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Plasma membrane H⁺-ATPase regulation is required for auxin gradient formation preceding phototropic growth.

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Abstract
Phototropism is a growth response allowing plants to align their photosynthetic organs towards incoming light and thereby to optimize photosynthetic activity. Formation of a lateral gradient of the phytohormone auxin is a key step to trigger asymmetric growth of the shoot leading to phototropic reorientation. To identify important regulators of auxin gradient formation we developed an auxin flux model that enabled us to test in silico the impact of different morphological and biophysical parameters on gradient formation, including the contribution of the extracellular space (cell wall) or apoplast. Our model indicates that cell size, cell distributions and apoplast thickness are all important factors affecting gradient formation. Among all tested variables regulation of apoplastic pH was the most important to enable the formation of a lateral auxin gradient. To test this prediction we interfered with the activity of plasma membrane H⁺-ATPases that are required to control apoplastic pH. Our results show that H⁺-ATPases are indeed important for the establishment of a lateral auxin gradient and phototropism. Moreover, we show that during phototropism H⁺-ATPase activity is regulated by the phototropin photoreceptors, providing a mechanism by which light influences apoplastic pH.

Keywords: phototropism / plasma membrane H⁺-ATPase / phototropins / auxin / modeling.
**Introduction**

The ability of plants to adjust their growth to the direction of incoming light already intrigued Greek philosophers in ancient times (Whippo & Hangarter, 2006) and lost nothing of its fascination today. Several key steps of this process termed phototropism starting with light perception and leading to directional growth are well understood (Christie & Murphy, 2013; Hohm et al, 2013; Sakai & Haga, 2012). The formation of a lateral gradient of the phytohormone auxin across a unilaterally irradiated hypocotyl (embryonic stem) is necessary and sufficient to cause asymmetric growth and subsequent phototropic bending (Baskin et al, 1986; Friml et al, 2002; Haga & Sakai, 2012). Yet, the mechanisms for forming this gradient remain elusive. Several auxin carriers including members of the PIN-FORMED (PIN), ATP-binding cassette transporters subfamily B (ABCB), and AUXIN-RESISTANT1 (AUX1) families have been implicated in auxin gradient formation (Blakeslee et al, 2004; Christie et al, 2011; Ding et al, 2011; Friml et al, 2002; Stone et al, 2008; Willige et al, 2013). Light perception by the photoreceptor phototropin1 (phot1) leads to inhibition of ABCB19 activity, which controls the basipetal flux of auxin in the hypocotyl and thereby indirectly modulates lateral auxin gradient formation (Christie et al, 2011). The auxin transporters that have most prominently been implicated in formation of a lateral auxin gradient are members of the PIN family (Ding et al, 2011; Haga & Sakai, 2012; Willige et al, 2013). However, how light affects PIN activity and the importance of intracellular localization of PIN proteins upon phototropic stimuli is still a matter of debate (Christie et al, 2011; Ding et al, 2011; Hohm et al, 2013; Sakai & Haga, 2012).

In addition to being actively transported, protonated auxin is able to diffuse across the plasma membrane (Krupinski & Jonsson, 2010). The protonated fraction of the weak
acid auxin (pKₐ 4.8, (Delbarre et al, 1996)) depends on the environmental pH. Because of contrasted pH between cytoplasmic and apoplastic compartments (estimated at 7 and 5.5, respectively) (Bibikova et al, 1998; Kramer & Bennett, 2006; Krupinski & Jonsson, 2010; Kurkdjian & Guern, 1989; Yu et al, 2000 ), an auxin fraction can be passively imported by the cell, while only active transport allows for auxin export. Whether regulation of apoplastic pH is required for auxin gradient formation and phototropic bending, to our knowledge, has not been thoroughly investigated so far. Regulation and maintenance of the proton gradient across the plasma membrane and apoplastic pH requires the activity of plasma-membrane localized proton pumps of the AHA family (H⁺-ATPases) (Palmgren, 2001). H⁺-ATPase activity is crucial for a large variety of physiological processes such as stomatal opening, nutrient uptake, or hypocotyl and root growth (Haruta et al, 2010; Haruta & Sussman, 2012; Palmgren, 2001). Interestingly, the role of H⁺-ATPases has been linked to cell elongation by the acid growth theory (Cosgrove, 2005; Hager, 2003; Rayle & Cleland, 1992). It stipulates that cell elongation requires apoplastic acidification to activate cell-wall loosening proteins (Hager, 2003). Recently it has been shown that auxin-induced cell elongation involves auxin-mediated regulation of H⁺-ATPase activity by phosphorylation (Spartz et al, 2014; Takahashi et al, 2012). Therefore, regulation of H⁺-ATPase activity might play a dual role during phototropism: to modulate the portion of protonated auxin and thus auxin influx, and to promote cell wall acidification and thus cell elongation.

To shed further light on auxin gradient formation during phototropism, we established an auxin flux model based on the morphology of the hypocotyl of an Arabidopsis thaliana seedling enabling us to test in silico the impact of various parameters:
hypocotyl topology, apoplast thickness, and apoplastic pH changes. Our model predicted that regulation of apoplastic pH is a key step for the establishment of a lateral auxin gradient, a prediction that we supported experimentally. Finally, we provide results suggesting a mechanism explaining how light can regulate H⁺-ATPases and thereby potentially apoplastic pH at the molecular level.
**Results**

**An in silico model for auxin flux during hypocotyl phototropism**

Overall auxin fluxes include active and passive cellular efflux and influx, and free auxin diffusion within the apoplastic compartment (Kramer, 2007; Krupinski & Jonsson, 2010). While the apoplastic diffusion distance depends on the actual apoplastic thickness and pH (Kramer, 2006), passive efflux and influx depend on compartmental pH and cell surface (Krupinski & Jonsson, 2010). Moreover, active fluxes are subject to carrier expression levels and localization.

To test *in silico* the impact of these various contributions on auxin gradient formation during phototropism we used ordinary differential equations to build an auxin flux model. We considered active efflux contributions from both ABCBs and PINs (Table S1), because members of both transporter families have been proposed to control auxin gradient formation upon phototropic stimulation (Christie et al, 2011; Ding et al, 2011; Haga & Sakai, 2012; Willige et al, 2013). We also explicitly considered fluxes resulting not only from passive in- and effluxes in the cells but also from free diffusion in the apoplast. Concerning active auxin transport, a starting modeling assumptions in our model supported by experimental evidence is that upon unilateral blue light irradiation PIN3 is polar in the endodermal cells on the lit side (Ding et al, 2011). In all other tissues PINs and ABCBs are expressed apolarly. We did not consider active IAA (auxin indole-3-acetic acid) influx contributions resulting from AUX1/LAX for the following reasons: (i) a previous study showed that phototropism in the aux1lax1lax3 triple mutant is not significantly different from the wild type (Christie et al, 2011), (ii) this triple mutant lacks the expression of *AUX1* and *LAX3* which were the most highly expressed members of the *AUX1/LAX* family in the
hypocotyl (Fig. S1), and (iii) we observed that different double, triple and the aux1lax1lax2lax3 quadruple mutant showed a normal final phototropic response although in the quadruple mutant there was a slight growth re-orientation delay (Fig. S1). Possible implications of including an AUX1/LAX term in our model are further evaluated in the discussion.

In etiolated Arabidopsis seedlings, light sensing occurs at the site of asymmetric growth suggesting that formation of a lateral auxin gradient occurs locally (Iino, 2001; Preuten et al, 2013; Yamamoto et al, 2014). Thus, we assumed locality of gradient formation and used a realistic hypocotyl cross section to model gradient formation (Fig. 1A and B). We tested the effect of a change in apoplastic pH, because small variations around the estimated resting apoplastic pH of 5.5 have a big impact on the protonation state of auxin influencing passive diffusion (the pKa of IAA is 4.8) (Bibikova et al, 1998; Kramer & Bennett, 2006; Krupinski & Jonsson, 2010; Kurkdjian & Guern, 1989; Yu et al, 2000) (Fig. 1C). In all our simulations, pH modulation was treated as an exogenous variable, i.e. a variable that is not affected by the model. pH modulation during phototropism therefore was imposed manually by modifying the apoplastic pH around cells on the shaded and/or lit side of the cross section. As we will discuss later on, a potential mechanism to create such a pH modulation is a light triggered and phototropin mediated modulation of H⁺-ATPase activity (see below).

