ubi-HaloTag-3mCherry



pTH-Ubi-CLoG1-HaloTag

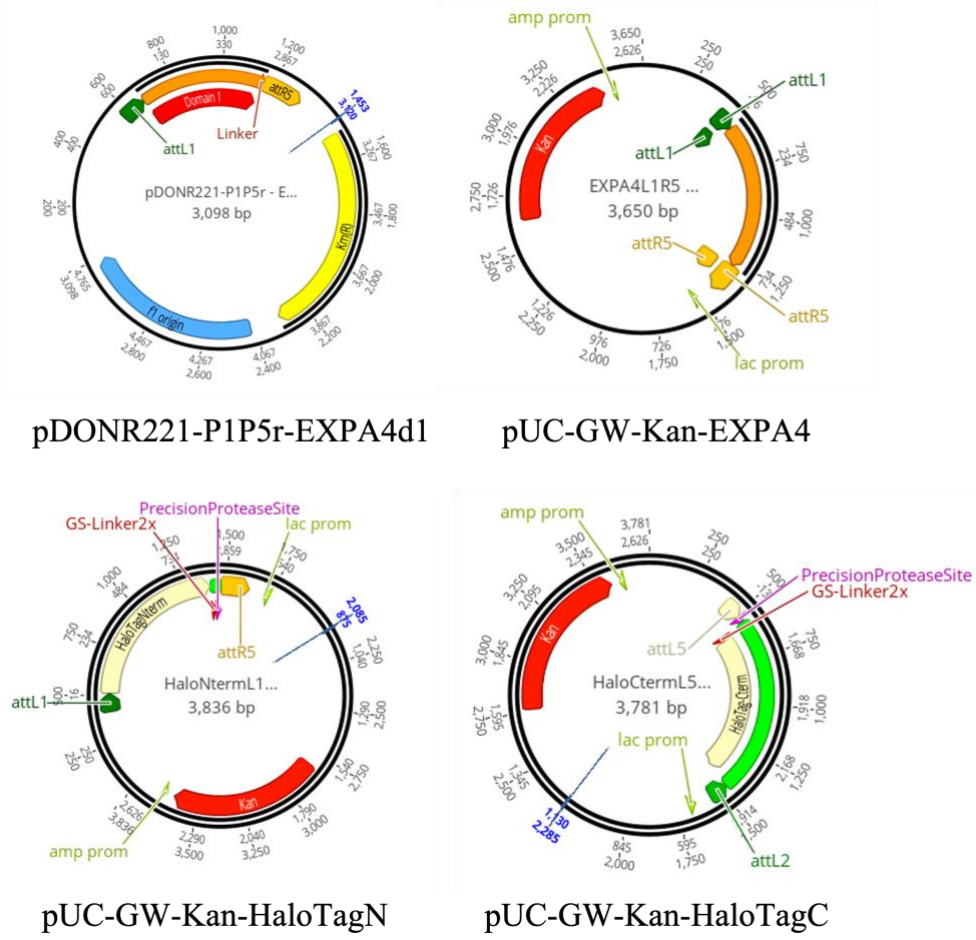


pTH-Ubi-EXPA4d1-3mCherry



pTH-Ubi-EXPA4d1-HaloTag

Figure 20. pTH-Ubi constructs created throughout the project.



pDONR221-P1P5r-EXPA4d1 pUC-GW-Kan-EXPA4

pUC-GW-Kan-HaloTagN pUC-GW-Kan-HaloTagC

Figure 4. Entry clones used to create constructs in this project.

