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Auxin transport in algae
Transport auxinu v řasách

Master thesis

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I hereby declare I carried out this study by myself under the supervision of RNDr. Jan Petrášek, Ph.D. and professional guidance of the same, Ing. Petr Skůpa, Ph.D. and Ing. Karel Müller, Ph.D and Mgr. Stanislav Vosolsobě. It documents my own work on the subject unless explicitly mentioned otherwise. I have acknowledged and cited all used sources. This work is not a part of any other defending procedure.

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Abstract

Phytohormone auxin plays an important role in regulating plant development. Directional (polar) cell-to-cell auxin transport creates auxin gradients within plant tissues, which trigger a specific developmental response. The vast majority of available data concerns angiosperms. Lower land plants have been much less explored in this regard, but the important auxin-related mechanisms (including polar auxin transport) are already present in mosses. To uncover the origins of auxin action, one must focus on green algae, especially of clade *Streptophyta*, which are the direct ancestors of all land plants.

In this study, the possible effects of auxins, both native and synthetic, were investigated on two algae: basal, unicellular *Chlorella lobophora* and advanced, filamentous *Spirogyra sp*. The latter received comparably more attention, since it belongs to a clade now acknowledged as a sister group to land plants. *Chlorella lobophora* culture growth was irresponsive to synthetic auxin NAA. The average *Spirogyra sp.* cell length was, however, changed by auxins at high concentrations. By conducting accumulation assays of radioactively labelled auxins and HPLC analysis, auxin metabolism and transport was investigated in *Spirogyra sp*. This alga was able to metabolize the plant-native IAA, but not synthetic auxins NAA and 2,4-D. Auxin efflux, if present, was unaffected by land-plant auxin efflux inhibitors. By contrast, there was inhibition of auxin influx, which is presumably driven by ABC carriers. ABC family are evolutionary ancient and diverse transporters. Using available transcriptomic data for *Spirogyra* genus, an incomplete gene sequence orthologous to *Arabidopsis thaliana* ABCB19 auxin efflux carrier was identified. A partial sequence ortologous to *At*ABCB19 was isolated from *Spirogyra sp.*

While no effect of auxin on *Chlorella lobophora* was observed, *Spirogyra sp.* is able to metabolize IAA, but not NAA and 2,4D. Furthermore, it contains an auxin influx mechanism and its cells responded to high exogenous auxin concentrations by changes in cell elongation. These results suggest that some of the mechanisms of auxin action are already present in the streptophyte *Spirogyra sp.*

Keywords: auxin, auxin transport, green algae, *Spirogyra*, *Chlorella*, cell length, metabolism, ABC, IAA, NAA, 2,4-D

Abstrakt

Fytohormon auxin hraje důležitou roli v regulaci rostlinného vývoje. Směrovaný (polární) transport auxinu mezi buňkami vytváří jeho gradienty v rostlinných pletivech, které spouští specifickou vývojovou odpověď. Valná většina dostupných dat se týká krytosemenných rostlin. Nižší rostliny jsou v tomto ohledu mnohem méně prozkoumány, ale důležité auxinové mechanismy (včetně polárního transportu) jsou přítomny již v meších. Abychom odhalili počátky role auxinu v rostlinách, musíme se zaměřit na zelené řasy – obzvláště řasy ze skupiny *Streptophyta*, které jsou přímými předchůdci všech rostlin.

V této studii byl zkoumán možný vliv auxinů, nativních i syntetických, na dvě řasy: původní, jednobuněčnou *Chlorella lobophora* a pokročilou, vláknitou *Spirogyra sp.* (šroubatka). Šroubatka dostála více pozornosti, neboť se řadí do skupiny dnes uznávané jako sesterská k rostlinám. Růst kultur *Chlorella lobophora* nebyl ovlivněn syntetickým auxinem NAA. Naproti tomu, průměrná délka buněk šroubatky byla ovlivněna auxiny ve vysokých koncentracích. Prostřednictvím akumulačních esejí radioaktivně značených auxinů a HPLC analýzy byl pozorován metabolismus a transport auxinů ve šroubatce. Šroubatka byla schopna metabolizovat nativní auxin IAA, nikoli však syntetické NAA a 2,4-D. Výdej auxinu buňkami, pokud je přítomen, nebyl ovlivněn jeho inhibitory používanými pro rostlinný materiál. Byla ovšem zaznamenána inhibice příjmu auxinu, jenž je možná zprostředkováván prostřednictvím ABC proteinů. ABC je prastará rodina různorodých proteinových transportérů. S užitím dostupných transkriptomických dat pro rod *Spirogyra* byl identifikována neúplná sekvence genu ortologní proteinu ABCB19 z *Arabidopsis thaliana*, který je znám jako transportér auxinu ven z buňky. Částečná sekvence genu ortologního AtABCB19 byla izolována ze *Spirogyra sp.*

Zatímco vliv auxinu na *Chlorella lobophora* nebyl pozorován, šroubatka *(Spirogyra sp.)* je schopna metabolizovat IAA, nikoli však NAA a 2,4-D. Dále obsahuje mechanismus aktivního příjmu auxinu a její buňky odpovídají na vysoké hladiny exogenních auxinů změnami elongace. Tyto výsledky naznačují, že některé mechanismy auxinového působení jsou již přítomny ve šroubatce.

Klíčová slova: auxin, transport auxinu, zelené řasy, *Spirogyra,* šroubatka, *Chlorella*, délka buněk, metabolismus, ABC, IAA, NAA, 2,4-D

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Abbreviations

ABCB/PGP – ATP-binding cassette subfamily B/Poly-glycoproteins, sometimes also called MDR (Multi-drug resistance)

ABP1 – Auxin binding protein 1

ARF – Auxin Response Factor

Aux/IAA – Auxin/Indole-3-acetic acid (Aux/IAA)

AUX1/LAX – Auxin 1 / Like Auxin 1

CHPAA – 3-chloro-4-hydroxyphenylacetic acid (CHPAA)

ER – endoplasmic reticulum

IAA – indole-3-acetic acid

KDEL – Lys-Asp-Glu-Leu, an ER-retention amino-acid sequence

Mya – million years ago

NAA – 1-napthaleneacetic acid

NPA – 1-naphthylphthalamic acid

PAT – polar auxin transport

PIN – PIN-FORMED

PILS – PIN-LIKES

PM – plasma membrane

TIR1/AFB - Transport Inhibitor Response 1/Auxin Signaling F-box

2,4-D – 2,4-dichlorophenoxyacetic acid

1 Introduction

1.1 Green algae

The clade *Viridiplantae* (green plants) comprises all land plants and green algae. There are two groups of green algae*: Chlorophyta* and *Streptophyta;* colloquially chlorophytes and streptophytes (reviewed in Lewis & McCourt, 2004). Aside from green algal members, clade *Streptophyta* also includes all land plants (their evolutionary descendants). Streptophyte green algae are sometimes referred to as charophytes (*Charophyta*; not in this study). Chlorophytes are a basal, species-rich, primarily marine clade. Their unicellular representatives comprise marine phytoplankton, but various complex morphologies are also present. Streptophytes originated in freshwater and have been restricted to it since. Today they are found in basic, non-eutrophic still waters, though a few can tolerate various water conditions and successfully dominate their habitat, like pond scum from the *Zygnematophyceae* clade. Though not as rich in species, streptophytes possess extraordinary diversity in life forms, ranging from unicellular representatives to ones with true, complex multicellularity and even plant-like morphology. The relative low species richness of streptophytes, compared to chlorophytes or land plants, may be the result of getting out-competed in their natural freshwater habitat by various groups of organisms which came to dominate it later (Figure 1; Becker & Marin, 2009). While showing some distinctive differences (i.e. gametophytic algal vs. sporophytic plant bodies), streptophyte algae share some apomorphies with land plants (e.g. sporopollenin Delwiche *et al.,* 1989, phragmoplast Graham *et al.,* 2000 or glycolate pathway Stabenau & Winkler, 2005). Some of them may have played a crucial role in decisive evolutionary divergences.

Most research on green algae has been conducted on unicellular chlorophytes, likely because of their comparably easier cultivation and widespread distribution and importance in freshwater habitats. Several chlorophyte genomes have been sequenced. By contrast, streptophytes are not widely studied and not a single genome has been sequenced. Their genomic evidence is restricted to fragmented transcriptomic analyses.

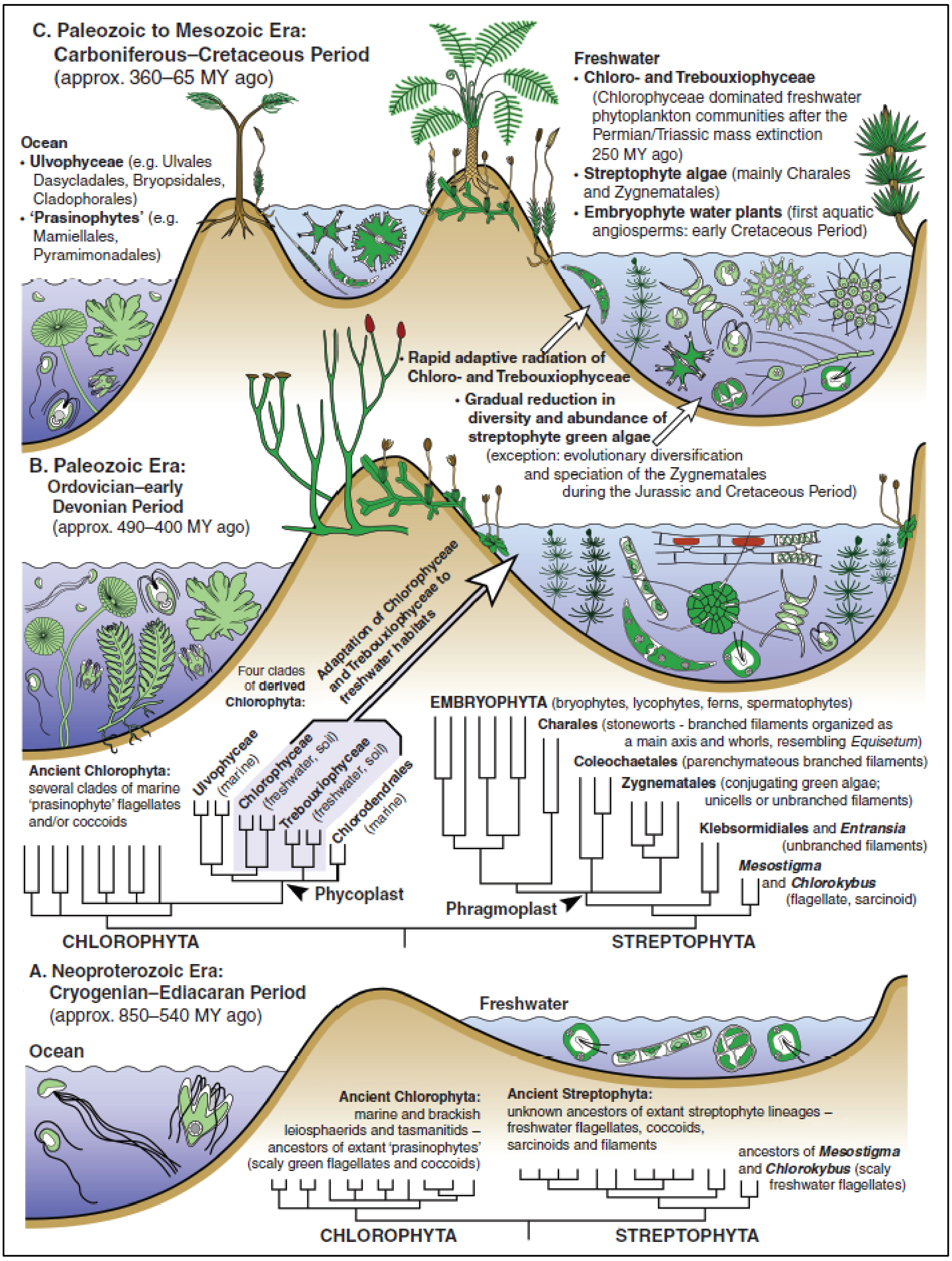


Figure 1. Diversification of green plants *(Viridiplantae)* and colonization of terrestrial habitats by streptophyte algae. Three stages are shown (from bottom): (**A**) early evolution of green algae during the Neoproterozoic era; (**B**) colonization of terrestrial habitats by streptophyte algae during the Palaeozoic era; and (**C**) origin of current freshwater ecosystems during the Palaeozoic to Mesozoic era. The smaller freshwater body illustrates an acidic bog-pool with conjugating green algae. Green algae and embryophytes (land plants) are illustrated in different tones of green: dark *(Streptophyta)* vs. bright green *(Chlorophyta)*. Note that placing *Charales* as sister group to embryophytes is now outdated. Figure reproduced from Becker & Marin (2009).

1.2 Early plant evolution

The estimates on divergence times of the early green & land plant groups, based on both fossil evidence and molecular clock, vary by different sources (Sanderson *et al.,* 2004; Becker & Marin, 2009; Rubinstein *et al.,* 2010; Becker, 2013; Ruhfel *et al.,* 2014). One of the groups that emerged after primary endosymbiosis some 1.5 billion years ago were the green plants *(Viridiplantae)*, by then green algae. It was around or before 700 million years ago (Mya) that these split into two groups: *Chlorophyta* and *Streptophyta*. The latter have lived in freshwater, which was likely crucial for them to colonize land some 450 Mya (Ordovican). All land plants are descended solely from streptophyte algae. The common ancestor of land plants was likely similar to extant conjugating green algae *(Zygnematophyceae)*, which are now recognized as a sister group to land plants (Turmel *et al.,* 2006; Wodniok *et al.,* 2011; Ruhfel *et al.,* 2014).

Transition to land was followed by radiation of moss-like organisms (Graham *et al.,* 2000): liverworts *(Marchantiophyta)*, then mosses *(Bryophyta)* and hornworts *(Anthocerotophyta)* (reviewed in Qiu, 2008). Vascular plants are traced to the border of Silurian and Devonian (420 Mya; Edwards & Feehan, 1980). The Devonian period marked an incredible rise in vascular plant diversity (Kenrick & Crane, 1997; Niklas & Kutschera, 2009).

Recent years allowed us to complement fossil evidence with genetic in search for the origins and evolution of plant body plants and developmental processes (Dolan, 2009; Niklas & Kutschera, 2009; Pires & Dolan, 2012). Many morphological, developmental and cellular features evolved independently multiple times in plants, but the genetic machinery behind their regulation is generally shared. Phytohormone auxin has a crucial role in regulation of essential developmental processes in plants (see chapter 1.4), including the regulation of plant morphogenesis. The evolutional history of plant development therefore can be tracked by following the evolution of gene families for proteins that mediate auxin action (Finet & Jaillais, 2012).

Physcomitrella patens is a moss model organism, which is established in culture and its genome was fully sequenced (Rensing *et al.,* 2008). Mosses are the second earliest land plant organisms to have diverged, therefore the data from *Physcomitrella patens* regularly represent the basal-most evolutionary evidence in land plant research. This includes some mechanisms of auxin action (see chapter 1.5).

1.3 What is auxin?

There is a plethora of compounds recognized as "plant hormones", i.e. substances (signaling molecules), which can cause developmental changes or physiological reactions based on their local concentration. Unlike animal hormones, they can be synthesised by almost any plant cell rather than in special glands, induce pleiotropic effects and their effective concentration range is rather broad. Phytohormones have been detected in various groups of organisms including green algae (reviewed in Tarakhovskaya *et al.*, 2007), though the credibility of some older data has been disputed (Evans & Trewavas, 1991; Sztein *et al.*, 1999). One group of phytohormones is called auxins.

There are multiple molecules with auxin activity, both endogenous (native) compounds in plants and synthetic ones (Figure 2). Many developmental processes in plants are subject to auxin regulation, so the definition of “auxin activity” has been discussed through history (reviewed in Skůpa *et al.*, 2014).

The most widely studied native auxin in plants is indole-3-acetic acid (IAA), a tryptophan derivate. A few other native auxins are known, but much less explored: 4-Cl-IAA (Reinecke *et al.*, 1995; Reinecke 1999), phenylacetic acid (PAA) (for discussion see Simon & Petrášek, 2011) and arguably indole-3-butyric acid (IBA), which is now increasingly being recognized as a storage metabolite of IAA (Bartel *et al.*, 2001; Woodward & Bartel, 2005; Simon & Petrášek, 2011). Widely used synthetic auxins are 1-naphtaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). NAA is a commercial rooting agent. 2,4-D is industrially used as herbicide and was as a part of the infamous defoliant Agent Orange. Both NAA and 2,4-D are particularly useful in experiments on auxin transport, because their dynamics of auxin uptake and efflux by plant cells are different from the native IAA (Delbarre *et al.*, 1996). Known auxin molecules are discussed in Skůpa *et al.* (2014).

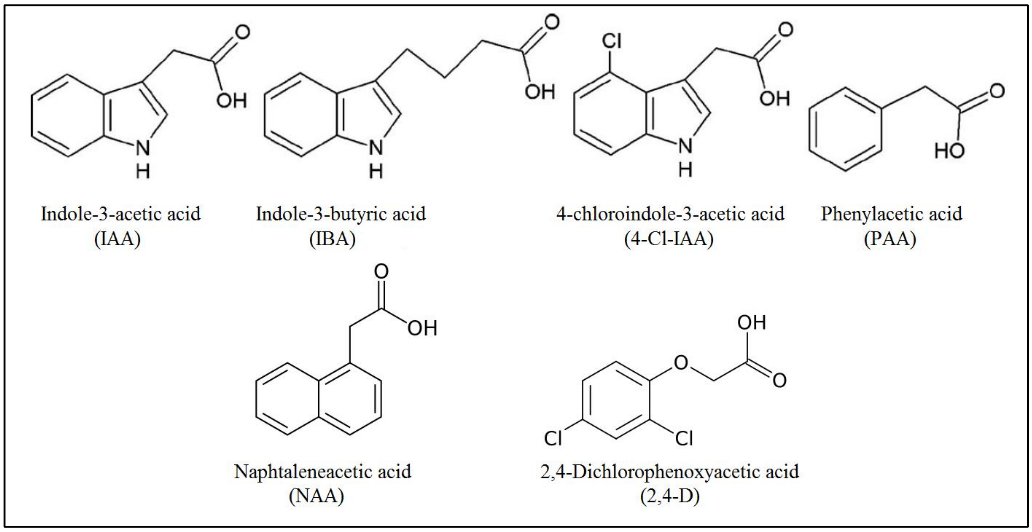


Figure 2. Native auxins (top) and two synthetic ones (bottom). Top four formulae are reproduced from Simon & Petrášek (2011), bottom two formulae were taken from Wikimedia Commons.

1.4 Auxin regulates land plant development

Auxin regulates land plant development at all its stages *via* differential spatial concentration distribution (gradients). It regulates many processes in land plants, but because land plants are not a subject of this study, only some of the most renowned examples will be mentioned.

