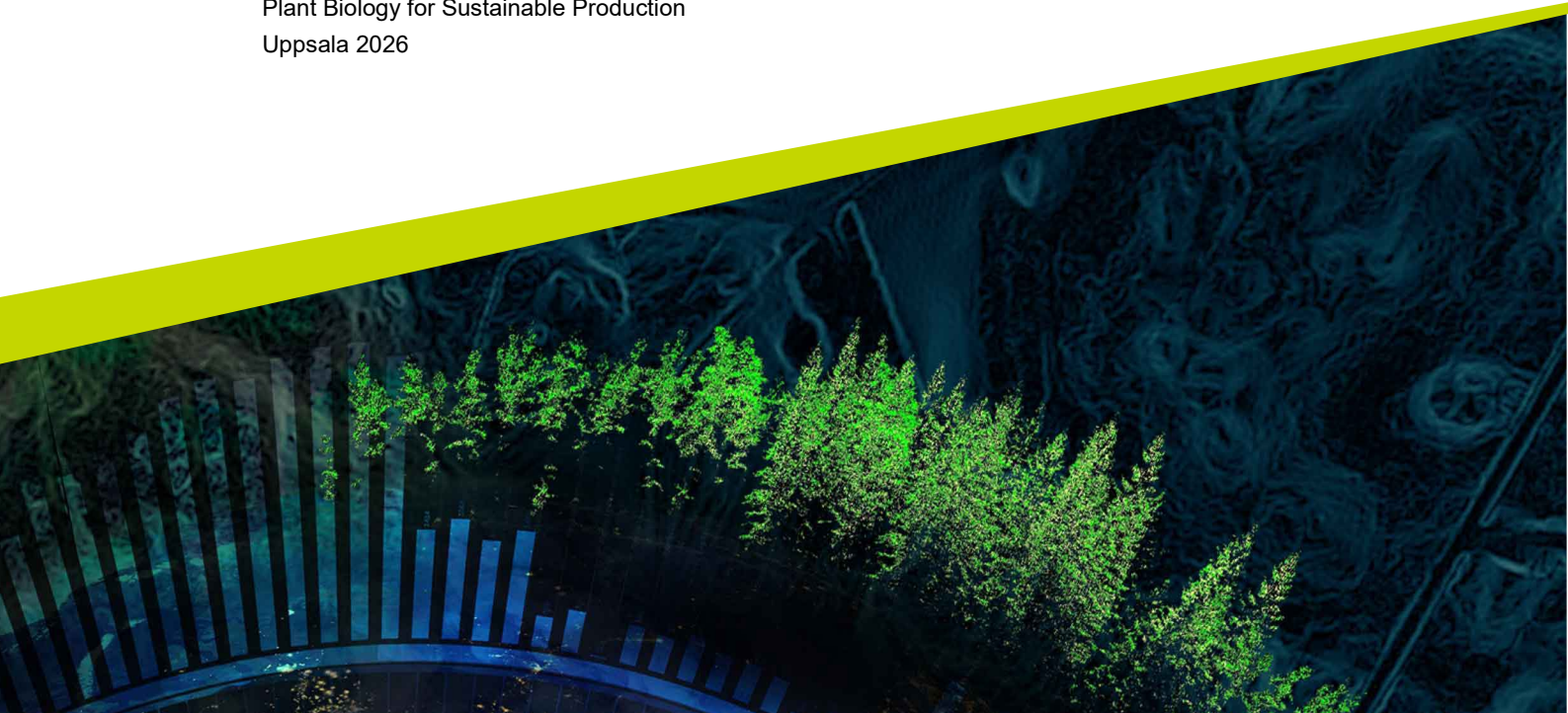




Auxin is involved in the post fertilization development of archegonia and sporophytes in *Physcomitrium patens*

Simon Koetsier

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Swedish University of Agricultural Sciences, SLU
Department of Plant Biology
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Auxin is involved in the post fertilization development of archegonia and sporophytes in *Physcomitrium patens*

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Keywords:	Auxin, Auxin biosynthesis inhibitor, Auxin transport inhibitor, Archegonium, Sporophyte, <i>P. patens</i> , TAR, YUC, PIN
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Abstract

Research on the growth and development of model organism *P. patens* gives insight into the evolutionary history of developmental processes in land plants. Auxin is a phytohormone involved in regulating several developmental processes in the vegetative stage of the moss gametophyte. We investigate the understudied role of auxin in the early development of the sporophyte and that of the female sexual organ (archegonium) post fertilization. We treat *P. patens* with auxin and auxin related compounds, study lines with knockout mutations in auxin biosynthesis and transport genes and study reporter lines of those same genes. We found that auxin treatment can trigger archegonium enlargement independent of fertilization and that auxin treatment interferes with the ability for sporophytes to develop from the fertilized egg cell. We also found that auxin biosynthesis and transport gene expression changes and shifts location as the egg cell is fertilized and develops into a mature sporophyte. Knocking out biosynthesis and transport genes causes different defects in sporophyte development. This research gives an increased insight into these processes and simultaneously raises several new questions.

Keywords: Auxin, Auxin biosynthesis inhibitor, Auxin transport inhibitor, Archegonium, Sporophyte, *P. patens*, *TAR*, *YUC*, *PIN*

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Abbreviations

Abbreviation	Description
TARs	TAA1-related enzymes
YUCs	YUCCA-related enzymes
PIN	PIN-FORMED
NPA	N-1-Naphthaleneacetic acid
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
<i>yucabf</i>	<i>yuca yucb yucf</i> triple knockout mutant
<i>yucbcf</i>	<i>yucb yucc yucf</i> triple knockout mutant
<i>tarac</i>	<i>tara tarc</i> double knockout mutant
<i>pinab</i>	<i>pina pinb</i> double knockout mutant

1. Introduction

Land plants are monophyletic in origin, with bryophytes being the first land plants (Qui and Palmer, 1999). Bryophyte is a group name for liverworts, hornworts and mosses. Given that seed plants have the same common ancestor as mosses, they have the same origin for their developmental processes. This allows for comparisons between seed plants and mosses, which can be helpful for increasing understanding of the developmental mechanisms in both.

Model organisms are often used in developmental research, since they have certain qualities that make them easy to research. A model organism is easy to handle in the laboratory, due to qualities such as short life cycles, ease of cultivation and relatively simple genetics. The moss *Physcomitrium patens* (previously known as *Physcomitrella patens*) has these qualities in addition to being easy to genetically modify and holding an informative phylogenetic position as an early diverging land plant, which were the initial reasons for *P. patens* to gain popularity as a model organism (Rensing et al., 2020). The ability to easily transform *P. patens* makes it easy to produce and study knock-out mutants, a valuable technique in developmental research. *P. patens*' entire genome has been sequenced (Rensing et al., 2008), allowing for tracing of key events in the evolutionary history of land plants.

The vegetative stage of *P. patens* right after spore germination starts with the development of protonema, a filament structure that branches out to form a weblike network. The protonema cells start out as chloronema, that contain many chloroplasts and are separated by transverse cell walls. These transition into caulonema later on, which have fewer chloroplasts and have oblique cell walls separating the cells. Caulonema are thinner and grow faster, allowing for the protonema to extend quickly to find light and other resources. On the caulonema, buds can form and grow into 3D structures, forming gametophores. Gametophores are leaf-like structures that under the right conditions develop sexual organs at the apex.

For the formation of the gametangia, the sexual reproductive organs, cold- and short day conditions are required (Rensing et al., 2020). The male sexual organs, the antheridia, release spermatozoids that fertilize the egg cell located inside of the female sexual organ, known as the archegonium. The sporophyte grows from the egg cell after fertilization. A mature archegonium contains an elongated neck at the apical side with canal cells inside. The basal part is swollen, containing an egg cell at the center with venter cells on the outside (Rensing et al., 2020). To reach maturity, the archegonium undergoes 10 stages of development. It starts out as a bud that undergoes cell divisions that cause it to enlarge, forming inside and outside cell layers. The egg cell starts forming in the basal inner cell at stage 5. By

stage 9 the canal leading into the mature egg cell at the center opens up. Once the archegonium reaches full maturity, its development arrests for as long as it remains unfertilized (Landberg et al., 2013). After fertilization, the zygote grows into an embryo with the archegonium venter expanding around it. The embryo develops into a sporophyte. Lopez-Obando et al. (2022) describes a 15 stage development process for the sporophyte. At stage 4 a ridge forms around the middle part of the archegonium. Right under that bulge the archegonium ruptures at stage 8. The remaining archegonial tissue around the sporophyte following this rupture is referred to as the calyptra. Around stage 9 the sporophyte head starts transitioning from an elongated shape to a more globular shape. Once almost fully mature, around stage 12, the sporophyte turns from light green to dark brown. Throughout the entire life cycle of the sporophyte, it remains basally attached to the gametophyte with the foot. The foot is lined with transfer cells at the outsides, which are used to exchange nutrients with the gametophyte, on which the sporophyte is dependent for survival (Regmi, 2017; Noroozi and Budke, 2025).

Auxin is a phytohormone that plays a major role in plant development. Some of the many processes that auxin is involved in are branching of shoots and roots, differentiation of organs, gravitropism and phototropism (Woodward, 2005).

In flowering plants, the levels of auxin in any given tissue is regulated by de novo synthesis, transport, reversible inactivation by conjugation and degradation. These systems regulate auxin levels in mosses as well (Thelander et al., 2018).

The major auxin biosynthesis pathway in flowering plants is the indole-3-pyruvate (IPyA) pathway. Here, tryptophan is converted into IPyA by TAA1-related enzymes (TARs), which are then converted into auxin by YUCCA-related enzymes (YUCs). *P. patens* has a homologous pathway, acting as the main pathway for auxin biosynthesis in *P. patens* (K. Landberg et al., 2021). The protein family PIN-FORMED (PIN) is responsible for polar transport of auxin in all land plants (Lüth VM et al., 2023). *P. patens* has 3 canonical PIN proteins called PpPINA, PpPINB and PpPINC, in addition to a non-canonical PpPIND. Knocking out PpPINA and PpPINB gives similar effects as applying transport inhibitors. PpPINC can not compensate for the lacking function of PpPINA and PpPINB on its own (Lüth VM et al., 2023).

In *P. patens*, extensive research has been performed on the role of auxin in the vegetative tissues. Auxin stimulates the differentiation from chloronema to caulonema (Jang G & Dolan L, 2011). Auxin also inhibits the growth and initiation of secondary chloronema, which are the chloronema cells that branch off of the primary chloronema (Thelander M et al., 2018). Additionally, auxin initiates the formation of buds on caulonema that later develop into gametophores. In the gametophores, addition of different auxin concentrations cause different phenotypes. Lower concentrations cause elongated shoots with more roots, while

high concentrations cause fewer and narrower leaves, or no leaf formation at all. High concentrations of auxin can also cause termination of the apical growth in a gametophore, resulting in no sexual organs being able to develop (Thelander M et al., 2018).

Auxins' role in moss sporophyte and gametophyte development has seen less research compared to its role in the vegetative stage of gametophytes. PIN-mediated transport of auxin suppresses branching in the sporophyte (Bennett et al., 2014). Auxin is required for the opening of the apical part of the archegonium, once it matures. The maturation of the egg cell also requires auxin. Auxin signalling can be observed in the apical part of the archegonium, the canal cells and the egg cell (Landberg et al., 2013). During early embryo development, auxin signalling is observed uniformly throughout the zygote. In the early stages of sporophyte development, auxin signalling is mainly observed in the apical part of the embryo. In the later stages, the signal becomes stronger in the basal part. In the mature sporophyte, the signal has been reported exclusive to the seta and the foot (Fujita T et al., 2008).

N-1-Naphthaleneacetic acid (NPA) is an auxin transport inhibitor and can cause auxin to accumulate in the tissue it was synthesized. This accumulation can influence developmental processes that depend on auxin levels in a given tissue. As an example, NPA application can cause bifurcation at the margin of the gametophore leaves due to this accumulation of auxin caused by NPA (Robinson and Ashton, 2024). We can gain insight into in what tissue auxin levels are important for a given developmental process using NPA application.