Appendix D – Other figures

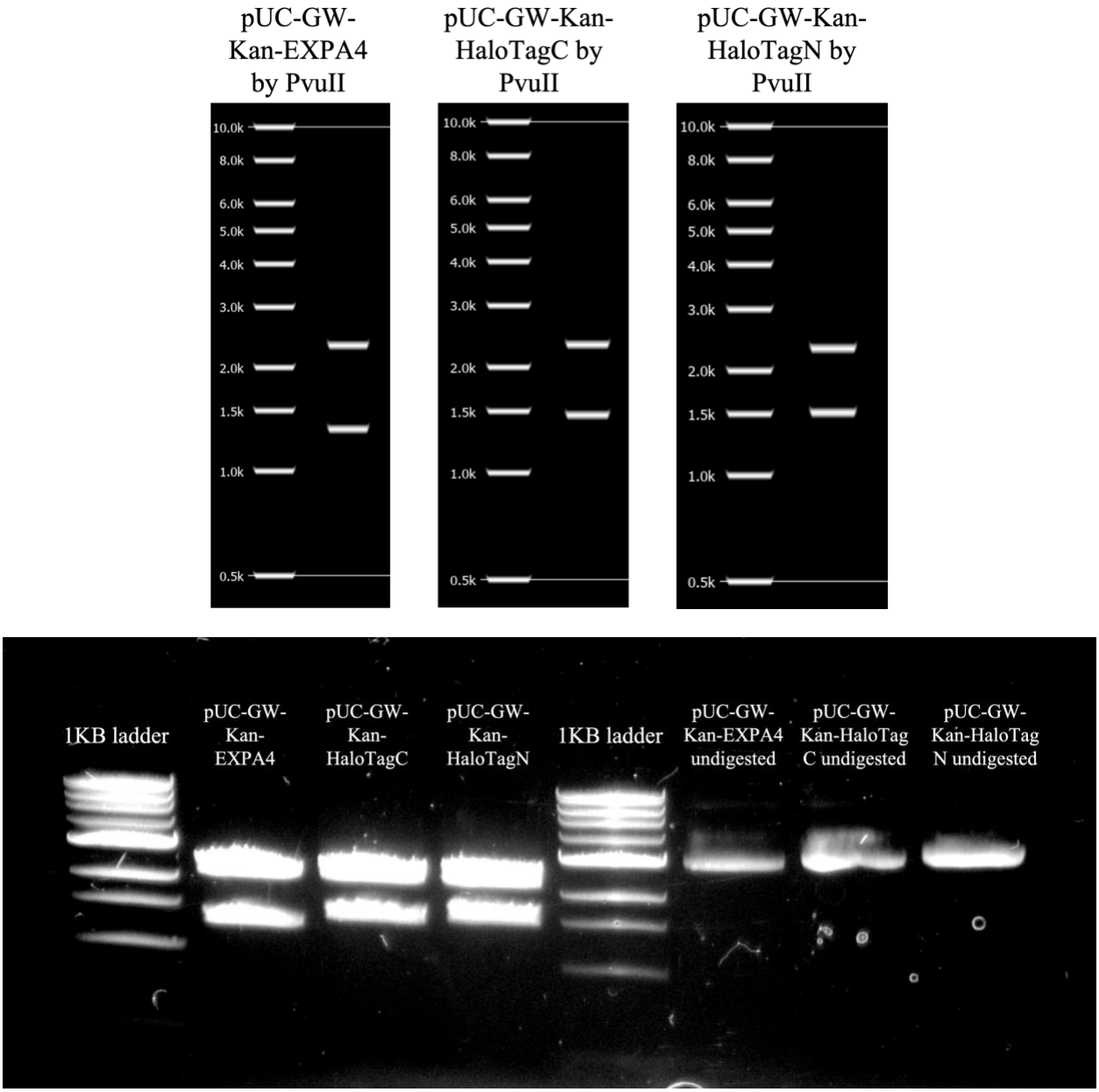


Figure 22. (a) Virtual gels of the expected bands from Geneious. (b) The first gel run after *E. coli* transformation and DNA miniprep of pUC-GW-Kan-EXPA4, pUC-GW-Kan-HaloTagC, and pUC-GW-Kan-HaloTagN, obtained from Genewiz, digested using PvuII. Undigested DNA of each vector was also run in the gel.