Auxin gradients in embryonic development mark cells for specific fates (e.g. root, shoot, cotyledons) and regulate cell division polarity, defining shape of the growing embryo (Figure 4). Though all families of cellular auxin transporter proteins are active in this process, the plasma membrane localized PIN-FORMED (PIN) auxin efflux transporters are the most important (Benková *et al.,* 2003; Friml *et al.,* 2002b; Friml *et al.,* 2003).

Local auxin maxima mark the emergence of lateral organs – roots, leaves and flowers (Figure 3C, D; Benková *et al.,* 2003; Dubrovsky *et al.,* 2008; Reinhardt *et al.,* 2000). Auxin regulates the development of lateral roots both at their priming in root apical meristhem and their eventual emergence in the elongation zone. Lateral root emerges by shifts in cell division polarity in the main root pericycle and goes from being a local auxin sink to becoming a grown root with the specific fountain pattern of auxin fluxes (Dubrovsky *et al.,* 2001; De Smet *et al.,* 2007; Dubrovsky *et al.,* 2008). The emergence of leaves happens by a similar mechanism (reviewed in Reinhardt *et al.,* 2005). By being local auxin sinks, the existing leaf primordium prevents formation of other primordia, resulting in a phyllotactic pattern.

Leaf vein formation follows the “canalization hypothesis” (Sachs, 1981). Narrow auxin maxima, generated by long PIN1 in conjunction with three known short PINs, mark the emergence of future veins (Reinhardt *et al.,* 2003; Scarpella *et al.,* 2006; Sawchuk & Scarpella, 2013).

Gravitropic bending of shoots and roots is also mediated by auxin gradients mediated by the plasma membrane localized PIN3 auxin efflux transporter (Friml *et al.,* 2002a; Rakusová *et al.,* 2011). In both shoot and root auxin is predominantly fluxed to the lower side on the organ. Comparably higher auxin concentrations promote growth of the lower side in shoot, bending it upwards, but inhibit growth in root, resulting in downward bending. General “fountain” auxin fluxes (see chapter 1.5.2) are not changed, only concentrations are shifted.

A similar mechanism to auxin-mediated gravitropic response is expected for phototropic bending, which is not as well explored. Phototropin blue light receptors belong in the same protein family as PINOID kinase, which regulates polar localization of PIN auxin transporters, thereby regulating the direction of auxin flux. Connections between phototropins and PINOID have been both supported and disputed (Galvan-Ampudia & Offringa, 2007; Haga & Sakai, 2012). Rapid phototropic response was shown to be mediated by PIN3-dependent auxin gradient (Ding *et al.,* 2011), but not long term phototropic growth (Haga & Sakai, 2012).

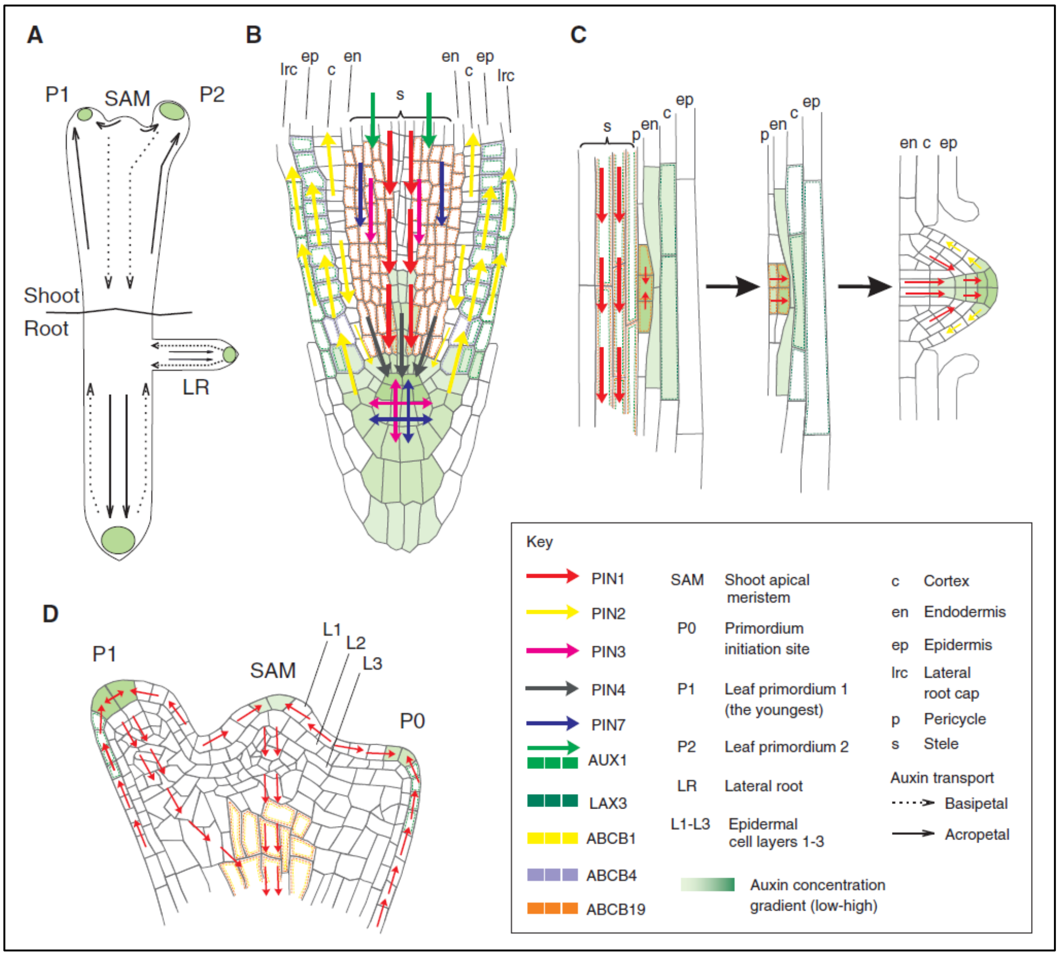


Figure 3. Auxin gradients and transporters in shoot and root morphogenesis. (**A**) The fountain model of predominant auxin fluxes within a plant body. (**B**) Auxin gradients and transporters in the root tip, (**C**) developing lateral roots and (**D**) shoot apical meristem during phyllotaxis. Coloured arrows: directional auxin transport by a particular transporter. Coloured dotted lines: apolar auxin transport by a particular transporter. Figure reproduced from Petrášek & Friml (2009).

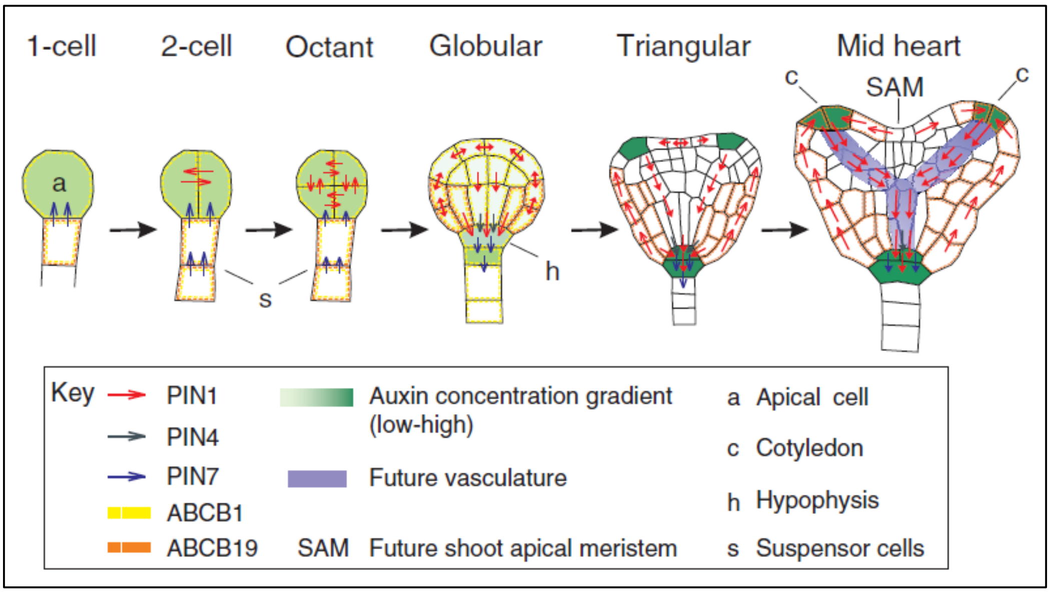


Figure 4. Auxin gradients and transporters during embryogenesis. Figure reproduced from Petrášek & Friml (2009).

1.5 Mechanisms of auxin action in angiosperms

Most of what we know about auxin and the processes it regulates in plants has been described in angiosperms. Auxin regulates plant development *via* its differential distribution within plant tissues (Benková *et al.,* 2003). These concentration gradients trigger specific developmental responses. There are both constitutive (e.g. root collumella) and transient (e.g. lateral root primordium) auxin gradients and they can be visualized *in vivo* (Benková *et al.,* 2003). In each sub-chapter below I provide a brief introduction into the mechanisms that generate, maintain or contribute to these gradients or act upon their stimulus. These are complemented by evidence of these mechanisms in lower land plants and green algae.

1.5.1 Auxin metabolism

Metabolism comprises synthesis, conjugation and de-conjugation and degradation. Auxin metabolism and transport generate, maintain and change auxin gradients within plant tissues (Normanly, 2010; Ludwig-Müller, 2011; Korasick *et al.,* 2013 and Ljung (2013).

IAA can be produced in nearly any living plant tissue, but young leaves show the highest biosynthetic capacity (Ljung *et al.,* 2001). IAA is a tryptophane (Trp) derivate. There are five biosynthetic pathways more or less described to date: four Trp-dependent and one Trp-independent (reviewed in Woodward & Bartel, 2005; Normanly, 2010; Ljung, 2013). Most of IAA is synthetized in a Trp-dependent manner (Ljung, 2013). YUCCA enzyme family of the tryptamine (TAM) Trp-dependent biosynthetic pathway is essential for creating local auxin maxima in various developmental processes (Cheng *et al.,* 2006; Cheng *et al.,* 2007). Trp-dependent auxin biosynthesis is also utilized to readily create high auxin levels for quick responses, e.g. after wounding (Sztein *et al.,* 2002).

Most of cellular IAA is conjugated to other molecules, thereby deactivated (Ludwig-Müller, 2011; Korasick *et al.,* 2013). This deactivation is either reversible (storage metabolites), or irreversible (first step of a degradation pathway). There are two major groups of IAA conjugates: amide-linked and ester-linked. Amide-linked conjugates comprise IAA bound to amino-acids, peptides and small proteins. Amide-binding inactivates IAA (Staswick *et al.,* 2005) except for IAA-Trp, which is an active IAA antagonist (Staswick, 2009). Ester-linked conjugates are bound to carbohydrates, like sugars (glucose) or sugary alcohols (*myo*-inositol) (Ludwig-Müller, 2011). In *Arabidopsis thaliana* some 1% of total IAA is present in its free state. The rest is bound in conjugates, of which around 10% are ester-linked and 90% amide-linked (Tam *et al.,* 2000). Monocotyledonous maize *(Zea mays)*, on the other hand, prefers ester-binding of IAA (Cohen & Bandurski, 1982). These ratios and amounts, however, vary by tissues, intrinsic conditions and species (Ludwig-Müller, 2011).

Some modifications are irreversible and mark IAA for degradation, like oxidation of both free and conjugated IAA (reviewed in Woodward & Bartel, 2005; Korasick *et al.,* 2013). Conjugates of IAA with Asp and Glu amino acids and with glucose have also been suggested as the first steps for degradation, but these are likely variable patterns and conjugates destined for hydrolysis and catabolism cannot be strictly separated (Ludwig-Müller , 2011).

IAA and its plant metabolite forms were found in multiple groups of organisms including lower land plants and green algae (reviewed in Cooke *et al.,* 2002; Tarakhovskaya *et al.,* 2007). Basal land plants (liverworts, mosses, spike mosses) synthetize IAA predominantly *via* Trp-independent pathways (Sztein *et al.,* 2000), in contrast to higher land plants. IAA is predominantly amide-conjugated in both higher and lower land plants except for liverworts, which seem to predominantly use biosynthesis-degradation strategy to regulate free IAA levels rather than conjugation (Sztein *et al.,* 1999).

There are reports of chlorophytes utilizing auxin produced by environmental bacteria (de-Bashan *et al.,* 2008), but also of the algae themselves producing auxin (Mazur *et al.,* 2001). Genomes of some, mostly unicellular chlorophytes possess orthologs to some land-plant genes for auxin metabolism, especially biosynthesis (reviewed in De Smet *et al.,* 2011). IAA in chlorophytes was also suggested by Lau *et al.,* 2009 to be a by-product of tryptophan metabolism. Growth of *Chlorella*, a unicellular chlorophyte, was shown to respond to exogenously applied auxin (Yin, 1937; Pratt, 1938; Saygideger & Okkay, 2008; Hunt *et al.,* 2011). Much less about auxin metabolism is known for streptophyte algae. An advanced streptophyte *Nitella sp. (Charales)* contains native IAA in its growing tips and controls its levels by degradation rather than conjugation (Sztein *et al.,* 2000), similarly to liverworts.

1.5.2 Auxin signaling

Auxin signaling pathways convey the information of auxin stimulus to response mechanisms. The best characterized auxin signaling pathway is the TIR1/AFB–Aux/IAA–ARF pathway, further just TIR1-pathway (Figure 5; reviewed in Guilfoyle & Hagen, 2007; Mockaitis & Estelle, 2008; Chapman & Estelle, 2009; Hayashi, 2012). It is named after three protein families which take part: Transport Inhibitor Response 1/Auxin Signaling F-box (TIR1/AFB), Auxin/Indole-3-acetic acid (Aux/IAA) and Auxin Response Factor (ARF). This pathway modulates the expression of auxin-responsive genes. ARFs are the transcriptional regulators of auxin-responsive genes. Their action is repressed by Aux/IAAs in the absence of auxin stimulus. After auxin enters the cell (see chapter 1.5.3), it is perceived by nuclear, soluble TIR1/AFB receptors. These auxin receptors can also bind to Aux/IAAs. As suggested by Tan *et al.* (2007), auxin would mediate the bond between TIR1/AFB and Aux/IAA, acting as a ‘molecular glue’. More recent research suggests that an already existing TIR1/AFB-Aux/IAA co-receptor complex actually allows for efficient auxin binding and various member combinations of both protein families constitute a regulatory mechanism of auxin-modulated gene expression (Villalobos *et al.,* 2012). Either way, besides being auxin receptors, TIR1/AFBs are also subunits (F-box containing proteins) of an E3 ubiquitin-ligase complex. Auxin binding to a TIR1/AFB-Aux/IAA complex promotes poly-ubiquitinilation of the Aux/IAA, marking it for degradation in a 26S proteasome. Free of its repressor, the respective ARF can regulate transcription of an auxin-responsive gene.

Auxin-responsive genes to TIR-pathway are defined by a characteristic TGTCTC sequence called auxin-responsive element (AuxRE), first identified in Gretchen-Hagen 3 (GH3) genes (Hagen & Guilfoyle, 1985; Ulmasov *et al.*, 1995). Synthetic auxin response element DR5 contains multiple repeats of the TGTCTC sequence and is a useful tool for artificial auxin-induced over-expression of chosen genes (Ulmasov *et al.*, 1997). ARFs bind to TGTCTC, likely as dimers, which is disturbed by dimerizing instead with Aux/IAAs in the absence of auxin (Ulmasov *et al.,* 1999). GH3 enzymes are an example of TIR-pathway mediated regulation. They perform amide-conjugation of IAA (Staswick *et al.,* 2002). By up-regulating their expression, auxin eventually attenuates its own signal. Auxin also regulates the expression of genes for some auxin transporter proteins via TIR1-pathway (see chapter 1.5.3). Key players of TIR1-pathway have been first identified in mutant phenotypes, which clearly demonstrate the pathway's crucial importance in plant development (e.g. Hardtke & Berleth, 1998; Hamann *et al.,* 2002).

Processes not known to be mediated by TIR-pathway are largely being attributed to Auxin-Binding Protein 1 (ABP1) auxin receptor (reviewed in Timpte, 2001; Tromas *et al.,* 2010; Hayashi, 2012). ABP1 was shown to be involved in a myriad of auxin-regulated processes, both including and not including gene expression. Auxin-induced ABP1-mediated protoplast swelling and cell enlargement through PM hyperpolarization, apoplast acidification and subsequent cell wall loosening by wall hydrolases does not involve gene expression (reviewed in Tromas *et al.*, 2010). Another non-expressional effect involving ABP1 is the auxin-induced inhibition of clathrin-mediated endocytosis (Paciorek *et al.,* 2005; Robert *et al.,* 2010). ABP1 promotes clathrin recruitment to the inner side of PM, which is disturbed by auxin binding. ABP1-regulation of cell elongation and division involves gene expression, e.g. of D-type cyclins (Tromas *et al.,* 2009). *Arabidopsis thaliana abp1* null mutant phenotype is an example of the protein's crucial role in cell division and elongation (Chen *et al.,* 2001). This mutant is embryonic lethal, which used to hamper further research of ABP1. It was eventually overcome by ABP1 conditional repression. The results showed the importance of ABP1 for cell division and elongation in post-embryonic development of shoot (Braun *et al.,* 2008) and root (Tromas *et al.,* 2009). The actual signaling pathways of ABP1 are, however, largely unknown.

The vast majority of ABP1 is retained in the lumen of ER, because of the characteristic KDEL amino-acid sequence, but a small portion is present at the outer PM (Henderson *et al.*, 1997; Shimomura *et al.*, 1999). It is presumably bound to the PM *via* a "docking protein", since ABP1 lacks trans-membrane domains or the necessary post-translational modifications (Klambt, 1990). Some candidates are being investigated (Xu et al., 2014). Though marginal, only the PM-localized portion of ABP1 was considered active in auxin signaling, because the relatively high pH of ER lumen does not support auxin binding (Tian *et al.,* 1995). However, there is evidence of the importance of ER-localized pool of ABP1 (Robert *et al.,* 2010). ABP1 may be an upstream regulator of TIR1-pathway by influencing Aux/IAA stability (Tromas *et al.,* 2013).

Physcomitrella patens (moss) and *Selaginella moellendorffii* (spike moss, an early vascular plant) already possess all important gene families for the ‘canonical’ transcriptional TIR1-pathway, though with much lesser members than *Arabidopsis thaliana* (Lau *et al.*, 2008; Paponov *et al.*, 2009). The ability of *Physcomitrella* TIR1-pathway to actually affect transcription like in angiosperms is doubted by some (Paponov *et al.*, 2009). The expansion and retention of Aux/IAA and ARF genes during vascular plant evolution may reflect rising structural and developmental complexity. ABP1 putative auxin receptor is likewise already present in mosses, though it mostly lacks the KDEL ER-retention motif (Panigrahi *et al.*, 2009; Tromas *et al.*, 2010). PM-localization of ABP1 may therefore be ancient.