Preliminary unpublished results in the lab suggest that external treatment with the synthetic auxins 1-Naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) break the pre-fertilization arrest of archegonium growth in the essentially sterile *P. patens* ecotype Gransden. Usually, in a fertile ecotype, the archegonium venter starts to enlarge as soon as the egg cell is fertilized and the embryo starts to grow and develop. In this case, application of auxin caused the archegonium venter to enlarge independent of fertilization. This phenotype was observed in *P. patens* grown on medium containing either 1 μ M NAA, 5 μ M 2,4-D or 10 μ M 2,4-D. This suggests auxin may be important for archegonium enlargement following fertilization.

For this project, we hypothesized that the developing embryo, and later the sporophyte foot, use auxin as a signal to break the pre-fertilization arrest of the archegonium and induce its enlargement. This hypothesis was tested using three approaches: application and phenotypic analysis of auxin and auxin inhibitors; expression pattern analysis of several key auxin biosynthesis and transport genes;

and phenotypic analysis of auxin biosynthesis and transport gene knockout mutants.

2. Materials & Methods

2.1 General growing conditions

This project investigated the role that auxin has in archegonium and sporophyte development with three different approaches. Firstly, two *P. patens* ecotypes (Gransden and Reute) were treated with different auxin and auxin inhibitor concentrations, after which the anatomy of the sporophyte was observed under a Leica DMI4000 B microscope at 20x magnification at an early development stage. Secondly, The anatomy of the sporophyte was observed in an early development stage from various different knockout-mutants under a Leica DMI4000 B microscope at 10x magnification. Thirdly, reporter lines were observed of some of the same genes studied in the knock-out mutants under a confocal microscope.

The conditions for the moss grown in all 3 parts of the research were as follows: 4 or 6 *P. patens* colonies were grown in 90x25 mm petri dishes on BCD medium (1 mM MgSO₄, 1.85 mM KH₂PO₄, 10 mM KNO₃, 45 µM FeSO₄, 1 mM CaCl₂, 0.22 µM CuSO₄, 0.19 µM ZnSO₄, 10 µM H₃BO₄, 0.10 µM Na₂MoO₄, 2 µM MnCl₂, 0.23 µM CoCl₂ and 0.17 µM KI). After 5 weeks in the growth chamber (25 degrees celsius, constant light, light intensity of 30 µmol/m²/s) they were transferred to a short photoperiod growth chamber (16 degrees celsius, 8 hours light, light intensity of 30 µmol/m²/s). 20 days after transfer to the short photoperiod growth chamber, the petri dishes were watered. This synchronizes fertilization by spreading out all the spermatozoids around in the entire dish, fertilizing the archegonia.

2.2 Auxin and inhibitor treatments

The auxin treatment was applied during two different points in *P. patens* development and with 2 different synthetic auxins: NAA and 2,4-D. One group received NAA treatment from the start of the colonies, in the BCD medium it was grown on (start treatment). The other group received either an NAA, 2,4-D or NPA treatment from the water used to synchronize fertilization (watering treatment), or was transferred to BCD medium containing auxin right after the fertilization (transfer treatment). This transfer was done by growing this particular group on a BCD medium covered with cellophane. The cellophane was transferred while carrying the moss colonies to a new petri dish with BCD medium containing auxin. This group was watered by spraying water on the colonies, rather than pouring water in the petri dish. The treatments with NAA and NPA had 3 different concentrations. For NAA, those concentrations were 0.1, 1 and 10 µM. For NPA, it was 10, 20 and 40 µM. Only one concentration of 2,4-D

was used, that being 10 μ M. 4 to 6 days after watering, the archegonia were harvested. When possible, 10 gametophores containing archegonia were collected per petri dish. The harvested gametophores were photographed under a microscope with 20x magnification. The entire experiment was performed in duplicate.

For every microscope picture taken, we judged whether or not at least one of the archegonia on a gametophore had enlarged or not. This scoring was done subjectively, with the assumption that all fertilized archegonia would have had plenty of time after fertilization synchronization to develop large enough to be visibly and discretely distinct from non-developed archegonia (Figure 1). The proportions of enlarged and non-enlarged archegonia were analysed with the Fisher's exact test for each treatment.

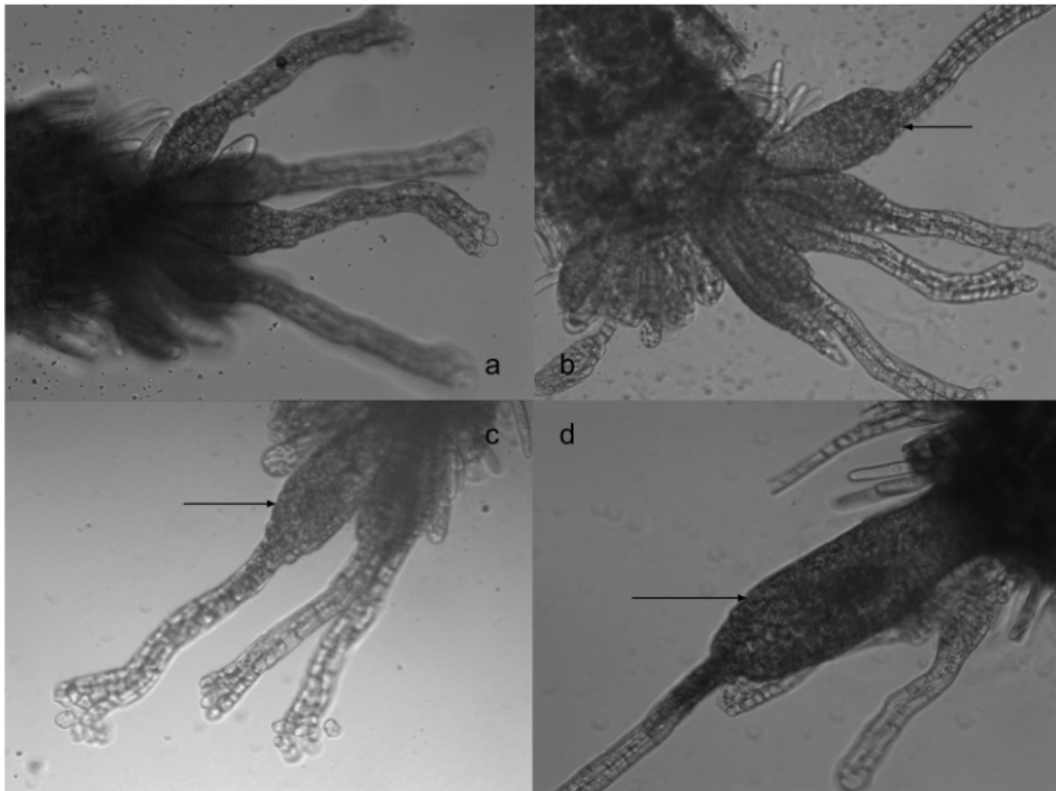


Figure 1: Some examples of how the archegonia were scored. 1a was scored as non-enlarged. 1b was scored as non-enlarged, despite the archegonium with an arrow being slightly larger than the other archegonia in the same image. 1c was scored as enlarged, despite being a relative edge case. Besides this archegonia being clearly larger than the other non-enlarged archegonia, a developing sporophyte can be observed in the center of the venter. 1d is representative of what most enlarged archegonia looked like, where the archegonia has very clearly become much larger in addition to a developing sporophyte being visible at the venter center.

2.3 Knockout mutant analysis

The auxin biosynthesis mutants *tara tarac* double knockout (*tarac*), *yuca yucb yucf* triple knockout (*yucabf*), *yucb yucc yucf* triple knockout (*yucbcf*) and the auxin transport mutant *pina pinb* double knockout (*pinab*) were analyzed. All mutants were previously produced by the group using Reute ecotype as genetic background. After three days post watering, the mutants were occasionally checked for any developed sporophytes. After a maximum of 57 days, any developed sporophytes were harvested and observed under a 10x magnification microscope.

2.4 Reporter line analysis

The expression patterns of reporter lines TARA, TARC, YUCB, YUCF (Reute background) and PINA (Gransden background) were analyzed (K. Landberg et al., 2021). After around 9 days post watering a couple shoots were harvested, with the intention of finding sporophytes of different stages. The samples were observed under a Nikon AxR confocal microscope with an excitation wavelength of 488 nm and a detection range of 499 to 551 nm. YUCB specifically was observed under the Zeiss LSM800 confocal microscope with an excitation wavelength of 488 nm and a detection range of 481 to 578 nm. When possible, the transmitted light images were combined with the fluorescent channel using Fiji.

3. Results

3.1 Auxin treatment reduces sporophyte development frequency and induces archegonium growth independent of fertilization

In order to find out whether auxin and auxin inhibitors have an effect on archegonium and/or sporophyte development, we used several treatment methods on *P. patens* with NAA, 2,4-D and NPA. As there is not much information available on what concentrations of these compounds are best used on mature colonies, we try to cover a wide range of concentrations. We tested these in two different ecotypes: Gransden and Reute. Gransden is male sterile and thus essentially sterile. It was therefore used to test whether auxin treatments might have an effect on the archegonium independent of fertilization. Reute has a high fertility, meaning that we could test whether auxin has a suppressing effect on archegonium and sporophyte development or not. The effect tested for in these treatments was the enlargement of the archegonium venter.

3.1.1 *P. patens* has a reduced frequency of developing sporophytes when treated with NAA

To test the effect of external auxin application, *P. Patens* was grown on medium containing a wide range of NAA concentrations based on previous literature: 0.1, 1 and 10 μM (Le Bail et al., 2013; Rawat et al., 2017; Bascom et al., 2023; Lavy et al., 2016). We used three different concentrations to test whether any observed effect becomes stronger with increasing concentrations, which is generally to be expected if the treatment is the cause of an observed effect.

Reute showed a negative correlation between archegonium enlargement frequency and NAA concentration (Figure 2). Fisher's exact test gave the recorded frequency of enlarged archegonia a p-value of $8 \cdot 10^{-6}$ ($n = 49$), suggesting that NAA interferes with the initiation of sporophyte development. The moss with the 10 μM treatment did not develop any archegonia at all, demonstrating that growing moss on high NAA concentration containing medium severely affects its ability to produce reproductive organs. Even in the 1 μM treatment the amount of archegonia found was extremely limited. None of the archegonia found in this concentration had enlarged.

Start NAA

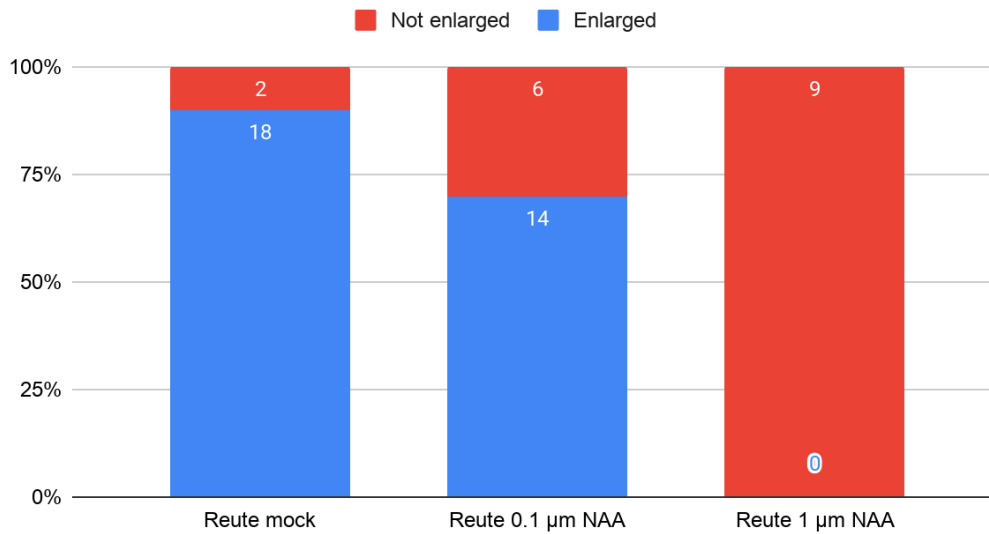


Figure 2: Visualization of the proportions of enlarged archegonia found in the NAA watering treatment. The proportion decreased as NAA concentration increased with an exponential looking trend.

To investigate whether the aforementioned effects of NAA on archegonial and sporophyte development was due to an indirect effect in the earlier vegetative stages or due to a direct involvement of NAA in the reproductive organs, *P. patens* was treated with 0.1, 1 and 10 µM NAA, which was added to the water used to synchronize fertilization. This way, *P. patens* is only treated with NAA once the archegonia are already mature and being fertilized.

