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RESEARCH PAPER

Polar auxin transport: an early invention

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Abstract

In higher plants, cell-to-cell polar auxin transport (PAT) of the phytohormone auxin, indole-3-acetic acid (IAA), generates maxima and minima that direct growth and development. Although IAA is present in all plant phyla, PAT has only been detected in land plants, the earliest being the Bryophytes. Charophyta, a group of freshwater green algae, are among the first multicellular algae with a land plant-like phenotype and are ancestors to land plants. IAA has been detected in members of Charophyta, but its developmental role and the occurrence of PAT are unknown. We show that naphthylphthalamic acid (NPA)-sensitive PAT occurs in internodal cells of *Chara corallina*. The relatively high velocity (at least 4–5 cm/h) of auxin transport through the giant (3–5 cm) *Chara* cells does not occur by simple diffusion and is not sensitive to a specific cytoplasmic streaming inhibitor. The results demonstrate that PAT evolved early in multicellular plant life. The giant *Chara* cells provide a unique new model system to study PAT, as *Chara* allows the combining of real-time measurements and mathematical modelling with molecular, developmental, cellular, and electrophysiological studies.

Key words: cytoplasmic streaming, *Chara corallina*, IAA, polar auxin transport.

Introduction

In higher plants, cell-to-cell polar auxin transport (PAT) of the phytohormone auxin, indole-3-acetic acid (IAA), generates maxima and minima that direct growth and development (Boutte *et al.*, 2007; Zazimalova *et al.*, 2007; Vanneste and Friml, 2009; Grunewald and Friml, 2010; Peer *et al.*, 2011). Although IAA is present in all plant phyla (Cooke *et al.*, 2002; Galvan-Ampudia and Offringa, 2007), including members of the Charophyta such as *Nitella* species (Ross and Reid, 2010), PAT has only been detected in land plants, the earliest being the Bryophytes (Fujita *et al.*, 2008) and most PAT studies have been done in angiosperms and predominantly in *Arabidopsis thaliana*. These studies indicate that, in angiosperms, auxin is produced in the young developing aerial organs and in shoot apical and root meristems, and also that IAA is transported from shoots to roots by files of specialized parenchyma cells in the vasculature. These transport cells express several classes of auxin influx- and efflux-supporting proteins (Vieten *et al.*, 2007; Petrášek and Friml, 2009). Among the influx carriers are members of the AUXIN RESISTANT1/LIKE AUX (AUX1/LAX) proteins, while members of the PIN-FORMED (PIN) and MULTIDRUG RESISTANCE/P-GLYCOPROTEIN ABCD (ABCB/PGP) class are involved in auxin flux out of cells. Auxin efflux can be impaired by specific PAT inhibitors such as 1-N-naphthylphthalamic acid (NPA).

In the Chlorophyta unicellular green algae, ABCB/PGP members have been identified by genome analysis, but these algae have no sequence homology to PIN orthologues (Galvan-Ampudia and Offringa, 2007). The earliest PIN orthologues, based on sequence data, were found in multicellular green algae *Spirogyra pratensis* expressed sequence tag libraries (De Smet *et al.*, 2011). There are few PAT measurements in the pteridophyte and bryophyte lower land plants. Polar IAA transport could be measured in elongating rachis from *Osmunda cinnamomea* fern leaves

(Steeves and Briggs, 1960). PAT was also detected in sporophytes of the mosses *Bartrania pomiformis*, *Physcomitrella patens*, and *Dawsonia superba*, but not in the gametophores of *P. patens* (Galvan-Ampudia and Offringa, 2007; Fujita *et al.*, 2008; Fujita and Hasebe, 2009; Lau *et al.*, 2009).

