Converging light, energy and hormonal signalling control meristem activity, leaf initiation and growth

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One sentence summary: Development of leaves requires photoreceptors to initiate auxin export, cytokinin action and sugar-dependent signalling at dividing cells, energy signalling further adjusting growth to available light.

Author contributions:

BM and SFB performed the majority of experiments. RD, EG, SR, FD and ELJ performed essential experiments. BM, SFB, RD, FD, LB and ELJ analysed and discussed data. KP, LB and ELJ supervised work. ELJ wrote draft manuscript. All authors contributed to final manuscript.

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Abstract

The development of leaf primordia is subject to light control of meristematic activity. Light regulates the expression of thousands of genes with roles in cell proliferation, organ development and differentiation of photosynthetic cells. Previous work has highlighted roles for hormone homeostasis and the energy-dependent TOR kinase in meristematic activity, yet a picture of how these two regulatory mechanisms depend on light perception and interact with each other is yet to emerge. Their relevance beyond leaf initiation is also unclear. Here we report the discovery that the dark-arrested meristematic region experiences a local energy-deprivation state and confirm previous findings that the PIN1 auxin transporter is diffusely localised in the dark. Light triggers a rapid removal of the starvation state and the establishment of PIN1 polar membrane localisation consistent with auxin export, both preceding the induction of cell cycle and cytoplasmic growth-associated genes. We demonstrate that shoot meristematic activity can occur in the dark through manipulation of auxin and cytokinin activity, as well as through the activation of energy signalling, both targets of photomorphogenesis action, but the organ developmental outcomes differ: while TOR-dependent energy signals alone stimulate cell proliferation, development of a normal leaf lamina requires photomorphogenesis-like hormonal responses. We further show that energy signalling adjusts the extent of cell cycle activity and growth of young leaves non-cell-autonomously to available photosynthates, and leads to organs constituted of a greater number of cells developing under higher irradiance. This makes energy signalling perhaps the most important biomass growth determinant under natural, unstressed conditions.
Leaves are biological solar panels, the development of which begins as primordia at the flanks of the shoot apical meristem (Tsukaya, 2005; Kalve et al., 2014). This meristem consists of a pool of stem cells and their close descendants, is organised during embryogenesis and arrests as the embryo enters dormancy, becoming protected within the seed. Following germination, which frequently occurs underground, the development of leaf primordia is arrested in darkness (Chory, 2010). This constitutes part of the skotomorphogenesis developmental programme, which helps young seedlings to emerge through the ground, before the photomorphogenesis programme commences above ground. Emergence into light reinitiates leaf development, including that of leaf mesophyll cells filled with chloroplasts (Nemhauser and Chory, 2002). In most gymnosperm plants however, leaves can develop and cells with chloroplasts can differentiate in the dark, suggesting that the skotomorphogenesis programme is an evolutionary innovation to assist seedling establishment (Hills et al., 2015). As a consequence, upon first exposure to light photosynthesis cannot immediately commence, instead photomorphogenesis is activated by informational photoreceptors, most notably the phytochrome and cryptochrome families (Chory, 2010) that detect the presence, quality and quantity of light. Accordingly, combined deficiency of phytochromes and cryptochromes prevents leaf initiation in the light (López-Juez et al., 2008). Repressors of photomorphogenesis, including DET1 and COP1, target light signalling proteins for degradation in the dark, as revealed by the fact that their loss of function leads to constitutive photomorphogenic development (Chory et al., 1994; Lau and Deng, 2012).

The response of seedlings to the first light exposure post-germination is so dramatic that it constituted the very first target for large-scale gene expression profiling, followed by many subsequent genome-wide studies (Jiao et al., 2007). However these studies were of limited use to understand the initiation of leaves at the meristem in response to light, since the various organs show distinct responses to light; e.g. the cotyledons undergo expansion, while hypocotyls cease to elongate. A developmental and transcriptome-wide analysis of dissected, etiolated shoot apices when the growth of leaf primordia is initiated upon the first exposure to light addressed this question (López-Juez et al., 2008). This analysis revealed a dramatic stimulation of cell proliferation, peaking between 6 and 24h after light exposure. Gene expression signatures associated with cell proliferation and cytoplasmic growth (protein translation) peaked at 6h and were followed by expansion growth-associated signatures, including cell wall remodelling and water influx. A direct regulation of cell cycle-associated E2F transcription factors by photoreceptors, under DET1 and COP1 control, provided a possible mechanism for meristem activation by light (López-Juez et al., 2008; Berckmans et al., 2011). Furthermore, based on diagnostic gene expression signatures, a transient downregulation of auxin and ethylene signalling at the apex was postulated, one which preceded an upregulation of cytokinin responses. The latter coincided with the peak in cell cycle and ribosome-related gene expression activity.

Hormonal responses are central to leaf initiation. Consecutive leaves develop at the flanks of the shoot meristem in striking geometric arrangement, known as phyllotaxy, which can be explained by inhibitory fields generated by emerging leaves. Elegant experiments have revealed those fields to be based on the self-regulating dynamics of auxin transport (Braybrook and Kuhlemeier, 2010). Positions for leaf primordia on the epidermis at the peripheral zone of the meristem are selected where the auxin concentration is high and these points become sinks for further auxin transported from nearby epidermal cells, due to
the polar re-localisation of the PIN1 auxin transport protein. Cells at this position then enter rapid cell proliferation, but leaf emergence requires a second re-localisation of PIN1 proteins to export auxin away from the primordium into the rib meristem (Reinhardt et al., 2003). These events constitute the first steps in leaf development. Auxin is further involved in the proliferation of leaf cells and in the differentiation of vasculature (Scarpella et al., 2006; Scarpella et al., 2010). Auxin thus plays fundamental and separate roles in the positioning and early development of leaves (Capua and Eshed, 2017).

An elegant study carried out in tomato shoot meristems showed that the auxin efflux transporter, PIN1, became internalised when light-grown shoot apices were transferred to the dark, while in the light the auxin maxima established by plasma membrane-localised PIN1 determined the positions for cytokinin action to drive leaf initiation (Yoshida et al., 2011). A subsequent study (Pfeiffer et al., 2016) has demonstrated that sugars acting through the Target of Rapamycin (TOR) kinase pathway, together with cytokinin activity, lead to induction of \textit{WUS} expression and subsequent meristem activation in the light.

Growth is the most resource-consuming process living organisms undertake, and it is not surprising that mechanisms have evolved to sense and interpret the availability of energy and nutrients. Besides their role as reduced carbon source for oxidative phosphorylation, both glucose and sucrose can trigger direct responses in plants. Exhaustion of reduced carbon has been shown to trigger a common set of genes, named "starvation genes", regardless of the means by which the exhaustion takes place; e.g. change of media composition or loss of available starch in leaves after an unexpectedly-long night. Starvation genes are turned off when reduced carbon becomes available (Usadel et al., 2008; Sulpice et al., 2009). The starvation state is perceived as deficiency in the metabolite trehalose-6-phosphate and acts through the SNF-related protein kinase, SNRK (Robaglia et al., 2012; Tsai and Gazzarrini, 2014), which negatively regulates Target of Rapamicin (TOR), a central kinase that universally mediates "resource" signals in eukaryotes (Laplante and Sabatini, 2012; Nukarinen et al., 2016). TOR is a master regulator, controlling a number of growth signalling cascades, which responds to sugar and amino acid availability. One of the fundamental outputs of TOR activity is an enhanced ability to manufacture cellular components through an increase in the cellular translation capacity. TOR also promotes cell proliferation (Xiong et al., 2013). In plants TOR responds both to sugar signals (Baena-González et al., 2007; Deprost et al., 2007; Xiong et al., 2013; Dobrenel et al., 2016), and to auxin (Schepetilnikov et al., 2013; Schepetilnikov et al., 2017). It has recently been shown that in shoot meristems light stimulates the TOR activity via two parallel pathways, through photosynthates and through light signalling linked to auxin biosynthesis (Li et al., 2017).

