An Analysis of Auxin Distribution and Activity during Photomorphogenesis and Skotomorphogenesis in the moss, *Physcomitrella patens*

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Shawn Riley Robinson, candidate for the degree of Master of Science in Biology, has presented a thesis titled, An Analysis of Auxin Distribution and Activity during Photomorphogenesis and Skotomorphogenesis in the moss, Physcomitrella patens, in an oral examination held on July 31, 2015. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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ABSTRACT

Plant development varies depending on whether it occurs in the light (photomorphogenesis) or in the dark (skotomorphogenesis). This phenomenon has been studied in depth in flowering plants such as Arabidopsis thaliana but remains largely unexplored in more ancient plant groups such as the bryophytes, which include the model moss, Physcomitrella patens, despite decades-old awareness of the markedly different gametophytic morphologies that result from photomorphogenesis and skotomorphogenesis in this moss. Research undertaken with Arabidopsis has elucidated in detail roles for various phytohormones, including auxin, in regulating the reversible developmental interconversion of photomorphogenesis and skotomorphogenesis. By contrast, although auxin has previously been shown to be active at many stages of Physcomitrella development, its specific involvement in photomorphogenesis and skotomorphogenesis and their interconversion, especially in relation to gametophore development, is unknown.

Through the use of transgenic auxin-responsive GUS reporter strains in conjunction with auxin signalling and polar transport inhibitors it was shown that auxin is critically important for the development of gametophore stems and leaves in Physcomitrella during photomorphogenesis but it has a diminished role in leaf development and is not required for stem elongation during skotomorphogenesis. However, auxin is required for the gametophore bud to leafy gametophore transition in a dark-dependent manner and for protonemal development both in the dark and in the light. The roles of auxin in development
of the moss gametophore are very similar to those in hypocotyl elongation in
*Arabidopsis*, implying that they are ancient and have been highly conserved
during land plant evolution.
ACKNOWLEDGEMENTS

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>4-Cl-IAA</td>
<td>4-chloroindole-3-acetic acid</td>
</tr>
<tr>
<td>ABCB</td>
<td>ATP BINDING CASSETTE SUBFAMILY B</td>
</tr>
<tr>
<td>ABP1</td>
<td>Auxin binding protein 1</td>
</tr>
<tr>
<td>ARF</td>
<td>AUXIN RESPONSE FACTOR</td>
</tr>
<tr>
<td>Aux/IAA</td>
<td>Auxin/INDOLE-3-ACETIC ACID</td>
</tr>
<tr>
<td>AUX1/LAX</td>
<td>AUXIN RESISTANT1/LIKE AUX1</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>BL</td>
<td>Blue light</td>
</tr>
<tr>
<td>COP</td>
<td>Constitutive photomorphogenic</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GH3</td>
<td>Gretchen Hagen 3</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>mas</td>
<td>Mannopine synthase</td>
</tr>
<tr>
<td>NAA</td>
<td>1-Naphthaleneacetic acid</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Monosodium dihydrogen orthophosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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xiv
<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>NPA</td>
<td>N-1-naphthylptalamic acid</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAA</td>
<td>Phenyl-acetic acid</td>
</tr>
<tr>
<td>PEO-IAA</td>
<td>α-(phenylethyl-2-oxo)-indole-3-acetic acid</td>
</tr>
<tr>
<td>PIF</td>
<td>Phytochrome-interacting factor</td>
</tr>
<tr>
<td>PILS</td>
<td>PIN-likes</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PIN</td>
<td>PIN-FORMED</td>
</tr>
<tr>
<td>RL</td>
<td>Red light</td>
</tr>
<tr>
<td>SBI</td>
<td>Side branch initial</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transferred DNA</td>
</tr>
<tr>
<td>Ti</td>
<td>Tumor-inducing</td>
</tr>
<tr>
<td>TIBA</td>
<td>2,3,5-triiodobenzoic acid</td>
</tr>
<tr>
<td>TIR1/AFB</td>
<td>TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WL</td>
<td>White light</td>
</tr>
<tr>
<td>X-Gluc</td>
<td>5-bromo-4-chloro-3-indolyl- β-D-glucuronic acid</td>
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1. INTRODUCTION

1.1 The moss *Physcomitrella patens*

*Physcomitrella patens* (Hedw.) Bruch, Schimp & W. Gümbel is a bryophyte belonging to the Funariaceae, a moss family comprising approximately 300 species. It has become a popular plant model system, which has been employed to investigate a broad spectrum of botanical phenomena. Its popularity and utility stem in part from its ease of culture, small size and rapid life cycle, which can be completed in about three months (Engel, 1968), and its high efficiency of targeted homologous recombination (Cove and Knight, 1993; Schaefer and Zryd, 1997). The majority of *Physcomitrella* research is conducted with plants that originated from a single wild-type spore isolated from nature in Gransden Wood, Huntingdonshire, U.K. in 1962 by H.L.K. Whitehouse (Engel, 1968).

Like that of other mosses, the life cycle of *Physcomitrella* consists of a dominant gametophytic phase that alternates with a sporophytic phase. The former is usually haploid while the latter is usually diploid. The haploid gametophytic phase often begins with germination of a spore or regeneration of a protoplast, from which emerges a primary chloronemal filament. Chloronemata contain large chloroplasts and their primary function is carbon assimilation by photosynthesis. The apical cells of some primary chloronemata differentiate into caulonemal apical cells that give rise to caulonemata, which contain fewer chloroplasts and have as their main roles habitat exploration and colony expansion. Subapical cells of caulonemata can divide to form single-celled side
branch initials (SBIs) with four optional developmental fates. They may remain SBIs or differentiate into chloronemal or caulonemal apical cells, which give rise to secondary chloronemata and secondary caulonemata, or differentiate into gametophore buds from which leafy shoots develop (Fig. 1) (Cove, 2005; Cove and Knight, 1993, Roberts et al., 2012).

The *Physcomitrella* mature gametophore is analogous to the shoot of a flowering plant such as *Arabidopsis thaliana*. It comprises a multicellular stem with leaf-like appendages, which have a midrib but lack the true vascular tissues, xylem and phloem, present in the leaves of vascular plants. Consequently, these appendages are not true leaves in the purest sense. Nevertheless, for the sake of simplicity, they will be referred to as leaves throughout this thesis.

*Physcomitrella* is monoecious with both antheridia and archegonia being formed at the apex of each of its leafy gametophores. Within these sex organs, antherozoids and oogonia develop respectively. Fertilization of the single egg within an archegonium produces a zygote, which develops into a sporophyte consisting of capsule, seta and foot. Spore mother cells in each sporophyte capsule undergo meiosis giving rise to approximately 5000 spores (Cove and Knight, 1993).

1.2 Photomorphogenesis and skotomorphogenesis in plants

1.2.1 Differing morphologies of plants during photomorphogenesis and skotomorphogenesis

Plant morphologies, including those of *Physcomitrella*, differ dramatically depending on whether development has occurred in the light
**Fig. 1. The life cycle of *Physcomitrella patens***

Development usually begins with a germinating spore or protoplast from which a primary chloronema emerges. A chloronema may differentiate into a caulonema from which SBIs (side branch initials) arise. Some SBIs become bud initials, which in turn develop into leafy gametophores on which antheridia and archegonia are formed. Fertilization of an egg within an archegonium results in a zygote, which develops into the sporophyte. Meiosis of spore mother cells in the sporophyte gives rise to approximately 5000 spores. Figure reproduced from Roberts *et al.*, 2012.
(photomorphogenesis) or in the dark (skotomorphogenesis). To date, these processes have been studied much more intensively in angiosperms than in more ancient plants, including Physcomitrella.

In Arabidopsis thaliana, a model angiosperm, short hypocotyls, open cotyledons, the production of true leaves, and a fully functional photosynthetic apparatus typify photomorphogenesis (Mohr, 1987). In skotomorphogenesis, the plants become etiolated with an elongated hypocotyl. The cotyledons remain closed, true leaves are not produced, and an apical hook forms to protect the shoot apical meristem. Hypocotyl elongation is negatively gravitropic in the dark, while light stimulation reduces gravitropic orientation and shoots become positively phototropic (Poppe et al., 1996). The morphology of the plant in the dark maximizes the use of storage materials in an effort to reach a light source (as reviewed in Mohr, 1987; Josse and Halliday, 2008).

In Physcomitrella, analogous differences can be discerned when development in the light is compared with that in the dark. During photomorphogenesis, protonemal and gametophore development occurs as previously described. During skotomorphogenesis, the predominant protonemal developmental pathway is the production of caulonemal runners, which do not form SBIs and thus do not branch or give rise to buds. If gametophore buds are present on a colony when it is transferred into the dark, they develop into shoots with etiolated stems and small, scale-like leaves. Whereas colonies grown in the light are agravitropic, both gametophores and caulonemal runners are negatively gravitropic in the dark (Cove et al., 1978). Thus, the Physcomitrella gametophore
responds similarly to the hypocotyl and cotyledons of *Arabidopsis*, implying modulation of morphogenesis in response to the presence or absence of light may be achieved in both model systems via an ancient regulatory mechanism that has been evolutionarily conserved in plants.

### 1.2.2 Light signal transduction in photomorphogenesis

The means of light signal transduction by plants depends upon the wavelength of the incoming light signal. Different receptors are present in plants to receive red/far-red, blue, UV-A, and UV-B wavelengths of light. Phytochromes are responsible for the detection of red and far red light, cryptochromes and phototropins for the detection of blue and UV-A light, and the recently discovered UVR8 receptor is responsible for the detection of UV-B wavelengths (Mohr, 1987; Tilbrook *et al.*, 2013).

In *Arabidopsis*, the phytochrome family of receptors includes five members, PHYA to E (phytochrome A-E), each of which alternate between the inactive P<sub>r</sub> form and the active P<sub>fr</sub> form. Red light signals a transition from P<sub>r</sub> to P<sub>fr</sub> and bioactivity, while far-red light reverses this effect (reviewed in Vierstra, 1993). Active phytochromes mediate photomorphogenesis over skotomorphogenesis through three mechanisms: inhibition of proteins responsible for the maintenance of skotomorphogenesis and for the inhibition of photomorphogenesis, upregulation of photomorphogenic genes, and downregulation of skotomorphogenic genes. Proteins targeted for degradation include COP1 (constitutive photomorphogenic 1) and PIF (phytochrome-interacting factor) proteins. COP1 is a RING (really interesting new gene) finger
E3 ubiquitin ligase that targets regulator proteins (including multiple transcription factors as well as phytochromes themselves) that induce photomorphogenesis for degradation by the 26S proteasome, thereby supporting skotomorphogenesis (Lau and Deng, 2012; Li et al., 2012; Smirnova et al., 2012). PIF proteins are transcription factors that promote the etiolated state in darkness and directly attenuate phytochrome signaling (Fig. 2; reviewed in Josse and Halliday, 2008). Genomic expression profiles after red light treatment indicate that PHYA plays a dominant role in early upregulation of photomorphogenic genes, a role supported by PHYB. PHYC, D and E may be involved in the downregulation of skotomorphogenic genes (Tepperman et al., 2006).

In Arabidopsis, cryptochromes have two members, CRY1 and CRY2 (cryptochromes 1 and cryptochrome 2), which mediate photomorphogenesis in response to blue-light signaling. Cryptochromes inhibit hypocotyl elongation and promote cotyledon and root development (Wu and Spalding, 2007). Both CRY1 and CRY2 interact with COP1 and block its activity by removing it from the nucleus, thereby suppressing skotomorphogenesis (reviewed in Fortunato et al., 2015 and Li and Yang, 2007).

The roles of phytochrome and cryptochrome signaling in Physcomitrella have not been as extensively studied as in Arabidopsis. However there are indications that these light receptors have roles in regulating photomorphogenesis in the moss. Physcomitrella possesses four phytochromes and two cryptochromes (Imaizumi et al., 2002; Mittman et al., 2004). Although a direct role for these photoreceptors in regulating photomorphogenesis has not
The functions of receptors and proteins in the regulation of photomorphogenesis and skotomorphogenesis in *Arabidopsis thaliana*. Light stimulates phytochromes (red/far red light) and cryptochromes (blue/UV-A light), which act through various transcription factors to upregulate photomorphogenic genes and downregulate skotomorphogenic genes. COP1 and PIF proteins act to attenuate phytochrome and cryptochrome activity via targeted ubiquitination of these light receptors as well as the downstream transcription factors. Reciprocally, COP1 and PIF protein activity is attenuated by active phytochrome and cryptochrome.