Lastly, we took into account topological parameters like different apoplast thickness distributions as observed during seedling elongation (Derbyshire et al, 2007) as well as modifications of the available cell surface for the different cell layers (Fig. 1D and
The former was tested because apoplast thickness impacts auxin travel distances in the apoplast while the latter is of interest since changes in size of the active interface between cells and apoplast modify absolute auxin flux contributions via a membrane. The cell surface variation was realized by modifying the classical cell size distribution of low diameter endodermal and epidermal cells and large diameter cortical cells and thereby affecting the relation between cell volume and cell surface (Fig. 1D). We judged our models based on their ability to generate an auxin concentration difference in epidermal cells of opposing sides because the epidermis is considered as limiting for growth (Kutschera & Niklas, 2007; Savaldi-Goldstein et al, 2007) and auxin gradients were observed in the epidermis of photo stimulated seedlings (Haga & Iino, 2006). To calculate concentrations we used the cell and apoplast volumes and surfaces as obtained from a hypocotyl cross-section (Fig. 1D and 1E). We arbitrarily considered that a gradient was established when more than 1% concentration difference between opposing sides was obtained in the model.

**Apoplastic pH modulation is necessary for auxin gradient formation**

Among all variables tested, auxin gradient formation depended most critically on a modulation of the pH in the apoplast. Gradients could only be formed when the apoplastic pH around epidermal cells on the shaded side was lowered (Fig. 2A; Fig. S2). Before acidification we assumed an initial apoplastic pH of 5.5 (Bibikova et al, 1998; Kramer, 2006; Krupinski & Jonsson, 2010; Kurkdjian & Guern, 1989; Yu et al, 2000 ) and we assumed a drop in pH by 0.7 units. Such a drop in apoplastic pH has been observed previously (Boonsirichai et al, 2003; Fasano et al, 2001; Monshausen et al, 2011). Moreover, this has a serious impact on the protonation state of the naturally occurring IAA: while at a resting pH of 5.5 only ~16% of the apoplastic
auxin is protonated and is therefore able to permeate cell membranes, after acidification (to pH 4.8) the protonated fraction increases to 50%. Therefore, a drop in apoplastic pH creates a trap for apoplastic auxin and boosts the intracellular auxin concentration of surrounding cells. On the contrary, simulation of apoplast basification on the lit side was not sufficient to induce auxin gradient formation (Fig. 2A). This might be explainable by the fact that increasing the apoplastic pH is only able to affect the protonation state of the ~16% of IAA already protonated (Fig. 1C). We also modeled the effect of basification of the apoplast on the lit side while lowering the initial pH in etiolated seedlings from 5.5 to 4.8, however this did not increase the resulting gradients (Fig. S2). Finally, concomitant apoplast acidification and basification on opposing sides enhanced the gradients observed in the acidification-only scenario (Fig. 2A).

**Topological parameters strongly modulate gradient formation**

Cellular topology has the potential to contribute to the formation of lateral auxin gradients for the following reasons. To result in an equivalent change in auxin concentration more auxin molecules need to be transported in or out of a cell with a larger volume than a cell with a smaller volume. In addition, for the nearly cylindrical cells found in etiolated hypocotyls, the ratio of cell surface to cell volume decreases with increasing diameter. Considering that the cell surface is the interface via which auxin has to be moved, small diameter cells can change their auxin concentration more easily and faster. In addition the cellular geometry impacts the apoplastic volume (see below).

We quantified the impact of hypocotyl morphology on gradient formation in our model. For calculations of auxin concentrations we used cell and apoplast volumes
and surfaces determined from the cross sections shown in Fig 1D. We found that topological features indeed have a strong impact on gradient formation. For simplification we only considered cell size variations using idealized topologies (Fig. 1D, topologies T2-4). When testing the impact of cell size distributions we started with the natural cell size distribution with small cells (~15 µm in diameter) in epi- and endodermis and big cells (~30 µm in diameter) in the cortex (Fig. 1D, topology T2), and tested inverted cell size distributions (Fig. 1D, topology T3) as well as only small cells (Fig. 1D, topology T4) and only big cells (Fig. 1D, topology T5). According to these simulations the natural cell size distribution is beneficial for gradient formation (Fig. 2B).

Notably, the idealized topology with realistic cell size distribution yielded a relatively similar gradient to the gradient simulated in the natural topology (Figure 2B), indicating that potential asymmetries in the realistic topology do not have a strong influence on gradient formation. To test this further we also simulated gradient formation in a situation where light comes from a different side (rotated by 90 degrees) than in the original simulations. As our hypocotyl cross-section and others that we found in the literature (Crowell et al, 2011; Gendreau et al, 1997) are not perfectly symmetric under (discrete) rotations, different directions could have led to different outcomes in our model prediction. Yet, our simulations showed that the observed differences were small (a few percent at most), indicating that asymmetries in our cross-section do not affect our results significantly (Fig. S3).

In contrast, an inverted cell size distribution prevented gradient formation, as did a distribution consisting of only big cells. On the other hand, formation of an auxin gradient was possible using only small cells. In addition, we observed that further decreasing the cell volume while maintaining the cell surface constant further
enhanced the steepness of gradients. This is a likely scenario in hypocotyl cells of etiolated seedlings that primarily consist of a large vacuole. Assuming that auxin is excluded from the vacuoles and that vacuoles in fully pressurized hypocotyl cells make up for at least 90% of the cell volume, simulations predict resulting gradients reaching up to 12% difference between shaded and lit side opposed to 8% when ignoring vacuoles and otherwise using the same settings. This corresponds to a 50% increase in gradient strength by considering potential effects of compartmentalization of the cells.

Apart from cell sizes and volumes, apoplast thickness also plays a potential role in auxin gradient formation. This is due to the fact that the apoplast potentially provides a mode of long distance auxin transport depending on its diameter (Kramer, 2006; Kramer, 2007). Considering that, depending on the elongation status and thus on the cellular geometry of hypocotyl cells their surrounding, apoplast thicknesses vary considerably (with apoplast thicknesses decreasing with increasing cell elongation) (Derbyshire et al, 2007), cell elongation and thickness might contribute to auxin gradient formation. To test this in our model, we compared thickness distributions documented during different elongation states of hypocotyl cells (Derbyshire et al, 2007). We particularly considered the early stages of hypocotyl elongation that all show relatively thick outer epidermal apoplasts and considerably thinner apoplasts on the inside. Using the thickness distributions reported by Derbyshire and colleagues (Derbyshire et al, 2007), we considered non-elongated cells (IIa), partly elongated cells (IIb), and strongly elongated cells (IIc) (Derbyshire et al, 2007) (Fig. 2C and 1E). We contrasted these measured thicknesses with homogeneous thickness distributions within the range of those measurements (Derbyshire et al, 2007) (Fig. 2C and S3).
The strongest gradients were found in scenarios using thick apoplasts (IIa, 800 nm, and a bit less in case of 400 nm) (Fig. 2C). This supports the hypothesis that the apoplast constitutes an important mode of long distance auxin flux during lateral gradient formation. Our analysis indicated that a thick epidermal layer was particularly favorable for gradient formation since thickness distribution IIa features a very thick outer epidermal apoplast (1250 nm) but all the other layers are thinner than in the 400 nm scenario (Fig. 1E, Fig. 2C). Despite thinner apoplast in the inner cell layers, gradients formed in scenario IIa were stronger than in case of the homogeneous 400 nm apoplast.

**Regulation of plasma membrane H⁺-ATPase activity is required for phototropism**

Our *in silico* study predicted that the apoplast and in particular apoplastic pH regulation upon unilateral light perception is a fundamental parameter for auxin gradient establishment (Fig. 2A). Apoplastic pH is regulated by the activity of plasma membrane localized H⁺-ATPases (Palmgren, 2001). To test this prediction experimentally, we first analyzed the kinetics of phototropic bending in conditions with an altered regulation of the apoplastic pH, by modulating the plasma membrane H⁺-ATPase activity. Treatment with increasing concentrations of the proton pumps inhibitor dicyclohexylcarbodiimide (DCCD) progressively inhibited the phototropic response in the wild type (Fig. 3A). Accordingly, we observed a delayed phototropic response of mutants lacking the expression of the two most expressed AHA proteins, *aha1* and *aha2* (Fig. S4). Inhibition of H⁺-ATPase activity by genetic or pharmacological approaches resulted in reduction of the hypocotyl growth rate (Fig.
S5A and B), which could be the cause of reduced phototropism. However, we note that in a previous study the growth rate of decapitated seedlings was similarly reduced as in seedlings treated with 50 μM DCCD but decapitated seedlings still showed robust phototropism while DCCD treated seedlings did not (Fig. 3A) (Preuten et al, 2013). This indicates that reduced growth rate alone is not the reason for phototropism inhibition observed when H⁺-ATPase activity is reduced.