In this study we demonstrate that cell proliferation can be arrested in young primordia by dark exposure, or reduced at low light, through a local starvation state in the meristem, and reinitiated by transfer to light, which rapidly overcomes such state. We show that upon light exposure of dark-arrested leaf primordia, PIN becomes rapidly polarized and this precedes cell proliferation and growth gene responses. We also show that shoot meristematic activity can be induced in the dark by exposure to cytokinin, and more efficiently so under reduced auxin sensitivity. It can also occur in the dark by direct access of the meristem to sugar, in a TOR-dependent manner, i.e. through the activation of energy signalling, the second target of photomorphogenesis action, but with differing results: energy signals stimulate cell proliferation, but development of a normal leaf lamina requires photomorphogenesis-like hormonal responses. Lastly we show that available photosynthates impact energy signalling.
and adjust the extent of cell cycle activity in meristematic cells in a non-cell-autonomous manner, which under higher irradiance leads to organs constituted of a greater number of cells.
RESULTS

The shoot meristem and arrested primordia of dark grown seedlings experience local energy starvation

In our earlier analysis of light responses at the shoot apex upon first exposure of dark-grown seedlings we identified nearly 6000 differentially-expressed genes (López-Juez et al., 2008). Among these genes, we identified one cluster that was comprised of hundreds which responded rapidly, within an hour, and negatively to light, exclusively in the shoot apex, not in the cotyledons. Subsequent analysis revealed that this cluster was highly enriched in common carbon-repressed "starvation" genes, as classified by a previous study (Usadel et al., 2008). We re-examined the expression of all such genes in our transcriptome data. The resulting expression plot of the complete set of "starvation"-defined genes shows a generalised, rapid down-regulation of transcript levels (Figure 1). More than 50% of starvation genes were expressed 2 fold or higher in the dark than after 1h in the light in the shoot apex, with more than 20% being 10 fold or higher. Because the etiolated cotyledons are unlikely to become photosynthetically competent in the short time interval of 1 hour, we postulate that the rapid repression of starvation genes in the shoot apex is a consequence of rapid mobilisation of reserves stored in the cotyledons upon light exposure. This is in contrast to growth of the hypocotyl, which occurs rapidly at the etiolated stage, demonstrating that resources do not limit growth of another organ in the dark. Interestingly, down-regulation of starvation genes in the shoot apex was in most cases transient, expression becoming high again 24h later. The reason for this is not clear but it might represent the fact that the carbon supply could not keep up with the rapid growth taking place within the shoot apex. In contrast carbon-induced genes, which we refer to as "feast" genes, exhibited the opposite expression pattern, a strong expression between 1 and 6h after light exposure (Figure 1). We conclude that skotomorphogenesis in the dark imposes a starvation state specifically on cells within the shoot apex, and that this state is rapidly released upon light exposure.

Light triggers the polar localisation of PIN1 to the plasma membrane, allowing auxin export that precedes primordia growth

Auxin responsive genes were shown to be highly expressed in the dark-arrested shoot apex, and upon light exposure the expression of these genes was rapidly and transiently reduced (López-Juez et al., 2008). This could be explained if light activates auxin export from emerging leaf primordia. To test this hypothesis, we examined the localisation of the PIN1 protein in the arrested meristems of dark-germinated seedlings, before and after their first exposure to light, using immunofluorescence labelling. Confirming a previous report (Yoshida et al., 2011), the PIN1 signal was weak, with limited membrane localisation, largely diffused inside the cells and difficult to distinguish from background in the dark-arrested shoot apex. We found that upon light exposure of dark arrested meristems the PIN1 localisation became polar on the plasma membrane within 2h, in a pattern pointing towards the tips of emerging leaf primordia at the epidermal cell layer, and away from the tip of primordia in a cell file at the centre of the leaf lamina. This pattern was particularly evident 24h after exposure to light, the position of the PIN1 signal indicating auxin transport towards the primordia tips, and export towards the rib meristem (Figure 2A).
Consistently with the diffuse PIN1 localisation pattern in dark, the DR5:GUS auxin activity reporter (Ulmasov et al., 1997) revealed a relatively high but delocalised auxin response in dark-grown shoot apices, including the meristem and the arrested primordia. Given that the GUS protein is stable, we could not monitor the changes of GUS signal in a similar time scale to that used for PIN1 localisation. However, 24h after transfer to light, the DR5:GUS activity was no-longer diffuse and coincided with known, strong auxin maxima at the primordia tips, and a distinct signal accompanying the differentiation of provascular cells emerged in the future mid vein (Figure 2B).

**Reduction of auxin sensitivity enhances the ability of cytokinin to induce leaf initiation in the dark**

The expression of auxin and cytokinin signature genes when the dark-arrested meristem was exposed to light suggested that in the dark auxin might prevent leaf primordia growth, and this auxin action is rapidly removed upon light exposure, to be replaced by cytokinin to drive growth (López-Juez et al., 2008; Yoshida et al., 2011; Pfeiffer et al., 2016). In agreement, it has been shown that an auxin partially-insensitive mutant (axr1-12, Leyser et al., 1993) and a cytokinin overproducing one (amp1, Chaudhury et al., 1993) exhibit a de-etiolated state in the dark, manifest as short hypocotyl and open cotyledons. It has also been shown that exposure of wild type Arabidopsis to the synthetic cytokinin 6-benzylaminopurine
(BAP) causes leaf initiation in the dark (Chory et al., 1994). We attempted to experimentally transform the hormonal balance characteristic of dark-arrested meristems (high auxin, low cytokinin activity) into the one normally found after light exposure (low auxin, high cytokinin activity) and asked whether such manipulation would allow leaf initiation in the dark. To this end, we exposed the axr1-12 mutant to BAP on sucrose-containing plates in the dark. Without BAP the leaf primordia remained arrested in the dark, while a substantial increase in leaf primordia size was observed in the axr1-12 mutant grown in the same conditions (Figure 3). As expected, cytokinin could stimulate wild type leaf primordia growth in the dark, but the size of primordia observed after the addition of BAP was further increased in the axr1 mutant. After 5 days in the dark the leaf primordium size of BAP-treated axr1 seedlings reached about one third of that of the wild type in the light in the absence of exogenous hormones. Data obtained from these experiments are consistent with the idea that removal of auxin and activation of cytokinin response are required for leaf primordia growth.

Active cell proliferation in young leaf primordia can be reversibly arrested in the dark

Skotomorphogenesis facilitates seedling establishment upon germination in soil, but photoreceptors remain active throughout the life of the plant. We asked whether the control of leaf development by photomorphogenic pathways remained active after the establishment of leaf primordia, using the well-established CYCLINB1;1:DB-GUS mitotic reporter (Colón-Carmona et al., 1999; Donnelly et al., 1999). 7 days old, light-grown seedlings displayed leaves 1 and 2, which were about 0.5 mm in length, and which exhibited abundant mitotic activity in the proximal region (Figure 4A; Supplemental Figure S1). Flow cytometric ploidy analysis of these leaf primordia showed that around 60% of cells had 2N and 40% had 4N nuclear DNA content (Figure 4C). Cell cycle analysis of the flow cytometry data revealed that a high proportion of nuclei were undergoing DNA synthesis (Figure 4B; extended data in

Figure 2. Light exposure of dark-grown seedlings triggers a rapid PIN1 polarisation at the leaf primordia and the establishment of localised auxin maxima. A, PIN1 immunofluorescence localisation in the first two leaf primordia of wild type seedlings. PIN1 (green), and DAPI (blue). Seedlings were germinated in the dark for three days, then examined immediately or after exposure to continuous white light for the times indicated (in hours). B, enlargements of a primordium tip from the PIN1 localisation images in A, after 0, 2 and 24h. C, DR5:GUS reporter activity of seedlings in dark and exposed to white light for 24h. Scale bars: 10 μm (A), 5 μm (B) and 50 μm (C).
Supplemental Figure S2A), indicating that these cells are very actively proliferating. A further three days in the light led to a pronounced increase in organ size as cells exited proliferation and entered cellular expansion. Flow cytometry confirmed an increase in the number of cells with higher ploidy levels, including cells which entered endoreduplication (with 8N nuclei, Figure 4C; Supplemental Figure S2B). In contrast, transfer to dark for three days led to the almost complete losses of mitotic activity, organ expansion and endoreduplication; instead, an

