Nyctinastic thallus movement in the liverwort *Marchantia polymorpha* is regulated by a circadian clock

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The circadian clock coordinates an organism’s growth, development and physiology with environmental factors. One illuminating example is the rhythmic growth of hypocotyls and cotyledons in *Arabidopsis thaliana*. Such daily oscillations in leaf position are often referred to as sleep movements or nyctinasty. Here, we report that plantlets of the liverwort *Marchantia polymorpha* show analogous rhythmic movements of thallus lobes, and that the circadian clock controls this rhythm, with auxin a likely output pathway affecting these movements. The mechanisms of this circadian clock are partly conserved as compared to angiosperms, with homologs to the core clock genes PRR, RVE and TOC1 forming a core transcriptional feedback loop also in *M. polymorpha*.

Rhythmic movements of plant organs were documented already several centuries BC, but the first known experiments searching for the origin of such rhythms were conducted by the French astronomer de Mairan. Working with a sensitive plant (likely *Mimosa pudica*), he could show that leaves moving in day/night conditions continued to move in constant darkness. During the following centuries, experiments with what Linnaeus later termed “sleep movements” resulted in both the concept of the circadian clock and that of osmotic motors. These so called nyctinastic movements often occur in non-growing tissue and are reversible as in several legumes. The reversible movements involve osmotic motors in the pulvinus organ, but rhythmic leaf movements can also be growth associated and thus non-reversible. Such rhythms are evident in the movement of leaves in tobacco and cotyledons in *Arabidopsis thaliana*. The irreversibility of this process is probably due to deposition of new cell wall material and decreased wall extensibility, but tissue expansion likely results from mechanisms in common with those in pulvinus tissue.

Since the introduction of the concept of a circadian or endogenous biological clock great progress has been achieved in understanding the mechanisms behind such internal rhythms. In plants most of this work has been performed in the flowering plant *Arabidopsis*. A working model of the plant circadian clock comprises a self-sustaining oscillator with an approximately 24-hour rhythm resulting mainly from transcriptional and translational feedback loops. In short, the main components in such models are a set of single MYB domain transcription factors, a family of PSEUDO-RESPONSE REGULATORs (PRRs), and a few plant specific genes with unknown biochemical function. The early morning phased genes *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) encode two MYB-like transcription factors that function mainly as repressors of day- and evening-phased genes. A second sub-family of related MYB-like transcription factors including *REVEILLE4* (*RVE4*), *RVE6* and *RVE8* has an opposite function, enhancing clock pace through the activation of several core clock genes.

The family of PRR genes comprise five members in *Arabidopsis*: PRR1, PRR3, PRR5, PRR7 and PRR9. PRR1 is also known as *TIMING OF CAB EXPRESSION 1* (*TOC1*) that together with *CCA1* constituted the first conceptual model of the Arabidopsis clock. The expression of PRR genes ranges from morning to evening, with PRR9 peaking in the morning, PRR5 and PRR7 around noon, and PRR3 and TOC1 around dusk. PRR proteins are in recent models incorporated as transcriptional repressors of *CCA1/LHY* and other PRR genes.

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An additional crucial component of the circadian clock in Arabidopsis is the so-called evening complex (EC), that consists of three proteins: EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX) 18–20. In contrast to other clock genes, knockout mutants of the genes coding for these proteins all show an arrhythmic phenotype in Arabidopsis. Previous studies in Arabidopsis have revealed that the EC mainly function as repressors of transcription. Within the circadian clock, the EC repress TOC1, PRR7, PRR9, and LUX during the night21–23, and a transcriptional feedback loop is formed through the repression of EC genes by CCA1/LHY 20,21,24–26.

Important components of the Arabidopsis circadian clock also include ZEITLUPE (ZTL), an F-box protein, and GIGANTEA (GI)27,28. Homologs of ELF3, ELF4, LUX, PRR3,7,9, TOC1, RVE4,6,8, ZTL and GI have been reported in M. polymorpha29. However, a CCA1/LHY homolog is not present in the genome, suggesting that many, but not all, known clock gene families are also present in M. polymorpha.

