Control of leaf cellular proliferation, differentiation and growth by light: establishing and distinguishing the roles of hormonal- and sugar-signalling

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This thesis was submitted for the degree of Doctor of Philosophy at Royal Holloway University of London, September 2019

Declaration of authorship

I (Sara Farahi Bilooei) hereby declare that the work presented in this thesis is the original work of the author unless otherwise stated. Original material used in the creation of this thesis has not been previously submitted either in part or whole for a degree of any description from any institution.

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Acknowledgments

A very special gratitude goes out to my supervisors, Dr Enrique Lopez-Juez, and co-supervisor, Professor Laszlo Bogre, for the opportunity to do this PhD. This opportunity has provided me with a learning curve from which I have gained academically and personally.

I am grateful to Ehsan Ahmadian who has provided me with emotional supports throughout my PhD.

And a special thanks to my best friend Jia He who has supported me along the way.

And finally, I would like to express my gratitude to my parents and siblings (Azam, Samira, Meti, Nima, and Anahi) for providing me with constant support and encouragement throughout my years of study and through the process of researching and writing this thesis.

Abstract

Light induces the shoot apical meristem to initiate the production of leaf primordia and eventually leaves, but this process is arrested in the dark. Light energy is itself required for leaf growth. Previous studies showed the role of hormones and energy-dependent TOR kinase in meristem activity, but how these two regulatory mechanisms crosstalk or how they are controlled by light is not known yet. We have in the past observed that the arrested meristem and primordia in the dark show a strong response to auxin (López-Juez et al. 2008). I now report that they also show a strong "starvation" gene expression profile. These signatures are rapidly turned by light into strong cytokinin and strong "feast" gene expression. Both coincide with ribosomal protein gene expression and simultaneous cell proliferation, key components of leaf initiation. The leaf primordia transfer to dark leads to disappearance of mitotic reporter activity, but this will reappear in the light. Our data suggest that the seedling meristem and young leaf primordia may specifically experience carbon starvation in the dark, this being quickly repressed when transferred to the light. My results demonstrate that manipulating auxin and cytokinin or, alternatively, activating energy signalling in the dark leads to reactivated meristematic activity, although the developmental outcomes differ, the development of light grown-like leaves requires a light-like hormonal response. Plants' transfer from low light (LL) to high light (HL) also results in extra proliferation and growth. A leaf grown in HL is composed of several layers of larger cells. Transfer from LL to HL leads to the growth of larger lamina. Thus, both multiple layers, and a larger lamina composed of more cells in two dimensions, occur in HL. From those observations, I propose that energy signalling processes are also central to leaf growth under natural, varying light conditions. The study of TOR and MAPK signalling pathway showed, they are both involved in regulation of meristem activity and leaf initiation. However, both signalling pathways have different roles. The research on MKK7, indicates that other than its role in stress signalling, also involved in developmental regulatory function. *mkk7* seedlings de-etiolation is faster showing MKK7 acts as suppressive regulator of meristem activity. RAPTOR1-1 and E2Fa are both working downstream of TOR pathway and have regulatory role in growth. However, in this research it was shown they probably have inhibitory role in growth.

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Introduction

1. Chapter 1: Introduction

1.1. Light

Light is one of the most important environmental factors influencing photosynthetic organisms and plants depend on it to direct and power their morphogenesis, development, and physiology. Consequently, many aspects of plant development from seed germination to morphological changes and floral induction are strongly regulated by light. Once a seed has germinated, depending on the availability of light, plants will go through skotomorphogenesis or photomorphogenesis (Pfeiffer et al., 2016). In the presence of light, hypocotyl elongation is reduced, the apical hook opens, cotyledons unfold, cells expand, leaves develop, dark-grown plastids change to chloroplasts, and many light inducible genes are up-regulated. Light has different effects in various organs of the plant such as cell division, cell expansion, and leaf growth (Fankhauser and Chory, 1997). Plants are constantly regulate their growth and development according to environmental signals whereby they undergo dynamic changes to adjust photosynthesis and prevent photodamage (Galvão and Fankhauser, 2015).

1.1.1 Skotomorphogenesis

Seedlings of higher plants can adapt to the absence of light and exhibit skotomorphogenesis and become etiolated. During etiolation, in many dicot plants, hypocotyls grow very long, seedlings are pale yellow or white and cotyledons remain folded to protect the meristematic region. The rapid extension of the hypocotyl serves the purpose of reaching light. The etiolation process is achieved by the active repression of the photomorphogenesis development genes, as demonstrated by the fact that loss-of-function mutations abolish it, causing constitutive photomorphogenesis (Josse and Halliday, 2008).

1.1.2 Light Photoreceptors

Plants perceive light energy through absorption by specialized photoreceptors. Photoreceptors are light-responsive proteins that are specifically designed to couple the specific light to a downstream signal transduction event, in order to initiate appropriate modifications to plant development (Schmidt and Cho, 2015). There are several photoreceptor systems that perceive the quantity, quality, direction, and duration of light. The absorption properties of photoreceptors match their molecular structures that are sensitive to specific wavelengths of light. Red/far red light (600-750 nm) is captured through the phytochrome (phys) family (Chen and Chory, 2011), Blue/UVA (320-500

nm) by the phototropins (phots) (Christie, 2007) and by cryptochromes (crys) (Whitelam et al., 1998). Phytochrome captures red/far-red light and subsequently changes gene expression, which results in growth responses. *Arabidopsis thaliana* contains five phytochrome apoprotein genes (*PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE*) that have different but overlapping functions. Phytochromes perceive light via the chromophore, phytochromobilin, which consists of a linear tetrapyrrole molecule which is synthesised as a haem oxidation product**.** Phytochromobilin is the chromophore common to all phytochromes (Terry and East, 1997).

Phytochromes are involved in seed germination, stomata development, flowering, shade avoidance, and inhibiting hypocotyl elongation in *Arabidopsis* (Casal, 2012). Phytochromes reversibly photoconvert between Pr (inactive, the ground state) and P_{fr} (active) forms, and regulate shade avoidance in response to perception of red-rich or far red-rich wavelengths of light (Devlin, 2016). Upon light irradiation, phytochrome promotes degradation of PHYTOCHROME INTERACTING FACTORS (PIFs) and inhibits COP1 nuclear accumulation and thereby leads to stabilization of transcription factor HY5 (Fig 1.1) (Lu et al., 2015). A constitutive version of phyB called phytochrome B-Y276H (YHB) triggers light responses in the absence of light and carries a P_{fr} conformation even in plants grown in dark. YHB separates photoconversion from signalling, allows phytochrome signalling to be regulated in a way free from light quantity and quality (Hu and Lagarias, 2017; Jones et al., 2015). The circadian clock is no longer sensitive to the different wavelength of light in this mutant (Jones et al., 2015).

Cryptochromes are, together with phytochromes, the major regulators of photomorphogenic development (de-etiolation) and have several important functions in different stages of plant growth including seedlings' early growth, synchronisation of the circadian clock, and activation of genes expression by light (Liu et al., 2011). Like phytochromes, cryptochromes consist of an apoprotein and a chromophore. Each cryptochrome molecule carries two blue light-absorbing chromophores, a flavin and a pterin or two flavins. Analysis shows that cryptochrome's family of *Arabidopsis* contains three members, *CRY1*, *CRY2*, and *CRY3* (Cashmore et al., 1999; Kleiner et al., 1999). CRY1 and CRY2 are predominantly localised to the nucleus and act mainly by their effect on gene expression under blue light (Chaves et al., 2011; Liu et al., 2011). Their transcriptional regulation is carried out by controlling positive regulators such as the bZIP transcription factor HY5. CRY1 is largely involved in the process of de-

etiolation, while CRY2 plays a role as a regulator of photoperiodic flowering and functions mainly under LOW LIGHT (LL) (El-Din El-Assal et al., 2003; Lin et al., 1998). CRY3 is a CRY-DASH (Drosophila – *Arabidopsis*–Synechocystis–Human) type protein thought to be localised to chloroplasts or mitochondria and functions to repair the damage caused by UV lesions on single-stranded DNA (Brudler et al., 2003; Pokorny et al., 2008).

Phototropins (PHOT1 and PHOT2) are light quantity dependent and involved in directional growth of plants toward the light source (Phototropism), chloroplast movement (Sakai et al., 2001), opening of stomata (Kinoshita et al., 2001) and leaf expansion. In contrast to other photoreceptors, phototropins act through phosphorylation events at the plasma membrane (Christie et al., 2015). UV-B RESISTANT 8 (UVR8) mediates the photomorphogenic responses to UV-B light (UV-B; 280–315 nm) of the solar spectrum. The only known UV-B photoreceptor is UVR8, which is involved in transcriptional activation of genes that lead to photomorphogenic responses (Velanis et al., 2016). The most active family of photoreceptors upon first light exposure are phytochromes, with the assistance of cryptochromes, as they are responsible for enormous changes in gene expression; this is particularly evident from the role of combined loss-of-function mutants (López-Juez et al., 2008). The need for photosensory mechanism is the requirement of the plant to absorb light for photosynthesis and to adapt to different light intensities.

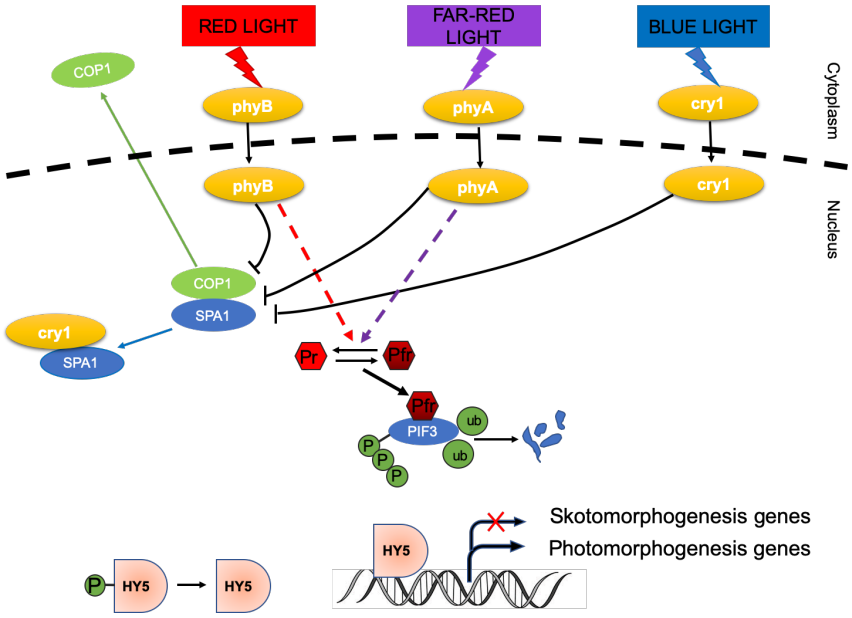

Figure 1. 1 The scheme shows the signalling during photomorphogenesis through upon light absorbance. Light is sensed through photoreceptors. The photoreceptors migrate to the nucleus in their active form. Phytochromes interact with COP1-SPA1 complex which thereby leads to stabilization of transcription factor HY5. Phytochromes also reduce the activity of PIF transcription factors which promote skotomorphogenesis: in light they are ubiquitinated and degraded. Once HY5 is increased in the nucleus, it activates transcriptional networks that lead to photomorphogenesis.

1.1.3 Photomorphogenesis

Upon light exposure, various developmental responses that are referred to as photomorphogenesis lead to growth and development in plants. Photomorphogenesis is a complex process that follows the perception of light by photoreceptor systems (Arsovski et al., 2012). This process requires complex coordination of global gene expression reprogramming and selective degradation of several proteins (Jang et al., 2019). Two central negative regulators of the photomorphogenic pathway are COP1 and DE-ETIOLATED1 (DET1). In *Arabidopsis*, COP1 and DET1 are the key repressors that target the photomorphogenesis promoting transcription factors for degradation in the absence of light (Lau and Deng, 2012). *COP1* and *DET1* belong to a group of genes named *CONSTITUTIVE PHOTOMORPHOGENIC/ DE-ETIOLATED/FUSCA* (*COP/DET/FUS*). COP1 is a RING E3 Ubiquitin ligase that has many other roles downstream of light signalling pathways including the circadian rhythm, flowering, plant defense, and shade avoidance responses. DET1 plays a role in transcriptional repression. The involvement of COP1 in most of these processes is through direct interaction and ubiquitination of key transcription factors such as

LONG AFTER FAR-RED LIGHT 1 (LAF1), ELONGATED HYPOCOTYL 5 (HY5), and LONG HYPOCOTYL IN FAR RED (HFR1) (Kim et al., 2017). However, when light is present, the photoreceptors inhibit COP1 function by a poorly understood mechanism which includes its export from the nucleus, and permit the accumulation of transcription factors involved in photomorphogenesis responses such as HY5. The exception to the general rule of photoreceptor activation being followed by COP1 inactivation is ultraviolet light perception: the photoreceptor UVR8 interacts with COP1 once it receives UV-B radiation, and this results in expression of HY5 (Favory et al., 2009).

1.1.4 Light Quantity

Plants can monitor environmental changes, such as nutrition, temperature, and light. Among these environmental cues, light is undoubtedly the most fluctuating factor influencing plant growth. The intensity and quality of light is fundamental for plant growth and development (Weston et al., 2000) and strongly impacts leaf physiology, anatomy, and morphology (Fukuda et al., 2008). Phytochromes function in a light quantity-dependent manner. Normal light in this project is considered as 100 µmol m^{-2} s^{-1}. This irradiance appears low for a natural environment, however it is not low for *Arabidopsis* growth requirement (which in nature can occur in dense, mutually shading stands in regions with frequent cloudy conditions) and is also supplied as continuous light for 24 hours. Similar approaches have been used before as extreme as low as 15 µmol m^{-2} s^{-1} or as high as 600 µmol m^{-2} s^{-1}(Weston et al., 2000). A plant in the LOW LIGHT (LL) (in this study is 40 µmol m^{-2} s^{-1}) condition is more susceptible to photoinhibition and reduction in expansion rate in a perpendicular direction to the leaf surface, because LL results in development of a thin leaf (Long and Humphries, 1994). The ability of the leaf to change its physiology and morphology under different light quantity demonstrates that plants have evolved mechanisms to adapt to different environmental conditions (Long and Bernacchi, 2003). Morphological and physiological changes occur in response to HIGH LIGHT (HL) (in this study is 300 µmol m^{-2} s^{-1}), including reducing specific leaf area, increasing in stomatal index, increasing the number and extension of palisade mesophyll cells to thicken the leaf and provide for a better levels of carbon fixation capacity (Walters, 2005), increase in the number of chloroplasts per cell (Anderson, 1986), increase electron transport rate, and increase in the capacity of energy dissipation (López-Juez et al., 2007; Mishra et al., 2012). The composition of chloroplasts and the morphology of palisade cells react to

the fluence rate of light by maximising photosynthetic activity while minimising photodamage. The underlying process is poorly understood and probably involves several mechanisms. Studies have shown that blue light receptors have a role in leaf morphology changes under HL. In *Arabidopsis*, loss of both phototropins receptors (*phot1*, *phot2*) leads to severe growth reduction in a light intensity-dependent manner (Schuerger et al., 1997; Takemiya et al., 2005). The phototropin mutant seedlings lose the chloroplast movement responses and therefore are prone to photodamage in presence of HL due to increase in photoinhibition and reduced recovery (Kasahara et al., 2002). A palisade elongation response under strong light requires blue light, but even loss of both phototropins does not abolish the response, so a full expansion response must have a non-photoreceptor element. The best candidate is energy signalling (López-Juez et al., 2007; Tan et al., 2008). This shows that higher plants can undertake a huge variety of acclimation responses to survive highly variable environmental conditions. However, one widely shared response targets the functional antenna size of PHOTOSYSTEM II (PSII) (Bielczynski et al., 2016). The PSII could be adjusted in amount, composition, and functionality in response to changes in light intensity (Ballottari et al., 2007; Suorsa et al., 2015).

1.2. The Shoot Apical Meristem

The SHOOT APICAL MERISTEM (SAM) is a population of stem cells that are placed at the tip of shoots and are responsible for development of all parts of the plant above ground (Shapiro et al., 2015). The stem cells divide into two daughter cells; one maintains as a stem cell, and the other differentiates according to positional signals (Clark, 2001). The SAM is involved in initiating the development of aerial organs continuously from daughter cells and maintains a balance between source of undifferentiated (stem) cells and cells going through differentiation (Baumann, 2013; Reyes-Olalde and de Folter, 2019). This complex structure organised during embryogenesis is maintained throughout the plants' life. The meristem contains three different zones based on molecular markers, developmental potential, and rate of cell division (Veit, 2009); these are named CENTRAL ZONE (CZ), PERIPHERAL ZONE (PZ), and RIB ZONE (RZ). CZ is where cells divide at a low rate and preserve their stem cell nature. In the PZ, cells divide at a faster rate and differentiate to form new primordial organs. Beneath these two zones is the RZ where cells are also rapidly

dividing (Braybrook and Kuhlemeier, 2010). In dicotyledonous plants, the SAM is made of three different layers, each with a different fate. The outermost layer is called L1 and comes to be the epidermis, L2 makes sub-epidermal cells and L3 forms the inner layer. Cells of L2 and L3 layers are contributing to the formation of internal structures, the mesophyll (the L2 and L3), and vascular structures (primarily the L3) (Kalve et al., 2014). The small group of cells called ORGANIZING CENTER (OC) underneath the CZ have a role in sustaining the fate of overlying stem cells and thus maintains a constant stem cells population despite the continuous departure of the daughter cells into lateral organs (Gregory et al., 2018; Williams and Fletcher, 2005). In order to maintain the make-up of the SAM, cell division and growth must adjust to accommodate the different cell sizes in all three layers (Kalve et al., 2014).

1.2.1 The meristem regulators

A tight regulation of the meristem is necessary to keep a balance between slowly dividing stem cells and departure of cells. In *Arabidopsis*, organ initiation happens through cells closer to the PZ and form the primordia. The rapidly dividing cells in the nearby regions differentiate and promote organ initiation (Schoof et al., 2000). The SAM maintains the fate of cells by their position, showing that cells respond to local signals from their neighbours and to long-range signals (Fletcher, 1999). The homeodomain transcription factor *WUSCHEL* (*WUS*) is involved in activating stem cell identity in the CZ and is expressed in a group of cells below the presumed location of the stem cells, and modulates the pattern of cell division. The distinction between stem cells and daughter cells is under the control of pathways containing prominent transcription factor families, including ARP proteins and KNOX1. In *Arabidopsis*, leaf primordia initiation is under control of *KNOTTED 1- LIKE HOMEOBOX* (*KNOX1*) and *SHOOTMERISTEMLESS* (*STM*) genes, which function antagonistically with *ASYMMETRIC LEAF1/ROUGH SHEA TH2/PHANTASTICA* (*ARP*) genes (Long et al., 1996). *ARP*s promote cell proliferation a fixed number of times and form a determinate organ such as a leaf. *KNOX* genes are expressed in the SAM to maintain stem cells and increase the expression of cytokinin hormone (Cytokinin is described later in this introduction) but decrease in the daughter cells at the time of leaf initiation (Byrne et al., 2000). Under KNOX influence cells divide indefinitely, ensuring indeterminate growth and KNOX also have a role in inhibition of cell differentiation and expansion related to organogenesis (Scofield and Murray, 2006). Notably, *WUSCHEL-LIKE*

HOMEOBOX (*WOX*) genes have a crucial role in sustaining stem cells and are necessary for SAM activities towards leaf primordia (Costanzo et al., 2014). The regulatory loop of *CLAVATA* (*CLV1*, *CLV2*, and *CLV3*) gene products is required to promote meristem cells towards differentiation at the shoot and restrict the proliferation of cells at the centre of the SAM (Clark et al., 1996; Kayes and Clark, 1998). The antagonistic relation between WUS and CLV in the layers of the shoot is necessary to preserve stem cells (Fig. 1.2) (Adibi et al., 2016; Schoof et al., 2000). A seedling carrying a mutation in *WUS* would suffer premature termination of the meristem (Endrizzi et al., 1996). *CLV3* encodes a ligand present in L1 and L2 that moves to L3 cells and once there, binds CLV1/CLV2 proteins. The binding of the ligand to CLV1/CLV2 results in inhibition of WUS activity. *CLV3* expression is activated by WUS and *WUS* expression is positively controlled by cytokinin hormone. Once stem cells are activated, this leads to expression of *CLV3*, which inhibits *WUS* expression (Kalve et al., 2014; Schoof et al., 2000). The anticlinal division of cells (the new wall being perpendicular to the surface of the meristem) in L1 and L2 regulate size of the meristem and the number of stem cells, while cells in L3 divide periclinally and anticlinally (Braybrook and Kuhlemeier, 2010). CLV3 and CLV1 have a very important function in maintaining the balance between stem cells and differentiated cells (Fletcher, 1999). The *clv* mutation results in accumulation of excess undifferentiated stem cells and therefore a delay in developing organs (Clark et al., 1996). Expression of *WUS* is activated by light through a signalling pathway that depends on the TOR kinase (Pfeiffer et al., 2016).

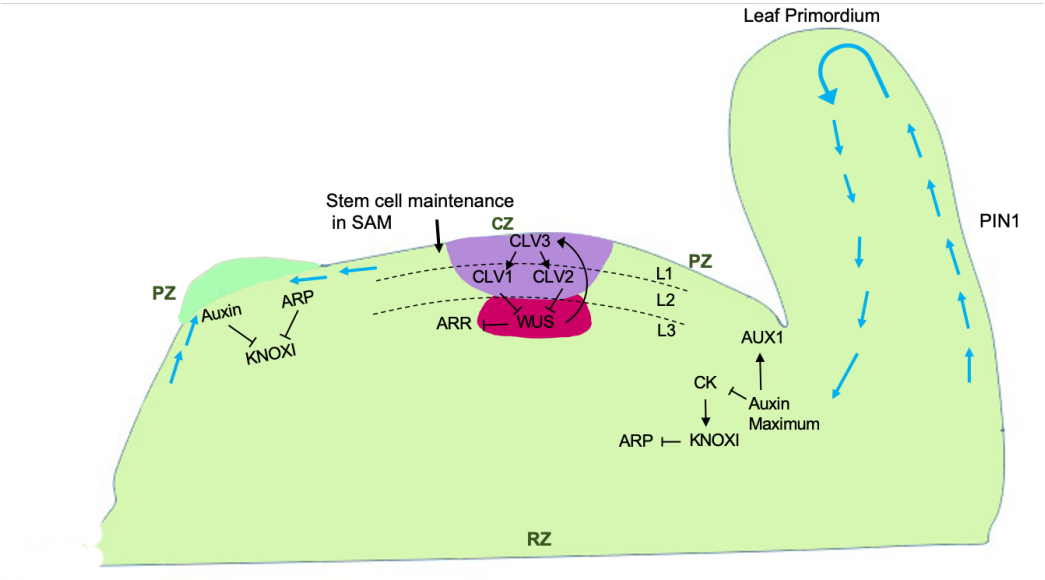

Figure 1. 2 The SAM is organized in three zones, CZ, PZ, and RZ.CLV and WUS function antagonistically to preserve cells in the SAM. Once CLV3 is activated by WUS, it will bind with CLV1/2 and *WUS* expression is repressed. WUS expression is positively regulated by cytokinin and WUS repress type A ARRs, negative regulators of cytokinin action. Cytokinin also positively regulates KNOX1 to maintain stem cells, and KNOX1 inhibits ARP that is involved in initiation of differentiation in the leaf primordium. The *KNOX1* expression is repressed by high auxin levels and ARP to restrain biosynthesis of cytokinin in all meristem cells except the ones in the site where new organ is emerging. PIN1 leads the flow of auxin in the epidermis towards the tip and then flows back through the inner tissues (future vasculature) at centre of the young leaf primordium. The polar distribution of PIN1/AUX1 results in formation of an auxin maximum, which specifies the formation of a leaf primordium, the leaf tip, and lobes at leaf margins.

1.3. How does a plant build leaves?

Just after cells leave the stem cell niche, displaced by other slowly dividing and growing stem cells, they are either contributing to the main axis or will differentiate to develop lateral organs such as a leaf primordium (Bar and Ori, 2014). The cells' decision to go through a particular path involves a complex regulatory network. (Floyd and Bowman, 2010). Leaf development starts from initiation in the flank of the SAM involving the determination of axes of symmetry, and later progresses into a structure of variable sizes and shapes. The laminae expand after leaf initiation and the formation of leaf primordia is determined by primary morphogenesis, then cells undergo fate determination and differentiation. The complex processes of leaf formation and growth are under regulation and interaction between plant hormones, transcriptional regulators, and nutrient availability (Bar and Ori, 2014). Auxin transport is the central regulator influencing the initiation of leaves (Auxin will be introduced in more detail

in the auxin section below) (Cheng et al., 2007). Auxin influx carrier AUXIN RESISTANT (AUX1) (Bennett et al., 1996) and efflux transporter PINFORMED1 (PIN1), transport auxin to different organs of the plant (Okada et al., 1991; Reinhardt et al., 2003). Organ initiation is closely associated with vascular strand formation, which is marked by the auxin maxima, small areas of locally highest auxin concentration and action (Scarpella et al., 2006). PIN1 activity is present in the epidermal layer (L1) and directs auxin flow to the tip and from tip to the base of the leaf (through the future midvein) to induce differentiation of vascular strands, whereas AUX1 leads to the accumulation mostly in L1 (De Reuille et al., 2006; Kalve et al., 2014). The effect of auxin on growth is mediated through the transcription factors of auxin named auxin response factors (ARFs) (Bar and Ori, 2014). The position of new leaf primordium is determined by high auxin concentration at the PZ on the apical side of epidermal cells, this position becomes a sink for more auxin from adjacent epidermal cells by PIN1 auxin efflux protein activity. Rapid cell division then starts at this position, however a second relocalisation of PIN1 is required to transport auxin away from the leaf primordium to the RZ. The primary cells of the new leaf primordium are founder cells that are recruited from PZ (Fig. 1.3) (Reinhardt et al., 2003). Organ initiation is prevented in the seedlings carrying *pin1* mutation or experiencing auxin polar transport (PAT) inhibition (Braybrook and Kuhlemeier, 2010). The interaction and delicate balance between plant hormones are fundamental in organ initiation and SAM maintenance (Kurakawa et al., 2007). Light is an essential factor in leaf primordia initiation and growth by mediating its effect through auxin and cytokinin (López-Juez et al., 2008; Yoshida et al., 2011). In maize, expression of the phyllotaxy response regulator *ABPHYL1* together with *PIN1* takes place at the spot where the new leaf primordium will be initiated, and both are induced by cytokinin (Giullni et al., 2004; Lee et al., 2009). Auxin is a positive regulator of leaf initiation; however, the response of cytokinin is more complex, and its influences on organ initiation highly dependent on the species and developmental perspective. Furthermore, relative levels of cytokinin, as well as the ratio of both hormones, play a role in regulating leaf initiation (Bar and Ori, 2014).