To further investigate the role of H⁺-ATPases we treated seedlings with the specific activator fusicoccin (FC). General activation of H⁺-ATPase activity upon FC treatment increased hypocotyl growth rate but reduced phototropism (Fig. 3B and Fig. S5C). The fact that both inhibition and activation of H⁺-ATPases during unilateral light treatment affect the amplitude of hypocotyl bending suggests that regulation of H⁺-ATPase activity is required for optimal phototropism.

**H⁺-ATPase regulation is necessary for auxin gradient establishment**

We then tested whether H⁺-ATPase regulation is required for auxin gradient establishment during phototropism. We evaluated the distribution of auxin across the hypocotyl upon phototropic stimulation using the auxin sensor DII-Venus, a synthetic protein degraded directly upon auxin perception (Brunoud et al, 2012). The signal of DII-Venus was homogeneously distributed in the hypocotyl before phototropic stimulation. Following a 1-hour unilateral blue light treatment, which precedes phototropic re-orientation in our conditions, we observed an asymmetric DII-Venus signal across the hypocotyl (Fig. 4A and S6). The signal was stronger on the lit side compared to the shaded side, indicating a higher accumulation of auxin on the shaded side (Fig. 4A and B). Treating seedlings with the auxin efflux inhibitor NPA
completely abolished the formation of the gradient (Figure 4 and S6). Importantly, FC treatment strongly impaired auxin gradient establishment (Figure 4 and S6), demonstrating that misregulation of H⁺-ATPase activity and consequently misregulation of apoplastic pH during phototropic stimulation prevents auxin relocalisation.

Phototropins regulate plasma membrane H⁺-ATPase phosphorylation during phototropism

Since regulation of H⁺-ATPase activity is required for auxin gradient formation preceding phototropic bending, we examined how AHA proteins are regulated upon light perception in hypocotyls. Phosphorylation of H⁺-ATPases occurs at multiple sites and is an important mechanism regulating their activity (Duby & Boutry, 2009; Rudashevskaya et al, 2012). Phosphorylation at the penultimate residue, a threonine (Thr947 in Arabidopsis AHA2), is a primary step for the activation of H⁺-ATPases (Duby & Boutry, 2009). To evaluate the activity of H⁺-ATPases in hypocotyls upon light perception, H⁺-ATPases phosphorylation levels were analyzed by immunoblotting using an antibody recognizing the catalytic region of H⁺-ATPases and an antibody specifically recognizing the phosphorylated threonine (pThr947). These antibodies recognize several members of the AHA family (Hayashi et al, 2010). A decreased phosphorylation of H⁺-ATPases was detected in dissected hypocotyls when the seedlings were irradiated unilaterally with blue light (Fig. 5A, S7). Importantly, while the level of H⁺-ATPase phosphorylation at the penultimate amino acid was similar between wild type and the phot1 phot2 mutant in the dark, we did not observe any blue light regulation of H⁺-ATPases phosphorylation in the absence of phototropins (Fig. 5B). Altogether our data indicate that phototropins regulate H⁺-
ATPase activity in the hypocotyl during phototropism. Consequently, these data provide a potential molecular explanation for the need of specific regulation of H⁺-ATPase activity during phototropism and the stimulus-induced pH modulation predicted by our model.
**Discussion**

We investigated the importance of different factors on lateral auxin gradient formation *in silico* by modeling auxin fluxes in an *Arabidopsis thaliana* hypocotyl cross section. We thereby assume locality of perception and response, which was recently demonstrated in Arabidopsis (Iino, 2001; Preuten et al, 2013; Yamamoto et al, 2014). The cross section used represents a natural topology including the apoplastic space surrounding the cells, which was explicitly represented because it provides a potentially important aspect of auxin transport (Kramer, 2007) and has also commonly been neglected in otherwise comparable models (Grieneisen et al, 2007; Santuari et al, 2011; Wabnik et al, 2010).

**Impact of morphological parameters on auxin gradient formation**

Phototropic bending happens in the short hypocotyl cells in the elongation zone and it is usually assumed that this is due to the lack of growth potential in elongated cells (Kami et al, 2012; Preuten et al, 2013; Yamamoto et al, 2014). Our results suggest that in addition to a reduced ability to grow, elongated cells with concomitantly reduced apoplast thickness also have a reduced potential to form a lateral auxin gradient (Fig. 2C, S2 and S3) (Derbyshire et al, 2007). During phototropic bending of the hypocotyl the epidermis on the shaded side elongates the most of all layers (MacLeod et al, 1985; Orbovic & Poff, 1993) and it corresponds to the layer with the most wall material (Derbyshire et al, 2007). Thereby, the naturally observed topology favors both gradient formation and rapid elongation without the prior need for new cell wall synthesis.
Our model also predicts a strong impact of cell size distributions on gradient formation (Fig. 2B, S2). Seedling morphology with small cells enhances the potential to form a lateral auxin gradient. Moreover a layer of small epidermal cells, followed by large cortex cells and small endodermal cells is favorable for the establishment of a lateral auxin gradient while inverting the sizes of the cells in the different cell types prevents gradient formation (Fig. 2B). As expected for an embryonic organ the hypocotyl cellular arrangement is stereotypical with 33-36 epidermal, 13 outer cortex, 8 inner cortex and 8-9 endodermis cells (Fig. 1) (Crowell et al, 2011; Gendreau et al, 1997). Intriguingly the cell size distribution present in Arabidopsis hypocotyls appears to be conserved among angiosperms suggesting the possibility of selection of morphological features that favor tropic hypocotyl growth (Busse et al, 2005; Meyer, 1971).

Our findings highlight the importance of cellular morphology that may constrain the formation of auxin gradients. Several recent studies have provided evidence for links between mechanical/cellular constraints and auxin-mediated growth processes (Heisler et al, 2010; Lindeboom et al, 2013; Lucas et al, 2013; Nakayama et al, 2012; Peret et al, 2012; Vermeer et al, 2014). Further investigating the relationship between cellular morphology, the associated biophysical constraints and auxin-mediated growth processes is important if we want to understand fundamental aspects of plant growth.

**A role for regulated H⁺-ATPase activity in phototropism**

We designed our starting modeling assumptions based on a paper that showed polar distribution of PIN3 in the endodermis of the lit side during phototropism (Ding et al, 2011).
Our model suggests that polarization of PIN3 in the endodermis is not sufficient to create an auxin gradient (Fig. 2 and S2). This does not mean that light-induced PIN3 relocalization is unimportant but suggests that additional mechanisms are required to promote auxin gradient formation in photostimulated hypocotyls (see below). By testing a number of model parameters we identified modulation of apoplastic pH as a key step to form a lateral auxin gradient across the hypocotyl of photo-stimulated seedlings. Apoplastic basification on the lit side and apoplastic acidification on the shaded side is the optimal combination to establish a lateral auxin gradient (Fig. 2A). We propose that this leads to the concomitant increase and decrease of growth rates on opposing sides of the stem that has been documented previously (MacLeod et al, 1985; Orbovic & Poff, 1993). Interestingly, pH changes have been observed on the surface of gravi-stimulated Arabidopsis roots (Monshausen et al, 2011) and appear to be linked to intracellular pH modulation (Boonsirichai et al, 2003; Fasano et al, 2001; Monshausen et al, 2011). Cytoplasmic basification (that correlates with extracellular acidification) occurs within 2 minutes of gravi stimulation and precedes asymmetric distribution of PIN efflux carriers (Boonsirichai et al, 2003; Fasano et al, 2001; Monshausen et al, 2011).

We provide experimental evidence supporting this important modeling prediction; to modulate apoplastic pH, we interfered with the plasma membrane H⁺-ATPase activity and showed that this disrupts formation of a lateral auxin gradient preceding phototropism (Fig. 4B and S6). The fact that both H⁺-ATPase activation and inhibition negatively influenced phototropic bending suggests that not only the proton pump activity but also an appropriate regulation of its activity is required for an optimal growth response during phototropic stimulation (Fig. 3 and S6). We therefore
propose that differential apoplastic pH regulation is achieved by a differential regulation of the H\(^{+}\)-ATPase activity on opposing hypocotyl sides, i.e. activation on the shaded side and inhibition on the lit side.