Although IAA occurs in members of the Charophyta (Sztein *et al.*, 2000) and Charophyta have a 'plant-like' morphology i.e. they have rhizoids, a stem, and branches (Fig. 1), the role of IAA in its growth and development is unresolved. In *Chara*, one study concluded that IAA uptake was not affected by NPA (Dibb-Fuller and Morris, 1992), whereas another reported an enhanced IAA accumulation after addition of NPA to *Chara* explants (Klämbt *et al.*, 1992). These data suggest that proteins involved in IAA transport are present in *Chara*; however, polar cell-to-cell transport of auxin has not been demonstrated in these multicellular algae. The present study investigated the possible presence of PAT in single *Chara* internode cells.

Materials and methods

Materials and growth conditions

Most experiments used *Chara corallina* (Fig. 1), which was originally obtained from Dr. R. J. Reid (University of Adelaide, Australia). *C. corallina* was grown at room temperature in aquaria filled with deionized water and forest soil, under 8/16 light/dark conditions as previously described (Berecki *et al.*, 1999). *Chara vulgaris* var. *longibracteata* was taken from a pond in the botanical garden of Leiden University, Leiden, The Netherlands.

Polar auxin transport

PAT was performed on excised internodes containing an intact nodal cell on both ends. Measurements were performed in Petri dishes filled with molten paraffin in which grooves between two wells were cut. The grooves were sealed at the donor and receiver ends by embedding the internode just before the nodes in a small amount of silicon grease, covered by a small amount of grafting wax, thus obtaining a water-tight seal (Fig. 2). The wells and bridge compartment were filled with MA medium (half-strength MS Macro I and II, FeNa EDTA, B_5 microelements, 1% sucrose, Masson and Paszkowski, 1992) supplemented with 50 mM MES (pH 4.8). ^{3}H-labelled IAA (specific activity 23 Ci/mmol) was added at a final concentration of 2×10^{-7} M to either the bridge compartment or the donor well. Radioactivity of the samples was measured in a LKB liquid scintillation counter.

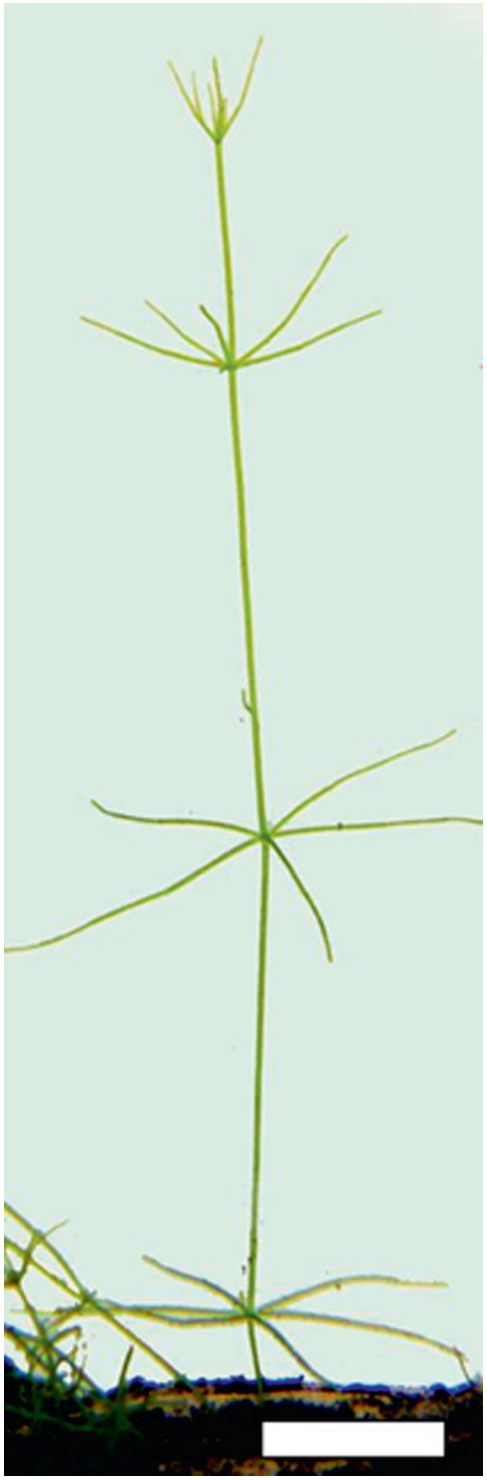

Fig. 1. *C. corallina* as grown in the culture facility used in this study. Isolated internode cells were used in the auxin transport assays. Bar, about 2.5 cm.