Genomic evidence suggests that green algae lack TIR1-pathway (Lau *et al.*, 2009; De Smet *et al.*, 2011). By contrast, ABP1 orthologs are already found in unicellular chlorophytes (Lau *et al.*, 2009; Tromas *et al.*, 2010). Again, most lack ER-retention motifs. Rapid auxin-regulated non-transcriptional responses therefore might already be present in chlorophyte algae. Yin (1937) observed auxin-induced cell enlargement of *Chlorella*, a unicellular chlorophyte, though it was disputed a year later (Pratt, 1938). If this was again reliably demonstrated, it might be speculated that native ABP1 homologs are involved, as it happens in auxin-mediated cell enlargement in land plant cells.

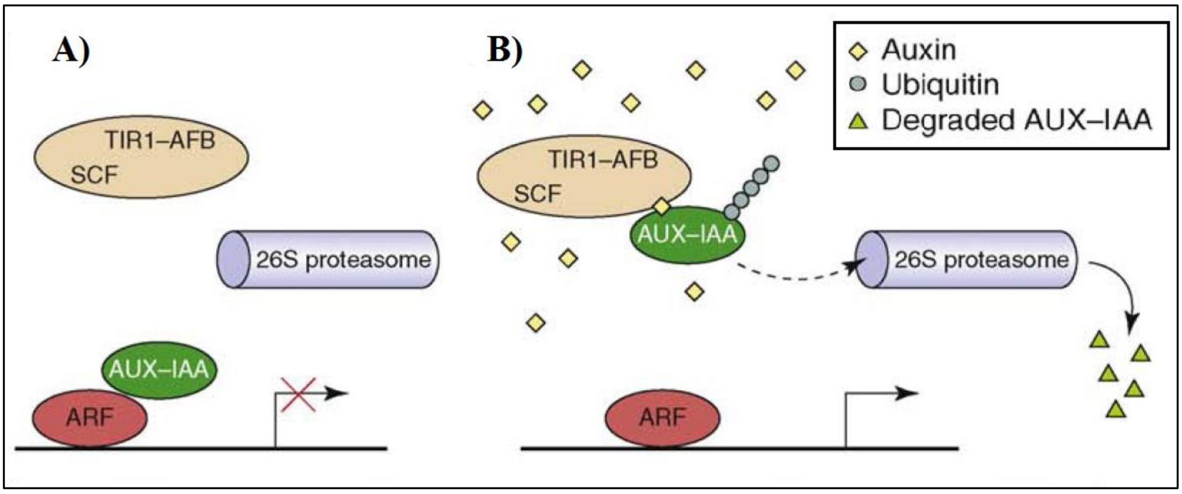


Figure 5. A simple representation of the nuclear TIR1/AFB-Aux/IAA-ARF pathway and its effect on the transcription of auxin-responsive genes. (**A**) In the absence of auxin, the ARF transcription factors of auxin-responsive genes are bound to their Aux/IAA repressors and no transcription takes place. (**B**) A complex is formed between TIR1/AFB and Aux/IAAs, which binds auxin. TIR1/AFB are F-boxes of an ubiquitin-ligase complex. Aux/IAAs are poly-ubiquitinated and marked for degradation in a 26S proteasome. ARFs are now free to regulate transcription. Figure reproduced from Lau *et al.* (2009).

1.5.3 Auxin transport

Auxin is transported through a plant in two ways. Firstly, it can be carried from source to sink *via* phloem bulk flow (Marchant *et al.*, 2002). Secondly, there is a cell-to-cell transport within parenchymatic tissues, known as Polar Auxin Transport (PAT; Goldsmith, 1977). It is slower, directional and regulated, carried out by cellular PM-localized transporters. PAT contributes to generation, maintenance and changes of auxin gradients in plant tissues, which trigger specific developmental responses. For review see Petrášek & Friml (2009); Vanneste & Friml (2009) and Peer *et al.* (2011).

The overall pattern of auxin flow within a plant could perhaps be best described as a "root fountain" and "shoot reverse fountain" (Benková *et al.*, 2003; Figure 3A): from the source in shoot apical meristem, auxin flows downward through the inner parts of shoot and root. Eventually it reaches the quiescent centre and columella initials within the root cap. From there, auxin is refluxed back up, now through the periphery. It goes through the outer layers of root and shoot, all the way back to the source. The resulting “fountain” flux model matches its name in shoot and root, respectively. Along the flux a new organ primordium may form (in shoot periphery or root pericycle), which becomes a temporary auxin sink and eventually gets connected to nearby vasculature. Auxin fluxes are the result of uptake and efflux, largely by the action of PM-localized cellular transporters.

“Uptake" represents the ways auxin gets into a plant cell. An accurate and so far valid model has been proposed in the 1970’s, called the "chemiosmotic hypothesis" (Rubery & Sheldrak, 1974; Raven 1975). It proposes the pH gradient between a cell cytoplasm (7.0) and the surrounding apoplast (5.5) as the crucial background behind auxin uptake (corroborated later e.g. by Li *et al.*, 2005). IAA is a weak acid (pKa = 4.75). In a relatively acidic apoplast, about 15% of its molecules (Vanneste & Friml, 2009) is present un-dissociated form (H-IAA). This form is lipophilic enough to pass through the PM by simple diffusion. Once inside a cell, the neutral cytoplasmic pH turns most IAA molecules into polar anions (IAA^-). These cannot pass through the PM by diffusion and become trapped inside a cell in so called "anion trap".

The apoplastic portion of IAA^- anions is transported inside cells by PM-localized influx carriers from AUX1/Like AUX1 (AUX1/LAX) family. They serve as H^+/IAA^- symporters (Pickett *et al.*, 1990; Yang *et al.*, 2006). Altogether, there are four AUX1/LAX members in *Arabidopsis thaliana* (AUX1 and LAX 1-3) and they have distinct developmental functions (Swarup *et al.*, 2008; Peret *et al.*, 2012).

Auxin influx function was also demonstrated for the ATP-binding cassette subfamily B/Permeability glycoproteins (ABCB/PGP), which were for a time thought to be only involved in auxin efflux (see further). ABCB4 serves both as auxin efflux and influx carrier (Terasaka *et al.*, 2005; Kubeš *et al.*, 2012; Cho *et al.*, 2012).

The chemiosmotic hypothesis suggested auxin molecules get trapped inside the cell after entry, unable to get out by diffusion. Hence, it predicted the existence of PM-localized efflux carriers to address the issue.

PIN-FORMED (PIN) family trans-membrane proteins are likely the most intensively studied auxin efflux carriers. They took their name from the *Arabidopsis thaliana* mutant phenotype where PIN1 protein was first identified (Galweiler *et al.*, 1998), showing needle-like inflorescence instead of a normal floral organ, caused by defective auxin transport. They are secondary active type transporters, i.e. use an H^+-gradient established by proton pumps to power up transport (Křeček *et al.*, 2009). Altogether, there are 8 PIN members in *Arabidopsis thaliana*. Although there is some level of functional redundancy and expression overlap (Vieten *et al.,* 2005; Wisniewska *et al.,* 2006), the individual PINs' expression and localization are generally specific and perform distinct functions in plant development (reviewed in Petrášek & Friml, 2009 or Křeček *et al.*, 2009). All PINs share five trans-membrane helices at each of their termini and a variable loop between them. Based on the length of this loop, they have been generally divided into "long" and "short" PINs. The loop is reduced or virtually absent in the latter (Křeček *et al.*, 2009). This view was recently challenged by Bennett *et al.,* 2014, proposing a new division of PINs based on conservation of their genes, because loop length may not necessarily reflect structure.

Long PINs are the crucial, rate limiting auxin carriers responsible for polar auxin transport (Petrášek *et al.,* 2006; Figures 3 and 4). These encompass PIN1, 2, 3, 4 and 7 in *Arabidopsis thaliana*. They are found at the PM and can be preferentially localized to one side of it, which gives PAT its directionality (Wisniewska *et al.,* 2006). They are subject to constitutive recycling by clathrin-mediated endocytosis (Dhonukshe *et al.,* 2007; Kitakura *et al.,* 2011) and ARF GEF GNOM-mediated exocytosis (Geldner *et al.,* 2001, Kleine-Vehn *et al.,* 2008). This allows for a relatively rapid reassembly of PINs and the establishment of their localization polarity. Auxin inhibits clathrin-mediated endocytosis *via* ABP1 (see chapter 1.5.2), but not exocytosis, resulting in increased amount of PINs on the PM and greater auxin efflux. Auxin further promotes its efflux by up-regulating long PIN expression (Vieten *et al.,* 2005) while also down-regulating the expression of short PINs (Mravec *et al.* 2009), which compete with them for their mutual intracellular substrate. PINs' apical or basal PM-localization is determined by PINOID (PID) kinase and Protein Phosphatase P2A (PP2A) (Friml *et al.,* 2004; Michniewicz *et al.,* 2007).

Short PINs encompass PIN5, 8 and arguably PIN6 as well in *Arabidopsis thaliana*. They localize to the membrane of ER and, by transporting auxin between ER-lumen and cytoplasm, are likely primarily involved in intracellular auxin homeostasis. PIN5 was the first characterized short PIN (Mravec et al., 2009); it probably transports auxin from cytoplasm into the lumen of ER, where some auxin-metabolic enzymes are located. PIN8 was shown to be expressed in pollen and to regulate its development (Ding et al., 2012). Finally, all three short PINs are involved in regulation of leaf vein formation, otherwise mastered by the "long" PIN1. It appears that PIN6 and 8 transport auxin from ER-lumen into cytoplasm, providing additional substrate for PIN1 and thereby contributing to the creating of local auxin maxima and subsequent vein formation (Sawchuk & Scarpella, 2013).

ATP-binding cassette subfamily B/Permeability glycoproteins (ABCB/PGP) family transporters serve as auxin transporters in land plants, predominantly as efflux carriers (reviewed in Geisler & Murphy, 2006; Cho & Cho, 2013). They use the energy from ATP hydrolysis to power up transport. Three ABC carriers of subfamily B have been shown to transport auxin in plants: ABCB1, 4 and 19 (Figure 3). ABCB1 and ABCB19 are efflux carriers (Noh *et al.*, 2001; Geisler *et al.*, 2005). ABCB4 can perform both efflux and influx function (Terasaka *et al.*, 2005; Kubeš *et al.*, 2012; Cho *et al.*, 2012). ABCB are localized on the PM very stably and do not undergo vesicular cycling like long PINs (Geisler *et al.*, 2003; Cho *et al.*, 2012). Their localization on the PM is largely apolar, though it can be polarized in some specific tissues and in specific conditions (Terasaka *et al.*, 2005). While ABCB are not regarded as important players in PAT, they can co-localize with long PINs and stabilize them in the PM, thereby contributing to PAT (Blakeslee *et al.*, 2007; Mravec *et al.*, 2008; Titapiwatanakun *et al.*, 2009). ABCB14 and 15 were also proposed as auxin transporters (Kaneda *et al.*, 2011). Considering the high substrate variability and often the ability to carry multiple substrates by a single protein, the Members of another ABC subfamily, ABCG36 and 37 were shown to specifically transport IBA (see chapter 1.3), but not IAA (Strader & Bartel, 2009 and 2011). ABC carriers could perhaps be considered diverse-utility cellular transporters that also happen to be involved in auxin transport, unlike the substrate and function specific PIN proteins (Cho & Cho, 2013).

Recently, a new group of auxin carriers has been identified. They have been named PIN-LIKES (PILS), because their predicted protein topology is similar to PIN proteins. Like short PINs they localize to the ER membrane and affect intracellular auxin homeostasis (Barbez *et al.*, 2012).

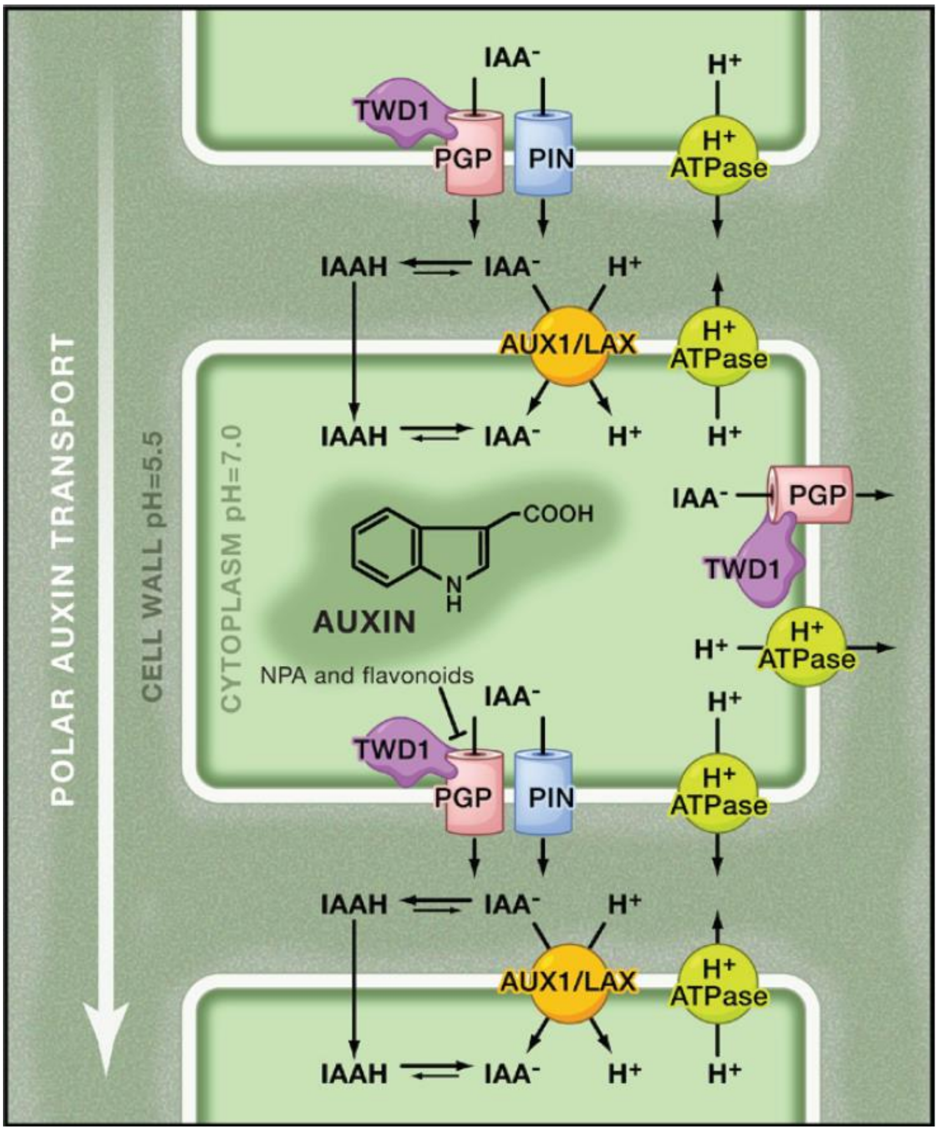


Figure 6. Chemiosmotic hypothesis for polar auxin transport. In the acidic apoplastic pH of 5.5 maintained by proton pumps, a small portion of IAA molecules remains undissociated (H-IAA). These can pass through the PM and into the cell by diffusion. The dissociated form (anion, IAA^-) cannot enter the cell by diffusion and is imported by active influx carriers. Once inside, almost all IAA molecules become dissociated in the neutral cytoplasmic pH of 7.0. This form is exported out of the cell by PM-localized ABCB/PGP and PIN efflux carriers. ABCB/PGP are predominantly uniformly localized. PIN can assume polar localization and determine the direction of auxin efflux, resulting in polar auxin transport. Reproduced from Vanneste & Friml (2009).

The earliest fossil evidence for auxin transport tracks the evolution of secondary vascular tissues (xylem) in late Devonian progymnosperms (Rothwell & Lev-Yadun, 2005). Auxin transport has been experimentally demonstrated in extant liverworts and mosses, the basal-most land plants with gametophyte-dominant life cycles. Earlier studies clearly state there is polar auxin transport (PAT) in the gametophytes of liverwort *Marchantia polymorpha* and moss *Funaria hygrometrica* (Gaal et al., 1982; Rose & Bopp, 1983; Rose *et al.*, 1983). Later investigation of PAT in a liverwort, moss and a hornwort provided mixed results (Poli et al., 2003) and finally, recent research restricts PAT to moss sporophytes (Fujita *et al.*, 2008).

Auxin was shown to enter the cells of *Chlorella*, a unicellular chlorophyte, by diffusion, without the involvement of influx carriers (Dibbfuller & Morris, 1992). Though one genomic study identified very clear orthologs of angiosperm AUX1/LAX carriers in two *Chlorella* strains (De Smet *et al.*, 2011), this is likely the result of horizontal gene transfer by dsDNA viruses (Tromas *et al.*, 2010). Possible function of these orthologs in *Chlorella* is unknown. No other green algae were reported to contain AUX1/LAX genes. ABCB and PIN-LIKES were also reported in organisms as basal as unicellular chlorophytes. Though present in algal genomes (Feraru *et al.*, 2012), the function of PIN-LIKES in algae yet remains to be resolved. ABC proteins are extraordinarily diversified in plants (Jasinski *et al.*, 2003; Kang *et al.*, 2011), but they are a very ancient family present in all domains of living organisms (Dean & Allikmets, 1995; Albers *et al.*, 2004). They are also very substrate non-specific. Even if some ABC proteins were found capable of transporting auxin in green algae, it still might not reflect their native function.

Being the principal determinants of polar auxin transport, PIN-FORMED carriers have received much attention in evolutionary analyses (Křeček *et al.*, 2009; Mravec *et al.*, 2009; Carraro *et al.*, 2012; Forestan *et al.*, 2012; Viaene *et al.*, 2013; Bennett *et al.*, 2014; Clouse & Carraro, 2014; Wang *et al.*, 2014). In theory, the diversification of PINs should track the evolution of PAT. Previously, the short PIN-regulated intracellular homeostasis was considered ancient (Mravec *et al.*, 2009). Recent studies, however, identify long PINs as ancestral, probably present in the last common ancestor of land plants (Bennett *et al.*, 2014; Clouse & Carraro, 2014). Putative orthologs of long PINs were identified in two evolutionary distinct, but morphologically similar streptophyte algae: *Klebsormidium* (Viaene *et al.*, 2013) and *Spirogyra* (De Smet *et al.*, 2011). The latter belongs to clade now identified as sister group to land plants (see chapter 1.2).

Auxin transport was experimentally studied in *Chara*, an advanced multicellular streptophyte alga with plant-like morphology. Dibbfuller & Morris, 1992 report there is both influx and efflux, influx is dominant and efflux is not affected by 1-N-naphthylphtalamic acid (NPA), an auxin efflux inhibitor used in land plants (Thomson *et al.*, 1973). Klambt *et al.*, 1992 showed promoted growth of decapitated *Chara* rhizoids in reaction to exogenously applied auxins, which was inhibited by NPA. Recent study clearly demonstrates there is polar auxin transport in *Chara* (Boot *et al.*, 2012). It goes from thallus to rhizoid, is subject to NPA inhibition and is independent of cytoplasmic streaming. PAT in *Chara* is presumed to utilize other than acto-myosin molecular motors, which power cytoplasmic streaming (Raven (2013). PAT presence in *Chara*, which life cycle is completely gametophyte-dominant, contrasts with the reported absence of PAT in moss gametophytes.