Figure 3. Loss of auxin sensitivity and supplementation of cytokinin cause leaf initiation in the dark. A, seedlings of the axr1-12 mutant or Col wild type (WT) were germinated and grown in the dark for five days, on media with or without benzylaminopurin (BAP) at the concentrations indicated, or for the WT in the light for five days, on media without BAP. Area of one of the first two leaf primordia is indicated. Error bars represent standard error of the mean. Asterisks reflect significance of differences between axr1 and WT. B-F, images of leaf primordia of representative shoot apical regions of seedlings as in A: B-D, wild type. E-F, axr1-12 mutant. B and E, dark, no BAP. C and F, dark, 10 μM BAP. D, light. Scale bar: 200 μm.
increase in the proportion of 2N nuclei occurred, indicating a widespread G1 arrest in the dark (Figure 4A-C; Supplemental Figure S2). Re-exposure to light triggered a re-initiation of cell proliferation as indicated at 12h by the increased mitotic activity (note the GUS mitotic signal, Figure 4A), increased number of cells undergoing DNA synthesis as measured by flow cytometry (note the increase of S phase nuclei, Figure 4B; Supplemental Figure S2), and an increased percentage of 4N nuclei, indicating cells which had passed through DNA synthesis (Figure 4C). At a later time point of 48h cells with 8N nuclei also appeared, indicating the start of endoreduplication-associated cell expansion (Supplemental Figure S2B).
The above observations were made on seedlings grown on sucrose-containing plates, but similar phenomena took place in the absence of exogenous sucrose as well. While some aspects of the response, like the increase in the proportion of nuclei in S phase in the light, were not as pronounced (Supplemental Figure S2), others, like the re-initiation of mitotic events, were even more so (Supplemental Figure S3). These experiments suggest that prolonged dark exposure of young, developing leaves leads to G1 arrest and block of endoreduplication irrespective of whether the seedlings are grown on sucrose-free or sucrose-containing plates. Upon light exposure the arrest in G1 cell cycle phase is reversed and cells rapidly enter into S-phase and mitosis.

**Dark-arrested and light-reactivated leaf primordia exhibit an arrest/growth gene expression programme**

We previously observed a programme of rapid up-regulation of the expression of growth-related genes at the shoot apex, as leaves initiated development in the light (López-Juez et al., 2008). Having established a system of dark-arrest, light-reactivation of leaf growth, we made use of it to monitor the expression of genes selected to represent DNA synthesis and mitosis, and translation capacity/ribosome build-up (Table 1). We assessed whether a comparable gene expression programme to that seen during deetiolation took place during dark arrest and light reactivation of growth in the dissected first leaf pair. We performed these experiments on seedlings grown on sucrose-containing media.

Genes associated with mitosis (CYCB1;1), DNA synthesis (RNR2A, H2A) and translation (RPS6, EBP1) were all repressed during the 3-day dark period, and were upregulated in the first leaf pair within 8h following re-exposure to light; in several cases upregulation could be detected already at 3h after re-exposure (Figure 5).

The originally-observed, rapid changes in hormonal responses in the shoot apex also took place in the developing leaves: transfer to dark caused a mild elevation of auxin responses as indicated by the auxin-responsive AUX1 gene, while light exposure brought about within 1h a transient, substantial drop, which preceded a mild upregulation of cytokinin-responsive gene expression (ARR5). At later time points during light-reinitiated leaf growth, between 3 and 24h, both the expression of auxin biosynthesis genes (TAR2, TAA1) and that of auxin-responsive AUX1 and HAT2 increased. In contrast, the expression of two genes representing ethylene response (EIN3 and EBP) was consistently elevated in the dark and reduced in the light.

As expected, the expression of "starvation" genes became upregulated in the dark, reflecting the establishment of a starvation state, and rapidly dropped upon transfer to light, within 1h (Figure 5). Since this happened in spite of the fact that the seedlings were grown on sucrose-containing plates, the dark-induced elevation of transcript levels of starvation genes and their rapid decrease upon light exposure might be under photomorphogenic control in young developing leaves. The gene expression changes upon dark-arrest and light re-exposure on plates with sucrose were also largely replicated when seedlings were grown in the absence of exogenous sucrose (Supplemental Fig. S4). A notable difference between the experiments on sucrose-containing and sucrose-free plates was that, in the latter, cell cycle- and growth-associated genes declined both in the dark and when seedlings remained in the light. This might relate to differences in the leaf growth kinetics under these two conditions. However a clear, further suppression during dark-acclimation occurs in both
Figure 5. Expression of signature genes during dark arrest and subsequent light exposure in young leaf primordia. The dark arrest blocks the cell proliferation and growth genetic programme and activates starvation genetic responses. Light reverses these and brings about hormonal resetting. WT seedlings were grown in light on sucrose-containing plates, transferred to dark and returned to light under conditions and times identical to those for Figure 4, or after 8dL. Seedlings harvested at the corresponding times had the primordia of leaves 1 and 2 dissected and expression of the genes shown, representing the biological process indicated above each graph and in Table 1, was monitored by quantitative real time PCR (qPCR). Error bars indicate standard error of the mean (between biological replicates).

conditions. These gene expression changes are unlikely to be circadian-regulated. Although 8 out of the 20 selected genes monitored in this study were reported to exhibit circadian expression, the circadian pattern of expression of only one (ARR5) would coincide with the
observed pattern in our experiment, an elevation at the start of light exposure (dawn, Zeitgeber 0h) (Supplemental Table 1). The extended, slightly-finer time course examined for seedlings in the absence of sucrose also showed that the changes occurring did not fit an underlying endogenous, circadian control, and were most likely a direct consequence of the light exposure.

The re-initiation of leaf development necessitates the differentiation of all cell types which, in essence, consist of an epidermis enclosing a combination of photosynthetic mesophyll and vascular cells. We could indeed observe that the dark arrest was accompanied by a reduction of expression of marker genes for early chloroplast biogenesis (GC1; ARC5) and for the initiation of vascular development (VND6, ATHB8), and that both types of cellular differentiation were promoted by re-exposure to light (Supplemental Figure S5A).

**The starvation/growth-arrest gene expression programme is largely under the control of the COP1-dependent photomorphogenic pathway**

To address whether the gene expression program upon dark arrest and light re-exposure of young developing leaves is imposed by photosynthetic activity status or light signalling, we performed these experiments using the cop1-1 mutant. In the dark this mutant maintains active photomorphogenic signalling pathways, even though photosynthesis is completely absent. Transfer of cop1-1 seedlings to dark did not cause a leaf growth arrest, as revealed by the additional area of white tissue produced in the young leaves during the dark exposure, proximal to the green tip developed prior to the dark transfer (Figure 6, insert). We then monitored gene expression signatures associated with growth, hormones (auxin, ethylene and cytokinin) and starvation in cop1-1 mutant seedlings compared to wild type upon three days in dark (Figure 6A). Compared to the wild type, cell proliferation and growth gene expression signatures were less impacted by the dark-adaptation in cop1-1 (Figure 6A). The reduced expression of a gene involved in auxin synthesis, as well as the upregulation of ethylene action and of the starvation response in the dark, were all attenuated in cop1 (Figure 6A). The same difference in expression was observed for the cell type-specific signature genes (Supplemental Figure S5B). We then examined the kinetics of the auxin response monitoring AUX1 gene expression both during dark arrest and light re-activation. The rapid, transient downregulation of auxin response following re-exposure to light was present in the cop1-1 mutant (Figure 6B). This implies that a COP1-dependent photomorphogenic pathway is responsible for the bulk of the gene expression programme in the dark. However, the transient downregulation of auxin signalling during the dark to light transition appears to be independent of COP1 action.

**Direct sucrose access to the shoot apex activates cell proliferation and the growth gene expression programme in the dark**

We have shown that the shoot apex in the dark locally experiences a starvation state, which is rapidly terminated by light in a way that cannot be explained by photosynthetic activity. Intriguing observations have shown that exposure of the meristem to sucrose or glucose can trigger the further growth of organs in the dark (Roldán et al., 1999; Li et al., 2017). We made sucrose available to the shoot apices of seedlings in the dark using the following strategy: 7d light-grown seedlings, exhibiting active meristematic activity, were arrested by transferring to dark in sucrose-free liquid culture, and after three days the culture medium was replaced, under very dim green safe light, with sucrose-containing medium, in which the
seedlings continued to grow. Monitoring of the CYCB1;1:DB-GUS reporter demonstrated that the mitotic activity of young developing leaves in light (see Figure 4) all but disappeared during dark adaptation in the absence of sucrose, while exposure to sucrose resulted in a re-emergence of mitotic activity, which was most pronounced after 24h (Figure 7, Supplemental Figure S6A). The most frequent localisation of such events was the proximal region of leaf primordia (Figure 7).