Key: blue arrows indicate upregulation/induction, red arrows indicate inhibition/degradation/downregulation. Known *Physcomitrella patens* orthologs, whose functions in this pathway have yet to be demonstrated, are indicated with an asterisk, while putative *Physcomitrella* orthologs of an uncharacterized function are indicated with a double asterisk.
been proven, some data suggest this may be the case. The skotomorphogenic morphology of *Physcomitrella* is abolished by application of a 23 h dark/1 h red light cycle, implying phytochrome involvement (Cove *et al.*, 1978). Cryptochrome knockout and over-expresser strains indicate that cryptochrome signaling induces leaf growth and inhibits stem growth, developmental processes typically associated with photomorphogenesis in *Physcomitrella* (Imaizumi *et al.*, 2002). Orthologs of COP1 have been found in the *Physcomitrella* genome (Ranjan *et al.*, 2014), as has an uncharacterized PIF-like sequence (Jeong and Choi, 2013). However, the specific roles of the proteins encoded by these *Physcomitrella* genes have not been determined.

In *Arabidopsis*, UV-B light induces photomorphogenesis via the UVR8 receptor. Following activation, this receptor interacts with COP1 to induce some photomorphogenic changes, particularly inhibition of hypocotyl growth and perhaps cotyledon expansion. The involvement of COP1 in inducing photomorphogenesis following UV-B stimulation is a contrast to this molecule’s role in promoting skotomorphogenesis (Tilbrook *et al.*, 2013). *Physcomitrella* has a *UVR8* ortholog and can respond to UV-B stimulation (Wolf *et al.*, 2010). No details about the regulation of *Physcomitrella* photomorphogenesis via UV-B signaling have been reported.

### 1.2.3 Hormonal regulation of photomorphogenesis and skotomorphogenesis

Phytohormones play critical roles regulating plant morphology in response to light stimulation. In *Arabidopsis*, gibberellins, ethylene, auxin and
brassinosteroids work synergistically to maintain skotomorphogenesis including apical hook formation (De Grauwe et al., 2005; Gallego-Bartolome et al., 2011; Lehman et al., 1996; Li et al., 2004) and hypocotyl elongation (Azpiroz et al., 1998; Lincoln et al., 1990, Nagata et al., 2000). The phytohormones also support the activity of COP1 and PIF proteins (Alabadi et al., 2008; Oh et al., 2012; Song et al., 2009) and regulate photomorphogenic and skotomorphogenic gene transcription (Alabadi et al., 2004; Song et al., 2009). A role may also exist for cytokinin in the regulation of photomorphogenesis. Application of exogenous cytokinin or overexpression of endogenous cytokinin results in photomorphogenic growth characteristics in plants grown in the dark, including leaf and chloroplast development, inhibition of hypocotyl elongation, and upregulation of light-mediated genes (Chin-Atkins et al., 1996; Chory et al., 1994; Lochmanova et al., 2008). The involvement of phytohormones in regulating skotomorphogenesis or photomorphogenesis has not been extensively studied in Physcomitrella. A priori, in this regard at least some differences must exist between Arabidopsis and Physcomitrella since both the brassinosteroid and gibberellin signaling systems are absent in the latter (Anterola et al., 2009; Prigge and Bezanilla, 2010; Rensing et al., 2008; Yasumura et al., 2007)

1.3 Auxins

1.3.1 Active auxins and auxin homeostasis

Auxins were discovered as regulators of plant growth and development in the early 20th century. Since their discovery, several compounds have been
identified as active auxins. These include IAA (indole-3-acetic acid), 4-Cl-IAA and (4-chloroindole-3-acetic) PAA (phenyl-acetic acid) (reviewed in Korasick et al., 2013). IAA has been detected in Physcomitrella (Ashton et al., 1985). Though not naturally occurring in plants, 2,4-D (2,4-Dichlorophenoxy-acetic acid) and NAA (1-naphthaleneacetic acid) are examples of synthetic chemicals that elicit an auxin response from plants (Korasick et al., 2013) including Physcomitrella (Ashton, 1998).

Auxin homeostasis can be mediated through three mechanisms – auxin biosynthesis, storage and inactivation. Auxin biosynthesis can occur via tryptophan-dependent or independent pathways, with the latter being less understood and its contribution to the IAA pool debated. IAA can be removed from the active pool and stored in plant cells via methylation or conjugation with carbohydrates, amino acids or larger peptides. These conjugations are reversible, returning IAA to an active pool, or are instead targeted for IAA catabolism and thereby permanently removing these molecules from the cell (Korasick et al., 2013).

1.3.2 Roles of auxin in plant development

Auxin activity is involved at multiple stages of development throughout the plant kingdom, from bryophytes to angiosperms. In angiosperms such as Arabidopsis, auxin plays a role in leaf initiation and patterning (as reviewed in Li et al., 2007), as well as in root and shoot development, maintenance of apical dominance, fruit formation, senescence, and tropic responses to light and gravity (reviewed in Sauer et al., 2013). In skotomorphogenesis, auxin is required for
formation of the apical hook (Lehman et al., 1996) and is involved in generalized cell expansion in the hypocotyl (Lincoln et al., 1990). In Physcomitrella, auxin promotes caulonemal development and bud initiation (Ashton et al., 1990) and is involved in shoot and leaf development in the light, in particular in elongation of these tissues (Barker and Ashton, 2013; Decker et al., 2006; Hayashi et al., 2008). However, little is known about auxin’s involvement in skotomorphogenesis in Physcomitrella.

1.3.3 Auxin signaling mechanisms

In Arabidopsis, auxin signaling can occur by two currently known pathways: the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) pathway and the AUXIN BINDING PROTEIN1 (ABP1) pathway. In TIR1/AFB signaling, Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) proteins bind to AUXIN RESPONSE FACTOR (ARF) transcription factors, blocking them from the upregulation of auxin responsive genes. In the presence of auxin, TIR1/AFB acts as a co-receptor with Aux/IAA for auxin. Upon auxin binding, Aux/IAA is targeted for ubiquitination and degradation by the 26S proteasome, freeing ARF to act upon auxin responsive genes. In ABP1 signaling, auxin binds to ABP1 anchored on the external surface of the plasma membrane. Signal transduction following this binding leads to effects including cytoskeletal rearrangement, changes to auxin transport protein localizations and cell expansion (reviewed by Enders and Strader, 2015).

Recently, molecules have been designed to act antagonistically to auxin signaling. PEO-IAA [α-(phenylethyl-2-oxo)-IAA] and auxinole (a derivative of
PEO-IAA incorporating methylation of the phenyl ring) were devised via in silico screening of ligand binding to TIR1. Both PEO-IAA and auxinole bind to the same location as IAA on TIR1 and thereby can act as competitive inhibitors to auxin binding, decreasing the cellular auxin response (Hayashi et al., 2008; Hayashi et al., 2012).

The Physcomitrella genome contains TIR1/AFB, Aux/IAA, and ABP1 homologues (Rensing et al., 2008). Using auxin-resistant mutants of Physcomitrella it has been shown that the TIR1/AFB signaling pathway behaves in Physcomitrella as it does in Arabidopsis (Prigge et al., 2010). Both PEO-IAA and auxinole inhibit auxin signaling in Physcomitrella (Hayashi et al., 2008; Hayashi et al., 2012).

1.3.4 Polar auxin transport

1.3.4.1 Mechanisms of polar auxin transport

While auxin is capable of moving through plant tissues by simple diffusion or through the phloem in vascular plants, specialized uptake and export from cells via transporter proteins allows for rapid cell-to-cell transport. The polar alignment of these proteins allows for directional transport, and is termed polar auxin transport. Polar auxin transport is critically important in Arabidopsis for embryogenesis, organogenesis, gravitropism and phototropism, apical hook maintenance, root and shoot development and vascularization (reviewed in Adamowski and Friml, 2015).

The passage of IAA across the plasma membrane (PM) is pH-dependent since IAA can exist both undissociated as an acid, IAAH, which diffuses readily
across the PM, or, after dissociation of a proton from its carboxylic acid group, as an anion, IAA\(^{-}\), which cannot freely pass across the hydrophobic PM. The pKa of IAA is approximately 4.75 and in the apoplast of the plant cell wall, which typically has a pH of about 5.5, approximately 17% of IAA molecules will be undissociated and will diffuse across the PM down a concentration gradient into the cell. In addition to passive diffusion, auxin uptake proteins, AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX), can facilitate uptake of auxin. Once inside the cell, IAAH will ionize since the pH inside plant cells is typically about 7.00. Thus, in the absence of an exporter protein, the diffused or transferred IAA will be trapped as IAA\(^{-}\) anions and this ion trapping mechanism will maintain the gradient of IAAH across the PM. Exporter proteins, which facilitate the passage of IAA\(^{-}\) anions across the PM may be localized to a particular portion of the PM thereby facilitating the directional passage of IAA out of the cell down an IAA\(^{-}\) concentration gradient (as reviewed by Zazimalova et al., 2010).

Three major classes of auxin export proteins are currently known to exist in plants - the PIN-FORMED (PIN), ATP BINDING CASSETTE SUBFAMILY B (ABCB) and the more recently discovered PIN-likes (PILS). PIN proteins are separated into two groups – the canonical “long” form PIN proteins that localize to the PM and are responsible for auxin transport out of the cell and non-canonical “short” PINs that localize to the endoplasmic reticulum and are responsible for regulating auxin homeostasis within the cell. PIN localization is mediated by endocytosis and recycling to the PM in a regulated manner, which results in polar localization of PINs within a cell. Two ABCB proteins are
necessary for polar auxin transport. However, it is not clear how ABCB proteins function and it remains possible that they do not interact directly with IAA (as reviewed by Enders and Strader, 2015). PILS appear to have a similar function to that of the non-canonical PINs. They too are localized to the ER and are involved in the maintenance of cellular auxin homeostasis (reviewed in Adamowski and Friml, 2015).

The *Physcomitrella* genome contains gene homologues for both PIN proteins and AUX1/LAX influx transporters (Rensing *et al*., 2008). *Physcomitrella* PINA to C are homologous to the canonical “long” PIN proteins in *Arabidopsis* while PIND is homologous to the non-canonical “short” PINs (Bennett *et al*., 2014a). PINA and PINB localize to the plasma membrane, much like their homologues in *Arabidopsis* (Bennett *et al*., 2014b; Viaene *et al*., 2014), while PIND localizes to the ER (Viaene *et al*., 2014).

**1.3.4.2 Inhibition of polar auxin transport**

Application of NPA (*N*-1-naphthylphthalamic acid) or TIBA (2,3,5-triiodobenzoic acid) results in an inhibition of polar auxin transport. Polar auxin transport inhibitors work by inhibiting rapid cycling of canonical PIN proteins to and from the PM (Geldner *et al*., 2001). NPA affects the activity of PM-based PINs but neither those in the ER nor similarly localized PILS proteins (Barbez *et al*., 2013).
1.3.5 Detection of auxin distribution with β-glucuronidase histochemical assays

Transgenic lines of plants have been developed for the purpose of determining auxin distribution. Several versions of these contain, stably inserted into their chromosomal DNA, a copy of the *E. coli* β-glucuronidase (GUS) *uidA* gene (Jefferson *et al.*, 1987) fused to an auxin-responsive promoter. The GUS gene can be used as a reporter for the presence of auxin: staining procedures reveal indigo coloration where auxin is present. Two transgenic auxin-responsive GUS reporter lines of *Physcomitrella* have been utilized in this research.

1.3.5.1 LTII-2 – A *Physcomitrella* GUS reporter strain with an auxin-responsive *mas* promoter

The *mas* promoter is derived from the Ti plasmid of the plant pathogen, *Agrobacterium tumefaciens*. This soil bacterium transfers part of its Ti plasmid, T-DNA, into the chromosomal DNA of dicotyledonous plants, causing the development of tumours and crown gall disease. A 479 base pair section of T-DNA comprises the *mas* promoter: a bidirectional, dual 1’2’ promoter which regulates the transcription of two open reading frames (ORFs) in the T-DNA (Velten *et al.*, 1984). The ORFs, which encode two mannopine synthase polypeptides, are positioned on opposite sides of the promoter on opposite DNA strands.

The *mas* promoter is active in transgenic plant tissues and the spatial distribution of *mas* promoter activity has been investigated in transgenic *Nicotiana tabacum* (tobacco) (Langridge *et al.*, 1989) stably transformed with
pPCV701luxA&B, which contains the bacterial luciferase luxA and luxB genes fused to the 1' and 2' ends of the mas promoter as reporter genes (Koncz et al., 1987). It was found, for example, that there is a gradient consisting of a 30-fold increase in activity from the leaf base to the tip. In the same study it was shown that the mas promoter is auxin-inducible. Tissue sections excised from the stem apex and the ninth internode below the apex were treated with 10 µM NAA, resulting in a 130-fold and 50-fold increase in reporter activity respectively. Treatment of sections from the apex of the stem with water produced a 50-fold increase in luciferase. Treatment of the stem excised at the ninth internode with 1 µM BAP resulted in an increase in reporter activity equal to 50% of that observed with the auxin treatment. Addition of BAP to NAA-treated sections of tissue saw no further increase in reporter activity. Leaf discs incubated with 0.3 µM BAP demonstrated minimal reporter activity, but addition of NAA to the BAP-treated discs increased reporter activity in a dose-dependent manner. These findings show that the mas promoter responds to wounding and also imply that the auxin-responsiveness of the mas promoter may be enhanced by cytokinin (Langridge et al., 1989).