When searching for rhythmic growth patterns in the liverwort M. polymorpha we discovered that gemmalings (asexually produced plantlets) displayed rhythmic thallus movements as the lobes of young thalli waves up and down with a 24 hour rhythm in conditions of 12 hour light and 12 hour darkness (neutral days (ND); Fig. 1a,b; Supplementary Video S1). Furthermore, in gemmalings of different accessions these rhythmic movements are
of these genes can be used to study the role of the circadian clock in the control of growth and development. Knockout mutants of MpPRR, MpRVE and MpTOC1 affects each other’s expression. qRT-PCRs measuring expression of M. polymorpha clock genes during two consecutive days of constant light in wild type (Tak1, Mpprrko, Mprrveko and Mptoc1ko). Plants were entrained in ND and sampling was conducted every six hours starting 24 hours after switch to LL. Graphs show the average expression of MpPRR (a), MpTOC1 (b) and MpRVE (c) in three replicates of wild type or mutant line for each time point. Green and orange lines combined with filled symbols indicate significantly overall higher or lower expression as compared to wild type (bold line), respectively. Small symbols indicate the value of each replicate.

maintained in LL (continuous light) conditions with an approximate average period of 26.1–26.5 hours for several days, supporting that they are controlled by a circadian clock (Fig. 1c–e). One key characteristic of circadian rhythms is temperature compensation, i.e., that the free-running period does not change much with ambient temperature. We thus estimated the free-running period of nyctinastic thallus movement at different temperatures. Consistent with circadian regulation, we found no significant difference in period for temperatures ranging from 18 to 24°C (Fig. 1c).

To further investigate the role of a circadian clock for this movement, we first obtained a more detailed view on the role of MpPRR, MpRVE and MpTOC1 as core circadian clock components. Because transcriptional feedback loops are crucial for angiosperm circadian clocks, we examined temporal expression patterns of these genes using qRT-PCR over a 48-hour period. As previously reported, MpPRR display rhythmic expression in the wild type in LL conditions (Fig. 2a). In Mptoc1ko mutants, expression of MpPRR was continuously high and arrhythmic, as indicated by highly significant effects of both genotype (G) and genotype x time interaction (GxT) terms in ANOVA (P < 10^-11), suggesting that MpTOC1 represses MpPRR. Conversely, expression of MpPRR was low with limited amplitude in Mprveko (P-values for both G and GxT terms were < 10^-9), indicating that MpRVE promote the expression of MpPRR (Fig. 2a). The rhythmic expression of MpTOC1 in LL is weak, hampering interpretations of changes in its expression in mutant genotypes. Still, the expression of MpTOC1 in wild type, Mpprrko and Mprveko is consistent with an activating role of MpRVE and a repressing role of MpPRR analogous to the role of the Arabidopsis homologs on TOC1 expression (Fig. 2b; both G (P < 10^-9) and GxT (P = 0.026) were significant for Mpprrko and G was significant for Mprveko with P = 0.017). Furthermore, in Mpprrko and Mptoc1ko, expression of MpRVE remains high and arrhythmic indicating that MpRVE is repressed by both MpPRR and MpTOC1 (Fig. 2c; P-values for G were less than 10^-9 in both cases, while GxT was marginally significant with P = 0.04 and 0.07 for Mpprrko and Mptoc1ko, respectively). These data collectively suggest that MpPRR, MpRVE and MpTOC1 are part of a core transcriptional feedback loop of the M. polymorpha circadian clock, and that knock-out mutants of these genes can be used to study the role of the circadian clock in the control of growth and development.

We then analyzed rhythm movements during constant conditions in knockout mutants of MpPRR and Mptoc1 that we identified as part of core feedback loops in the M. polymorpha circadian clock29 (Fig. 2). In Mpprrko and Mptoc1ko mutants the rhythm is completely lost in LL (Fig. 1d,e). Because mutations of EC members result in an arrhythmic Arabidopsis clock we also created two independent mutant alleles for MpLUX, the single M. polymorpha homolog to the Arabidopsis EC component LUX (Supplementary Fig. S1). The two mutants, Mploxko-9 and Mploxko-19, also showed the arrhythmic phenotype in LL observed in the other clock mutants (Fig. 1f,g). These results strongly support that the circadian clock controls “gemmaling waving”, and that estimates of this movement can be used to monitor the M. polymorpha circadian clock.