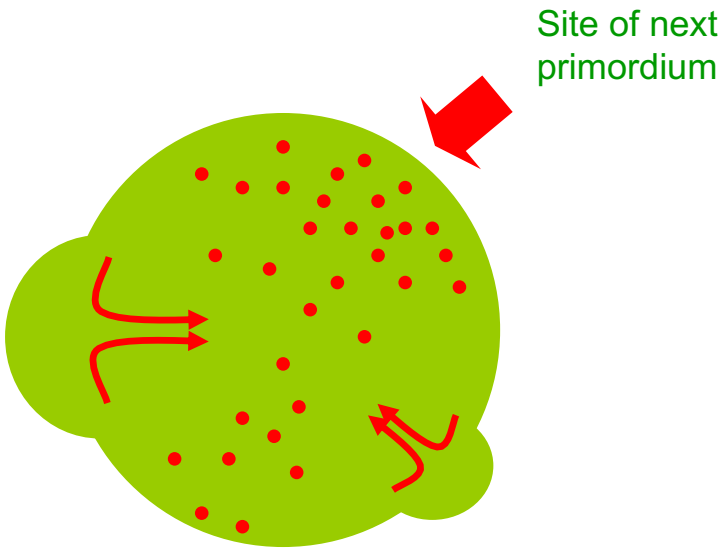

Figure 1. 3 Auxin is the main modulator of leaf initiation. New leaf primordia emerge at the site of highest auxin response. Leaf initiation occurs as far away as possible from existing leaf primordia. The red dots in the diagram shows auxin polar transport towards the site of new leaf primordium. The red arrows show the auxin transported away from the developed primordia.

The leaf cell size and number are fundamental to determine organ size and vary hugely between species. In *Arabidopsis*, the increase in the number and size of cells in the leaf primordia are regulated by members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene family (Tsukaya, 2014; Usami et al., 2009). The coordination between the cell size and cell population is established by endoreduplication of cells (Komaki and Sugimoto, 2012), however, in *Arabidopsis*, it was found that no direct link exists between ploidy level and increase in the cell size (Tsukaya, 2014). However, the expanding cells increase their size by expanding their vacuole content (Kalve et al., 2014). Ultimately, cell growth relies highly on macromolecular synthesis, cell cycle, wall extension, and endocycle. Macromolecular synthesis is regulated by the TARGET OF RAPMAYCIN (TOR) when adequate sugar is available (Sablowski, 2016). A very important target of TOR is ribosome biogenesis, a highly energy demanding process, thus linked to the energy status (Sablowski and Carnier Dornelas, 2014). Accordingly, protein synthesis is an important part of cell growth, which is regulated through translation initiation and involves modulating activity of the TOR and translation initiation factors (Sesma et al., 2017). The two main downstream factors of TOR are RIBOSOMAL S6 PROTEIN

KINASE1 (S6K) and the ErbB-3 BINDING PROTEIN 1 (EBP1) that regulates mRNA translation (Horváth et al., 2006).

1.3.1 Dorsoventrality

In higher plants, leaf formation occurs in a wide array of sizes and shapes (Szakonyi et al., 2010). One feature of leaf development is dorsoventrality, the creation of distinct abaxial (lower) and adaxial (upper, that faces the sun) sides. Leaf development starts with the initiation of primordia, the establishment of dorsoventrality, and growth of a marginal meristem (Xiong et al., 2013). In dicot plants, primordia grow outward from the site of leaf initiation to establish a proximodistal axis. After this axis is formed, the symmetrical primordium flattens to form the dorsoventral axis. *YABBY* and *KANADI* gene families are responsible for abaxialization, whereas the class lll HD-ZIP genes are central to the formation of adaxialization (De Reuille et al., 2006; Siegfried et al., 1999). *KANADI* genes are expressed in the abaxial regions of emerging lateral organs and their delocalized action results in the complete abaxialization of those organs (Eshed et al., 2001; Hawker, 2004). In most dicot species, the abaxial side differentiates into cells more specialised for gas exchange and the adaxial surface differentiates under the epidermis into palisade cells. A further, new, lateral meristem is located between the adaxial sides of the base of the leaf and the stem (Roldán et al., 1999).

1.4. Cell cycle

Plants have a unique style of continuous growth and development by repeating the initiation of new organs during their whole life (Komaki and Sugimoto, 2012). Growth and development of tissues in multicellular organisms are driven by cell division, and the basic control mechanisms underlying the cell cycle progression are conserved process among all eukaryotes (Nieuwland et al., 2009a). In plants, a large amount of cell division activities are initiated in the SAM (Guiterrez, 2009) under the control of several factors such as cytokinin hormone and nutrient availability (Riou-Khamlichi et al., 1999). Our understanding of the importance of cytokinins' role in cell division comes from observations of *Arabidopsis* seedlings with no cytokinin sensing ability, which either fail to develop the SAM, or develop only a very small SAM (Leene et al., 2010). The mitotic cell cycle is created from four distinct, ordered phases. The synthesis period (S phase) is the DNA synthetic phase, where the cell replicates genetic material, the mitotic (M phase) sees the segregation of genetic material into two sets, with two

gap phases (G_1 and G_2) occurring between the M phase and the entry into the S phase (Komaki and Sugimoto, 2012; Scofield et al., 2014). The gap phases are required to control whether or not the previous phase has been accurately and thoroughly completed (Koyama et al., 2009). The G_1 phase starts after the last cell division and prepares the cell to undergo the next division. It is in this phase that transcription and translation of genes necessary for the synthesis of DNA begins. During the G_2 phase, the cell coordinates transcription and translation to make proteins that are required for mitosis. Lastly, DNA condenses, and the cell divides into two daughter cells in the M phase. The progression through each phase of the cell cycle is driven by the limited time activation of CYCLIN-DEPENDENT KINASES (CDKs) that work with different CYCLINS (CYC) (Komaki and Sugimoto, 2012; Magyar et al., 2005).

1.4.1 Cell cycle regulators

Progression through the cell cycle and its checkpoints is regulated in plants by CDKs together with activator CYCs. Both are products of multigene families. Cell cycle regulators are fundamental for the phase-specific activation of CYC-CDK (Berckmans and De Veylder, 2009). The tissue-specific activity of CDKs and the vast possible combinations with their activity partner CYCs allows a cell to deal with the complexity related to multicellularity and provides a more extensive range of signals to regulate cell division (Dewitte et al., 2007). The different types of CYC-CDK complexes phosphorylate substrates in the transition from G_1 to S phase and G_2 to M phase. Thus, they result in the activation of the beginning of DNA replication and of mitosis, respectively (Komaki and Sugimoto, 2012). For example, the progression of the cell cycle to mitosis is reliant on the build-up of CDK with CYCB (Baena-González et al., 2007). The essential role of CYC-CDK is not limited to proliferation but also, while inactive, they lead to differentiation, as they are controlled at the transcriptional and posttranslational level (Stals et al., 2000). Plants contain a great number of CDKs and CYCs; *Arabidopsis* contains 10A (CYCA), 11B (CYCB), and 10D type (CYCD) CYCs (Zhao et al., 2012). Primarily CDKA and D-type CYC have been associated with regulation of G_1 to S phase, while A- and B-type CYCs most probably regulate G_2 to M phase (Dewitte et al., 2007). CYCDs play significant roles through cell division in *Arabidopsis*. The CYC-B subtype has a role in the initiation of entry into the M phase as CYCBs contain a mitosis-specific activator sequence within their promoter (Ito et al., 2001). In this thesis, CYCB1;1 has been used to visualize cells going through

division by a cyclin-GUS reporter (the promoter and first three exons of *CYCB1;1* gene were translationally fused to the Escherichia coli *uidA* (GUS) gene). *Arabidopsis* transformed with *CYCB1;1::GUS* was used to monitor growth-related responses within individual cells. The histochemical activity of this chimeric reporter shows individual cells going through mitosis. The promoter turns on at the start of mitosis, but the protein "turns off" at the end and is degraded in anaphase, this has allowed a detailed analysis of the patterns of mitosis during organ initiation (Colón-Carmona et al., 1999; Donnelly et al., 1999). The CYCD family form a complex with CDKA and act in a pathway that leads to phosphorylation of RETINOBLASTOMA-RELATED protein (RBR). Phosphorylated RBR protein allows the activity of E2F transcription factors to initiate S phase (Dewitte et al., 2007). In plants, as in animals, some of the cyclins react to external signals such as developmental and hormonal ones. For example, CYCD3 genes show responses to cytokinin (Dewitte et al., 2007) and CYCD2 to the levels and availability of sucrose (Nieuwland et al., 2009b).

1.4.2 Importance of RBR and E2Fs in regulating cell cycle

The RB protein was first identified as a human tumour suppressor that regulates cell proliferation by interacting with E2F transcription factors. Consistent with its role as a tumour suppressor, RB was long known as a negative modulator of the cell cycle, therefore a critical regulator of entry to cell division. However, later research has shown RB is also involved in cell differentiation, preventing cell division, endoreduplication, and regulation of stem cell behaviour (Harashima and Sugimoto, 2016). RB-RELATED (RBR), the homolog of RB in *Arabidopsis*, controls cell proliferation by forming a complex with core cell cycle regulator, E2F transcription factor and thus restrict the transcription of genes that are E2F-dependent to prevent uncontrolled cell divisions (Harashima and Sugimoto, 2016; Magyar et al., 2005). *Arabidopsis* contains a single *RBR* gene and three *E2F*s (*E2Fa*, *E2Fb*, and *E2Fc*). The E2Fs contain DNA-binding, transactivation, dimerization partner (DP) heterodimerization, and RBR-binding domains (Desvoyes et al., 2006; Shen, 2002). In *Arabidopsis*, G_1 CYCs contain a sequence of LXCXE (X being any amino acid), which is also present in mammals and has been shown to interact with RBR (Dick and Rubin, 2013). The CYCDs are acting as the sensors of conditions promoting cell division (Perrot-rechenmann, 2010). E2Fa/DPa complex after heterodimerization functions as a transcriptional activator to regulate cell proliferation and endoreplication. However, the formation of E2F/DP

complex is promoted by RBR, which then blocks the activity of E2F target genes required for progression to S phase. RBR is phosphorylated by CYCD-CDKs during G_1 phase, which then results in its inactivation and dissociation from the E2F-DP heterodimer complex. The E2Fa stays in complex with RBR and suppresses cell differentiation to maintain the meristem, while E2Fb is dissociated from RBR and promotes cell proliferation (Fig 1.4) (Horvath et al., 2017). It is suggested that RBR is in control of all three E2F transcription factors that regulate plant cell cycle and differentiation (Henriques et al., 2013). The transcriptional activation of E2F is switched on in animals by CYCE, which in turn leads to more RBR phosphorylation, hence E2F activity is within a positive feedback loop (Dick and Rubin, 2013). CYCE expression levels increase in the transition from G_1 to S phase and are destroyed later in S phase (Woo and Poon, 2003) However; plants have CYCD3 instead of CYCE (Wang et al., 2004). Mutation in *CYCD3* causes the early exit from cell cycle, greater endoreduplication, and results in development of larger cells (Nieuwland et al., 2009b). It is clear that RBR has a fundamental role in cell proliferation, and recently, a study showed it is also involved in regulating symmetry of division (Desvoyes et al., 2015).

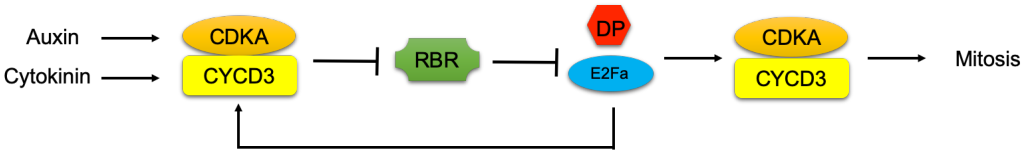

Figure 1. 4 The mitosis process is regulated through CDKA-CYCD kinase activity Auxin is necessary for activation of CDKA complex with CYCD3 and cytokinin regulates CYCD3 expression. RBR supress the E2F-DP complex, once RBR is phosphorylated and therefore inactivated, E2Fa_DP complex promotes mitosis. Adopted from (Nieuwland et al., 2009b).

1.4.3 Importance of auxin and cytokinin in cell cycle regulation

Hormones will be introduced in greater detail below. However, it is important to note that most of the known plant hormones are involved in the cell cycle and/or expansion. Cytokinin and auxin are both involved in cell proliferation. Research has shown auxin is in part acting through stabilization of the E2FB protein that promotes mitosis (Magyar et al., 2005). The ectopic expression of stabilized E2FB is enough to promote cell division in culture (Scofield et al., 2014). Cytokinin also induces the proliferation of undifferentiated cells and works with other signals in the SAM and then later auxin, which will promote organ outgrowth (Schaller et al., 2014). Cytokinin has been

associated with the regulation of G_1/S and G_2/M transitions. Cytokinin hormone induces three *CYCD3* genes' expression, but this requires 20 hours of a high level of cytokinin. Mutation in all three *CYCD3* genes will block the action of cytokinin in promoting greening and shoot development from callus. This suggests key targets of cytokinin in regulating cell cycle are CYCD3s (Perrot-rechenmann, 2010). Cytokinin is essential for transition through the cell cycle, as it affects the time a typical cell spends in each phase of the cell cycle. Importantly, applying exogenous cytokinin could also inhibit the cell cycle by blocking the cell cycle at G_1 or delay the exit from the G_2 phase (Riou-Khamlichi et al., 1999).

1.5. Hormones

1.5.1 Auxin and plant development

While we have referred earlier to the role of auxin and its transport in relation to the SAM function and the initiation of leaf primordia, a more detailed look at auxin function is required to understand the basis of this project. Auxin is an essential plant growth hormone that probably has fundamental roles as a general coordinator in almost all aspects of development including those in response to gravity or light. The main naturally and primary occurring auxin in the plant is indole-3-acetic acid (IAA) (Zhao, 2010). Auxin has many growth-related roles in the plant, such as acting as coordinating organs' development, lateral organ formation, vascular patterning, cell expansion, gametogenesis, tropism and embryogenesis (Fabregas et al., 2015; Friml et al., 2002). However, auxin is better known for its elongation effect in the shoot and proliferation (including the establishment of new lateral root meristems) in the root (Rayle and Cleland, 1992; Perrot-Rechenmann, 2010). Significant progress has been reached in understanding auxin signalling, but auxin biosynthesis has remained a target of research for plant biologists. Uncovering the molecular mechanisms underlying biosynthesis of auxin is crucial in better understanding of roles auxin plays in growth. Auxin biosynthesis is a very complex process, and together with its signalling pathways it is connected to other plant hormones such as cytokinin, ethylene, and brassinosteroids. It was long suggested that IAA is synthesized through two pathways, the TRYPTOPHAN (TRP) dependent and independent routes (Normanly et al., 1993). Most of IAA biosynthesis is derived from the INDOLE-3-PYRUVIC ACID (IPyA) pathway, which is a TRP-dependent pathway and called the YUCCA and TAA pathway. IPyA is made

from TRP by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) and YUCCA family convert IPyA to IAA (Korasick et al., 2013). Expression of genes responding to auxin mediates a wide range of patterning processes in the plant. Thus, changes in transcription are the main mechanism by which auxin levels are transformed to cellular responses (Paponov et al., 2008). Auxin causes changes in transcription by facilitating the binding of the family of F-box proteins including TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) auxin receptors to ligate ubiquitin to Aux/IAA proteins, thus allowing them to be degraded (Tan et al., 2007). Therefore, any variations in the auxin levels will be transformed to changes in Aux/IAA levels. Several features of auxin action depend on its dispersal inside plant tissues, where it can form gradients or local maxima in different tissues (Robert and Friml, 2009). Auxin moves directionally within tissues; this polar transport is unique to auxin and necessary to direct auxin flow and to arrange a gradient vital for establishing a developmental pattern (Korasick et al., 2013).

1.5.2 Auxin transport route

Auxin transport occurs via two distinct pathways; a passive fast, non-directional transport through phloem vasculature cells from young primordia and apex to root. Second, directional cell to cell movement over plasma membrane referred to as PAT (Park et al., 2017). Most IAA is possibly transported away from young leaves and flowers to root through phloem associated cells, through vascular parenchyma, cell to cell which is maintained by the action of PIN-dependent route. The unloading of auxin is through AUX1 into protophloem cells in the root apex (Petrášek and Friml, 2009). There are three families of proteins that transport auxin between cells: auxin influx AUX, LIKE AUX1 (AUX1/LAX), auxin efflux PIN-FORMED (PIN) proteins, and B type ATP BINDING CASSETTE (ABCB) superfamily transporter (ABCB MULTIDRUG RESISTANCE (MDR)-p-glycoprotein (PGP) proteins) (Fig 1.5) (Fabregas et al., 2015; Su et al., 2011). AUX1/LAX and ABCB transporters facilitate auxin sinks and regulates auxin levels to contribute to lateral root formation. The extremely organised distribution of auxin is facilitated through the cooperative action of AUX and LAX (Petrášek and Friml, 2009). The PIN proteins are predicted to have structure similar to the secondary transporters (Membrane transport protein) (Křeček et al., 2009). PIN members are often located asymmetrically in cells, which corresponds with the routes of auxin flow (Friml et al., 2002). In *Arabidopsis*, the PIN family is

referred to as PIN1 to PIN8, among which PIN5, PIN6, and PIN8 act as auxin efflux carriers between the cytosol and endoplasmic reticulum (Mravec et al., 2009; Petrášek et al., 2006). The remaining PIN proteins are localized in the plasma membrane and pump auxin out of the cell. It is well known that efflux transporters play a critical function in shoot vascular patterning, but the role of influx carriers in the shoot vascular patterning is not known yet. Among PIN members, in *Arabidopsis*, PIN1 is the most critical member controlling shoot development (Bennett et al., 2016). *PIN1* expression is strong in the epidermal cells of the SAM, and its apical polarization leads to an auxin gradient that flows towards the site of the primordia initiation (Sassi and Vernoux, 2013). Differential auxin distribution within tissues is transport-dependent and essential to facilitate multiple aspects of development (Petrášek and Friml, 2009)

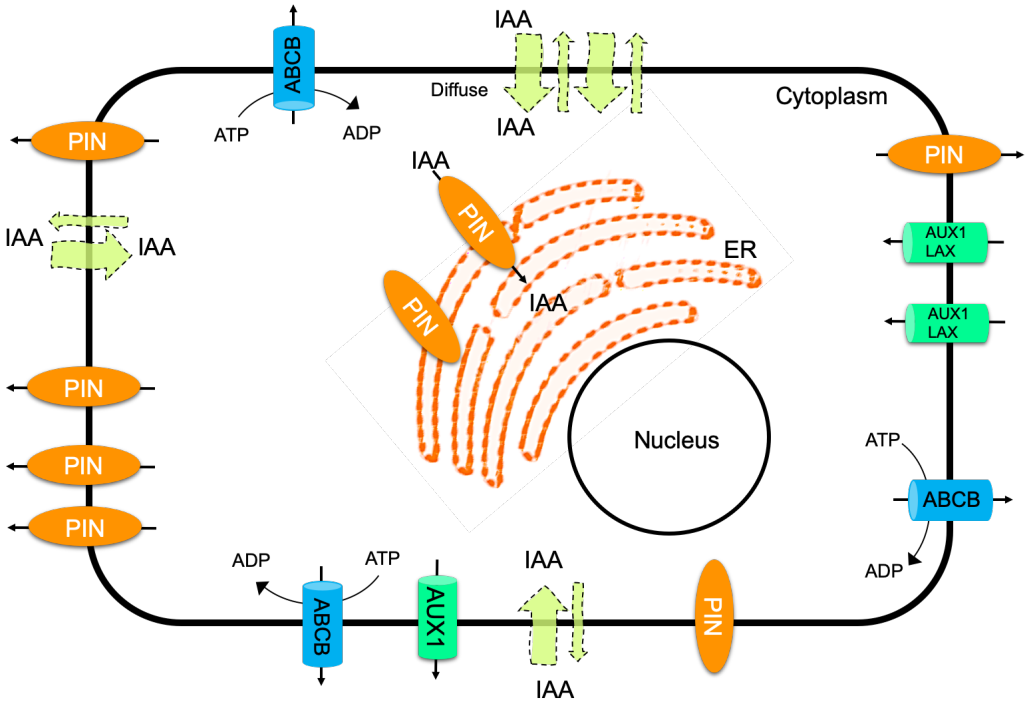

Figure 1. 5 Auxin transport route. The scheme shows the proteins involved in transport of auxin. PIN family and AUX1/LX are involved in auxin transport. The direction of IAA transport is shown by black arrow. IAA could also enter cell across the plasma membrane through lipophilic diffusion. ABCB auxin transport activity is dependent on ATP activity.

1.5.3 Regulation of auxin transport and MAPK pathway

Various aspects of the spatiotemporal regulation of plant growth and development are mediated by transport-dependent auxin movement. Thus, a tight regulation of its pattern of distribution and activity is necessary. Several mechanisms can be combined to regulate auxin-dependent distribution. The most important regulator of the auxin

transporting system is auxin itself. Proteins transporting auxin can be modulated at three levels through the regulation of the levels of transporter, localising auxin carriers to a specific location within the plasma membrane, and by regulating carriers' transcription, translation and degradation levels (Petrášek and Friml, 2009). The PAT is an asymmetric directional distribution of auxin from cell to cell, unique in manner that creates local auxin maxima (Armengot et al., 2016). PIN proteins and protein kinases that reversibly phosphorylate them are involved in regulating PAT (Dai et al., 2006; Dory et al., 2018). Localisation of PIN proteins to the plasma membrane is negatively regulated by MAP kinases, including MPK4 and MKK7/MPK6 (Dory et al., 2018). MKK7/MPK6 cascade regulates shoot branching by phosphorylating PIN1 and affects PIN1 basal localization and polar auxin distribution (Jia et al., 2016). The involvement of MAPK pathway in connecting auxin to development has been elucidated but the regulatory connections yet to be discovered. MAPK pathway is involved in signalling of growth factors, hormone pathway, and stress-related molecular patterns and thus, is fundamental for plant signalling transduction (Sinha et al., 2011). The signal transduction is carried out by converting the external stimuli into intracellular signals. YODA kinase (MAPKKK4) and MPK6 are involved in root development by auxin-dependent modulation of cell division (Jagodzik et al., 2018; Smékalová et al., 2014). MAP kinases specific and transient expression establishes leaf venation patterns, which is induced by auxin in *Arabidopsis* by amending the efficiency of PAT (Stanko et al., 2014).

1.5.4 Auxin transport and phyllotactic patterning

Phyllotaxis is the production of leaves on the flank of the SAM in an ordered pattern. Plants have different phyllotactic forms (Sassi and Vernoux, 2013), the most frequently observed patterns of phyllotactic leaf arrangement are spiral or otherwise decussate (in opposing pairs), alternate, and whorled (multiple leaves emerging at the same distance from the meristem). In all the patterns the new leaf tends to form as far away as possible from the previous leaf. The molecular mechanisms related to the position of a leaf coincide with low expression of KNOX genes. The feedback loop of the auxin hormone and its transporter protein PIN1, establish the pattern of auxin maxima where presumptive primordia are positioned (Braybrook and Kuhlemeier, 2010). The specification of a site for leaf initiation is also attended by the expression of genes involved in modulating the balance between differentiated fate initiation and meristem

identity. KNOX1 is involved in regulating auxin responses (Tsiantis et al., 1999) and the balance between cytokinin (which promotes meristematic cell fate) and gibberellic acid (which promotes differentiation) (Bar and Ori, 2014; Scofield et al., 2013). Therefore, KNOX1 coordinates the response and activity of several plant hormones within the SAM to keep the balance among leaf initiation and SAM function. This transcription factor is repressed at the site of leaf initiation by other transcription factors involved in promoting specification of the leaf initiation site such as ASYMMETRIC LEAF1 (AS1) (Bar and Ori, 2014; Byrne et al., 2000; Hayat et al., 2013). MYB91/AS1 regulates shoot morphogenesis and leaf patterning through its antagonistic actions with KNOX proteins. The MYB transcription factors facilitate the separation of primordium cells from the shoot meristem. The specification of leaf initiation site allows primordium cells to differentiate and develop and determines the shape of a leaf (Dubos et al., 2010).

1.5.5 Cytokinin

The phytohormone cytokinin influences multiple aspects of plant growth and development. Cytokinin was first identified as a factor promoting the cell proliferation and shoot formation, it is also involved in chloroplast development, vascular cambium activity, and response to nutrients (Wen et al., 2017). In a developing plant, cytokinin acts as a positive regulator of the SAM by triggering the proliferation of undifferentiated cells (Schaller et al., 2014) (Su et al., 2011). Cytokinin is sensed by *ARABIDOPSIS* HISTIDINE KINASE (AHK) family receptors that auto phosphorylate and transmit the signal to *ARABIDOPSIS* RESPONSE REGULATORS (ARRs) (To et al., 2007). The vast majority of cytokinin receptors are in the endoplasmic reticulum (ER) and cytokinin bind to the CYCLASE HISTIDINE KINASE ASSOCIATED SENSORY EXTRACELLULAR (CHASE) domain of the receptor. Type A-ARRs, are negative regulators of cytokinin signalling to reduce cytokinin responses (To et al., 2007), and type B-ARRs lead to the activation of cytokinin-induced genes (Gordon et al., 2009). Type-A ARRs disruption results in an increase in meristem size, which is consistent with type-A ARRs function to reduce cytokinin response levels and thus inhibit meristem function. A cytokinin producing enzyme, *LONELY GUY* (*LOG*), is required for meristem maintenance, and *log* mutant in the rice results in a smaller shoot meristem in rice (Yoshida et al., 2011). This information indicates that cytokinin controls the size of the *Arabidopsis* shoot meristem; in contrast, it negatively regulates

the root meristem by modulation of cell differentiation (Su et al., 2011). Cytokinin is not uniform and is instead mostly present in the centre of the meristem. It was also found that cytokinin and WUS reinforce each other in multiple positive feedback loops (Gordon et al., 2009). When Cytokinin level is above a critical threshold, *WUS* expression will be increased and will repress *CLV1* (Yoshida et al., 2011). Cytokinin impacts auxin transport through multiple mechanisms, by modulating efflux (PIN) and influx (LAX) transporters and through regulation of auxin synthesis. Auxin in turn regulates cytokinin through regulation of *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*), which acts as a cytokinin inhibitor, and through type A-ARRs (Schaller et al., 2014). While A-ARRs are negative regulators of cytokinin action, they are strongly cytokinin-induced, so they can be used to monitor cytokinin action. Thus, type A-ARRs are good readouts of the presence of active cytokinin. One particular member of type A, ARR5, was used as signature of cytokinin signalling in this thesis. The *ARR5* mRNA level is almost untraceable when cytokinin is not present and will shortly accumulate once cytokinin is present (Romanov et al., 2002).

1.5.6 Ethylene

The gaseous plant hormone ethylene is highly stimulated during mechanical stresses and effects morphogenesis accordingly (Liu et al., 2017). Ethylene is known as a natural product produced by plant tissues and coordinates many aspects of cellular and developmental responses, including seed germination, growth, cell expansion, differentiation, hypocotyl development, and reaction to stresses (Ceusters and Van de Poel, 2018). Ethylene is perceived by ETHYLENE RECEPTORS (ETRs), whereby suggested by several studies to act as inhibitors and removed by ethylene. This model suggested by several studies is supported in tomato where level of ETR1 gain of function is related to level of insensitivity to ethylene. This indicates, the level of ETRs may be a critical factor in determining ethylene signalling (Gallie, 2010). The signal transduction pathway includes other proteins such as ETHYLENE INSENSITIVE 2 (EIN2) and CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). Once EIN2 moves to nucleus, a specific transcription factor, EIN3, is activated and promotes expression of genes dependent on ethylene (Y. Chen et al., 2018).