Phototropins are the primary photoreceptors triggering phototropism but how their activation lead to auxin gradient formation remains elusive (Christie & Murphy, 2013; Hohm et al, 2013; Sakai & Haga, 2012). Phototropin (phot1) interacts with and regulates the phosphorylation status of several proteins involved in phototropism (Christie et al, 2011; Demarsy et al, 2012; Pedmale & Liscum, 2007; Takemiya et al, 2013). Here we demonstrated that phosphorylation of the plasma membrane H\(^{+}\)-ATPases in the hypocotyl is regulated by the phototropins (Fig. 5B). We propose that the phototropin-mediated control of H\(^{+}\)-ATPase phosphorylation is important to establish asymmetric hypocotyl growth during phototropism. Asymmetric activation of the phototropins has been observed during unilateral seedling irradiation (Salomon et al, 1997), this may lead to differential phosphorylation/regulation of H\(^{+}\)-ATPases across a hypocotyl section.

We showed that in the context of hypocotyl phototropism, phototropin activation inhibits H\(^{+}\)-ATPase phosphorylation. Phototropin-mediated regulation of H\(^{+}\)-ATPase phosphorylation has been observed for other physiological responses. For example, during light-induced kidney bean movements phototropin activation also leads to a dephosphorylation of H\(^{+}\)-ATPases in pulvini cells (Inoue et al, 2005). In contrast in stomata phototropin activation leads to enhanced phosphorylation of H\(^{+}\)-ATPases (Kinoshita & Shimazaki, 1999). In both cases activation of phot1 leads to changes in the phosphorylation status of the penultimate threonine of AHA proteins which
regulates their activity. However, the steps leading from phototropin activation to the regulation of H+ -ATPases phosphorylation are not fully understood and depend on the context (Takemiya et al, 2013).

**Working model for lateral auxin gradient formation during phototropism**

Our data highlight the importance of regulated H+-ATPase activity in the establishment of an auxin gradient preceding phototropism (Figs 2-4). As auxin promotes H+-ATPase activity (Chen et al, 2010; Takahashi et al, 2012), we propose a model that includes feedback and feed-forward loops between auxin transport and H+-ATPase regulation thereby promoting auxin gradient formation. On the lit side reduced proton pump activity leads to apoplast basification decreasing auxin uptake, and in turn the decrease in intracellular auxin further reduces proton pump activity. In contrast, on the shaded side auxin uptake increases with H+-ATPase activity leading to apoplast acidification. In turn auxin accumulation in the cell further enhances proton pump activity. Thus a network of interlaced regulatory loops controls auxin gradient formation during the phototropic response. Consistent with this idea, we show that interference with auxin transport (NPA) and with H+ ATPase activity (FC) disrupts lateral auxin gradient formation (Fig. 4 and Fig. S6). This reciprocal regulation of auxin concentration and H+-ATPase activity is reminiscent of the complex relation between auxin concentration regulation and auxin transport as auxin regulates the expression and localization of its own transporters (Krecek et al, 2009).

Our mathematical model identified a novel mechanism required for auxin gradient formation that was validated experimentally (Figures 2-4). The strongest auxin gradients predicted by our model (12% by considering vacuolated cells) was lower
than what was measured in maize coleoptiles and pea epicotyls but nevertheless comparable to the 20% gradient determined in hypocotyls of Brassica which are closely related to Arabidopsis (Esmon et al, 2006; Haga & Iino, 2006; Iino, 1992). The relatively shallow gradient predicted by our simulation contrasts with the large difference in DII-Venus signal between the shaded and lit sides of the hypocotyl observed here (Fig. 4). However, we do not know how the in vivo auxin concentration relates to the DII-Venus signal. Hence, it cannot be concluded that a three-fold change in DII-Venus signal corresponds to a three-fold change in auxin concentration. In our model gradient strength is sensitive to auxin efflux carrier density, pumping capacity, and coupling of the modeled cross section to cross sections above and below not explicitly represented in the model (see supporting information). Thereby the steepness of the gradient depends on these parameters. And while these parameters can have a strong effect on gradient strength, they do not impact the qualitative behavior of the model. We unfortunately lack precise measurements for these parameters, however the sensitivity of our model to efflux carrier density and pumping capacity is in accordance with the experimental evidence showing that mutants lacking several PINs show delayed and reduced phototropic responses (Ding et al, 2011; Friml et al, 2002; Haga & Sakai, 2012; Willige et al, 2013). Additional factors that likely favor gradient formation are the newly identified plasmodesmatal gating mechanism (Han et al, 2014), and the activity of AUX1/LAX carriers (Band et al, 2014). Indeed, since AUX1/LAX carriers are proton symporters one can hypothesize that they contribute to reinforcing the auxin gradient formation: apoplastic acidification and increase of H⁺ concentration on the shaded side potentially increases AUX1/LAX mediated active auxin uptake on the shaded side (Carrier et al, 2008; Lomax et al; Steinacher et al, 2012). This could explain the delay.
in phototropism observed in the quadruple auxlax1lax2lax3 mutant (Fig S1). Importantly, a recent study has shown that within the root tip members of the AUX1/LAX family are essential to determine which cells have high auxin levels (Band et al, 2014). Taken together with our results we conclude that further studying of mechanisms controlling entry of auxin into cells is very important to understand the distribution of this hormone within plants. To extend our model and to refine our hypotheses it would be interesting to include the contribution of the AUX1/LAX family and the feedbacks between auxin transport and pH regulation (Carrier et al, 2008; Krecek et al, 2009; Lomax et al; Steinacher et al, 2012). Finally, once the link between phototropin activation and H\(^+\) ATPase activity is better understood it could be included directly into the model (similarly to the implementation of auxin induced apoplastic acidification described by Steinacher and colleagues (Steinacher et al, 2012)) instead of treating pH change as an exogenous variable.
Material and Methods

**Model.** Description of the model, parameters and equations are provided in Supporting Text.

**Plant material and growth conditions**

The Columbia (Col-O) ecotype of *A. thaliana* was used as the WT. All the following transgenic line and mutant alleles were in the Col-O background: *aha1-6, aha2-4* (Haruta et al, 2010), *phot1-5phot2-1* (de Carbonnel et al, 2010), DII-Venus (Brunoud et al, 2012). Seeds were surface-sterilized, sown on agar plates (½ strength MS pH5.7 buffered with MES, 0.8% agar) and treated as described (Lariguet & Fankhauser, 2004). For pharmacological treatments seeds were sown on nylon mesh (160 µm, Micropore) placed on the surface of the plate. Seedlings were grown for 3 days in darkness at 22°C before indicated treatment. Light intensities were determined with an International Light IL1400A photometer (Newburyport, MA) equipped with an SEL033 probe with appropriate light filters.

**Pharmacological treatments**

Nylon meshes with 3-day-old etiolated seedlings were transferred 1 hour before indicated light treatment onto freshly prepared plates supplemented by 0, 5, or 10µM fusicoccin (FC, Sigma) and 0.01% DMSO, or 0, 10, 50 µM dicyclohexylcarbodiimide (DCCD, Sigma) and 0.01% ethanol, or 10 µM 1-N-Naphthylphthalamic acid (NPA, Duchéfa).

**Phototropism**

3 day-old etiolated seedlings (6 to 9 mm-long hypocotyls) grown on vertical agar plates were irradiated with 10 µmol m⁻² s⁻¹ unilateral blue light for 24 hours. Pictures were taken with an infrared camera at different time points. Angles formed by the
hypocotyl relative to vertical were measured with the NIH image software. Means, standard errors and Student’s t test were performed on 50 seedlings minimum.

**DII-Venus signal visualization and quantification**

Seedlings were grown as described for phototropism except that an additional 24h-treatment with white light (25 µmol m\(^{-2}\) s\(^{-1}\)) was applied to induce de-etiolation. This treatment was necessary to allow detection of DII-Venus signal in the hypocotyl, and the seedlings were still responding to phototropic stimulation and sensitive to FC treatment (Fig. S6B and C). Imaging was performed on an LSM-510 laser-scanning confocal microscope (Zeiss). Serial optical sections were acquired and quantification was performed as described in Fig. S6A.