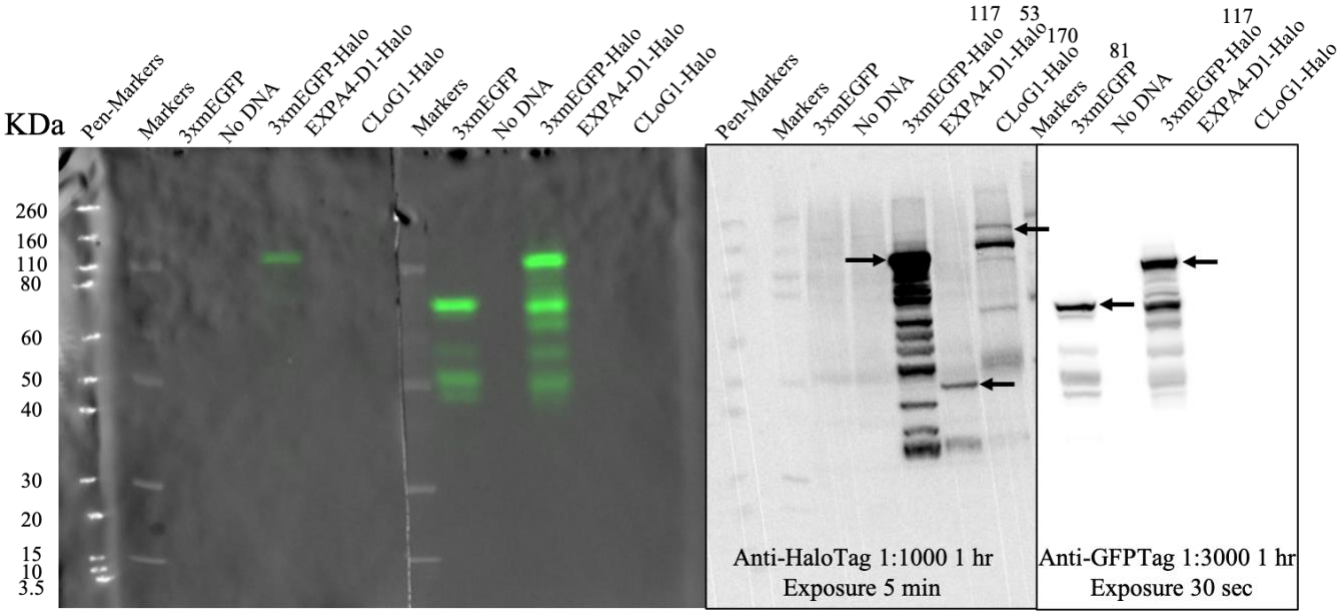


Figure 23. The first western that was performed in this project, done with both the anti-HaloTag antibody (1:1000) and a control anti-GFP antibody (1:3000). We obtained similar results for the 3xmEGFP-HaloTag, EXPA4d1-HaloTag, and CLoG1-HaloTag tagging in our second western (**Figure 16**).