We monitored the gene expression programme initiated by direct exposure of the meristem to sucrose in the dark (Figure 8). As expected from the observation of reactivation of mitotic activity visualised by the CYCB1;1:DB-GUS reporter, the cell proliferation- and growth-associated gene expression was also strongly stimulated by direct sucrose access, with a simultaneous rapid downregulation of starvation signature genes (Figure 8). The induction of genes associated with plastid biogenesis and vasculature development also exhibited light-like responses (Supplemental Figure S5C). Three notable differences could, however, be observed in comparison with the response to light. First, the response of growth-related genes to direct sucrose supply was somewhat slower than that to light, generally clear after 8h rather than 3h. Second, the rapid, transient downregulation of auxin responses upon dark to light transition was not seen when dark-adapted seedlings were exposed to sucrose, only a strong increase of such responses was observed, as confirmed by three separate
signature genes, suggesting that a rapid activation of auxin export had not taken place under sucrose influence, only the upregulation of auxin synthesis had. Thirdly, ethylene responses, which were rapidly down-regulated by light, were only mildly reduced after sucrose exposure in the dark (Figure 8). We conclude that during leaf development cell proliferation, cytoplasmic growth, aspects of plastid biogenesis and vasculature differentiation are all under sucrose control and can occur in the dark.

The organs developed by meristem activation through direct access to sugar differ in the dark

Following an extended 6d incubation in sucrose-containing liquid medium in the dark, we observed the appearance of an internode between the youngest leaf primordia and the point of cotyledon emergence (Supplemental Figure S7). To examine this further, we administered a prolonged exposure of the meristem to sucrose in the dark while avoiding hypoxia that characteristically occurs in liquid culture, by growing seedlings on vertical sucrose-containing solid media, where apices of seedlings contact the medium’s surface, as carried out by Roldán and collaborators (Roldán et al., 1999). The shoot apex of seedlings grown on horizontal, sucrose-containing media developed leaves only in the light but were completely arrested in the dark (Figure 9A and B). The meristem of seedlings grown in the dark in liquid medium without sucrose was also arrested, while if the medium contained sucrose the leaf primordia developed (Figure 9 C and D). This indicated that direct sugar access is required for leaf initiation. Prolonged growth of shoot apices in contact with sucrose led to extraordinarily elongated seedlings (Figure 9E), with unusually long petioles of cotyledons.
and new leaves, and internodes (Figure 9 E-H). Elongation of the internodes reflects premature activation of the rib meristem. Leaf lamina barely developed (Figure 9G), however the transition to flowering occurred (Figure 9H). Addition of BAP to the medium of seedlings whose shoot meristems were not in contact with sucrose also initiated leaf development, both in WT and in the axr1 mutant background. In addition, we noted in the axr1 mutant occasional tumour-like growths on some leaf primordia when exposed to cytokinin (Figure 9K). The cop1 mutant also developed leaf primordia in dark without direct contact with sucrose-containing media (Figure 9L). We conclude that sugar can promote leaf initiation in the dark only through direct access to the shoot apex, and that the dark arrest can also be overcome by a light-like shift in hormonal activity or by removal of COP1, thus activating photomorphogenic signalling.

The strategy of enhancing sucrose access through growth of seedlings on sucrose-containing vertical plates maintains full exposure of the seedlings to ambient air. This allowed us to also test whether the growth response of the meristem and young leaf primordia relies on photosynthesis-generated sucrose in the light. To this end we designed an experimental set-up that depletes CO₂ in air (see materials and methods, Supplemental

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**Figure 8.** Direct sucrose access activates a proliferation and growth gene expression programme. WT seedlings grown for 7dL on solid media were transferred to sucrose-free liquid media in D for 3d, then transferred to sucrose-containing media for the times indicated. Seedling shoot apices were dissected and gene expression quantified and displayed as in Figure 5.
Figure S8: The transfer of seedlings for three days into darkness on vertical plates without sucrose led to the almost complete cessation of cell proliferation activity, while on sucrose-containing vertical plates, with apices being in contact with the plate, cell proliferation remained active. Eight hour light exposure reactivated cell proliferation in the shoot meristem of seedlings grown in sucrose-free medium, but this was prevented in CO₂-free air where photosynthesis cannot take place (Supplemental Fig. S8, quantitation in Supplemental Figure S6B). We conclude that the photomorphogenic response of the meristem and leaf primordia to light requires photosynthesis-generated reduced carbon.

The growth response to sucrose is mediated by the TOR pathway
Having observed key aspects of the genetic programme which are initiated by exposure to sucrose, we used a pharmacological approach to determine which of those aspects were TOR-dependent. TOR is a structurally- and functionally-conserved protein kinase belonging to the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family (Dobrenel et al., 2016a). Because of this conservation, highly specific ATP-competitive TOR inhibitors developed in animal or yeast cells (Liu et al., 2012), including AZD-8055, have been shown effective in plants (Montané and Menand, 2013; Dong et al., 2015; Kravchenko et al., 2015; Schepetilnikov et al., 2017). The fact that AtTOR heterozygous KO plants are hypersensitive to AZD-8055 in terms of root growth (the TOR gene becomes haploinsufficient, Montané and Menand, 2013) is a strong indication that TOR is the genuine target. This was experimentally proven by measuring the activity of direct downstream TOR targets, S6 kinase and S6 phosphorylation, both of which were strongly inhibited by AZD-8055 (Dobrenel et al., 2016b; Schepetilnikov et al., 2017). We carried out treatment with this selective TOR inhibitor, at previously-used concentrations, and observed that it dramatically reduced the mitotic activity in young leaf primordia (Figure 7, Supplemental Figure S6C). It also reduced the sucrose-induced expression of cell cycle- and cell growth-signature genes, confirming that these processes are to a large extent mediated by the TOR pathway (Figure 10, note the log scale). Remarkably, the upregulation of two out of three auxin-response genes was also found to be partially TOR-dependent. Interestingly, we found genes involved in plastid biogenesis to be particularly sensitive to TOR inhibition (Supplemental Figure SSD). In the dark, addition of sucrose repressed the expression of starvation genes in leaf primordia, but only to some extent after 24h (Figure 10). Unexpectedly, addition of AZD further reduced their expression, indicating that the sugar repression of starvation genes is modulated, but not dependent on TOR signalling. We conclude that sucrose access acts on the meristem in a TOR pathway-dependent manner which leads to the bulk of responses impacting on cell and organ growth.

**Light fluence rate increases lead to an accelerated development of leaves with more cells**

One obvious advantage for plants to utilise energy signalling to determine meristematic activity would be that it would allow them to adjust organ growth to the constantly changing level of available resources, the products of photosynthetic activity. It is known that under high irradiance leaves develop with a multilayer palisade mesophyll to support photosynthetic performance (for example López-Juez et al., 2007; Kalve et al., 2014). Here we tested how the mitotic activity becomes modulated in response to changing light intensity throughout the leaf, by analysing the CYCB1;1:DB-GUS reporter in the palisade layer. We found a rapid increase of mitotic activity soon after the transfer from low light (LL) to high light (HL, Figure 11A) as well as an increased S-phase proportion measured by flow cytometry (Supplemental Fig. S9). The mitotic events occurred in the competent, proximal region of young leaf primordia (leaves 3 onwards), but a few were visible even in leaves 1 and 2 only under HL (Figure 11B). Cells of leaves 3 and 4 also entered endoreduplication at an accelerated rate in HL (Supplemental Figure S9), as could be expected given the greater extent of cell expansion under those conditions.

As a result of an increased mitotic activity, the cell number across the leaf, as calculated by dividing leaf area by weighted, average palisade cell areas at proximal, middle and distal regions, over a longer time course, also increased (Supplemental Figure S10A). The average size of mesophyll cells was much smaller in the proximal than in the middle and...
Correspondingly with the immediate increase in cell proliferation activity upon transfer of seedlings from LL to HL, the expression of cell cycle and cell growth signature genes in young developing leaves of the seedling apex also showed up-regulation (Figure 12). Notably the auxin-responsive AUX1 expression also increased in HL, while starvation gene transcript levels decreased, showing that light quantity sensitively modulates hormone and energy signalling in developing leaves (Figure 12). Genes for chloroplast biogenesis and vascular differentiation, ARC5 and VND6 respectively, showed a transient decrease followed by an increase upon HL transfer, indicating that the transient burst in cell proliferation is accompanied by an early but transient arrest in cellular differentiation (Supplemental Figure S5E). We conclude that, like the dark to light transition, a change in light intensity rapidly alters the energy, hormonal, cell proliferation and differentiation programmes.