1.6 Why we should study the evolution of auxin mechanisms in green algae

If we summarize the so far provided information, the mechanisms of auxin action are already well developed even in basal land plants like mosses or liverworts (Figure 7). The genome of model moss *Physcomitrella patens* already possesses the important genes for mechanisms of auxin action. Its development is affected by auxin and other phytohormones (Decker *et al.,* 2006; Eklund *et al.,* 2010) and polar auxin transport is present in sporophyte (Fujita et al., 2008). Though no complete genome is known, auxin transport and other mechanisms are also present in liverworts (Poli et al., 2003; Bennett et al., 2014). The next lower step in plant evolution is green algae, especially the members of clade *Streptophyta*, from which all land plants originated.

Basal unicellular green algae contain orthologs of PIN-LIKES and some genes for auxin biosynthesis (Feraru *et al.*, 2012; De Smet *et al.*, 2011). Auxin conjugation is not expected, since it is believed to have evolved in land plants after the split of liverworts (Sztein *et al.*, 1999). As such, homeostatic regulation by PIN-LIKES makes little sense, unless they transport auxin into the ER for degradation. Auxin biosynthesis might represent an ancient mechanisms, though it is not clear what single cells would need auxin for. If auxin efflux was present in unicellular chlorophytes, possibly originally mediated by non-specific ABC carriers, it might convey some kind of information to neighbouring cells. One possible result of this colony-level signaling might be the experimentally observed promotion of *Chlorella* culture growth by exogenously applied auxins (Yin, 1937; Pratt, 1938; Saygideger & Okkay, 2008; Hunt *et al.*, 2011).

TIR1-pathway is absent in green algae and is very simple in mosses, compared to vascular plants (Lau et al., 2009, Paponov et al., 2009). Perhaps its evolution coincided with transition to land and/or development of vascular tissues, maybe even with transition to sporophyte domination in land plant life cycle. By contrast, the presence of ABP1 appears to be as ancient as unicellular chlorophytes (Tromas *et al.*, 2010). ABP1 is involved in many developmental processes. There might be some parallels between land plants and algae, like auxin-induced inhibition of clathrin-mediated endocytosis or swelling of cells, similar to what Yin (1937) observed in *Chlorella* after exogenous auxin treatment.

Experimental evidence of polar auxin transport (PAT) in *Chara* (Boot *et al.,* 2012), coupled with putative PIN-FORMED orthologs identified in *Klebsormidium* and *Spirogyra* (Viaene *et al.,* 2013; De Smet *et al.,* 2011), suggest that origins of PAT lie in streptophytes. All of the mentioned algae are multicellular; *Chara* has complex multicellularity and plant-like morphology, *Klebsordmidium* and *Spirogyra* cells are arranged in unbranched filaments. Though the latter two do not possess complex morphology, they live as a tightly aggregated biomass. Perhaps the origins of auxin transport follow the evolution of complex multicellularity. All of the three mentioned algae are aquatic (except for some *Klebsormidium* species), which argues that PAT was already present before the first algae made their transition to land.

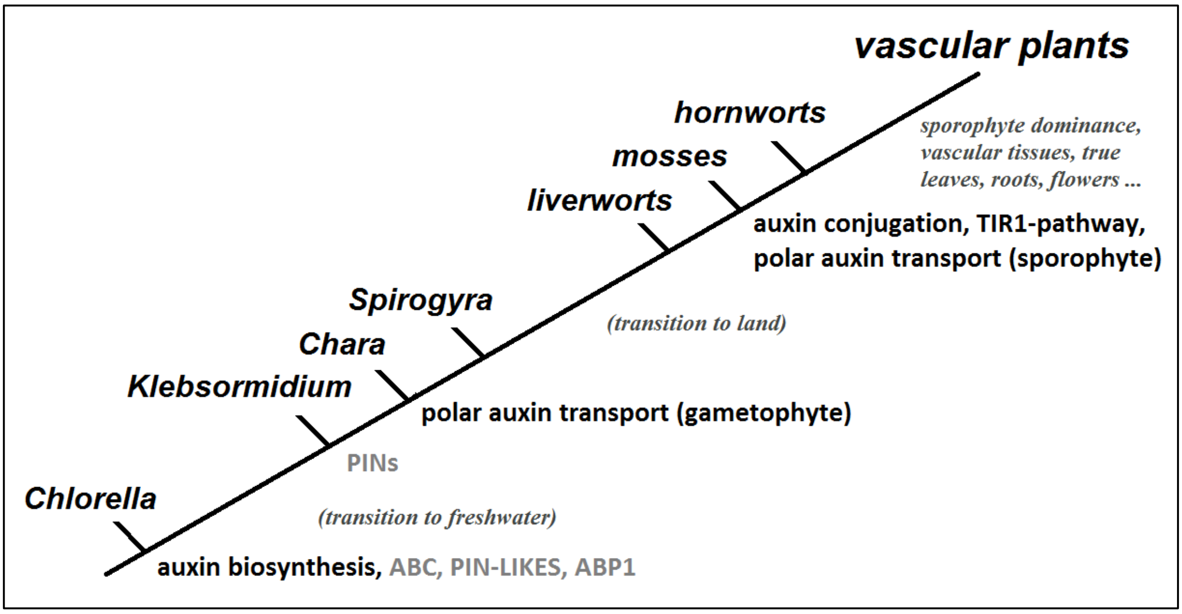


Figure 7. A simplified cladogram of early land plant evolution with respect to the evolution of mechanisms of auxin action. ABC, PIN-LIKES, ABP1 and PINs are present in green algal genomes, but their native function (or connection to auxin transport in case of ABC family) has not yet been demonstrated in green algae.

2 Main objectives

2.1 Establishment of cell cultures from representative chlorophyte and streptophyte algae

Two algal representatives were chosen for experiments, *Chlorella lobophora (Chlorophyta)* and *Spirogyra sp. (Streptophyta)*. Selected strains are kept in the algae collection, but there was no information on the parameters of growth under *in vitro* conditions in liquid cell cultures. Therefore, this objective includes optimization of *in vitro* growth including determination of cell population growth parameters (cell and optical density).

2.2 Studying of growth response of algae to exogenous auxin application

There is only scarce evidence on the growth response to auxin in *Chlorophyta* and *Streptophyta*. This objective is focused on the quantification of changes in the growth parameter (cell division and cell elongation) after the application of native auxin IAA and synthetic auxin 1-NAA, as well as auxin transport inhibitors.

2.3 Determination of auxin metabolism and transport in *Spirogyra sp.*

The goal of this part of the work is to experimentally test auxin influx and efflux kinetics in the axially growing *Spirogyra sp.* by technique that is optimized in the laboratory for the cells of tobacco or *Arabidopsis thaliana*. In parallel, metabolic profiles of exogenously applied native and synthetic auxins will be analysed.

2.4 Identification and cloning of plant auxin carrier orthologs from *Spirogyra sp.*

Available DNA sequence information from *Spirogyra sp.* from the *Zygnematophyceae* clade, a likely sister group to land plants, suggests the presence of sequences orthologous to the *Arabidopsis PIN* and *ABCB* genes. Within this objective, potential candidates will be cloned from DNA of *Spirogyra sp.* using specific primers and sequenced. Inversion PCR will be used to clone whole coding sequences for future expression studies.

3 Materials and methods

3.1 Plant material

Chlorella lobophora Andreeva (strain H 1995) and *Spirogyra sp.* (strain K 902) were obtained from CAUP Culture Collection of Algae of Charles University in Prague and cultured in liquid Bold’s Basal Medium (Cox & Bold, 1966; Table 1) at 24°C, 16/8 (l/d) photoperiod and continuous shaking (120 rpm, GFL 3015 Orbital Shaker, 30 mm diameter). The algae obtained from the collection suffered from a slight bacterial contamination. Therefore, they were initially cultured in BBM in the presence of claforan (cefatoximum natricum, 0.3 g/l. The length of the subculture interval was set to six weeks for both algae. The biomass densities of algal inocula were 8 mil cells/ml for *Chlorella lobophora* and 0.05 g fresh weight/10 ml for *Spirogyra sp.* Both algal cultures were inoculated and kept as 30 ml suspensions in 100 ml Erlenmeyer flasks.

Stock solutions *per* 400 ml		
1	$NaNO_3$	10 g
2	$CaCl_2 \cdot 2H_2O$	1 g
3	$MgSO_4 \cdot 7H_2O$	3 g
4	$K_2HPO_4 \cdot 3H_2O$	3 g
5	KH_2PO_4	7 g
6	NaCl	1 g
Stock solutions *per* 1000 ml		
7	H_3BO_3	11.42 g
8	$FeSO_4 \cdot 7H2O$	4.98 g
	conc. H_2SO_4	1 ml
9	$ZnSO_4 \cdot 7H_2O$	8.82 g
	$MnCl_2 \cdot 7H_2O$	1.44 g
	$(NH_4)_6Mo_7O_{24} \cdot 4H2O$	0.88 g
	$CuSO_4 \cdot 5H_2O$	1.57 g
	$Co(NO_3)_2 \cdot 6H_2O$	0.49 g
10	EDTA	50 g
	KOH	31 g

Table 1. Stock solutions for Bold’s Basal medium. For one litre of medium, add 20 ml of stock solution 1, 10 ml of stock solutions 2–6 and 1 ml of stock solutions 7–10 into 1 l of distilled water.

3.2 Chemicals and services

Unless stated otherwise, all chemicals were supplied by Sigma-Aldrich, Inc. (St. Louis, USA). NPA was supplied by OlChemIm Ltd. (Olomouc, Czech Republic). ^{3}H-IAA, ^{3}H-NAA (both of specific radioactivity 20 Ci/mmol) and ^{3}H-2,4-D (10 Ci/mmol) were supplied by American Radiolabeled Chemicals, Inc. (St. Louis, USA). Claforan was supplied by ICN Polfa Rzeszów S.A.

Primers for PCR were produced by Sigma-Aldrich Inc. DNA sequencing was carried out by GATC Biotech.

Auxins and auxin transport inhibitors were kept as following stock solutions: CHPAA (30 mM, EtOH), CHPAA (100 mM, DMSO), gravacin (15 mM, EtOH), IAA (30 mM, EtOH), NAA (10 mM, EtOH), NAA (30 mM, EtOH), NPA (30 mM, EtOH), 2,4-D (10 mM, EtOH), 4-Cl-IAA (10 mM, EtOH).

3.3 Determination of growth parameters

The growth of *Chlorella lobophora* was followed by determining cell densities during the subculture interval. This was performed by microscopical counting of cells in Fuchs-Rosenthal hemocytometer slide. The slide is ruled with sixteen 1 mm^{2} areas that form together a 4 x 4 mm square, where the total number of cells is counted. The number of cells/ml (P) is recalculated from values according to the following formula: P = number of cells in the chamber x 1000/3.2. A cell suspension was diluted before counting to decrease measurement errors. This dilution was between 5x and 20x, depending on the density of the culture. For the construction of growth curves, multiple samples from each culture were measured and their average values were used. The optical density of *Chlorella lobophora* cultures was measured by spectroscopy with DU 730 UV/Vis Spectrophotometer (Beckman Coulter) at 600 nm wavelength.

Spirogyra sp. cells are arranged in filaments and suspension homogeneity cannot be achieved at volumes. The growth of *Spirogyra sp.* cultures was therefore measured as biomass increase (dry weight) during a subculture interval. Each day biomass samples were harvested and dry weight determined after 24h at 80°C in dry heat oven (Gallenkamp Sanyo OMT, Schoeller Instruments). Dry weight was then determined by weighing. The pH of culture media was measured at Orion DUAL STAR pH/ISE Benchtop with Orion electrode (both Thermo Scientific).

The microscopy was performed with Nikon Eclipse E600 microscope (Nikon, Japan). Using colour digital camera (DVC 1310C, USA), the images from microscope were saved into computer and later analysed using LUCIA image analysis software (Laboratory Imaging, Prague, Czech Republic).

3.4 Auxin accumulation assays in *Spirogyra sp.*

Three-week-old *Spirogyra sp.* biomass was used for radioactively-labelled auxin accumulation assays. While this does not correspond to the most rapid growth phase of its cultures, there is higher amount of available biomass at the time and the biomass does not yet aggregate, which would reduce suspension homogeneity. Accumulations of radiolabelled auxins were followed the procedures adapted from Delbarre *et al.* (1996) and Petrášek *et al.* (2006). All biomass available for the experiment was mixed together and moved into a fresh BBM medium of pH 5.5, which served as an auxin uptake buffer, and left that way for about 15 minutes. The final density of this suspension was set to 0.1 g fresh weight/10 ml. The suspension was then divided into multiple flasks representing individual experimental samples, always with a reserve of 30% extra suspension volume. At all stages, the flasks were always shaken just enough to keep the suspension homogenous. The addition of a radioactively-labelled auxin (final concentration 2 nM) always marked the start of an experiment. Auxin transport inhibitors and other auxins were added either together with a radiolabelled auxin, or at the defined time i.e. "in-flight". Suspension samples were taken during the accumulation period. At each time, four 5-ml suspension samples were taken and their biomass harvested by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. Biomass samples were immediately transferred into scintillation vials with 2 ml 96% ethanol and incubated for at least 30 min. 12 ml of scintillation solution (EcoLite Liquid Scintilation Fluid, MP Biomedicals, Solon, USA) was added into each vial and all vials were shaken at 480 rpm (Ika KS 130 shaker, 2 mm diameter) for 5 min. All vials were then kept in a dark place for two weeks to allow for degradation of chlorophyll, which would impair scintillation measurements. To receive reliable data, another 12-16 ml of scintillation solution had to be added into each vial before measuring to clear up the solution. The samples with dead biomass were bathed in 96% EtOH for 30 minutes and then resuspended in the uptake medium and tread the same way as biomass samples. Radioactivity of samples in vials was then determined with liquid scintillation counter Packard Tri-Carb 2900TR (Packard-Canberra, Meridian, CT, USA). The amount of radioactivity being present in samples (dpm) was expressed as pmol/fresh weight of biomass, using specific radioactivity values for each of the hot auxin stock solutions. Besides multiple recognized auxin molecules (reviewed in *Skůpa et al.*, 2014), the following auxin land-plant auxin transport inhibitors were used: 3-chloro-4-hydroxyphenylacetic acid (CHPAA, influx inhibitor; Laňková *et al.*, 2010), gravacin (ABCB inhibitor; Rojas-Pierce *et al.*, 2007) and 1-naphthylphthalamic acid (NPA, efflux inhibitor; Thomson *et al.*, 1973).

3.5 Auxin metabolic profiling in *Spirogyra sp.*

Spirogyra sp. biomass suspension was loaded with radiolabelled auxins in the same way as described for auxin accumulation assays, except 20 nM radiolabelled auxin concentrations were used. Each sample was represented by 12 ml of biomass suspension (or uptake medium). After a biomass sample was taken by filtration, it was immediately frozen in liquid nitrogen and stored at -80°C until further use. Extraction and purification of auxin metabolites in biomass and uptake media samples were performed according to Dobrev & Kamínek (2002) and Dobrev *et al.* (2005) protocols for purification of auxins and cytokinins. Auxins were purified and eluted by methanol using Oasis MCX 1 cc (30 mg) columns. For the extraction, 500 µl of pBielski extraction solvent (methanol/water/formic acid: 12/2/1 v/v/v; solvent stored at -20°C) was added and samples homogenized with 2-mm iron balls in the mixer mill (Retsch MM 301; 30 Hz, 3 min). After addition of another 1 ml of pBielski, samples were kept at -20°C overnight. After centrifugation (18 000 rcf, 4°C, 30 min), supernatant was collected, 500 µl of pBielski added to the residual pellet and this step repeated. Supernatants were pooled together, frozen at -80°C for 30 min and evaporated at +40°C to dryness or near-dryness. Evaporated samples were dissolved in 500 µl 1M formic acid and applied to the activated column (1 ml H_2O followed by 1 ml 1M formic acid). After several washing steps, auxins were eluted into clean tubes with 2x 500 µl 100% methanol and evaporated to dryness at -40°C. The radioactive metabolites were separated on HPLC. For this method column LunaC18 (2), 150 × 4.6 mm, 3 µm (Phenomenex, Torrance, CA, USA) was used, mobile phase A was 40 mM CH_3COONH_4, (pH 4.0) and mobile phase B was CH_3CN/CH_3OH, 1/1, (v/v). Flow rate was 0.6 ml min^{-1} with linear gradient 30–50 % B for 10 min, 50–100 % B for 1 min, 100 % B for 2 min, 10–30 % B for 1 min. The column eluate was monitored by a Ramona 2000 flow-through radioactivity detector (Raytest GmbH, Straubenhardt, Germany) after online mixing with three volumes (1.8 ml.min^{-1}) of liquid scintillation cocktail (Flo-Scint III, Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). The radioactive metabolites were identified on the basis of comparison of their retention times with authentic standards.

3.6 Nucleic acid isolation from *Spirogyra sp.*

About 100 mg fresh weight of *Spirogyra sp.* biomass was put into a ceramic mortar and frozen by pouring liquid nitrogen on it. While frozen, it was ground with a pestle. This was repeated until the biomass was transformed into a fine powder. Total DNA was separated from the homogenate and eluted using NucleoSpin® Plant II Mini kit (Macherey-Nagel). Nucleic acid concentrations were measured on NanoDrop ND-2000c spectrophotometer at 260nm wavelength. Though DNA was always isolated in relatively low concentrations (ca. 10 ng/µl), they were of sufficient quality for PCR.

3.7 PCR and cloning of *Spirogyra sp.* orthologs

Transcriptome shotgun assembly (TSA) sequences available in the National Center for Biotechnology Information (NCBI) database were used to design primers for the isolation of putative auxin carrier orthologs. Namely, TSA: *Spirogyra pratensis* strain UTEX 928 spra_Contig1371 mRNA sequence for *Arabidopsis thaliana ABCB19* and TSA: *Spirogyra pratensis* strain UTEX 928 spra_contig01436_c_s mRNA sequence for *Arabidopsis* *thaliana PIN1*. Primers were designed using Primer3, a freely available online tool and are listed in Table 2. Primers used for the PCR amplification of *Spirogyra sp.* Sequences are specified in Table 3. Polymerase chain reaction (PCR) with Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc.) was performed with 35 cycles under conditions specified in Table 4 *Spirogyra sp.* DNA, 20x diluted in water after isolation, was used as reaction template. Reactions were conducted on TProfessional Basic Thermocycler (Biometra).

PCR Products were visualized on agarose gel electrophoresis (1.5% for our expected product sizes). Phage Lambda DNA PstI marker (Thermo Fisher Scientific) was used as ladder and SYBR® Safe DNA Gel Stain (Life Technologies) as a dye to visualize DNA under UV light. Final PCR products were manually excised from the agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen).