EIN3 is the primary downstream transcription factor in the ethylene pathway and is activated in response to abiotic stresses (Liu et al., 2017). The family of EIN3

transcription factors regulate the transcription of the majority of genes that ethylene rapidly induces during plant stress, such as *ETHYLENE RESPONSE FACTOR 1* (*ERF1*) (Cheng et al., 2013). Once EIN3 and ERFs are activated, they will drive changes in gene expression to exert an effect in the cell functions in response to ethylene. Ethylene signalling and sensing are tightly interconnected with abscisic acid (ABA) and sugar signalling (Matsoukas, 2014). Ethylene has a role in maintaining the root meristem by a mechanism that possibly involves auxin. How sugar signalling is involved in preservation of the root meristem by ethylene and auxin remains to be discovered.

1.6. Energy signalling

1.6.1 Sugar transport from source to sink

The plant requires both long and short distance signalling mechanisms to coordinate growth within the plant and to environmental changes. The photosynthetic organs represent the sugar exporting (source) tissues, while non-photosynthetic organs and meristems represent the importing (sink) tissues. Sink organs are dependent on the sucrose provided by the source tissues through the phloem for growth and development (Osorio et al., 2014). Transport of photosynthetically fixed carbon depends on sucrose supply and sink demand. An increase in the sucrose contents in leaves could have an inhibitory outcome on the activity of SUT and consequently, sucrose unloading is inhibited. This was observed by providing sucrose to the cut sugar-beet leaves (Chiou and Bush, 1998). The main form of sugar that is photosynthetically produced from triose-phosphates and exported, is sucrose, and the hydrolytic products of sucrose are hexoses, glucose, and fructose. These sugars account for many sugar signalling actions that could impact growth. Sucrose is imported to the sink tissues through the cell wall or plasmodesmata. The excess photosynthate generated during the day is stored as starch synthesised by ADP-glucose pyrophosphorylase (AGPase) enzyme. Most of the glucose present in photosynthetic cells throughout the night is generated from starch stored in chloroplasts (Hendriks et al., 2003).

1.6.2 Sugars and plant growth

The synthesis and degradation of carbohydrates are diurnally adjusted to maintain the balance during day and night. Energy condition is reflected by a sugar's levels; thus, a monitoring network is required to respond to variations in nutrient availability (Nunes-Nesi et al., 2010). The growth-promoting regulators are TOR kinase, HXK glucose sensor, and the sugar trehalose 6-phosphate (T6P) and the inhibitors are SNF1-RELATED KINASE 1 (SnRK1) complex (Gazzarrini and Tsai, 2014) and C/S1 bZIP transcription factors. These are the central regulators to respond to changes in energy status to either promote or inhibit growth (Jamsheer et al., 2019). TOR-kinase plays role in stimulation of protein synthesis mainly by phosphorylating S6K1 and eIF4E BINDING PROTEIN (4EBP), and through this it regulates metabolism and growth (Dobrenel et al., 2016). SnRK1 is an energy sensing kinase (Baena-González et al., 2007) that controls the expression of over a thousand genes coding for transcription factors and proteins (Crozet et al., 2016). The catalytic activities in the SnRK1 complex are carried out by KIN10/11 protein kinases in *Arabidopsis* (Gao et al., 2016). SnRK1 is involved in coordinating the environmental signals with plant growth by regulating the enzymes that have a function in metabolism. SnRK1 kinase functions as a heterotrimeric complex and has an important role in sensing sugar and energy deprivation. TOR and SnRK1 have been proposed to act antagonistically in response to primary sugar and coordination of energy status (Tomé et al., 2014). There is a correlation between the expression of genes regulated by the KIN10 and the genes expressed in response to carbon starvation (Sakr et al., 2018). TOR is active when nutrients and reduced carbon are available, and the plant is in a favourable condition for growth and development, while SnRK1 is active in response to metabolic stress to control starch and carbohydrate catabolism (Jossier et al., 2009). The crosstalk between TOR and SnRK1 is mandatory for plants to adapt to different conditions through protein synthesis and metabolism according to the available resource. TOR and SnRK1 both function downstream of sugar sensing pathways, and their activity is controlled according to the plant condition (Sakr et al., 2018). SnRK1 induces changes in metabolic regulation by mobilisation of starch during low energy conditions (Thelander et al., 2004), phosphorylation of several enzymes including SUCROSE PHOSPHATE SYNTHASE (SPS) (Kulma et al., 2004), and through transcriptional regulation of an enormous set of genes such as the ones encoding sucrose synthase (Baena-González et al., 2007; Jossier et al., 2009). It is still unknown how a single protein kinase acts as a

main regulator of a huge number of cellular activities via partners and effectors in complex signalling pathways (Xiong and Sheen, 2014). A pharmacological treatment used in the study of the TOR pathway is the ATP-competitive selective inhibitor of TOR (AZD-8055) (Chresta et al., 2010; Thoreen et al., 2009; Werth et al., 2019). AZD-8055 has proved to be effective in plants (Dong et al., 2015; Montané and Menand, 2013; Xiong et al., 2013) and reduces the mitotic activity and inhibits the cell proliferation capacity of root meristem (Montané and Menand, 2013).

1.6.3 Carbon starvation and energy stress

Plant growth depends on inputs such as light and nutrient availability. Exposure to the limitation in any of these factors has multiple metabolic and physiological effects that lead to plant starvation (Honig et al., 2012). A plant suffering from energy stress shows alteration in growth. The energy stress could be induced in seedlings grown in extended darkness, in which seedlings adapt to the stress by inhibiting growth (Lastdrager et al., 2014).

1.6.4 TOR signalling

The central controller of growth-related processes is the TOR signalling pathway, which is evolutionarily conserved in all eukaryotic organisms. TOR is a serine-threonine kinase, which senses and regulates energy and nutrient availability to stimulate changes required for cell proliferation, growth, survival, and synthesis of protein (Bakshi et al., 2019; Xiong et al., 2013). The TOR protein kinase is part of the family of PHOSPHATIDYLINOSITOL KINASE-RELATED KINASES (PIKK) and contains distinct domains such as the FAT domain, Kinase domain, and the FATC domain. TOR exists as two different protein complexes, TOR COMPLEX 1 AND 2 (TORC1) and (TORC2) in animals and yeast. Mammalian TORC1 is comprised of TOR, LST8, REGULATORY ASSOCIATE PROTEIN OF TOR (RAPTOR), and FK506-BINDING PROTEIN 12 (FKBP12) (Dobrenel et al., 2016). *Arabidopsis* only contains a single copy of the *TOR* gene that encodes an unusually large protein of 280 kDa (Cafferkey et al., 1993). The activation of TOR signalling by glucose regulates genes involved in cell proliferation and activates genes involved in DNA and RNA synthesis during the cell cycle (Xiong et al., 2013). A family of Rho GTPases known as ROP leads to the activation of TOR (Schepetilnikov et al., 2017; Bakshi et al., 2018). Auxin and light activate the ROP2-GTPase, which leads to the activation of TOR to

initiate cell proliferation in the SAM (Li et al., 2017). The SAM requires both light and glucose for TOR activation (Pfeiffer et al., 2016; Xiong et al., 2013). TOR kinase has a role as a central regulator of stem cell activation at the SAM through *WUS* expression (Pfeiffer et al., 2016). Activation of TOR could even happen in the absence of light in the constitutively activated ROP2 plants (Li et al., 2017). The only known downstream effectors of TOR are TAP46, an important regulator of TOR signalling pathway whose silencing will lead to programmed cell death in tobacco and *Arabidopsis* (Hu et al., 2014), S6K1, and E2Fa in *Arabidopsis*. TOR kinase links photosynthesis-produced glucose status to plant growth programme and reduction in TOR activity results in a significant build-up of amino acids and sugars. If the environmental conditions are favourable, catabolic processes are muted through TOR signalling. How sugars affect TOR activity and how the signal is sensed and integrated is not clear yet. One surprising way in which TOR promotes the cell cycle activation is by directly phosphorylating transcription factors E2Fa and E2Fb in both the shoot and root meristem (Li et al., 2017; Xiong et al., 2013). In this case E2F transcription factors are regulated irrespective of cyclin or RBR function. In addition, S6K1 plays a function in reinitiating translation (Li et al., 2017). S6Ks belong to the AGC family of protein kinases and are conserved in plants and animals. S6Ks function downstream of TOR kinase and act as central effectors in this pathway. Plants contain two types of S6Ks, S6K1 localised to the cytosol and S6K2 localises to the nucleus. In *Arabidopsis*, some of the activation effects of TOR occur through phosphorylation of S6K1. Auxin could stimulate S6K1 phosphorylation by TOR and, by doing so, regulate translation initiation through RPS6 (Fig. 1.6) (Schepetilnikov et al., 2013). The S6Ks in *Arabidopsis* have essential roles in maintaining chromosome stability and ploidy level. S6K1 represses cell proliferation in *Arabidopsis* by forming a complex with the RBR-E2Fb and localizes RBR to the nucleus and potentiates E2Fb repression (Henriques et al., 2010) and also tunes translational capacity of cells by phosphorylating its major substrate, RPS6. The knockout mutation of *s6k* in Drosophila and mice led to a reduction of cell size (Montagne et al., 1999), while mutation of the phosphorylation site of *s6k* on RPS6 showed no effect on protein synthesis, indicating RPS6 is a determinant of cell size (Ruvinsky et al., 2005). In contrast with this level of understanding, TOR kinase regulatory mechanisms and the molecular function of TOR kinase remains much less in plants (Xiong et al., 2013).

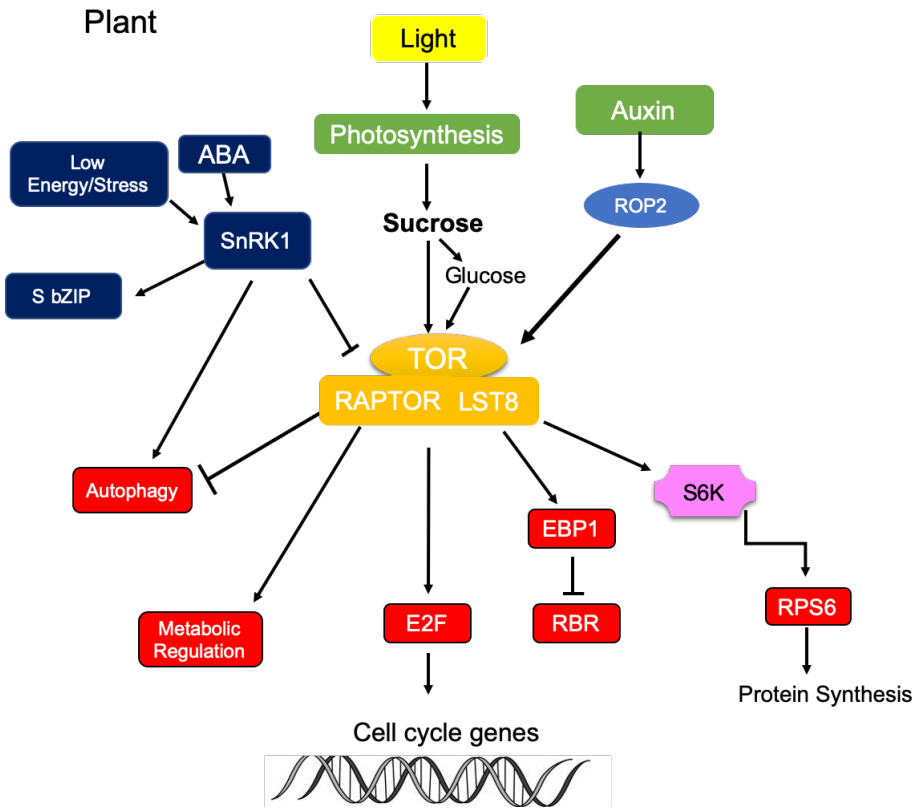

Figure 1. 6 TOR is a master regulator of sensing energy status, sucrose/glucose, stress, and hormones. RAPTOR and LST8 are cofactors part of the TOR cascade. Light activates TOR directly through photoreceptors, including phyA, and indirectly through photosynthesis, and auxin activates it through ROP2. TOR regulates the cell cycle through E2Fa. The pathway of TOR-S6K-RPS6 contributes to increase in translation that is activated by light. S6K phosphorylation is TOR dependent and once phosphorylated regulates translation through RPS6. The phosphorylation of E2Fa by TOR promotes organ growth once sucrose is available. TOR also positively effects transcription of cell cycle modulator, EBP1 that negatively regulates RBR. Autophagy is activated by SnRK1 and could be regulated by TOR depending on environmental condition. The SnRK1 pathway is activated in response to stress such as starvation and regulates S/bZIP transcription factors to mediate starvation response.

1.6.5 Sugar sensors

The adjustment of primary metabolism to the availability of nutrients and environmental factors is the first important factor to maintain cell homeostasis. Sugars act as a nutrient source and signalling molecule to regulate gene expression toward plant growth (Eveland and Jackson, 2011). If the sugar level drops, genes involved in photosynthesis, nitrogen metabolism, and carbohydrate export will be up-regulated in order to increase the sugar level. Alternatively, sugar abundance will give the plant energy to go through fundamental processes required for its growth, such as a typical organ activity. Plants contain both intracellular and extracellular sugar sensors. G-protein signalling 1 (RGS1) is a key plasma membrane protein that senses extracellular

sugar and responds to the changes in the level of sucrose, fructose, and glucose (Phan et al., 2013). One intracellular glucose sensor is metabolic enzymes HEXOKINASE 1 (HXK1) (Sakr et al., 2018). The HXK1 role as a glucose sensor was characterized by *Arabidopsis* mutant *glucose insensitive 2* (*gin2*). The HXK1 role as a glucose sensor was characterized by *Arabidopsis mutant glucose insensitive 2* (*gin2*). HXK acts as a member of the metabolic pathway that is involved in catalysing the first step of glycolysis and as a hexose sensor to send the signals established on the availability of sugar. In seed development processes, HXK was suggested to regulate metabolism and growth (Moore et al., 2003). The fructose sensor is FRUCTOSE 1-6-BISPHOSPHATASE/ FRUCTOSE-INSENSITIVE 1 (FBP/FIS1) and sucrose transporter (SUT2/SUC3) was proposed to be a sucrose sensor (Sakr et al., 2018). There is now plenty of evidence that TREHALOSE-6-PHOSPHATE (T6P) is an important signal and a proxy for sucrose. T6P levels are determined by sucrose concentration levels and therefore, this makes it possible for it to be a fundamental signal metabolite (Carillo et al., 2013). A large increase in sucrose level results in an increase in T6P and represses SnrK1 and stimulates growth (Lawlor and Paul, 2014). T6P contributes to maintain the level of sucrose, depending on several factors (Sakr et al., 2018). In general, sucrose (a transported disaccharide) tends to be involved in differentiation and maturation, but hexoses (monosaccharides) are associated with signalling that promotes cell proliferation and organ growth (Koch, 2004). Sucrose metabolic enzymes, together with their transporters, act to establish sugar gradients. Previous studies have shown that differential glucose concentration is related to an increase in mitotic activity; this suggests there is a link between cell cycle activity and hexoses (Eveland and Jackson, 2011).

1.6.6 Sugar signalling and hormones

Sugar signalling cross-talks with hormonal signalling to regulate key biological processes of plant growth. The interaction between sugars and hormones underlies the central regulatory role of sugar signalling in the growth, development, and physiology of plants. Plants can adjust the carbohydrate level and by doing so, integrate the influence of environmental conditions with internal developmental processes that are controlled directly by hormones (Ciereszko, 2018). Studies have revealed a deep connection between sugar and hormones ethylene and ABA, signals regulating processes such as seed germination and growth. Plants with mutations of ABA

synthesis and/or ethylene signalling, will exhibit distorted sugar response phenotypes during seedling development (Eveland and Jackson, 2011). ABA deficient mutant (*aba2*, *aba3*) and ABA-insensitive (*abi4*) exhibit glucose insensitivity (Arenas-Huertero et al., 2000). Another hormone that cooperates with sugar signalling is auxin. The first connection between auxin and sugars came from *gin2*/*hxk* mutant plant that are insensitive to auxin induction of cell proliferation and root development and *gin2* is hypersensitive to cytokinin (Mishra et al., 2009). Accordingly, auxin resistance-mutant (*tir1*, *axr1*, and *axr2*) are also insensitive to the high level of glucose (Dharmasiri et al., 2005; Moore et al., 2003; Rolland et al., 2006). Glucose regulates several auxin related genes such as *TIR1* and *YUCCA*, however some of the genes require the presence of both auxin and glucose to be able to respond to glucose (Mishra et al., 2009). However, as the experiments described in this thesis will show, many aspects of the sugar signalling pathway and how it relates to hormone signalling are yet to be revealed.

1.7. Aims and objectives

The overall aim of this thesis is to understand how leaves develop from the SAM under the control of light. The aims of each individual chapter are described below.

My aim in chapter 3 is to understand how the absence or presence of light could switch the meristem from an arrested state to an active one, primarily by monitoring the underlying changes in transcript level of readouts of both presumed regulators and some of main biological processes that take place in both states.

My aim in chapter 4 is to understand how meristematic growth arrest takes place in the dark and the role energy signalling plays in growth and specifically if the "starvation state in the dark" can be confirmed and is important for growth arrest. An associated aim is to find out if the transcript responses to direct exposure of the SAM to sucrose mimic, as a whole or in part, the genetic responses to light re-exposure observed in the chapter 3.

In the last part of the chapter 4, I have asked whether the energy limitation is a determinant of the extent of leaf growth under different irradiances of light.

My aim in chapter 5 is to understand the role of MAPK signalling pathways, for which previous evidence showed transcriptional control by light specifically in the shoot apex (López-Juez et al., 2008), in the regulation of meristematic activity.

Chapter 2

Materials and Methods

2. Chapter 2: Materials and Methods

2.1. Plant lines and sources

The information about the plant line used in this project together with the source pf seeds are provided in the table below.

Table 2. 1 The table below gives the details of all plant line and their source.

Plant line	Source
Wild-type Arabidopsis (Col)	Nottingham Arabidopsis Stock Centre
axr1-12	Nottingham Arabidopsis Stock Centre
cop1-4	From X. Deng, Univ. California [a]
CYCLINB1;1:Dbox-GUS	From A. Colón-Carmona, Salk Institute [b]
DR5:GUS	From T. Ulmasov, Univ. Missouri [c]
E2fa	From Zoltan Magyar, Univ. Szeged [d]
E2fb	From Zoltan Magyar, Univ. Szeged [e]
Raptor1.1	From L. Bogre, Univ. Royal Holloway [f]
Raptor2.1	From L. Bogre, Univ. Royal Holloway [g]
S6K1	From L. Bogre, Univ. Royal Holloway [h]
S6K2	From L. Bogre, Univ. Royal Holloway [j]
MKK7	From R. Doczi, Univ. Szeged [k]

[a] (Deng et al., 1991)
[b] (Colón-Carmona et al., 1999)
[c] (Ulmasov et al., 1997)
[d] (Magyar et al., 2012)
[e] (Magyar et al., 2012)
[f] (Henriques et al., 2014)
[g] (Henriques et al., 2014)
[h] (Henriques et al., 2013)
[j] (Henriques et al., 2013)
[k] (Dóczi et al., 2019)

2.2. Arabidopsis sterile culture

2.2.1 Media preparation

Murashige and Skoog solid medium (MS) (Duchefa, solidified with 0.8% plant agar) (pH to 5.7 adjusted with KOH) with Gamborg's vitamins (Duchefa) was prepared (agar was not used for the liquid medium). The medium was autoclaved and poured (in a laminar flow hood) about 35-40 ml into each 9 cm round plate. The plates were left in the hood to solidify and to get rid of any moisture with no lids. After the plates were completely solid the lids were closed, and they were carefully transferred to a sterile plastic bag. They were kept upside down in the bag to reduce evaporation and avoid any water condensation that could lead to infections.

Table 2. 2 Reagents used for the media preparation.

Reagents	grams/Litre
Phyto-agar (Duchefa)	8
MS salts with Gamborg's vitamins (Duchefa)	4.4
MES	0.5
Sucrose	10
dH_2O	1L

2.2.2 Liquid media preparation

To make the liquid media, all the above reagents were added except Phyto-agar to prevent solidification of medium, all the rest were added exactly in the same amount.

2.2.3 Seed sterilization and sowing

Arabidopsis seeds (1000 seeds = 20-25 mg) up to 1000, were transferred to 1.5ml centrifuge tube, and kept in (95-100%) ethanol for 1 minute. Ethanol was removed and the seeds were resuspended in the diluted household bleach (1:2 water) and incubated for 10 minutes, with infrequent mixing with pipette, then the bleach removed. The seeds were washed with 1ml of sterile water 5 times and

then 500 µl of 0.1% agar (autoclaved) was added. Seeds were distributed evenly in the plate by pipetting about 100 µl of agar containing seeds. The plates were left to dry in the hood with their lids left open for about 20-30 minutes. The lids of petri plates were sealed with parafilm and then transferred to a fridge at 4°C for three days to synchronise and boost the germination. After three days of cold treatment, the plates were transferred to a Percival I-30 growth chamber, 20 to 22°C, and 100 µmol m^{-2} s^{-1} of continuous fluorescent light. This was considered day 0. Then depending on experiment seedlings were treated with light or dark. Once they were 7 days old, the seedlings were transferred to either dark collected and rapidly transferred to RNAlater (Sigma-Aldrich) in six well plate and kept at 4°C until dissection.

2.2.4 Light cabinets and growth conditions

The same light cabinet was used for all experiments; however, the light intensity was changed for light quantity experiments. light cabinets contained fluorescent white light (colour 840) lamps with ~100 µmol m^{-2} s^{-1} (+/-10 %) and a constant temperature of 21 °C. For experiments which required seedlings to stay in dark, a dark cabinet was used, at identical temperature, for plates which were also covered in aluminium foil and held in opaque boxes.

2.2.5 Growth conditions and soil preparation

The soil was prepared using 1-part perlite, 6-parts Levington M3, and 6-parts John Innes number 3. The incubator-grown seedlings on plates were transferred to soil trays. The tray was placed in a light incubator to be able to monitor the light quantity. A lid was placed on the tray to cover the seedlings for a few days and the lid was opened a few times for a short period of time for acclimatization.

2.2.6 Seedling mounting

To observe the seedlings under microscope, they were mounted with a few drops of Hoyer's solution (80 g chloral hydrate, 10 ml glycerol in 30 ml water) on microscope slides to make them transparent. The seedlings were placed on the slide one by one and cotyledons were opened to expose primordia under the stereomicroscope using two fine forceps. A cover slip was placed over the slide.

2.2.7 Images of seedlings

The leaf primordia images were taken using Optiphot 2 DIC microscope (Nikon) equipped with a Nikon DXM1200 or a Micropublisher 5 camera.

2.3. qRT-PCR steps: seedling dissection

The seedlings required for RT-PCR gene expression analysis study were harvested with forceps and stored into six well plate consisting of RNAlater solution (To stabilize RNA for one week) at 4ºC for not more than one week to obtain good quality RNA. On the day of dissection, they were kept on ice and, using two fine forceps, the SAM region and very early leaf primordia (the tissue between the cotyledon petioles) were harvested together in triplicates, each biological replicate containing 200 seedlings under a stereomicroscope (Nikon SMZ-2T) on a clean microscope slide. The slide was also covered by RNAlater solution and few seedlings were dissected at a time. The dissected seedlings were transferred to a 1.5ml microcentrifuge tube before flash-freezing in liquid nitrogen in order to avoid RNA degradation. Samples were stored at -80 ºC.

2.3.1 Grinding of material

The harvested seedlings were grounded by adding N_2 to 1.5ml microcentrifuge tube containing samples and grinded by using a clean and chilled plastic pestle using a mechanical drill until a fine powder was apparent.

2.3.2 Total RNA extraction

The total RNA was extracted using the Plant RNA mini spin kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The manufacturer's protocol was carried out with some modifications. The frozen tissue collected previously

was ground. A mixture of 400 µl of RA1 and β-mercaptoethanol (β-ME) was added. Then the sample was spun at 16000 g for 2 minutes. 400µl of ethanol was added to the filtrate without disturbing the pellet. Then the centrifugation steps were used according to the protocol. The rDNase reaction mixture was applied to the nucleic acid to eliminate any traces of genomic DNA and when three washes were done, the RNA was eluted in 60 µl RNase-free water. The RNA concentration in each sample was determined using nanodrop. The RNA samples were stored at -80°C.

2.3.3 Quantification of RNA

The RNA concentration available in the samples was quantified using the ND1000 spectrophotometer. The device can measure RNA concentration from 1µl sample at absorbance of 260 and 280 nm. It will automatically convert the RNA absorbance at 260 nm to give the true estimation of RNA concentration in ng/µl. In addition, the ratio of $A_{260/280}$ can be used to assess the purity of RNA. A high-quality RNA (protein free) will show a ratio of $A_{260/280}$ of 1.9 to 2.5. RNA samples were stored at -80ºC.

2.3.4 Electrophoresis Gel Preparation for RNA quality check

The gel electrophoreses method was used to separate macromolecules such as RNA according to their fragment size and charge. The quality of RNA was checked using electrophoresed in a 1.2% agarose gel. To make the gel, 1.44g agarose powder was added to 120ml Tris/Borate/EDTA (TBE). TBE and agarose were mixed and microwaved until the agarose powder melted completely and then cooled down, 6µl Ethidium bromide (EtBr, 1 mg/ml) was added to the mixture in the hood and mixed. Gel was poured into a prepared tray with inserted comb and left until it solidified. 6µl of DNA ladder (10Kb DNA ladder) diluted with 6X blue/green dye was loaded alongside samples. A required amount of sample was added to the water and 6X loading dye and then transferred to the gel. Loading dye was used to make the sample visible to the eyes and also weigh down the sample in each well. The gel was placed inside an electrophoresis tank filled with TBE buffer and ran at 90 volts voltage for 40 minutes. After completion of electrophoresis, the samples were visualised using a UV camera to check if

discrete bands corresponding to those of ribosomal RNA were observed, as evidence of absence of RNA degradation.

Table 2. 3 Preparation of Gel electrophoresis.