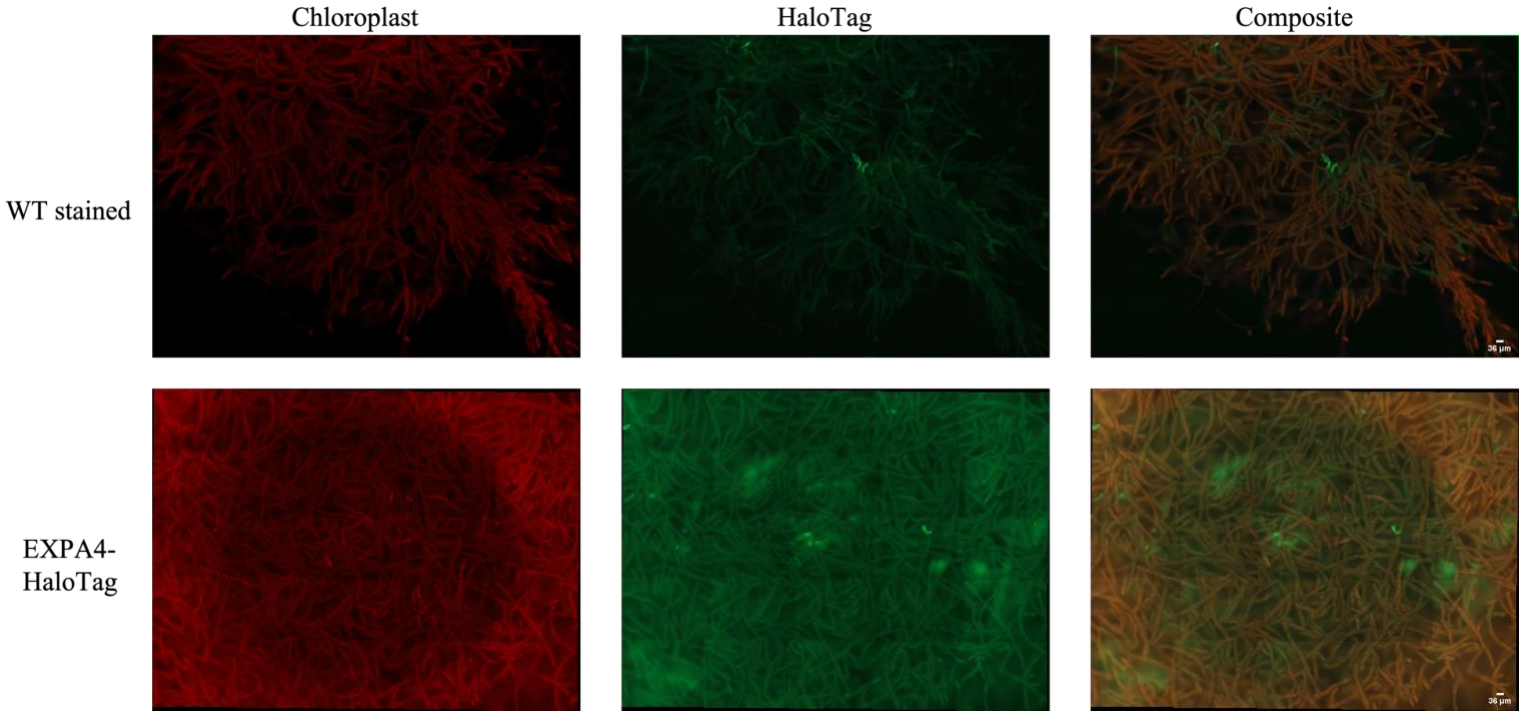


Figure 24. External staining using HaloAlexa488 of wild type Gransden moss and pTH-Ubi-EXPA4-HaloTag moss of developed plants. Background levels in the HaloTag channel are high in pTH-Ubi-EXPA4-HaloTag moss, most likely from not being washed enough. Images taken farther zoomed in seen in **Figure 25**.