The circadian clock regulates IAA levels and expression of the auxin biosynthesis gene MpTAA. Most likely, the rhythmic movement is growth related and similar to cotyledon movement in Arabidopsis, suggesting a role for rhythmic auxin production, transport and/or signaling in its control. Available data suggest that most auxin in liverworts such as Lumaria crassata and M. polymorpha is produced in the apical region and transported basipetally through the midrib region producing an auxin gradient. To assay the role of MpPRR in MpTAA, we first obtained a more detailed view on the role of MpPRR, MpRVE and MpTOC1 as core circadian clock components.
Mptoc1ko, respectively. For MpYUC2 expression in the wild type we could not detect a rhythmic pattern in LL, and only Mprveko showed a slightly higher overall expression level with \( P = 0.006 \) for G term (Fig. 3b).

Because MpTAA share expression domain in the apical part of the thallus with MpPRR, MpTOC1 and MpRVE, as well as with other putative clock genes such as MpLUX and MpELF3, it seems plausible that the clock could regulate IAA levels at least in part via MpTAA. To test if IAA levels display circadian oscillations we measured free IAA in whole young gemmalings of wild type (Upp14) and MpLUX-9 and MpLUX-19 lines compared to wild type (Upp1 and Upp5). For qRT-PCR experiments plants were entrained in ND and sampling was conducted every four (d) or six (a,b) hours starting 12 (d) or 24 (a,b) hours after switch to LL. qRT-PCR graphs show the average expression of three replicates of wild type or mutant line (a,b), or four replicates (two for each of the genotypes) (d), for each time point. Green and red lines combined with filled symbols indicate significantly overall higher or lower expression as compared to wild type (bold line), respectively. Small symbols indicate the value of each replicate.

**Figure 3.** MpTAA expression levels as well as levels of free IAA display circadian oscillations that are disrupted in knockout mutants of *Marchantia polymorpha* clock genes. (a,b) qRT-PCRs measuring expression of *M. polymorpha* auxin biosynthesis genes during two consecutive days of constant light in wild type (Tak1), Mpprrko, Mprveko and Mptoc1ko. (a) MpTAA expression. (b) MpYUC2 expression. (c) IAA measurements of whole wild type (Upp14) and MpLUX-9 gemmalings in LL over 32 hours. (d) MpPRR expression in the MpLUX-9 and MpLUX-19 lines compared to wild type (Upp1 and Upp5). For qRT-PCR experiments plants were entrained in ND and sampling was conducted every four (d) or six (a,b) hours starting 12 (d) or 24 (a,b) hours after switch to LL. qRT-PCR graphs show the average expression of three replicates of wild type or mutant line (a,b), or four replicates (two for each of the genotypes) (d), for each time point. Green and red lines combined with filled symbols indicate significantly overall higher or lower expression as compared to wild type (bold line), respectively. Small symbols indicate the value of each replicate.

**Auxin could be a mediator of circadian control of thallus movements.** To further evaluate a role for auxin in nyctinasty, we conducted nyctinastic movement experiments manipulating auxin levels, distribution and response. For chemical treatments, wild-type gemmae were first grown on standard growth medium for five days to allow for initiation of growth and dorsiventral polarity establishment. Actively growing gemmalings
were subsequently transferred to media supplemented with the auxin transport inhibitor 2-[4-(diethylami-
no)]-2-hydroxybenzoyl benzoic acid (BUM)\textsuperscript{37}, or low concentrations of indole 3-acetic acid (IAA).

Low doses of IAA (10 and 100 nM) resulted in a reduced angle of growth and thus a more flattened thallus
(Fig. 4a,c). Rhythmic waving was detectable throughout the experiment on mock and 10 nM IAA, but dampened
earlier on 100 nM IAA (Supplementary Videos S2–S4). We cannot exclude that this dampening is due to contact
with the solid medium (Supplementary Video S4). This dampening hampered statistical analyses of rhythmic-
ity resulting in a very low sample size of rhythmic plants at 100 nM IAA (n = 3). Still, analyses with Spectrum