Primer name	**Orientation (Forward vs. Reverse)**	**5´ to 3´nucleotide sequence**
PIN917	F	ACGGCACATTAGCCAGAACT
	R	AATCATCATCGTCCACGTCA
PIN1038	F	TGAAATCTGGGCAGCAAAA
	R	AGCATCAGTGGGAAACACAA
PIN1123	F	TGTACGGCACATTAGCCAGA
	R	AGCATCAGTGGGAAACACAA
ABC2053	F	AACTGTCAGGAGGGCAGAAA
	R	ACCATCAAACGATCCAAAGC
ABC2073	F	AGGCAACGTCAGCGTTAGAT
	R	TGCCCTGCTCCACTATCTTT
ABC2105	F	ATCCAAACATTGCCAGAAGG
	R	ACCATCAAACGATCCAAAGC
ABC2326	F	ATCCAAACATTGCCAGAAGG
	R	ACGCAAACACCAAACAGACA

Table 2. Primers used in the initial attempts for the isolation of *Spirogyra sp.* ortholog fragments.

Primer name	**Orientation (Forward vs. Reverse)**	**5´to 3´nucleotide sequence**
*Spiro*ABC-New	F	CGGTTCGATCAATCCAATGT
	R	CGTGGTTCTTCCAACCATTA
*Spiro*ABC-Q1	F	AGAACAGTGCAGGAGCCATT
*Spiro*ABC-Q2	F	AGCTCTTTCCCGAACCTGAC

Table 3. Primers designed for *Spirogyra sp.* ABCB19 ortholog.

PCR reaction mixture (volume: 20 µl)		PCR protocol (35 cycles)	
Phusion High-Fidelity Buffer	4 µl	Initialization	98°C, 2 min
dNTPs (10 mM, aq. solution)	0.4 µl	Denaturation	98°C, 15s
of each primer (10 µM, aq. solution)	1 µl	Annealing	58°C, 20s
Phusion High-Fidelity Polymerase	0.2 µl	Extension	72°C, 20 s per 1 kb
DNA	2 µl	Final extension	72°C, 5 min
water	11.4 µl	hold at 4°C until sample recovery	

Table 4. Conditions and reaction mixture for Phusion PCR reaction.

Approximately 100 ng of clean PCR product was ligated into pJET1.2 vector using CloneJET PCR Cloning Kit (Thermo Fisher Scientific). The vector confers resistance to ampicillin, which serves as selection of positive clones. Ligation was done at 4°C for 15 hours followed by 15 min at 65°C to inactivate the reaction. 8 µl of ligation product was used to transform 100 µl of *Escherichia coli* XL1-Blue competent cells by 42°C heat shock for one minute. Colonies grown overnight at 37°C on plates containing Luria-Bertani (LB) medium (Bertani, 1951) and ampicillin (100 mg/l) were tested for presence of the insert by PCR after XhoI/XbaI digestion. Plasmids were isolated from positive clones using QIAprep Spin Miniprep Kit (Qiagen) and the insert was sequenced using pJET1.2 vector specific primers.

3.8 Statistics

The significance of growth difference and accumulation results was tested for using ANOVA test and Tukey's HSD test in R programme (R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

4 Results

4.1 Establishment of *in vitro* cell cultures of algae

Both *Chlorella lobophora* and *Spirogyra sp.* were kept in a liquid medium containing antibiotics (claforan) for the first few subculture intervals to prevent slight bacterial contamination that was detected in the culture samples obtained from the algae collection. Since there were no growth curves available for both obtained algae strains, cell population density and biomass growth has been followed during a subculture interval in *Chlorella lobophora* and *Spirogyra sp.*, respectively.

4.1.1 Growth characteristics of *Chlorella lobophora*

The growth of cell population of *Chlorella lobophora* was followed microscopically by counting cells in a Fuchs-Rosenthal hemocytometer slide. In parallel, the optical density (absorbance) was measured by spectrometry to establish less time-consuming method for the cell density determinations.

The most rapid growth was observed within the first week after inoculation, after a one-day lag phase, and cell culture reaches stationary phase around 20th day (Figures 8A and 8B). The most remarkable relative increase of cellular density is in between 1st and 2nd day of cultivation (Figure 8D). The progression of subculture interval of *Chlorella lobophora* culture might reflect subtle variation in the manipulation with the inoculum. Therefore, cell densities were determined in either cultures inoculated from the same inoculum (Fig. 8A and B) or from the inoculum of different age (Figures 8C and D). From these experiments, it could be concluded that, in general, basic characteristics of the *Chlorella lobophora* growth curves are always the same. To further standardize the construction of growth curves, optical density (OD) of the cell population was used to track differences in cell densities in cultures inoculated from the same inoculum. As shown in Figure 9A, the number of cells corresponding to the OD=1 changes during the subculture interval and it also reflects the age of the inoculum (Figure 9B). The initial high peaks correspond to the cultures being vivid dark-green, while the eventual decrease corresponds to the cultures gradually becoming more yellow and brighter. Absorbance values could be used to detect larger differences in cellular densities, provided the cultures came from the same inoculum.

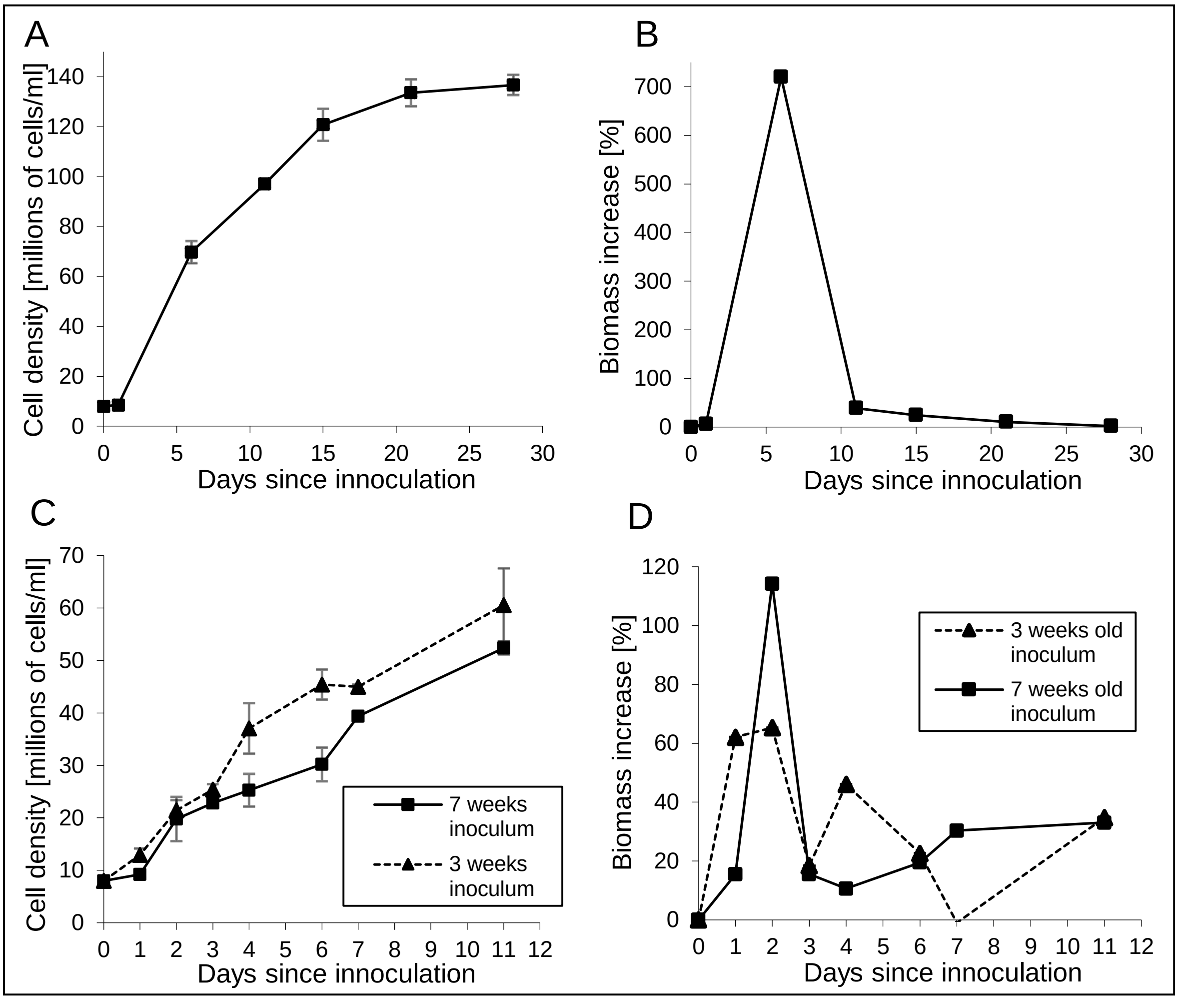


Figure 8. Cell densities and their relative changes during a subculture interval of *Chlorella lobophora*. (**A**) Average cell density in two cultures inoculated from the same inoculum. The inoculum was a 6 weeks old culture. 28-day interval. (**B**) Relative changes of average cell density in between measurements in 2 cultures inoculated from the same inoculum. The inoculum was a 6 weeks old culture. 28-day interval. (**C**) Cell densities in 2 cultures inoculated from separate inocula, which were in different stages of subculture interval. 11-day interval. (**D**) Relative changes of cell density in between measurements in 2 cultures inoculated from separate inocula, which were in different stages of the subculture interval. 11-day interval.

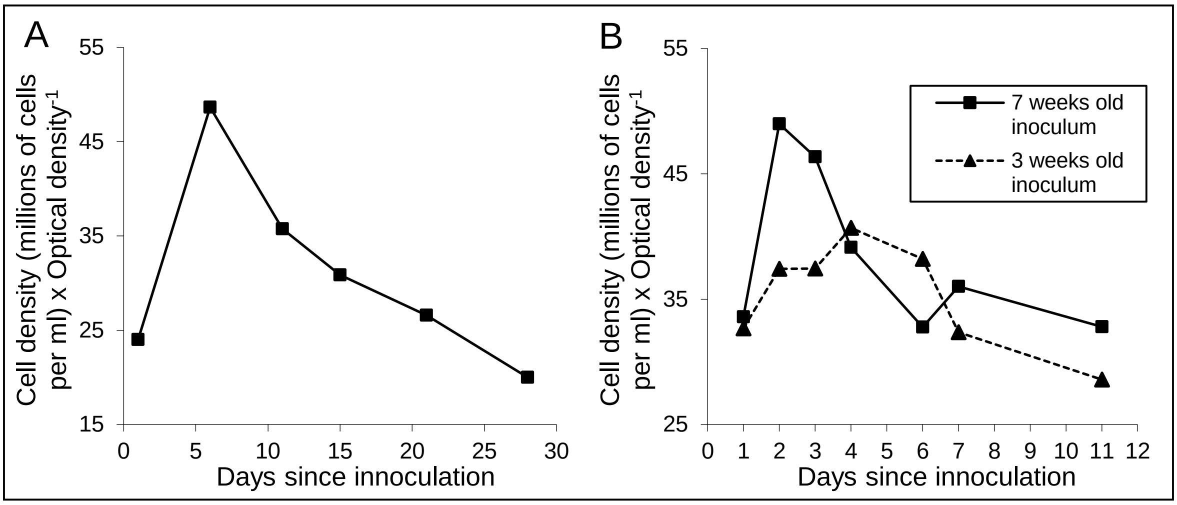


Figure 9. Relation between suspension optical density and cell population density in *Chlorella lobophora* cultures. (**A**) Calibration curves showing the kinetics of number of cells that correspond to the value of OD=1 in 2 cultures inoculated from the same inoculum over a 28-day interval. The inoculum was a 6 weeks old culture. (**B**) Calibration curves showing the kinetics of number of cells that correspond to the value of OD=1 in 2 cultures inoculated from separate inocula, which were in different stages of the subculture interval, over a 11-day interval. OD was measured at 600 nm wavelength.

4.1.2 Growth characteristics of *Spirogyra sp.*

The growth *Spirogyra sp*. biomass was measured by determining average biomass dry weight in different days of a subculture interval. Each measurement represented three separate biomass samples. The most rapid growth was observed around the 10th day after inoculation (Figure 10A and B). The culture experiences another boost in biomass growth about a month after inoculation (Figures 10A and B). However, the biomass starts clumping together at this time and the suspension loses its relative homogeneity.

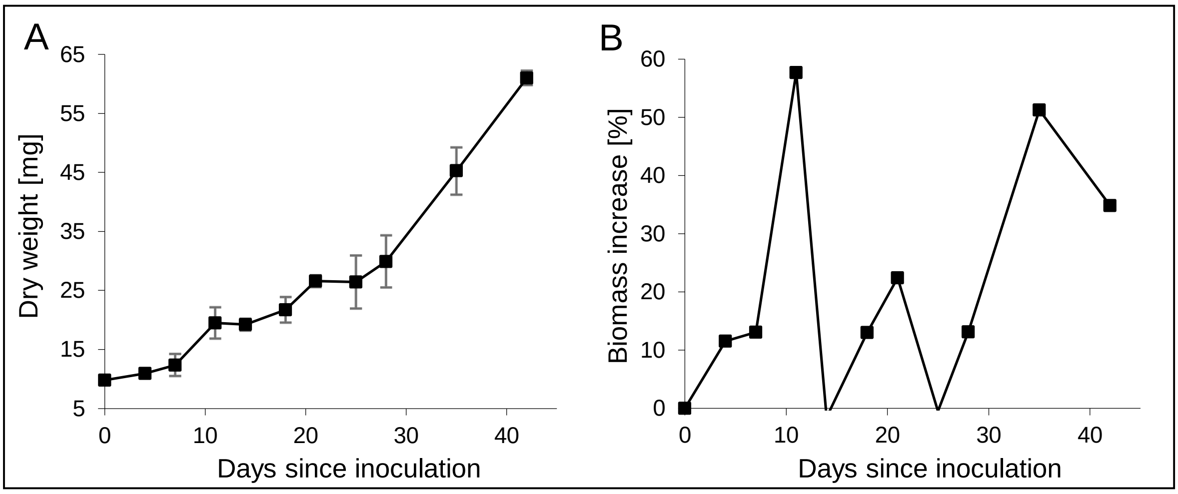


Figure 10. Biomass dry weight and its relative changes during the subculture interval of *Spirogyra sp.* (**A**) Gradual increase in average biomass dry weight during a subculture interval. 42-day interval. (**B**) Relative changes of average biomass dry weight in between measurements. 42-day interval.

Spirogyra sp. was markedly increasing the pH of cultivation medium during the subculture interval (Table 5). While most culture samples follow the pattern in Table 5, the medium pH value of a few individual samples was occasionally found to be as high as 10. This was happening at any stage of life cycle past the 14th day and the cultures were always visibly very green and vivid. Medium pH changes can be prevented by adding 20 mM 2-(N-morpholino)ethanesulfonic acid (MES buffer) into the medium (Table 6).

Days since inoculation	0	3	7	10	14	17	21
Average suspension pH	6.6	7.2	7.3	7.7	8.0	8.3	8.0

Table 5. pH changes of culture medium during a part of *Spirogyra sp.* subculture interval. Each measurement represents three different cultures, but all originating from the same inoculum.

Day since inoculation	0	6	14	28
Average suspension pH	5.5	5.9	5.9	5.7

Table 6. pH changes of culture medium with 20 mM MES buffer during a part of *Spirogyra sp.* subculture interval. 10 different cultures, all from the same inoculum, were measured in a 28-day period.

4.2 Growth response of algal cultures to long-term exogenous auxin exposure

4.2.1 Effects of exogenously applied NAA and some auxin transport inhibitors on culture growth of *Chlorella lobophora*

NAA, NPA and gravacin were added into *Chlorella lobophora* culture media. NAA was added both separately and together with each auxin transport inhibitor. Growth response of algal cultures to these combinations was observed by measuring their optical density.

No changes in optical density of *Chlorella lobophora* cultures were induced by their exposure to NAA, NPA and gravacin during period of 21 days (Figure 11A, B, C).

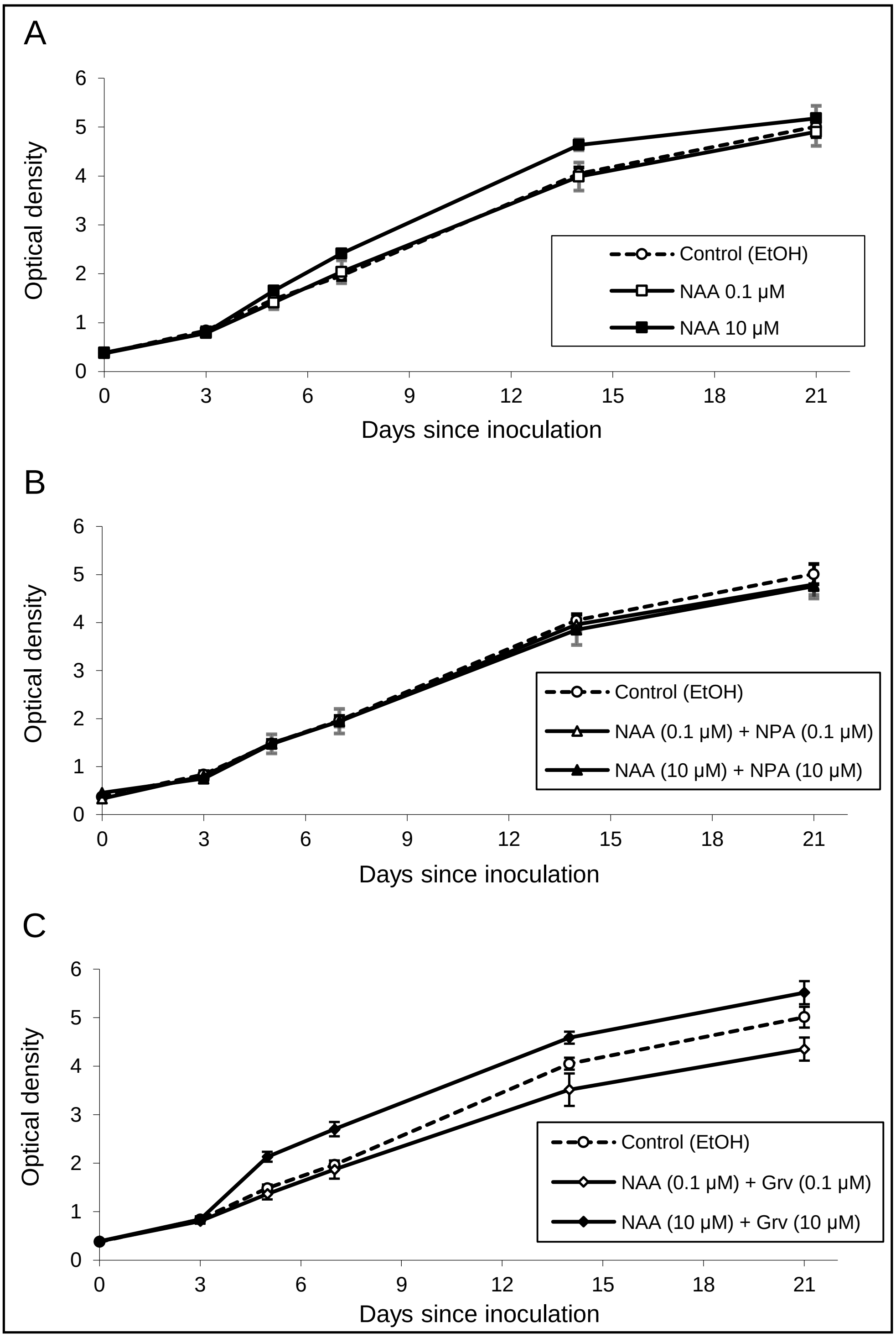


Figure 11. Optical density and its changes in *Chlorella lobophora* cultures exposed to exogenously applied NAA, NPA and gravacin. (**A**) Average optical densities for cultures cultivated in a medium with NAA (0/0.1/10 µM). 21-day interval. (**B**) Average optical densities for cultures cultivated in a medium with equal concentrations of NAA and NPA (0/0.1/10 µM). 21-day interval. (**C**) Average optical densities for cultures cultivated in a medium with equal concentrations of NAA and gravacin (0/0.1/10 µM). 21-day interval. All cultures came from the same inoculum. Control is the same for A, B and C. Each variant (curve) represents average optical density values of three separate cultures.