Reute showed a negative correlation between NAA concentration and the proportion of enlarged archegonia (Figure 3). Fisher's exact test gave the data a highly significant p-value of 0.005 ($n = 80$). In archegonia that did enlarge, usually a developing embryo was observed, showing a correlation between archegonium enlargement and the initiation of embryo development.

Water NAA

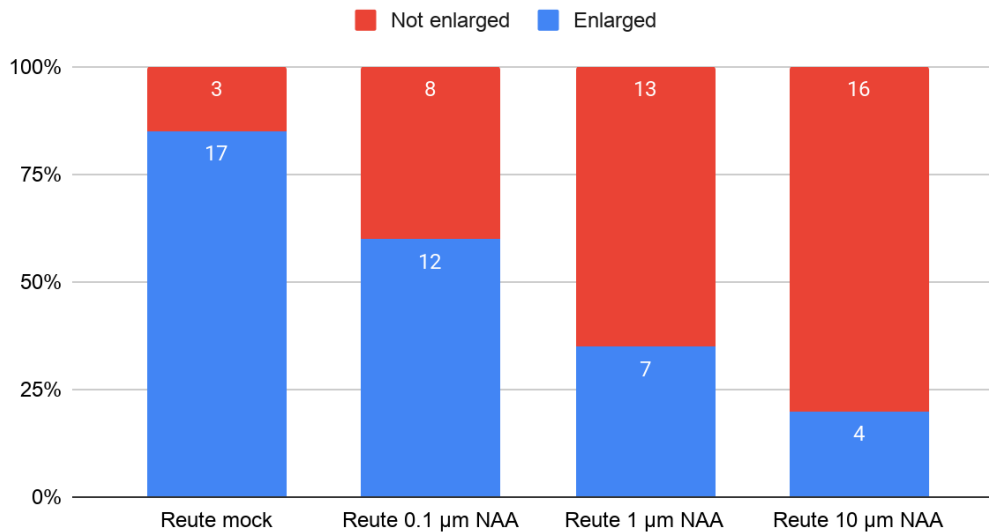


Figure 3: Visualization of the proportions of enlarged archegonia found in the NAA watering treatment. The proportion decreased as NAA concentration increased with a linear looking trend.

Not much is known about effective methods of applying auxin to *P. patens* in later stages of its life cycle. It is possible that treating *P. patens* with auxin by watering does not allow the moss colonies to efficiently take up auxin. Therefore, we also tried using cellophane to transfer the moss colonies to a new medium containing 0.1, 1 or 10 µM of NAA, right before watering to synchronize fertilization.

The proportions of enlarged archegonia fluctuated across concentrations for the Reute in the transfer treatment (Figure 4). Still, there seems to generally be a negative correlation between NAA concentration and archegonium elongation frequency. Fisher's exact test performed on these values gives a p-value of <0.0002 ($n = 78$).

The cellophane had a clear effect on the moss. The colonies looked a lot different and less healthy than their non-cellophane-grown counterparts (Appendix 1). Also, the frequency of enlarged archegonia was significantly lower in the Reute mock transfer treatment compared to the Reute mock watering treatment, having a P-value of 0.003 with Fisher's exact test ($n = 39$). All instances of enlarging archegonium in the three different application methods of NAA seemed to be caused by the initiation of sporophyte development. These results show that NAA interferes with *P. patens*' ability to initiate embryo development. This effect was observed when applying NAA both from the start of colony growth as well as during fertilization. This hints at the observed effect from NAA being due to a direct effect on the archegonium or egg cell, rather than due to an indirect effect on a different tissue earlier on in the life

cycle. NAA treatment did not have any effect on Gransden archegonia both when applied from the start and during fertilization.

Transfer NAA

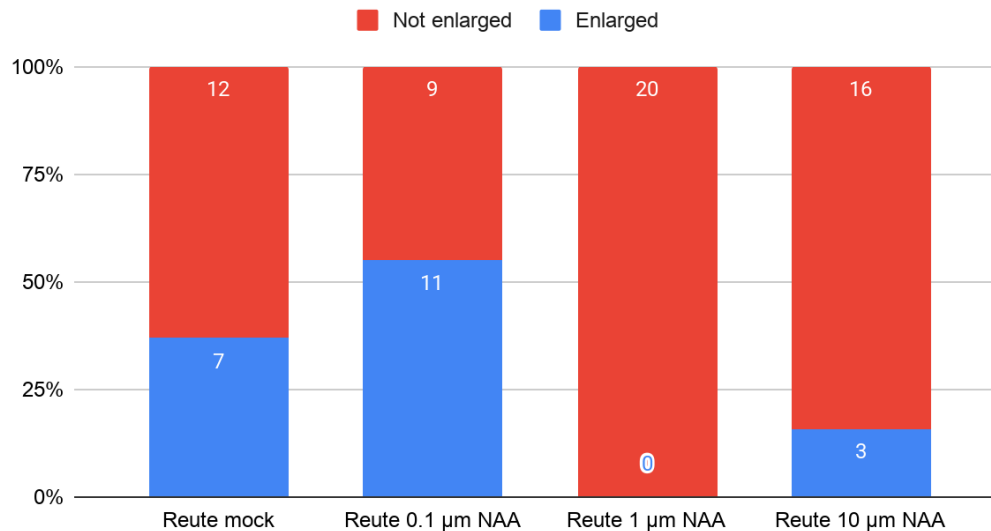


Figure 4: Visualization of the proportions of enlarged archegonia in Reute found in the NAA transfer treatment. The proportion fluctuates up and down with rising NAA concentration, but generally the higher concentrations correlate with a lower proportion of enlarged archegonia. The proportion of enlarged archegonia for all concentrations was lower than that found in the watering treatment for the corresponding concentrations, suggesting that growing on cellophane reduced the amount of enlarging archegonia.

3.1.2 Applying 2,4-D during fertilization interferes with sporophyte development and causes archegonium enlargement independent of fertilization

To compare the effects of different synthetic auxins, 10 µM 2,4-D was also applied with water used for fertilization synchronization. Interestingly, 2,4-D and NAA showed different effects. While in all other treatments the archegonia in the Gransden ecotype seemed unresponsive, adding 2,4-D caused Gransden archegonia to increase in size despite the egg cell not being fertilized. In mock-treated Gransden, none of the archegonia enlarged, suggesting that auxin is important for venter expansion, and that auxin might be produced by the developing embryo and transported to the surrounding archegonium.

These enlarged archegonia had a different shape compared to the shape observed in mock and NAA treatments, with bulges on the sides, similar to the bulge forming right above the rupturing point of the calyptra in a normally developing fertilized archegonium (Figure 5d). Reute treated with 2,4-D showed a very similar phenotype (Figure 5c). Additionally, the enlargement observed in both ecotypes was less than that observed in mock Reute or even Reute treated with

high concentrations of NAA (Figure 5a). In contrast to NAA and mock treated Reute, there was no sporophyte developing inside any of the enlarged archegonia found in the 2,4-D treatments, regardless of the ecotype.

The proportion of enlarged archegonia in the 2,4-D treated Gransden was similar to the Reute mock (Figure 6). On the other hand, the Reute 2,4-D treatment had a significantly lower proportion of enlarged archegonia compared to the Reute mock, with a p-value of about 0.008 as tested with Fisher's exact test ($n = 40$).

Also noteworthy is that the proportion of enlarged archegonia in Reute 2,4-D was lower than the proportion in Gransden 2,4-D. The P-value for this difference was about 0.053 ($n = 40$).

Taken together, it appears that 2,4-D had a stronger effect on *P. patens* than NAA at the same concentrations, interfering with the initiation of embryo development. The experiments show that proper initiation of embryo development is sensitive to auxin concentration and that enlargement of the archegonium venter surrounding the embryo can be triggered by auxin. They also suggest that 2,4-D may affect the archegonia differently depending on ecotype.

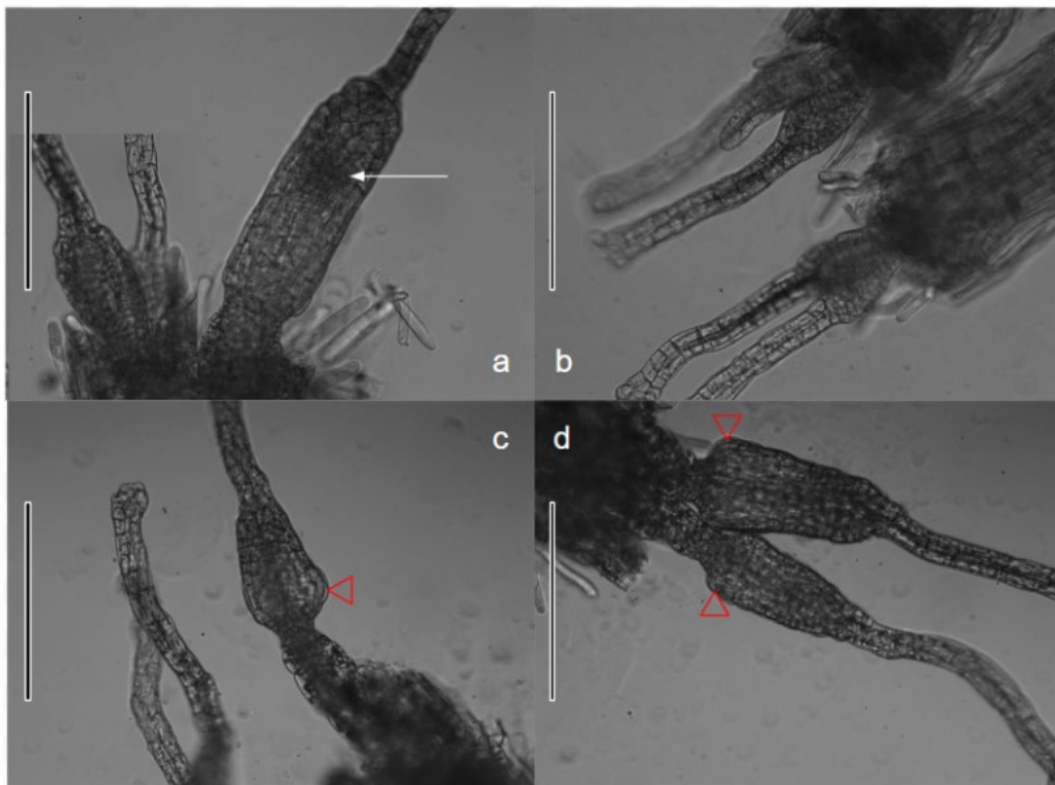


Figure 5: Comparison of archegonia in the 10 μ M NAA treatment of Reute (5a), the 10 μ M NAA treatment of Gransden (5b), the 10 μ M 2,4-D treatment of Reute (5c) and the 10 μ M 2,4-D treatment of Gransden (5d). Visible from these pictures is how the archegonia treated with NAA had a remarkably different shape and size compared to the archegonia treated with 2,4-D. Reute treated with 10 μ M NAA grew its archegonia similarly to the mock treatment, albeit with lower frequency. A developing sporophyte appeared as a faint looking darker circle in the Reute 10 μ M NAA treatment (white arrow). On the left

side of 5a, a smaller picture was edited in that depicts a non-enlarged archegonium from the 10 μM NAA treated Reute. Gransden treated with 10 μM NAA looks like a regular unfertilized archegonium as seen in the mock treatment. However, both Reute and Gransden treated with 2,4-D do not contain a sporophyte within the archegonia, despite those archegonia clearly being larger than the undeveloped 10 μM NAA treated Gransden archegonia. They also have a remarkable shape that is broader at the base (red arrowheads). Both the 10 μM NAA treated Reute and Gransden archegonia look the same as their respective mock treatment counterparts. Bars on the left represent 335 μm .