**Quantification of H\(^+\)-ATPase phosphorylation level at Thr947**

3 day-old etiolated seedlings (6-9 mm-long hypocotyls) grown on vertical agar plates were irradiated with 10 µmol m\(^{-2}\) s\(^{-1}\) unilateral blue light or 10 µmol m\(^{-2}\) s\(^{-1}\) blue light from above for indicated time (0 to 30 minutes). Seedlings were fixed in EtOH-Acetic acid solution (3:1) for 15 minutes and transferred into 75 % EtOH for 1 to 3 hours. Proteins were extracted from 25 hypocotyls sections ground with a plastic pestles in 20 µl 1X Phosphate-Buffered Saline (PBS) containing 6M Urea and overnight incubation at room temperature. After addition of 30 µl 2X Laemmlli buffer, proteins (10 µl/lane) were separated on 9% SDS-poly-acrylamide gels and transfer onto nitrocellulose with Tris-glycine buffer. The blots were probed with antibodies raised against the catalytic domain of AHA2, or antibodies that recognize peptide containing the phosphorylated Thr947 in AHA2 (Hayashi et al, 2010). These antibodies recognize not only AHA2 but also other H\(^+\)-ATPase isoforms in Arabidopsis (Hayashi et al, 2010). Membranes were blocked in PBS, 0.1% Tween 20,
and 5% nonfat milk (PBS-T-M) for 1 hour at room temperature, incubated in presence of the primary antibodies overnight at 4°C, washed 3 times in PBS-T-M, incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase for 1 hour at room temperature and washed 3 times in PBS-T-M. Chemiluminescence signals were generated using Immobilon Western HRP Substrate (Millipore). Signals were captured with a Fujifilm ImageQuant LAS 4000 mini CCD camera system and quantifications were performed with ImageQuant TL software (GE Healthcare) (Fig. S7).

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Author Contribution
T. H. Designed research, performed research, analyzed data and wrote the article.
E. D. Designed research, performed research, analyzed data and wrote the article.

C. Q. Performed research and analyzed data.

T. P. Performed research and analyzed data.

L. A. Performed research and analyzed data.

T. V. Provided important material.

S. B. Designed research and analyzed data.

C.F. Designed research, analyzed data and wrote the article.

Conflict of Interest

none
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**Figure Legends**

**Figure 1:** Overview on model domain, formed gradients, topological parameters, and biophysical parameters tested in the model.

A. Modeled domain representing a cross section through the elongation zone of a 3-day-old etiolated *Arabidopsis thaliana* seedling.

B. Example of an auxin concentration gradient formed within a cross section showing apoplastic auxin gradient and cellular auxin gradient.

C. Dissociation curve for IAA based on its pK$_a$ of 4.8 showing protonated fractions for different compartmental pH values.

D. Different topologies tested during model parameter exploration: a realistic cross section (T1), a rotational symmetric cross section model with a cell size distribution over the different layers as found in the realistic cross section (T2), a rotational symmetric cross section model with an inverted cell size distribution (T3), rotational symmetric cross section model where all cells have the same size (either small (T4) like cells found in epi- and endodermis or big (T5) like cells found in the cortex). Here, small cells have a diameter of ~15 µm while big cells have a diameter of ~30 µm.

E. Illustration of the exact localization of the different apoplast layers, outer epidermis (OE), inner epidermis (IE), outer cortex (OC), inner cortex (IC), and endodermis (EN) and their measured thicknesses for different elongation states as reported by Derbyshire and colleagues (Derbyshire et al, 2007).

**Figure 2:** Impact of different parameters on *in silico* auxin gradient formation.
As base scenario for a realistic cross section with apoplast thickness distribution IIa (corresponding to short cells), full PIN activity and concomitant acidification and basification was used.

A. Impact of modulations in apoplast pH distributions. Here, only the subset of scenarios in which we applied apoplast acidification shows lateral gradient formation.

B. Impact of different cell size distributions. Only the realistic, symmetrized realistic, and only-small-cells topologies are able to form lateral gradients.

C. Impact of apoplast thickness on gradient formation. Tested apoplast thickness distributions were distributions with relatively thick epidermal walls and thinner internal apoplast starting from very thick (IIa) ranging over medium (IIb) to small (IIc) and homogeneous apoplast thicknesses of 800 nm, 400 nm, and 100 nm respectively. Only scenarios IIa, 400 nm, and 800 nm are able to form lateral gradients.

Figure 3: Regulation of H⁺-ATPase activity is required for optimal phototropism

A. Inhibition of proton pump activity by the proton pump inhibitor dicyclohexylcarbodiimide (DCCD) represses phototropism.

B. Enhancement of proton pump activity by fusicoccin (FC) treatment represses phototropism. Data represent the rate of hypocotyl growth curvature upon unilateral blue light irradiation with a fluence rate of 10 µmol m⁻² s⁻¹. Values are means +/- 2XSE, n>20.

Figure 4: Regulation of H⁺-ATPase activity is required for lateral auxin gradient formation.
A. DII-Venus signal in hypocotyls was examined before (left) or after (right) 1 hour blue light (BL) irradiation with a fluence rate of 10 µmol m$^{-2}$ s$^{-1}$.

B. General activation of H$^+$-ATPases and inhibition of auxin transport prevent auxin gradient formation. Seedlings treated with DMSO, NPA or FC were analyzed as in A. Quantification of DII-Venus signal was performed on 11 to 13 seedlings for each treatment, and data represent the ratio of the DII-Venus fluorescence signal between the lit side and the shaded side. Values are means, and error bars represent standard errors.

Figure 5: Phototropins regulate H$^+$-ATPase phosphorylation in hypocotyls upon light perception.

A. Unilateral blue light decreases H$^+$-ATPase phosphorylation levels in hypocotyls. Three-day-old (WT) seedlings were either kept in darkness (0) or irradiated with 10 µmol m$^{-2}$ s$^{-1}$ unilateral blue light for the indicated time. Total proteins from dissected hypocotyls were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Accumulation of total H$^+$-ATPases and H$^+$-ATPases phosphorylated at the penultimate amino acid was analyzed by immunobloting using anti-H$^+$-ATPases (H$^+$-ATPases) and anti-phosphorylated-threonine-947 (pThr947) antibodies, respectively. Quantifications of pThr947 signal relative to the H$^+$-ATPases total signal were performed on three biological replicates. Values are means, and error bars represent standard errors. * indicates significant difference between means of light treated samples compared to dark control (P<0.05).

B. Regulation of H$^+$-ATPase phosphorylation levels at pThr947 depends on phototropins. Three-day-old seedlings of Col-0 (WT) or phot1phot2 mutant were either kept in darkness (0) or irradiated with 10 µmol m$^{-2}$ s$^{-1}$ unilateral blue light for
30 minutes. Proteins were analyzed as described in A. * indicates significant difference between means of light treated samples compared to dark control (P<0.05).

**Expanded view (Supplementary Information)**

Supplementary Material and Methods

**Supplemental Figure 1:** Phototropic response of various aux1lax mutants and expression of AUX1, LAX1, LAX2 and LAX3 in etiolated hypocotyls.

**Supplemental Figure 2:** Impact of initial apoplastic pH on simulated auxin gradients.

**Supplemental Figure 3:** Impact of different irradiation directions on simulated auxin gradients.

**Supplemental Figure 4:** Phototropic response of aha1-6 and aha2-4 mutants.

**Supplemental Figure 5:** Hypocotyl elongation rate during phototropism.

**Supplemental Figure 6:** Analysis of auxin distribution in hypocotyls.

**Supplemental Figure 7:** Quantification of H⁺-ATPase phosphorylation levels at the penultimate Thr.
Supplemental Figure 8: Phototropic response of the auxin biosynthesis mutants sav3 and yuc1yuc4.

