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Clathrin-Mediated Auxin Efflux and Maxima Regulate Hypocotyl Hook Formation and Light-Stimulated Hook Opening in *Arabidopsis*

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ABSTRACT

The establishment of auxin maxima by PIN-FORMED 3 (PIN3)- and AUXIN RESISTANT 1/LIKE AUX1 (LAX) 3 (AUX1/LAX3)-mediated auxin transport is essential for hook formation in *Arabidopsis* hypocotyls. Until now, however, the underlying regulatory mechanism has remained poorly understood. Here, we show that loss of function of clathrin light chain *CLC2* and *CLC3* genes enhanced auxin maxima and thereby hook curvature, alleviated the inhibitory effect of auxin overproduction on auxin maxima and hook curvature, and delayed blue light-stimulated auxin maxima reduction and hook opening. Moreover, pharmacological experiments revealed that auxin maxima formation and hook curvature in *clc2 clc3* were sensitive to auxin efflux inhibitors 1-naphthylphthalamic acid and 2,3,5-triiodobenzoic acid but not to the auxin influx inhibitor 1-naphthoxyacetic acid. Live-cell imaging analysis further uncovered that loss of *CLC2* and *CLC3* function impaired PIN3 endocytosis and promoted its lateralization in the cortical cells but did not affect AUX1 localization. Taken together, these results suggest that clathrin regulates auxin maxima and thereby hook formation through modulating PIN3 localization and auxin efflux, providing a novel mechanism that integrates developmental signals and environmental cues to regulate plant skotomorphogenesis and photomorphogenesis.

Key words: auxin maxima, clathrin, hook formation, hypocotyl, *Arabidopsis*

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INTRODUCTION

In dicotyledonous plants, after seed germination in soil, a darkness/soil-induced hook at the top of the hypocotyl is formed to protect the two cotyledons and shoot meristem from damage by soil particles (Von Arnim and Deng, 1996; Vandenbussche et al., 2005; Zhong et al., 2014). Hook formation is caused by differential cell elongation at two sides of the hypocotyl apex. In etiolated *Arabidopsis* seedlings, it has been shown that hook formation is predominantly regulated by two major phytohormones auxin and ethylene (Raz and Ecker, 1999; Li et al., 2004; Abbas et al., 2013; Mazzella et al., 2014; Zhong et al., 2014). Auxin maxima at the concave (inner) side of the hook, essential for hook formation and maintenance, are largely determined by AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX)-mediated auxin influx (Vandenbussche et al., 2010), PIN-FORMED (PIN)-mediated auxin efflux (Zádníková et al., 2010), and TRYPTOPHAN AMINOTRANSFERASE 1/TAA RELATED 2 (TAA1/TAR2)-mediated local auxin biosynthesis (Stepanova et al., 2008). During hook formation, auxin influx transporters mediate the proper basipetal auxin transport from the two cotyledons and shoot meristem to the hook (Vandenbussche et al., 2010), while auxin efflux transporters determine the establishment of auxin maxima in the hook region (Zádníková et al., 2010). Once the seedling emerges from the soil, different types of light, including blue, red, and far-red light, trigger hook opening (Liscum and Hangarter, 1993a, 1993b; Wu et al., 2010), and consequently, seedling development will switch from skotomorphogenesis to photomorphogenesis. To date, however, how auxin maxima are regulated by light to influence hook formation, maintenance, and opening has remained poorly understood.

Clathrin, a triskelion-shaped complex consisting of three heavy chains (CHCs) and three light chains (CLCs), regulates the

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formation of clathrin-coated vesicles and, thereby, membrane trafficking in animal cells (Bitsikas et al., 2014; Kirchhausen et al., 2014). In plants, clathrin-mediated trafficking has been found to functionally regulate many plant developmental processes including embryogenesis (Kitakura et al., 2011), gametogenesis (Backues et al., 2010), pollen tube elongation (Zhao et al., 2010), root growth and gravitropism (Robert et al., 2010; Chen et al., 2012; Wang et al., 2013), and leaf development (Xu et al., 2010), as well as defense or stress responses (Hao et al., 2014; Smith et al., 2014) and nutrient uptake (Barberon et al., 2011, 2014). Recent studies have demonstrated that auxin transport and distribution in plants are highly dependent on clathrin-mediated endocytosis, which facilitates the internalization of plasma membrane (PM)-resident PIN proteins and their polar localization (Dhonukshe et al., 2007; Kitakura et al., 2011; Wang et al., 2013). Mutations in *Arabidopsis CHC* or *CLC* cause defects in PIN trafficking, auxin distribution, root gravitropism, and other auxin-related processes (Kitakura et al., 2011; Wang et al., 2013). However, it remains to be determined whether clathrin functions in the establishment of auxin maxima during hook formation.

In this study, we have characterized the functional roles of clathrin in hook formation and blue light-stimulated hook opening. Our results show that clathrin modulates hook formation and blue light-triggered hook opening through its effects on PIN3 localization and consequently on auxin maxima, revealing an important regulatory role for clathrin-mediated trafficking during plant skotomorphogenesis and photomorphogenesis.