Water 2,4-D

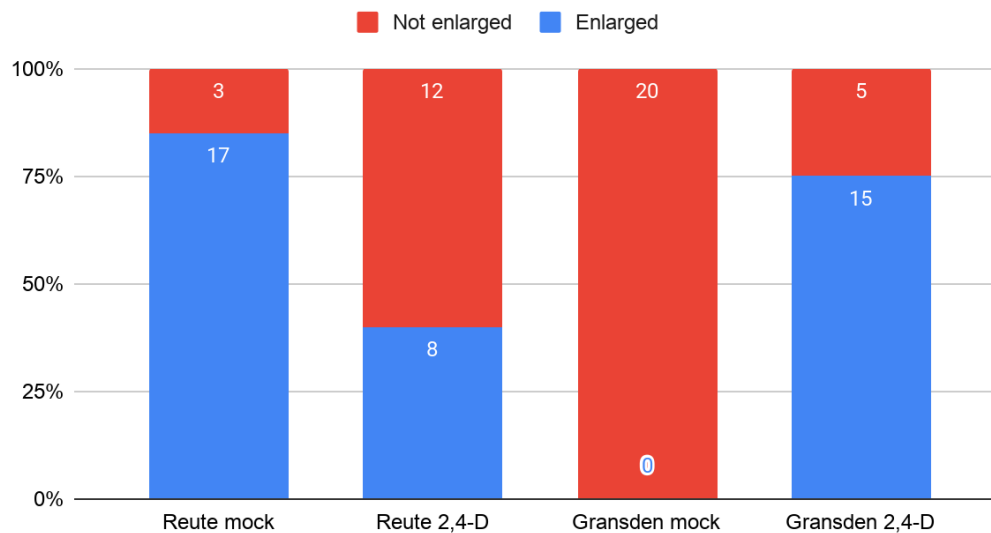


Figure 6: Visualization of the proportions of enlarged archegonia found in the 2,4-D watering treatment. Gransden 2,4-D had a similar proportion of enlarged archegonia as Reute mock, although none of the enlarged archegonia contained a developing sporophyte, in contrast to Reute mock. Reute 2,4-D had no developing sporophytes either and a statistically significant lower frequency of enlarged archegonia than Gransden 2,4-D.

3.1.3 Applying NPA during fertilization may have no effect on *P. patens* archegonium enlargement

If auxin does get actively transported from the developing sporophyte to the archegonium to induce the archegonium to enlarge, we would expect to see this process disrupted with treatment of auxin transport inhibitor NPA. Based on previous research, we used three different concentrations: 10 μM , 20 μM and 40 μM (Nemec-Venza et al., 2022; Fujita et al., 2008; Rawat et al., 2017).

A slight negative correlation can be observed between NPA concentration and enlarged archegonium frequency (Figure 7). However, Fisher's exact test gives these values a very high p-value of 0.518 ($n = 70$). Since there were no visual differences in the colonies across different NPA concentrations, it is unclear whether the NPA was even taken up into the moss in the first place (Appendix 2). Overall these results are inconclusive.

Water NPA

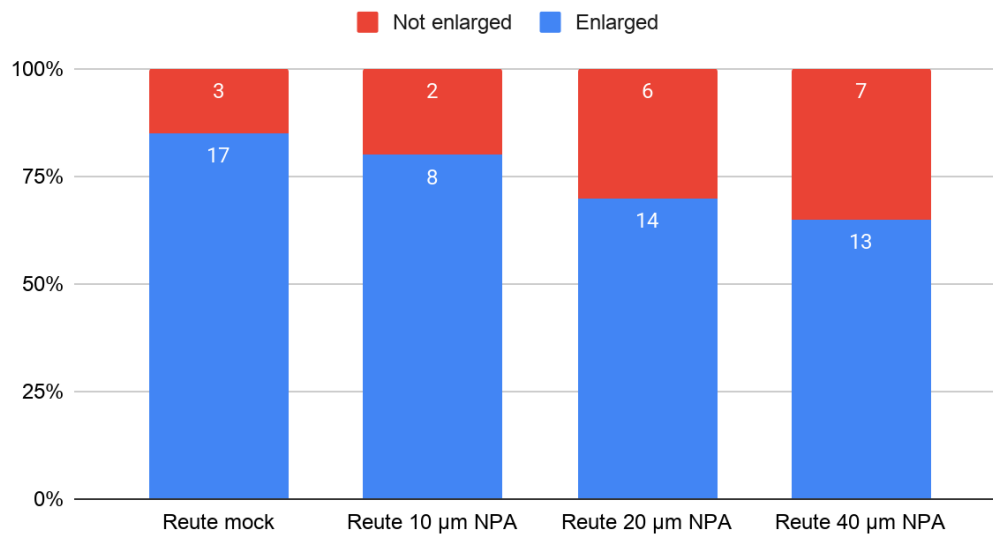


Figure 7: Visualization of the proportions of enlarged archegonia found in the NPA watering treatment (excluding the outlier, see Appendix 5). The proportions seem to slightly decrease with increasing NPA concentration, although this decrease is statistically insignificant.

3.1.4 Conclusion

All methods of NAA treatment had the same trend. In Reute, the frequency of archegonium enlargement and with that sporophyte development decreased as NAA concentration increases. In Gransden, NAA treatment had no effect. 2,4-D treatment caused archegonia in both Reute and Gransden to enlarge despite no sporophyte developing in any of the samples treated with 2,4-D. NPA treatment had no effect on the archegonia nor on the treated moss colony as a whole suggesting that blocking active auxin transport does not affect archegonia and/or sporophyte development, or that NPA simply was not properly taken up by the moss tissue. The effects of auxin on archegonium development were observed both when the hormone was applied from the start and when it was applied at the time of fertilization. These observations support a direct role for auxin in regulating archegonium enlargement, rather than an indirect effect mediated by elevated auxin levels in vegetative tissues.

3.2 Sporophyte development is stunted in auxin biosynthesis and transport knockout mutants

3.2.1 Sporophytes in *tarac* and *yucabf* have stunted development and *yucbcf* develops no sporophytes

TARs and YUCs are both involved downstream of each other in the main auxin biosynthesis pathway in *P. patens*. TAR has four homologs and YUC has six homologs (K. Landberg et al., 2021). In order to gain more insight into which of these homologs are important for sporophyte development, we tested three different unpublished knockout mutants: *tarac*, *yucabf* and *yucbcf*.

Developing sporophytes were much less common in the YUC and TAR mutants compared to the wild type. The images of the sporophytes were taken 57 days post watering for fertilization synchronization. Most of the *tarac* sporophytes were around stage 10, with one being around stage 7 (Figure 8). *yucabf* mostly had sporophytes around stage 9, with one being around stage 11 (Figure 9). No sporophytes were found in the *yucbcf* mutant.

While analyzing the rare sporophytes developing in *tarac* and *yucabf*, it was clear that their development was heavily stunted, considering they still were not fully mature 57 days after watering. In the wild type, it takes around 16 days to reach stage 11 and around 31 days to full maturity (Lopez-Obando et al., 2022). They were also considerably smaller than the wild type sporophytes of the same developmental stage, around 200 μM wide, compared to wild type stage 10 sporophytes that are usually over 400 μM wide (Figure 10). Also noteworthy is the fact that some sporophytes detached from the mother tissue very easily and that the basal tip of the foot was pigmented, having a reddish colour. Lastly, in *yucabf* the archegonium necks were noticeably shorter than in the wild type (Figure 9).

It seems that while it is possible for *P. patens* to start the development of some sporophytes without the TARac or the YUCabf genes, they are still important for the developmental process to normally function and required for full maturation of the sporophyte. Since *yucbcf* did not seem to develop any sporophytes at all, YUCc is likely essential for the initiation of sporophyte development, at least

without functional YUCb and YUCf.

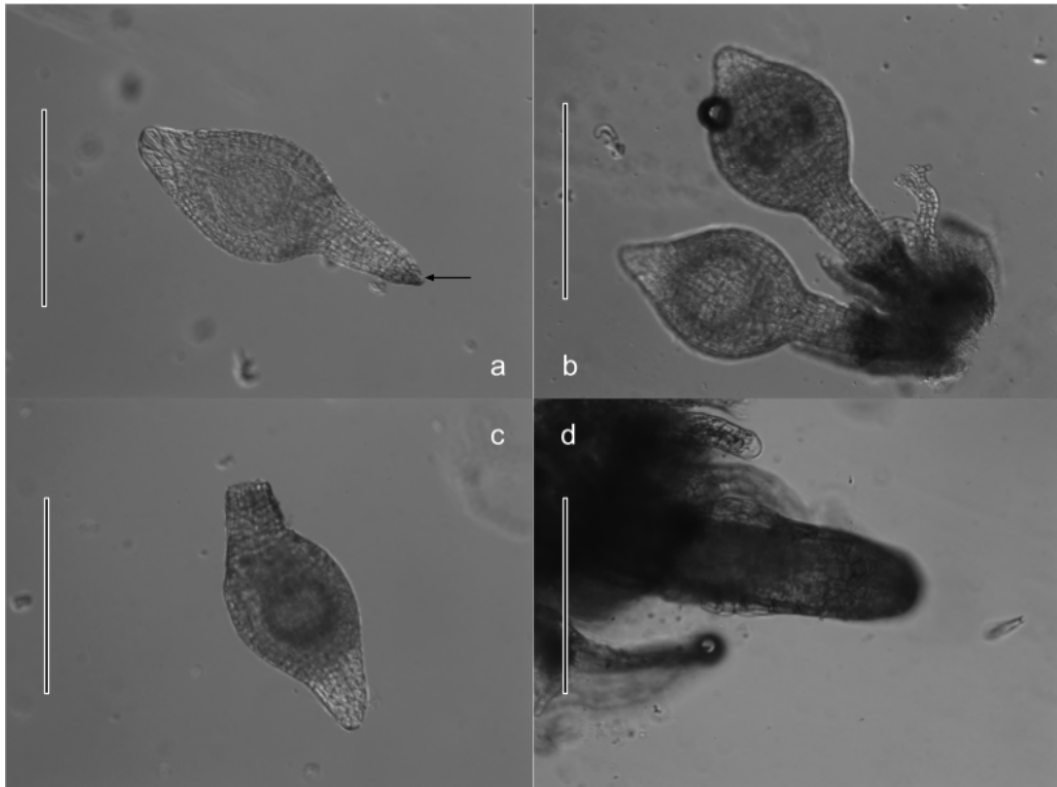


Figure 8: Sporophytes of the *tarac dko* mutant. 8a, 8b and 8c are around stage 10. 8d is around stage 7. The black arrow (8a) points at a pigmented tip in the foot. These sporophytes were less than half the size of wild type sporophytes. Bars on the left side of the image represent 335 μm .

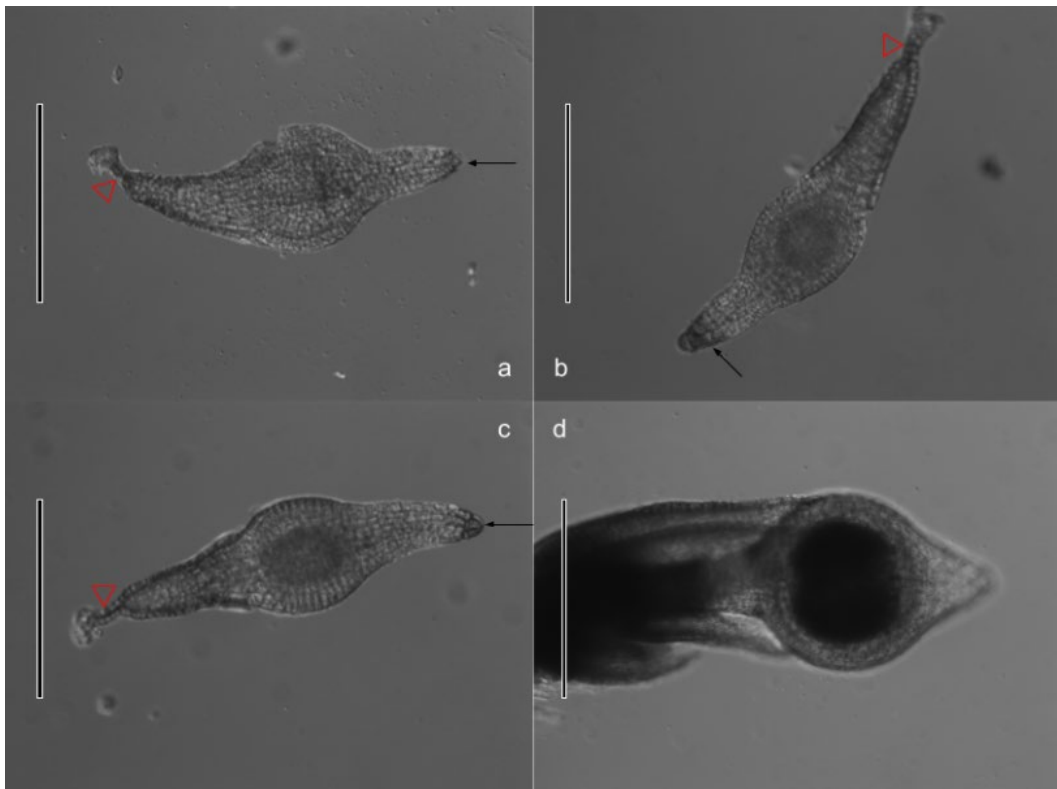


Figure 9: Sporophytes of the *yucabf tko* mutant. 9a, 9b and 9c are around stage 9 and 9d around stage 11. Black arrows point at pigmented tips in the foot. Red arrowheads point at the archegonium canals, which are much shorter than in the wildtype. These sporophytes were less than half the size of wild type sporophytes. Bars on the left side represent 335 μ m.