**Supplemental Table I:** Parameters impacting auxin flux kinetics including their assumed values.

**Supplemental Table II:** List of primers used in this study.
A

Apoplastic acidification  

| gradient strength |
|-------------------|
| no gradient       |
| weak gradient     |
| strong gradient   |

Apoplastic basification  

B

Apoplastic basi-/acidification on opposing sides  

C

Auxin concentration difference of opposing sides (in %)
A. DCCD (µM)

- 0
- 10
- 50

B. FC (µM)

- 0
- 5
- 10

hypocotyl curvature (°)

Time under unilateral blue light irradiation (hours)
A

B

fluorescence lit/shaded side

|       | NO blue light   | unilateral blue light |
|-------|----------------|----------------------|
| DMSO  |                |                      |
| NPA   |                |                      |
| FC    |                |                      |
Expanded view (Supplementary information)

Supplemental Material and Methods

RNA extraction and quantitative RT-PCR

50 Hypocotyls from 3 day-old etiolated seedlings were dissected using a dissection scissor under green safe light and immediately immersed into liquid N2. RNA extraction and quantitative real time PCR were performed as described in (Lorrain et al. 2009), except that 170 ng of RNA were used for the reverse transcription reaction. Primer efficiency was preliminary determined using a dilution serie of the cDNA preparation. Primers sequences are given in table S2. Data from three technical and three biological replicates were then analyzed and normalized with the value of the expression levels of AUX1 of one of the replicate.

The model

To investigate lateral auxin gradient formation underlying phototropic bending in Arabidopsis thaliana hypocotyls, we constructed an auxin flux model that tracks auxin relocation as a function of different topological as well as biophysical parameters. The geometry of the model includes a transverse cross section through the hypocotyl explicitly featuring cellular compartments as well as apoplastic compartments. This cross section was determined using microscopy data and an overview on the modeled domain is given in Fig. IA.

The model is based on a system of ordinary differential equations simulating the flux contributions between neighboring compartments. Free diffusion is assumed between the apoplast compartments, while we consider protonation state dependent auxin
diffusion and active auxin efflux based on carriers, i.e. those of the PIN and PGP families, on the interface between cellular compartments and the apoplastic space. We did not consider active IAA influx contributions resulting from AUX/LAX for the following reasons that are explained in the main manuscript.

Apart from the fluxes within the cross section, we explicitly allowed for exchange with the apoplastic space just above and below the considered cross section. Since gradient formation is assumed to happen locally (Iino 2001; Preuten et al. 2013), auxin concentrations in the apoplast just above and below are kept constant, thereby functioning as an auxin source or sink depending on auxin redistribution in the modeled cross section. Our simulations have shown that this exchange with surrounding cross sections has a cushioning effect on the strength of gradients predicted by our model: For example, setting the exchange between the modeled cross section and cross sections above and below to zero results in qualitatively similar results but stronger gradients (increasing gradient strength from 12% for vacuolated cells to 82%). And although this documents a considerable effect of coupling strength on gradient strength, the fact that the qualitative behavior in both extremes is similar lets us not further pursue this parameter.

In addition, we incorporate the fact that the stele is the major mode of basipetal auxin transport and therefore has to be considered an auxin source. Further incorporating the fact that auxin transport in the stele is faster than in the apoplast (Kramer 2006), for our model we assume that auxin concentrations in the stele are high and kept constant. The model thereby neglects a potential contribution of modulated auxin biosynthesis within cells of the modeled cross section. This assumption seems to be confirmed by phototropism assays in two auxin biosynthesis mutants that were previously shown to be defective for shade-induced auxin-dependent hypocotyl elongation sav3 and
but show normal phototropic responses (see Fig S8). Nevertheless, considering the redundancy in the TAA1/TAR and YUC gene families, a contribution of local auxin biosynthesis cannot be ruled out completely.

In this framework we define a lateral auxin gradient as follows: First, due to the type of manipulations we use in the simulations (e.g. side-directed pH manipulation, side-directed PIN localization changes) the modeled cross section can be divided in shaded and lit side just like for hypocotyls in experiments. Under this assumption, a gradient is defined as difference in intracellular auxin concentrations comparing epidermal cells on lit and shaded side by building ratios between the two. Whenever their ratio differs from ‘1’ a gradient has formed. Its steepness is expressed in terms of percentage difference in auxin concentration between the two sides.

**Model parameters**

The model comprises three different types of parameters. First there is a set of kinetic constants that are inherently necessary to translate auxin fluxes into mathematical expressions. Although these constants ideally would be determined experimentally, many of them are difficult to measure and therefore have to be treated as free parameters (Kramer 2007). An overview on these parameters is given in Table S1.

All of the listed parameters apart from the decay rate $\mu$ had been part of a sensitivity analysis in which we varied each reported value separately by $\pm 1$ and $\pm 2$ orders of magnitude. During this analysis it turned that the dissociation constants $K_{\text{PIN}}$ and $K_{\text{PGP}}$ as well as the membrane permeability of membranes for auxin ($P_{\text{diff}}$) are rather negligible because varying them had no strong impact on the forming gradients. Instead, the model was sensitive to changes of the remaining three parameters, transport capacities of PIN carriers $C_{\text{PIN}}$, transport capacity of PGP carriers $C_{\text{PGP}}$, and
the diffusion constant of auxin \( D_{\text{IAA}} \). It responded approximately linearly to changes of these parameters with pumping capacities increased by 2 orders of magnitude resulting in gradients of about 70% concentration difference between sides and a change of 2 orders of magnitude in the diffusion rate resulting in gradients increased by about 50% as compared to the reported diffusion constants.

The second class of considered parameters are topological parameters. And although the cross section shown in Fig. 1A is generated copying an experimentally determined hypocotyl cross section (determined using microscopy data) that has to be considered a naturally occurring topology, it remains open if the inherent asymmetries of this topology introduce a non-negligible bias into the model. We therefore tested the impact of these asymmetries and other features of this topology by comparing it to a range of altered topologies shown in Fig. 1E (including different cell size distributions).

In addition to shape and cell size distribution across layers (which at the same time influence cell volumes and thereby impact resulting auxin concentrations), the distribution of apoplast thicknesses across the different cell layers has to be considered as another important topological parameter because the thickness of the apoplastic space modifies auxin storage capabilities as well as possible auxin travel distances in the apoplast (again influencing apoplastic volumes and thereby impacting resulting auxin concentrations). To choose plausible thickness distributions for the simulations we relied on data gathered by Derbyshire and colleagues (Derbyshire et al. 2007). And in order to match the reported thickness distributions with the size of our seedlings, we used the growth stages IIA-c (Hayashi et al. 2010; Steinacher et al. 2012; Derbyshire et al. 2007). In addition to these reported distributions we tested different artificial and homogeneous apoplast thickness distributions of 100 nm, 400
nm, and 800 nm throughout all layers to avoid introducing unnecessary bias by potential peculiarities of the reported distributions.

In addition, auxin fluxes are subject to expression levels and localization of auxin carriers (Noh et al. 2003; Friml et al. 2002; Kerr & Bennett 2007; Yang et al. 2006). Here, especially the efflux carriers of the PIN family are of interest since they are known to show polar distributions as well as knock out experiments show a significant defect in phototropism (Friml et al. 2002; Ding et al. 2011; Christie et al. 2011). In addition to the PINs we explicitly incorporated potential contributions of carriers of the PGP family as well since they as well have been implicated to be of potential importance for phototropism (Noh et al. 2003; Christie et al. 2011; Nagashima et al. 2008). With respect to modeling auxin fluxes the other carriers and especially the carriers belonging to the AUX/LAX family were neglected due to their negligible effect on phototropism (Fig. S1).

Lastly, auxin fluxes are impacted by compartmental pH (Kramer & Bennett 2006; Kramer 2006; Krupinski & Jönsson 2010; Steinacher et al. 2012). Here, predominantly extracellular pH distributions are of interest because the natural occurring auxin indole-3-acetic acid (IAA) is a weak acid with a pK_a value of 4.8 (Delbarre et al. 1996). This means that for an assumed apoplast pH of around 5.5 (Kramer 2006; Krupinski & Jönsson 2010; Yu et al. 2000; Bibikova et al. 1998; Kurkdjian & Guern 1989), the protonation state of IAA crucially depends on the exact pH value (e.g. at pH 5.5 only ~16% of the IAA are protonated while at pH 4.8 already 50% are protonated, Fig. 1C). And considering that only protonated IAA is able to permeate cell membranes, the pH and protonation state potentially have a significant impact on auxin uptake by the cells. In this regard, we considered pH as an exogenous variable, i.e. one that follows an assumed distribution (e.g. homogeneous apoplastic...
pH throughout the whole cross section during model equilibration and differential distributions as described during the simulations of gradient formation during photo stimulation). In other words, this means that the model does not consider inherent means on how photo stimulation impacts pH, e.g. by the hypothesized photo stimulus dependent regulation of H⁺-ATPases or other yet uncovered means of regulation. Especially the hypothesis about a phototropin based regulation of H⁺-ATPase provides a possible link between phototropism and apoplastic pH and could be included in a future version of the model inspired by the implementation by Steinacher and colleagues for a link between H⁺-ATPase activity and pH.