4.2.2 Effects of exogenously applied auxins on *Spirogyra sp.* cell length

Spirogyra sp. cultures were cultivated in media enriched with NAA or IAA in multiple concentrations from 0.1 µM to 50 µM. Controls were shared for both treatments. The cultures were observed by microscopy and their cell length measured by image analysis.

Very high concentrations (compared to land plants) of auxins exhibited a significant effect on *Spirogyra sp.* cell length. NAA (50 µM) markedly increased the average cell length at all stages of cultivation (Figure 12). By contrast, IAA (50 µM) decreased the average cell length at all stages of cultivation, except for the 3rd day of cultivation where an opposite effect was shown (Figure 13). For statistical evaluation for individual auxin concentrations see Tables 7 and 8.

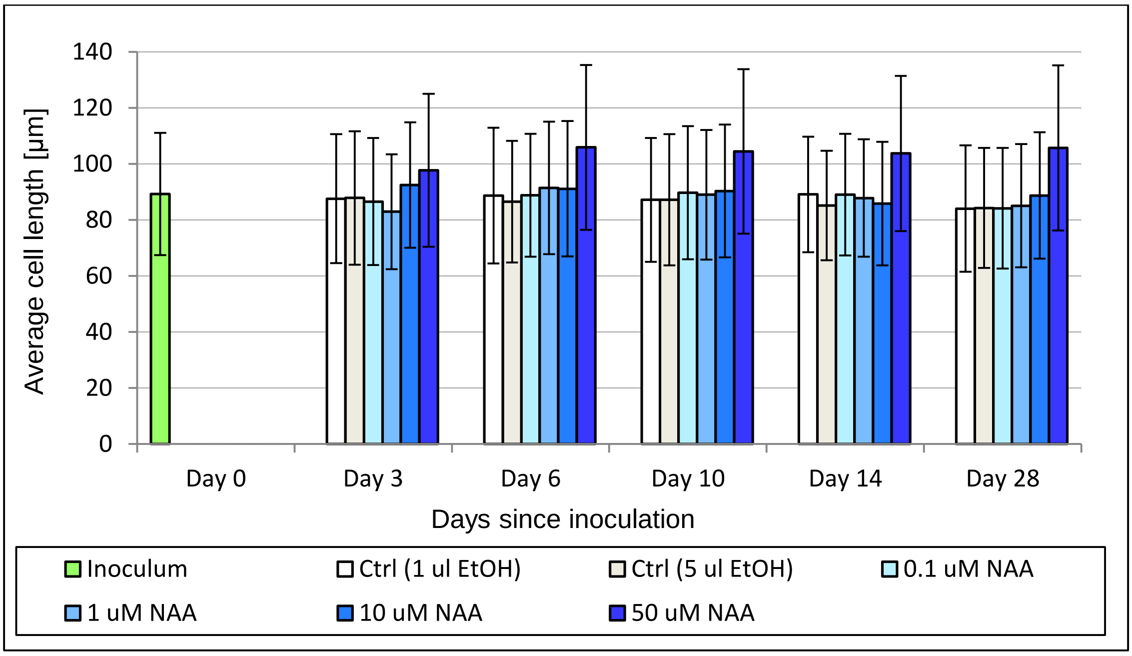


Figure 12. Changes in cell length of *Spirogyra sp.* cultures during a long-term exposure to exogenous NAA. Each concentration sample represents one culture, which average cell length was observed during a 28-day interval.

	NAA concentration [µM]				
	0	0,1	1	10	50
Day 3			**	**	***
Day 6			*		***
Day 10					***
Day 14					***
Day 28				**	***

Table 7. Statistical evaluation of cell length differences between NAA-treated samples and the average of the two used controls by Tukey's HSD test. *** stands for P<0.001, ** for P<0.01 and * for P<0.05, which is the threshold of statistical significance.

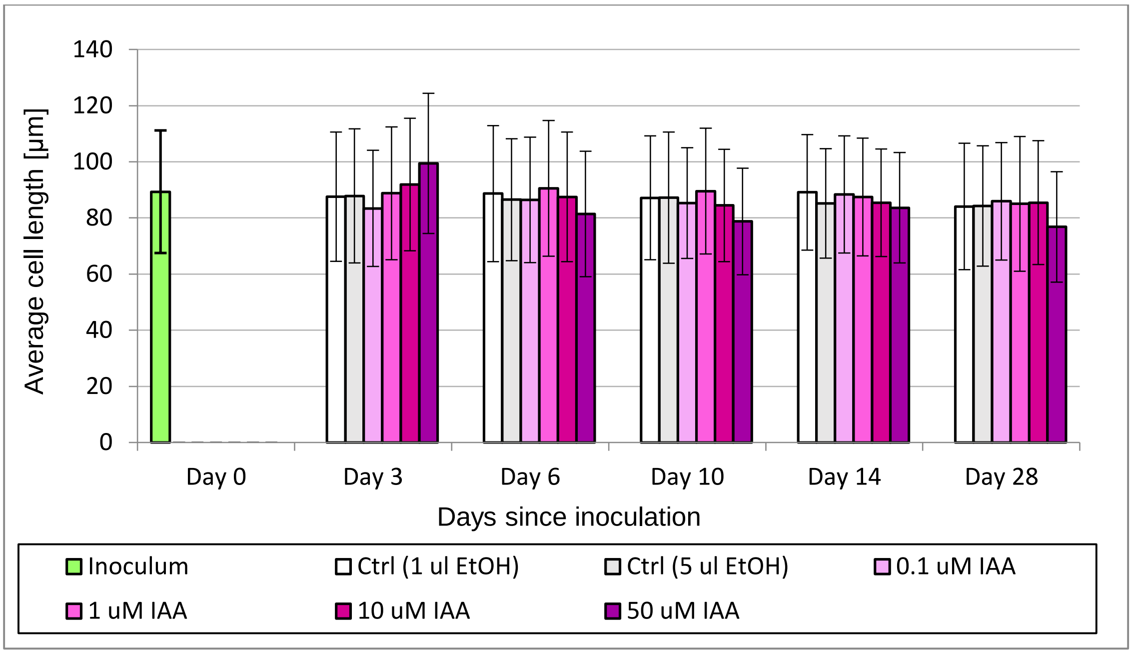


Figure 13. Changes in cell length of *Spirogyra sp.* cultures during a long-term exposure to exogenous IAA. Each concentration sample represents one culture, which average cell length was observed during a 28-day interval.

	IAA concentration [µM]				
	0	0,1	1	10	50
Day 3		*		*	***
Day 6					***
Day 10					***
Day 14					*
Day 28					***

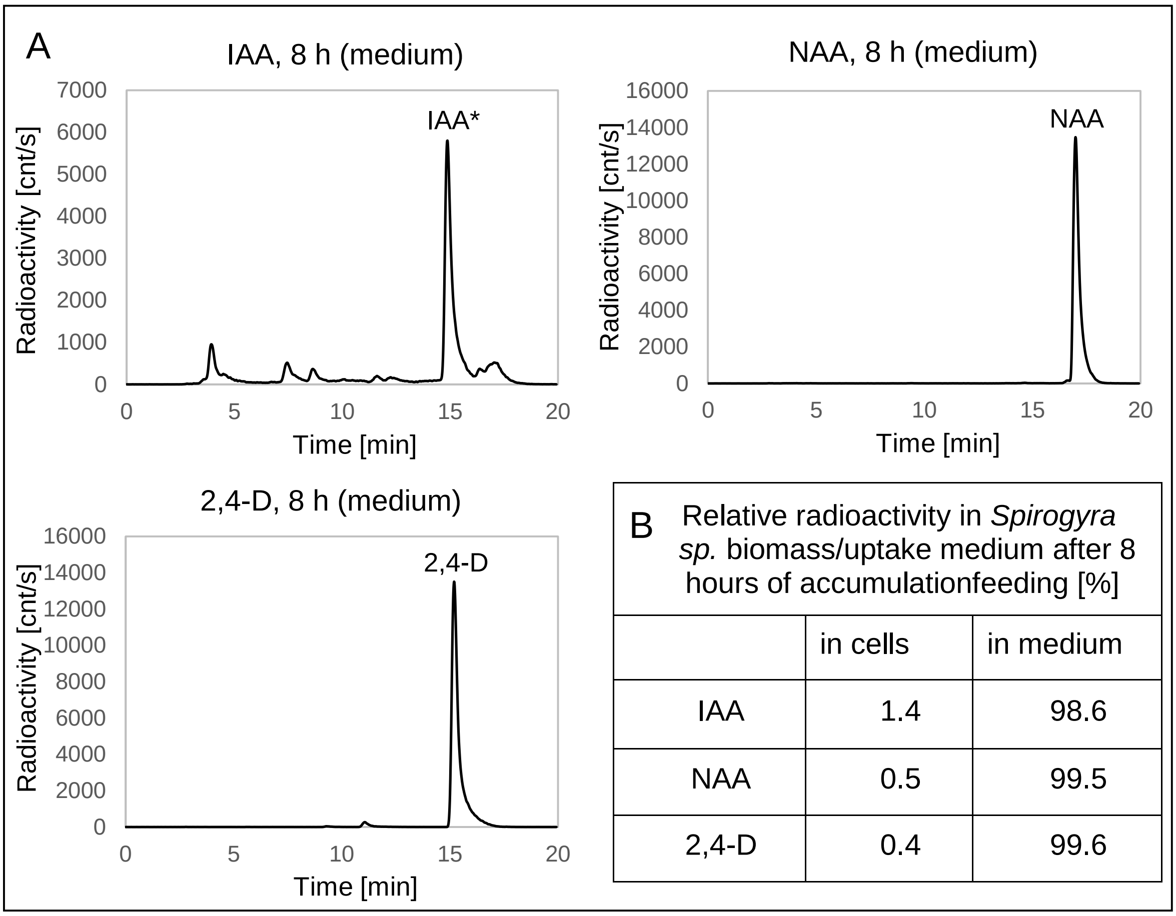
Table 8. Statistical evaluation of cell length differences between NAA-treated samples and the average of the two used controls by Tukey's HSD test. *** stands for P<0.001, ** for P<0.01 and * for P<0.05, which is the threshold of statistical significance.

4.3 Determination of auxin metabolism and transport in *Spirogyra sp.*

4.3.1 Metabolic changes of exogenously applied auxins in *Spirogyra sp.*

Auxin metabolic profiles in *Spirogyra sp.* biomass were investigated by feeding it radioactively-labelled auxin, taking samples at different times since addition, then purifying the auxin fractions from samples and separating them by HPLC. Medium samples were analysed as well.

2,4-D and NAA and IAA did not undergo metabolic changes in the uptake medium after 8 hours of incubation (Figure 14A, B, C). There were none other than 2,4 D and NAA peaks present in the *Spirogyra sp.* biomass profiles for these auxins (Figure 15A), implying that no metabolic changes took place. By contrast, there were multiple peaks present in the IAA *Spirogyra sp*. biomass profile (Figure 15) at different times of auxin feeding. The biomass took up relatively small fraction of auxins after 8 hours of feeding (Figure 14B).



B Relative radioactivity in *Spirogyra sp.* biomass/uptake medium after 8 hours of accumulationfeeding [%]

	in cells	in medium
IAA	1.4	98.6
NAA	0.5	99.5
2,4-D	0.4	99.6

Figure 14. HPLC chromatograms of auxin metabolic profiles in the uptake media for *Spirogyra sp.* biomass and comparison of auxin fractions in cells vs. in media after 8 hours of auxin feeding. (A) Metabolic profiles of radioactively-labelled auxins in the *Spirogyra sp.* biomass auxin uptake media. The position of the peak here identified as IAA does not match its standard retention time, for which an explanation has not yet been found. (**B**) Relative values of radioactivity in biomass/uptake medium after 8 hours of auxin feeding (%).

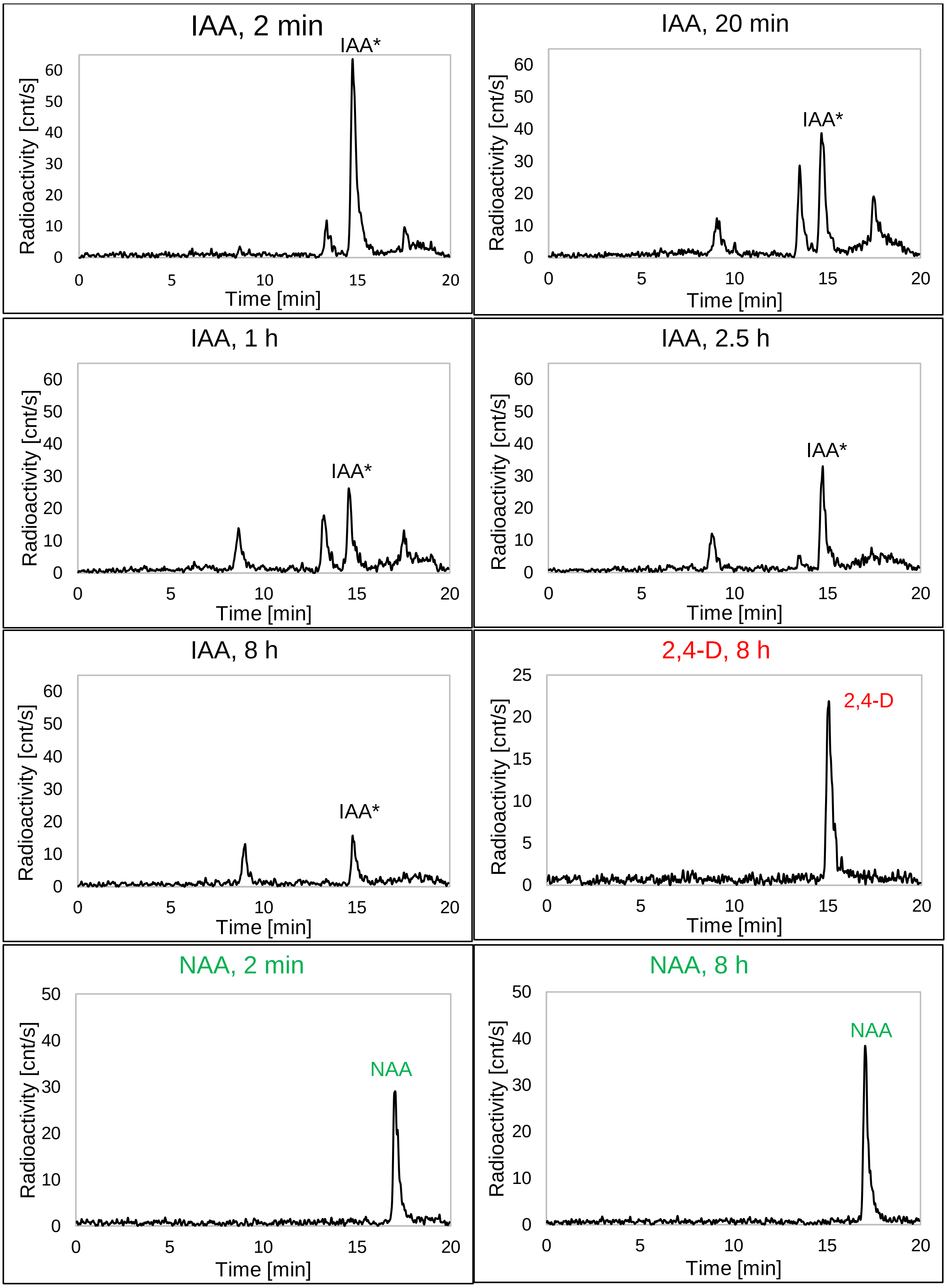


Figure 15. HPLC chromatogram of auxin metabolic profiles in *Spirogyra sp.* biomass. Retention times at labelled peaks correspond to particular auxins, except for IAA, which profile seems to be shifted by one minute. The times in chart headlines represent the time *Spirogyra sp.* biomass was fed with the particular auxin.

4.3.2 Auxin transport in *Spirogyra sp.*

Accumulation assays of radioactively-labelled ^{3}H-NAA and ^{3}H-IAA in *Spirogyra sp.* biomass were performed. It was tested for whether unlabelled auxins and auxin transport inhibitors used in land plant material would affect auxin accumulation in *Spirogyra sp.* by competition or inhibition, respectively. Decreased radioactively-labelled auxin accumulation by another auxin would hint at competition among them for a shared uptake mechanism. If the same was caused by an auxin transport inhibitor, this uptake mechanism would be possibly inhibited by a similar mechanism it happens in land plants. The same would be true for efflux mechanisms, if increased auxin accumulation was observed. The significance of obtained results was tested for by ANOVA statistical testing.

The uptake of ^{3}H-NAA showed quick saturation (Figures 16, 17, 18). The uptake of ^{3}H-IAA continued for the entire duration of measurements (Figures. 19 and 20), suggesting IAA undergoes some change in *Spirogyra sp.* cells, which allows for continued uptake. Results that follow are summarized in Table 9. No inhibition of auxin efflux was identified at all. By contrast, auxin uptake inhibition caused by inhibitors and unlabelled auxin molecules was observed. The uptake of ^{3}H-NAA is inhibited by gravacin, unlabelled NAA (Figure 17), 2,4-D, 4-Cl-IAA (Figure 18) and unlabelled NAA. The uptake of IAA was inhibited by CHPAA, 2,4-D, NAA (Figure 20), and likely by unlabelled IAA and gravacin (Figure 19) as well. Collectively, these results suggest a non-specific auxin uptake mechanism. Judging from gravacin inhibition, it might be mediated by ATP-binding cassette transporters. Dead *Spirogyra sp.* biomass accumulated about 10 to 20% of ^{3}H-NAA compared to live cells (Figure 21).

Auxin	**Inhibition of:**	**By:**
^{3}H-NAA	uptake	gravacin, 2,4-D, 4-Cl-IAA, NAA, IAA
	efflux	none
^{3}H-IAA	uptake	CHPAA, 2,4-D, NAA, IAA*, gravacin*
	efflux	none

Table 9. Summary of auxin transport assays in *Spirogyra sp.* ^{3}H-IAA uptake inhibition by IAA and gravacin was not shown as statistically significant ($P<0.05$), but this was likely because of insufficient number of samples.