10x TBE		Gel		Loading	
Tris Base	242 g	1x TBE	120 ml	Ladder	6 µl
Glacial acetic acid	57.1 ml	Agarose (1.2%)	1.4 g	RNA	1µg
0.5M EDTA (pH 8.0)	100 ml	EtBr (10 mg/ml)	6 µl	Loading dye	6 µl
dH_2O (total)	1000 ml			dH_2O (total)	15 µl

2.3.5 Reverse transcription cDNA synthesis

RNA was reverse-transcribed into cDNA by Thermo scientific revertAid first strand cDNA synthesis kit. The protocol was followed according to the kit manual, summarized in the tables bellow. The following reagents were added to an RNase-free tube:

RNA	1 µg
10X Reaction buffer with $MgCl_2$	1 µl
DNase I	1 µl
H_2O, nuclease-free	To 10 µl

This was incubated at 37ºC for 30 minutes, 1µl of 50 mM EDTA was added and incubated again at 65ºC for 10 minutes. The following reagents were added into a sterile tube on ice:

Template RNA	0.1 ng- 5µg
primer	2 µl
DNase I	1 µl
H_2O, nuclease-free	To 12 µl
Total volume	12 µl

The following components were added:

10X Reaction buffer	4 µl
RNase inhibitor (20U/µL)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuL V RT (200 U/µL)	1 µl
Total volume	20 µl

The tubes were mixed gently, centrifuged briefly and then incubated for 60 minutes at 42ºC and the reaction was terminated by heating at 70ºC for 5 minutes. The samples were stored at -80ºC.

2.3.6 Primer design strategy and Real-Time Polymerase Chain Reaction (qRT-PCR)

The primers to be used in the qRT-PCR were designed as follows: The AGI code for each gene of interes was obtained from TAIR (www.arabidopsis.org), , then Quantprime website (Arvidsson et al., 2008) was used to design the primers using those codes. The organism chosen was *Arabidopsis thaliana* and annotation chosen was TAIR release 10 (genome+) (splice variants). The quantification protocol was SYBR Green real-time qRT-PCR (accept splice variant hits). Once these options were chosen, the new project was created. Upon entering the AGI code from TAIR the primer sequence was obtained.

2.3.7 Primer preparation

The primer for each gene was hydrated using the required amount of water, to make the concentration 100µM stock. 10µl of forward primer was mixed in a new tube with 90µl of distilled water to have 10mM concentration of primer. A no template control as a point of comparison for the data was required. One housekeeping gene, *UBQ10*, that is constitutively expressed in *Arabidopsis* was used as the control.

2.3.8 Quantitative polymerase chain reaction (qRT-PCR)

Quantitative polymerase chain reaction (qRT-PCR) is a powerful method that is used for quantification of relative or absolute level of gene expression. qPCR is a specific and sensitive technique that measures the amount of DNA by the use of fluorescent dye after each cycle. RNA was extracted from 200 seedlings per

biological replicate and cDNA was made using the messenger RNA (mRNA) in extracted RNA. qRT-PCR was used to find the relative expression of interesting genes in the *Arabidopsis* meristem. In this technique a highly sensitive fluorescence dye termed SYGreen, which binds to the minor grove of double stranded DNA molecules and then emits a fluorescent signal was used. This allows the amplification process to be measured in the real time. SYGreen Mix Lo-ROX (2x qPCRBIO, SyGreen Mix Lo-ROX, PCRBIOSYSTEMS, UK) is very specific in binding to double stranded DNA (dsDNA) therefore, the amount of DNA target sequence is inversely, logarithmically proportional to the timing of the cycle at which emitted fluoresce signal reaches the threshold level. The cDNA concentration of the target gene can be calculated by normalization with the control housekeeping gene.

2.3.9 Standard preparation

A 1:100 fold dilution of the original stock of cDNA (made in two 1:10 steps) and RNase-free water were prepared. 2 µl of diluted cDNA from each sample and 18 µl of master-mix (Primer, RNase-free water, and SYGreen mix) added to the reaction tube (Applied Biosystems) in triplicate. No template control (RNase-free water and master-mix) was also added to check for any contamination in the master-mix. The following reaction volumes were prepared for the master-mix.

Table 2. 4 The reagents required to prepare master mix for a qRT-PCR run.

Reagents	**Volume (µl)**
SyGreen Mix	10 µl
RNase-free Water (Promega)	6.4 µl
Forward 10mM primer	0.8 µl
Reverse 10mM primer	0.8 µl
Template (DNA/ cDNA)	2 µl
Total Volume	18 µl

The prepared tubes containing samples were closed and placed in the Rotor-gene qRT-PCR Rotor-Gene instrument (Qiagen). The tubes were incubated at 95ºC for 2 minutes to denature the sample and activate the polymerase. The number of cycles (35 – 40) was adjusted based on the cycle of signal. Melt curve analysis

following PCR cycles was performed at the end of qRT-PCR in the same reaction tubes. Melt curve was run to confirm the samples amplified a single, specific sequence. The program used to run qRT-PCR is provided in the table below.

Table 2. 5 qRT-PCR run program.

qRT-PCR Program		
Hold	95°C 2'	
Cycling	95°C 5''	rep 35
	60°C 20''	
Melt	72 °C - 95°C	
Channels	Green source: 470 gain: 7-10 detector: 510	

2.3.10 Data Analysis

Results of each gene of interest expression was normalised to the expression levels of a house keeping gene (*UBIQUITIN10, AT4G05320*). The housekeeping gene known as internal control and most important criteria to choose this gene is that its expression level should remain constant throughout all the samples. *UBQ10* was stable with no variation in the expression under different conditions (Different light quantity and dark), as judged from its qRT-PCR threshold cycle from identical amounts of cDNA accross very different sample types. The mean of the two technical replicates each of three biological replicates (six samples) expression value of the gene of interest was normalized, relative to housekeeping gene (calculated as $E^{-Ct_{test}}/E^{-Ct_{house\ keeping}}$, where Ct is takeoff value and E is efficiency). Evaluation of the possible circadian behavior of genes studied in this project was by using the LL_LLHC data series available at the Diurnal tool (http://diurnal.mocklerlab.org/).

2.4. CyclinB1;1::DB-GUS Assay

The fusion of the promoter and destruction box of CYCB1;1 to GUS (CYCB1;1::DB-GUS) was used as a mitotic reporter to visualize the cells going through cell proliferation. The outcome of the enzymatic reaction was a blue coloured cell that shows they were undergoing mitosis. The principle is that of Colón-Carmona et al. (1999) and Donnelly et al. (1999). The detailed method was adapted from the lab of Jim Murray, University of Cardiff.

2.4.1 Harvesting of sample

Seedlings were collected and transferred into the centrifuge tubes containing 1ml of ice-cold 90% acetone and incubated for 15 minutes in +4°C. Then samples were washed two times with sodium phosphate buffer (pH 7). The 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) reaction solution was added (~1 ml) after the last sodium phosphate wash. The X-Gluc substrate used was a sodium salt.

2.4.2 Vacuum infiltration and incubation

After addition of X-Gluc reaction solution, a vacuum infiltration was performed. An ordinary speed-vacuum instrument (DNA-star) was used where a vacuum of 1⁄2 atm pressure was used, for 10 minutes, as recommended by Donnelly et al, (1999). The samples were incubated in the dark at 37 °C for ~15-21 hours.

Table 2. 6 The reactions used for preparation of X-Gluc solution.

J.Murray Lab (Cardiff University)		
Chemical Reagent	**Stock**	**Working**
X-Gluc	50mg/ml	0.3mg/ml
Sodium Phosphate Buffer	0.2M	100mM
Potassium Ferricyanide	0.1M	0.5mM
Potassium Ferrocyanide	0.1M	0.5mM
Tween 20	10%	0.1%

2.4.3 Post (X-Gluc) incubation protocol

After ~21 hours of incubation the reaction was stopped by removal of X-Gluc reaction solution and washed with 70% ethanol.

2.4.4 Mounting of samples

Seedlings were mounted as quickly as possible and left for no longer than two weeks in the 1.5 ml microfuge tube containing of Hoyer's solution. Seedlings were dissected under a stereo microscope by fine tweezers and mounted on a microscope slide with Hoyer's solution. Cover slip was placed and sealed with clear nail polish for long term use.

2.4.5 Quantitation

Quantitation of the blue precipitate was carried out using the ImageJ software - 'http://imagej.nih.gov/ij/'. Quantitation was based on percentage GUS per leaf area:

$$\%GUS = \frac{Area^{GUS}}{Area^{Leaf}}$$

2.5. Scanning electron microscopy

Seedling were grown in different conditions such as continuous light or dark or grown on liquid medium as individually indicated, and then collected and 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).

2.6. CO_2-deprivation experiment

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). The 5 g of soda lime was thousands of folds in excess of the amount sufficient to quench the amount of CO_2 in the atmosphere inside the bag.

This was experimentally confirmed by generating additional CO_2 through the addition of 5 g of $NaHCO_3$ and drops of 10M HCl: the bags inflated but soda lime-containing bags were rapidly deflated.

Chapter 3

Roles of light and hormones in leaf growth

3. Chapter 3: Roles of light and hormones in leaf growth

3.1. Introduction

3.1.1 Light

Light is a source of energy as well as a signal received by plants and drives plant growth and development. It has been shown that light is required for leaf initiation in tomato and that this is mediated by the plant hormones auxin and cytokinin (Yoshida et al., 2011). A previous study observed the transcriptional response changes in the cell cycle (Fig. 3.1 A) and ribosomal (Fig. 3.1B) protein genes during dark to light transfer in the shoot meristem and cotyledon. The heat map shows that both ribosomal and cell cycle genes are highly up-regulated and upon transfer to light, the transcriptional responses are increased within 1 hour in the shoot meristem (López-Juez et al., 2008).

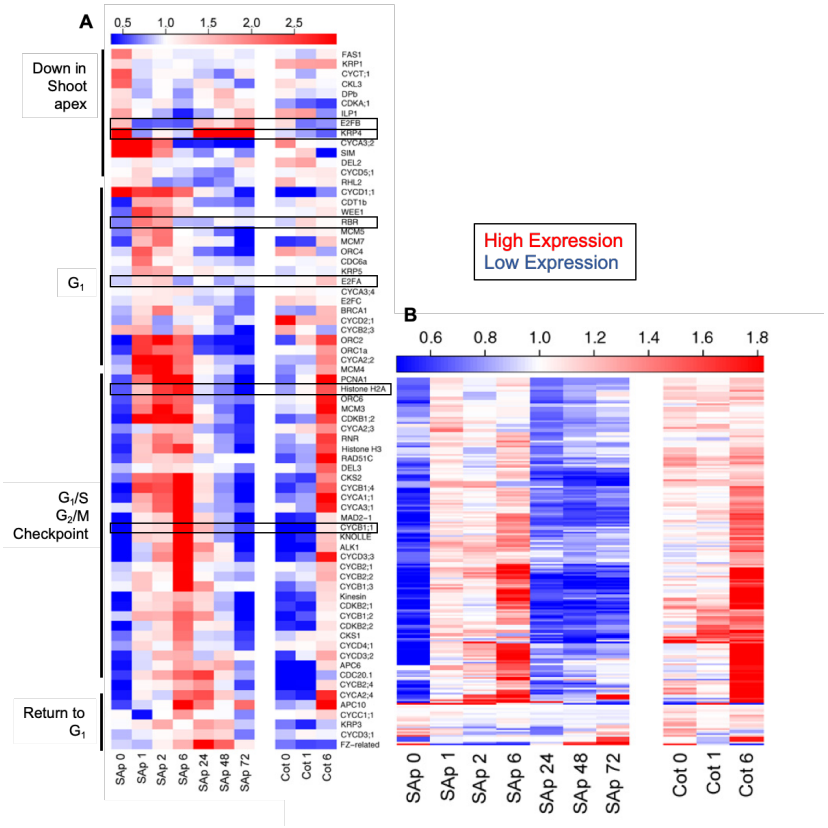

Figure 3. 1 The transcriptional responses in the SAM plus leaf primordia (SAp) and cotyledon (Cot) activated upon first light exposure after 3 days in the dark is hypothesized to be similar to the light grown primordia arrested by transfer to dark and growth upon re-exposure to light. The x axis shows the time, 0 represent the expression in the seedlings germinated in dark for 3 days and further hours shows the time course when seedlings were transferred to the light (SAp 0, 1, 2, 6, 24, 48, 72 hours). **A**, groups of cell cycle genes show initial lower expression (negative), early up-regulation in light (positive) and coordinated peak expression at around 6 hours compared to the time course average. **B**, Robust, unexpected, highly coordinated up-regulation of translation activity in the shoot apex and cotyledons, peaking 6 hours after transfer to light. The heat map is from (López-Juez et al., 2008).

3.1.2 Leaf initiation and auxin

Auxin acts as a central regulator of growth and organ initiation. Auxin maxima is generated at the point of primordia initiation in the SAM and its transport is facilitated by PIN1 auxin transporter. In the event of auxin transport inhibition or *pin1* mutation, organ initiation is inhibited, but providing meristem with exogenous auxin is enough to trigger organ initiation (Pinon et al., 2012). Auxin plays a vital role in triggering vascular cell differentiation and induces vascular strand formation (Scarpella et al., 2010). Initiation of leaf vascular development requires tissue specific local auxin biosynthesis (Nishimura et al., 2014), the seedlings with auxin biosynthesis mutant (*tar2*), have fewer veins that are more widely spread out and frequently disorganized and disjointed (Kneuper et al., 2017). Auxin transport through PIN1 in the epidermis has provided a mechanism that directs auxin to the vein development (Petrášek and Friml, 2009). Thus, development of both leaf and vasculature requires directional auxin transport and PIN1 regulating factors. The auxin biosynthesis gene *TAR2* is expressed in the sites that make veins and *TAA1* is expressed in pre-provascular cells at the regions where vein development has not yet happened, and reduced when PIN1 accumulates in provascular cells (Kneuper et al., 2017). TAA1 and its close homolog TAR2 are essential for maintaining proper local auxin levels in apical hooks and specific cell-types of root and additionally promoted by ethylene. Production of auxin by TAA1 is necessary for hormonal cross talk, analysis of TAA1 showed presence of a connection between local auxin biosynthesis, specific organ effect by ethylene and development of organ (Stepanova et al., 2008).

3.1.3 Auxin and cytokinin cross talk

Hormones are involved in every aspect of plant biology. Plant growth and development is regulated by extensive crosstalk among different hormones. A fine coordination and balance between cytokinin responses and regulation of local auxin stabilizes leaf initiation and development. Auxin has a positive role in leaf initiation, however the role of cytokinin is more complex (Czesnick and Lenhard, 2015). Hormonal heat maps of gene-expression signatures reveal unexpectedly large “swings” in the SAM. The expression levels of genes regulated by auxin show early lower expression (negative), early up-regulation (positive) and coordinated peak expression at around 6 hours of light compared to the time course average. Interestingly, a group of auxin induced genes

was highly expressed in the SAM after 24 to 72h in the light, during the time of leaf primordia development, but had transiently declined immediately after transfer to light. Contrary to the auxin responsive genes, highly coordinated up-regulation of cytokinin regulated genes activity in the shoot apex and cotyledons was observed shortly after transfer to the light (Fig. 3.2). Light for 24h brings a reduction in auxin signalling pattern shown by auxin reporter (DR5:GUS), a strong induction in the tips of the primordia and a disappearance elsewhere but in veins (Fig 3.3). Cytokinin responsive genes were induced with several reaching their highest response at 6 h, at the time when, cell cycle genes expression reached maximum (Fig 3.2).

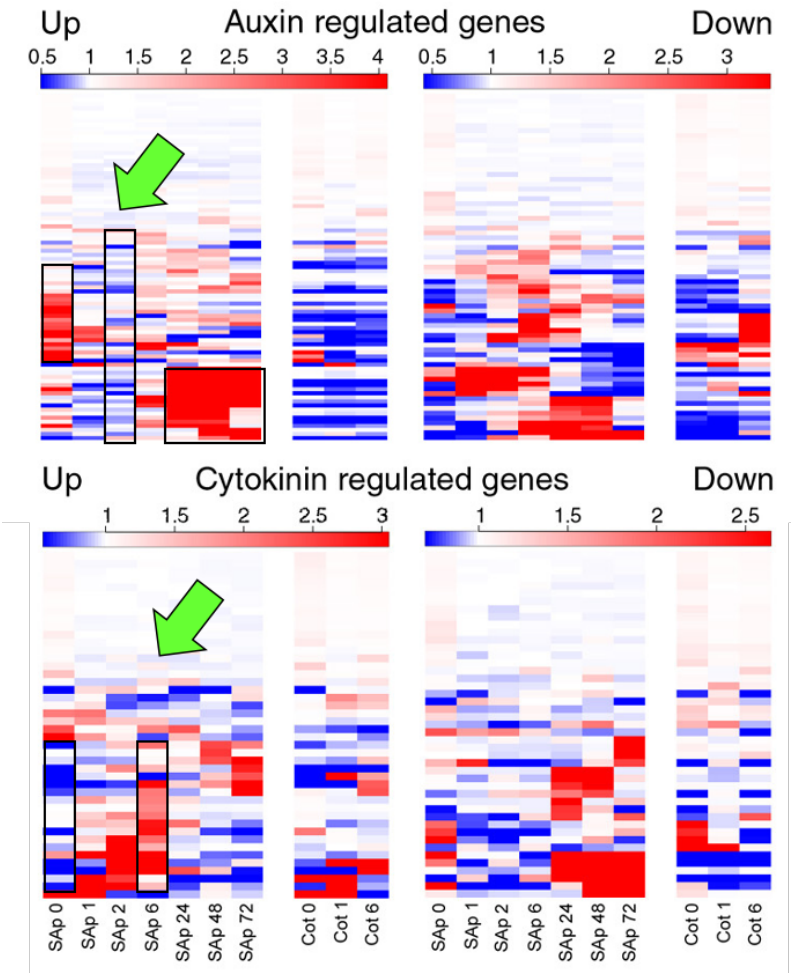

Figure 3. 2 Hormonal heat maps of gene-expression signatures reveal unexpectedly large "swings" in the SAM. The transcriptional responses in the SAM plus leaf primordia (SAp) and cotyledon (Cot) activated upon first light exposure after 3 days in the dark. The heat map displays expression level of genes previously classified as upregulated or downregulated by auxin and cytokinin. The scale of expression (ratio to mean across samples) is presented above the heat maps. The x axis shows the time, 0 represent the expression in the seedlings germinated in dark for 3 days and the number shows the hours after transfer to light (SAp 0, 1, 2, 6, 24, 48, 72 hours). The heat map reveals a transient drop of auxin and surge in cytokinin responses in the shoot apex shortly after transfer to light, and is from (López-Juez et al., 2008).

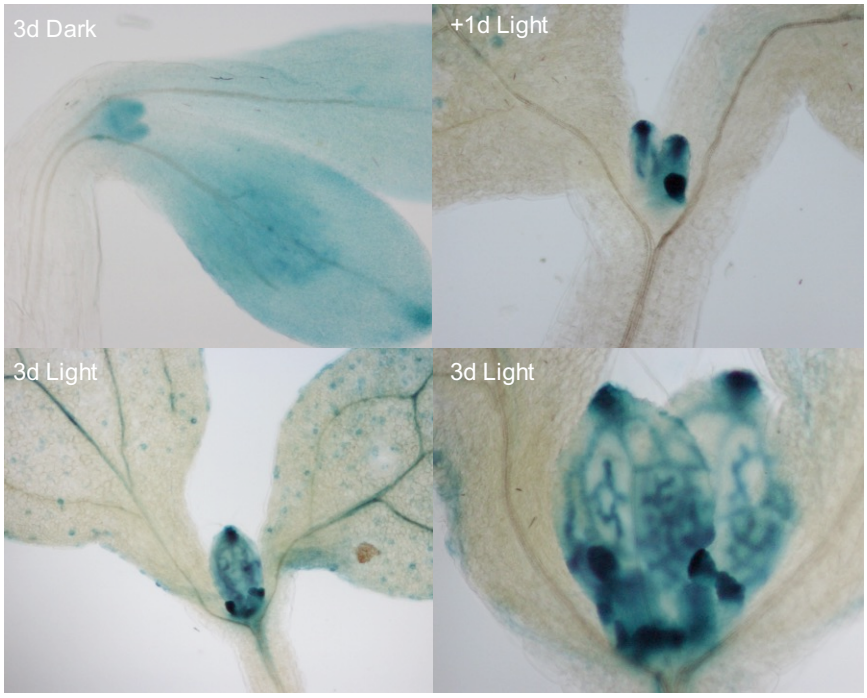

Figure 3. 3 The auxin signalling pattern shown by auxin reporter (DR5:GUS) in the seedlings grown in dark for 3 days, transferred to light for 3 days.The experiment carried out by Mr. Rajat Yadav Dr. Lopez-Juez.

3.1.4 Constitutive Photomorphogenic 1

COP1 is a RING E3 Ubiquitin ligase that functions as a main repressor of light signalling pathway in the absence of light and central modulator of growth (Lau and Deng, 2012). *COP1* regulates many other roles downstream of light signalling pathway including circadian rhythm, flowering and shade avoidance responses (Crocco et al., 2010). COP1 is enriched in the nucleus in the dark to act as a suppressor of photomorphogenesis and upon transition to light will exit from the nucleus by a specific nucleocytoplasmic partitioning (Von Arnim and Deng, 1994). COP1 functions by targeting several photomorphogenesis promoting factors for degradation in the absence of light. Hence, COP1 mutated seedlings show a constitutively photomorphogenic development in the dark (Lian et al., 2017). However, when light is present, photoreceptors inhibit *COP1* function by its export from the nucleus and permit expression of *ELONGATED HYPOCOTYL 5* (*HY5*). HY5 activates accumulation of transcription factors involved in photomorphogenesis responses. Among the photoreceptors, phyA, phyB, and cry1 are involved in starting the pathway that regulates COP1 abundance in the nucleus. COP1 is involved in regulating flowering

time via phyB and mediates day length input to the oscillator. The photoreceptor UVR8 also interacts with COP1, once it receives UVB radiation, and this results in expression of *HY5*, therefore COP1 plays a positive, not a negative, role in the responses to UVB light. COP1 has a role in the input of light from photoreceptors to the clock, however its regulation is dependent on photoreceptors (Oakenfull and Davis, 2017).

3.1.5 Role of translation control in growth regulation

The process of translation is a high energy-consuming process, therefore it is essential to finely regulate this process to provide a mechanism to regulate production of protein in the cell (Bailey-Serres and Juntawong, 2012). Modulation at the translational level has fundamental role in regulating gene expression. The translational machinery includes ribosome and transfer RNA (tRNA). Ribosome is a large 'molecular system' that its main function is to translate information from mRNA into the protein. Ribosome in the eukaryotes is made of two subunits, 70-80 different ribosomal proteins (RPs) and four ribosomal RNA (rRNA) (Hummel et al., 2012). Ribosomal translation is defined of three steps: initiation, elongation, and termination (Schmitt et al., 2019). Loss of RPs in the seedlings of *Arabidopsis*, decrease shoot and root growth and cell proliferation (Degenhardt and Bonham-Smith, 2008). Regulation of translation machinery takes place largely, during the initiation phase by regulating TOR activity throughout plant growth and development (Sesma et al., 2017). There are some mechanisms affecting regulation of translation machinery such as stress. The translational machinery could be regulated through altering ribosome composition, such as phosphorylation of RPs. Light is a very important factor in regulating translation, the translation activity is higher in the light compared to the dark (Floris et al., 2013). Whole genome studies have revealed that transfer of plants from dark to light, experience a huge up-regulation in translation (Liu et al., 2013; López-Juez et al., 2008). Given that translation is an expensive process in the sense of energy cost, availability of sucrose is essential for this process. the seedlings in the sucrose limiting conditions, show in a general a reduction in translation (Merchante et al., 2017).

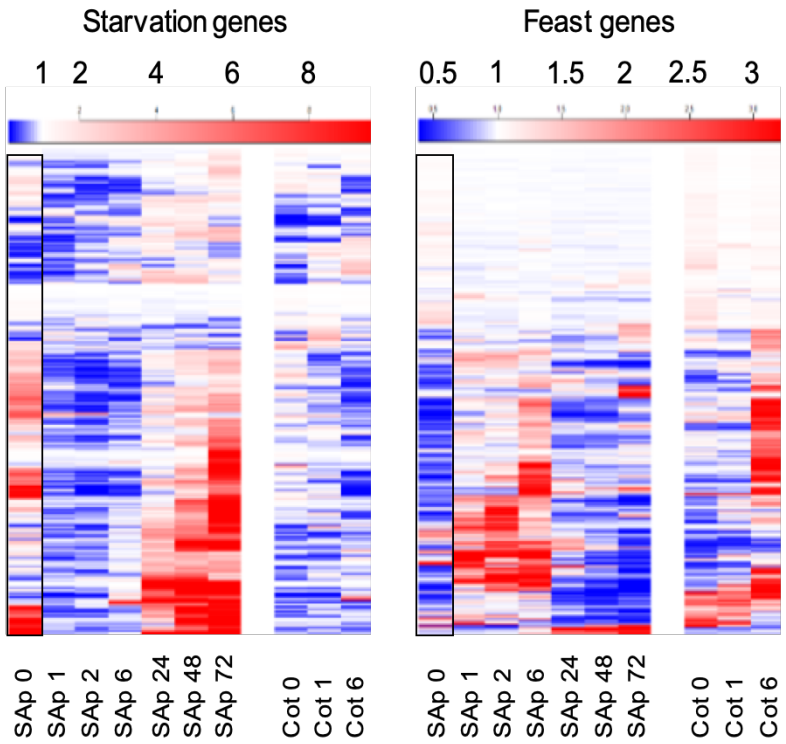

Figure 3. 4 The transcriptional responses in the SAM plus leaf primordia (SAp) and cotyledon (Cot) activated upon first light exposure after 3 days in the dark. The x axis shows the time, 0 represent the expression in the seedlings germinated in dark for 3 days and further hours shows the time course in the light (SAp 0, 1, 2, 6, 24, 48, 72 hours and Cot 0, 1, 6 hours). The transcript levels of the cluster of starvation genes in SAp show at time 0 the expression of these genes is high and upon transfer to light they are down-regulated compared to the time course average. The feast genes are responding in the opposite to starvation genes, they are down-regulated in the dark and up-regulated upon transfer to light compared to the time course average (López-Juez et al., 2008).

The analysis of light responses at the SAM showed a Cluster of genes identified as starvation genes transcript levels are elevated in the dark and responded very quickly and negatively to light (Fig 3.4). However, this was not observed in the cotyledon, since etiolated cotyledons are unlikely to be able photosynthetically active within 1 hour in the light.

3.2. Aims and objectives

My aim is to understand how absence or presence of light could switch the meristem from an arrested state to active, leading to growth. This can provide us with fundamental information to better understand the basic biological phenomenon underlying leaf development. In summary, the hypotheses reflect the transcriptional response changes observed in the dataset generated by López-Juez (2008) in the SAM plus first leaf primordia.

Hypothesis 1:

The transcriptional response changes activated upon first light exposure after 3 days in the dark (etiolated state) is hypothesized to be comparable to the dark arrest and light reactivation of emerging first pair of leaf primordia.

Hypothesis 2:

A strong (but diffuse) auxin response is associated with leaf growth arrest. Auxin response removal upon light exposure and the upregulation of cytokinin response are necessary for leaf primordia initiation and growth.

Hypothesis 3:

Cell cycle transcripts that work in favour of growth, increase in the same pattern as cyclin B-reporting GUS expression. Expression of cell cycle signature genes response level are repressed in the dark and a dramatic increase once in light again.