The effect of high light on cell proliferation in young leaves is non-cell-autonomous

If available photosynthates, produced by photosynthetically-competent leaves, are indeed the proliferative signal in young leaf primordia, one would predict that the exposure of mature leaves to HL would be sufficient to stimulate cell proliferation in primordia emerging from the meristem. To test this hypothesis we allowed Arabidopsis rosettes to develop to a larger size...
and acclimated them to LL. We then used local shading of only the meristematic region including young primordia, or of the entire seedling except that region, during a shift from LL to HL for 8h (Supplemental Figure S11), and monitored the expression of the mitotic reporter. We found an increase in the mitotic activity in the young leaf 8 even when it was itself shaded (remained under LL) and only the mature leaves became exposed to HL. This increase was similar to that when the whole plant was uniformly exposed to HL, indicating a systemic action of the HL effect from mature leaves to very young ones (Supplemental Figure S11).

We then asked whether the ability to respond to the HL signal was restricted to a developmental window. We showed earlier that the first leaf primordia pair of light-grown seedlings on sucrose-containing media exhibited extensive numbers of cells undergoing mitotic activity at day 7, a much reduced number if development continued until day 10 in the light, and almost none if development was arrested for 3 days in the dark (Fig. 4). We exposed identically-grown, 10 day, constant-light grown seedlings to HL for 48h. This did lead to a few extra events of mitotic activity (Fig. S12), but their number was minimal.

Figure 11. During growth in the light, exposure to high light for 8 or 24h increases cell proliferation. A, CYCB1;1::DB-GUS-expressing seedlings were grown for seven days in continuous light (7dL), transferred to soil, adapted to low light (LL, 40 μmol m⁻² s⁻¹) until day 11, then harvested immediately or after transfer to high light (HL, 300 μmol m⁻² s⁻¹, upper panels) or maintaining at LL (lower panels) for the times indicated, and visualised for GUS reporter activity. Leaf 3 shown. Scale bar: 200 μm. B, apical region, displaying primordia of leaves 3 and 4, 8h after the light transfer, visualised for the GUS reporter. Arrow: mitotic events in the primordium of leaf 2.
compared to that caused by the light exposure of dark-arrested primordia of 7 day-old seedlings (Fig. 4). Interestingly, such events at this later stage tended to be associated with provascular or vascular cells throughout the leaf lamina, not just the proximal region. We conclude that most leaf primordia cells are competent to respond to light signals with increased mitotic activity only during a very early developmental window, and that at a later time point, when most cells have already exited the cell cycle during normal development, only vascular cells are competent to respond to HL exposure through cell division.
DISCUSSION

How leaves form at the shoot meristem is a central developmental question. Understanding how light, as a natural trigger, brings about the transition from meristem arrest to activity, or how light intensity changes modulate leaf emergence can provide fundamental clues to this basic biological phenomenon. Taking together the results of this and previous studies (López-Juez et al., 2008; Yoshida et al., 2011; Pfeiffer et al., 2016; Li et al., 2017), a picture of how light, hormonal and energy-signalling mechanisms regulate leaf development emerges.

The hormonal switch centres on the biology of auxin. Auxin has a complex role in leaf initiation (Braybrook and Kuhlemeier, 2010; Capua and Eshed, 2017), both growth-promoting and growth-inhibiting, but it appears from our data that in the dark auxin becomes diffusely localised in the meristem and this inhibits the emergence of primordia. One outcome of such activity is to prevent the occurrence of auxin maxima, while another may be to prevent cytokinin action. Such an antagonistic action would be consistent with the unexpected observation of occasional, tumour-like growths in primordia of the auxin-resistant mutant exposed to cytokinin. At least two mechanisms are known by which this auxin/cytokinin antagonism could take place: the auxin response factor MP inhibits CK signalling (Pacifici et al., 2015; Pfeiffer et al., 2016), and auxin also promotes the expression of CKX6, a gene for cytokinin inactivation, in young leaves under simulated shade (Carabelli et al., 2007). A close homologue of this gene, CKX5, is also repressed in the shoot apex by the first light exposure (López-Juez et al., 2008), and simultaneous inactivation of CKX5 and CKX6 enhances expression of the meristem-organising WUSCHEL gene (Pfeiffer et al., 2016), helping to explain, at least part, the initial meristem-repressive auxin role. Meanwhile the absence of auxin maxima prevents the initiation of auxin export, necessary for leaf initiation (Reinhardt et al., 2003). Indeed we observed simultaneous establishment of polar localisation of PIN1 towards primordia tip maxima in the epidermis and away towards the rib meristem in the developing mid vein. Once maxima are established, auxin clearly plays a positive role, needed to direct the expansion of primordia and the differentiation of vasculature (Scarpella et al., 2006; Scarpella et al., 2010). As part of the complex action of auxin, we confirmed that a strong, localised auxin activity occurs at the tips of emerging primordia in the light, and that light promotes the expression of at least some auxin biosynthesis genes. Meanwhile cytokinin plays an unambiguously positive role, as previously demonstrated (Chory et al., 1994; Yoshida et al., 2011; Pfeiffer et al., 2016), and our data show that reduced auxin and enhanced cytokinin activity not only phenocopy a photomorphogenic state but they form an intrinsic part of the endogenous, early photomorphogenic programme, under direct light regulation. Our results further show that their effects interact, confirming their shared underlying growth output.

A finding, surprising at first, in our experiments was the fact that energy signalling through direct exposure of the meristem to sucrose is itself capable of promoting at least some auxin responses, as evidenced by the regulation of signature genes (Figure 8). This action was, for two out of three genes tested, TOR-dependent (Figure 10). It has been demonstrated that the TOR kinase, in addition to mediating cell proliferation and protein synthesis in response to sugar, also mediates the translational control of expression of several auxin response factors (ARFs) in response to auxin (Schepetilnikov et al., 2013). The activation of TOR by auxin occurs through a family of small GTPases (Schepetilnikov et al., 2017). Therefore this
central growth kinase may occupy a crucible of growth actions underpinning energy and auxin signalling, and explain some of their partly-shared responses.

Energy signalling plays a central role in the control of both cellular growth (Dobrenel et al., 2016a; Dobrenel et al., 2016b) and cell proliferation (Xiong et al., 2013). It can boost meristematic activity (Pfeiffer et al., 2016; Li et al., 2017), and indeed, through direct sugar access to the meristem, override the dark-repression completely. However it cannot on its own lead to photomorphogenic-like rosette leaves. Instead the meristem overwhelmingly produces petioles and internodes (Figure 9, Supplemental Fig. S7). While such developmental behaviour resembles the phenotype of auxin overproducing seedlings (Chen et al., 2014), a central key factor may be ethylene, responses to which are strong in the dark, and are barely affected by sucrose exposure. Ethylene signalling is necessary for hypocotyl hook formation, a component of the skotomorphogenic programme (Marin-de la Rosa et al., 2014) and pea phytochrome mutants have been shown to exhibit strong ethylene responses (Foo et al., 2006). Auxin synthesis genes were identified in genetic screens for weak ethylene insensitivity (Stepanova et al., 2008), because the ethylene actions under observation were mediated by newly-synthesised auxin. Tellingly, pea phytochrome mutants produced leaves with limited laminae (Weller et al., 2015), as do phytochrome mutants of Arabidopsis (Tsukaya, 2005), and loss of an ethylene-dependent transcription factor gene restored in those pea mutants the wild type leaf phenotype (Weller et al., 2015). Our observations not only confirm a fundamental role for auxin in leaf organ differentiation, but also support a role for ethylene in directing the meristematic cellular activity towards elongating organs, like internodes and petioles in the dark, when ethylene response is high, or towards leaf laminae, with their distinct epidermal and mesophyll cellular make-up in the light, when ethylene responses are repressed. Whether this possible ethylene switch of the proliferative potential acts solely through auxin activities is at present unknown. An elegant genetic screen has recently identified the LEAFLESS tomato gene, deficiency in which results in meristem cells producing only elongating internodes under auxin action (Capua and Eshed, 2017). The role of such genes in photomorphogenic leaf initiation also awaits further study. We should, nevertheless, note that following a substantially extended period of dark growth on sucrose, after the transition to flowering, one could observe comparatively normal cauline leaves, as well as floral buds (Figure 9H). This could reflect environmental plasticity early in development, fully subjected to skotomorphogenic or photomorphogenic regulation, yet enhanced homeostasis of development following the transition to flowering. Whether this in any way relates to ethylene signalling, or competence to respond to it, is at present only a matter of conjecture.