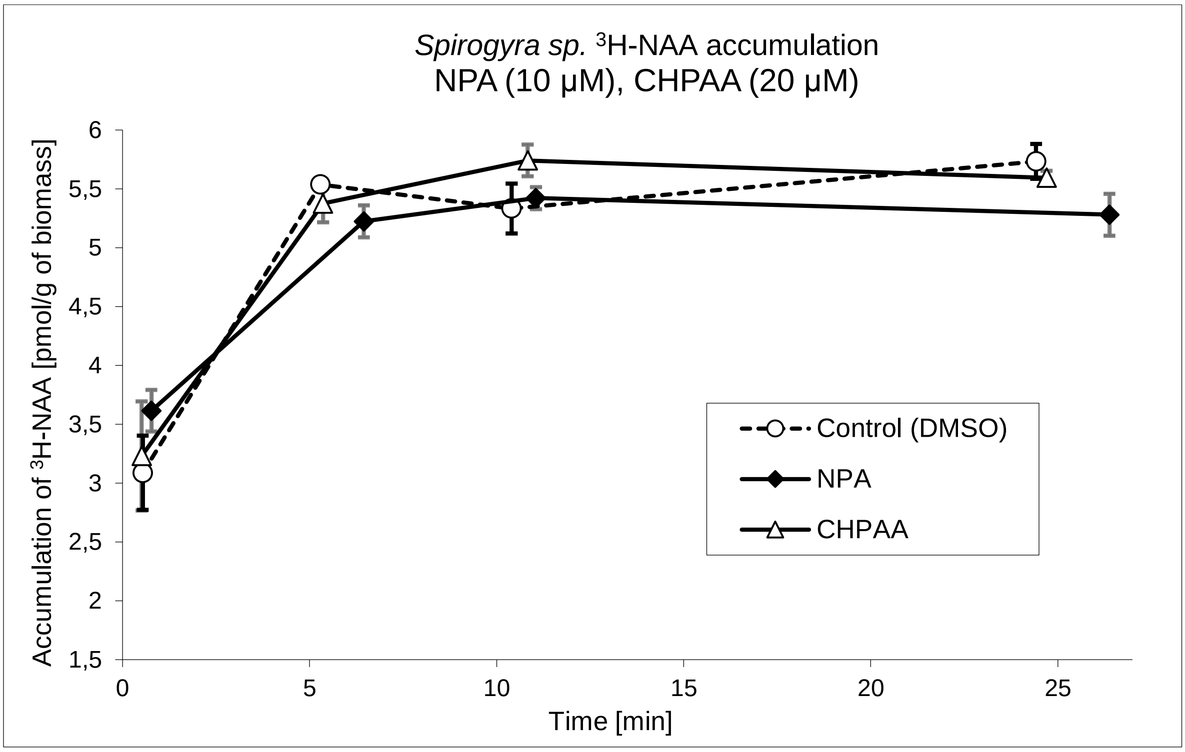


Figure 16. The effect of NPA and CHPAA on the accumulation of radioactively labelled ^{3}H-NAA in *Spirogyra sp*. No effects were observed.

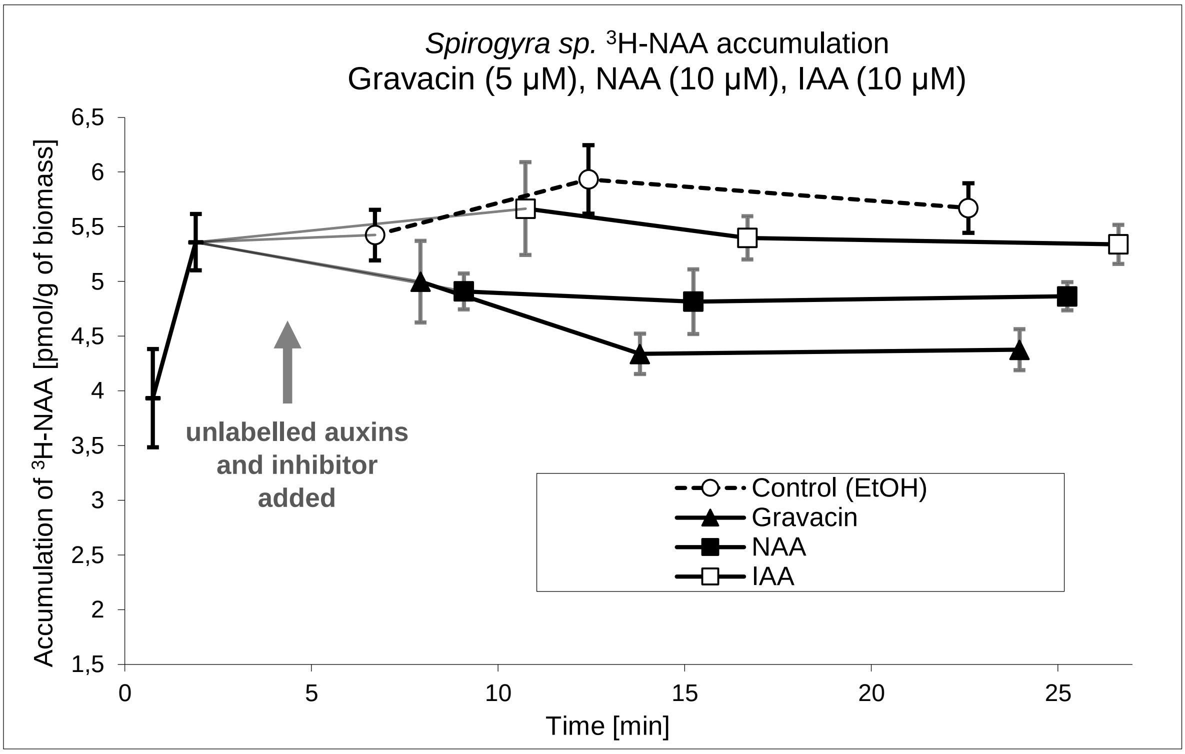


Figure 17. The effect of gravacin and NAA and IAA on the accumulation of radioactively labelled ^{3}H-NAA in *Spirogyra sp*. Gravacin and unlabelled NAA inhibit the accumulation of ^{3}H-NAA.

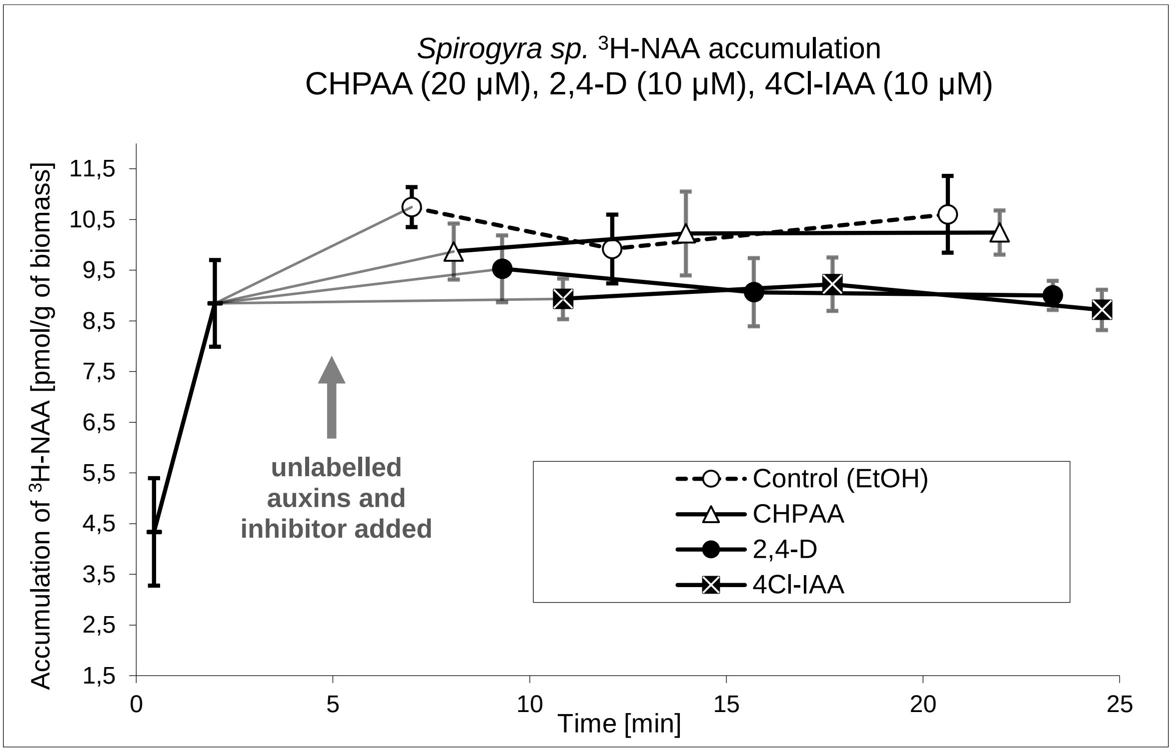


Figure 18. The effect of CHPAA and unlabelled 2,4-D and 4Cl-IAA on the accumulation of radioactively labelled ^{3}H-NAA in *Spirogyra sp.* Unlabelled 2,4-D and 4Cl-IAA inhibit the accumulation of ^{3}H-NAA.

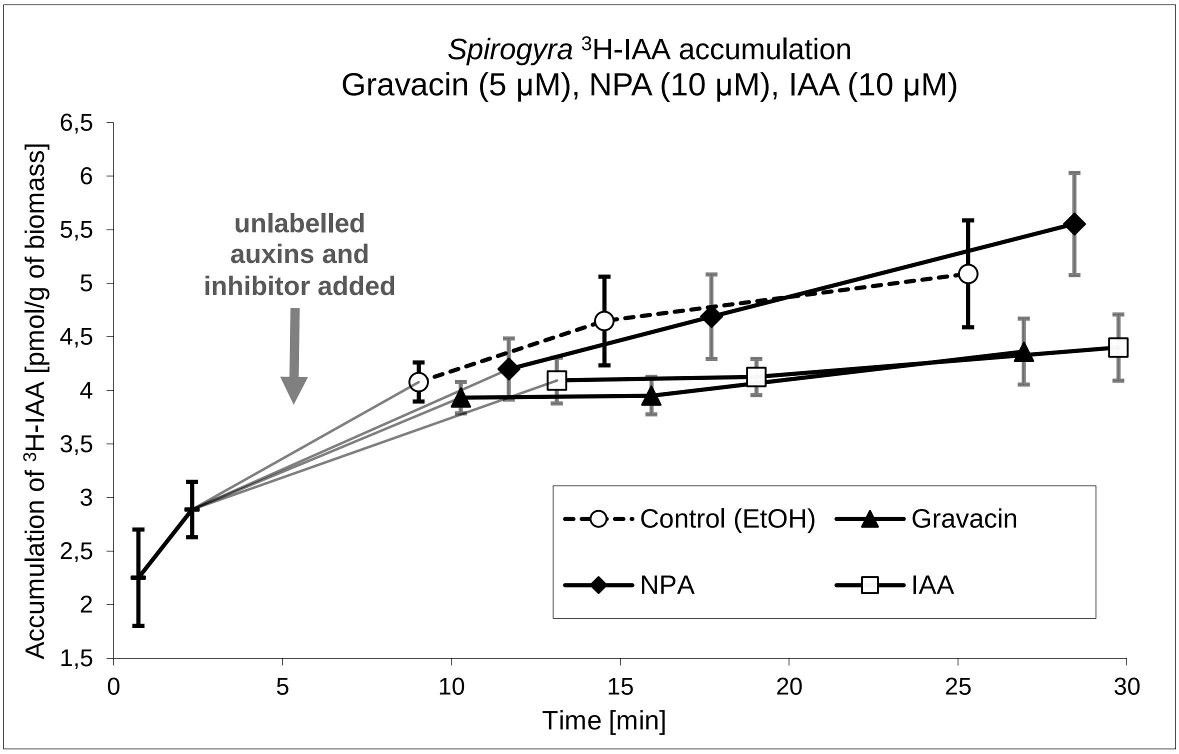


Figure 19. The effect of gravacin, NPA and unlabelled IAA on the accumulation of radioactively labelled ^{3}H-IAA in *Spirogyra sp.* IAA and gravacin do not inhibit the accumulation of ^{3}H-NAA significantly ($P>0.05$) in statistical testing, though this was proposed by to be the result of an insufficient number of samples.

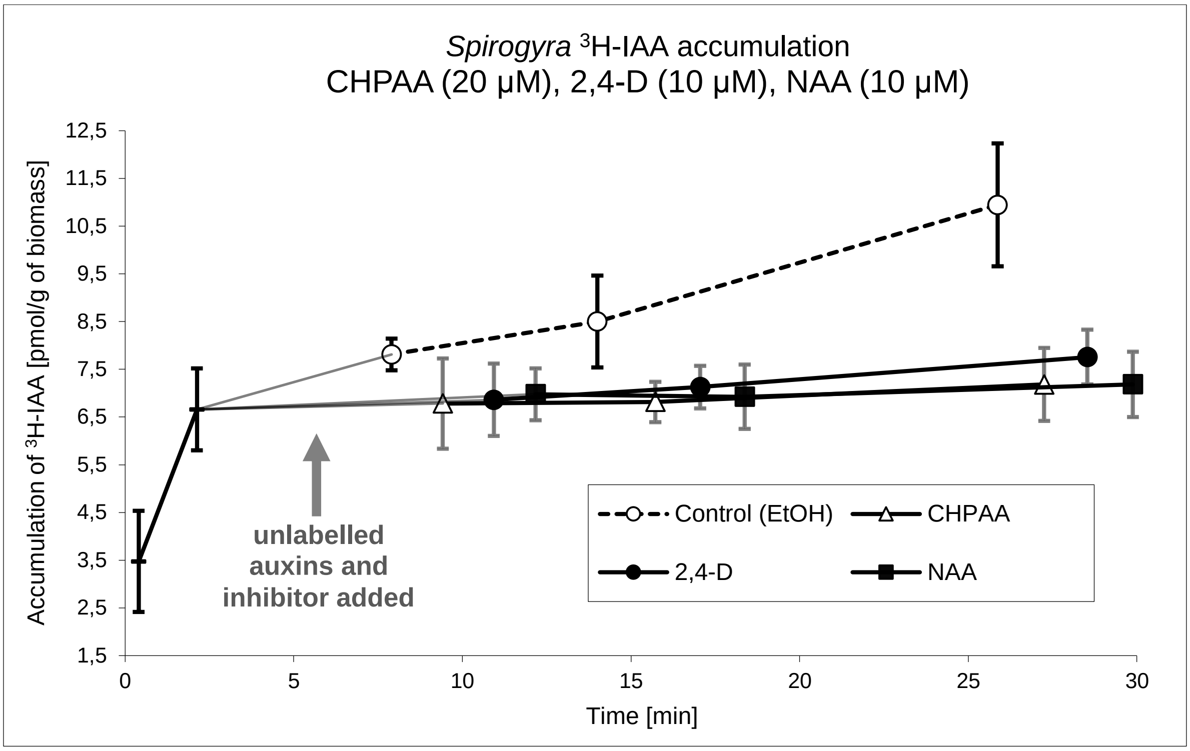


Figure 20. The effect of CHPAA and unlabelled 2,4-D and NAA on the accumulation of radioactively labelled ^{3}H-IAA in *Spirogyra sp.* CHPAA, unlabelled 2,4-D and NAA all inhibit the accumulation of ^{3}H NAA.

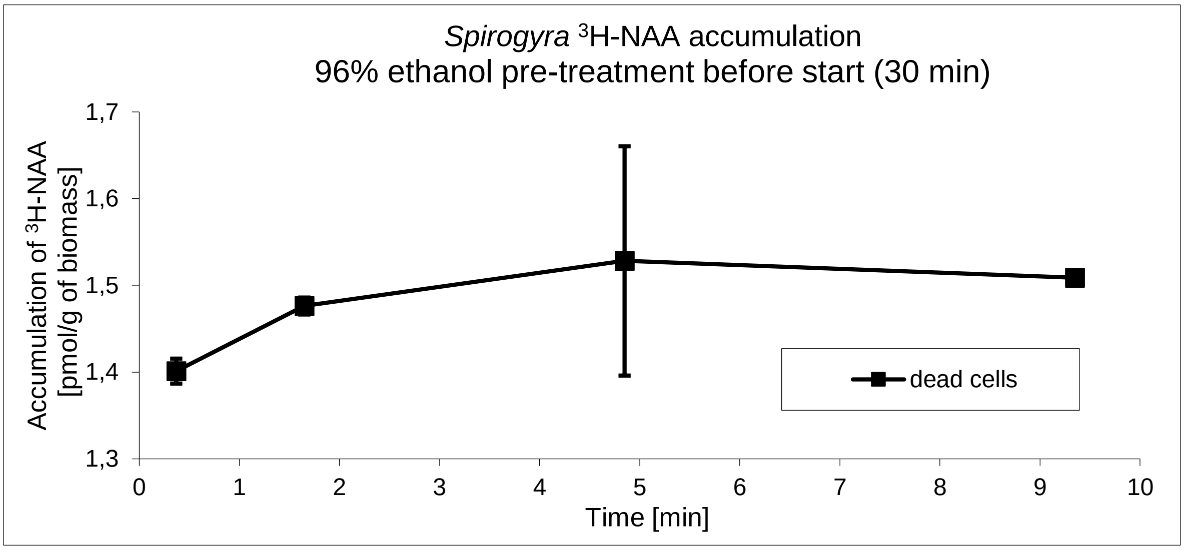


Figure 21. The accumulation of radioactively labelled ^{3}H-NAA by a dead *Spirogyra* sp. biomass. This experiment set the 1.5 pmol/g threshold that serves as the minimum value for our ^{3}H-NAA charts and was also adopted for the ^{3}H-IAA charts.

4.4 Isolation of plant auxin transporter protein orthologs from *Spirogyra sp.*

Isolation of putative orthologs of *Arabidopsis thaliana* PIN1 and ABCB19 proteins from *Spirogyra sp*. DNA was attempted. For sequences of used primers see Tables 2 and 3 in chapter 3.7.

Primers for PIN1 ortholog failed to produce PCR products (not shown). One set of primers for the ABCB19 ortholog *(Primers ABC2073)* did produce a product (Figure 22A). This product was amplified by PCR and sequenced. A part of sequence on each side of the fragment was obtained, allowing for designing new primers specific to *Spirogyra sp.* strain in our culture *(Primers SpiroABC-New)*. Using these, PCR product was obtained (Figure 22 B), cloned into pJET plasmid vector and transformed into bacteria. After plasmid isolation and excision, it showed that one bacterial colony was transformed with the insert (Figure 22 C). It was sequenced using pJET1.2 vector specific primers. About1 kbp sequence on each side of the fragment was obtained. To determine the still missing middle region, the obtained peripheral sequences were blasted to assess the positions of exons and new primers were designed close to the unknown middle region *(Primers SpiroABC-Q1-F, SpiroABC-Q2-F)*. The following sequencing provided the middle region sequence. The sequence of *Spirogyra sp.* ABCB19 ortholog fragment is now a 2830 bp long (Figure 23), still lacking start or stop kodon. For alignment overview with *Arabidopsis thaliana* ABCB19, see Figure 24.