Hypothesis 4:

Removal of photomorphogenesis repressors such as COP1 will mimic the hormonal and energy-related light responses in the dark.

Table 3. 1 The table below contains list of genes used in this thesis as the readout for biological processes.

Process	Gene	Arabidopsis Genome Initiative Code	Encoded product
Cell cycle entry block	*KRP4*	At2g32710	Kip-related protein4 is a CDK inhibitor, negatively regulates the transfer from G1 to S phase[a] and is quickly affected by light.[b]
DNA synthesis (S phase)	*RNR2A*	At3g23580	Ribonucleoside-diphosphate reductase small chain A encodes a small subunit of nucleotide biosynthesis. Important for cell cycle progression and repair of DNA damage [c]. RNR2A is accumulated in the S phase of cell cycle in tobacco BY2 cells.[d]
	H2A	At1g51060	Histone 2A synthesis gene expression is during S phase and associate with mitotic cell division and DNA replication.[e]
Mitosis (M phase)	*CYCB1;1*	At4g37490	Cyclin B1;1 is expressed during mitotic cell cycle transition.[fg]
Ribosome biosynthesis	*RPS6*	At4g31700	40S ribosomal protein S6-1 is part of the 40S ribosomal unit and is modulated by S6K. The phosphorylation of RPS6 modulate the translation of ribosomal proteins, which increase translational capacity.[h]
	EBP1	At3g51800	ERBB-3 binding protein 1 expression is correlated with the ribosome biogenesis and sustains protein translation.[i,k]
Auxin response	*AUX1*	At2g38120	Auxin resistant1 is auxin influx transporter and is also involved in apical hook development.[l]
	IAA1	At4g14560	indole-3-acetic acid inducible1 is one of the first genes induced by auxin.[m]
	HAT2	At5g47370	Homeobox-Leu zipper protein2 is induced by auxin and regulated morphogenesis mediated by auxin.[n]
Auxin synthesis	*TAA1*	At1g70560	Trp aminotransferase of Arabidopsis 1 belongs to the IPA auxin biosynthesis pathway. IPA pathway is important in generating strong auxin gradient.[o]
	TAR2	At4g24670	Trp aminotransferase-related protein 2 is auxin biosynthesis gene.[p]

Cytokinin response	*ARR5*	At3g48100	Arabidopsis two-component response regulator5 is a cytokinin induced gene. [q] ARR5 is used as a readout of active cytokinin. it has a high specificity induction in response to cytokinin.
Epidermis	*GL2*	At1g79840	Glabra 2 is involved in development of epidermal cell identity such as trichome. [r]
Vasculature	*ATHB8*	At4g32880	Homeobox-leucine zipper protein8 is expressed in vasculature and promoting specification and differentiation of the vascular cells (procambial cells). [s]
	VND6	At5g62380	Vein deficient6 is involved in inducing differentiation of cells in xylem. [t]
Plastid/ mesophyll	*LHW*	At2g27230	Lonesome highway is required to maintain and determine number of vascular cell types. [u]
	GC1	At2g21280	Giant chloproplast1 is essential for plastid division. [v]
	ARC5	At3g19720	Accumulation and replication of chloroplasts5 is involved in accumulation and replication of chloroplasts5 is a chloroplast division protein. [w]
Starvation	*bZIP*	At5g49450	Basic Leu zipper1 is activated by low energy stress and involved in the transcriptional reprogramming in dark-induced starvation responses.
	TPS9	At1g23870	Trehalose-6-phosphatase/synthase9 is responding to sugar availability in plant

[a] (Zhao et al., 2012) [b] (López-Juez et al., 2008)
[c] (Huang et al., 1998) [d] (Philipps et al., 1995)
[e] (Yi et al., 2006)
[f] (Donnelly et al., 1999) g (Fung and Poon, 2005)
[h] (Henriques et al., 2013) [i] (Squatrito et al., 2004) [k] (Squatrito et al., 2006)
[j] (Horváth et al., 2006) l (Filip Vandenbussche et al., 2010)
[m] (Park et al., 2002) n (Sawa et al., 2002)
[o] (Stepanova et al., 2008) p (Ma et al., 2014)
[q] *reviewed by* (To and Kieber, 2008) [r] (Masucci et al., 1996)
[s](Miyashima et al., 2013) [t] (Fukuda, 2016)
[u] (Ohashi-Ito and Bergmann, 2007) [v] (Maple et al., 2004)
[w] (Glynn et al., 2009)

3.3. Results

3.3.1 Reduction of auxin sensitivity enhances the leaf initiation in the dark caused by cytokinin action

To test the growth control hypothesis with hormonal balance characteristic of auxin and cytokinin in the early stages of leaf initiation in the light (low auxin and high cytokinin response) and dark arrested seedlings (high auxin and low cytokinin response), an auxin partially insensitive mutant plant (*axr1*-12) was used to determine role of auxin in early developmental stages of leaf (Leyser et al., 1993). *axr1*-12 mutant was exposed to different concentrations of synthetic cytokinin 6-benzylaminopurine (BAP), on sucrose-containing plates. Seedlings grown in the continuous dark and one group in the continuous light, both for 5 and 7 days.

The *axr1*-12 seedlings showed an increase in leaf primordia area compared to the wildtype (WT) in the dark (Fig. 3.5). By increasing the concentration of BAP, the leaf area of *axr1*-12 seedlings in dark was increased. The difference between *axr1*-12 and WT leaf primordia size could be seen more clearly in 7-day dark (Fig. 3.5D). The dose-dependent increase in the leaf area shows the positive effect of cytokinin and of reduced response of auxin in the leaf primordia growth. The *axr1*-12 plant in the light with no BAP showed a reduction in growth compared to WT. By increasing concentration of BAP in the light, differences in the leaf primordia size between WT and *axr1*-12 mutant were reduced at 7 days in the light. A substantial increase in the leaf primordia size in BAP-treated *axr1*-12 seedlings could be observed compared to WT in 7dD (Fig. 3.5). Looking at the leaf area of seedlings in light shows that absence or presence of BAP highly impacted the extent of primordia growth (Fig 3.5A and B).

The difference between the *axr1*-12 and WT was significantly reduced by growing the *axr1*-12 seedlings in presence of 2 μM BAP in 5-day light (Fig. 3.16 D-F). However, comparing the two in the absence of BAP, showed a huge reduction in primordia size of *axr1*-12, which may indicate the hormonal crosstalk and balance is necessary for normal primordia cell proliferation and growth (Fig. 3.6). The present data (Fig. 3.5) are consistent with the hypothesis of auxin export from shoot meristem upon light exposure and the upregulation of cytokinin response are necessary for leaf primordia initiation and growth.

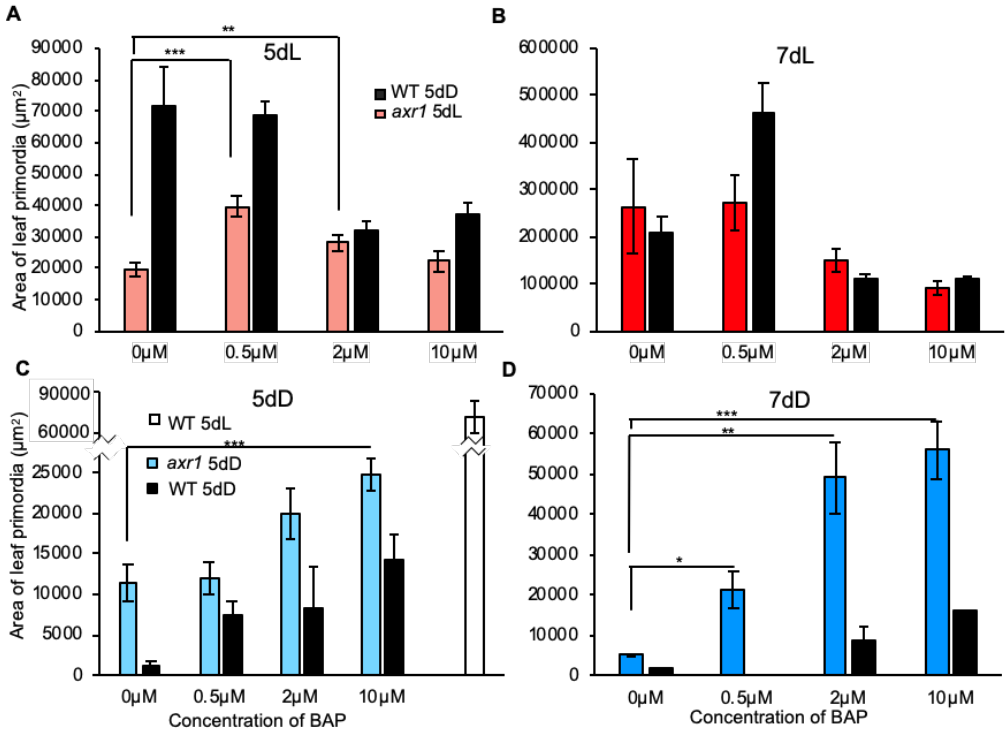

Figure 3. 5 Providing exogenous cytokinin to seedlings with reduced auxin sensitivity results in initiation of leaf development in the dark. The *axr1*-12 mutant and Columbia WT were grown in the continuous light or dark for 5 and 7 days, on 1% Suc-containing medium with or without BAP at the concentrations of 0, 0.5, 2, and 10 µM. The area of one of the first two leaf primordia was measured. Error bars show standard error of the mean (SEM). Number of samples:15-25. Asterisks represent the significance of differences between the WT and *axr1*-12: Anova followed by post-hoc Tukey tests was used and asterisks indicate level of significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

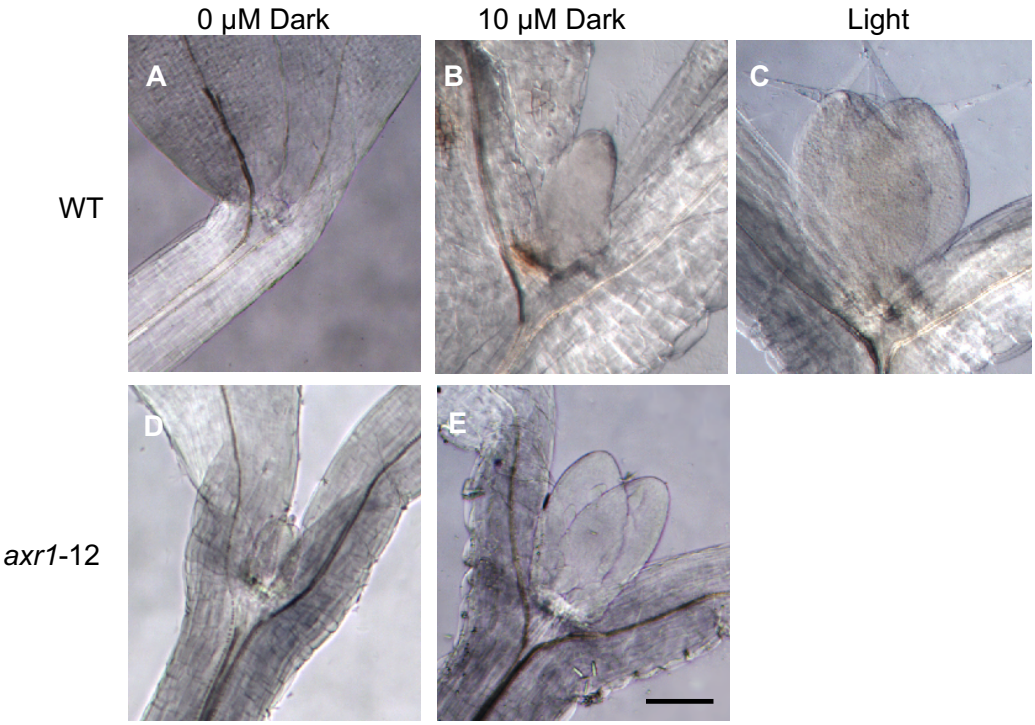

Figure 3. 6 The above seedlings represent development of primordia in presence and absence of BAP. A-C, WT.D-E, *axr1*-12 mutant. A and D, 5 days dark, 0 µM BAP. B and E, 5 days dark, 10 µM BAP. C, 5 d light 0 µM BAP. Scale bar: 200 µm.

3.3.2 Active cell proliferation is arrested in dark and re-initiated in light

Skotomorphogenesis is triggered in the dark where cell proliferation is inhibited; once in the light, growth and production of organs is activated by promoting cell proliferation and expansion (Li and Sheen, 2016). To follow growth through cell proliferation activity in young leaf primordia, seedlings were grown in standard white light (21ºC, continuous fluorescent white light (100 µmol m^{-2} s^{-1}) for 7 days, at this point the leaf primordia are about 0.5 mm in length and display a high mitotic reporter activity. After 7 days in the light, seedlings were transferred to the dark (21ºC) for 3 days and some continued in light. After 3 days in dark, they were transferred back to light and samples were collected according to a time course (1-24h). By using CYCB1;1::DB-GUS seedlings (Colón-Carmona et al., 1999; Donnelly et al., 1999) in the dark and light system, I aimed to measure the fraction of area of leaf in which cells are going through mitosis in the whole leaf area. The seedlings were grown on 1% sucrose plates. Looking at the level of cells going through mitosis after 3 days in the dark, the level of mitosis is nearly zero (Fig. 3.7). Upon transferring to light, a slow increase in the level of GUS

areas could be seen and the highest point was observed at 12h. A further 3 days in the light led to increase in the leaf size, which indicates that the leaf exited cell proliferation and entered cell expansion.

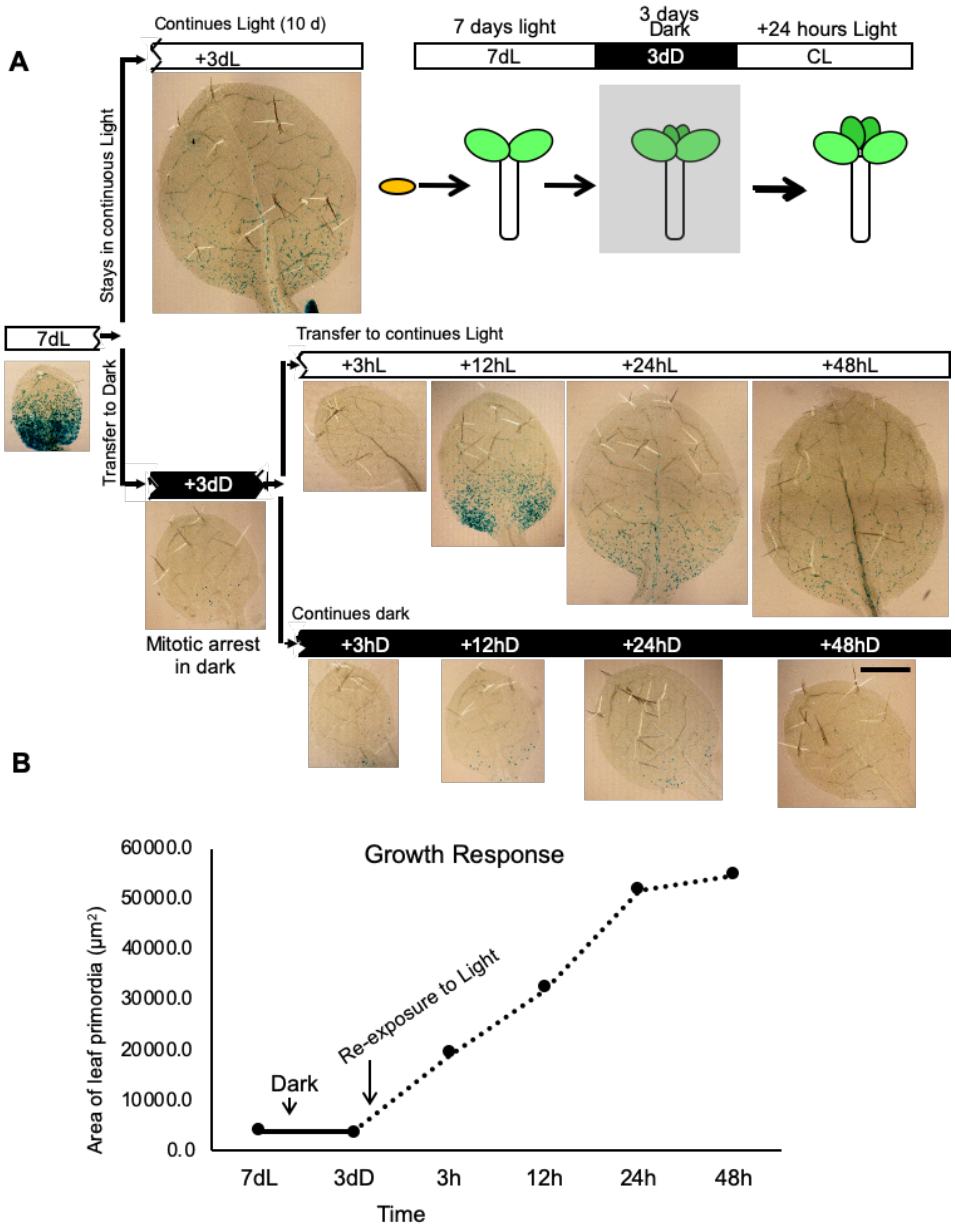

Figure 3. 7 The blue GUS stain in the cell indicates that cell is going through mitosis and therefore cell proliferation. **A,** 7-day old light grown CYCB1;1::DB-GUS expressing seedlings were transferred to continuous light (3 days), continuous dark and one group to dark for 3 days and later were transferred back to light and harvested at the time indicated above. The first leaf primordium is dissected after visualizing the GUS reporter. B, shows the growth response. Scale bar = 500 µm.

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

The SAM initiates the production of leaf primordia in the light, but this process is arrested in dark. The transcriptional responses in the shoot apex plus primordia and separately on cotyledon were studied. The microarray data produced from this study showed the transcriptional responses in the shoot meristem and cotyledon indicating strong auxin-signature and strong "starvation" gene expression responses in the dark (Fig. 3.2, Fig. 3.4). These signatures are quickly turned by light into strong cytokinin and strong "feast" gene expression. The intention of the experiments described below was to reproduce these responses in a new experimental set-up of growth arrest, by growing the seedlings in light until seedlings are large enough for manageable laboratory work involving dissections, and then arrest the growth by transferring them to dark for 3 days. The first gene expression pattern was followed in the whole seedling looking at the signatures of cell cycle, hormones, and starvation (Fig. 3.8). Upon 3 days of dark transfer, gene signatures of cell cycle were down-regulated and hormonal signatures were up-regulated. The mesophyll gene *GIANT CHLOROPLAST 1* (*GC1)* was down-regulated. The cell cycle genes are down-regulated during the dark arrest as expected and observed in the SAM in the microarray data (Fig. 3.1). On the other hand, the responses observed based on the microarray data from Lopez-Juez and colleagues, showed the cell cycle and starvation genes responses take place specifically in the SAM like *CYCB1* and *TPS9*, however in the whole seedling study, their expression is barely visible (López-Juez et al., 2008). Therefore, it is important to study gene expressions in the specific tissues rather than whole seedling study. This makes the dissection of specific tissue to study them valuable and crucial to learn new biology.

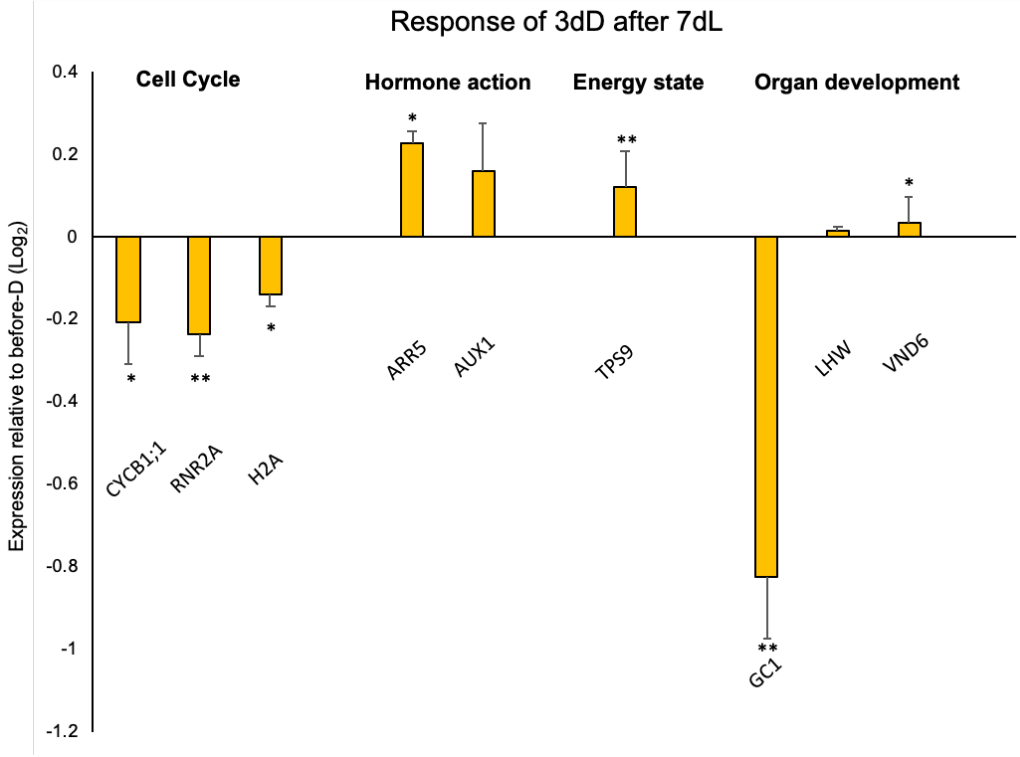

Figure 3. 8 The gene expression programme changes were monitored in light grown WT seedling for 7 days, transferred to 3 days in dark. Whole seedlings were collected after the dark arrest and gene expression quantification was produced using quantitative real time polymerase chain reaction (qRT-PCR). The values of each time point in the graphs represent averages of 3 biological replicates (15 seedlings for each replicate), each quantified through two technical replicates. T.test was used to compare with the time before dark transfer and asterisks indicate level of significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

After confirming the importance of using the right tissue to study gene expression and also confirming the system of dark arrest works, this was used to monitor the expression of genes representing DNA synthesis, mitosis, translational capacity, and organ development in the SAM and emerging leaf primordia. These responses were reviewed by growing seedlings in standard light incubator (21ºC, continuous fluorescent white light 100 µmol m^{-2} s^{-1}), for 7 days to allow leaf primordia to develop to a sufficient size for tissue dissection and further work. The time courses started from day 8, when the first seedlings were harvested. Then the rest of the seedlings were transferred to the dark (21ºC) for 3 days. This programme was used to observe whatever a comparable gene expression to that observed during skotomorphogenesis took place in 3-days dark arrest (Fig. 3.8). The results indicate a meristematic growth arrest while seedlings were transferred to the dark. This phenomenon was tested for similarities and differences in

responses to absence or presence of light in the shoot apices via gene expression analysis of signature genes.

3.3.4 Light up-regulates genes involved in DNA synthesis

As previously shown by Lopez-Juez et al. (2008), there is an immediate upregulation in the transcript levels of genes involved in S phase, mitosis, and translation in the dissected SAM. According to previous evidence and also the results of GUS quantification from CYCB1;1::DB:GUS seedling (Fig. 3.7), I hypothesized cell cycle transcript that are working in favour of growth will increase in the same pattern as GUS expression. Here the mitosis gene *CYCB1;1* shows a dramatic drop in new leaves of seedlings having been in dark for 3 days, but upon re-transferring to light, there is a gradual increase from time 0 to 3 hours and then substantial increase in transcript levels within 24 hours, reaching the same level of expression as originally after 8 days light earlier in the time course. The S phase signature genes used are *RIBONUCLEOTIDE REDUCTASE 2A* (*RNR2A*) and *HISTONE-2A* (*H2A*). As hypothesized, both genes' transcript level is repressed in dark and a dramatic increase once in light is seen again. The transcript levels reach the same level as during first light exposure (7day light time point) within 8 hours of light re-exposure (Fig. 3.9). The cells arrest *KIP-RELATED PROTEIN 4* (*KRP4*) which, as expected, had a high transcript level during meristematic growth arrest (3-day dark) and was down-regulated during transfer to light and re-activation of growth. However, it was gradually up-regulated over 24 hours in the light.

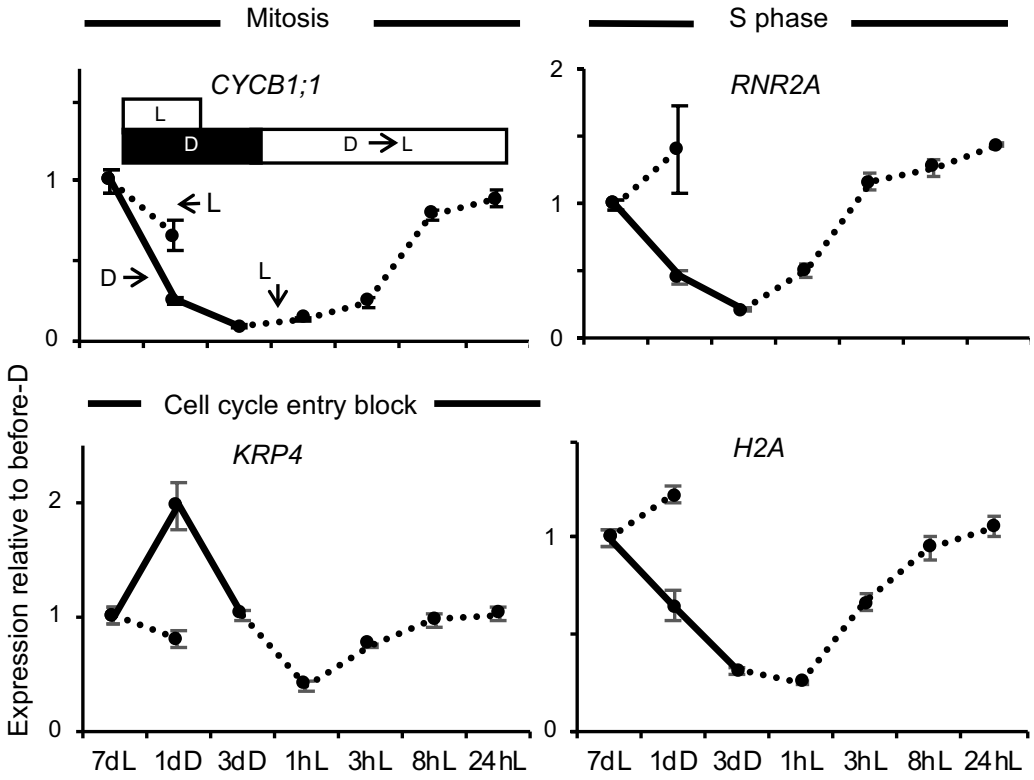

Figure 3. 9 Active cell proliferation in young leaf primordia can be reversibly arrested in the dark. The gene expression programme changes were monitored in 7-day old continuous light grown WT seedling in Suc-containing medium, transferred to the dark for 3 days, and re-transferred to light for 24 hours. Leaves 1 and 2 including the SAM were collected at the times before dark transfer, 1 and 3 days in the dark and 1, 3, 8, and 24 h in the light re-exposure. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

3.3.5 Light expands the cells' translation capacity

Growth involves producing new cellular components, building cytoplasm, and this is carried out primarily through the activity of ribosomes. Translation, like transcription, is an important mechanism of modulating protein expression during growth (Kalve et al., 2014). The translation genes *RIBOSOMAL PROTEIN S6* (*RPS6*) and *ERBB-3 BINDING PROTEIN 1 (EBP1)* transcript levels declined in the 3 days dark period and rapidly responded to light and reached their highest peak within 8 hours and dropped a little between 8 to 24 hours. The expression level changes upon dark to light transfer indicates growth of new leaves involves cell cycle genes together with translation capacity and ribosome biogenesis (Fig. 3.10).