Figure 10: image of a wild type stage 10 sporophyte under a 5x magnification microscope. The bar on the left represents 335 μ m. This sporophyte is significantly larger than those of similar stages in *yucabf* and *tarac*.

3.2.2 PIN-mediated auxin transport is important but not essential for sporophyte development

Only 2 sporophytes were found in the *pinab*. 3 days post watering, when sporophytes usually already have started to develop in the wild type, only non-enlarged archegonia were found (Figure 11a). 35 days post watering, one developing sporophyte of around stage 8 was found. This same sporophyte was harvested and photographed 57 days post watering, at which point it was still stage 8, indicating that its development had stagnated (Figure 11c). Another sporophyte of around stage 10 was also found 57 days post watering, of which it is unclear whether its development had stagnated (Figure 11d).

Just like how the treatment with the auxin transport inhibitor NPA looked similar to the mock, nothing about the morphology of *pinab* seemed different from the wild type. These observations suggest that *pinab* double mutants are severely affected in the fertilization process or during early sporophyte

development. However, at a very low frequency, they can overcome this and continue sporophyte development to maturity.

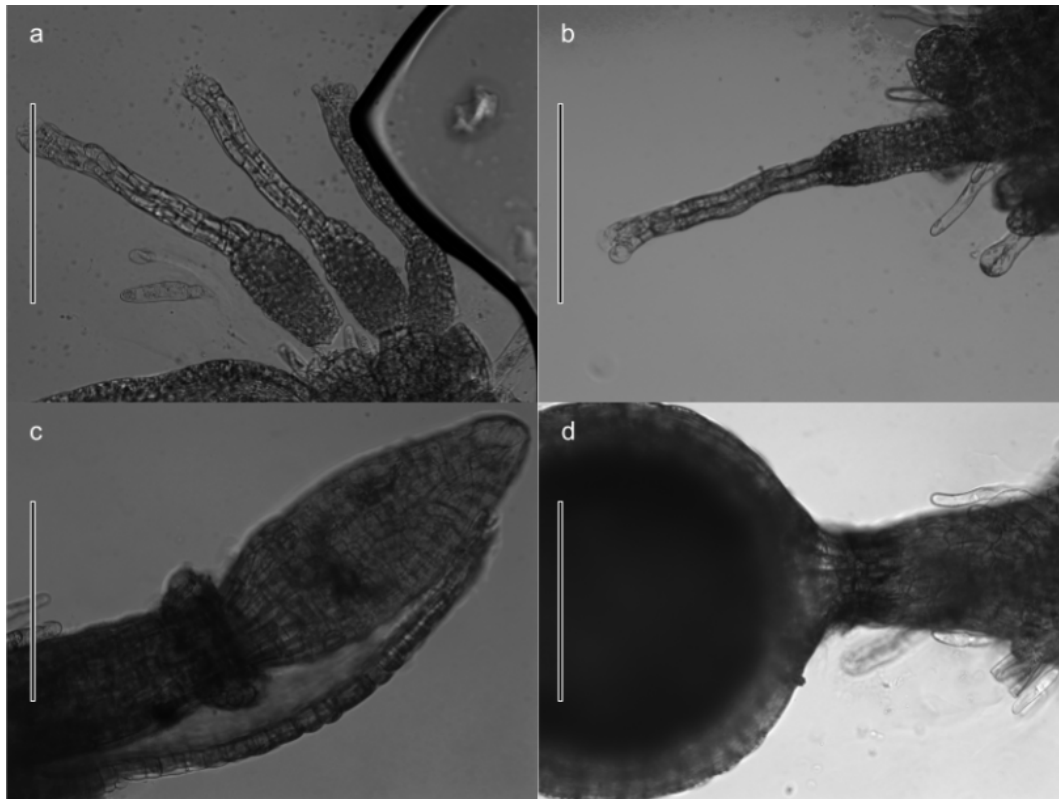


Figure 11: Archegonia and sporophytes of the PINab dko mutant. 11a was taken 3 days post watering, 11b 50 days post watering, 11c and 11d both 57 days post watering. Sporophyte development in pinab was much slower than the wild type and stagnated before reaching maturity. Bars on the left side represent 335 μ m.

3.3 Reporter lines hint at the possibility of exchange of auxin signals between sporophyte and archegonium

To reveal where and when auxin biosynthesis genes are expressed during sporophyte development, we used confocal microscopy to analyze transcriptional reporter lines TARA::GFP, TARC::GFP, YUCB::GFP and YUCF::GFP. We also analyzed the expression of the PINA::GFP reporter line to monitor auxin transport.

3.3.1 Auxin biosynthesis genes are expressed in fertilized egg cell, and apical and basal parts of developing sporophyte

TARA displayed a strong apical signal that decreases towards the basal region forming a clear discrete gradient (Figure 12a). A faint signal was also visible in the foot (Figure 12b). TARC also mainly displays an apical signal in early stages,

along with a fainter signal in the rest of the embryo (Figure 13a,b). As it matures, this signal becomes more homogeneous over the whole embryo, including the foot (Figure 13c,d). Both in TARC and TARA there is usually a faint signal in the basal part of the archegonium still wrapped around the sporophyte as well (Figures 12 and 13c).

YUCB has a relatively homogenous expression in early stages (Figure 14a). This signal later shifts to only the apical and basal parts (Figure 14b), and in the latest stages is very clearly expressed in the foot (Figure 14c,d). YUCF shows a clear signal in the foot throughout the developmental stages (Figure 15). Both YUCB and YUCF express uniformly throughout the fertilized egg cell, and not at all in an unfertilized egg cell (Figure 16). In all reporter lines, strong signalling can be observed in elongated structures growing on the gametophyte around the base of the sporophyte. These are axillary hairs, and it is unknown why these have high expression of auxin biosynthesis genes.

These observations suggest that TAR and YUC genes have a changing role during sporophyte development.

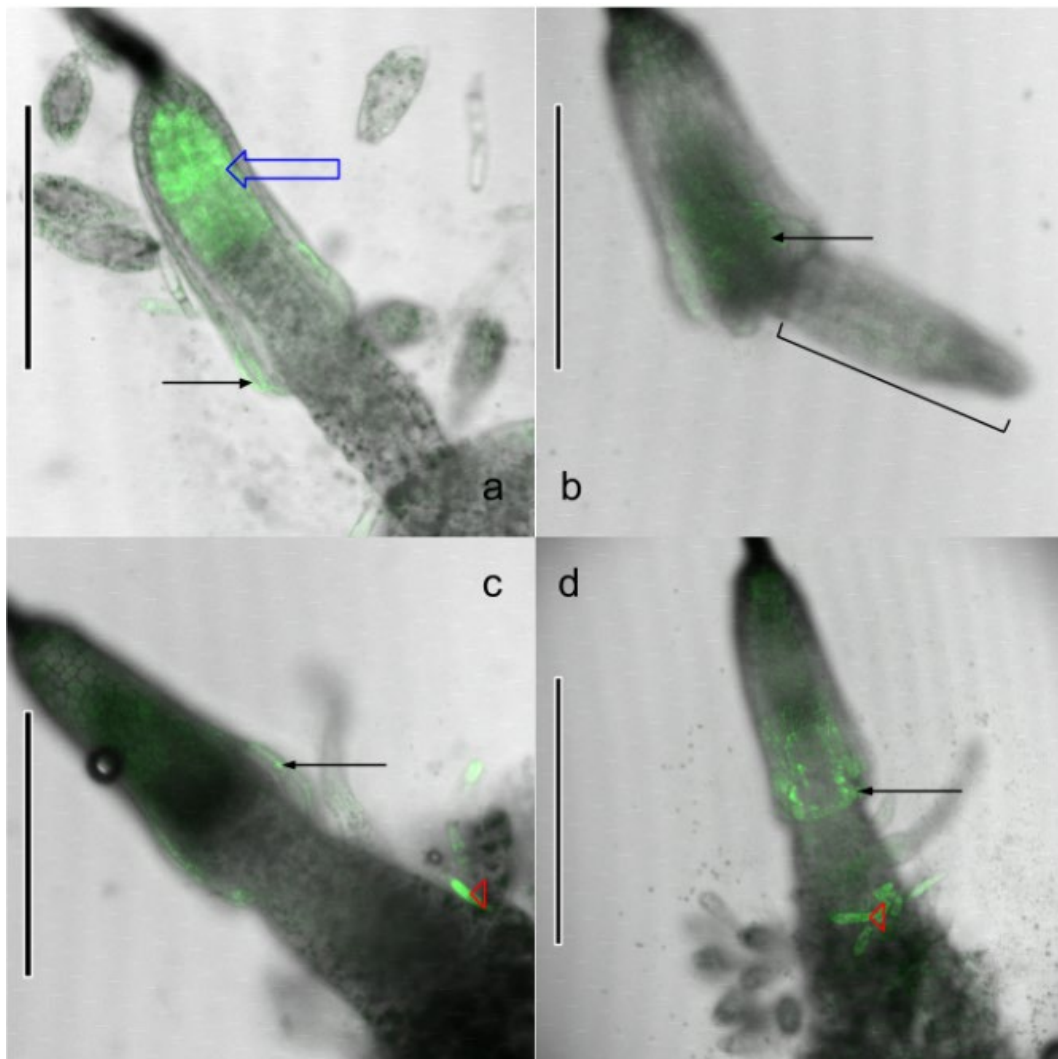


Figure 12: Signalling patterns of the *TARA::GFP* reporter line. All these images are from around stage 7. Broad blue arrows show signalling in the sporophyte, thin black arrows in the mother tissue and red arrowheads in axillary hairs. 12a shows signal in the apical part in the sporophyte, which decreases in intensity towards the basal direction following a discrete spectrum. A similar signal in the sporophyte was not found in the other three samples. In 12b, some faint signalling can be observed in the foot, which is indicated with a bracket. Both 12c and 12d do not have clear signalling in the sporophyte. In all four samples signalling in the calyptra can be observed. The left bars represent 240 μm for 12a, 293 μm for 12b, 300 μm for 12c and 440 μm for 12d.