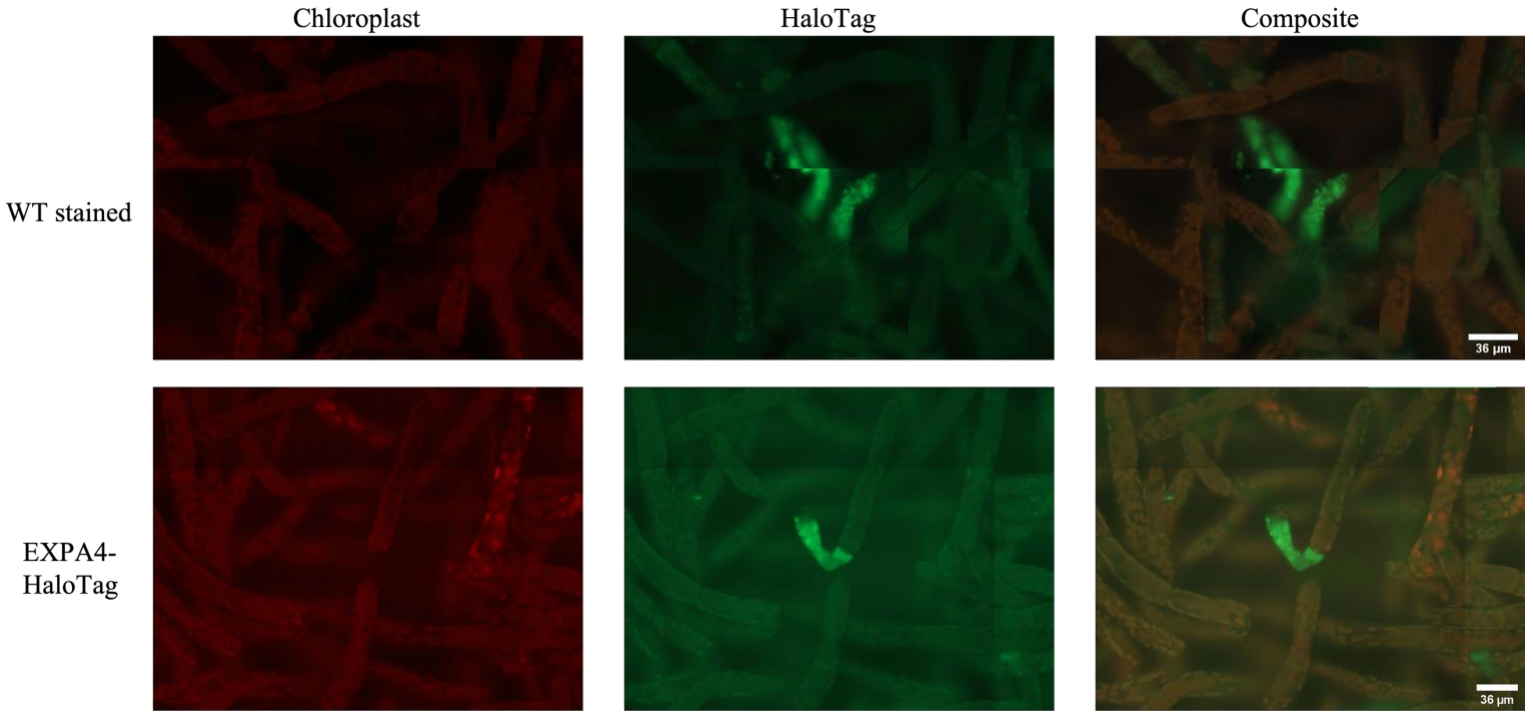


Figure 25. External staining using HaloAlexa488 of wild type Gransden moss and pTH-Ubi-EXPA4-HaloTag moss. WT stained moss shows background levels in the HaloTag channel from a dead cell. pTH-Ubi-EXPA4-HaloTag stained moss shows unsuccessful staining and only background in the HaloTag channel from a dead cell.