**Figure 4.** Manipulation of auxin levels, distribution and response affects nyctinastic movements. (a)
Gemmalings growing on different concentrations of IAA. (b) Gemmalings growing on different concentrations
of BUM. (c) Plot of growth angle change over time for gemmalings growing on different concentrations of IAA.
(d) Plot of growth angle over time for gemmalings growing on different concentrations of BUM. Angle was
measured as indicated in (a). Plants (a–d) were entrained in ND for three to five days, then transferred to IAA
(a,c) or BUM (b,d) for one and three days in ND, respectively, before transfer to LL. (e,f) Average amplitude
of nyctinastic movement for various genotypes (e) and for wild type (Upp5) growing on L-Kyn (f). WT (wild
type) M (male) and F (female) (e) are of the Australian accession\textsuperscript{39}. Experiments (e,f) were performed using
younger gemmalings in (f), which is why the controls are not identical. Graphs (e,f) shows means \pm SE of three
replicates. **P < 0.01, ***P < 0.001 for two-tailed t-test against control (wild type or mock).
Resampling\textsuperscript{38} indicated an increased period and RAE with increasing IAA doses ($P < 0.001$ and $P < 0.01$, respectively).

Conversely, an increased growth angle was observed for growth on BUM in a dose-dependent manner (Fig. 4b,d). The apparent early dampening of rhythmic waving on BUM could be the result of contact between the two lobes due to the high growth angle. Statistical analysis indicated an increased period and RAE but reduced amplitude with increased BUM doses ($P < 0.05, P < 0.05$ and $P < 0.01$, respectively).

Even though these chemical treatment data does not show very convincing changes in rhythmicity, an important conclusion is that IAA and BUM have clear opposing effects on growth angle.

To study the effect of reduced auxin levels, we analysed waving in MpSHI\textsubscript{pro:iaaL} plants. These lines express the bacterial auxin-conjugating enzyme IaaL from a promoter mainly active in the apical notch, that result in plants displaying e.g., slow growth and narrow thalli\textsuperscript{39}. No difference in period of the rhythmic waving, as compared to wild type, was observed for MpSHI\textsubscript{pro:iaaL} plants ($P > 0.75$), but the analysed lines displayed a significantly lower amplitude ($P < 9.2 \times 10^{-10}$; Fig. 4e). Growth on high concentrations of the TAA inhibitor L-Kynurenic acid (L-Kyn: 250 μM) gave a similar result: a significantly reduced amplitude ($P < 1.4 \times 10^{-11}$; Fig. 4f) but no significant change in period ($P > 0.09$). Similarly, a reduced amplitude but similar period was observed for EF1\textsubscript{pro:amiR-MpARF1\textsubscript{35pro:amiR}} plants ($P > 0.96$ and $P < 2 \times 10^{-10}$, for period and amplitude, respectively; Fig. 4e), suggesting that reduced auxin sensitivity also attenuate rhythmic waving. Collectively these results support a role for auxin as an output pathway for the M. polymorpha circadian clock.

Discussion

In angiosperms the circadian clock regulates a wide range of processes, including those affecting metabolism, growth, abiotic and biotic stress, and various photoperiodic responses (\textsuperscript{40} and references therein). Our previous work suggests an early acquisition of a complex circadian network in plant evolution, but also important differences in the wiring and function of the A. thaliana circadian clock\textsuperscript{29}.

One unexpected handle of the M. polymorpha circadian clock is the rhythmic waving of gemmaling thallus lobes. This movement is most likely related to the non-reversible alternating growth of adaxial and abaxial sides of e.g., cotyledons of Arabidopsis\textsuperscript{4}. It has been suggested that this type of nycinastic movement was ancestral, and that motor cells and pulvini developed later as a means of enhancing leaf movement\textsuperscript{41}. If so, our results suggest an early acquisition of nycinasty already in the ancestor of bryophytes and seed plants. The cause of this type of alternating growth is poorly known, but may be related to the circadian elongation response of hypocotyls\textsuperscript{42}. This rhythmic elongation involves circadian regulated hormone production, transport and/or signaling\textsuperscript{43}. Auxin is a good candidate for such a hormone in Arabidopsis and also in M. polymorpha. Early studies showed that plants respond differently to applied auxin depending on time of day\textsuperscript{44}, and subsequent experiments revealed that auxin levels oscillate in several species\textsuperscript{45,46}. Furthermore, the circadian clock controls many genes that affect auxin synthesis and signaling in Arabidopsis\textsuperscript{47–49}.