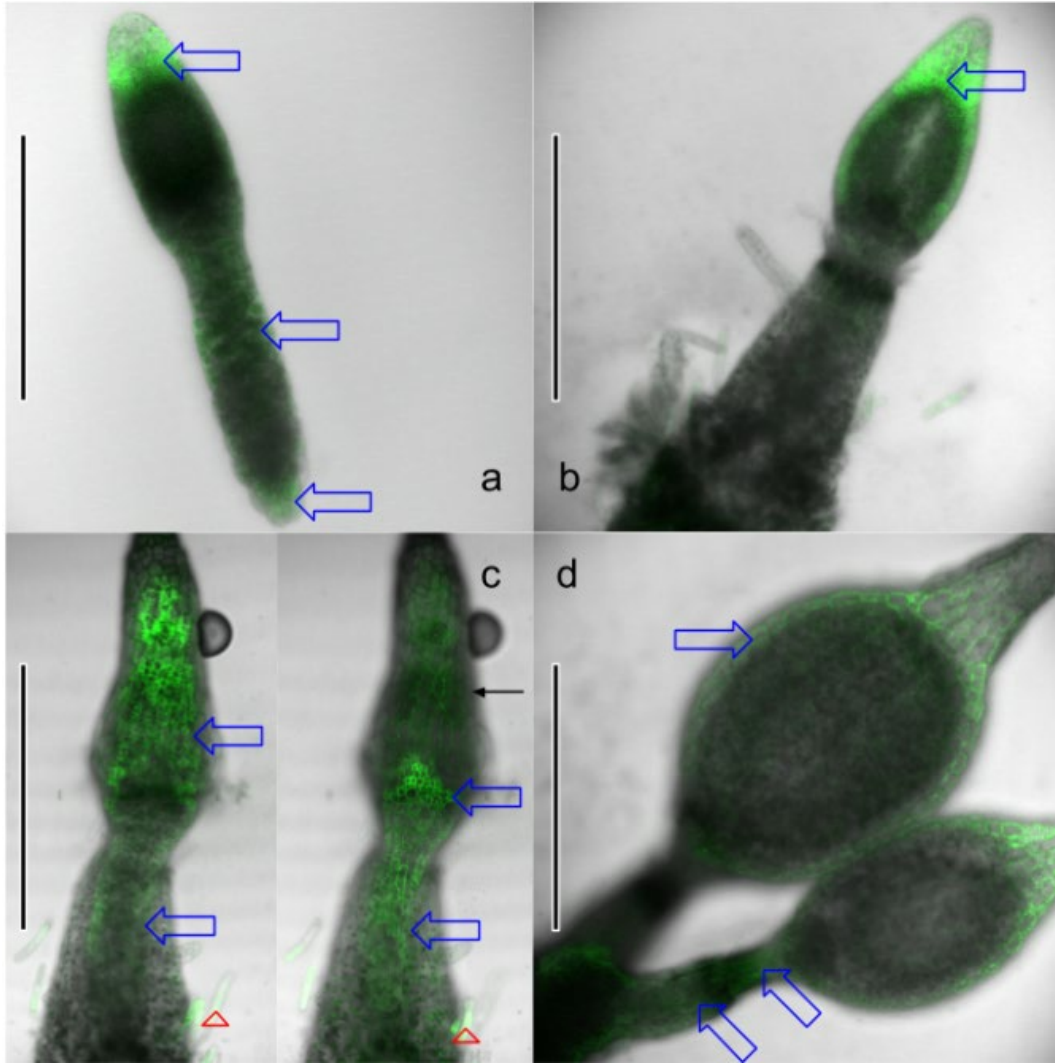


Figure 13: Signalling patterns of the *TARC::GFP* reporter line. 13a is stage 7, 13b stage 8, 13c stage 9 and 13d stage 10. Broad blue arrows show signalling in the sporophyte, thin black arrows in the mother tissue and red arrowheads in axillary hairs. In 13c two different planes are visible. 13a and 13b both showed strong signal in the apical part of the sporophyte, with 13a also showing a relatively weaker signal in the foot. 13c shows signalling throughout the entire sporophyte and across the entire calyptra. 13d shows a signal in the outer layers of the sporophyte head and some signal in the foot (bottom arrow) and the seta (arrow right above the foot). Bars on the left represent 353 μm for 13a and 440 μm for 13b, 13c and 13d.

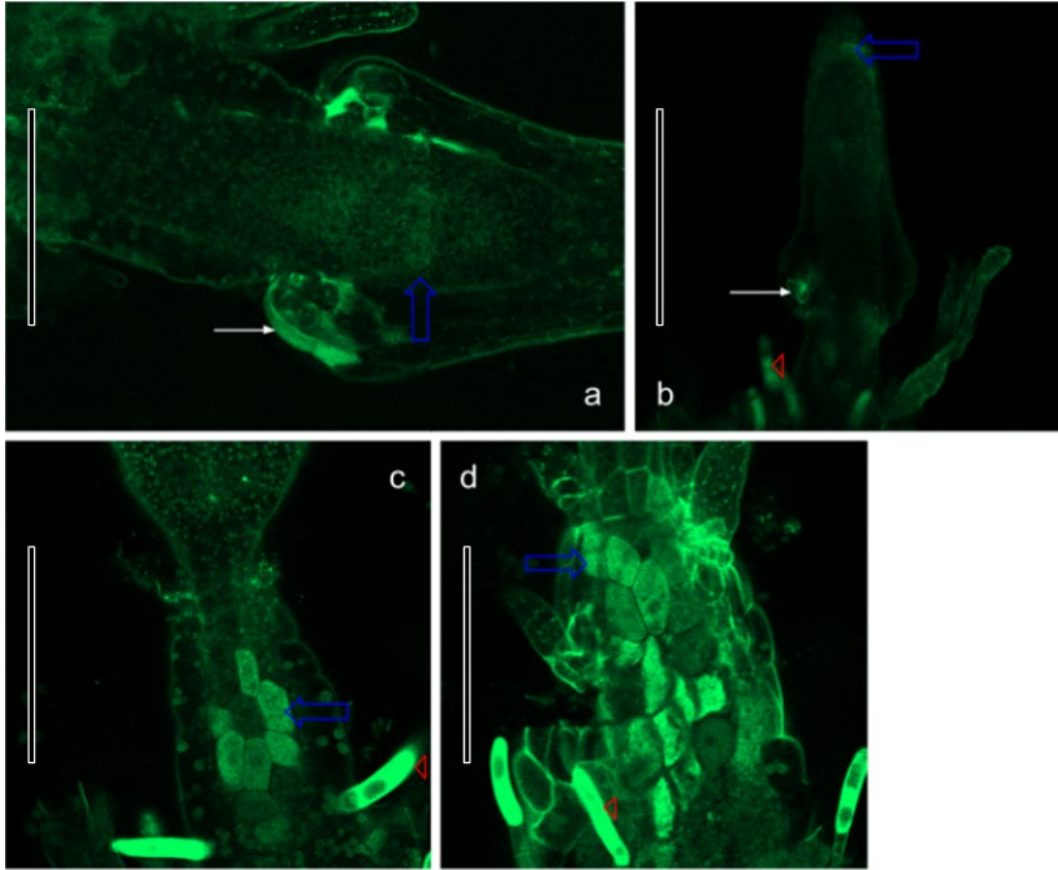


Figure 14: Signalling patterns of the YUCB::GFP reporter line. Image 14a is composed of two images stitched together to get more coverage of the sporophyte. This one is around stage 5. 14b is stage 7, 14c stage 9 and 14d stage 10. Broad blue arrows show signalling in the sporophyte, thin white arrows in the mother tissue and red arrowheads in axillary hairs. 14a has weak expression throughout the entire sporophyte and a stronger expression in the lower calyptra. 14b has overall relatively weak signalling, showing the highest expression in the apical part. It also shows slight expression in the basal part of the calyptra. 14c has a very clear signal in the foot, specifically in the outermost cells, that are likely the transfer cells. In 14d this signal can also be observed, although being photographed in a plane closer to the surface. Bars on the left represent 160 μm for 14a, 14c and 14d and 320 μm for 14b.

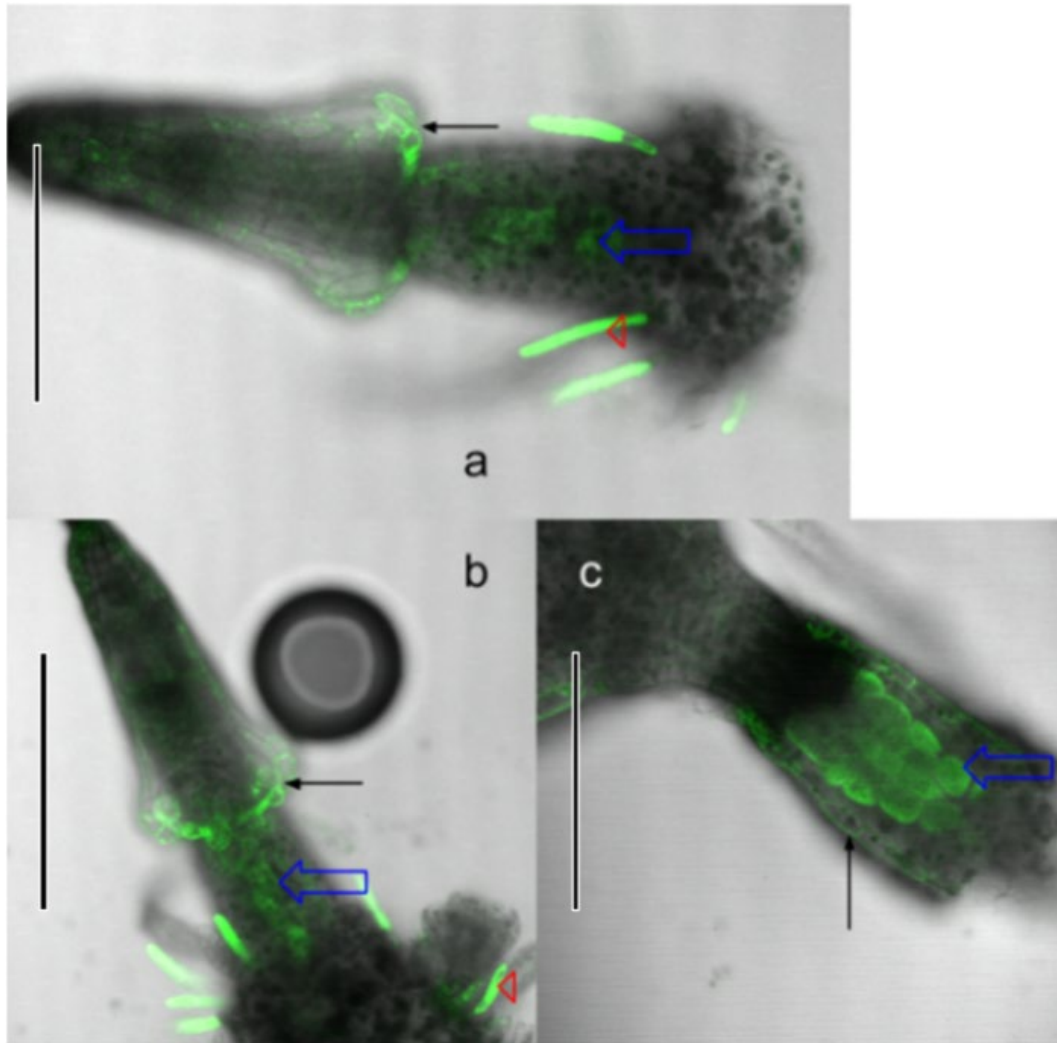


Figure 15: Signalling patterns of YUCF::GFP reporter line. Image 15a is stage 6, 15b stage 7 and 15c stage 10. Broad blue arrows show signalling in the sporophyte, thin black arrows in the mother tissue and red arrowheads in axillary hairs. In 15a, a weak signal can be observed in the foot. This signal is even more apparent in 15b. Both 15a and 15b also show a clear signal in the lower calyptra, as well as a weaker signal in the rest of the calyptra. 15c Shows a clear signal in the foot that seems to get stronger on the periphery. There is also weaker expression in the mother tissue surrounding the foot. Left bar represents 232 μm in 15a, 322 μm in 15b and 220 μm in 15c.