Differential promoter activity of the mas 1' and 2' ends has been investigated with transgenic tobacco lines stably transformed with either pBDH001 cl or pBDH001 cII. In the former case, an E. coli uidA and a firefly luciferase (luc) reporter gene had been fused to the 1' and 2' ends of the promoter respectively. In the latter case, the orientation of the mas promoter had been reversed. Quantitative expression studies with these transgenic lines
revealed the 2′ end of the mas promoter is approximately 7- to 8-fold more active than the 1′ end (Leung et al. 1991).

Transgenic *Physcomitrella* lines stably transformed with pBDH001 cl or cII have been made. A study using LTII-2, a wild-type line transformed with pBDH001 cII, has demonstrated auxin activity in apical and marginal cells of leaves, as well as in caulonemata, indicating the potential utility of this transgenic line for studying the distribution patterns of auxin in *Physcomitrella* (McDonald, 1999).

1.3.5.2 GH3::GUS – A *Physcomitrella* GUS reporter strain with an auxin-responsive GH3 promoter

*GH3* genes encode a family of enzymes that catalyze the amino acid conjugation of small molecules including IAA (reviewed in Okrent and Wildermuth, 2011). They were first demonstrated to be responsive to auxin in soybean hypocotyls (Hagen *et al.*, 1984). The *GH3* promoter contains three auxin-inducible elements (Liu *et al.*, 1994) and, if fused to a reporter gene such as *uidA* and stably inserted into a plant’s genomic DNA, it becomes a useful tool for probing the distribution of auxins in the tissues and organs of transgenic plant lines (Hagen *et al.*, 1991).

A *GH3::GUS*-containing transgenic line of *Physcomitrella* has been used to probe auxin distribution in light-grown moss gametophytes. Auxin activity was observed in protonemata, gametophore buds and the stems of leafy gametophores. However, the leaves of mature gametophores showed no activity unless treated with 10 µM NAA, when the entire shoot became stained for GUS.
Application of 5 µM BAP had no effect on GUS staining, implying the \textit{GH3} promoter is unresponsive to this phytohormone (Bierfreund \textit{et al.}, 2003).

1.4 Leaf Terminology

Throughout this thesis, reference will be made to leaf position along a gametophore. Consideration of leaf position is critical in the undertaking of leaf development in \textit{Physcomitrella}, as the leaf series is heteroblastic, with small, additive differences in leaf shape and structure between successive leaves (Barker and Ashton, 2013). Leaf position on a shoot stem can be referenced in two ways. From the base of the stem, leaves are assigned a leaf number (L number). The most basal leaf is L1, the leaf above L1 will be L2 and so on (Sylvester \textit{et al.}, 1990). From the shoot apex, leaves are assigned a plastochron number. The plastochron is derived from the time period between leaf initiations (Erikson and Michelini, 1957). In this numbering system, P1 is the newest leaf, P2 the second youngest and so on (Sharman, 1942).

Overall leaf shape is the output of strict regulation of a variety of exogenous and endogenous signals. Leaf index is a measure of elongation of a leaf relative to expansion of its width. It is calculated as the leaf length divided by the leaf width, and provides a useful metric for following the overall changes to leaf shape after experimental treatment.

1.5 Objectives

While the regulation of photomorphogenesis and skotomorphogenesis has been extensively studied in angiosperms, little research has been conducted in this area in ancient plant groups including mosses, despite decades-old
knowledge of the existence of these two developmental pathways in Physcomitrella (Cove et al., 1978). Due to its demonstrated involvement in photomorphogenesis and skotomorphogenesis in Arabidopsis as well as in the growth and development of Physcomitrella in the light, auxin has emerged as a potential candidate for regulating the reversible transition between photomorphogenesis and skotomorphogenesis in mosses. Auxin becomes even more attractive as a potential regulator of this developmental switch because of the absence of other main categories of phytohormones in Physcomitrella, namely the gibberellins and brassinosteroids. Elucidation of the mechanisms of development in Physcomitrella is compelling not only from the standpoint of understanding the morphogenesis of this model moss, but because it may inform and direct experimentation with other plants and bring us closer to a rigorous and authentic comprehension of the evolution of land plant development.

Based primarily on an analysis of the phenotypes of several categories of Physcomitrella auxin- or cytokinin-repairable or insensitive developmental mutants, a model has previously been proposed in which skotomorphogenesis is regarded as the default or most fundamental developmental pathway in Physcomitrella and other mosses. The model further contends that auxins and cytokinins are not required by the default developmental pathway; they are needed only to attenuate the developmental influences of light during photomorphogenesis. Light strongly favours the production of chloronemata over caulonemata and gametophore buds, which are in turn induced by the activities of auxin and cytokinin (Ashton et al., 1990). Given this information, the proposed
model predicts that, in the light, if the moss cannot make (or alternatively respond to) auxin and/or cytokinin, its phenotype will consist entirely of chloronemata. Conversely, in the dark, the moss will comprise mainly caulonemata since their formation now does not require auxin or cytokinin whereas production of chloronemata is light-dependent. These studies were in part designed to test the veracity of this developmental model, with the hypothesis being that auxin is required for the normal development of caulonemata as well as gametophore shoots and leaves during photomorphogenesis, but is not required for the development and growth of these tissues during skotomorphogenesis.

To address this hypothesis, transgenic auxin-responsive GUS reporter lines, the synthetic auxin NAA, and auxin signaling inhibitors were utilized to identify regions of auxin activity and associated effects on the morphology of the moss grown in the light or in the dark. Auxin transport inhibitors were used to probe the involvement of polar auxin transport. The experiments were performed in the presence and absence of light in order to provide data that contribute to a meaningful understanding of the extent to which differential regulation of auxin activity effects reversible switching between photomorphogenesis and skotomorphogenesis.
2. MATERIALS AND METHODS

2.1 *Physcomitrella patens* strains

When provided with *p*-aminobenzoic acid, the auxotrophic mutant, *pabB4*, is morphologically indistinguishable from wild-type *Physcomitrella patens* (Ashton and Cove, 1977). It was used in experiments reported herein either alone or with other strains where it acted as a control strain whose phenotype provided the standard of comparison with the those of the other strains.

Investigation of the distribution of endogenous and exogenous auxins within tissues and organs of the moss were pursued with two transgenic strains each with a *GUS* reporter gene (*E. coli* β-glucuronidase [GUS] gene, *uidA*) driven by one of two different auxin-inducible promoters. LTII-2 (*MAS::GUS*) (McDonald, 1999) contains a *GUS* gene fused to the 2′ side of the 1′,2′ dual promoter that lies between the two mannopine synthase genes (*mas*) in the T-DNA of *Agrobacterium tumefaciens* Ti plasmid. The other auxin-inducible reporter strain (*GH3::GUS*) possesses a *GUS* gene controlled by the *GH3* promoter (Bierfreund *et al*., 2003) and was kindly provided by the International Moss Stock Center in Freiburg, Germany. A third transgenic *GUS* reporter strain (*HSP::GUS*), in which the *GUS* gene is regulated by the soybean *Gmhsp17.3B* heat shock promoter, was employed in β-glucuronidase (GUS) histochemical assays as a positive control strain and was kindly provided by Dr. Pierre Goloubinof of the University of Lausanne, Lausanne, Switzerland. (Saidi *et al*., 2005).
2.2 Initial *Physcomitrella patens* growth medium and culture conditions

Cultures of *pabB4*, LTII-2, *GH3::GUS* and *HSP::GUS* were grown axenically on solid ABC medium (Knight *et al.*, 1988) supplemented with p-aminobenzoic acid (1.8 µM). Individual leaves from mature gametophores on 14-60 day-old colonies, previously grown on supplemented ABC medium, were used to inoculate fresh medium overlaid with cellophane discs (325P Cellulose, A.A. Packaging Ltd, Preston, United Kingdom) with three equally spaced inocula per dish. Cellophane overlays allowed water and nutrient transfer from the medium to the developing moss colonies but prevented the colonies from growing into the solid medium. This in turn facilitated subsequent transfer from one medium (initial growth/culture medium) to another (treatment medium) without physically damaging the moss. The inocula were incubated at approximately 21ºC in continuous white light (WL) from fluorescent tubes (Sylvania Supersaver Cool White). Petri dishes were covered with a layer of clear resin filter (Roscolux No. 114, Hamburg frost, MacPhon Industries, Calgary, AB, Canada) to reduce the rate of evaporative loss of water from the medium. Photon flux at the surface of the medium under these conditions was approximately 30 µmol m⁻² s⁻¹.

2.3 Transfer to treatment media and/or different culture conditions

After 21-25 days in the conditions described above, inocula had grown into moss colonies with gametophores, most of which were still at the bud stage, i.e. without leaves and a stem. Colonies containing gametophores that had advanced past the bud stage were discarded. At this time, the cellophane
overlays with moss colonies were transferred from the original growth medium to fresh treatment media containing various plant hormones and hormone antagonists/inhibitors. These substances were added to autoclaved and cooled medium to avoid their thermal degradation. Various different culture conditions, specifically relating to the quality (photon wavelengths) and/or quantity (photon flux) of light used to illuminate the cultures, were also employed.

2.3.1 Preparation of treatment media containing synthetic hormones and/or hormone antagonists/inhibitors

Stock solutions of the synthetic auxin, NAA (naphth-1-ylacetic acid) (Sigma-Aldrich, St. Louis, MO, USA), were prepared in absolute ethanol and stored at -4 °C. Working solutions were diluted in absolute ethanol and prepared as needed. Concentrations of NAA in the medium ranged from 10 to 100 nM as noted in the Results section, with a final concentration of 0.05% ethanol.

Concentrated stock solutions of auxinole (50-75 mM) and PEO-IAA (150-200 mM), both of which are auxin signal inhibitors (Hayashi et al., 2008; Hayashi et al., 2012), and NPA (N-1-naphthylphthalamic acid) (Naptalam, Pestanal, Sigma-Aldrich) (50 mM), an auxin transport inhibitor, were prepared in a solution of 50% dimethyl sulfoxide (DMSO) and 50% ethanol (v/v) and stored at -20°C. Working solutions were prepared as necessary by diluting stock solutions with 50% DMSO 50% ethanol (v/v). Concentrations of auxinole and PEO-IAA used in test media were between 75 and 150 μM while those of NPA ranged from 30 to 50 μM as noted in the Results section, with final concentrations of 0.05% DMSO and 0.05% ethanol.
Working solutions of the synthetic cytokinin, BAP (6-benzylaminopurine), were prepared fresh for each use since BAP quickly comes out of solution when stored. BAP was initially dissolved in 1M NaOH and diluted with ddH₂O so that the final concentration of NaOH was 50 mM. The solution was filter sterilized and brought to working concentrations of BAP with the addition of sterile 50 mM NaOH. Working concentrations of BAP in 50 mM NaOH were added to test media to give concentrations in the medium ranging from 10 to 100 nM as noted in the Results. The concentration of NaOH in test media was 50 µM.

2.3.2 Preparation of cultures for growth in darkness

Cultures with gametophore buds grown in WL were transferred to fresh medium containing 0.5% sucrose (Thermo Fisher Scientific, Waltham, MA, USA). Parafilm was used to seal the Petri plates along the bottom edge to prevent water leakage, which can provide a conduit for contaminant organisms. Plates were wrapped in aluminum foil and placed vertically on their edges. Moss colonies were incubated for a further 21-27 days in the dark.

2.3.3 Polychromatic white light (WL), red light (RL) and blue light (BL) illumination

WL illumination of cultures was described earlier and included covering the Petri dishes with one layer of clear resin filter (Roscolux No. 114, Hamburg frost). Photon flux at the surface of the medium was approximately 30 µmol m⁻² s⁻¹. RL and BL illumination were achieved respectively by substituting the clear resin filter with a red filter (Roscolux No. 27, medium red, MacPhon Industries), resulting in a photon flux of 0.95 µmol m⁻² s⁻¹, or a blue filter (Lee Filters No. 713
J. Winter Blue, Ruggieri Lighting and Staging Ltd., Regina, Saskatchewan, Canada) resulting in a photon flux of 0.78 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

Moss colonies previously grown in darkness were returned to WL, RL and BL and incubated for a further 7-21 days.