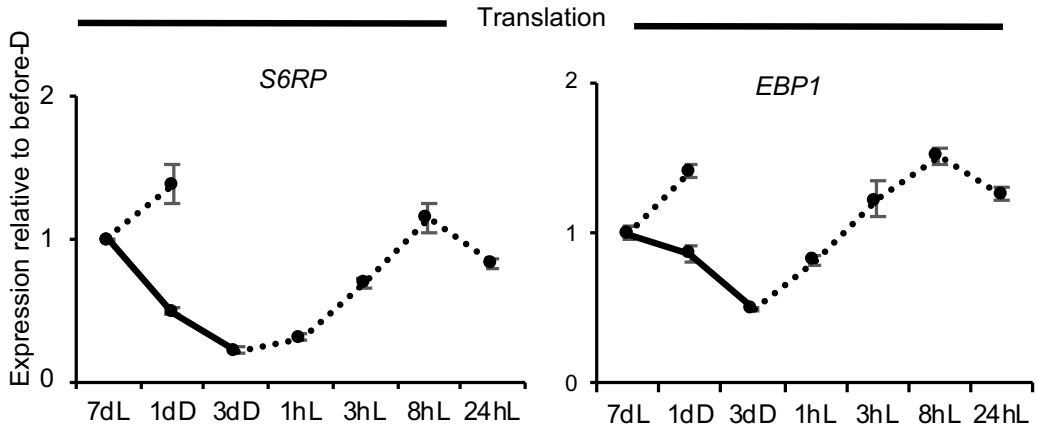

Figure 3. 10 Active cell proliferation in young leaf primordia can be reversibly arrested in the dark. The gene expression programme changes were monitored in 7-day old continuous light grown WT seedling in Suc-containing medium, transferred to the dark for 3 days, and re-transferred to light for 24 hours. Leaves 1 and 2 including the SAM were collected at the times before dark transfer, 1 and 3 days in the dark and 1, 3, 8, and 24 h in the light re-exposure. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

3.3.6 Dark and the starvation response

A previous transcriptional responses study of starvation and energy state genes in the shoot meristem confirmed an upregulation during the dark period (López-Juez et al., 2008). Here it was hypothesized that representative starvation genes will be up-regulated in the dark but will drop in response to light. The carbon starvation response genes *TREHALOSE-6-PHOSPHATASE9* (*TPS9*) and *BASIC LEUCINE ZIPPER1* (*bZIP1*) increased in the dark as hypothesized and rapidly drop within 1 hour upon transfer to light (Fig. 3.11). The up regulation of starvation genes in the dark manifests the establishment of a starvation state, and promptly drops within 1 hour in the light. Despite presence of sucrose in media, the starvation state was induced during dark and rapidly dropped in the presence of light. This suggests this process may be regulated by the photomorphogenic pathway in young leaf primordia.

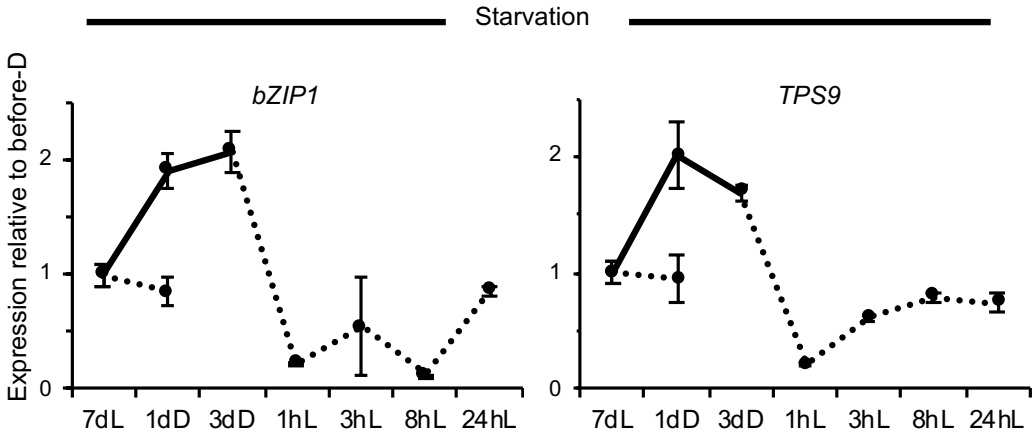

Figure 3. 11 The dark transfer showed an increase in transcriptional responses of starvation signature genes. The gene expression programme changes were monitored in 7-day old continuous light grown WT seedling in Suc-containing medium, transferred to the dark for 3 days, and re-transferred to light for 24 hours. Leaves 1 and 2 including the SAM were collected at the times before dark transfer, 1 and 3 days in the dark and 1, 3, 8, and 24 h in the light re-exposure. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

3.3.7 Light and Dark and the role of hormones

A rapid change in hormonal responses also took place in leaf primordia as previously observed in the shoot meristem. The hormonal changes were also observed in tomato shoot meristem, which gives rise to new organs (Yoshida et al., 2011). Here the pattern of hormonal response changes in the new primordia in *Arabidopsis* in the presence and absence of light are shown by observing gene signatures. Auxin is an important hormone involved in all developmental processes in plant. Auxin representative gene *AUX1* (AT2G38120, also known as *AUXIN RESISTANT1* but not to be confused with *AXR1*, AT1G05180) response level upon transfer to dark was increased as seen previously, independently in DR5;:GUS reporter seedling, which shows presence of auxin gene expression activity in leaf primordia and shoot meristem. Interestingly response level rapidly dropped within 1 hour of transferring to light. This may indicate the meristematic growth arrest in the dark could to some extent be due to high auxin level. An auxin inducible gene that reflects responses of auxin in *Arabidopsis*, *HOMEOBOX-LUCINE ZIPPER PROTEIN 2* (*HAT2*) showed no change during dark arrest period, however compared to 8 days continuous light, the response is higher in the first day of dark arrest. The expression pattern of auxin synthesis genes was also investigated. The purpose of looking at auxin synthesis was to find out if increase in

auxin level is due to its synthesis or to auxin being trapped in the shoot meristem and primordia to inhibit growth. Auxin synthesis genes *TRYPTOPHAN AMINOTRANSFERASE RELATED 2* (*TAR2)* and *TRYPTOPHAN AMINOTRANSFERASE* (*TAA1)* expression level drops in dark and remains low in light, later gradually increases between 8h to 24h in light. The TAA1 response in the first day of dark is similar to 8 days in the light. According to the previous evidence it was hypothesized cytokinin level will drop in the dark as auxin increases, and gradually increase in the light to mediate growth. There was a mild but smaller increase in the cytokinin-signature gene expression *ARABIDOPSIS RESPONSE REGULATOR* (*ARR5*) level in dark than without dark, and then a gradual increase upon re-exposure to light. Expression of ethylene response genes *ETHYLENE RESPONSIVE ELEMENT BINDING PROTEIN* (*EBP*) and *ETHYLENE INSENSITIVE 3* (*EIN3*) was elevated for both in the dark and reduced in the light (Fig. 3.12).

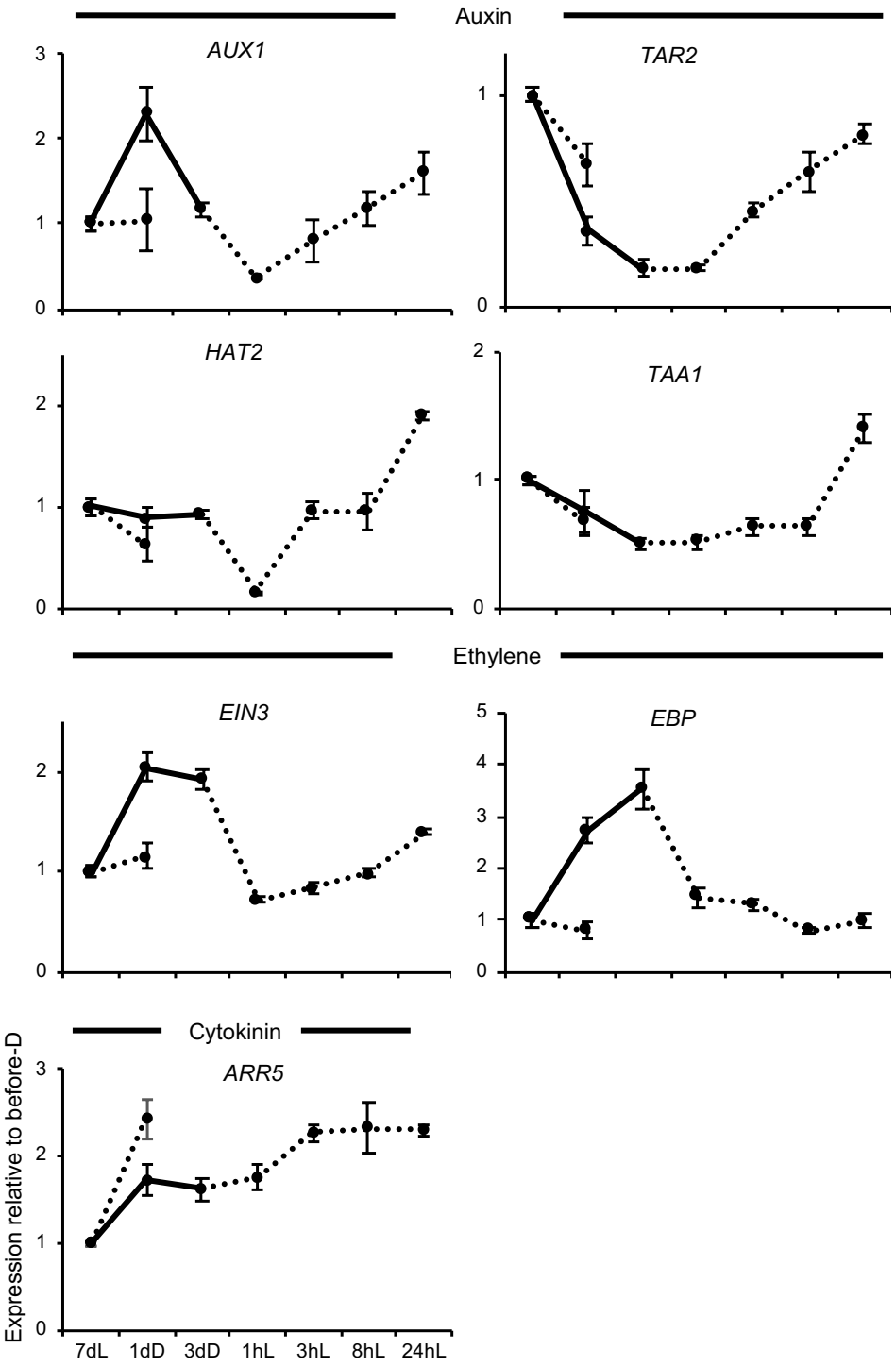

Figure 3. 12 The hormonal markers responded to dark arrest and light re-exposure. The gene expression programme changes were monitored in 7-day old continuous light grown WT seedling in Suc-containing medium, transferred to the dark for 3 days, and re-transferred to light for 24 hours. Leaves 1 and 2 including the SAM were collected at the times before dark transfer, 1 and 3 days in the dark and 1, 3, 8, and 24 h in the light re-exposure. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

3.3.8 Organ gene expression Dark-arrested or light-reactivated meristems and leaf primordia

In order to understand the development further, signatures of different types of cells that make up a leaf were chosen to track their expression. *VEIN DEFICIENT 6* (*VND6*) and *HOMEOBOX-LEUCINE ZIPPER PROTEIN 8* (*ATHB8*) were chosen to represent the initiation of vascular development and pattering. These signature genes showed a reduction in expression level in dark and a gradual increase once re-exposed to light. The drop in the response in the dark could indicate that vascular patterning and development requires light. The mesophyll and plastid signature genes *GIANT CHLOROPLAST 1* (*GC1*) and *LONESOME HIGHWAY* (*LHW*) are both involved in plastid biogenesis. Their expression level dropped in dark and gradually increased in light re-exposure. *GLABRA 2* (*GL2*) controls epidermal cell identity, being involved in trichome development. Upon dark arrest transcript level mildly elevated but less so than in the light, and within 3 days in dark decreased. Then it showed a transient decrease during the first hour and increased later in the light (Fig. 3.13). The organ development genes involved in cellular differentiation were broadly promoted by light, but to different extents.

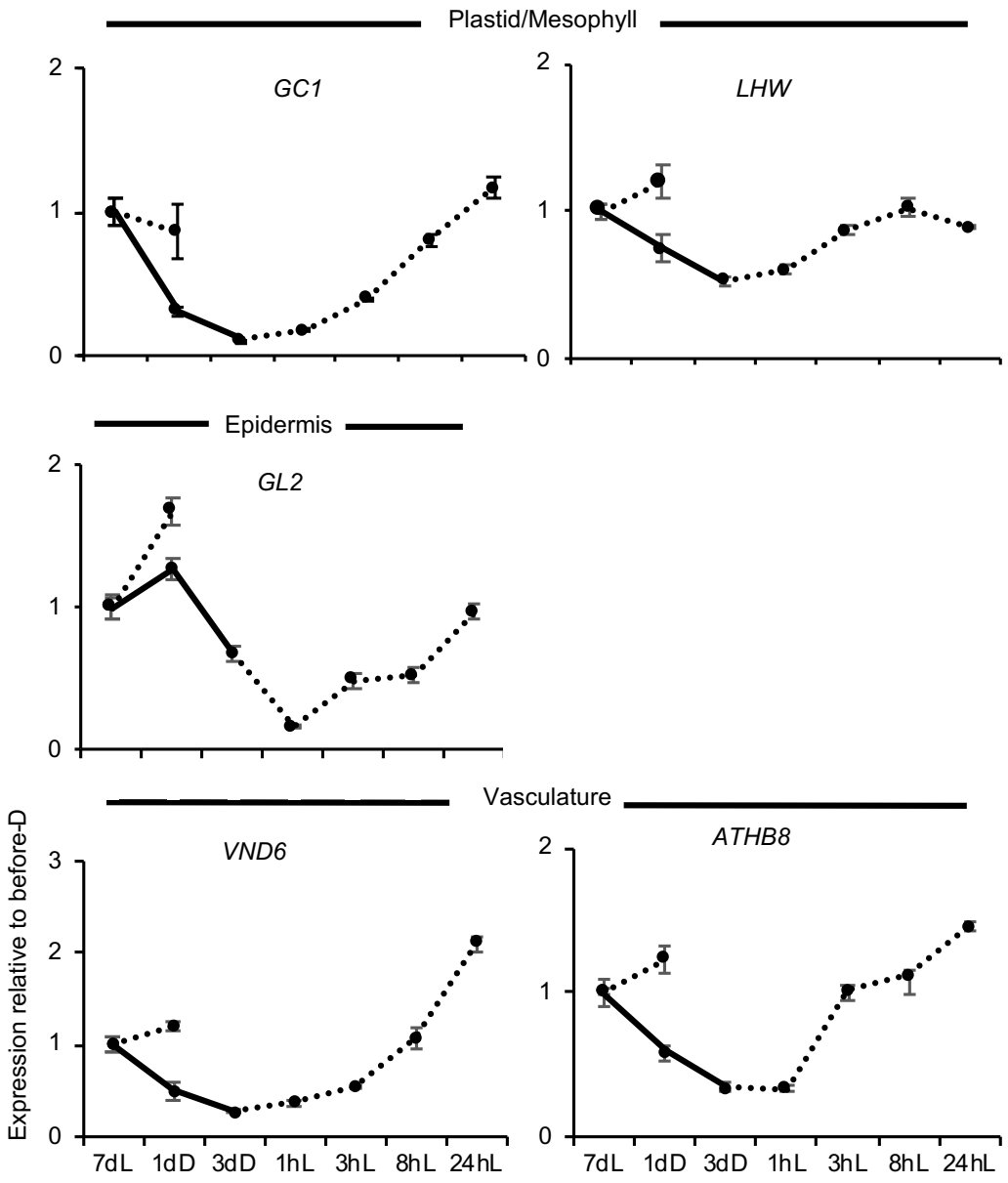

Figure 3. 13 The cellular differentiation markers were elevated in the light exposure. The gene expression programme changes were monitored in 7-day old continuous light grown WT seedling in Suc-containing medium, transferred to the dark for 3 days, and re-transferred to light for 24 hours. Leaves 1 and 2 including the SAM were collected at the times before dark transfer, 1 and 3 days in the dark and 1, 3, 8, and 24 h in the light re-exposure. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

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

The results provided from the gene expression study indicate intervention of a double mechanism involved in the meristematic growth arrest in the dark. One mechanism involving the absence of energy signalling could be observed from the high expression level of starvation genes in dark and the second mechanism involves a growth inhibitory hormonal state at the SAM. Therefore, the next question was to test whatever the absence of energy signalling is the result of a shortage of photosynthetic activity in the dark, or a result of direct photomorphogenic photoreceptor action. To answer this question, *cop1*-4 mutant in the dark was examined. The *cop1*-4 mutant seedling exhibits photomorphogenic pathway activity in the dark. COP1 is an E3 ubiquitin ligase that is necessary for etiolation and to repress positive regulators of photomorphogenesis in the dark. *cop1*-4 seedlings were grown in light for 7 days to get them to a good size for experimental work and then transferred to dark to monitor growth, hormonal and starvation gene expression. Microscopic observation of the seedling transferred to dark showed leaf growth is not arrested. However, growth happens but in contrast to the light grown plant, the tissue grown in the dark is white, while the tip of leaf that was differentiated prior transferring to dark was green (Fig. 3.14B). The gene expression levels observed in developing primordia of *cop1*-4 seedlings were compared to the WT. The gene expression signatures of cell cycle and growth were less affected by 3-day dark period in *cop1*-4 (Fig. 3.14A). The cytokinin and auxin synthesis genes also showed a similar pattern to WT. The difference between WT and *cop1*-4 seedling transcriptional responses may indicate that a COP1-dependent photomorphogenic pathway is responsible for much of the expression changes of genes involved in repression in the dark and reactivation of leaf growth and development in light (including the growth, starvation and ethylene responses), but that the (not immediate) auxin and cytokinin-related changes may be largely independent of COP1.

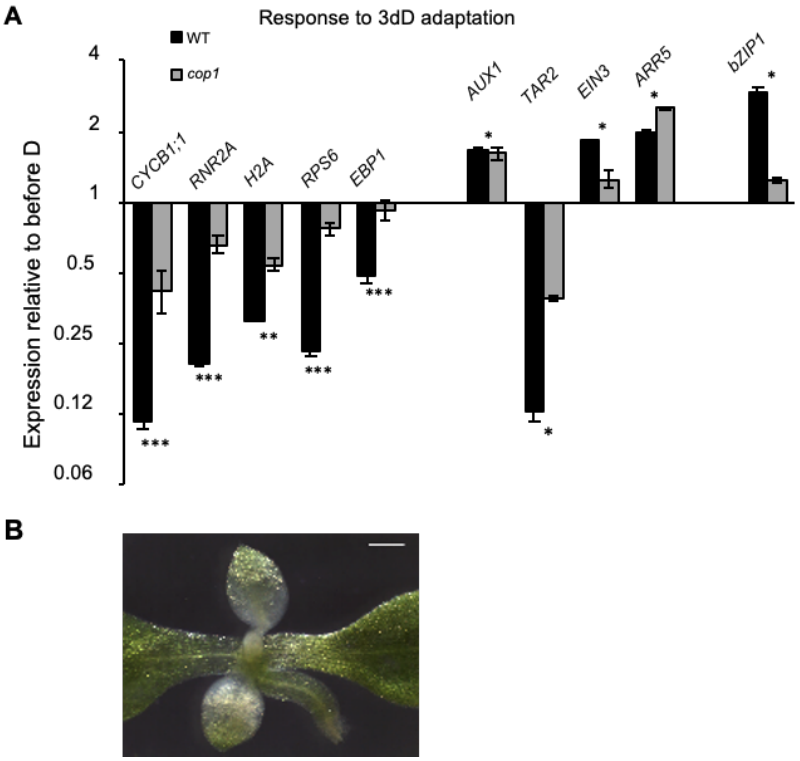

Figure 3. 14 The gene expression programme at the shoot meristem and first pair of leaf primordia was monitored on seedlings carrying the *cop1*-4 mutation. The expression changes were monitored in light grown WT seedling after 7 days in continuous light, transfer to 3 days in dark, and then light re-exposure for 24 hours. **A**, the graph is plotted on a $\log_2$ scale relative to the expression levels 7 days in light in *cop1*-4 and WT. **B**, shows young leaf primordia of *cop1*-4 seedlings adapted to 3day dark. The leaves 1 and 2 including the SAM were dissected at the times indicated on the graph and gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates). T.test was used, and asterisks indicate level of significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3.3.10 Organ Genes expression

As it was seen with other transcriptional responses, the genes involved in organ differentiations (Vascular genes: *ATHB8* and *VND6*, Plastid/mesophyll genes: *ARC5* and *GC1*), also dropped in *cop1*-4 mutant seedlings. But compared to WT seedlings the expression level response was milder (Fig. 3.15).

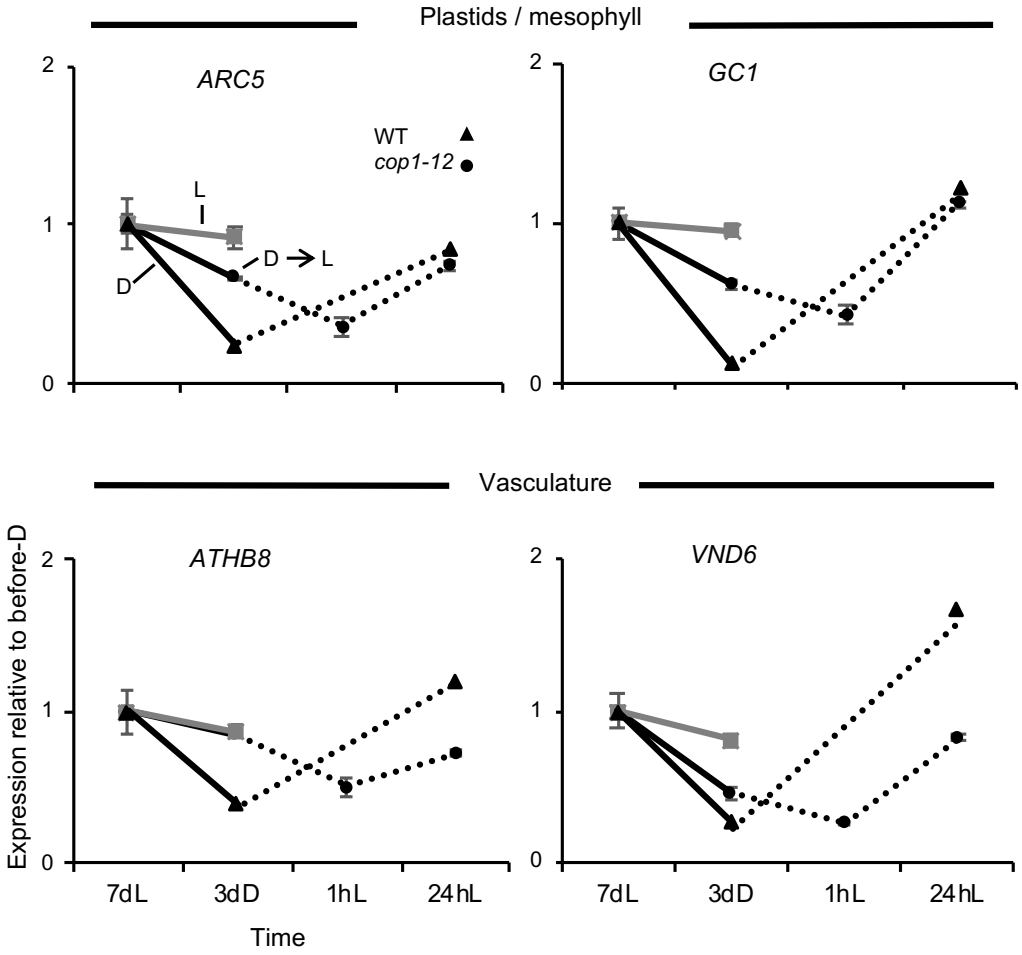

Figure 3. 15 The organ gene expression programme on *cop1*-4 mutant seedling. The expression changes were monitored in light grown WT seedling after 7 days in continuous light, transfer to 3 days in dark, and then light re-exposure for 24 hours. The leaves 1 and 2 including the SAM were dissected at the times indicated on the graph and gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

3.3.11 The dark arrested shoot meristem could be activated and begin leaf initiation by direct access to sucrose, or by manipulating hormone homeostasis.

The dark arrested shoot meristem could be activated and begin leaf initiation by direct access to sucrose, or by manipulating hormone homeostasis. However, the developed organs under these conditions differ from the light grown seedling. The seedling grown in the sucrose-containing medium in horizontal plates showed leaf primordia growth only in the light which was absent in the dark grown ones (Fig. 3.16, A and B). The seedling grown in the dark in liquid medium with no sucrose also showed no leaf growth and was arrested (Fig. 3.16C). The presence of BAP in the horizontal plates led to the growth of leaf in the WT and *axr1*-12 mutant seedlings (Fig. 3.16, D and E). The *axr1*-12 mutant seedlings in BAP containing medium showed occasional tumour like growth (Fig. 3.16F). The *cop1*-4 mutant seedling grown in the medium developed leaf unlike WT in the dark (Fig. 3.16G).