Results and discussion

To explore auxin transport properties of *C. corallina* internodes, we measured auxin transport through single and coupled internodal cells placed in a donor–receiver compartment set up (Fig. 2). ^{3}H-IAA could be added to any of the three compartments (donor, receiver, or bridge) and the appearance of the radio-labelled IAA could be measured in samples from the other compartments.

To determine the transport polarity, radio-labelled auxin was added to the bridge compartment and the amount of IAA secreted into either the donor or receiver compartment was measured. *Chara* internode cells showed a strong polarity with respect to IAA secretion (Fig. 3A). The basal side of the thallus internode (directed towards the rhizoid) exported about 50-times more IAA after 1 h than the distal side (Fig. 3A, about 18 and 0.3 pmol, respectively). After incubation with the PAT inhibitor NPA, IAA export was similar in the basal and distal direction (Fig. 3A). Although

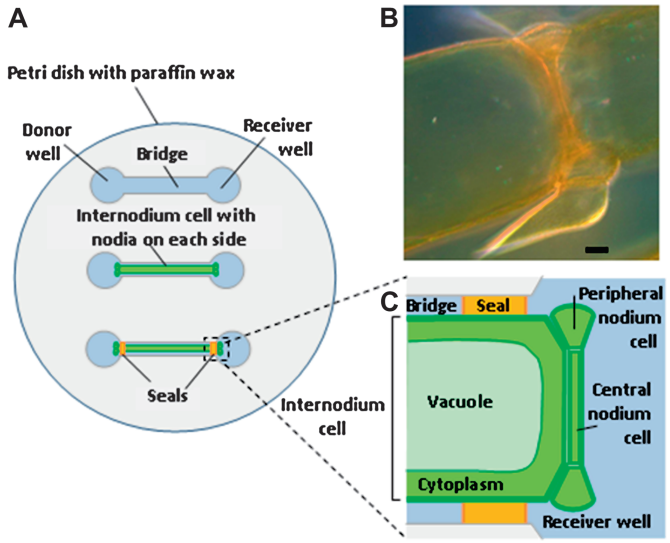

Fig. 2. Experimental set up for measuring polar auxin transport in *Chara* internodes. (A) Schematic representation of the experimental set up. (B) A *Chara* internode–node boundary with attached peripheral cells: bar, 5 μm. (C) Detailed schematic representation of a seal at the receiver well.