2.4 Histological detection of GUS

After culturing and the various treatments described earlier, gametophores from LTII-2 (MAS::GUS), GH3::GUS, HSP::GUS (positive control) and \( pabB4 \) (negative control) were harvested for histochemical GUS assays. Approximately 10 mg of tissue were introduced into 200 \( \mu \text{L} \) of 2mM 5-Bromo-4-chloro-3-indolyl-\( \beta \)-D-glucuronic acid (X-Gluc; B-7300, Biosynth International Inc., Naperville, IL., USA) in the wells of a 96-well microtitre plate. X-Gluc (with a final concentration of 2 mM) was dissolved in dimethyformamide (DMF) and brought to volume with 50 mM NaH\(_2\)PO\(_4\), pH 7. Samples were incubated at 37°C in the dark for 48 hours (Bierfreund et al., 2003). Gametophores from dark-grown colonies were harvested at 3 day intervals for 21 days and tested under a green safe light to avoid light stimulation of the moss tissues (Imaizumi et al., 2002).

2.5 Preparation and storage of gametophores prior to photomicrography.

After harvesting and being assayed for GUS, gametophores were fixed prior to storage. The gametophores were treated with 5% formaldehyde for 15 minutes followed by 5% acetic acid for 15 minutes (Bierfreund et al., 2003). Gametophores not assayed for GUS were washed with NaH\(_2\)PO\(_4\) buffer, pH 7, three times for 30 minutes. Gametophores, which had been subjected to the GUS assay protocol, were fixed, and then decolorized with an ethanol series of
30%, 50% and 70% ethanol (for 30 minutes each), and twice with 95% ethanol for 1 hour (Bierfreund et al., 2003). Following fixation, washing and decolorization, gametophores were stored in NaH$_2$PO$_4$ buffer, pH 7, at 4°C until examined, photographed and measured.

2.6 Bright field photomicrography and measurement of moss colonies and gametophores

Bright field microscopy was employed for all photographs. Whole moss colonies were photographed using a Nikon DS-Fi1 camera attached to a Nikon SMZ1500 stereo-microscope. Individual shoots and leaves were photographed with a Nikon DS-Ri1 camera fitted to a Nikon Eclipse 80i compound microscope. Measurements were made using NIS-Elements BR 3.22.11 software (Nikon, Tokyo, Japan).

Intact leafy gametophores were used for internode and leaf measurements. To standardize comparisons, L6 leaves were chosen for leaf measurements after culturing in both light and darkness with the exception of dark-grown gametophores treated with exogenous auxin or cytokinin, in which cases multiple leaves were measured as noted in the results. Average internode length was based upon the internodes from the bottom-most (most proximal) leaf to the top (most distal) non-apical leaf. The 4-5 uppermost (apical) leaves develop in the absence of any measurable internodes, and therefore were not included in the analysis. Average cell size was calculated by dividing the total leaf surface area by the number of cells in the leaf. Leaf areas were determined
using particle analysis in ImageJ (Rasband, 2014). Graphs were created and statistical analyses (t-tests) were performed using GraphPad Prism 6.0.
3. RESULTS

3.1 Analysis of auxin activity in WL-grown gametophores

3.1.1 Distribution of auxin in WL-grown gametophores

To gain an understanding of when and where auxin may play a role in gametophore development in the light, samples of gametophores were taken at 3 day intervals from WL-grown cultures of the transgenic auxin-responsive GUS reporter strains, LTII-2 (MAS::GUS) and GH3::GUS, the ubiquitously expressing GUS strain, HSP::GUS (positive control), and pabB4 (negative control). Sampling was conducted over a developmental time span beginning with gametophore buds and ending with fully mature gametophores comprising leafy shoots. The length of the observation period was 21 days in total.

By day 3, histological staining revealed no or little GUS activity in the sample of LTII-2 gametophores (buds and small, immature shoots with a few leaves). Where GUS staining (blue coloration) was observed, it was always in the leaves of the most developmentally advanced gametophores present at this time. Staining was more pronounced by day 6 and occurred most often in the distal halves of young (most recently formed) leaves. By day 15, the lamina and midrib of mature leaves near the apices of leafy gametophores were stained with the most intense staining occurring in the midrib (Fig. 1). A pattern of GUS staining in the leaves of more mature gametophores emerged. In general, the youngest, i.e. most recently formed at the shoot apex, leaves (generally P1-2 or 3) showed no GUS activity. In P4 leaves, GUS staining was usually present at the tip (distal end) of each leaf. In successively older leaves, from P5 to P9 or
Fig. 3. Histological staining for GUS in gametophores of WL-grown LTII-2 (MAS::GUS), pabB4, and HSP::GUS.

The images are of gametophores sampled at day 3, 6 and 15 of the 21 day observation period. The scale bar represents 500 µm.
P10, staining expanded from the tip towards the middle and the base of the leaves while decreasing in the distal portion. Leaves with P>10 displayed little or no GUS activity (Fig. 2). The regions of leaves with GUS staining, indicative of the presence of auxin, corresponded quite closely to regions in which cells had recently expanded or were in the process of expanding.

By day 3 of the 21 day observation period, some gametophore buds of GH3::GUS displayed GUS staining. Older buds, from which small leaves had arisen, were stained in the bud but not the leaf portions of the developing gametophores. By day 6, as the buds continued their development into leafy shoots, the stems of the leafy shoots showed intense GUS staining. Incipient leaves on some very young leafy gametophores also had GUS activity. By day 9, GUS activity was limited predominantly to the stems of the developing leafy gametophores, a trend that continued to day 21 (Fig. 3). These observations match those reported by Bierfruend et al. (2003).

3.1.2 Inhibition of auxin signaling and transport in WL-grown gametophores

Since the experiments with auxin-responsive GUS reporter strains implicated auxin in moss gametophore development in WL, the roles of endogenous auxin were explored further using two auxin antagonists: (a) PEO-IAA, an inhibitor of auxin signaling and (b) NPA, an auxin transport inhibitor.

Both substances had significant effects on the growth and development of leaves on gametophores grown in WL for 21 days (Fig. 4). 150 µM PEO-IAA decreased by 34% (compared to the untreated control) the average length of L6.
Fig. 4. Histological staining for GUS in leaves taken from a 21 day-old LTII-2 (MAS::GUS) gametophore grown in WL

(a) P1-3; (b) P4; (c) P6; (d) P9; (e) P11; (f) P14 (L1) The scale bar represents 500 µm.
Fig 5. Histological staining for GUS in gametophores of WL-grown GH3::GUS (following page)

The images are of gametophores sampled at day 3 (a, b), day 6 (c) day 9 (d) and day 21 (e) of the 21 day observation period. The scale bar represents 500 µm.
Fig. 6. L6 leaves removed from 21 day-old gametophores of *pabB4* cultures exposed to the auxin signal inhibitor, PEO-IAA, or the auxin transport inhibitor, NPA (following page)

Moss colonies were grown on minimal medium for 23 to 25 days in WL and then incubated for a further 21 d in WL after transferring them to fresh media: (a) minimal medium (control medium); (b) medium containing 150 µM PEO-IAA; (c, d) medium containing 50 µM NPA. The arrow in (d) points to a bifurcated marginal apical outgrowth. The scale bar represents 500 µm.
leaves, while 30 μM NPA resulted in a 23% increase (Fig. 5a). Leaf width, at the widest part of the leaf, was not significantly changed by PEO-IAA but NPA decreased the width of L6 leaves by 10% (Fig. 5b).

These effects on leaf length and width resulted in a 36% decrease in leaf index in PEO-IAA-treated leaves and a 37% increase in NPA-treated leaves (Fig. 5c). The altered leaf shape caused by PEO-IAA was due to a 35% decrease in calculated leaf cell size (surface area) combined with a 16% increase in leaf cell number. By contrast, the changed leaf shape caused by NPA was caused exclusively by a 29% increase in calculated leaf cell size with no significant effect on leaf cell number (Fig. 6a and b).

NPA also induced the formation of additional apices on leaves of treated gametophores. These additional apices usually, but not exclusively, arose from the leaf margins and were sometimes bifurcated. (Fig. 4).

PEO-IAA and NPA also affected the length of internodes of leafy gametophores (Fig. 7). 150 μM PEO-IAA and 30 μM NPA resulted respectively in a 48% decrease and a 42% increase in mean internode length of leafy shoots from WL-grown cultures (Fig. 8).

In WL, although gametophore morphogenesis was altered as described above, neither PEO-IAA nor NPA appeared to affect the transition of gametophore buds into shoots. Since new buds are produced continuously in the light, the number of buds present prior to treatments that had transitioned into leafy shoots by the end of the treatments could not be readily calculated.
Fig. 7. Leaf length, width and index of L6 leaves removed from 21 day-old gametophores of *pabB4* cultures exposed to PEO-IAA or NPA (following page)

(a) Leaf length; (b) leaf width at the widest point; (c) leaf index (length/width).

Moss colonies were grown in WL for 21 to 23 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred to fresh media, which contained 150 µM PEO-IAA, 30 µM NPA, or neither substance (control medium), for a further 21 days in WL. Error bars represent 1 standard deviation above and below the mean. N = 19 (control), 13 (PEO-IAA), 9 (NPA). Significant differences from the control where p is less than 0.05 are indicated with an asterisk. Significant differences from the control where p is less than 0.001 are signified with a double asterisk.
(a) Leaf Length (µm)

<table>
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<tr>
<th>Treatment</th>
<th>Value (µm)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>150 µM PEO-IAA</td>
<td></td>
</tr>
<tr>
<td>30 µM NPA</td>
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(b) Leaf Width (µm)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Value (µm)</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>150 µM PEO-IAA</td>
<td></td>
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<tr>
<td>30 µM NPA</td>
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</table>
Fig. 8. Number of cells per leaf and calculated leaf cell size of L6 leaves removed from 21 day-old gametophores of pabB4 cultures exposed to PEO-IAA or NPA (following page)

(a) Number of cells per leaf; (b) calculated leaf cell size (surface area).

Moss colonies were grown for 21 to 23 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred to fresh media, which contained 150 µM PEO-IAA or 30 µM NPA or neither substance (control medium) and incubated for a further 21 days in WL. Error bars represent 1 standard deviation above and below the mean. N = 18 for number of cells and 9 for calculated leaf cell size (control), 13 for number of cells and 10 for calculated leaf cell size (PEO-IAA), 9 for both number of cells and calculated leaf cell size (NPA). Significant differences from the control where p is less than 0.05 are indicated with an asterisk.
Fig. 9. Mature, 21 day-old leafy gametophores from *pabB4* cultures exposed to PEO-IAA or NPA (following page)

Moss colonies were grown for 23 to 25 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred to fresh media, which contained (a) neither inhibitor (control medium), (b) 50 µM NPA or (c) 150 µM PEO-IAA and incubated for a further 21 days in WL. The scale bar represents 1 mm.
Fig. 10. Internode lengths of gametophores from *pabB4* cultures exposed to PEO-IAA or NPA

Moss colonies were grown for 23 to 25 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred to fresh media, which contained 150 µM PEO-IAA, or 30 µM NPA or neither inhibitor (control medium), and incubated for a further 21 days in WL. Error bars represent 1 standard deviation above and below the mean value. N = 40 (control), 15 (PEO-IAA), 10 (NPA). Significant differences from the control where *p* is less than 0.0001 are indicated with an asterisk.
However, the moss colonies seemed to produce a similar number of leafy shoots independent of treatments. Protonemal development was affected and diameters of the moss colonies were decreased by treatment with either of the antagonists. (Fig. 9).

The inhibition of gametophore elongation and caulonemal development upon exposure to PEO-IAA match the results previously reported with this inhibitor (Hayashi et al., 2008; Hayashi et al., 2012).

3.1.3 Distribution of auxin activity in gametophores of WL-grown LTII-2 (MAS::GUS) and GH3::GUS cultured on media containing PEO-IAA or NPA

GUS staining patterns in leaves of both LTII-2 and GH3::GUS cultured for 21 days on media containing PEO-IAA or NPA were similar to those in leaves of gametophores of an equivalent age from gametophytes grown on control medium containing neither substance. The single difference was that GUS staining was present in the multiple apices of misshapen leaves resulting from exposure to NPA (Fig. 10).

3.2 Development of gametophores in the dark

The morphology of leafy gametophores that had developed in darkness from WL-grown buds was markedly different from that of leafy gametophores of cultures grown continuously in WL. The dark-grown negatively gravitropic gametophores were etiolated and possessed very small scale-like leaves, which resembled the most basal leaves (L1) of WL-grown leafy gametophores.
Moss colonies were grown for 23 to 25 days in WL by which time gametophore buds, but no leafy shoots, had developed. The moss colonies were then transferred to fresh media, which contained (a) neither inhibitor (control medium), (b) 50 µM NPA or (c) 150 µM PEO-IAA, and incubated for a further 21 days in WL. The scale bar represents 5 mm.
Moss colonies were grown in WL for 25 days by which time gametophore buds but no leafy shoots had been formed. (a, b) LTII-2 colonies were then transferred to fresh treatment medium containing 50 µM NPA and grown another 21 days. This treatment sometimes resulted in misshapen leaves with extra apices (arrowed), which stained weakly. For comparison, leaves from (c) HSP::GUS and (d) pabB4 grown on control medium for a further 21 days. The bar represents 500 µm.