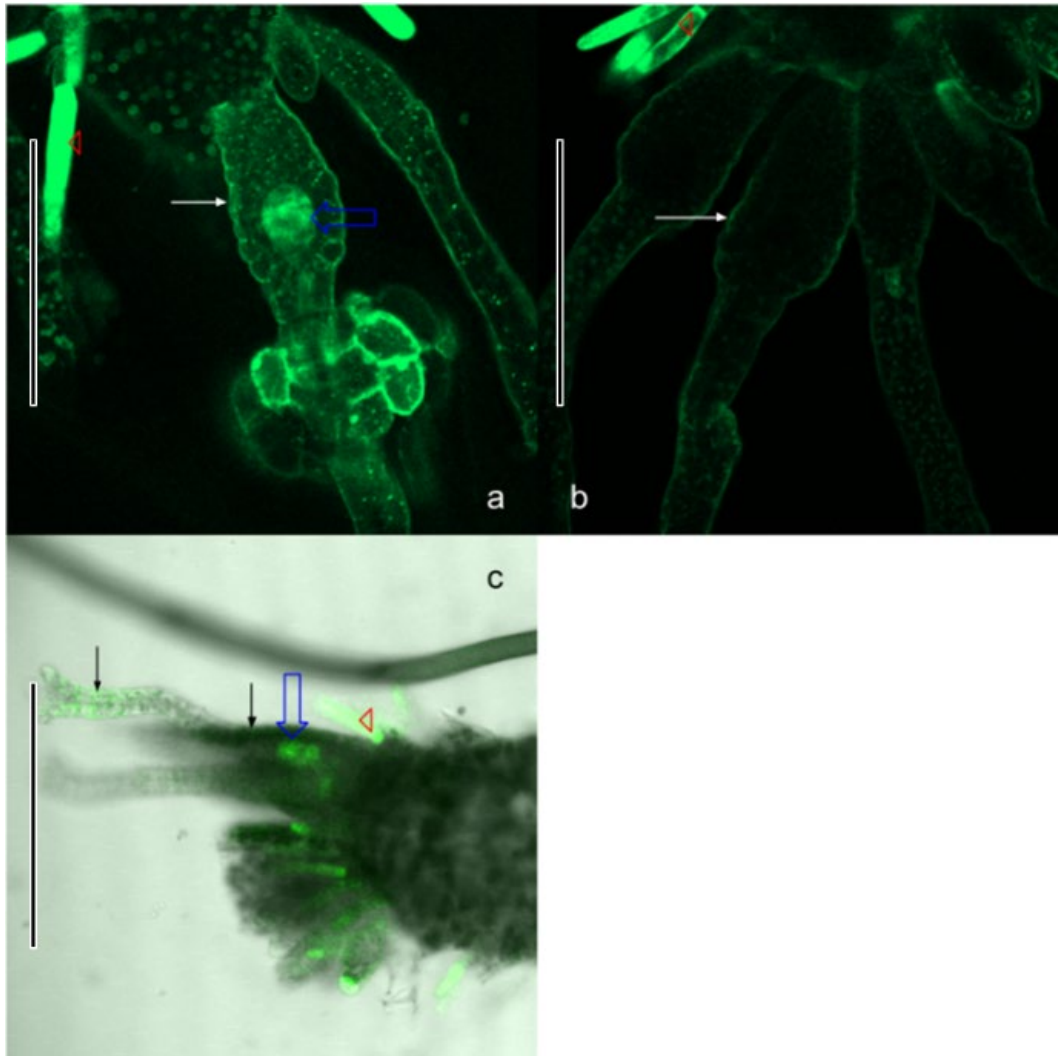


Figure 16: Expression pattern of YUCB (16a and 16b) and YUCF (16c) in archegonia. 16a and 16c seemed to be fertilized, while 16b was not. Broad blue arrows show signalling in the sporophyte, thin black/white arrows in the mother tissue and red arrowheads in axillary hairs. The egg cell shows a strong uniform YUCB expression signal upon fertilization. Both fertilized (16a) and unfertilized (16b) show a YUCB signal in the outermost cell layer. YUCF is also expressed in the fertilized egg cell. A faint signal can also be seen in the surrounding venter and upper canal. Left bars in 16a and 16b are 160 μm , left bar in 16c is 266 μm .

3.3.2 PINA reporter line reveals expression in unfertilized egg cell

To explore the possibility of PIN involvement in the transport of auxin from the zygote to the archegonium, we investigated a PINA::GFP reporter line.

This line was produced in a Gransden background, meaning only unfertilized egg cells could be analyzed. A uniform signal could be observed in both a mature egg cell (Figure 17a) and an egg cell that recently separated from the upper basal cell (Figure 17b). This suggests that PINA is already expressed before the egg cell

is fertilized and can potentially start transporting the auxin that it produces after fertilization into the surrounding archegonium as soon as it is fertilized.

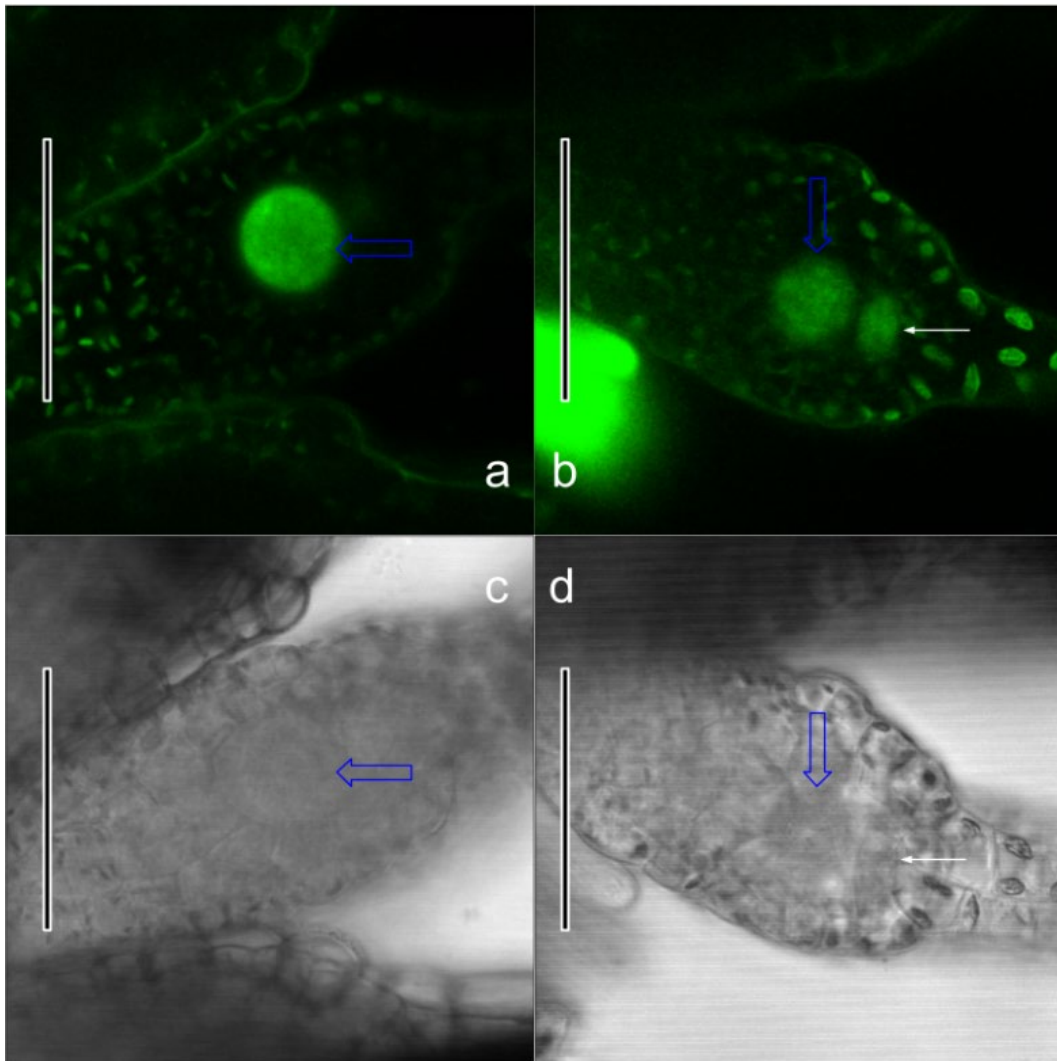


Figure 17: Expression patterns of PINA reporter line. 17a and 17c showed a mature egg cell (blue broad arrow), 17b and 17d showed an egg cell (blue broad arrow) that has recently separated from the upper basal cell (thin white arrow). PINA is uniformly expressed in the entire egg cell prior to fertilization. Bars on the left represent 50 μ m.

4. Discussion

4.1 NAA and 2,4-D treatments had different effects on archegonial development

The infertile Gransden ecotype archegonia did not respond to NAA in any treatment. Reute showed a consistent and significant NAA response across the three application methods, with archegonia venters either remaining small or becoming visibly enlarged. The enlarged archegonia contained a developing sporophyte while the small archegonia did not. This suggests that NAA may interfere either with fertilization itself or with the earliest stages of sporophyte development after fertilization. In both the treatment from start of growth and the fertilization-stage treatments, increasing NAA concentrations negatively correlated with archegonium enlargement frequency. Together, these results indicate that NAA acts at or after fertilization and directly affects sporophyte development. NAA does not appear to have an effect on archegonium venter expansion when fertilization does not occur.

In Reute, similar to NAA, 2,4-D treatment blocked fertilization or early embryo development. In addition, treatment with 2,4-D caused venter enlargement of unfertilized archegonia in both Gransden and Reute. It mimicked the expansion of the venter that normally only happens after fertilization, as the embryo starts to grow within the archegonium. This supports the hypothesis that the embryo may produce auxin that can influence the surrounding archegonium to start expansion.

The reason behind the difference between the effects of NAA and 2,4-D is unclear but may reflect differences in the activity range of the two auxin analogues. It is also unclear as to why 2,4-D has a different effect on Reute compared to Gransden, which might have to do with ecotype specific differences in responsiveness.

The *pinab* knockout mutant had, just like Reute treated with high auxin concentrations, a low frequency of developing sporophytes. This is possibly explained by auxin accumulation in the tissues where auxin is produced, caused by a decreased ability to transport auxin to surrounding tissues. This causes a high concentration of auxin, which might simulate a high external application of auxin and therefore result in similar effects.

4.2 NPA treatment did not have any effect

The NPA treatment results should not be taken at face value, since there was no clear evidence that NPA was even taken up by the moss in the first place. Everything about the moss treated with NPA looked indistinguishable from the

mock treatment. It is possible that other methods of applying NPA are necessary for this treatment to properly work.

The results from the NPA treatment suggest that NPA does not affect archegonium enlargement. This suggests that PIN proteins, being blocked by NPA, do not have an important role in the enlargement of archegonia. This could be explained by possible PIN independent auxin transport, which would allow the zygote to still exchange auxin with the surrounding archegonium.

4.3 TAR, YUC and PIN signalling suggests auxin secreted from zygote induces archegonium enlargement

Both the TAR and YUC genes are expressed directly after the egg cell fertilization, followed by a shifting of expression patterns throughout sporophyte development. Additionally, PINA is expressed in the egg cell prior to fertilization. These patterns suggest that the egg cell starts producing auxin upon fertilization, at which point auxin is immediately transported into the archegonium via PIN mediated transport. This auxin can then induce archegonium venter enlargement, as demonstrated by the 2,4-D treatments.

An observation that challenges this notion is that TAR and YUC do not seem to be expressed in the foot of early developmental stage embryos. Since the foot would likely be where the embryo and the mother tissue exchange auxin signals, it is unlikely that the archegonium receives a constant supply of auxin that induces archegonial expansion throughout sporophyte development. Another observation challenging this notion is the calyptra producing its own auxin, as evidenced by the YUC and TAR expression patterns. A possible explanation for this is that the archegonium, upon receiving the initial auxin signal from a fertilized egg cell that causes it to start enlarging, starts producing its own auxin to induce enlarging and therefore does not need additional auxin from the embryo.

While the expression of PINA in the unfertilized egg cell makes it tempting to conclude that PIN mediated transport is required for transporting auxin from the zygote to the archegonium, *pinab* demonstrates that PINA is not essential for sporophyte development. However, the frequency of developed sporophytes was clearly heavily reduced in *pinab* compared to the wild type. It is possible that while PINA is involved in transporting auxin from the zygote to the archegonium, other forms of auxin transport can be sufficient for the induction of archegonium enlargement.

Since the *pinab* knockout mutant was able to produce sporophytes despite both PINA and PINB being dysfunctional, this explanation seems plausible. According to Lüth VM et al. (2023), PINC alone can not replace the function of PINA and

PINB in *pinab*. However, it may be possible that in rare instances PINC, possibly in combination with apolar auxin transport, can rescue embryo initiation in *pinab*.