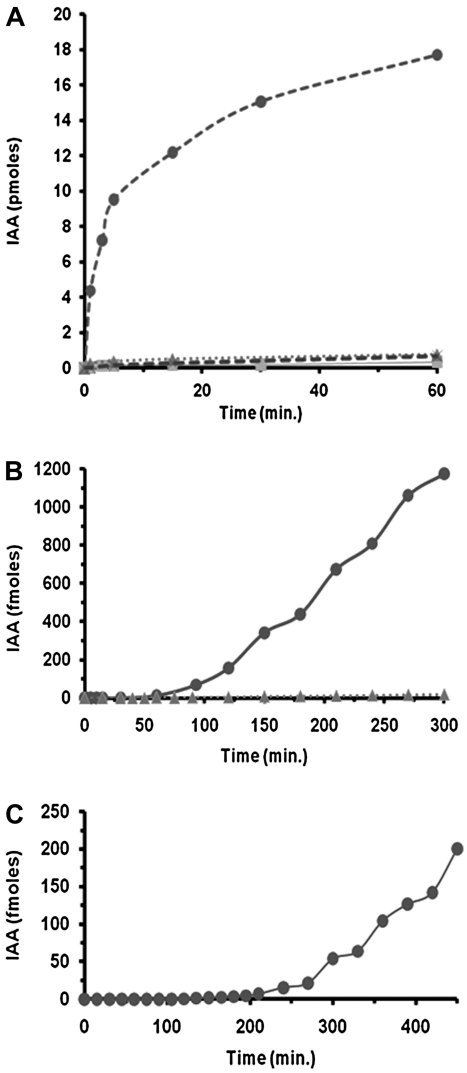

Fig. 3. Polarity of auxin efflux in *Chara* internodes. (A) A typical example showing ^{3}H-indole-3-acetic acid (IAA) added to the bridge compartment and the efflux into the donor and receiver wells was monitored for 1 h. The cumulative accumulation of auxin is plotted against time for the apical side (donor compartment, filled square) and the basal side (receiver compartment, filled circle) of the internode. Besides untreated controls, internodes of *Chara* were pre-incubated for 30 min with 5×10^{-6} M naphthylphthalamic acid. Subsequently, ^{3}H-IAA was added to the bridge compartment and the efflux of IAA in donor (filled triangle) and receiver (×) compartments was determined. (B) A representative example of ^{3}H-IAA added to the donor well, then the receiver well was sampled and ^{3}H-IAA determined by LSC. Cumulative IAA accumulation in the receiver well was only observed in internodes placed in a polar orientation (filled circle). Reversion of the internode in a non-polar orientation displayed no transport of auxin through the internode (filled triangle). (C) Polar auxin transport in two connected internodes of *Chara*. Measurements of IAA accumulation in the receiver were performed as described in (B).

the results were highly reproducible, the amount of IAA accumulating in the receiver wells differed between experiments, with the accumulation ratio between donor and receiver wells ranging from 18 ± 2.5 pmol ($n = 2$) to 64 ± 19 pmol ($n = 2$). These differences may be due to variations in the internodal developmental stages.

In a classical donor–receiver experiment, IAA accumulation in the receiver compartment was measured after ^{3}H-IAA addition to the donor compartment. To confirm the presence of PAT in *Chara*, internode cells were oriented in both directions within the experimental set up. IAA accumulation was only observed with the basal side of the internode in the receiver compartment (Fig. 3B), clearly demonstrating that auxin is polarly transported in internodal cells of *C. corallina*. Similar to land plant stems, auxin was transported from the tip towards the rhizoid. ^{3}H-IAA could be detected in the receiver compartment ~45 min after addition of ^{3}H-IAA to the donor well. The amount of IAA that was polarly transported in different *Chara* internodes ranged from 1 ± 0.4 fmol/min ($n = 5$) to 5.4 ± 2.8 fmol/min ($n = 4$).

The same experimental set up was used to measure PAT in two internodes interconnected by a nodium cell. ^{3}H-IAA was first detected in the receiver wells ~120 min after its application to the donor wells, which was approximately twice as long as the 45 min taken for single cells (Fig. 3C). These results demonstrate that nodium cells support cell-to-cell PAT through the *Chara* thallus.

Interestingly, ^{3}H-IAA taken up from the donor compartment by the *Chara* internode was not only transported to the receiver compartment, but was also significantly lost to the bridge compartment (Fig. 4A). This leaking of IAA may indicate that *Chara* does not possess auxin-influx carrier proteins as observed in higher plants. This is in good agreement with the observation that addition of 2-naphthoxyacetic acid, an auxin influx inhibitor (Lankova *et al.*, 2010), had no effect on the uptake of IAA in *Chara* internode cells (data not shown).