Photomorphogenesis acts through a COP1-dependent pathway. Transcription factors which positively regulate light responses, including hypocotyl repression, cotyledon unfolding and the initiation of chloroplast biogenesis, are marked by COP1 for proteolysis and are degraded through a proteasome-dependent activity in the dark (Lau and Deng, 2012). Although we could observe some degree of response to dark adaptation by the cop1 mutant, overall those responses were clearly attenuated. It is a particularly intriguing aspect of the response to light that it can be overridden in terms of meristem activation, but not of developmental fate, by energy signalling. Light appears to play what could be described as a gating, or permissive, role towards energy signalling, in that the extent of meristem activity is seed reserves- or, later, photosynthates-dependent, but only when light is present this reduced carbon becomes accessible to the meristem. This light role is dependent on
photomorphogenic pathways, as it depends on photoreceptors (López-Juez et al., 2008) and COP1 (this study). One attractive hypothesis for the mechanism underlying the light gating phenomenon is that, in a manner analogous to auxin export, sugar import into the meristem is under photoreceptor control, in a COP1-dependent manner. This would explain the dramatic observations that direct sugar access to the meristem is capable of fully activating the meristem in the dark, which growth of seedlings on sucrose-containing solid medium alone cannot.

One exception to the involvement of COP1 is the transient drop of auxin responses in the light. COP1 in the dark is localised in the nucleus, where its targets are light-associated transcription factors. The transient auxin response drop is thus most probably a result of a posttranslational control of auxin export via PIN1, the control being mediated by PIN1 localisation. The nature of this control remains poorly understood, but post-translational signalling cascades, mediated by protein kinases, control PIN1 localisation under other developmental scenarios (Benjamins et al., 2001; Jia et al., 2016, Dory et al. 2017). Transcript levels of PIN1 are also activated in the shoot apical region by light exposure (López-Juez et al., 2008), further contributing to the establishment of fully-fledged auxin transport capacity in the light.

The role of energy signalling becomes most apparent in the control of the cellular make-up of leaves under different irradiances. This control is mediated by the regulation of cell proliferation and cell growth pathways, crucially dependent on the central, TOR pathway. It is well established that HL-grown leaves develop a multilayer palisade (Weston et al., 2000; Tsukaya, 2005), and it would be tempting to assume that further cell proliferation events occur in the mesophyll to generate such cellular anatomy. However the multilayer palisade is present in the youngest leaf primordia physically possible to examine, composed of just a few tens of cells (Kalve et al., 2014), suggesting that it may actually arise from the recruitment of a larger number of meristematic cells into the primordium. Given that a previous study demonstrated that the cellular anatomy of very young leaves is determined by light exposure of mature ones (Yano and Terashima, 2001), one can conclude that the recruitment of meristematic cells to primordia is under non-cell-autonomous, systemic control. Our observations complement those by showing that proliferation events in division-competent cells of the young leaves increase the number of cells observed and are followed by accelerated endoreduplication and cellular expansion, which thus increases the surface area of the solar panel. This contrasts with observations of high- and low-irradiance leaves of a different species, in which no change in the total number of palisade cells was observed (Yano and Terashima, 2004). Those authors suggested that in their experimental system light irradiance only controlled the angle of cell division, anticlinal to form extra palisade in sun (HL), periclinal to extend the lamina in shade (LL). This is clearly not the case in our observations (Figure 11, Supplemental Figure S9, Supplemental Figure S10), high irradiance does promote extra cell proliferation in our leaf primordia of HL-exposed plants. Our data show that this is also a systemic response, dependent on the irradiance received by mature leaves, adding a further dimension to the impact of photosynthetic signalling on meristematic activity. It has recently been shown (Van Dingenen et al., 2016) that the larger organs under HL are explained to an extent by an increased import of glucose into chloroplasts of young, meristematic, proximal leaf cells. This causes a downregulation of the overall transcriptional activity in chloroplasts, which in turn delays the exit of those cells from proliferation. The extended proliferative phase contributes to increasing the final organ size.
Such a mechanism would be expected to be cell-autonomous, while the response we observe is not. How these inter-organellar and energy-signalling regulatory mechanisms interact and delay or accelerate the exit into endoreduplication/differentiation remains to be answered.

CONCLUSIONS

Two stages in which the action of light determines meristematic activity become apparent in this study (see model, Supplemental Figure S13). Firstly, the presence of light plays a permissive role, i.e. no cell cycle and growth activities can occur in prolonged dark. This action utilises photomorphogenic pathways and is photoreceptor- and, largely, COP1-dependent and makes use of auxin, cytokinin and ethylene-dependent mechanisms of meristem organisation, leaf initiation and cell fate decision-making, together with a photomorphogenic "gating" control of energy signalling. The latter may be due to control of access to reduced carbon, activates the TOR signalling pathway, and has cell proliferation and growth as its output. Secondly, light irradiance determines the extent of cellular growth activities, adjusting the number of cells supplied and the extent of organ growth through the availability of photosynthates and its action is mediated by the TOR pathway.

Our present results, together with previous studies, contribute to untangling the complex role and interactions of hormonal and energy signalling, through the action of the TOR kinase, to determine the activity of meristematic and early-organ cells in the light. They have also opened many new questions. Understanding the means by which TOR action ensues the combined energy and auxin response, uncovering the mechanism of photomorphogenic energy-signalling gating, the way in which the starvation state is imposed in the absence of photoreceptor action, and unravelling the different cellular and organ fates produced by meristematic activity under light or energy-only signalling, should be among the matters addressed by further analyses. It is, nevertheless, apparent that energy signals may constitute the most important determinant of plant growth, and therefore biomass production, in non-stressed conditions.
MATERIALS AND METHODS

Plant materials, growth conditions and experimental treatments

Wild type Arabidopsis thaliana plants of the Columbia (Col) ecotype and axr1-12 mutant (Leyser et al., 1993) were obtained from the Nottingham Arabidopsis Stock Centre. The cop1-4 mutant (Deng et al., 1991), CYCLINB1;1:Dbox-GUS line (Colón-Carmona et al., 1999), DR5:GUS (Ulmasov et al., 1997) and ARR5:GUS (D'Agostino et al., 2000) were kind gifts of J. Gray, P. Doerner, T.J. Guilfoyle and J. Kieber respectively. Seedlings were plated on agar-solidified Murashige and Skoog (MS) medium, under continuous fluorescent white light (100 μmol m\(^{-2}\) s\(^{-1}\)) in Percival I-30 or I-35 (CLF Plant Climatics, Wertingen, Germany) or in a dark incubator, at 21°C, on horizontal plates containing 0.8% agar-solidified MS medium and 1% sucrose unless otherwise stated, and when required in the presence of benzylaminopurine (Duchefa/Melford Labs, Norwich, UK) at the indicated concentration, as previously described (López-Juez et al., 2008). In liquid culture experiments, unless stated, seedlings were grown for 7d on horizontal plates in the light as above, then transferred to 6-well microtiter plates containing liquid MS media devoid of sucrose, for a further 3d in dark, with shaking (80 rpm), at which point the medium was replaced under very dim green safe light with fresh sucrose-free or 1% sucrose-containing medium, with or without the addition of AZD-8055 (2 µM, Sigma Aldrich, Poole, UK). Liquid-cultured seedlings shown in Figure 9 were grown from germination in total absence of light. When indicated, seedlings were grown on 1.2% agar-solidified vertically-positioned square plates. For CO\(_2\)-deprivation experiments, seedlings were grown on sucrose-containing vertical plates in the light, and after 7d they were transferred to fresh sucrose-containing or sucrose-free vertical plates, double clear-bagged with or without 5g of indicator-containing soda lime (Fischer Scientific, Loughborough, UK), as previously described (Kircher and Schopfer, 2012). For light quantity experiments, seedlings grown for 7d on MS horizontal plates were transferred to soil, grown for 2d at 100 μmol m\(^{-2}\) s\(^{-1}\) and adapted for a further 2d to 40 μmol m\(^{-2}\) s\(^{-1}\), continuous low light (LL), before transfer if required to 300 μmol m\(^{-2}\) s\(^{-1}\) continuous high light (HL). For the cellular makeup experiment (Supplemental Figure S10), seedlings were transferred to soil and adapted to LL until day 14, before transfer to HL or being maintained in LL for a further 6 days. Leaf 5 was monitored. To assess local or systemic light effects, seedlings were transferred to soil after plate growth and kept at 150 μmol m\(^{-2}\) s\(^{-1}\) for 6d to achieve a sufficient rosette size, before adapting to LL for 3 more days, and then subjected to HL, local HL, systemic HL or LL, by exposure to HL and use of custom-sized neutral density celluloid filters. Leaves were collected 8h later for GUS reporter assay.