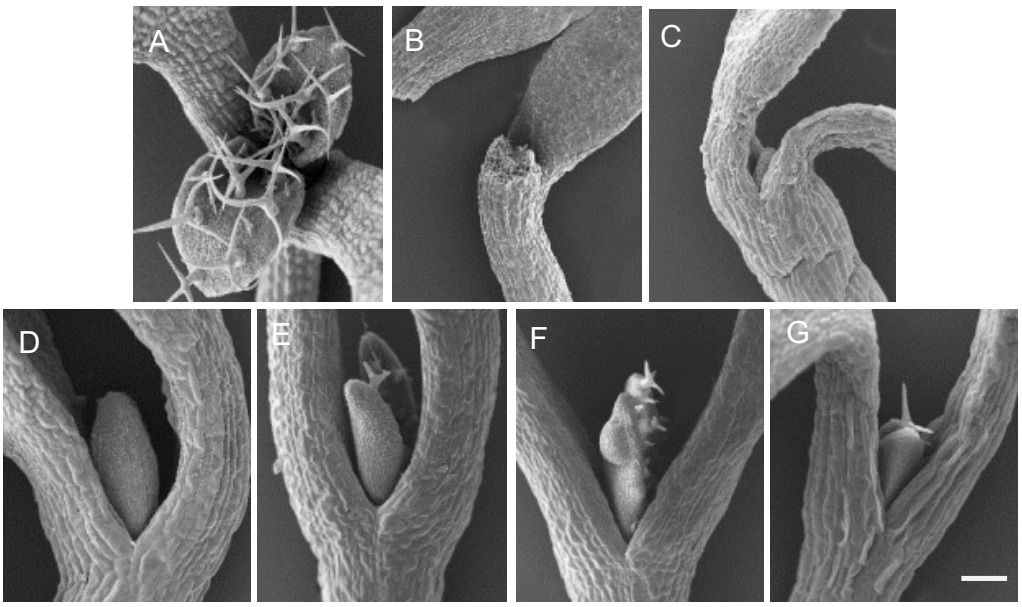

Figure 3. 16 The dark arrested shoot meristem could be activated and begin leaf initiation by direct access to sucrose, or by manipulating hormone homeostasis. All seedlings grown in continuous dark expect A. **A**, WT, continuous light, 7days light, horizontal sucrose-containing medium plate. **B**, same as 7 days dark. **C**, WT, 17 days sucrose-free liquid medium. **D**, WT, 7 days, horizontal sucrose-containing plate with 10 µM BAP. **E** and **F**, *axr1*-12 mutant, 7 days, horizontal sucrose-containing plates with 2 µM BAP. **G**, *cop1*-4 mutant, 7 days, horizontal sucrose containing plates. Scale bar: 100 µm.

3.4. Discussion

The environmental factors, development and growth processes of plants are connected. An important question I tried to answer in this project was how leaves develop from the shoot meristem. The aim was to understand how absence or presence of light could switch the meristem from an arrested state to an active state, which then leads to growth. This can provide fundamental information to better understand the basic biological phenomenon underlying leaf development. Light is perceived by photoreceptors and acts as a signal and an essential energy source for production of carbohydrates. The energy produced by the plant is in the form of sugar, which can be transported systemically as a source of energy and also itself as a signal. Previous studies have demonstrated a crosstalk between sugar signalling and photoreceptors. Therefore, the studies of environmental responses have the potential to provide understanding of developmental processes (López-Juez et al., 2008). López-Juez *et al. (2008)* observed a rapid upregulation of growth-related genes at the SAM during the development of leaves. An established system of dark arrest, light reactivation of plant growth in our lab was used by selecting representative genes. First a mitotic GUS reporter microscopic study in dark showed mitotic arrest and inhibition of proliferation, however mitotic activity was reactivated once in the light. Following this positive outcome, the gene expression profile was investigated using markers of proliferation and growth, energy and hormonal status, mesophyll, epidermis, and vascular-related development in the seedlings grown in light, during dark arrest and following re-exposure to light. Lopez-Juez and collaborators (2008) showed that markers of proliferation are expressed at a lower level or not at all in the dark in the SAM. The expression of markers of cell proliferation decreased in dark, confirming meristematic growth arrest in the dark. Therefore, in the absence of light, plants trigger skotomorphogenesis, where shoot apex cell proliferation is inactive (Li et al., 2017).

Auxin and growth

Changes in the gene expression are one of the most important biological processes, from cell differentiation to organ development (Merchante et al., 2017). It is well known that the interaction between auxin and cytokinin modulates and highly impacts organ growth in plant development (Kurepa et al., 2019). The hormonal auxin marker *AUX1* showed some increase in the dark, this result is consistent with Lopez-Juez et al.

(2008) findings. The auxin response (Fig 3.3) in the dark is both increased and delocalised in the SAM and new leaf primordia. The response of auxin transporters, to be lower and delocalised from the membrane in the dark, is consistent with the loss of auxin transport in the dark, and that would in turn explain the stronger but delocalised response in the dark. (Carabelli et al., 2007; Yoshida et al., 2011). Understanding how light switches skotomorphogenesis to morphogenesis, and how it leads to activation of the SAM, could give us a clue on organ development. The present results together with previous studies (López-Juez et al., 2008; Pfeiffer et al., 2016; Yoshida et al., 2011) provide evidence on the likelihood of the presence of a dual control switch. These two primary regulatory mechanisms are hormonal and energy signalling. In this project the hormones whose response was looked at closely were auxin, cytokinin, and ethylene.
The results presented here indicate that auxin promotes growth as well as inhibiting it. The inhibitory effect of auxin was concluded from data indicating the high (in the dark) or increased (on transfer from light to dark), but diffuse, auxin response in the meristem (Fig. 3.3, Fig 3.12) is one of the reason SAM activity is arrested in the dark. Thus, diffuse increase in the auxin level in primordia and meristem in the dark is growth inhibiting and a sudden drop in the first hour indicates auxin transported away from both organs and leads to the activation of growth by allowing cytokinin action to take place in the meristem. The local increase of diffuse auxin in the meristem, may occur to prevent the auxin maxima to take place, however, it could also prevent cytokinin action to take place in the meristem.

Cytokinin and growth

The positive role cytokinin plays in growth has been known for a long time, the data presented earlier indicates that reduced auxin and enhanced cytokinin action accompanies early regulation of a photomorphogenic state in light and can also mimic it in the absence of light. Comparing the control seedlings with *axr1*-12 in the absence of BAP, showed a huge reduction in primordia size of *axr1*-12, which may indicate the hormonal crosstalk and balance is necessary for normal primordia cell proliferation and growth. The presented data (Fig. 3.5) are consistent with the hypothesis of auxin removal upon light exposure and the upregulation of cytokinin response are necessary for leaf primordia initiation and growth. While cytokinin is necessary for the growth activity of the shoot meristem, excessive cytokinin actually reduces leaf primordia growth. It could be that cytokinin is antagonising the necessary auxin activity at the

primordia tip, it also could be that it is promoting cell proliferation but slowing the exit from proliferation to differentiation, which is what provides the visible change in organ area. E2FA form complex with dimerization partner (DP) family and when overexpressing plants have very active cell proliferation but produce tiny plants, made up of lots of very small cells (De Veylder et al., 2002).

Starvation state in presence and absence of light

The mechanisms that led to removal of the starvation state in the light are unknown, but one suggestion is that sucrose is blocked from entering into the meristem in the dark due to transporters being light-regulated. Sucrose can cause growth in the dark. Hence it is not that the meristem is insensitive to sugar in the dark, it senses it if sucrose is available. The starvation signatures showed an increase in the dark and a sudden drop in their expression in the light, which indicates carbohydrate deprivation in the meristem and young primordia in the dark, this state being quickly removed in the presence of light. The marker of mesophyll, *GC1*, was expressed at substantially lower levels in the dark compared to transcript levels in the light. GC1 has a role in division of chloroplasts and its reduction leads to inhibition of chloroplast division, with mesophyll cells comprising one or two giant chloroplasts (Maple et al., 2004). This indicates that the *GC1* response is light-dependent and required for development of photosynthetic organelles which is no longer necessary in the dark. The vascular-related genes *VND6* and *ATHB8*, showed no change in expression. *VND6* is a regulator of genes that are involved in secondary wall formation and programmed cell death. Mature xylem cells indeed undergo programmed cell death to produce a conducting, empty tracheid or vessel element.

COP1 and energy signalling

The above pieces of evidence that support the presence of dual mechanism of leaf initiation arrest in the dark led me to ask whether the absence of local sugar signalling is due to absence of photosynthesis in the dark or a result of photoreceptors' actions and their signalling pathways. The gene expression study of *cop1* seedlings in the dark showed a smaller degree of responses than that seen in the WT. The results are not sufficient to conclude how COP1 action regulates energy signalling. The starvation state of the meristem shown by gene expression responses in the absence of light suggests light may have a permissive role towards sugar access to the meristem. One

attractive suggestion is energy signalling may be regulated by photoreceptors in a COP1-dependent way. My hypothesis which was removal of photomorphogenesis repressors such as COP1 will mimic the hormonal and energy-related light responses in the dark. That turns out not to be completely true, the auxin transient drop is not apparent, which is consistent with it being the result of posttranslational modifications triggered by light (MPK), in which COP1 plays no role because they are not primarily gene expression responses.

Chapter 4

Energy signalling and TOR pathway in the regulation of leaf growth

4. Chapter 4: Energy signalling and TOR pathway in the regulation of leaf growth

4.1. Introduction

4.1.1 Carbon starvation and energy stress

Plant growth depends on several factors, such as light, stress, and nutrient availability. Exposure to the limitation in any of these factors has multiple metabolic and physiological effects that lead to plant starvation (Honig et al., 2012). A plant suffering from energy stress shows an alteration in growth. An energy stress could be induced in plants exposed to a regular light/dark cycle suddenly experience extended darkness at the time when light was anticipated, they adapt to the stress by inhibiting growth (Lastdrager et al., 2014). Arguably the most fundamental activity of the plant is the production of carbohydrate sugars which regulate expression of a vast number of genes. According to their response to sugar, genes are categorized as "starvation" or "feast" genes (Rolland et al., 2002). The sugar level is continuously monitored to coordinate growth to available sugar resources. The growth-promoting regulators are TOR kinase, HXK glucose sensor, and the T6P and the inhibitors are SnRK1 (Gazzarrini and Tsai, 2014) and C/S1 bZIP transcription factors (Smeekens et al., 2010). The TOR kinase is a master regulator that determines a good balance between nutrient and energy signalling to control cell division and growth (Mubeen et al., 2018; Schepetilnikov and Ryabova, 2018). TOR is involved in the regulation of transcriptional, translational, ribosomal biogenesis, nutrient and energy signalling of several pathways in all eukaryotes (Xiao and Grove, 2009). TOR regulates translational activity of the plant during abiotic stress by modulating the genes encoding ribosomal proteins to maintain balanced growth (Bakshi et al., 2019; Tomé et al., 2014). TOR is also involved in the activation of genes that have a role in S phase (DNA synthesis) by directly phosphorylating E2Fa (Li et al., 2017; Xiong et al., 2013). The involvement of sugar signalling through the TOR pathway in cell growth, proliferation and expansion implies a fundamental role in organ development and growth (Sablowski and Carnier Dornelas, 2014).

4.1.2 bZIP transcription factors

bZIPs are a family of transcription factors found in all eukaryotes. The family contains 78 members, allocated into 10 groups in *Arabidopsis*. They have been suggested to be targets of the SnRK1s pathway. The bZIPs are under the control of sucrose level both

translational and posttranslational, this suggests that their expression is highly correlated by the sucrose status of the cell. In this thesis, the physiological role of C and S_1 groups of the bZIP family was looked at in leaf size development. bZIP63 up-regulates the circadian oscillator in response to low sugar, and as a result during the starvation period, bZIP63 and PRR7 are required for correct circadian oscillator phase during light/dark cycles. bZIP1 belongs to the S_1 class of bZIPs. The S_1-bZIPs respond to the changes in carbon and nitrogen and by reprograming transcription in stress, development, and hormonal changes. Translation of bZIP11, another member of bZIP family, is regulated negatively by sucrose (Thalor et al., 2012; Wiese et al., 2004). bZIP1 forms heterodimers with group C bZIPs (bZIP9, bZIP10, bZIP25, and bZIP63). bZIP1 and bZIP63 are involved in regulating gene expression in response to carbohydrate limiting conditions. Applying 2% glucose to the seedlings of SnRK1 and S_1-bZIP would rescue plant growth, which suggests that these genes are particularly necessary during starvation conditions and are involved in managing the low-carbon conditions.

4.1.3 Ribosomal protein S6 kinase (S6K)

RAPTOR protein is conserved among all eukaryotes and a fundamental partner of TOR kinase, to activate the TOR complex (Dobrenel et al., 2016; Schepetilnikov et al., 2017). RAPTOR has important functions in stimulating responses to nutrient availability of S6K1 and maintaining cell size (Ramírez-Valle et al., 2010). TOR inhibitor rapamycin works as an allosteric inhibitor by disrupting the TOR-RAPTOR complex interaction (Kim et al., 2002). The *Arabidopsis* genome contains two *S6K* homologues, *S6K1* and *S6K2*. S6K is an effector downstream of TOR and up-regulates translational capacity of cells by phosphorylating RPS6 to stimulate protein synthesis and cell proliferation (Dobrenel et al., 2016; Roustan and Weckwerth, 2018). In the root, *S6K1* transcript builds up in the elongation zone and *S6K2* in the differentiation zone, suggesting they may involve in the exit from cell proliferation and thus negatively regulate mitosis. However, despite their importance in the TOR pathway, the mutation in *s6k1* and *s6k2* are still working fine and show no visible phenotype (Henriques et al., 2010).

4.2. Aims and Objectives:

My aim is understanding how sucrose/energy signalling could switch the meristem from arrested state to active, which then leads to growth in the dark. This can provide us with fundamental information to better understand the role of energy signalling in leaf development.

Hypothesis 1

Energy signalling regulates the extent of leaf growth and development. Previous studies demonstrated that SAM exposure to sucrose or glucose can initiate further growth of plant organs in the dark (Li et al., 2017; Roldán et al., 1999). My hypothesis is that both hormonal and starvation brake occur in the dark when no sucrose is present in the growth medium. Also, energy signalling activates and increases growth related transcript levels. The transcript responses to direct exposure of the shoot meristem to sucrose are hypothesized to mimic the genetic responses to light re-exposure observed in the previous chapter.

Hypothesis 2

The result of cell proliferation activity from the SAM could be different dependent on presence or absence of light. In the dark by providing exogenous sugar to the SAM, cell proliferation activity is initiated but the result of cell proliferation activity is becomes not new leaves, but rather organs appropriate for an extreme shade-avoidance reaction.

Hypothesis 3

A classic sugar sensing pathway, based on TOR signalling, is primarily in control of the activity of the meristem, involving both cell proliferation and translation. The TOR pathway as a major regulator of growth through nutrient and energy signalling, is hypothesized to be in control of the changes which take place during starvation as an unfavourable environmental condition. Also having observed the growth responses to sucrose, I hypothesize that growth response to sucrose is TOR-mediated.

Hypothesis 4

The starvation state that seedlings suffer from during an extended dark period is removed rapidly by light in a way that cannot be explained by photosynthesis. My hypothesis is that photosynthesis is not responsible for the primary cell proliferation response, which takes place immediately after exposure to light.

Hypothesis 5

The quantity of light causes key changes to the extent of organ growth. Leaves contain several layers of palisade mesophyll to support photosynthetic activity under HL quantity. Under HL, larger organs also develop. The question here is if the energy control is a determinant of the extent of leaf growth under different irradiances of light. By taking advantage of growth under different light quantities, my hypothesis is that transcript level of genes associated with growth will be elevated in HL and decreased under LL and the mechanism underlying these changes is hypothesized to be energy signalling.

4.3. Results

4.3.1 Direct sucrose access to the SAM reactivates cell proliferation and growth in a TOR-dependent manner in the dark

The results obtained from the gene expression study showed hormonal status in the absence of light correlate to leaf primordia arrest. The expression of starvation signature genes confirmed a local starvation state in the SAM, one rapidly removed by light. Previous research has shown that direct exposure of the meristem to sucrose activates the SAM and leads to the growth of organs in the dark (Li et al., 2017; Roldán et al., 1999). The question here was to what extent exogenous sucrose can override darkness and replace light. To investigate the effect of sucrose on meristematic activity and growth in dark, CYCB1;1::DB-GUS-expressing seedlings were used to observe mitotic activity. The seedlings were grown on sucrose-containing plates (solid medium, horizontal) for 7 days, then transferred to dark in sucrose-free liquid medium. The horizontal plate for the first part of experiment was chosen to grow seedlings in the light conditions without direct impact of nutrient supply to the meristem. Liquid medium was used to directly expose the shoot meristem to sucrose, examining responses in a time-course manner. Prior to exposure to sucrose, the first 3 days in the dark plates contained sucrose-free liquid medium to get them adapted to liquid medium in the dark while also starving the seedlings, in anticipation of sucrose exposure.

On day 3, the medium was replaced with a sucrose-containing liquid medium or sucrose-free liquid medium for control seedlings in the dark under dim green safelight (Fig. 4.1). The mitotic activity in the young leaf primordia adapted to dark was repressed, but amazingly within 1 hour in the presence of sucrose, the activity reemerged. The cell proliferation was most obvious after 24 hours and mainly spotted in the proximal region of leaf primordia (Fig. 4.1). The first leaf pair of the seedlings in presence of sucrose for 24 hours had increased in size, and leaves 3/4 emerged. The control seedlings in the dark with no sucrose for 4 days showed no cell proliferation activity. These experimental findings indicate that the SAM is activated, and cell proliferation activity begins upon exposure to sucrose while in the dark (Fig. 4.1). To find out if sucrose-activated mitotic activity in the dark is TOR-dependent, a TOR inhibitor (AZD-8055) together with sucrose were added to the medium to which the CYCB1;1::DB-GUS-expressing seedlings were exposed. Monitoring the seedlings after 24 hours confirmed mitotic activity being dramatically repressed in the young

leaf primordia (Fig. 4.1). This experimental result confirms that TOR to a large extent mediates the mitotic events.

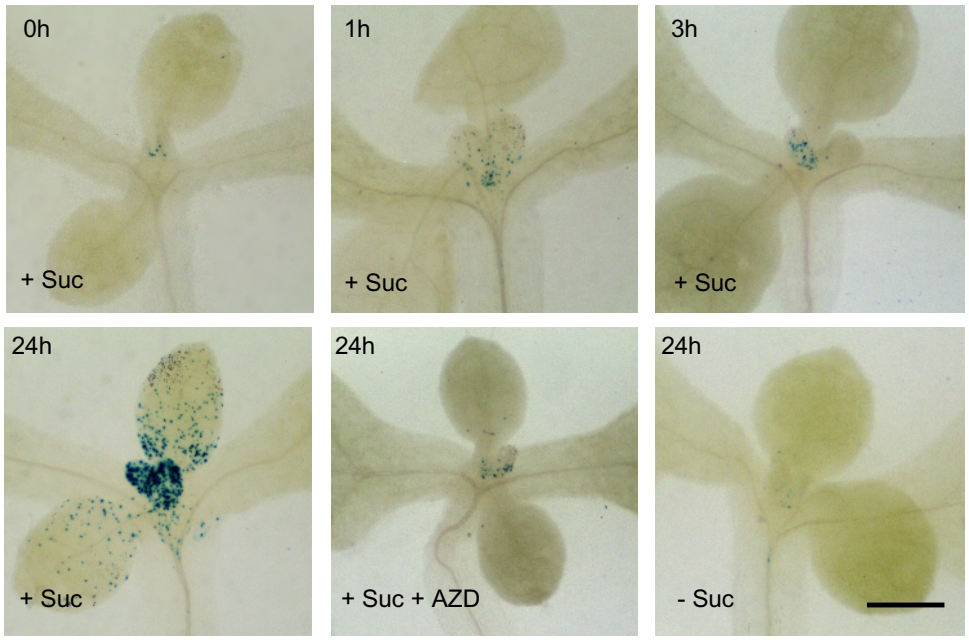

Figure 4. 1 Direct sucrose access to the SAM in the absence of light re-activates cell proliferation and growth in a TOR-dependent manner. CYCB1;1::DB-GUS-expressing seedlings were grown on solid medium for 7 days in continuous light, then transferred to liquid medium without sucrose (Suc) in the dark. Medium was replaced with Suc-containing medium, Suc-free medium, and to medium containing Suc plus AZD-8055, a specific TOR inhibitor, on the third day of dark treatment. Seedlings were harvested to visualize for GUS expression at the time points indicated on the images. Scale bar: 500 μm.

The percentage of GUS-occupied area of leaf 3 was measured upon adding sucrose to the seedlings using ImageJ software. The mitotic activity in the seedlings in the presence of TOR inhibitor (AZD-8055) and the seedlings with no sucrose showed nearly the same number of cells going through mitosis (P value= 0.02) (Fig. 4.2A). The result indicates the number of mitotic events was reduced to about 2% of leaf area by blocking TOR (P value= 0.01) pathway compared to seedlings in presence of sucrose with 34% of leaf area going through mitosis. The inhibition of cell proliferation by AZD-8055 treatment indicates stimulation of this process is facilitated by TOR pathway. The GUS activity indicates the cell proliferation re-initiated and gradually increased in 24 hours (Fig. 4.2B). The prolonged access of the meristem to external sucrose leads to activation of the SAM and thus gradual increase in the cell proliferation with highest peak at 24 hours (Fig. 4.2B). Interestingly, extending the dark period for 4 to 6 days showed access of the meristem to external sucrose leads to the growth of petiole and internode organs (Fig. 4.2C).

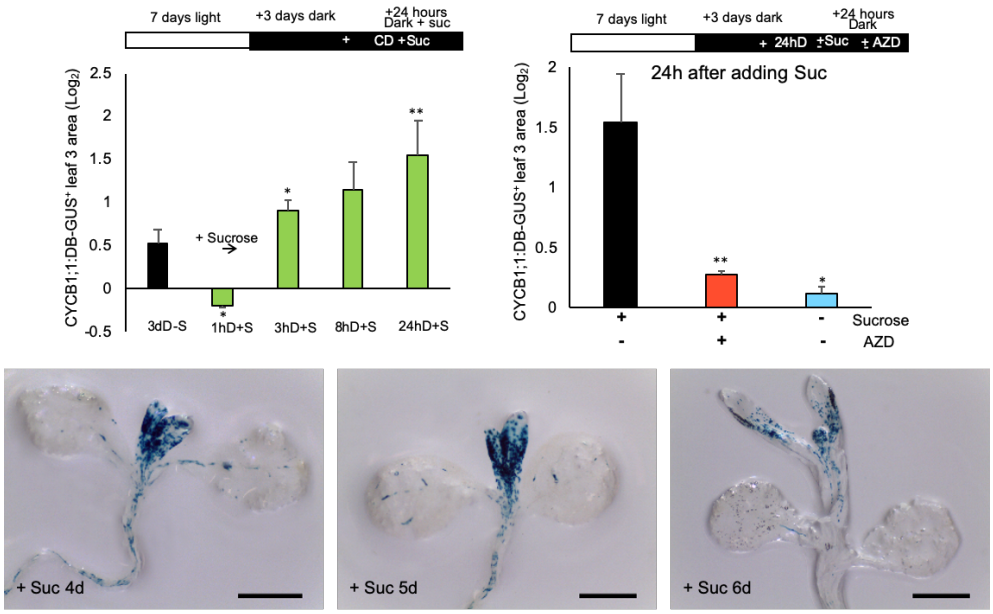

Figure 4. 2 Direct sucrose access to the SAM reactivates cell proliferation and growth in a TOR-dependent manner in the dark. The CYCB1;1::DB-GUS expressing Seedlings show the cells undergoing mitosis through a blue stain. 7-day old light-grown seedlings expressing CYCB1;1::DB-GUS were transferred to dark; after 3 days the medium was replaced with Suc-containing liquid medium with or without AZD-8055 or Suc-free medium and harvested after 24 hours. **A** and **B**, the percentage of GUS stained area of leaf 3 was quantified by ImageJ. 7-day old light-grown seedlings expressing CYCB1;1:DB-GUS were transferred to dark, after 3 days the medium was replaced with Suc-containing liquid medium. Seedlings were collected 24 hours after adding sucrose. **C**, 7 day light-grown seedling expressing CYCB1;1:DB-GUS, transferred to dark for 4, 5, and 6 days in Suc-containing liquid medium. Error bars indicate SEM. T.test was used and asterisks indicate level of significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Scale bar: 500 µm.

4.3.2 Direct sucrose access to the SAM activates cell proliferation and the growth gene expression programme in the dark.

The gene expression programme confirmed that the role of changes in hormonal responses in presence and absence of light cause leaf primordia initiation or arrest, respectively. The mitotic CYCB1;1::DB-GUS reporter uncovered the effect of direct sucrose access to the SAM, which re-activates cell proliferation and growth. The next step was to observe the effect of this energy status change on the transcript levels reflective of hormonal or energy status responses in the SAM and young leaf primordia. Given that energy signalling activates cell proliferation, I assumed there would be an increase in growth-related transcript responses. I tested this hypothesis by monitoring gene expression in the seedlings grown for 7 days on sucrose-containing medium, later transferred to sucrose-free liquid medium in the dark. On day 3, some of the seedlings were transferred to sucrose-containing liquid medium and the rest transferred to fresh sucrose-free liquid medium in the dark. The genes involved in the cell cycle, or representative of hormonal and starvation responses were monitored by measuring transcript levels. The cell cycle gene signatures containing mitosis *CYCB1;1*, S phase gene *H2A*, and translation genes *RPS6* and *EBP1* showed similar expression during dark adaptation. After transferring the seedlings to dark with no sucrose, the transcript levels sharply declined. However, the expression of mitosis representative gene (*CYCB1;1*) gradually increased upon sucrose supply. *H2A* and *RPS6* transcripts were increased and reached a higher level compared to the time before dark transfer. This indicates that translation genes are responding to sucrose. All the cell cycle and translational gene transcripts reached the highest peak in 24 hours, Contrary to other cell cycle or growth-related genes, *EBP1* reached the maximum peak within 3 hours (Fig. 4.3).

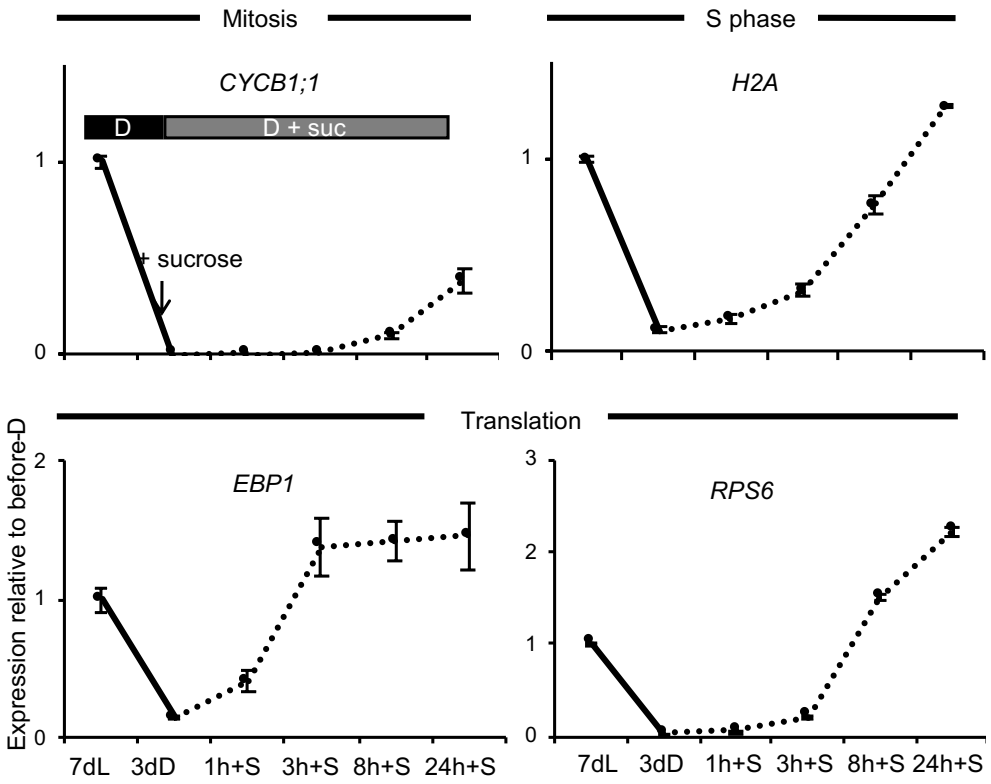

Figure 4. 3 Direct sucrose access to the SAM up-regulate transcript levels of cell proliferation and growth-related genes in the dark. The gene expression transcripts changes were monitored in 7-day old continuous light-grown WT seedlings on solid medium, transferred then to Suc-free liquid medium in the dark. on day 3, medium was replaced by Suc-containing medium for 24 hours. Leaves 1 and 2 including the SAM were harvested at each time: before dark transfer, 3 days in the dark and 1, 3, 8, and 24 h after adding Suc. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

4.3.3 Direct sucrose access to the SAM activates hormonal signature genes in the dark

The transcript levels of genes responsive to the hormones ethylene, auxin, and cytokinin in response to starvation and stimulation by sucrose were investigated. The expression of auxin-related genes (*AUX1*, *IAA1*, and *HAT2*) showed a drop in the dark (the highest effect was on *IAA1*, which sharply declined in the dark with no sucrose). However, presence of sucrose in the medium led to their elevation with the highest peak in 8 hours. Transcripts of the auxin synthesis-representing gene *TAA1* declined dramatically in dark and then slowly rose in the presence of sucrose. The expression of cytokinin gene *ARR5* was decreased once transferred to dark and then stimulated

upon sucrose supply and reached the highest peak within 8 hours (Fig. 4.4). The gene associated with ethylene hormone activity (*EIN3*) saw its transcripts increased in the dark adaptation and decreased slightly in presence of sucrose.