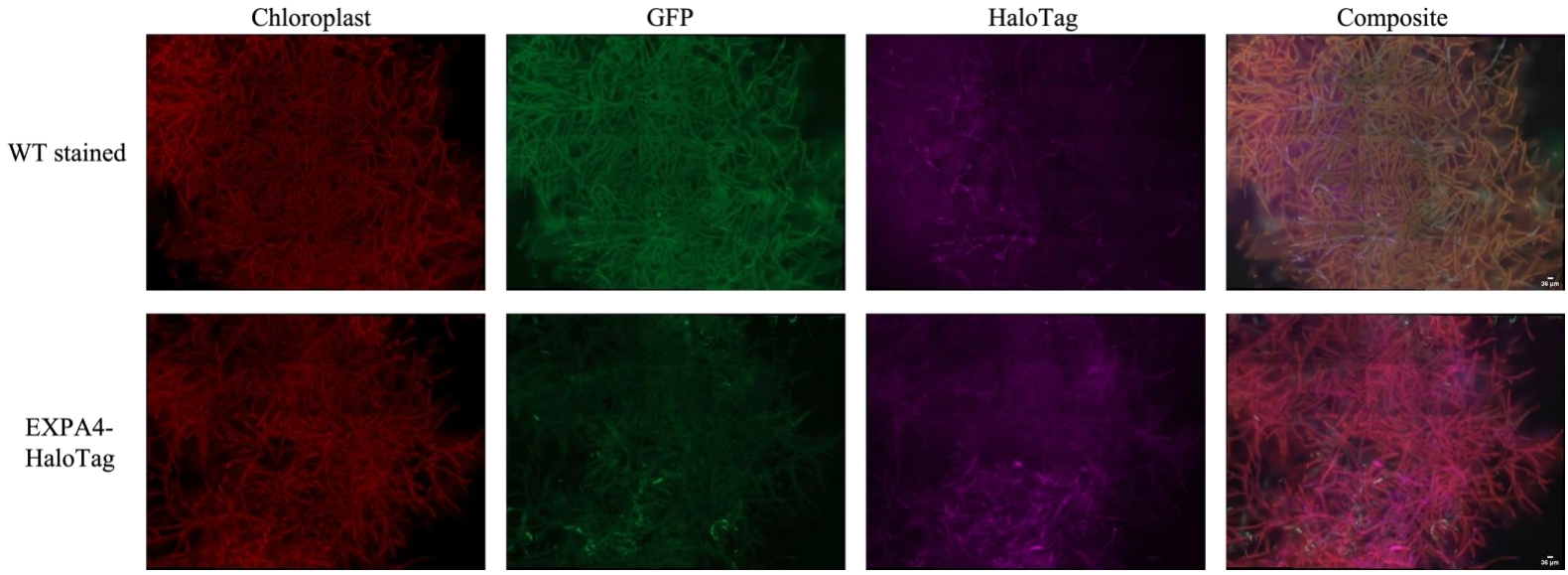


Figure 26. Internal staining using HaloTag Ligand TMR of wild type Gransden moss and pTH-Ubi-EXPA4-HaloTag moss. Images taken farther zoomed in seen in **Figure 27**.

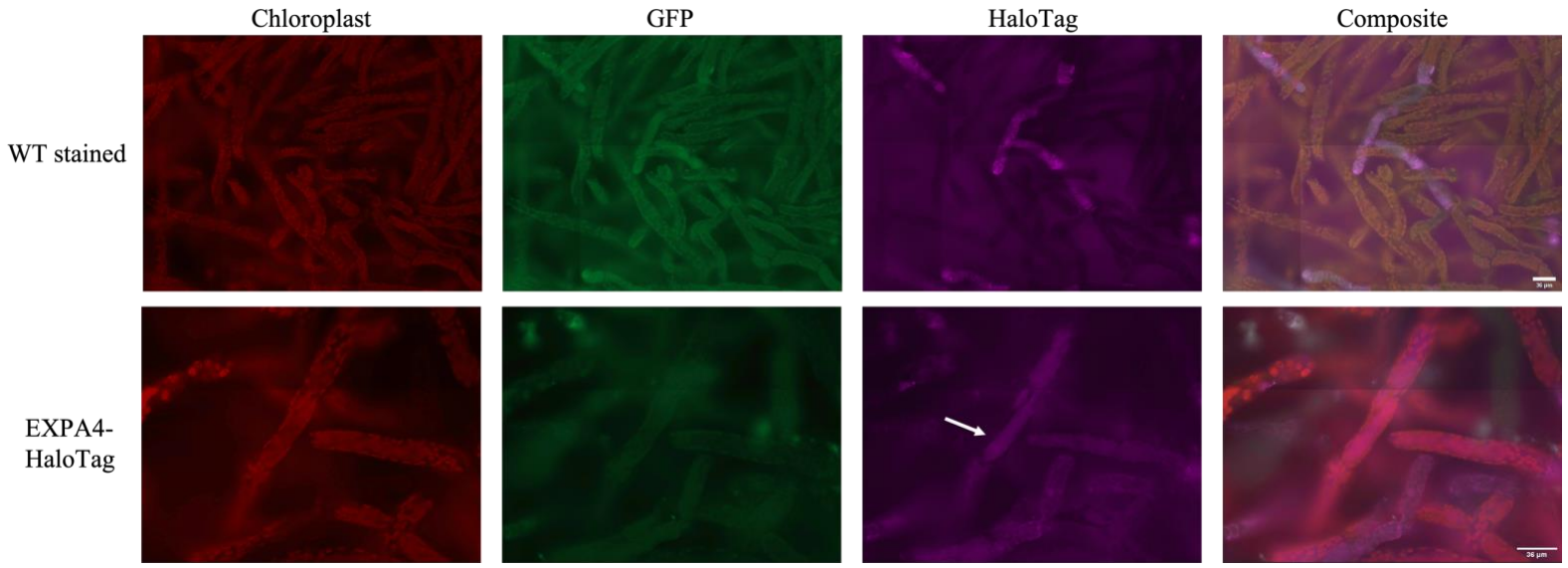


Figure 27. Internal staining using HaloTag Ligand TMR of wild type Gransden moss and pTH-Ubi-EXPA4-HaloTag moss. WT stained moss shows background in the HaloTag channel from dead cells (absent in chloroplast channel and slightly brighter in GFP channel). A positively stained cell (white arrow) in pTH-Ubi-EXPA4-HaloTag stable moss shows possible localization to the vacuole.