Fig. 12. Histological staining for GUS in LTII-2 leaves following NPA treatment
Gametophore length and number of leaves per gametophore increased in dark-grown \textit{pabB4} cultures until day 18 after which no further increases were observed (Fig. 11).

3.2.1 Distribution of auxin in leafy gametophores that had developed in the dark from WL-grown buds

By day 3 of a 21 day period of dark-culture, histological staining for GUS revealed little or no auxin in young buds and small leafy gametophores of LTII-2 (\textit{MAS::GUS}). At day 6, sub-apical leaves of a few of the larger gametophores were stained. This pattern of staining became more prominent by day 9, at which time the majority of gametophores possessed some stained leaves, with the shoot stem below the stained leaves sometimes being lightly stained as well. The stained leaves usually comprised the first to fourth leaf below the apical leaves, with only one or two leaves stained on any given gametophore. With increasing time in darkness, the proportion of gametophores with stained sub-apical leaves progressively decreased. By day 21, only one or two gametophores per culture demonstrated any GUS activity (Fig. 12).

Staining patterns in \textit{GH3::GUS} differed from those observed in LTII-2 (\textit{MAS::GUS}). On day 3, nearly all buds and young leafy gametophores displayed intense staining for GUS. This continued through day 6, when the gametophores were entirely stained. Staining was diminished by day 9, at which time the pattern of staining varied from gametophore to gametophore. Gametophores were stained at the stem apex or its base or both, and
Fig. 13. Gametophore length and number of leaves per gametophore during development of \textit{pabB4} gametophores in the dark

Moss colonies were grown for 25 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then incubated in the dark on medium containing 0.5% sucrose for another 27 days and samples of gametophores were removed at 3 day intervals. Cultures used for gametophore sampling were discarded. Error bars represent 1 standard deviation and for figure clarity have been provided either above (number of leaves per gametophore) or below (gametophore length) each mean value. N = 176 gametophores (day 6), 69 (day 9), 158 (day 12), 175 (day 15), 326 (day 18), 243 (day 21), 253 (day 24), 177 (day 27).
Fig. 14. 21 day time course of GUS activity in gametophores of dark-grown LTII-2 (*MAS::GUS*) (following page)

LTII-2 colonies were grown on minimal medium for 21 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose and incubated in the dark for another 21 days during which time gametophores were sampled at 3 day intervals. Cultures used for gametophore sampling were discarded.

(a) A gametophore bud at day 3 with no detectable staining. (b) A small leafy shoot at day 6 with GUS staining visible in the subapical leaf (arrowed) and associated node of the stem. (c) A leafy shoot at day 9 with stained subapical leaves (arrowed) and stained stem at the subtending nodes and the internode between them. (d) A gametophore at day 21 with no discernible staining. For comparison, gametophores of *pabB4* (e) and *HSP::GUS* (f), both collected at day 21 of the dark-growth period. The scale bar represents 500 μm.
sometimes in the leaves or the stem (particularly nodal regions), with differing levels of intensity. This varied pattern continued during development in the dark until the end of the 21 day observation period. However, in general, older gametophores were characterized by reduced levels of GUS activity (Fig. 13).

3.2.2 Auxin sensitivity of dark-grown gametophores

To determine if dark-grown gametophores remain sensitive to auxin, WL-grown colonies with buds were supplied with NAA and 0.5% sucrose and transferred into the dark. At concentrations as low as 10 nM, NAA elicited morphogenetic changes in pabB4 leaves that developed in the dark (Table 1). Higher concentrations (30 - 100 nM) of NAA inhibited the growth of gametophores and reduced the number of leaves they produced (Fig. 14). Of the leaves that were formed, L1 – L3 increased in length in response to all tested concentrations of NAA (Fig. 15a). Conversely, the width of leaves decreased but this was not statistically significant (Fig. 15b). The elongation and narrowing of the leaves resulted in increases in leaf index for L1 - L4 (Fig. 15c). With the exception of L2 and 10 nM NAA, the number cells per leaf was not significantly altered by NAA (Fig. 16a). By contrast, calculated leaf cell size was increased for L1 – L3 or L4 by treatment with 30 – 100 nM NAA (Fig. 16b).

None of the concentrations of NAA tested had a significant effect on internode length (Fig. 17). Only the first 3 to 4 internodes of gametophores that developed on control medium were measured for comparison to those of gametophores that developed on treatment medium containing high
Fig. 15. 21 day time course of GUS activity in gametophores of dark-grown GH3::GUS (following page)

GH3::GUS colonies were grown on minimal medium for 21 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose and incubated in the dark for another 21 days during which time gametophores were sampled at 3 day intervals. Cultures used for gametophore sampling were discarded. (a) A gametophore bud at day 3 with intense GUS staining. (b) A small leafy shoot at day 6 with intense GUS staining especially in the apex and base of the stem (arrowed). (c, d) Gametophores at day 9 with varied GUS staining patterns but often associated with the stem apex and base and in subapical leaves and at stem nodes (arrowed). (e) A gametophore at day 21 with only very faint blue staining. The scale bar represents 500 μm.
Table 1. Effects of NAA on dark-grown leaves

Concentrations of NAA (10 nM, 30 nM, 50 nM, 100 nM) that had a significant effect on leaf length, leaf width, leaf index, number of cells per leaf, and calculated cell size per leaf compared to the untreated control are noted. A blank cell indicates no significant change at any concentration of NAA. The p values for significant differences are given in parentheses. Leaves higher than L4 were not significantly altered or were not present.

<table>
<thead>
<tr>
<th>L No.</th>
<th>Significant Leaf Length Increases</th>
<th>Significant Leaf Width Increases</th>
<th>Significant Leaf Index Increases</th>
<th>Significant Number of Cells per Leaf Increases</th>
<th>Significant Average Cell Size per Leaf Increases</th>
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<tbody>
<tr>
<td>1</td>
<td>30 nM (0.0372) 50 nM (0.0164) 100 nM (0.001)</td>
<td>30 nM (0.0135) 50 nM (0.004) 100 nM (0.0029)</td>
<td>30 nM (0.0135) 50 nM (0.004) 100 nM (0.0029)</td>
<td>30 nM (0.0304) 50 nM (&lt;0.0001) 100 nM (0.0197)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10 nM (0.0217) 50 nM (0.0352) 100 nM (0.0164)</td>
<td>10 nM (0.0207) 30 nM (0.015) 50 nM (0.022) 100 nM (0.0227)</td>
<td>10 nM (0.0207) 30 nM (0.015) 50 nM (0.022) 100 nM (0.0227)</td>
<td>50 nM (0.0078) 100 nM (0.0043)</td>
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</tr>
<tr>
<td>3</td>
<td>100 nM (0.038)</td>
<td>50 nM (0.02) 100 nM (0.0005)</td>
<td></td>
<td>50 nM (0.02) 100 nM (0.0005)</td>
<td>50 nM (0.0062)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>100 nM (0.0009)</td>
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Fig. 16. The effect of NAA on a *pabB4* gametophore that developed in the dark (following page)

*PabB4* colonies were grown on minimal medium for 21 days in WL by which time gametophore buds, but not leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose (a) with 50 nM NAA or (b) without NAA and incubated in the dark for another 21 days. The scale bar represents 500 µm.
Figure 17: Leaf length, width and index of *pabB4* gametophores that developed in the dark in the presence of NAA (following page)

Length (a), width (b) and index (c) of leaves from gametophores allowed to develop for 21 days in the dark on media containing 0.5% sucrose and 10, 30, 50 or 100 nM NAA. Prior to incubating in darkness, the *pabB4* colonies were grown in WL on minimal medium for 21 days by which time gametophore buds, but no leafy shoots, had developed. Error bars represent 1 standard deviation. For figure clarity, only the error bar above each mean value has been shown. N = 47 for length, 42 for width and leaf index (control), 48 for length, 45 for width and leaf index (10 nM NAA), 39 for length, 36 for width and leaf index (30 nM NAA), 26 for length, width, and leaf index (50 nM NAA), 28 for length, width, and leaf index (100 nM NAA).
Fig. 18. Number of cells per leaf and calculated cell size in leaves of *pabB4* gametophores that developed in the dark in the presence of NAA (following page)

Number of cells per leaf (a) and calculated cell size (b) of leaves from gametophores allowed to develop for 21 days in the dark on media containing 0.5% sucrose and 10, 30, 50 or 100 nM NAA. Prior to incubating in darkness, the *pabB4* colonies were grown in WL on minimal medium for 21 days by which time gametophore buds, but no leafy shoots, had developed. Error bars represent 1 standard deviation. For figure clarity, only the error bar above the mean has been shown. N = 44 for number of cells, 39 for calculated cell size (control), 46 for number of cells, 42 for calculated cell size (10 nM NAA), 38 for number of cells and calculated cell size (30 nM NAA), 26 for number of cells and calculated cell size (50 nM NAA), 28 for number of cells and calculated cell size (100 nM NAA).
Fig. 19. Internode lengths of *pabB4* gametophores that developed in the dark in the presence of NAA (following page)

Internode lengths of *pabB4* gametophores that had been allowed to develop for 21 days in the dark on media containing 0.5% sucrose and 30 nM NAA (a), or 50 and 70 nM NAA (b). Prior to incubating in darkness, the *pabB4* colonies were grown in WL on minimal medium for 21 days by which time gametophore buds, but no leafy shoots, had developed. Error bars represent 1 standard deviation above and below the mean. None of the length differences were statistically significant. N = 18 (control for a), 12 (30 nM NAA), 10 (control for b), 12 (50 nM NAA), 12 (70 nM NAA).
concentrations, 50 and 70 nM, of NAA, since the latter gametophores were shorter and had correspondingly fewer nodes and internodes. For comparison with gametophores exposed to 30 nM NAA, all the internodes of gametophores that developed in the absence of the exogenous synthetic auxin were measured.

3.2.3 Inhibition of auxin signaling and transport in dark-grown gametophores

The roles of endogenous auxin in the normal development of gametophores in darkness were explored with (a) PEO-IAA, the inhibitor of auxin signaling and (b) NPA, the auxin transport inhibitor. Both substances had significant effects on the morphogenesis of leaves on gametophores grown in the dark as compared to dark-grown control gametophores. 150 µM PEO-IAA resulted in L6 leaves that were, on average, 16% shorter (Fig. 18a), with no significant change to their width (Fig. 18b). In turn, this resulted in a 14% decrease in the leaf index (Fig. 18c). The mean number of cells in a L6 leaf increased by 20% (Fig. 19a), with an associated 28% decrease in the mean cell size (Figure 19b). When auxin transport was inhibited with 30 µM NPA, the mean length of L6 leaves was not significantly altered (Fig. 18a). Although the mean leaf width decreased by 14% (Fig. 18b), this effect of NPA was insufficient to alter significantly the leaf index of L6 leaves (Fig. 18c). The mean number of cells per leaf was decreased by 20% (Fig. 19a) but the calculated leaf cell size was unaffected (Fig. 19b) by exposure to NPA.
Fig. 20. Leaf length, width and index of L6 leaves of *pabB4* gametophores that developed in the dark in the presence of the auxin signal inhibitor, PEO-IAA, or the auxin transport inhibitor, NPA (following page)

Mean leaf length (a), width (b) and index (c) of L6 leaves from *pabB4* gametophores that had developed in the dark. Moss colonies were grown in WL on minimal medium for 21 to 23 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose (control medium) and media containing 0.5% sucrose plus 150 µM PEO-IAA or 30 µM NPA and incubated in the dark for another 21 days. Error bars represent 1 standard deviation above and below the mean. Significant differences from the control where p values are <0.05 or <0.01 are indicated with an asterisk and double asterisk respectively. N = 29 (control), 18 (PEO-IAA), 15 (NPA).
Fig. 21. Total number and calculated cell size of L6 leaves from *pabB4* gametophores that developed in the dark in the presence of the auxin signal inhibitor, PEO-IAA, or the auxin transport inhibitor, NPA (following page)

Mean cell number (a) and calculated cell size (b) of L6 leaves from *pabB4* gametophores that had developed in the dark. Moss colonies were grown in WL on minimal medium for 21 to 23 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose (control medium) and media containing 0.5% sucrose plus 150 µM PEO-IAA or 30 µM NPA and incubated in the dark for another 21 days. Error bars represent 1 standard deviation above and below the mean. Significant differences from the control where p values are <0.05 or <0.0001 are indicated with an asterisk and double asterisk respectively. N = 29 for number of cells and 22 for calculated cell size (control), 18 for number of cells and 14 for calculated cell size (PEO-IAA), 15 for number of cells and calculated cell size (NPA).
Contrary to what happened with WL-grown gametophores, the internode length of dark-grown leafy shoots was unaffected by auxin signaling inhibitors, PEO-IAA and auxinole and the auxin transport inhibitor, NPA, at the concentrations tested (Fig. 20). Internodes of control leafy gametophores that developed in the dark were 109% longer than those of WL-grown gametophores.