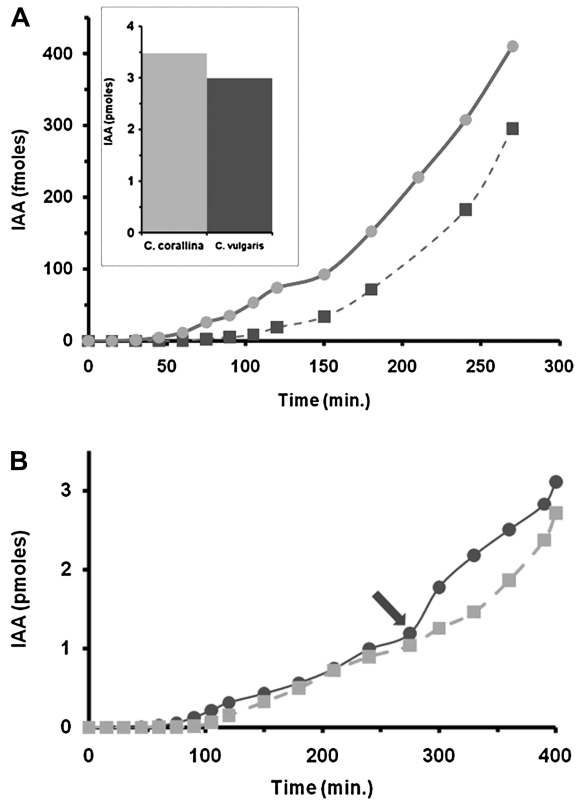

Fig. 4. (A) Polar auxin transport in two species of *Chara*. Auxin transport was measured in *Chara vulgaris* var. *longibracteata* (filled square), which contains cortex cells surrounding its internode cell, and in *Chara corallina* (filled circle), without a cortex. Inset shows the amount of indole-3-acetic acid (IAA) accumulated in the bridge compartments for *C. corallina* and *C. vulgaris* after 270 min. (B) Effect of cytochalasin H on polar auxin transport. Addition of the cytoplasmic streaming inhibitor cytochalasin H (30 μM) to *Chara* internodes after 270 min (arrow) did not inhibit auxin transport (filled circle). Pre-incubation (30 min) with cytochalasin H (30 μM) before ^{3}H-IAA was added in continued presence of this inhibitor did not alter polar auxin transport (filled square).

Blasting the available expressed sequence tag data from *Spirogyra* and *Coleochaete* (Timme and Delwiche, 2010) for AUX1/LAX1–3 influx carriers did not result in any significant homology. The AUX1/LAX auxin influx carriers in land plants are believed to play an important role as drivers of PAT by taking up leaked auxin and thereby preventing leakage to surrounding cells (Reinhardt *et al.*, 2003). This system may not be very efficient in the aquatic habitat of *C. corallina* where internode cells are in direct contact with a large extracellular volume of water. To test the possibility of IAA loss due to a large extracellular volume, the present study used *C. vulgaris* var. *longibracteata*, in which cortex cells around the large internode cell limit the free space around the cell. This variety had comparable PAT and IAA accumulation in the bridge compartment compared to *C. corallina* (Fig. 4A). Hence, the extra cortical cell layer surrounding the internode cell could not reduce the amount of IAA leakage into the medium, suggesting the lack of influx carriers along the internode cell.

Auxin transport velocity in *Chara* internodes varied between 4–5 cm/h, which was higher than the 1–2 cm/h that is routinely measured in *Arabidopsis* inflorescence stems. This ~4–5 cm/h is probably a minimum estimate taking into account the detection level of the first IAA molecules arriving in the receiver, the loss of auxin into the bridge compartment, and the passage of auxin from the internode through the node into the receiver well. In the bridge experiments, the distance from the bridge compartment to the node into the receiver well was typically ~5 mm (including the seal). Within 1 min, a substantial amount of IAA could be detected in the receiver. Assuming an arrival time of auxin of ~30 seconds, the transport velocity would be 57 cm/h. Therefore, the velocity of PAT in the internode is estimated to be somewhere between 3.6 and 36 cm/h.

Internode cell lengths varied between 3.5–5.0 cm, making it unlikely that auxin is transported intracellularly by simple diffusion. For diffusion the expected transport time of an IAA molecule between the donor and receiver end is at best $L^2/2D$, where L is the length of the internode and D is the diffusion constant of a molecule diffusing in a very thin hollow cylindrical region of the internode cell. The diffusion rate of IAA in agar ($D = 7 \times 10^{-10}$ m^2 s^{-1}) (Mitchison, 1980) is a reasonable estimation for diffusion of a 175-Da molecule in a cell (Mika and Poolman, 2011). An IAA molecule would therefore be expected to traverse a 4-cm-long cell in at least 317 h. Hence it is unlikely that, the PAT velocities measured are caused by diffusion.