**Model equations**

The model itself consists of ordinary differential equations modeling the auxin fluxes on the interfaces between the two considered types of compartments, cells and apoplast. This results in three different types of flux contributions: cellular auxin efflux, fluxes from the apoplast into adjacent cells, and fluxes between connected apoplast compartments. In turn these fluxes can be subsumed into one equation for each considered compartment type.

\[
\frac{d[\text{IAA}_c]}{dt} = -\mu[\text{IAA}_c] + \sum_{i \in \mathcal{N}_c(c)} \left( f_{i}^{\text{IAAH}}[\text{IAA}_i] - f_{c}^{\text{IAAH}}[\text{IAA}_c] \right) P_{\text{diff}} A_{c,i} \\
- \sum_{i \in \mathcal{N}_c(c)} \left( \frac{f_{c}^{\text{IAA}}[\text{IAA}_c]}{f_{\text{cell}}^{\text{IAA}}[\text{IAA}_c] + K_{\text{PIN}}} \right) C_{\text{PIN}} A_{c,i} \\
- \sum_{i \in \mathcal{N}_c(c)} \left( \frac{f_{c}^{\text{IAA}}[\text{IAA}_c]}{f_{\text{cell}}^{\text{IAA}}[\text{IAA}_c] + K_{\text{PGP}}} \right) C_{\text{PGP}} A_{c,i}
\]
\[
\frac{d[I_{A\alpha}]}{dt} = \sum_{i \in N(c)} \frac{[I_{A\alpha}]}{d(m_{\alpha}, m_i)} \cdot D_{I_{A\alpha}} A_{\alpha,i} \\
+ \sum_{i \in N(c)} \left( f_{i,1AAH}[I_{A\alpha}] - f_{i,1AAH}[I_{A\alpha}] \right) P_{\text{diff}} A_{\alpha,i} \\
- \sum_{i \in N(c)} \left( \frac{f_{i,1AAH}[I_{A\alpha}]}{f_{i,1AAH}[I_{A\alpha}] + K_{\text{PIN}}} \right) C_{\text{PIN}} A_{\alpha,i} \\
- \sum_{i \in N(c)} \left( \frac{f_{i,1AAH}[I_{A\alpha}]}{f_{i,1AAH}[I_{A\alpha}] + K_{\text{PGP}}} \right) C_{\text{PGP}} A_{\alpha,i} \\
\]

\[
f_{\text{comp}} = \frac{1}{1 + 10^{pH_{\text{comp}} - pK_{\text{IAA}}}} [I_{A\text{comp}}] \\
f_{\text{comp}}^{-} = (1 - f_{\text{comp}}^{-}) [I_{A\text{comp}}] 
\]

Here, \( f_{\text{comp}} \) and \( f_{\text{comp}}^{-} \) denote the protonated and deprotonated fractions of the total compartmental auxin concentration \( [I_{A\text{comp}}] \) in the compartment \( c \) as a function of the dissociation constant of auxin \( (pK_{\text{IAA}}) \) and the compartmental pH \( (pH_{\text{comp}}) \). Then, eq. 1 represents the cellular auxin concentration changes over time, subject to proportional decay with decay rate \( \mu \), diffusive fluxes of protonated auxin between the considered cellular compartment \( c \) and all neighboring apoplastic compartments \( N(c) \), proportional to the respective contact surface \( A_{c,i} \) and the membrane permeability for auxin \( (P_{\text{diff}}) \), and active efflux based on saturable transporters of the PIN or PGP family. The saturability of the active transport thereby
depends on the respective dissociation constants $K_{\text{PIN}}$ and $K_{\text{PGP}}$ and is subject to a transport capacity/density for the respective transporter combined with a constant reflecting membrane potential effects on active transport related fluxes ($C_{\text{PIN}}$ or $C_{\text{PGP}}$) and the contact surface $A_{c,i}$ between cell $c$ and apoplast compartment $i$. Conversely, the equation modeling the time evolution of auxin concentrations for apoplastic compartments ($[\text{IAA}_a]$) considers the same diffusive and active fluxes. Only the decay term is assumed to only act intracellularly but in addition to the already mentioned fluxes we assume free diffusion between neighboring apoplastic compartments $\mathcal{N}_a(a)$ guided by the concentration difference between compartments and relative to the distance $d(c,r)$ between centroids of the considered compartments ($m_a$ and $m_i$) and proportional to the diffusion constant $D_{\text{IAA}}$ and the contact surface $A_{a,i}$.

**Simulations**

The model was implemented in MATLAB® version 2012b relying on the ode23tb numerical integrator which is an implementation of an explicit Runge-Kutta scheme (Bogacki & Shampine 1989). Simulation runs were conducted in a two step process; first a run for 2.5h of simulated time (here, timescales are set via the time-dependent diffusion and permeability rates) allowing the system to reach steady state under dark conditions (i.e. no polar PIN or heterogeneous pH distributions) before in another run of 4h simulated time the respective differential pH and PIN distributions are applied. Within these 4h of simulated time, all simulations reached a steady state, usually already after about 1.5h-2h. Here, this time window fits well auxin gradient formation timescales seen in root gravitropism (Band et al. 2012), which can be seen as a gradient formation process similar to the one underlying phototropism and therefore
indicating that at least the timescales in the simulations a well in line with naturally occurring events.

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Supplemental figure legends

Supplemental Figure 1: Phototropic response of various aux1lax mutants and expression of AUX1, LAX1, LAX2 and LAX3 in etiolated hypocotyls.

(A) Time course of hypocotyl curvature upon unilateral blue light irradiation with a fluence rate of 10 μmol m⁻² s⁻¹. Values are means +/- 2XSE, n>20. (B) Final bending angle of the indicated genotypes upon 24 hours of unilateral blue light irradiation with a fluence rate of 10 μmol m⁻² s⁻¹. Values are means +/- 2XSE, n>40. (C) Relative expression of AUX1, LAX1, LAX2 and LAX3 in dissected etiolated hypocotyls. Data are averaged (+/- SD) from 3 biological replicates and expressed relative to one of the AUX1 values.

Supplemental Figure 2: Impact of initial apoplastic pH on simulated auxin gradients.

Decreasing the initial apoplastic pH from 5.5 to 4.8 overall results in approximately 50% weaker gradients. The impact on gradient formation is less in symmetric topologies, especially with smaller cells. This indicates that for a lowered base pH the cell size and thereby volume of cells might be of even greater importance than under higher resting pH of 5.5.

Supplemental Figure 3: Impact of different irradiation directions on simulated auxin gradients. To test the effect of topological features of our hypocotyl cross-section on auxin gradient formation we performed simulation following irradiation from different directions. Data are represented for the realistic cross section and an idealized cross section for the different apoplast configurations used in figure 2C.
Supplemental Figure 4: Phototropic response of aha1-6 and aha2-4 mutants

Data represent the hypocotyl curvature upon unilateral blue light irradiation with a fluence rate of 10 µmol m$^{-2}$ s$^{-1}$ for the indicated amount of time. Values are means +/- 2XSE, n>50.

Supplemental Figure 5: Hypocotyl elongation rate during phototropism

Average hypocotyl elongation rate during the first 4 hours of unilateral blue light irradiation (fluence rate of 10 µmol m$^{-2}$ s$^{-1}$) in Wild Type, aha1-6 and aha2-4 mutants (A); upon DCCD treatment (B); or upon FC treatment (C). Values are means +/- 2XSE, n > 20.

Supplemental Figure 6: Analysis of auxin distribution in hypocotyls

(A) Method used for quantification. (B) DII-Venus signal before (left) and after (right) unilateral blue light irradiation (fluence rate of 10 µmol m$^{-2}$ s$^{-1}$), in seedlings treated with DMSO, NPA and FC. (C) Kinetics of phototropism in conditions used for DII-Venus signal detection. Data represent the rate of hypocotyl growth curvature upon unilateral blue light irradiation with a fluence rate of 10 µmol m$^{-2}$ s$^{-1}$, for wild type seedlings grown for 4 days in darkness (4d etiolated), 3 days in darkness and 24 hours in white light (25 µmol m$^{-2}$ s$^{-1}$) and treated with DMSO, NPA or FC. For comparison, the data obtained using 3 day-old etiolated WT seedlings (figS3) are indicated (dashed line). Values are means +/- 2XSE, n>20.