Leaf cellular analysis, immunocytochemistry and reporter assay

Histochemical GUS assays took place largely as described (López-Juez et al., 2008) with minor modifications: after fixation (ice-cold 90% acetone) seedlings were infiltrated with GUS staining buffer with a final concentration of 0.3mg/ml X-Gluc under vacuum with ½ atm pressure for 10 min. Seedlings were then kept in dark at 37 °C for 14h followed by post-incubation in 3:1 methanol:acetic acid (v/v) for 2h and washed in 70% ethanol at 65°C for 10 min. Seedlings were mounted on slides in Hoyer’s solution. Digital images were recorded using Nikon SMZ1500 (Nikon, Kingston upon Thames, UK) equipped with a Nikon DXM1200 camera, or Leica EZ4HD (Leica Microsystems, Milton Keynes, UK) stereomicroscopes.
Primordia of BAP-treated axr1 or Col seedlings, or cellular anatomy of varying fluence rate leaves, were observed under Nomarski optics using a Nikon Optiphot 2 microscope equipped with a Nikon DXM1200 or a Micropublisher 5.0 RTV camera. The area of leaf primordia, of individual cells, or GUS-stained areas were measured using ImageJ software. Except where indicated, measurements used 10 seedlings. To quantify cellular anatomy, leaves were divided into basal, mid and distal thirds, average cell areas measured in each region, the number of cells for each region estimated as a third of the leaf area divided by the corresponding average cell area, and the resulting number of cells added for the three regions.

For immunocytochemistry samples were fixed and processed as described previously (Galweiler et al., 1998). PIN1 was detected in permeabilised seedlings incubated with an affinity-purified mouse anti-PIN1 monoclonal antibody (1:100) and monoclonal secondary antibody (Alexa 488-labelled goat anti-mouse at 1:1000 dilution). Fluorescence was analysed with a Zeiss (Oberkochen, Germany) LSM 5 DUO scanning microscope. Fluorescent labelled anti-PIN antibody and DAPI fluorescence were monitored using multi-tracking in frame mode. Alexa 488 was excited using the 488 nm laser line in conjunction with a 505–530 band-pass filter. DAPI was excited with the 405 nm laser line and collected using a 420-480 nm band-pass filter.

Flow cytometry analysis

To determine cell DNA content, leaf primordia of a minimum of 5 seedlings per sample were dissected on agar, transferred to a few drops of ice-cold nuclei extraction buffer (CyStain® UV Precise P kit, Sysmex Partec, Milton Keynes, UK), and cells chopped with a sharp razor blade as previously described (López-Juez et al., 2008). 1ml of DAPI DNA-staining solution (Partec) was added, the sample mixed, filtered and analysed through a PAS flow cytometer (Partec), the fluorescence of different ploidy peaks calibrated using Arabidopsis floral tissue. Proportions of peak areas at different ploidy levels were measured using Flomax software. Where only 2N and 4N peaks were present, ‘cell cycle analysis’ analysis mode was used to estimate the proportion of nuclei in S phase.

Analysis of gene expression

Seedlings were harvested into RNAlater (Sigma Aldrich) and stored for a maximum of 7d at 4ºC, before dissecting primordia under a stereomicroscope (Nikon SMZ-2T) and flash-freezing in liquid nitrogen. Dissected tissue consisted of the primordia of leaves 1 and 2 (dark-arrest, liquid culture, AZD or cop1 experiments), or the shoot apex including the meristem and all leaf primordia (light fluence rate experiments). Arabidopsis total RNA was extracted using the Plant RNA mini spin kit (Macherey-Nagel, Düren, Germany) following manufacturer's instructions, and quality-checked by agarose gel electrophoresis. Two µg aliquots were reverse-transcribed using the Maxima first strand complementary DNA synthesis kit (Thermo Fisher Scientific). DNA was used for real-time amplification as previously described (Hills et al., 2015). Three independent biological replicates, each containing 150-200 dissected apices, were used for each sample type or time point, and all reactions took place in duplicate. Relative quantitation for each target gene used the ∆Ct method against the expression of a constitutive gene, UBQ10. Primers were designed using QuantPrime (http://quantprime.mpimp-golm.mpg.de/). Gene identifiers and corresponding primers were as listed (Supplemental Table S2).
Assessment of the possible circadian behaviour of monitored genes (Supplemental Table S1) used the LL_LLHC data series available at the Diurnal tool (http://diurnal.mocklerlab.org/).

**Scanning electron microscopy**

Seedlings were placed in fixative (3% glutaraldehyde plus 4% formaldehyde in 0.1 M PIPES, pH 7.2) at room temperature and stored at 4 °C for 12 hrs. The primary fixative was removed and seedlings washed 2 × 10 min with 0.1 M PIPES, pH 7.2. Seedlings were dehydrated by immersion in 30, 50, 70, 95, and 95% ethanol, for 10 min each, followed by 2 × 20 min in 100% absolute ethanol. Fixed specimens were critical-point dried in CO₂, mounted on an aluminium SEM stub with conductive glue and sputter-coated with gold/palladium, before observation in a FEI Quanta 200 scanning electron microscope (Biomedical Imaging Unit, Southampton University Hospital).

**Accession numbers**

Accession numbers are listed in Table 1.

**SUPPLEMENTAL DATA**

The following supplemental data are available:

**Figure S1.** Quantitation of leaf area and *CYCB1;1:DB-GUS* expression in Figure 4.

**Figure S2.** Flow cytometric cell cycle parameters in cells of leaf primordia equivalent to those in Figure 4.

**Figure S3.** *CYCB1;1:DB-GUS* expression showing proliferation activity arrest following transfer to dark, and light re-initiation of mitotic activity in proliferation-competent cells, in seedlings grown on sucrose-free plates.

**Figure S4.** Gene expression analysis showing the dark arrest blocks the cell proliferation and growth genetic programme and activates starvation genetic responses at the shoot apex, in seedlings on sucrose-free plates.

**Figure S5.** Expression of genes associated with plastid biogenesis (primarily leaf mesophyll) and vascular development at the shoot apex, in the dark-arrest, *cop1*, sucrose-supply, TOR inhibitor and light fluence-rate experiments.

**Figure S6.** Quantitation of the proportion of GUS-positive leaf area, in Figures 7, S8 and 11.

**Figure S7.** *CYCB1;1:DB-GUS* expression showing prolonged access of the meristem to external sucrose (in liquid media) causes cell proliferation that extends petiole and internode organs.

**Figure S8.** *CYCB1;1:DB-GUS* expression showing access to external sucrose maintains cell proliferation in the dark; light activates cell proliferation in the absence of external sucrose, but this requires access to CO₂ for photosynthesis.
**Figure S9.** Flow cytometric cell cycle parameters showing transfer to high light rapidly promotes cell proliferation, and subsequently accelerates entry into endoreduplication.

**Figure S10.** Total two-dimensional leaf cell number, % of dividing cells and sample cell images showing that during growth in the light, exposure to high light produces organs composed of a greater number of cells.

**Figure S11.** Experimental set up and CYCB1;1:DB-GUS expression quantitation showing that high light acts systemically on cell proliferation in young leaf primordia, after perception by mature leaves.