Our gene expression data suggests that the circadian clock in M. polymorpha regulates the first step of auxin biosynthesis, which might contribute to rhythmic auxin levels primarily in the apical region where MpTAA is expressed and most auxin in M. polymorpha is found\textsuperscript{35}. If auxin levels were to control alternating growth of dorsal and ventral sides we would need to hypothesize a rhythm in dorsal/ventral distribution of the apically produced hormone during the 24-hour cycle. Such a role for auxin in these rhythmic movements is consistent with the results of our manipulations of auxin levels, distribution and response. Application of IAA and BUM had opposite effects on the angle of growth that in turn is directly connected to nycinastic movements. Assuming that BUM affects ABCB-mediated auxin transport as in Arabidopsis, the increased angle on gemmalings growing on BUM can be interpreted as a result of decreased auxin transport from the ventral to the dorsal side, leading to increased ventral auxin concentration and cell elongation. This hypothesis requires an initial uneven dorsal/ventral auxin distribution, perhaps through a basipetal transport of auxin mainly on the dorsal side. Under this scenario, addition of a significant portion of exogenous auxin (IAA) is expected to result in a more equal dorsal/ventral distribution and hence more flat growth (reduced growth angle in our experiments). This supports the epinastic growth of gemmalings on high concentrations of auxin or overexpression of the auxin synthesis enzyme MpYUC2\textsuperscript{55,39}. Conversely, L-Kyn-treatment, overexpression of IaaL and the use of amiRNAs to knock down the expression of auxin biosynthesis genes, resulted in hyponastic growth of gemmalings\textsuperscript{55,60}. In our experiments, directly decreasing the amount of active auxin by expressing IaaL or by reducing the function of MpTAA by adding L-Kyn resulted in lowered amplitude of nycinastic movement, further supporting the role for auxin in these movements.

Our present results support conservation of the function for the M. polymorpha homologs of PRR, TOC1 and RVE, each with only one copy in M. polymorpha (MpPRR, MpRVE and MpTOC1). Each of them seems to be crucial for maintaining a transcriptional feedback loop in constant light conditions. For MpPRR and MpTOC1 we also observed abolished circadian nycinastic thallus movement, verifying the importance of these genes in generating circadian rhythms in M. polymorpha. The stronger effects of mutating these genes in M. polymorpha, as compared to Arabidopsis, is likely due to the lack of functionally related paralogs in M. polymorpha.

Work on green algae suggest that one homolog of the PRR/TOC1 clade and one of the CCA/LHY/RVE clade constituted the core transcriptional feedback loop in the earliest plants\textsuperscript{50,51}. Our work thus supports a continuous use of pairs of CCA/LHY/RVE clade genes and PRR/TOC1 genes at the core of plant circadian clock networks.

The exact function of these genes within the network seems to have varied over time, partly due to addition of copies of existing genes or new genes to the network, or even deletion of core clock genes.

The only homolog in the whole CCA1/LHY/RVE clade present in M. polymorpha, MpRVE, belongs to the LCL sub-clade\textsuperscript{39}, as do RVE4, RVE6 and RVE8. Accordingly, the MpRVE gene does not show an early morning expression, nor acute light induction, which is typical of genes in the CCA1/LHY sub-clade\textsuperscript{29}. In addition, our data support a role for MpRVE as transcriptional activator as opposed to the role of CCA1/LHY genes as repressors\textsuperscript{51,13}.
Thus, MpRVE seems to have retained a function typical for the LCL subfamily, despite the loss of the CCA1/LHY gene that is absent in all liverworts.

Our identification of a circadian regulated thallus movement provides a practical and easy to use tool for further studies of the evolution of plant circadian clocks, including the effects of frequent gene duplication and circadian gene loss observed during land plant evolution.

**Methods**

**Plant growth and cultivation.** _Marchantia polymorpha_ ssp. _ruderalis_ Swedish accessions Uppsala (Upp) 1, 5 and 10 to 14, as well as Australian male and female, and Takaragaie (Tak) -1 and Tak-2 were grown aseptically on agar solidified Gamborg’s B5 medium (PhytoTechnology Laboratories, Lenexa, KS, USA), pH 5.5. Plants were grown under cool white fluorescent light (50–60 μmol photons m⁻² s⁻¹) in 16:8 hour, light: dark cycles at 20°C or as otherwise stated in the text. Plants were entrained in 12:12 hour, light: dark cycles at 20°C.