Supplemental Figure 7: Quantification of H+-ATPase phosphorylation levels at the penultimate Thr. Western blots used to generate the quantitative data presented on Figure 6 (a. u. arbitrary units).
Supplemental Figure 8: Phototropic response of the auxin biosynthesis mutants sav3 and yue1yue4. 3-day-old, etiolated seedlings grown on vertical plates with half-strength MS agar medium were irradiated with unilateral blue light at a fluence rate of 1 µmol m^{-2} s^{-1}. Curvature was determined at the indicated times after the start of irradiation. As both mutants are in the Col-0 background, bending rates were compared to Col-0 wild-type seedlings. Data shown are means +/- two standard errors of 136 (WT), 146 (yue1yue4), and 112 (sav3) measured seedlings.
Supplemental Table I: Parameters impacting auxin flux kinetics including their assumed values. In the model, capacities are subject to a base pumping rate combined with membrane potential effects (last factor, expressed as number of particles per time unit and per area unit of cell surface) and a loading density of membranes with the respective carrier (PGP or PIN). Because PIN carriers are assumed to potentially be polarly distributed, we assumed different base loadings (factors in the bracket) as carrier densities.

| Parameter | Function | Value |
|-----------|----------|-------|
| $D_{\text{IAA}}$ | Auxin diffusion constant | 0.0024 cm$^2$ h$^{-1}$ (1) |
| $P_{\text{diff}}$ | Auxin membrane permeability | 0.2 cm h$^{-1}$ (1) |
| $\mu$ | Auxin decay rate | 0.00075 s$^{-1}$ |
| $K_{\text{PIN}}$ | Dissociation constant of PINs and auxin | 30 |
| $C_{\text{PIN}}$ | Auxin transport capacity for PINs | {0.03, 0.07, 0.15, 0.65} * 4.7 molecules/surface unit |
| $K_{\text{PGP}}$ | Dissociation constant of PGP and auxin | 30 |
| $C_{\text{PGP}}$ | Auxin transport capacity for PGP | 0.5 * 4.7 molecules/surface unit |

(1) Kramer EM (2006) How far can a molecule of weak acid travel in the apoplast or xylem? Plant Physiol 141(4):1233-1236.
Supplemental Table II: List of primers used in this study

| Gene | name   | sequence                  | efficiency | r2  |
|------|--------|---------------------------|------------|-----|
| AUX1 | for    | CGGAAACCGTACCGGAAAAAG     |            |     |
|      | rev    | GAACCAAGCGTCCCCAGACAG     | 2          | 0.999 |
| LAX1 | for    | CCCTCCCTCTTCCATTAATC      |            |     |
|      | rev    | TTTCTCACCACGACATTAGC      | 1.93       | 0.996 |
| LAX2 | for    | AGATGGAGAACGGTGAGAAAGC    |            |     |
|      | rev    | TGAGAAGCTATGGCATGTC       | 1.994      | 0.993 |
| LAX3 | for    | CGCAGGGAATTACTTAGAAATG    |            |     |
|      | rev    | TGAGAAGCTATGGTAATG        | 1.98       | 0.992 |
A

WT
aux1
lax1
lax2
lax3

hypocotyl curvature (°)

Time under unilateral blue light irradiation (hours)

B

WT
aux1
lax1
lax2
lax3
aux1
lax1
lax3
aux1
lax1
lax2
lax3

hypocotyl curvature (°)

C

AUX1
LAX1
LAX2
LAX3

relative expression (a.u.)
Gradient formation results comparison between resting pH 5.5 and resting pH 4.8 based on scenario with concomitant epidermal pH change

| resting pH | PIN activity | pH 5.5 | pH 4.8 |
|------------|--------------|--------|--------|
|            | normal PIN activity | 6.7  | 5.1 |
|            | reduced PIN activity | 4.4  | 3.2 |
| pH 5.5     | normal PIN activity | 5.3  | 3.1 |
|            | reduced PIN activity | 5.3  | 1.1 |
| pH 4.8     | normal PIN activity | 4.3  | 1.6 |
|            | reduced PIN activity | 3.4  | 4.0 |

| concentration difference in percent (%) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|----------------------------------------|---|---|---|---|---|---|---|---|---|
| pH 5.5                                 | 6.1 | 6.5 | 7.4 | 7.9 | 8.4 | 9.1 | 9.6 | 9.9 | 10.2 |
| pH 4.8                                 | 5.3 | 5.6 | 6.2 | 6.9 | 7.4 | 8.0 | 8.5 | 8.9 | 9.3 |

Cell size and distribution

Apoplast thickness

- IIa (before elongation & thick)
- IIb (medium elongation & medium thickness)
- IIc (elongated & thin)
- 100nm (homogeneous & thin)
- 400nm (homogeneous & medium)
- 800nm (homogeneous & thick)
Gradient formation results comparison between different directions of light stimulus based on scenario with concomittant epidermal pH change at resting pH 5.5

| topology          | apoplast thickness | concentration difference in percent (%) |
|-------------------|--------------------|-----------------------------------------|
|                   | IIa (before elongation & thick) | 6.7 | 4.3 | 4.3 | 3.4 | 5.3 | 6.5 | 7.4 | 5.4 | 5.2 | 4.8 | 6.9 | 7.9 |
|                   | IIb (medium elongation & medium thickness) | 7.4 | 4.6 | 4.6 | 3.4 | 5.8 | 7.2 | 7.4 | 5.3 | 5.2 | 5.1 | 7.2 | 8.0 |
|                   | IIc (elongated & thin) | 6.7 | 4.3 | 4.3 | 3.4 | 5.3 | 6.5 | 7.4 | 5.4 | 5.2 | 4.8 | 6.9 | 7.9 |
|                   | 100nm (homogeneous & thin) | 7.4 | 4.6 | 4.6 | 3.4 | 5.8 | 7.2 | 7.4 | 5.3 | 5.2 | 5.1 | 7.2 | 8.0 |
|                   | 400nm (homogeneous & medium) | 7.4 | 4.6 | 4.6 | 3.4 | 5.8 | 7.2 | 7.4 | 5.3 | 5.2 | 5.1 | 7.2 | 8.0 |
|                   | 800nm (homogeneous & thick) | 7.4 | 4.6 | 4.6 | 3.4 | 5.8 | 7.2 | 7.4 | 5.3 | 5.2 | 5.1 | 7.2 | 8.0 |
hypocotyl curvature (°)

Time under unilateral blue light irradiation (hours)
A. Hypocotyl elongation rate (mm/hr) for WT, aha1, and aha2 genotypes.

B. Effect of DCCD (μM) on hypocotyl elongation rate.

C. Effect of FC (μM) on hypocotyl elongation rate.
A 6 μm slices

Z projection
SUM slices

ROI selection

Integrated density

1+3 : Lit side
2+4 : Shaded side

B

DMSO

NPA

FC

C

Time under unilateral blue light irradiation (hours)

hypocotyl curvature (°)

0 1 2 6 12 24

DMSO

NPA

FC

De-etiolated

3d Etiolated

4d Etiolated

3d Etiolated

4d Etiolated

FC

NPA
|                  | 0  | 0  | 0  | 5' | 5' | 5' | 30' | 30' | 30' |
|------------------|----|----|----|----|----|----|-----|-----|-----|
| total H+ ATPase  |    |    |    |    |    |    |     |     |     |
| signal (a.u.)    |    |    |    |    |    |    |     |     |     |
| phospho H+ ATPase|    |    |    |    |    |    | 0.67| 1.57| 0.78|
| signal (a.u.)    |    |    |    |    |    |    | 0.67| 1.57| 0.78|
| WT               |    |    |    |    |    |    |     |     |     |

|                  | 0  | 0  | 0  | 5' | 5' | 5' | 30' | 30' | 30' |
|------------------|----|----|----|----|----|----|-----|-----|-----|
| total H+ ATPase  |    |    |    |    |    |    |     |     |     |
| signal (a.u.)    |    |    |    |    |    |    |     |     |     |
| phospho H+ ATPase|    |    |    |    |    |    | 0.43| 1.97| 0.87|
| signal (a.u.)    |    |    |    |    |    |    | 0.43| 1.97| 0.87|
| phot1phot2       |    |    |    |    |    |    |     |     |     |

**WT**

**phot1phot2**

**signal (a.u.)**

**signal (a.u.)**