Inhibitors of auxin signaling, PEO-IAA (at 125 and 150 µM) and auxinole (at 75 µM), and of auxin transport, NPA (at 30 and 50 µM), markedly affected the transition from gametophore bud to leafy shoot in the dark. Inclusion of 75 nM NAA in growth media containing PEO-IAA or auxinole partially counteracted this effect of auxin signaling inhibitors. (Fig. 21).

As in WL, 150 µM PEO-IAA or 30 or 50 µM NPA reduced the number and length of caulonemal runners produced by colonies grown in darkness. The caulonemal runners that were produced in the dark, as well as any gametophores, remained negatively gravitropic (Fig. 22).

3.2.4 Distribution of auxin activity in dark-grown gametophores treated with PEO-IAA, NPA or NAA

No auxin activity, as indicated by GUS staining in LTII-2 (MAS::GUS), was discernible in 21-day-old gametophore shoots that had developed in the dark on media containing 0.5% sucrose with or without 150 µM PEO-IAA. PEO-IAA at 150 µM seemingly reduced GUS activity at the apices of dark-grown gametophores of the GH3::GUS strain. However, the significance of this is difficult to assess due to the varied and inconsistent GUS staining observed in
Fig. 22. Internode length of \textit{pabB4} gametophores that developed in the dark in the presence of an auxin signal inhibitor, PEO-IAA or auxinole, or the auxin transport inhibitor, NPA

Moss colonies were grown in WL on minimal medium for 21 to 23 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose (control medium) and media containing 0.5% sucrose plus 150 μM PEO-IAA or 75 μM auxinole or 30 μM NPA and incubated in the dark for another 21 days. Error bars represent 1 standard deviation above and below the mean. No significant differences were observed. \( N = 27 \) (control), 20 (PEO-IAA), 14 (auxinole), 11 (NPA).
Fig. 23. The transition of gametophore buds to leafy shoots in the dark in the presence of auxin signaling inhibitors, PEO-IAA or auxinole, or the auxin transport inhibitor, NPA (following page)

*PabB4* moss colonies were grown in WL on minimal medium for 21 to 23 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose (control medium) and media containing 0.5% sucrose plus 150 µM PEO-IAA +/- 75 nM NAA, 125 µM PEO-IAA +/- 75 nM NAA, 75 µM auxinole +/- 75 nM NAA, 30 µM NPA or 50 µM NPA and incubated in the dark for another 21 days. The number of buds that had transitioned into shoots after 21 days in the dark is reported in this histogram as a percentage of the total number of buds present at the time of transfer. Error bars represent 1 standard deviation above and below the mean. Significant differences from the control are shown directly above each treatment column. Significant differences between inhibitor and inhibitor plus NAA are shown above the lined-in groupings. A single asterisk indicates a p value <0.01, a double asterisk <0.001, and a triple asterisk <0.0001. N = 13 (control), 10 (150 µM PEO-IAA), 6 (150 µM PEO-IAA + NAA), 5 (125 µM PEO-IAA), 4 (125 µM PEO-IAA + NAA), 4 (auxinole), 3 (auxinole + NAA), 3 (30 µM NPA), 9 (50 µM NPA)
Fig. 24. The effect of PEO-IAA and NPA on the production of peripheral caulonemata (caulonemal runners) by *pabB4* gametophytes grown in the dark (following page)

Moss colonies were grown in WL on minimal medium for 21 to 23 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose (a) and media containing 0.5% sucrose plus 150 µM PEO-IAA (b) or 50 µM NPA (c) and incubated in the dark for another 21 days. The scale bar represents 5 mm.
this strain after growth in darkness. GUS staining patterns in \textit{GH3::GUS} gametophores that had developed in the dark in the presence and absence of 50 µM NPA were similar. Staining was intense in both cases and located predominantly at the gametophore apex and in the scale-like leaves. In LTII-2 grown in the dark on medium containing 50 µM NPA, only a single gametophore was produced. GUS staining in this LTII-2 gametophore was weaker than that in the \textit{GH3::GUS} strain. The distribution of stain, in the stem and some of the scale-like leaves, bore some resemblance to that in \textit{GH3::GUS}, thus raising the possibility that these auxin-responsive transgenic strains differ quantitatively rather than qualitatively in this respect (Fig. 23).

NAA at 50 nM elicited GUS activity in the gametophore leaves, which were elongated in response to NAA at this concentration, but not in the gametophore stem of dark-grown LTII-2 (Fig. 24).

3.3 Analysis of auxin activity in gametophores returned from the dark to the light

Colonies with gametophores that had developed in the dark for 21-22 days were placed for a further period of incubation in one of three light conditions: white light (WL), red light (RL) or blue light (BL).

Over the course of next 21 days, all three light conditions induced a return to photomorphogenesis of the dark-grown gametophores, characterized by renewed development with expanded leaves, thicker stems, shorter internodes, proliferation of rhizoids, loss of gravitropism and return of phototropism (Fig. 25), and of the gametophytic colonies, characterized by the formation of SBIs on the
Fig. 25. GUS activity in dark-grown gametophores of the auxin-responsive GUS reporter strains, LTII-2 (MAS::GUS) and GH3::GUS, exposed to PEO-IAA or NPA (following page)

Moss colonies were grown in WL on minimal medium for 25 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose or media containing 0.5% sucrose plus 150 µM PEO-IAA or 50 µM NPA and incubated in the dark for another 21 days. PabB4 and the HSP::GUS transgenic strain, negative and positive controls respectively, are shown for reference. The scale bar represents 500 μm.
Fig. 26. GUS activity in gametophores of LTII-2 grown in the dark in the presence of NAA (following page)

Moss colonies were grown in WL on minimal medium for 21 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose with (a) or without (b) 50 nM NAA and incubated in the dark for another 21 days. The scale bar represents 500 µm.
Fig. 27. Photomorphogenesis induced in dark-grown *pabB4* gametophores by exposure to white light, red light or blue light (following page)

*PabB4* colonies were cultured for 25 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose and incubated in the dark for another 22 days during which gametophore buds developed into negatively gravitropic leafy shoots with thin, etiolated stems and small scale-like leaves. The colonies were then placed in WL, RL or BL and cultured for a further 21 days. Gametophores were sampled every seven days. The scale bar represents 500 µm.
caulonemal runners from which secondary chloronemata and caulonemata and buds arose (Fig. 26). In terms of the number and size of leaves that developed on previously dark-grown gametophores and the rate at which new protonemata and gametophores were formed, WL was the most effective at inducing photomorphogenesis, followed by RL and finally BL.

Auxin activity also returned in dark-grown gametophores placed in the light. Since GH3::GUS gametophores displayed varied and inconsistent GUS staining patterns in the dark, only the LTII-2 auxin-responsive GUS reporter strain was used to assess auxin activity during the transition from skotomorphogenesis to photomorphogenesis. Auxin activity returned to LTII-2 gametophores within 72 hours of light induction in WL, RL or BL (Fig. 27). GUS staining was observed in shoot apices and sometimes in a sub-apical region of the stems as well as towards the distal ends of developing leaves. In some leaves, especially the first to be formed after induction of photomorphogenesis, the entire leaf was GUS stained. Dark-grown gametophores exposed to WL for 48 hours displayed noticeable apical growth and with thickening of the stem and leaf expansion, and loss of gravitropic response but without any discernible GUS activity (Fig. 28a). After 72 hours in WL, thickening of the stem, leaf expansion and loss of the negatively gravitropic response and return of a phototropic response are considerably more pronounced. By this time, GUS activity is readily visible in gametophore apices and at the distal ends of expanded leaves surrounding the stem apices (Fig. 28b and c).
Fig. 28. Photomorphogenesis induced in dark-grown \textit{pabB4} gametophytic colonies by exposure to white light, red light or blue light (following page)

\textit{PabB4} colonies were cultured for 25 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5\% sucrose and incubated in the dark for another 22 days during which gametophore buds developed into negatively gravitropic leafy shoots with thin, etiolated stems and small scale-like leaves and protonemata grew to become negatively gravitropic, unbranched caulonemal runners. The colonies were then placed in WL, RL or BL and cultured for a further 21 days. The bar represents 5 mm.
Fig. 29. GUS activity in dark-grown LTII-2 gametophores placed in the light
(following page)

LTII-2 colonies were cultured for 22 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were then transferred on to fresh medium containing 0.5% sucrose and incubated in the dark for another 22 days during which gametophore buds developed into negatively gravitropic leafy shoots with thin, etiolated stems and small scale-like leaves. Subsequently the colonies were placed in WL (a), RL (b) or BL (c) and cultured for an additional 7 days. The scale bar represents 500 µm.
Fig. 30. Time course for the return of GUS activity in dark-grown LTII-2 gametophores placed in white light

LTII-2 colonies were cultured for 21 days in WL before being transferred on to fresh medium containing 0.5% sucrose and allowed to develop in the dark for an additional 21 days during which gametophore buds developed into negatively gravitropic leafy shoots with thin, etiolated stems and small scale-like leaves. Subsequently the colonies were returned to WL and photographed after 48 hours (a) and 72 hours (b, c). GUS staining in stem apices and at the distal ends of expanded leaves is arrowed. A pabB4 (d) and HSP::GUS (e) gametophore are shown for reference. The scale bar represents 500 µm.
3.4 Cytokinin activity in dark and white light-grown gametophores

3.4.1 Analysis of cytokinin activity in dark-grown gametophores

To determine whether or not dark-grown gametophores remain sensitive to cytokinin, 21 day-old *pabB4* colonies with buds were transferred on to fresh medium containing 0.5% sucrose with and without the synthetic cytokinin, BAP and placed in the dark for a further 21 days. Leaf length and width increased significantly in response to 70 nM BAP (Fig. 29a and b). The increase in length was generally greater than the increase in width, leading to an increase in leaf index, significantly so for leaves L3 through L6 (Fig. 29c). The number of cells increased significantly for all leaves exposed to 70 nM BAP, while 10 nM BAP had little effect (Fig. 30a). Increases in average cell size were less pronounced and occurred in an unpredictable manner (leaves L2, L5 and L8 in response to 10 nM BAP and leaves L4 and L8 in response to 70 nM BAP) (Fig. 30b). Therefore, the observed increases in leaf length and width probably derived from increased cell numbers and not increased cell size. The increase in number of cells per leaf caused by 70 nM BAP occurred predominantly in the longitudinal direction rather than the transverse direction.

3.4.2 Effect of BAP treatment on GUS activity in LTII-2 and GH3::GUS dark-grown gametophores

LTII-2 colonies grown in WL for 21 days were transferred on to fresh medium containing 0.5% sucrose and 100 nM BAP and placed in the dark for a further 21 days. By the end of this period, no GUS activity was apparent in LTII-2 gametophores. The *GH3::GUS* strain displayed the varied and inconsistent GUS
Fig. 31. Leaf length, width and index of leaves of *pabB4* gametophores that developed in the dark from buds on media containing BAP (following page)

*PabB4* colonies were grown in WL for 21 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were transferred on to fresh medium containing 0.5% sucrose or media containing 0.5% sucrose and 10nM or 70 nM BAP and incubated in the dark for another 21 days when gametophores were collected and the length (a) and width (b) of leaves measured and leaf index (c) calculated. Error bars represent one standard deviation above and below the means. N= 62 for length, width, and index (control), 61 for length, width, and index (10 nM BAP), and 58 for length, width, and index (70 nM BAP).
(c)

Leaf Index

Leaf Number

- Control
- 10 nM BAP
- 70 nM BAP
Fig. 32. Number of cells per leaf and calculated cell size in leaves of *pabB4* gametophores that developed in the dark from buds on media containing BAP (following page)

*PabB4* colonies were grown in WL for 21 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were transferred on to fresh medium containing 0.5% sucrose or media containing 0.5% sucrose and 10nM or 70 nM BAP and incubated in the dark for another 21 days when gametophores were harvested and the number of cells per leaf (a) counted and average cell size (b) calculated. Error bars represent one standard deviation above and below the means. N = 61 for number of cells and 61 for calculated cell size (control), 61 for number of cells and 60 for calculated cell size (10 nM BAP), 58 for number of cells and 54 calculated cell size (70 nM BAP).
staining typical of this transgenic reporter line after developing in the dark in the absence of exogenous phytohormones (Fig. 31).