4.4 *yucabf* and *tarac* are likely mainly important around stage 10 in sporophyte development

Both *yucabf* and *tarac* have significantly smaller sporophytes than the wild type in addition to having slowed or arrested development, suggesting that *yucabf* and *tarac* play a role in proper development of the sporophyte. *yucabf* and *tarac* are able to initiate embryo development likely due to the fact that the remaining functional YUCs and TARs continue to produce sufficient auxin to initiate embryo development. Nevertheless, the frequency of developing sporophytes was much lower than in the wild type. Although this work lacks the data to conclude that the sporophytes had arrested development, it was notable that most of the sporophytes of these mutants were of similar stages. This suggests that something happens during this stage that requires these genes, and by proxy auxin, to properly continue development. Other relevant observations are that the TAR and YUC reporter lines consistently show expression in the foot around the same stages, in addition to the fact that the sporophytes of *yucabf* and *tarac* often had detached and had a pigmented foot tip. Overall, *yucabf* and *tarac* had very similar phenotypes, which is to be expected since YUC and TAR are downstream from each other in the same auxin biosynthesis pathway (K. Landberg et al., 2021). These observations together point to the expression in the foot in later stages to be essential for the survival and continued development of the sporophyte.

4.5 Cellophane mediated transfer is a not ideal yet viable method of delayed auxin application

Prior to this experiment it was not entirely clear what the best way to apply auxin treatment to moss is later in its life span. Auxin directly applied to the moss colonies with water might not be effectively taken up into the moss tissue. Therefore, we also applied auxin by using cellophane to transfer moss colonies from a control medium to a medium containing NAA. Although the moss colonies were less healthy when grown on cellophane, the auxin treatment was still applied successfully and showed the same overall effect as the other application methods. While applying auxin through watering may be a preferable method of application, cellophane transfer is a viable method for application of substances that may not get taken up effectively through watering. An example of this may be NPA, which in our experiment showed no evidence of being taken up by the moss colonies, but may be taken up more effectively when applied by cellophane transfer. It is advisable to start more colonies on cellophane to compensate for the lower frequency of healthy shoots. It is also important to limit the amount of

water added for fertilization synchronization, as the cellophane can start floating on the water.

4.6 Mistakes and difficulties

Due to a mix up with dates, the plates treated with 2,4-D were watered too early, before the archegonia had reached maturity. They were watered an additional time after that, in case the treatment might still have an effect despite being applied too early, after which they were harvesting again the next week. After this, there were still some interesting results despite the mistake, so we decided to include it in this report.

The first 10 μ M NPA treatment duplicate was a severe outlier, with at first 0 out of 10 observed archegonia having enlarged. Six more gametophore were investigated after these findings, resulting in only 2 out of 16 observed archegonia having enlarged. This outlier was discarded in the analysis, since the second replicate of 10 μ M NPA had the expected proportions, similar to the mock and 20 μ M NPA treatments. It is not clear what caused this outlier (Appendix 3).

4.7 Continuing this line of research

The results of this study give insight into the role auxin has in archegonium venter enlargement and sporophyte development. At the same time, this research raises more questions. For example, 2,4-D treatment did induce archegonium development without fertilization, as hypothesized. However, treatment with NAA did not produce this same effect. In addition, both NAA and 2,4-D treatments interfered with embryo initiation.

Useful for further investigation in the different role auxin has in archegonium and the sporophyte would be inducible mutants. This allows for reliable control over auxin levels throughout the entire organism, which can be activated at any desired point in development. For example, knockout mutants like *tarac* and *yucabf* could be induced right before fertilization, so that the observed phenotype of the developing sporophytes is not indirectly affected by stunted growth during the vegetative growth phase. This investigates the effects of inhibiting auxin biosynthesis only during fertilization. If our hypothesis is correct, we would expect this inhibition to prevent the archegonium from enlarging. It would also be interesting to investigate what happens if the gametophyte is an auxin biosynthesis mutant but the sporophyte is not. This might be achievable by fertilizing an auxin biosynthesis mutant with the spermatozoids of a wild type, which rescues the diploid sporophyte. This allows for investigation on whether archegonium enlargement is exclusively induced by auxin supplied from the sporophyte or not.

Although the role of some YUC, TAR and PIN genes were investigated in this review, all three have multiple variants that were not investigated (Thelander et al., 2022). PINC and PIND might have a role in transporting auxin from the zygote to the archegonium, explaining why *pinab* was able to produce sporophytes. YUCD, YUCE, TARB and TARD were not investigated and likely have a role in auxin production, explaining why the YUC and TAR mutants could produce sporophytes. Reporter lines and knockout mutants investigating different combinations of these genes should be studied to gain a full picture of the role of these genes and auxin in archegonium enlargement and sporophyte development.

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Popular science summary

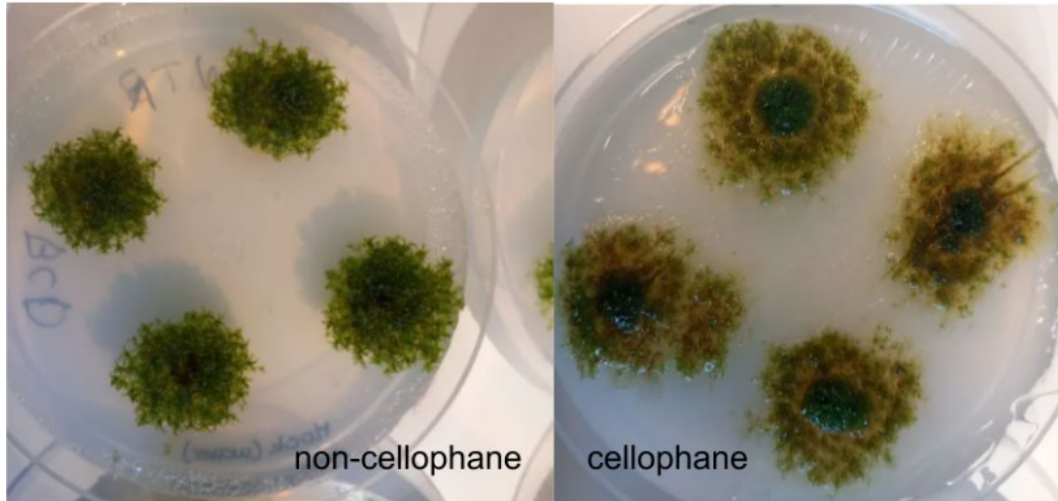
Övergången från gametofyt- till sporofytfasen hos mossar: Hur fungerar det egentligen?

Simon Koetsier

I landväxternas evolutionära historia har mossor en viktig plats. Alla landväxter härstammar från samma första gemensamma förfader, som var den första landväxten. Bryophyter, som mossor är en del av, liknar de första landväxterna mest. Det här gör så att vi kan få en bättre förståelse av de första landväxterna om vi förstår hur mossor fungerar. Mossor har två stadier i sin livcykel: gametofyt- och sporofytfasen. Gametofyten är den fas som representerar den största delen av mossan, som innebär den lövrika delen de flesta tänker på angående mossor. Sporofyten växer på gametofyten och är mycket mindre, och producerar sporer som sprids så att ny mossa kan växa någon annanstans. Auxin är ett växthormon som har olika roller i växtutvecklingen. Vi vet redan mycket om vad auxin gör i mossans gametofytfas, men inte så mycket om vilken roll det spelar i sporofytens utveckling. Därför har vi testat vad som händer med sporofyten och de honliga reproduktionsorgan runt sporofyten (ett arkegonium) när vi behandlar mossan med auxin. Vi har också tittat på några mutanter som har defekter i auxin produktionen och vi har observerat var auxintransport och -produktion händer i sporofyten med hjälp av reporterlinjer. Behandlingen med auxin hade två effekter. För det första var det svårare för sporofyten att utvecklas när auxinkoncentrationen var hög, samtidigt orsakade auxinbehandlingen arkegoniet att bli större för att ge plats åt en sporofyt, trots att sporofyten inte utvecklades alls. Vi hittade också ledtrådar hos mutanterna om att auxin är nödvändigt för sporofytens utveckling. I reporterlinjerna verkade det som att sporofyten transporterar auxin in i arkegoniet. Med allt taget tillsammans kan vi lära oss några olika saker om auxins roll i sporofytens utveckling. För det första vet vi att sporofyten behöver auxin för att börja utvecklas, men att för mycket auxin i kontrast förhindrar denna utveckling. Det verkar också som sporofytembryon producerar auxin och transporterar auxinet till arkegoniet, vilket orsakar att arkegoniet expanderar för att ge plats åt den växande sporofyten. Det kvarstår dock många frågor om hur säkra vi kan vara på dessa slutsatser. För att fortsätta denna forskningslinje behöver flera olika mutanter testas. Det skulle också vara en användbar utveckling att ha mutanter som kan induceras under ett senare utvecklingsstadium, så att den observerade effekten inte kan komma från en indirekt effekt orsakad av en tidigare utvecklingsdefekt hos mossan.

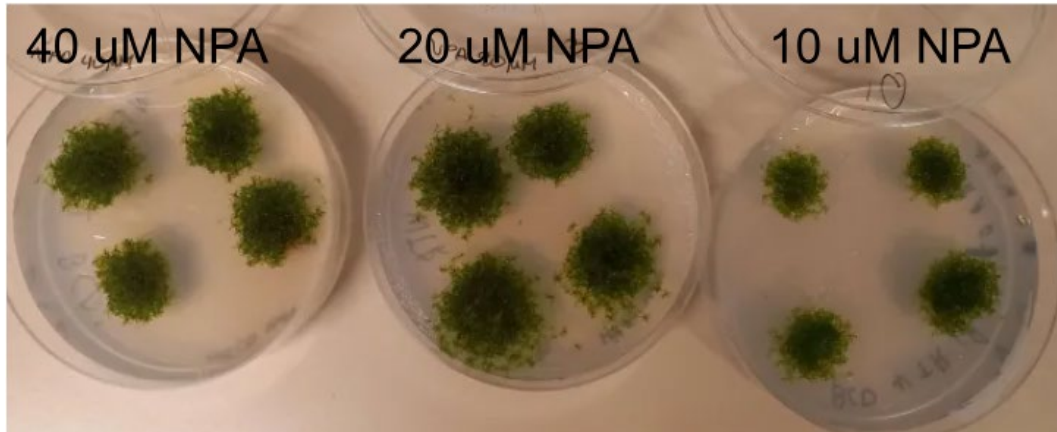
Appendix 1

Comparison between Reute mock colonies grown on naked medium and Reute mock colonies grown on medium with cellophane. The colonies grown on cellophane look less healthy than those grown on naked medium.



Appendix 2

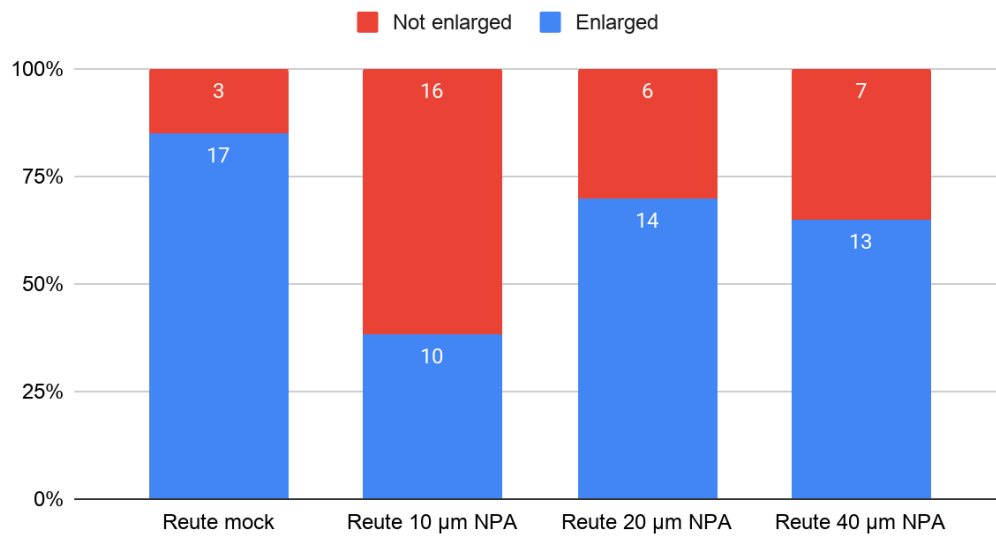
One of the Reute duplicates of the NPA watering treatment. No clear effect of NPA was observed on the colonies.



Appendix 3

The proportion of enlarged archegonia found in the NPA watering treatment, including the outlier. In the first duplicate of the 10 μ M NPA treatment, only 2 out of 16 observed gametophores contained an enlarged archegonium. The second duplicate contained a more expected frequency of 8 out of 10 enlarged archegonia.

Water NPA



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