**Figure S12.** Additional CYCB1;1:DB-GUS expression of partially developed leaves of 10dL-grown seedlings transferred to high light occurs almost exclusively in vascular cells.

**Figure S13.** Model of the impact of photoreceptor activation or exposure to high light and the occurrence or extent of leaf organ growth.

**Table S1.** Circadian response, if known, of genes subjected to expression analysis.

**Table S2.** Primers used for gene expression analysis.

**ACKNOWLEDGEMENTS**

We are indebted to J. Gray, P. Doerner, T.J. Guilfoyle, and the Nottingham Arabidopsis Stock Centre for the supply of materials, R. Yadav, J. Hall and M. Burke for skilful help with hormone-reporter and de-etiolation assays, A. Page and members of the Southampton Bioimaging Unit for excellent support for scanning electron microscopy, and C. Papdi and members of the ELJ and LB labs for constructive criticisms. Work funded in part by NSF/BBSRC bilateral grant BB/M025047 to LB.
Table 1. Genes monitored as representatives of biological growth processes, and products they encode.

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Figure 1. Shoot apices of 3 day old seedlings exhibit in the dark a starvation response, which disappears within one hour of light exposure. The expression levels of genes defined as carbon-repressed “starvation”, and carbon-induced “feast” (Usadel et al., 2008) were plotted using data from a previous microarray experiment (López-Juez et al., 2008). Heatmaps represent levels at each time point relative to the average level for the same gene across all time points (red, above; blue, below). SAP: shoot apex. Cot: cotyledons. Number after each sample type: hours after light exposure. Colour scales indicated over each plot.

Figure 2. Light exposure of dark-grown seedlings triggers a rapid PIN1 polarisation at the leaf primordia and the establishment of localised auxin maxima. A, PIN1 immunofluorescence localisation in the first two leaf primordia of wild type seedlings. PIN1 (green), and DAPI (blue). Seedlings were germinated in the dark for three days, then examined immediately or after exposure to continuous white light for the times indicated (in hours). B, enlargements of a primordium tip from the PIN1 localisation images in A, after 0, 2 and 24h. C, DR5:GUS reporter activity of seedlings in dark and exposed to white light for 24h. Scale bars: 10 µm (A), 5 µm (B) and 50 µm (C).

Figure 3. In the axr1-12 mutant leaves initiate in the dark, which is increased by the addition of cytokinin. A, seedlings of the or Col wild type (WT) and axr1-12 were germinated and grown in the dark for five days, on 1% sucrose-containing media with or without benzylaminopurin (BAP) at the concentrations indicated, or for the WT in the light for five days, on media without BAP. Area of one of the first two leaf primordia is indicated. Error bars represent standard error of the mean. Asterisks reflect significance of differences between axr1 and WT. B-F, images of leaf primordia of representative shoot apical regions of seedlings as in A: B-D, wild type. E-F, axr1-12 mutant. B and E, dark, no BAP. C and F, dark, 10 µM BAP. D, light. Scale bar: 200 µm.

Figure 4. Proliferation activity arrest following transfer to dark, and re-initiation of mitotic activity in the light in proliferation-competent cells at the leaf base. A, CYCB1;1::DB-GUS-expressing seedlings were grown for seven days in continuous light (7dL), harvested immediately or transferred to 3 days continuous light (+3dL) or continuous dark (+3dD), and the latter were transferred back to light, after which they were harvested at the times indicated in hours. A leaf of the first leaf pair, after visualising the GUS reporter, is shown. Blue GUS stain indicates cells undergoing mitosis, in an acropetal gradient. Scale bar: 500 µm. B, S-phase percentage of total nuclei determined by flow cytometry and cell cycle analysis, of nuclei from leaf primordia under the conditions indicated. C, percentage of nuclei undergoing S phase under the conditions indicated. Error bars represent standard deviation (n=3, each sample containing a pool of at least 5 leaves).

Figure 5. Expression of signature genes during dark arrest and subsequent light exposure in young leaf primordia. The dark arrest blocks the cell proliferation and growth genetic programme and activates starvation genetic responses. Light reverses these and brings about hormonal resetting. WT seedlings were grown in light on sucrose-containing plates, transferred to dark and returned to light under conditions and times identical to those for Figure 4, or after 8dL. Seedlings harvested at the corresponding times had the primordia of leaves 1 and 2 dissected and expression of the genes shown, representing the biological
process indicated above each graph and in Table 1, was monitored by quantitative real time PCR (qPCR). Error bars indicate standard error of the mean (between biological replicates).

**Figure 6.** The gene expression programme change in the light is brought about to a large extent by COP1-dependent photomorphogenesis pathways. Expression in leaf primordia of the genes indicated after 7dL + 3dD adaptation, plotted on a log₂ scale relative to the levels after 7dL, in the cop1 mutant and its WT grown on sucrose-containing plates. Inset: Leaf primordia of 3dD-adapted cop1 seedling. B, expression of AUX1 after 7dL, +3dD adaptation, and following transfer back to L (times indicated). Error bars indicate standard error of the mean.

**Figure 7.** Direct sucrose access to the meristem reactivates cell proliferation in the absence of light, in a TOR-dependent manner. CYCB1;1::DB-GUS-expressing seedlings were grown on solid media plates in light for 7d, transferred to sucrose-free liquid media in dark for 3d, and visualised for GUS expression as follows: after subsequent transfer to media containing sucrose, or to media containing sucrose plus AZD-8055, or to sucrose-free media, for the times indicated. Scale bar: 500 µm.

**Figure 8.** Direct sucrose access activates a proliferation and growth gene expression programme. WT seedlings grown for 7dL on solid media were transferred to sucrose-free liquid media in D for 3d, then transferred to sucrose-containing media for the times indicated. Seedling shoot apices were dissected and gene expression quantified and displayed as in Figure 5.

**Figure 9.** The dark-arrest of leaf initiation can be overcome by direct access to sucrose, change in auxin response and by the lack of COP1. Scanning electron micrographs of shoot apices of seedlings of WT (A-I), axr1-12 mutant (J and K) and cop1 mutant (L) genotypes. All panels except A grown in continuous dark. A, WT, continuous light, 7 days, horizontal sucrose-containing plate. B, as A but in continuous dark. C, WT, 17 days, sucrose-free liquid medium. D, WT, 7 days, sucrose-containing liquid medium. E, WT, 28 days, vertical sucrose-containing plate. F, detail of seedling equivalent to that in E. G, detail of seedling equivalent to that in E, but grown for 42 days. I, WT, 7 days, horizontal sucrose-containing plates with 10 µM benzylaminopurine (BAP). J, axr1 mutant, 7 days, horizontal sucrose-containing plates with 2 µM BAP. K, as J. L, cop1 mutant, 7 days, horizontal sucrose-containing plates. Arrows in B and C: leaf primordia. Arrow in K: tumour-like growth. Scale bar: 100 µm (A-D and I-L), 200 µm (F-H) and 2 mm (E).

**Figure 10.** The gene expression programme induced by sucrose in the dark is largely TOR-dependent. Expression in the shoot apex and leaf primordia of the genes shown following the growth treatment described for Fig. 7 (7dL solid media followed by 3dD sucrose-free liquid media), after transfer for a further 24h to media containing sucrose with or without AZD-8055, or without sucrose, as indicated. Expression quantitation by qPCR, displayed as in Figure 6.

**Figure 11.** During growth in the light, exposure to high light for 8h or 24h increases cell proliferation. A, CYCB1;1::DB-GUS-expressing seedlings were grown for seven days in continuous light (7dL), transferred to soil, adapted to low light (LL, 40 µmol m⁻² s⁻¹) until day 11, then harvested immediately or after transfer to high light (HL, 300 µmol m⁻² s⁻¹, upper panels) or maintaining at LL (lower panels) for the times indicated, and visualised for GUS.
reporter activity. Leaf 3 shown. Scale bar: 200 µm. B, apical region, displaying primordia of leaves 3 and 4, 8h after the light transfer, visualised for the GUS reporter. Arrow: mitotic events in the primordium of leaf 2.

**Figure 12.** Gene expression changes after transfer to high light. Expression of signature genes as indicated in the shoot apex and leaf primordia following the transfer to HL as described for Fig. 11. Expression quantitation by qPCR, displayed as for Figure 5.