3.4.3 The effect of BAP on GUS activity in gametophores of auxin-responsive lines cultured in white light

Moss colonies cultured for 21 days in WL were transferred on to fresh medium containing 100 nM BAP and incubated in WL for a further 21 days. By the end of this period, the cytokinin treatment had inhibited the development of LTII-2 gametophores but had not induced GUS staining in its morphologically altered leaves. GUS activity was present in the enlarged shoot and upper leaves, which is typical in WL-grown gametophores of this strain in the absence of phytohormones. As previously reported, BAP did not affect GUS expression in GH3::GUS grown under these conditions (Bierfreund et al., 2003) (Fig. 32).
Fig. 33: GUS activity in LTII-2 and GH3::GUS leafy gametophores that developed in the dark from buds on medium containing BAP (following page)

LTII-2 (a) and GH3::GUS (b and c) are auxin-responsive transgenic lines. PabB4 (d) and HSP::GUS (e), negative and positive control strains respectively, are included for reference. Moss colonies were grown in WL for 21 days by which time gametophore buds, but no leafy shoots, had developed. The colonies were transferred on to fresh medium containing 0.5% sucrose and 100 nM BAP and incubated in the dark for another 21 days when gametophores were collected and photographed. The scale bar represents 500 µm.
Fig. 34. GUS activity in LTII-2 and GH3::GUS leafy gametophores that developed from buds in white light on medium containing BAP (following page)

LTII-2 (a), GH3::GUS (b). PabB4 (c) and HSP::GUS (d), negative and positive control strains respectively, are included for reference. Moss colonies were cultured for 21 days in WL by which time gametophore buds, but no leafy shoots, had developed. The colonies were transferred on to fresh medium containing 100 nM BAP and incubated in WL for a further 21 days when gametophores were collected and photographed. Panels (e) and (f) show L2 leaves removed from LTII-2 gametophores that developed on media without BAP or with 100 nM BAP respectively. Although the morphology of the BAP-treated L2 leaf has been noticeably affected, only faint GUS staining in a small area at the base of the leaf is apparent. The scale bars in panel (a) and (b) represent 500 µm and 100 µm respectively.
4. DISCUSSION

4.1 Gametophore Photomorphogenesis

4.1.1 Auxin signaling is required for normal morphogenesis of gametophores and leaves in the light

Evidence has been accumulating for some time that auxin is involved in *Physcomitrella patens* gametophore development in the light. Auxin is required for the initiation of gametophore buds from caulonemal SBIs (Ashton and Cove, 1990). During gametophore growth, exogenous auxin causes stem and leaf elongation (Barker and Ashton, 2013; Decker et al., 2006; Hayashi et al., 2008). Disruption of cryptochromes, which have been implicated in the attenuation of auxin signaling, results in longer gametophores (Imaizumi et al., 2002). Conversely, knocking out auxin biosynthesis genes, *PpSHI1* or *PpSHI2*, results in shorter internodes in light-grown gametophores (Eklund et al., 2010). Consistent with these observations, auxin distribution studies using the auxin-sensitive *GH3::GUS* reporter strain reveal auxin activity in buds and the stems of leafy gametophores that have developed in the light (Bierfreund et al., 2003).

The roles of auxin in *Physcomitrella* photomorphogenesis are mirrored in the development of more complex land plants including the well-studied model plant, *Arabidopsis thaliana*. For example, auxin is involved in leaf initiation, development and vascularization (Aloni et al., 2003; Lincoln et al., 1990; Mattsson et al., 2003; Cheng et al., 2007) and hypocotyl and shoot elongation (Hayashi et al., 2008; Lincoln et al., 1990; Nishimura et al., 2014; Tao et al., 2008) during growth in the light.
The results reported here confirm a role for auxin in the photomorphogenesis of *Physcomitrella* gametophores. Auxin was implicated in photomorphogenesis via the two auxin sensitive GUS reporter strains. Previously reported auxin distribution data acquired with the *Physcomitrella* auxin-responsive *GH3::GUS* transgenic strain (Bierfreund *et al*., 2003) were replicated. Data obtained using another GUS reporter strain, LTII-2, containing an alternative auxin-sensitive promoter (*mas*) fused to the *uidA* gene were qualitatively and quantitatively different from those acquired with *GH3::GUS* and implied auxin has a different and more restricted distribution, occurring in apical and subapical regions of gametophore stems as well as in young, developing leaves with this last location being one not observed using *GH3::GUS*. During development of a young leaf, a region of auxin activity appeared initially at the distal end (tip) of the leaf and migrated basipetally to its base as the leaf aged. The regions displaying auxin activity corresponded quite closely with regions in which the cells had or were in the process of expanding. This spatio-temporal pattern of leaf cell expansion and its correspondence to basipetal migration of a wave of auxin activity had been proposed previously (Barker, 2013; Akister, 2002) and was supported here. Treatment with PEO, an inhibitor of auxin signaling, in the light produced shorter leaves containing an increased number of cells, as well as shorter gametophore stems due to reduced internode elongation. These results constitute additional evidence that auxin is involved in the elongation of both gametophore stems and leaves during photomorphogenesis.
The finding that inhibitors of auxin signaling increase the number of cells in gametophore leaves implies that auxin’s role in leaf photomorphogenesis in Physcomitrella may not be limited to stimulating cell expansion. Previous work indicates that cytokinins may be cell division regulators in *Physcomitrella* leaves (Barker and Ashton, 2013). Therefore, a possible explanation of the effects of auxin signaling inhibitors is that auxin down-regulates cytokinin activity in developing leaves. Discoveries that auxin regulates cytokinin biosynthesis in *Arabidopsis* (Nordstrom *et al*., 2004; Tanaka *et al*., 2006) and that auxin can induce cytokinin degradation in leaf primordia during canopy shading (Carabelli *et al*., 2007) provide traction for this hypothesis. In *Physcomitrella*, auxin activity and cytokinin activity are known to overlap spatio-temporally including during protonemal differentiation and gametophore bud initiation (Ashton and Cove 1990; Ashton *et al*., 1990). The hypothesis described above provides a starting point for the investigation of auxin-cytokinin cross-talk during *Physcomitrella* leaf morphogenesis. That said, the alternative possibility that auxin controls cell division independently i.e. via a mechanism that does not involve cross-talk with cytokinin, cannot yet be excluded.

### 4.1.2 Polar auxin transport has a role in gametophore shoot and leaf photomorphogenesis

Application of NPA resulted in longer, narrower gametophore leaves and gametophore shoots with longer internodes. There was no discernible effect on the number of cells in L6 leaves. Thus, the areas affected by inhibition of auxin transport matched the regions affected by auxin signaling inhibitors as well as
the regions of auxin activity identified using LTII-2. Furthermore, the results obtained with NPA treatment mimic those seen after application of exogenous NAA (Barker and Ashton, 2013; Hayashi et al., 2008). Collectively these data imply NPA is acting through modulation of auxin and not via some other mechanism.

The literature on the role of polar auxin transport in photomorphogenesis of Physcomitrella gametophores provides contradictory evidence and views. For example, Fujita and co-workers (Fujita et al., 2008) contended polar auxin transport is absent in moss gametophore shoots, while providing evidence that it is essential for the normal ontogeny of sporophytes. They also claimed that NPA has no discernible effect on gametophore morphogenesis. However, more recent data strongly supports the opposite view that polar auxin transport is involved in moss gametophytic growth and development. Physcomitrella PINA and PINB knockout and overexpression studies demonstrate that polar auxin transport is required for the differentiation of chloronemal apical cells into caulonemal apical cells and for normal leaf morphogenesis, with the absence of PIN activity resulting in longer leaves. It was also shown that this latter characteristic of the PIN knockout lines could be phenocopied in wild-type Physcomitrella by the application of NPA (Bennett et al., 2014b; Viaene et al., 2014), matching the findings presented here.

PINA and PINB promoter activity typically begins at the tips of Physcomitrella P3 leaves and localized expression moves basipetally as a wave in successively older leaves (Viaene et al., 2014). These data match perfectly
the basipetal wave of auxin activity observed with LTII-2 and reported here, suggesting that auxin transport and activity during leaf morphogenesis are closely allied. Surprisingly in this study, the basipetal wave of auxin activity in developing leaves observed with LTII-2 was unchanged by NPA treatment, a result that will require confirmation by further experimentation in the future.

The role of the ER-localized PIND has not been investigated in *Physcomitrella*. It is possible that auxin homeostasis as governed by PIND is required for leaf morphogenesis, in much the same as ER-localized PINs regulate auxin cellular homeostasis in higher plants. This is an attractive possibility given the morphological similarities between gametophores treated with NPA and those treated with exogenous auxin. While NPA had been shown to affect PM-localized PINs and not ER-localized *Arabidopsis* PINs in a tobacco BY-2 cell system (Barbez et al., 2013), the same is not necessarily true for *Physcomitrella*. Similarly, it is possible that the PM localized PINs are functioning to regulate auxin homeostasis or short range cellular exclusion of auxin, rather than the long range polar auxin transport observed in *Arabidopsis*.

4.2 Gametophore Skotomorphogenesis

4.2.1 Auxin involvement in gametophore stem and leaf skotomorphogenesis

Auxin activity as reported by the LTII-2 and *GH3::GUS* strains indicates auxin may be involved in leaf morphogenesis and stem internode elongation in the dark. The *GH3::GUS* reporter strain revealed putative auxin activity throughout gametophores including their leaves. GUS staining was not altered
by NPA, but PEO-IAA appeared to cause a moderate reduction in GUS expression, implicating auxin activity in these organs. The LTII-2 strain displayed auxin activity in one to two subapical leaves per gametophore. This activity ceased around day 21 in the dark, which is also when gametophores stopped elongating and producing new leaves. PEO-IAA did not alter GUS activity in 21-day-old leafy gametophores, a result that was expected since no auxin activity has been discerned in LTII-2 gametophores cultured for that long in the dark in the absence of exogenous auxin. Only a single leafy gametophore developed from dark-grown LTII-2 colonies exposed to NPA. This gametophore did have weak GUS activity throughout, suggesting that a low level of auxin is present during gametophore skotomorphogenesis. However, as only a single LTII-2 gametophore was produced under these conditions, replication of the experiment will be required to confirm these results.

Experiments in which NAA was supplied in the culture medium to moss colonies growing in the dark confirmed that gametophores remain sensitive to auxin, should it be present, during skotomorphogenesis. Thus, the altered appearance of dark-grown gametophores is not caused by a change to the auxin reception/response mechanisms. The LTII-2 strain displayed GUS activity in NAA-affected leaves that had developed in the dark, confirming that the mas promoter’s sensitivity to auxin is not altered by the absence of light.

The elongation response of leaves to exogenous NAA was similar in the light and in the dark showing that auxin probably acts in this organ in the same way under both circumstances. Gametophore stem internodes, however,
showed no increase in length in the dark when exposed to NAA. Correspondingly, dark-grown LTII-2 gametophore stems did not express the GUS reporter gene. These observations were in marked contrast to the NAA-induced increases in internode length induced by exogenous auxin seen during photomorphogenesis (Hayashi et al., 2008).

Inhibition of auxin signaling with PEO-IAA or auxin transport with NPA helped elucidate the involvement of endogenous auxin during skotomorphogenesis. PEO-IAA affected leaf development in a qualitatively similar manner to that which it did during photomorphogenesis: leaves were shorter and each was comprised of a larger number of cells. However, the effect in the dark was not as pronounced as that seen in the light. The effects of NPA were also less pronounced on leaves cultured in the dark. Thus, while active in the dark and the light, the involvement of auxin in (or its contribution to) skotomorphogenesis may be less crucial under normal circumstances than its involvement in (or contribution to) photomorphogenesis. How auxin is being differentially regulated or used in the dark as opposed to in the light remains unclear. It is possible that auxin is more readily conjugated in the dark than in the light, that auxin synthesis is turned off or reduced in the dark, or that auxin acts secondarily during leaf development and that an earlier step needed for full response to auxin does not occur in the dark. The discovery that fewer cells are present in dark-grown leaves and exogenous cytokinin stimulates cell division in these organs confirms that changes in the availability of or response to other developmental regulators besides auxin are influential during
skotomorphogenesis. Since PEO-I AA caused an increase in the number of cells comprising dark-grown and WL-grown leaves, it appears that whatever mechanism auxin uses to stimulate cell division is active in the dark and the light.

PEO-I AA, auxinole, and NPA had no effect on internode lengths of dark-grown gametophores. Taken together with the finding that NAA also had no effect on internode length in the dark, it becomes clear that auxin is not responsible for stem elongation, i.e. etiolation, during skotomorphogenesis. In this regard, auxin regulation of shoot elongation in *Physcomitrella* is very similar to that of hypocotyl elongation in *Arabidopsis*. Overexpression of auxin increases hypocotyl elongation only in the light. However, auxin-resistant mutants do display shorter hypocotyls during skotomorphogenesis, indicating auxin may still be important for generalized cell expansion (Lincoln *et al.*, 1990; Romano *et al.*, 1995). NPA inhibits hypocotyl elongation in the light but has no effect in the dark (Jensen *et al.*, 1998). This latter observation indicates auxin transport is not needed for hypocotyl elongation in the dark and strengthens the contention that auxin is not involved in this aspect of skotomorphogenesis.