**Gene expression analysis.** RNA was extracted using an Rneasy Plant Mini Kit (Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher) and analysed by qRT-PCR as previously described. Primers are listed in Supplementary Table S1. _MpEF1α_, _MpACT_ and _MpAPT3_ were used for normalization.

For sampling of RNA we used biological replicates (plants of individual transgenic lines or individual wild type lines), and technical replicates (pools of individually grown plants of the same transgenic line or wild type line). We did one cDNA from each RNA sample. In all sampling we used gemmalings harboring adult tissues, but with no visible gemma cups.

For time series experiments with wild type (Tak1), _Mpprko_, _Mpvko_ and _Mptoc1ko_, three replicate samples of each entity were harvested at six-hour intervals from the second day of LL for two days (in total eight time points). Test of statistically significant expression differences between lines were performed with a linear model in R. The model included time, genotype and phenotype interaction.

**Analyses of nyctinastic thallus movements.** 25-well square petri dishes (Fisher Scientific) were filled with Gamborg’s B5 medium, after which half of the medium in each well was removed to allow placement and growth of gemmalings. One gemmeling was placed in each well, and plates were placed vertically in a Sanyo growth cabinet (MLR-350) to allow imaging from the side (see Supplementary Fig. S2). Light was supplied from either cool white fluorescent light (experiments shown in Fig. 1a,b,f,g), or blue and red LEDs (experiments shown in Figs. 1d,e and 4) at 20°C constant temperature, or at the temperatures indicated in the text. Plants where entrained for three to five days in ND (12:0 hour, light: dark cycles) before exposure to constant conditions and imaging. Because younger (smaller) gemmalings waves with a higher amplitude than larger plants, the controls in the two experiments presented in Fig. 4e,f cannot be directly compared as we used smaller gemmalings for the L-Kyn experiment (Fig. 4f) than for the IaaL/ARF1 experiment (Fig. 4e). Within experiments all gemmalings were of the same age. For auxin related experiments, plants were exposed to the chemical in ND for one (IAA) or three (BUM) additional days before transfer to LL. Apex region position data was extracted from images using ImageJ. Images were converted to binary ones and a rectangular selection automatically following the apex region horizontally was used with the command “Analyse Particles” to extract the center of mass in each image. The obtained data on vertical position (relative center of mass of apex region) were de-trended using a cubic smoothing spline with 12 degrees of freedom with the R package smooth.spline. Estimates or circadian period, amplitude and RAE were conducted in MATLAB with a method based on Spectrum Resampling.

**Generation of _Mplux_— and _TOC1_—mutants.** Two gRNAs targeting the coding region of _MpLUX_ in or just upstream of the DNA-binding domain were constructed by annealing oligos CPEP60 + CPEP61 and CPEP62 + CPEP63, respectively (listed in Supplementary Table S1). Annealed oligos were inserted into the BsaI site of plasmid pMpGE En038. The constructs were subsequently cloned into pMpGE010 and pMpGW401, respectively, using GateWay LR cloning. All plasmids were sequenced.

Constructs were introduced into Agrobacterium strain GV3101. _M. polymorpha_ sporelings were transformed essentially as previously described. Transformed sporelings were plated on selective media: Gamborg’s B5 with B5 medium for 10 days in LD 20°C. Gemmalings were then entrained in ND 20°C for 3 days before being harvested every four hours over 32 hours (nine time points) sampling five replicates per genotype per time point, starting four hours after switch to LL conditions. Plant material (around 25–30 mg fresh weight) was purified as previously described, and 500 pg 13C6-IAA internal standard was added to each sample before homogenization and extraction. Free IAA was quantified in the purified samples using combined gas chromatography-tandem mass spectrometry.

**Data availability**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
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**Author contributions**
U.L. and D.M.E. designed the research; U.L., A.B., S.N.R. and D.M.E. performed research and analyzed data; K.L. measured IAA levels; U.L. and D.M.E. wrote the paper with the input of all authors.

**Competing interests**
The authors declare no competing interests.

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