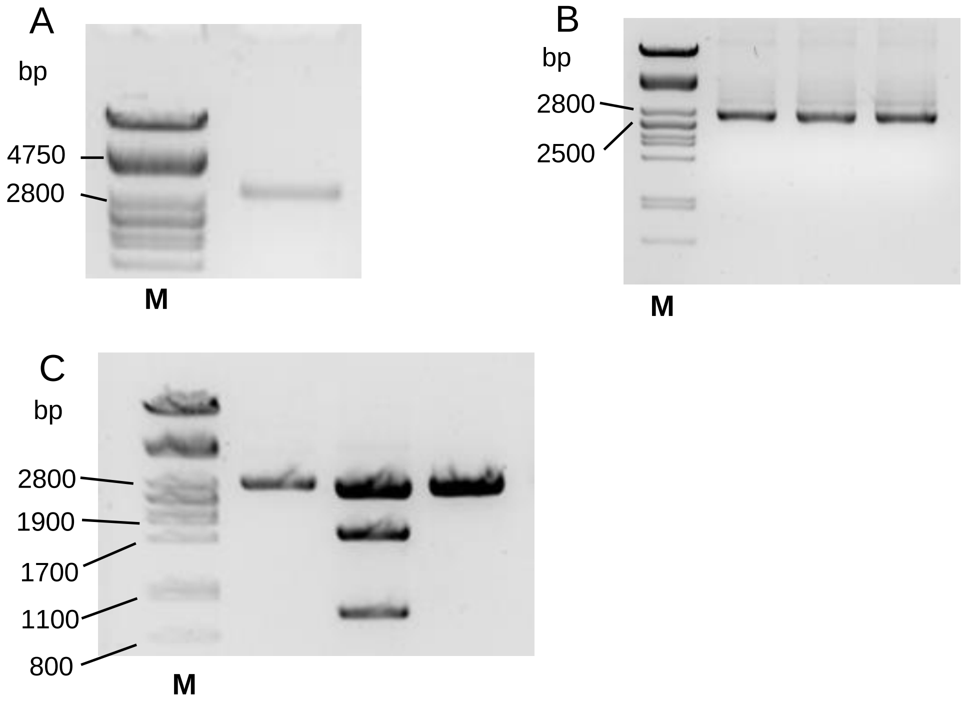


Figure 22. Isolation of *At*ABCB19-orthologous DNA sequence from *Spirogyra sp*. genomic DNA. (**A**) Product after using primers designed for a different *Spirogyra* strain than *Spirogyra sp.* (**B**) Product after using primers designed specifically for *Spirogyra sp.* strain. Left-to-right: 54-58-62°C annealing temperature gradient. Dim bottom bands is non-specific primer binding. (**C**) Amplification of the same product in bacteria. Top three bands are pJET vector. Two lower bands in the middle collectively correspond to *Spirogyra sp.* ABCB19 ortholog fragment, which was cut by restriction enzymes when excising the insert from a plasmid. M – marker.

Putative *Spirogyra sp.* ortholog of *Arabidopsis thaliana* ABCB19 (2830b):

```
5´-CGGTTCGATCAATCCAATGTTTGGATTATTCTTAACTTCGGTATGTTTTCGATAACAATCGTCTCTCGCTGTTCA
CCGTCTTTTTTTTCATTCTGCTTTTTTCTGTTAGCTTCCTTCCTTTTGCACAGTTGAGCTCGATCTAGCCCTTTCGAA
TTCATCCCTATCTTCATTTCAACCACCCCCATCATCGTACTTTCTCTTCATTAGTGATCGTCAAGGTCGAAGAGTTTA
ACACCAAAACTGTTTCTGTTCATGCTCTCCATTCATCCATTCTTTTCTTTTTGCTTCATTGAAAATGAACATCTCCCG
GATGGTTAACTCTGCCGTCTTCCTTGCATGACCCCTTCTTCCTCTTCTTCCTTCTCTTCCTCTCAGGTGATCGTTGCT
TATTACAAACCCGCTTCTGAGATGACTTCGGAGGTTCGGAAATGGTGCTTCGTGTTTATCGGCTTGGGATTCGTTTCC
ATCTTTGTTTACATTCTTCAGCATTATAACTTTGGAGCAATGGGGGAAAGACTCACCAAACACATCAGAGAGCTTATG
TTCGAATGTATGTTCGACTCAGTGATTCCCCTCTCTCTCTCTGATTTCATCTTTTTTACACCAATCCGATTCGATCAT
CGATGTTTCAACCATCAAATTGTATGTTTTGGTCATCGAATTCGATGTTTTTTGTCTTAATCTCCCTGTGTCTGTAAT
TTATGATTCTATTTTCCTTCATTTCATTCTGTTATGCACTCTATATGCATATCTCACAGTAAAACATACATGCCATTT
TACTCCATCTTCATGTTATTTCGATGTTTCCTCCATGGTTCTTGACCATTTCTAGTCTCACTCCCTTCACTTTTCTTT
ACTATTTTTCATTTTTTTTTCCGTTTTTCCTCTTCCTGTTCTCTTCAGCAATCTTAAAGAATGAGATTGGTTGGTTCG
ATCGAGATGAGAACAGTGCAGGAGCCATTACTTCTAGACTTGCTAGTGAAGCAACACTCATTAAAGCAGCTAGCAGTG
ATAGACTCTCTGTTCTCGCTCAGAATCTCTCGGTAGTGATAATCGCTTTCTCCATAGCCATTATCCTTCAATGGAAAA
TGTCCTTAGTTGTCATCGCCACCTTCCCTCTCCTCATCCTTGCAGCCATAGCTCAGGTACCACCATCCCTCTCCCTCT
TCCCACATCCCTCTCCTTTCCGATCACCTTTTTTTTATCAATCTGTATGGTTAAGTCTCTGGCCTTCAGGTGTGCATC
TACCCCGTGAACATTAACTTCATCCTTCTCCACCGCTTGTTTTTTTCAGCAAATGTTCCTGAAGGGGTTCGCAGGAGA
TTTGAAAAAGTCTCACGAAAAGGCAAGCCAAGTCGCAGGAGAGGCAGTCGGAAACATTCGAACTGTCGCTGCCTTCTC
TGCAGAGAGCAAGATCCTCCATCTGTTCAATCTCCAGCTCGAAGGACCTCTCAAACATTCCTTCAAGAAAGGCTTTGT
CGTAGGCTTGGCCTTTGGCTTATCTCAGGTACTATCCTCCTTCCGTTCGCATTATCCTCATCATCTTCGTCCTCATCT
CTCCTTCCCGATCGTCTTATTCTCCGTCTTCTTCACGAGCTCATTCCTGCGTGTTCGTCCTCTATTATTGCATATTCT
TTTAGCTATGCATGTACGGAGCCTACGGCTTAGGTCTGTACTACGGAACGACCCTGGTTCCGGACGAGACTCCTTTCG
ATCGCGTGATCAAAGTGTTCATGGTGTTGATCATCACCGCGTTCGCCATAGCGGAAACCATCACCTTAGCTCCGGACC
TGGCCAAGGGTGGGTTTGCGATCCGCTCGATCTTCGATCTGCTCGATCGACGGTCAGAGATCGATTCCGATGCTGCGG
AGGGGATCAAGCCGGATAAAATCGTCGGCCAGATCGAGCTTCGACGGATCAGATTCGCGTATCCATCTCGACCGGAGA
TTACGATTTTCGAGAGTTTCGATCTGTTGATTCCTGCGGGTCGGACTGTGGCTTTGGTAGGGGCGTCAGGTTCGGGAA
AGAGCTCCGTGATCTCCCTCGTCGAGAGGTTTTACGACCCTCTGGATGGGAAAGTGATGATCGACGGGAAGGATATTA
AGAGTTATAATCTGAGAGCCCTTCGCAAGTTCATCGGGTTGGTTAGTCAGGAACCTGCCCTCTTTTCATCCAGGTCTT
TCTTTATGACTTAACGTTATATTTTTCCTTTTTTTGCTTTCCAAGATGTTACAAGTCAGTTTTATGCTCCATGTGATA
TTGGTAATAATCGTTTTTTTTATCATATCGATTCCTTTTTGTATTAGCATTCGTGAGAATATACTCTATGGAAAAGAT
AATGCTTCGGAAAGCGAGGTTATGGAGGCAGCTAGGTCTGCAAATGCTCACAATTTCATCTCAAGCTTCCCTAATGGA
TATGAAACAGAGGTATGGTGCTTACTCAATTATTATATTTAGGTTTGGATATTGTATTTTTTAGTACTTCACATTTAT
TATTACTCTGGTTGACTTCAACTCCCCTTGTTCAGGTTGGTGAGAGAGGAGTTCAAATGTCTGGTGGTCAGAAGCAAA
GGATAGCCATTGCCAGGGCCGTGTTGAAGAATCCTTCCATCCTTCTCCTTGACGAAGTCTGAATTCTTTCCTTTTTGA
TGCCTCGTTTACGTTCTAATACCTTTGAATTCAAATGTGAGATTCGTGTTGTGAGTTTGCTGGGTGTTAAATCCTTAT
TTGTTAATCCGTTTTGATATCGCAGGCGACTTCTGCATTGGATTCTGAATCAGAAAAGGTGGTTCAAGAAGCTCTGGA
TAGATTAATGGTTGGAAGAACCACG-3´
```

Figure 23. A fragment of *Spirogyra sp*. gene orthologous to the *Arabidopsis thaliana* gene for ABCB19 auxin transporter, isolated from *Spirogyra sp.* DNA.

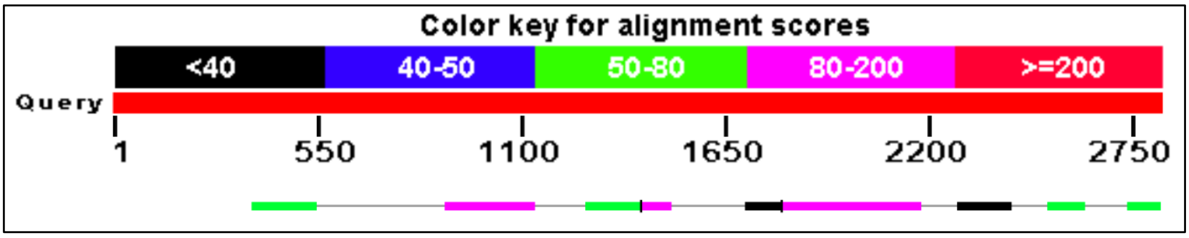


Figure 24. Alignment of the putative *Spirogyra sp.* ABCB19 ortholog to *Arabidopsis thaliana* ABCB19. Compared on the basis of translated nucleotides by tblastx.

5 Discussion

5.1 Establishment of *in vitro* cell cultures of algae

In nature, *Chlorella lobophora* lives in soil rather than water. It was decided to keep it in a liquid suspension in our culture to better control cultivation conditions. It did not seem to be detrimental to the algae in any way. The most rapid growth phase between 1st and 2nd day after inoculation represents an ideal point to conduct experiments on *Chlorella lobophora* culture growth and cell division. Because cell counting gets time consuming with growing number of samples, it was investigated whether the optical density of *Chlorella lobophora* cultures stably reflected cellular density during a subculture interval. It was found that the optical to cellular density ratio is different on different days of subculture interval, but the values are constant for multiple cultures coming from the same inoculum. As such, optical density can be used to observe notable growth differences among cultures from the same inoculum.

Spirogyra sp. naturally grows as a clumped biomass floating just under the water surface. It was kept it as a liquid suspension in our culture. Constant shaking prevented the biomass from floating to the water surface and aggregating, while also keeping all cells in the same conditions. *Spirogyra sp.* suspension cultivated in this manner can be pipetted nearly equally above 1 ml of volume using cut pipette-tips. It must be noted, however, that this is not the natural habitus of *Spirogyra sp*. Cultivation temperatures and perhaps the lack of winter diapause also may not be optimal. *Spirogyra sp.* cultures show the most rapid growth around the 10th day after inoculation, with another growth boost after about one month, when the biomass density is high and it starts aggregating. I speculate that increasing biomass density is a growth stimulus, conferring the need for filaments to outgrow each other to reach the sun just below water surface. Phototropic bending and growth would come to mind in this regard, but this has not yet been investigated. Furthermore, it was found that *Spirogyra sp.* biomass markedly increases the pH of culture medium. If the same happens in natural still water habitats, it may demonstrate a competitive mechanism. By increasing water pH, *Spirogyra sp.* would reduce photosynthetic carbon availability to other photosynthetic organisms (for photosynthesis in water environment see Raven, 1970).

5.2 Growth response of algal cultures to long-term exogenous auxin exposure

There was no variation in *Chlorella lobophora* culture growth if NAA was added into the medium. This is inconsistent with Hunt *et al*. (2011) study, where the growth of *Chlorella sorokiana* cultures positively responded to NAA. Other studies demonstrated that *Chlorella* growth responded to both exogenously applied IAA (Yin, 1937; Pratt, 1938) and 2,4-D (Saygideger & Okkay, 2008). NPA (polar auxin transport inhibitor) and gravacin (ABCB inhibitor, Rojas-Piece *et al.*, 2007), both added into culture media together with NAA, also exhibited no effects on *Chlorella lobophora* culture growth in our experiments. It seems that *Chlorella lobophora* either does not respond to NAA and auxin transport inhibitors, or it must be added in comparably higher concentrations than in other *Chlorella* species. Although a possible medium pH shifting by *Chlorella lobophora* was not investigated and a buffering agent was not added into the experimental medium, it is very unlikely a freshly inoculated culture could shift the culture medium quickly enough to hamper NAA uptake by diffusion for no growth effects to take place after the first day since inoculation.

The average cell length of *Spirogyra sp.* was subject to NAA- and IAA-mediated changes in our experiments, though only at a very high concentration compared to land plant material (50 µM). While NAA markedly increased the average cell length, IAA decreased it less notably, but still very significantly. It is not clear why NAA and IAA should act in the opposite fashion. NAA and IAA rather shifted the distribution of cell length categories to higher fractions of long and short cells, respectively, than affecting all cells equally. Adolfová & Vosolsobě (2011) likewise observed NAA-mediated cell elongation in *Klebsormidium* and in *Zygnema,* a close relative of *Spirogyra*, albeit at lower concentrations. In their experiment, culture growth was already inhibited by concentrations that affected cell elongation in this experiment. Their algal cultures started dying when exposed to high auxin concentrations. It was also observed in this study that the 50 µM NAA culture reached senescence sooner than other cultures, but a pronounced growth inhibition was never observed in *Spirogyra sp*.

5.3 Determination of auxin metabolism and transport in *Spirogyra sp.*

Metabolic profiles of *Spirogyra sp.* biomass were conducted after feeding it with radioactively-labelled auxins. The results clearly showed that NAA and 2,4-D were not metabolized by *Spirogyra sp.* By contrast, multiple peaks in the IAA profile suggest some metabolic changes did take place in *Spirogyra sp.* cells. While the peaks in IAA profile are somewhat confusing, it can be speculated from their position in front of the IAA peak that these are degradation intermediates. If *Spirogyra sp.* would indeed regulate IAA levels by degradation instead of conjugation, it would support the hypothesis that conjugation evolved in land plants after the split of liverworts (Sztein *et al.,* 1999). Being the only native auxin compound of the three, it would be interesting if *Spirogyra sp.* could only metabolize IAA. NAA is subject to conjugation in tobacco cell cultures (Hošek *et al.,* 2012), but this can hardly be compared as green algae do not perform auxin conjugation (Sztein *et al.,* 1999).

The accumulation curves of NAA showed a quick, saturable uptake by *Spirogyra* cells. By contrast, IAA was being taken up for as long as the measurements were conducted. This would corroborate the hypothesis that *Spirogyra sp.* is able to metabolize IAA: lowering the levels of active IAA inside the cells would allow for additional uptake. The inhibition of NAA and IAA uptake by different auxin molecules suggests a possible shared influx mechanism. No effects were observed for NPA, which is a known inhibitor of auxin efflux in land plants (Thomson *et al.,* 1973). There is both evidence for (Klambt *et al.,* 1992; Boot *et al.,* 2012) and against (Dibbfuller & Morris, 1992) the effect of NPA on polar auxin transport in streptophytes (*Chara*). Altogether, there was no auxin efflux inhibition by any compound observed used in this study. By contrast, uptake inhibition by CHPAA and gravacin was observed. CHPAA is a land-plant auxin influx inhibitor (Laňková *et al.*, 2010) and it only affected IAA accumulation in *Spirogyra sp.* Gravacin is an inhibitor of ABCB transporters and functions as auxin efflux inhibitor in land plants (Rojas-Pierce *et al.*, 2007). Interestingly, it inhibited auxin uptake in this study. ABCB transporters were shown to also mediate influx besides efflux in land plants (Kubeš et al., 2012). Altogether, these results indicate there may be auxin influx carriers present in *Spirogyra sp.*, possibly substrate non-specific ABC proteins.

5.4 Isolation of plant auxin carrier orthologs from *Spirogyra sp.*

Although a putative ortholog of Arabidopsis thaliana PIN1 was identified in the genome of a different strain of *Spirogyra* (De Smet et al., 2011), we could not isolate this fragment from *Spirogyra sp*. However, a fragmented sequence of a putative ortholog of *Arabidopsis thaliana* ABCB19 was isolated from the DNA *Spirogyra sp*. Next course of action is to investigate whether the gene is expressed and to obtain a complete sequence by inverse polymerase polymerase chain reaction. Future prospects are to observe the protein localization in *Spirogyra sp.* cells by using specific antibodies and to test its capability to transport auxin by heterologous expression in plant material. Even though it might not reflect its native function, the capability of an algal ABC protein to transport auxin would be an interesting discovery. The best approach would be, to generate mutants in the gene directly in *Spirogyra sp.*, if that would be possible. If the protein indeed could transport auxin in *Spirogyra sp.*, it would suggest that non-directional, possibly non-specific ABC-mediated auxin transport is an ancient mechanism.

6 Conclusions

The first objective of this study was to establish algal cultures. Growth curves for *Chlorella lobophora* and *Spirogyra sp.* were constructed. It was found that optical density of *Chlorella lobophora* suspensions can be used to compare cellular density between samples from the same inoculum. *Spirogyra sp.* suspension was optimalized for experimental conditions.

The second objective was to investigate the response of algal cultures to exogenously applied auxins. *Spirogyra sp.* cell elongation was found to be auxin responsive, but *Chlorella lobophora* growth did not respond to auxin.

The third objective was to investigate auxin metabolism and transport in *Spirogyra sp.* The alga is unable to metabolize synthetic auxins NAA and 2,4-D, but appears capable of metabolizing plant-native IAA. There is a shared uptake mechanism for auxins in *Spirogyra sp.*, possibly *via* substrate non-specific influx mediated by ATP-binding cassette family transporters.

The fourth objective was to isolate putative orthologs of land plant cellular auxin transporter proteins. A fragment of putative ABCB19 ortholog was isolated from *Spirogyra sp.*, though it yet has to be completed and its expression in the alga investigated.

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