**4.2.2 Auxin involvement in the gametophore bud to shoot transition in the dark**

This study demonstrated that auxin signaling is essential for the gametophore bud to shoot transition during skotomorphogenesis. PEO-I AA or auxinole reduced the percentage of buds that transitioned into leafy shoots in the dark. Inhibition was partially repairable with competing NAA. NPA mimicked the
auxin signaling inhibitors. None of the inhibitors affected this developmental step in colonies grown in light. Prior to this, it had been established with auxin-resistant and auxin-repairable mutants that auxin is needed for the formation of the buds that subsequently develop into shoots in the light (Ashton et al., 1979; Ashton et al., 1990; Ashton and Cove, 1990). Also, excessive auxin was known to inhibit this transition (Ashton et al., 1979). However, nothing was known about the roles of auxin signaling or redistribution during this important developmental step in the light or in the dark.

As buds developing in the dark are more sensitive to auxin perturbations than those in the light, it appears that auxin is differentially regulated or involved in the dark and light at this stage of development. A possibility is that endogenous auxin is continuously produced by or available to buds developing in light but buds transferred into the dark must rely on auxin accumulated while they were in the light, at least for this stage of development. If endogenous auxin is more readily available to buds in the light than in the dark, the observation that buds in the light are refractory to PEO-IAA, auxinole and NPA is easier to understand.

4.2.3 Auxin involvement in caulonemal runner development in the dark

Auxin had been shown previously to induce caulonemata formation in *Physcomitrella* in the light (Ashton 1998; Ashton et al., 1990) and auxin signaling inhibitors to reduce it (Hayashi et al., 2008). Auxin response mutants were utilized to demonstrate slower rates of runner development in the dark, implying a need for auxin in this process (Ashton and Cove, 1990). Recently auxin
transport has also been shown to be important in regulating caulonemal
development. PIN proteins are concentrated in the apical cells of developing
protonemata and a pinApinB double knockout develops at an increased rate
caulonemata in the light and caulonemal runners in the dark. However, colony
expansion was decreased (Viaene et al., 2014). The results presented here
confirm roles for auxin and auxin transport in the development of caulonemata
and colony expansion in both the light and the dark. PEO-IAA and NPA inhibited
the formation and elongation of caulonemal runners in the dark and altered
protonemal growth and development in the light.

4.2.4 Auxin signaling and transport inhibitors did not affect the gravitropic
responses of Physcomitrella gametophytes in the dark

While PEO-IAA and NPA inhibited the transition of buds to shoots and the
formation and growth of caulonemal runners in the dark, both shoots and
runners, once formed, became negatively gravitropic and their gravitropism was
refractory to the inhibitors. These latter findings contrast markedly with the
situation in Arabidopsis, in which auxin signaling and transport are required for
gravitropic responses in the hypotcotyl and root in both the dark and the light
(Jensen et al., 1998). Moreover, it has been shown very recently that
Physcomitrella pinApinB double knock out lines possess completely agravitropic
gametophores in the dark (Bennett et al., 2014b). No alteration of caulonemal
gravitropism was reported. The discrepancy between the data presented here
and those of Bennett and co-workers may be explained by a complete absence
of polar auxin transport in the double knock out strain and incomplete inhibition
of auxin transport by NPA at the concentration used. Conversely, as the application of the inhibitors did not occur until transfer to darkness, it is a possibility that the gravitational vector was established prior to the onset of any inhibitory effect. Further experimentation in the future is required to resolve this issue.

4.3 Auxin involvement in the transition from skotomorphogenesis to photomorphogenesis

Plants grown in the dark for a period of 21 days were returned to one of three light conditions – white light (WL), red light (RL) or blue light (BL). In all three cases, recovery of the photomorphogenetic phenotype occurred. These observations mirror what happens in Arabidopsis in which phytochrome and cryptochrome signaling are involved in the induction of photomorphogenesis during de-etiolation of plants transferred from the dark to red or blue light (Mazzella et al., 2001).

In all three light conditions, auxin activity reappeared in LTII-2 gametophores not until between 48 and 72 hours after cultures were reintroduced into the light. The reappearance of auxin activity was subsequent to the restoration of hallmark characteristics of photomorphogenesis, namely renewed growth of the gametophore apex accompanied by thickening of the shoot stem, leaf expansion, loss of gravitropism and recovery of phototropism. These observations suggest that auxin is not involved in earliest stages of induction of photomorphogenesis, but is required a little later in developmental programming to modulate growth. This model is supported by observations of
auxin activity in developing leaves of gametophores grown solely in the light. In LTII-2, auxin activity appears in P4 leaves, while P1, P2, and P3 leaves have no discernible auxin activity, implying the very earliest steps of leaf development are auxin-independent.

RL was noticeably more efficacious than BL at similar photon fluxes in inducing photomorphogenesis, suggesting a greater role for phytochromes than for cryptochromes. WL was more substantially more effective than RL and BL but the photon flux used for WL was much higher than for the other conditions. Therefore, the response of dark-grown *Physcomitrella* to WL cannot be attributed solely to the combined stimulation of multiple kinds of light receptors. It is particularly interesting that BL was able to induce auxin activity during a return to photomorphogenesis since it had been reported previously that signaling from cryptochromes attenuates auxin signaling (Imaizumi *et al.*, 2002). The indication that blue-light signaling can both induce and attenuate auxin activity suggests that the effects of light signaling on hormone regulation, in particular regulation of auxin, are varied and form a complex regulatory network.

4.4 Gametophores remain responsive to cytokinins in the dark

Fewer genetic tools are available at present for studying cytokinin in *Physcomitrella*. However, a simple experiment indicated that *Physcomitrella* gametophores developing in the dark remain responsive to cytokinin in a manner similar to that seen in the light, with cytokinin stimulating leaf cell division leading in turn to the production of larger leaves. Since gametophores clearly remain responsive to cytokinin in the dark, differential cytokinin synthesis, perhaps
coupled to differential auxin synthesis, during skotomorphogenesis and photomorphogenesis may be at least partially responsible for the different leaf morphologies associated with these developmental pathways.

4.5 Confidence in GUS reporting strains

Specificity of the responses of LTII-2 was initially a cause for concern since it had been reported that, although the activity of the mas promoter is primarily responsive to auxin, it is additionally enhanced by cytokinin (Langridge et al., 1989). Consequently, it was feasible that cytokinin alone might induce this promoter and generate GUS staining that could be mistakenly attributed to auxin activity. However, application of BAP to WL- and dark-grown LTII-2 failed to induce GUS activity in cytokinin-affected tissues, thereby allowing the attribution of GUS activity to auxin with increased confidence.

It is noteworthy that the GH3::GUS reporter strain does not express its GUS (uidA) gene in the leaves of untreated gametophores despite compelling evidence that auxin is active in these organs and, conversely, that GUS staining is produced in the shoot stems of dark-grown gametophores despite the results presented here that show auxin is not active in these organs. However, data supporting the notion that the GH3 promoter is auxin-sensitive have also been reported. The GH3 promoter contains three auxin responsive elements (Liu et al., 1994). Exogenous application of NAA markedly increases the measurable levels of GUS activity in the GH3::GUS strain, and the entire gametophore, including the leaves, becomes GUS stained in response to the application of NAA (Imaizumi et al., 2002; Bierfreund et al., 2003). Contradictory evidence has
also been presented questioning a direct link between auxin and GH3 expression in Physcomitrella. Neither exogenous auxin treatment nor mutations negatively affecting auxin response regulatory proteins altered GH3 expression (Prigge et al., 2010), suggesting that the GH3 promoter may not behave in Physcomitrella as it does in Arabidopsis, at least in terms of auxin responsiveness. It remains a possibility that sensitivity of the GH3 promoter is inadequate for detection of endogenous auxin levels since overexpression of the auxin biosynthesis gene, SHI1, in the GH3::GUS line results in weak GUS activity in some leaf cells (Eklund et al., 2010). Consequently, data obtained with GH3::GUS need to be interpreted cautiously. The results presented here strongly suggest that the LTII-2 strain containing the mas promoter is superior to the GH3::GUS strain in terms of detecting auxin distribution.

4.6 A model for the roles of auxin in Physcomitrella development

A model has previously been proposed, which suggests that auxin is required to attenuate the action of light during photomorphogenesis in suppressing the development of caulonemata and the growth of gametophores. In the absence of light stimulation, as in skotomorphogenesis, auxin is no longer required for the growth and development of these tissues. Therefore, skotomorphogenesis represents the default developmental pathway in Physcomitrella. It transpires that the data reported here are largely but not completely consistent with this model, which consequently may need to be modified. For example, auxin is active and required to at least some degree in the dark since auxin signaling and polar auxin transport inhibitors substantially
decrease the proliferation of caulonemal runners and affect, to a lesser extent than in the light, leaf morphogenesis. Auxin is also required in the dark to promote the initial transition of gametophore buds into shoots. Other aspects of development fit the original model more closely. Auxin seemingly has no role in the elongation of internodes in the dark, but is required in the light to counteract light-dependent inhibition of internode growth. Also, increased activity of auxin in gametophore leaves developing in the light can be discerned with auxin signaling and transport inhibitors as explained above. In summary, the results reported here support a model in which a low basal level of auxin is required for *Physcomitrella* skotomorphogenesis with a higher level of auxin involved and required during photomorphogenesis, possibly to counteract the effects of light signaling.

**4.7 Conclusions**

Auxin and polar auxin transport are required for normal gametophore morphogenesis in light. They are involved in leaf patterning, via effects on cell elongation and cell division, and in shoot elongation. Inhibitors of auxin transport increase leaf length and decrease width in a manner resembling that affected by the application of exogenous auxin. Whether this is due to altering the movement of auxin through the leaf or regulation of auxin homeostasis within individual cells or both has still to be determined. Results from gametophores grown continuously in the light or from those returned into the light from the dark indicate that auxin acts secondarily to other regulators of photomorphogenesis.
Auxin plays a lesser, though still active, role in development of *Physcomitrella* in the dark. Leaves responded to inhibitors of auxin signaling in a similar, though less intense, way to that seen in the light. It appears that modulation of auxin activity may play a part in establishing or maintaining the different morphologies of light and dark-grown leaves. It is uncertain whether auxin acts for a shorter period of time in the dark, if less auxin is synthesized, or if auxin acts secondarily through other regulators of leaf morphogenesis. Auxin doesn’t appear to be involved in elongation of the shoot stem in darkness and in inducing the etiolation response. Since neither exogenous auxin nor inhibitors of auxin signaling or transport altered the average internode length, the factors responsible for regulating gametophore stem elongation during skotomorphogenesis have still to be identified.

Auxin is involved in the gametophore bud to shoot transition. Inhibitors of auxin signaling or transport hinder this transition in a dark-dependent manner. This indicates that auxin is differentially regulated in the dark and in the light at this stage of development. The enhanced sensitivity of auxin activity to perturbations in the dark may be due to reduced biosynthesis of auxin in darkness.

Auxin and auxin transport are required for caulonemal development in both the light and the dark.

The roles of auxin in development of the gametophore in *Physcomitrella* and the hypocotyl in *Arabidopsis* are very similar. In both cases, both auxin signaling and transport appear to play a greater role during development in the
light than in the dark. This suggests that the roles and action mechanisms of auxin in development are evolutionarily ancient and highly conserved or have been co-opted in a similar way on several occasions during the course of land plant evolution.

4.8 Future directions

While a model of colony and gametophore development in *Physcomitrella* during photomorphogenesis and skotomorphogenesis as it relates to auxin has been modified and expanded based on the results of this research, much more remains to be discovered about reversible switching between these markedly different developmental programs. While phytochromes and cryptochromes have been implicated in regulating this switch in *Physcomitrella* and *Arabidopsis*, many details about the operation of the switch at the molecular level in *Physcomitrella* are still to be discovered. Investigations using known *Physcomitrella* orthologs of some elements, e.g. COP1, of the better understood *Arabidopsis* switch may be a good place to start.

How precisely auxin is being differentially regulated in the dark and in the light remains unknown. An investigation into the activity of auxin biosynthesis genes or auxin conjugation and degradation may help to elucidate this problem.

Since auxin is not responsible for *Physcomitrella* shoot elongation in the dark, other candidates for this role should be studied. Gibberellins and brassinosteroids are absent in *Physcomitrella*. However, *ent*-kaurene and *ent*-kaurenoic acid, two metabolites in the gibberrellin biosynthetic pathway in *Arabidopsis*, are involved in the chloronema to caulonema transition, which is a
point of overlap with auxin activity (Hayashi et al., 2010). Mutant strains of *Physcomitrella* defective in for the production of these metabolites have already been made (Hayashi et al., 2010), opening the door for exploration of the roles these gibberellin precursors during photomorphogenesis and skotomorphogenesis.

The role of auxin transport in *Physcomitrella* is an area of research that has only begun to be studied. How precisely PIN proteins regulate auxin distribution in *Physcomitrella* and whether this activity is based upon long range directional auxin transport or more localized movements and changes to auxin homesostasis needs to be determined.
5. LITERATURE CITED