Cytoplasmic streaming could be an alternative candidate mechanism for the transport of intracellular auxin in *Chara* internodes (Verchot-Lubicz and Goldstein, 2010). The present study measured velocities of ~12–20 cm/h (data not shown) for cytoplasmic streaming in *Chara*. Cytochalasin H, a cytoplasmic streaming inhibitor, has been extensively studied in *Chara* (Foissner and Wasteneys, 2007). In the present experiments, cytochalasin H inhibited cytoplasmic streaming in *Chara* internodes within 5 min, and replacement with buffer reinitiated cytoplasmic streaming after 15 min, with initial velocity regained after 30 min. The effect of cytochalasin H on PAT in *Chara* internodes was investigated by either pre-incubating the internodes with cytochalasin H within the bridge compartment or adding the inhibitor to the bridge compartment after PAT had been ongoing for 270 min. In both cases, no significant effect of cytochalasin H on PAT was observed (Fig. 4B), which is in agreement with the slightly reduced PAT rates using cytochalasin B in oat and maize coleoptiles (Cande *et al.*, 1973). However, cytochalasin H in *Chara* has been reported to only inhibit vesicle transport at the cortical part of the cytoplasm. Inhibition of cytoplasmic streaming of the subcortical cytoplasm takes several days of treatment with cytochalasin H before any effects can be measured (Foissner and Wasteneys, 2007). This subcortical cytoplasmic streaming can reach velocities of 18–36 cm/h, making it a good candidate for the observed intracellular auxin transport. The present study also tested the effect of cytochalasin H on

the subcortical cytoplasmic streaming and found that it could be completely and reversibly inhibited by the addition of the inhibitor. Therefore, cytoplasmic streaming is an unlikely mechanism for intracellular auxin transport in Chara cells. It remains an intriguing question as to what the driving force for this transport in *Chara* is.

The accumulation curve of IAA in the receiver in a classical donor–receiver transport experiment (Fig. 3B) can be described by the solution to a system of convection–diffusion equations with physiologically feasible values for the parameters. The corresponding accumulation curve for a bridge experiment (Fig. 3A), however, was quite different. During the first minute, the flux into the receiver was as high as ~73 mol/s on average, which within a few minutes levelled off to a steady-state value of ~2.5 fmol/s. Up till now, this dynamic behaviour could not be reconciled with the system of convection–diffusion equations describing the classical donor–receiver experiments. One interpretation of the data could be that there is a rapid downregulation of the transporters themselves or the activity of the proteins that are involved in the polar efflux of auxin. A systematic mathematical analysis of the transport data from specifically designed experiments is in progress.

Conclusion

Here we show that PAT is present in Charophyta, an evolutionary ancestor of higher plants (Finet *et al.*, 2010; Timme *et al.*, 2012). The ability to measure PAT at the cellular level provides a unique opportunity to further analyse PAT-related parameters such as membrane potential, cytosolic calcium, and pH levels that drive IAA transport inside the cell. It will be important to determine the proteins responsible for PAT in *Chara* and their relationship to the well-known PIN and ABCD/PGP classes of PAT efflux proteins found in higher plants. It will also be interesting to test the hypothesis that *Chara* lacks AUX1/LAX auxin influx proteins. Understanding the dynamics of PAT in an experimental unicellular system is easier than in a complex cell system such as *Arabidopsis* inflorescence stems. The former is better suited for mathematical modelling and analysis. Moreover, the extreme proportions of the *Chara* internode cell and the experimental set up reveal details of IAA transport that are relevant in reconstructing the transport mechanism and that are hard to observe in *Arabidopsis*. The similarity in PAT between *Chara* and *Arabidopsis* inflorescence stems indicates that PAT is a critical evolutionary early invention of multicellular plants that enables intercellular communication and directional growth.

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