RESULTS

Auxin Maxima and Hook Development Are Altered in Clathrin-Deficient Mutants

Although auxin influx and efflux transporters have been demonstrated to play a critical role in the establishment of auxin maxima and, thereby, hook formation, maintenance, and opening (Vandenbussche et al., 2010; Zádníková et al., 2010), detailed regulatory mechanisms are largely unknown. Here, we sought to explore whether clathrin-mediated trafficking functions in the establishment of auxin maxima and hook formation. Previous studies showed that the *Arabidopsis* genome contains three *CLC* homologs, namely *CLC1*, *CLC2*, and *CLC3*. Loss of *CLC1* function causes a pollen lethality phenotype, whereas loss of *CLC2* and *CLC3* function does not jeopardize viability and fertility of *clc2-1 clc3-1* double mutant plants (Wang et al., 2013). We therefore used *clc2-1 clc3-1* double mutants to investigate the roles of clathrin at different stages of hook development (72, 96, 120, and 144 h after sowing). As shown in Figure 1A–1C, the mean angle of hook curvature in the wild-type was maximal at 96 h and subsequently decreased at 120 and 144 h. At 72 h, there was no significant difference in the mean angle of the hook curvature between *clc2-1 clc3-1* and the wild-type. However, from 96 h onward, the double mutants exhibited larger hook curvature than that in the wild-type, suggesting that loss of *CLC2* and *CLC3* function enhances hook formation and impairs hook opening.

Next, to address whether auxin maxima essential for hook formation is altered in *clc2-1 clc3-1* double mutants, we examined the expression pattern of an auxin-responsive reporter *DR5*:*GFP* (green fluorescent protein; Benková et al., 2003) in the hook region of the wild-type and mutants. Consistent with previous observations using the *DR5*:*GUS* reporter (Vandenbussche et al., 2010; Zádníková et al., 2010), a specific GFP signal at the concave (inner) side, but not at the convex (outer) side, of the hook was observed in the *DR5*:*GFP* reporter line (Supplemental Figure 1), confirming the formation of auxin maxima at the concave side of the hook. Similar to dynamic changes of hook curvature (Figure 1C), the GFP intensity at the concave side peaked at 96 h in the wild-type and *clc2-1 clc3-1*, but was much stronger in the double mutants than in the wild-type (Figure 1D–1F), suggesting that loss of *CLC2* and *CLC3* function enhances auxin maxima formation at the concave side of the hook.

To rule out the possibility that loss of *CLC2* and *CLC3* function may affect seed germination and thereby causes a delay in hook development, we examined the germination rate of wild-type and *clc2-1 clc3-1* seeds. There was no significant difference between the wild-type and double mutants regardless of the light conditions (Supplemental Figure 2). Furthermore, quantitative real-time PCR (qRT-PCR) analysis showed that both *CLC2* and *CLC3* are expressed in the hook region, although *CLC3* is lowly expressed (Supplemental Figure 3). Together, these results suggest that clathrin regulates the establishment of auxin maxima and, thereby, hook development.

Effects of Auxin Overproduction on Auxin Maxima and Hook Curvature Are Alleviated in Clathrin-Deficient Mutants

Previous studies have shown that overexpression of *YUCs*, encoding a rate-limiting enzyme in auxin biosynthesis, significantly increases endogenous auxin levels and thereby inhibits hook formation (Zhao et al., 2001; Cheng et al., 2006). To further confirm whether auxin overproduction disrupts the establishment of auxin maxima in the hook region and whether loss of *CLC2* and *CLC3* function alters the inhibitory effects of auxin overproduction on auxin maxima and hook formation, we generated transgenic lines overexpressing *YUC1* (*35S*:*YUC1-GUS*; namely *YUC1-OE*) in the Col-0 background and subsequently generated *YUC1-OE clc2-1 clc3-1* (*YUC1-OE clc2 clc3*) lines. The *YUC1-OE* transgenic lines displayed characteristic auxin overproduction phenotypes including curled cotyledons, longer hypocotyls, and shorter primary roots with more root hairs relative to the wild-type (Supplemental Figure 4A and 4B), suggesting that YUC1-GUS is functional. Consistent with the previous finding in the *YUC1*-overexpressing lines (Zhao et al., 2001), at 96 h after sowing, hook formation in *YUC1-OE* transgenic lines was found to be dramatically inhibited relative to the wild-type (Supplemental Figure 4C). Furthermore, kinetic analysis of hook formation showed that dynamics of hook development were dramatically abolished in the *YUC1-OE* lines (Figure 2A and 2B), compared with the wild-type (Figure 1A and 1C). By contrast, loss of CLC2 and CLC3 enhanced hook curvature in *YUC1-OE clc2 clc3* lines relative to *YUC1-OE* lines from 72 to 144 h after sowing (Figure 2A–2C).

Next, we analyzed *DR5*:*GFP* expression pattern in the hook region of *YUC1-OE* and *YUC1-OE clc2 clc3* lines at 96 and 144 h