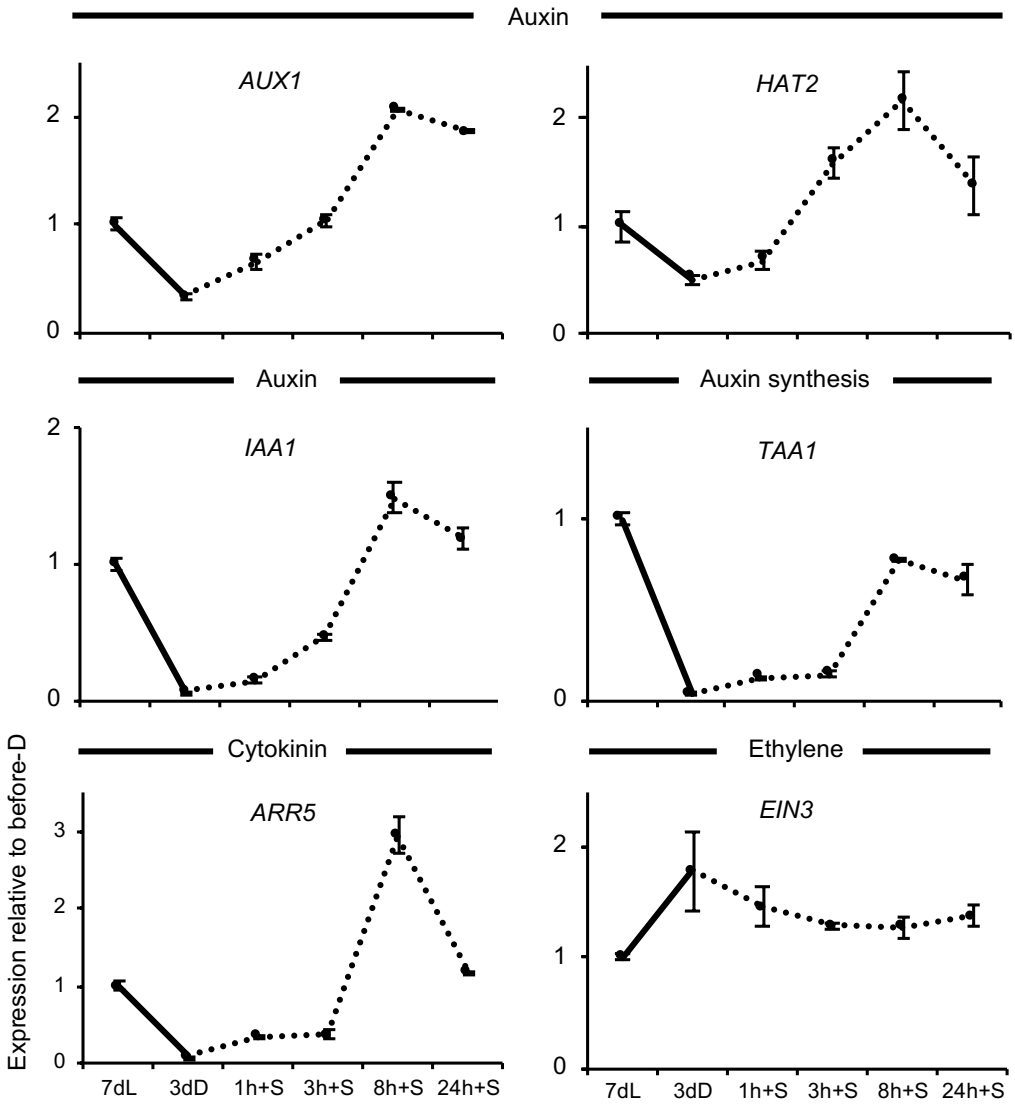

Figure 4. 4 Direct sucrose access to the meristem activates hormonal signature genes in the dark. The gene expression transcripts changes were monitored in 7-day old continuous light-grown WT seedlings on solid medium, transferred then to Suc-free liquid medium in the dark. on day 3, medium was replaced by Suc-containing medium for 24 hours. Leaves 1 and 2 including the SAM were harvested at each time: before dark transfer, 3 days in the dark and 1, 3, 8, and 24 h after adding Suc. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

4.3.4 Direct sucrose access to the SAM removes the starvation state in the dark

The genes known to respond to carbohydrates starved condition were expected to increase in 3 days dark adaptation with no sucrose. The transcripts level of starvation genes was elevated in the dark. However, after supplying sucrose, it was rapidly repressed within 3 hours and later increased (Fig. 4.5). The elevation in their transcripts level may indicate these genes have other roles in the energy signalling pathway.

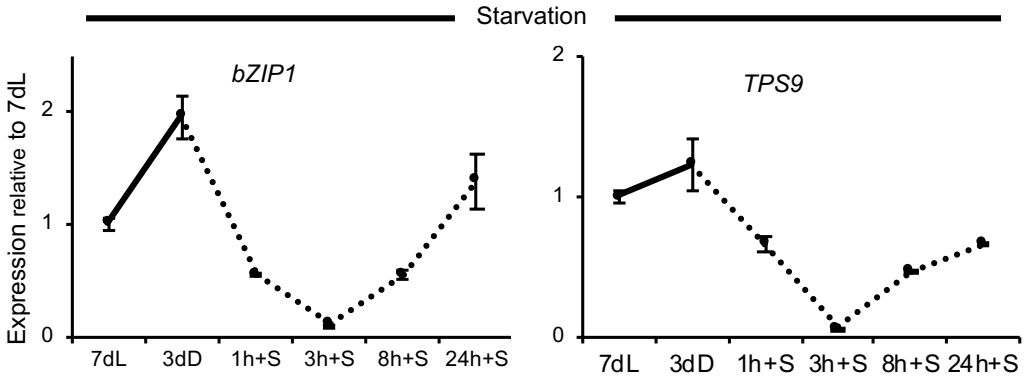

Figure 4. 5 The starvation marker genes respond to direct sucrose access to the shoot apex in dark. The gene expression transcripts changes were monitored in 7-day old continuous light-grown WT seedlings on solid medium, transferred then to Suc-free liquid medium in the dark. on day 3, medium was replaced by Suc-containing medium for 24 hours. Leaves 1 and 2 including the SAM were harvested at each time: before dark transfer, 3 days in the dark and 1, 3, 8, and 24 h after adding Suc. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

4.3.5 Organ 'readout' genes expression after direct sucrose access to the SAM

The organ-specific gene expression responded to sucrose supply in the dark. The plastid biogenesis markers *GC1* and *ARC5* decreased upon transferring to dark and gradually increased in response to sucrose. The expression of vasculature marker genes (*VND6* and *ATHB8*) showed a rapid reduction and gradually increased once sucrose was added and demonstrated a light-like response (Fig. 4.6).

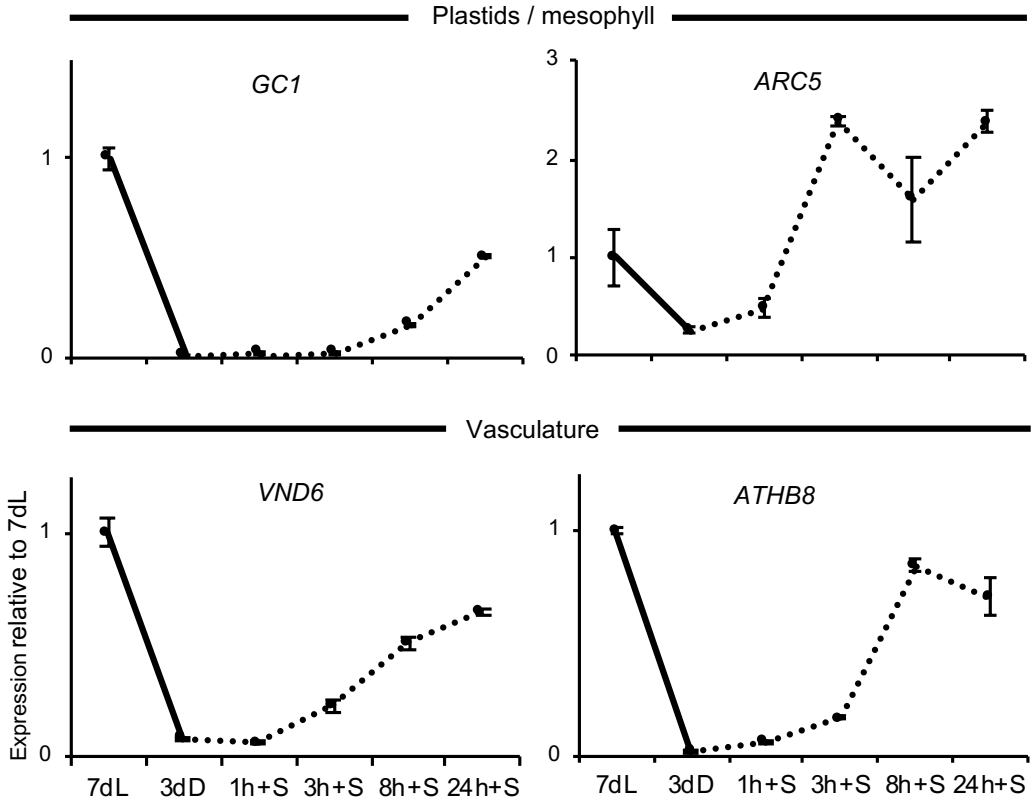

Figure 4. 6 Direct sucrose access to the SAM activates organ growth gene expression in the dark. The gene expression transcripts changes were monitored in 7-day old continuous light-grown WT seedlings on solid medium, transferred then to Suc-free liquid medium in the dark. on day 3, medium was replaced by Suc-containing medium for 24 hours. Leaves 1 and 2 including the SAM were harvested at each time: before dark transfer, 3 days in the dark and 1, 3, 8, and 24 h after adding Suc. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through two technical replicates. Error bars indicate SEM (between biological replicates).

4.3.6 The gene expression programme mediated by sucrose in the dark is mostly TOR-dependent

The results observed earlier, confirmed some critical aspects of the genetic programme, which responds to energy signalling. To establish which aspects of these responses were dependent on the TOR pathway, a pharmacological treatment with the ATP-competitive selective inhibitor of TOR (AZD-8055) was used. AZD-8055 is a highly specific competitive TOR-inhibitor which, was first developed in yeast or animal cells and also have been proven to be effective in plants (Montané and Menand, 2013; Schepetilnikov et al., 2017).

This was investigated by monitoring gene expression in the seedlings grown for 7 days in sucrose-containing horizontal plates, then transferred to sucrose-free liquid medium in the dark. On day 3 the seedlings were treated with sucrose-free liquid medium or medium containing sucrose with or without AZD-8055 in the dark for further 24 hours. The genes involved in the cell cycle, hormonal, and starvation responses were monitored. Expression of cell cycle marker genes *H2A*, *CYCB1;1*, and *EBP1* was reduced in the presence of TOR inhibitor (Fig. 4.7). Monitoring the effect of the TOR pathway on hormonal markers showed the ethylene signature gene *EIN3* was not sensitive to either sucrose or addition of AZD-8055. Auxin marker genes responded differently, *AUX1* was found to be partially TOR-dependent, *IAA1* was not affected, suggesting it is not TOR dependent (Fig. 4.7). The starvation genes response in the dark was minimal after 24 hours, yet they were repressed upon supply of sucrose to some degree after 24 hours. However, surprisingly, in the presence of TOR inhibitor that repression did not occur.

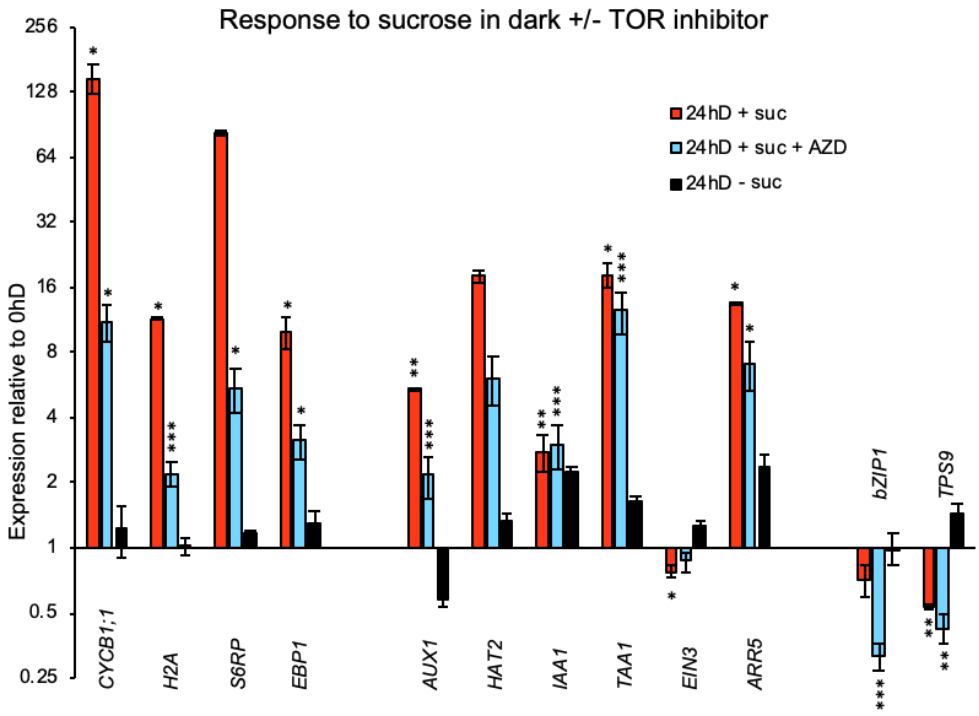

Figure 4. 7 TOR pathway is involved in controlling and sensing nutrient status and sucrose availability. The gene expression program changes were monitored in 7-day old continuous light grown WT seedlings in Suc-containing medium, they were then transferred to Suc-free liquid medium in the dark; on day 3, medium was replaced by either medium with or without Suc, or Suc-containing medium together with AZD-8055 for further 24 hours. Leaves 1 and 2 including the SAM were collected at times before dark transfer and 24 h after transfer. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through 2 technical replicates. T.test was used and asterisks indicate level of significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Error bars indicate SEM (between biological replicates).

4.3.7 The vasculature genes are partly TOR-dependent

Organ development gene expression signatures were monitored in presence of TOR inhibitor, and in this case demonstrated contrasting responses. It appears both organ gene classes are strongly stimulated by sucrose and down regulated in the absence of sucrose from medium in the dark. The expression of these marker genes decreased after 24 hours in the presence of sucrose and TOR inhibitor, but vasculature marker *ATHB8* mildly reduced compared to the seedlings in presence of sucrose (Fig. 4.8). The vasculature genes are less TOR-dependent than the plastid/mesophyll. Plastid-related genes *GC1* is particularly TOR-dependent.

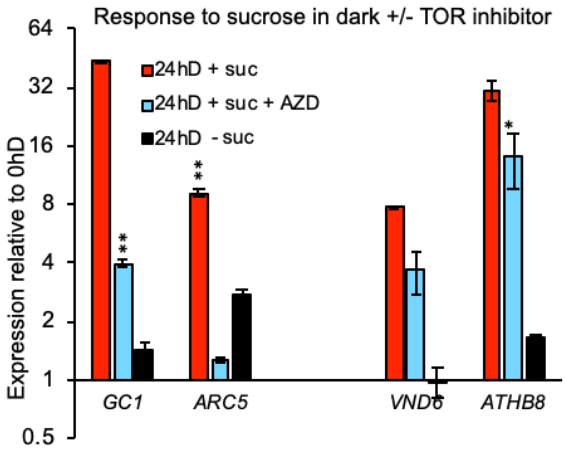

Figure 4. 8 Plastid/mesophyll marker genes expression confirmed to be extremely TOR-dependent. The gene expression program changes were monitored in 7-day old continuous light grown WT seedlings in Suc-containing medium, they were then transferred to Suc-free liquid medium in the dark; on day 3, medium was replaced by either medium with or without Suc, or Suc-containing medium together with AZD-8055 for further 24 hours. Leaves 1 and 2 including the SAM were collected at times before dark transfer and 24 h after transfer. The gene expression quantification was produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates, each quantified through 2 technical replicates. T.test was used and asterisks indicate level of significance $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Error bars indicate SEM (between biological replicates).

4.3.8 Cell proliferation in the presence of light could be blocked in CO_2 free air

Having confirmed (in the previous chapter) that meristematic growth activity and leaf development in response to light are COP1-dependent, in other words photomorphogenesis-dependent, the remaining question was if photosynthesis-generated sucrose is necessary for growth responses of young leaf primordia to the light. 7 day light-grown CYCB1;1::DB-GUS-carrying seedlings grown on vertical plates with or without sucrose were transferred to dark. This would cause an arrest of both photosynthesis and photomorphogenesis. The seedlings in the plates with no sucrose were arrested in presence or absence of CO_2 free air in the dark. This would allow or disallow photosynthesis upon subsequent transfer to light. By transferring seedlings on the vertical plates containing sucrose to dark (Vertical plates were used in order to put seedlings in direct contact with sucrose in the media), cell proliferation remained active, although this activity was reduced in the CO_2-free air plates by nearly half (Fig. 4.9 A and B). After transferring the vertical plates containing dark-adapted

seedlings with no sucrose to the light for 8 hours, they showed the re-activation of cell proliferation, with more than 40% of the leaf area exhibiting activity of the cell proliferation reporter (Fig. 4.9B). In contrast, in the absence of sucrose in the plates, the cell proliferation activity ceased in the dark. Cell proliferation activity was highly reduced in the CO_2-free air in the light, only in the presence of CO_2 it reappeared (Fig. 4.9, A and B). It is nevertheless interesting to mention the difference in the cell proliferation activity in the dark in presence of sucrose in the seedlings with and without CO_2, indicating that the supply of CO_2, and the consequent photosynthesis, has a more pronounced role on regulation of CYCB1;1 than the simple presence of exogenous carbohydrate.

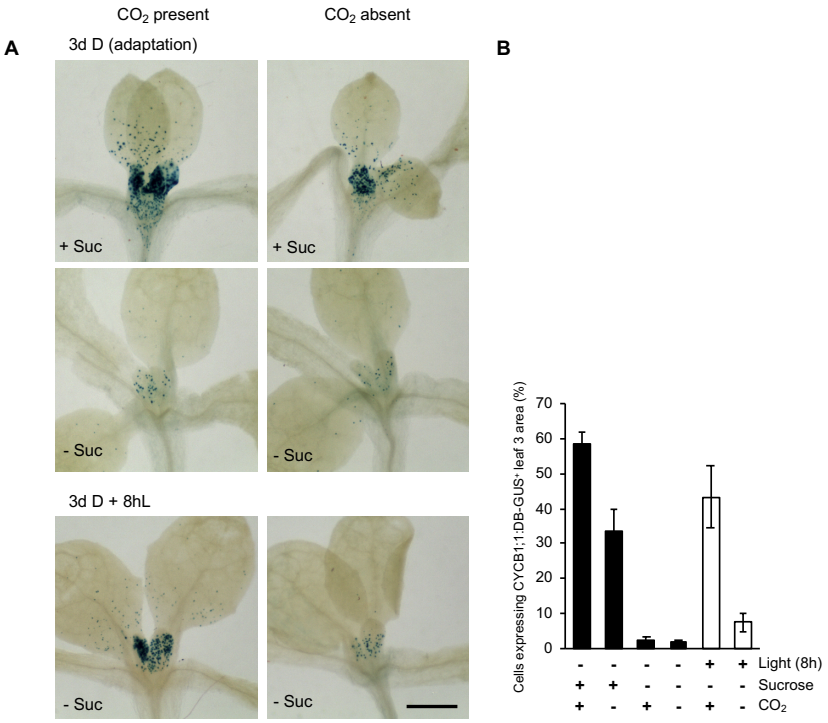

Figure 4. 9 Inhibiting photosynthesis in the presence of light will block cell proliferation. The CYCB1;1::DB-GUS-expressing seedlings were cultured on Suc-containing solid-medium vertical plates for 7 days in the light then transferred to new plates with or without Suc, in presence or deprivation of CO_2 in the dark. After dark adaptation, on day 3, seedlings on Suc-free medium were exposed to 8 h light (Number of samples: 20-25). **A**, Seedling were visualized for mitotic activity **B**, the area of mitotic activity was visualized for GUS expression and quantified using ImageJ software. Error bars indicate SEM. Scale bar: 500 µm.

4.3.9 Circadian responses of genes of interest

The circadian responses of genes used in this study, has been observed previously in, rhythmic temperature cycles, continuous light and after entrainment to light dark cycles. These genes are not likely to be regulated by the circadian clock. The previous studies indicated eight out of twenty genes selected to study in this project are demonstrating circadian expression. The only gene with circadian expression matching with the observed pattern in my study is ARR5, showing increase after transfer to the light. An extended time course ((published simultaneously, Mohammed et al. 2018)) experiment in sucrose free medium indicated that, the changes observed in the light did not fit an underlying endogenous, circadian control. It is the most likely a direct effect of presence of light. Evaluation of the possible circadian behavior of genes studied in this project was by using the LL_LLHC data series available at the Diurnal tool.

Table 4. 1 The table above illustrate previously observed circadian response of genes studied in this project. (http://diurnal.mocklerlab.org/).

Gene	AGI code	Circadian phase peak (ZT, hours)	Circadian response after start of day (ZT 0h)	Observed initial light response
KRP4	At2g32710	-	-	Down
RNR2A	At3g23580	20	Down	Up
H2A	At1g51060	-	-	Up
CYCB1;1	At4g37490	-	-	Up
RPS6	At4g31700	-	-	Up
EBP1	At3g51800	-	-	Up
AUX1	At2g38120	8	Up	Down
IAA1	At4g14560	5	Up	Down
HAT2	At5g47370	5	Up	Down
TAA1	At1g70560	-	-	Up
TAR2	At4g24670	-	-	Up
ARR5	At3g48100	8	Up	Up
EIN3	At3g20770	10	Up	Down
EBP	At3g16770	-	-	Down
bZIP1	At5g49450	-	-	Down
TPS9	At1g23870	10	Up	Down
GC1	At2g21280	-	-	Up
ARC5	At3g19720	0	Down	Up
VND6	At5g62380	-	-	Up
ATHB8	At4g32880	-	-	Up

4.3.10 RAPTORs disruption impact growth

Previous studies have concluded that *Arabidopsis TOR* gene mutation is embryo lethal (Menand et al., 2002) or mutation of its fundamental binding partner RAPTOR (RAPTOR1B) will impact growth and development (Salem et al., 2018). To assess the role of RAPTOR1 and RAPTOR2 in growth and development of leaf primordia, 3-day old dark grown seedlings of *raptor1*-1, *raptor2*-1, and WT were transferred to

continuous light for 1 and 2 days to follow growth and as a comparison, one set were kept in continuous dark for 5 days. The surface area of first leaf primordia were measured at various time points stated. *raptor1*-1 and *raptor2*-1 did not show any changes compared to WT in the dark. The *raptor1*-1 and *raptor2*-1 mutated seedlings have larger leaf primordia in the first day in light, however differences were not significant in the second day (Fig. 4.10).

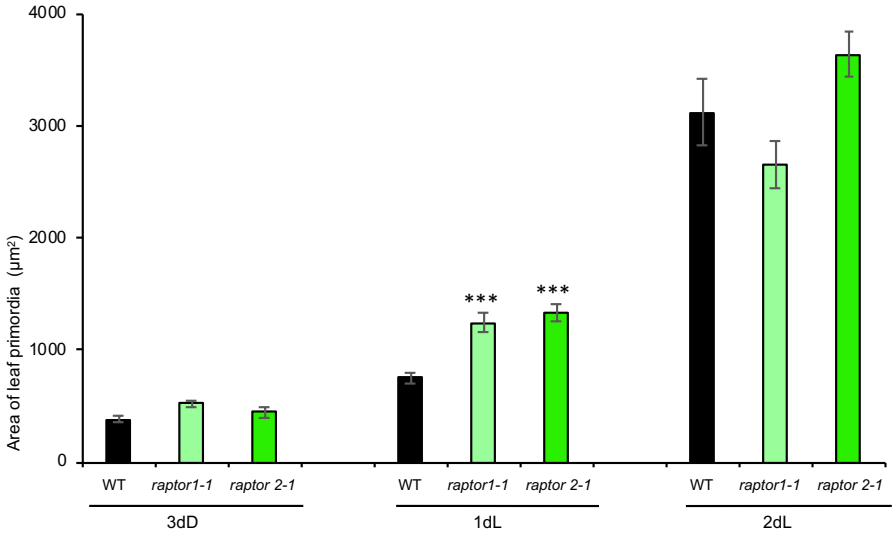

Figure 4. 10 The effect of RAPTOR disruption on growth of leaf primordia. 3-day old dark grown seedlings of *raptor1*-1, *raptor2*-1, and WT were transferred to the continuous light for 1 and 2 days to follow growth and one set were kept in continuous dark for 5 days. Horizontal plate contained medium with suc. The area of first leaf primordium was measured (Number of samples: 20-30). The error bars represent SEM. Asterisks represent the significance of differences between the WT and *raptor1*-1 and *raptor 2*-1: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

To investigate the functional significance of *raptor1*-1 on leaf development in the presence of sucrose but no light, the seedlings were germinated in continuous dark or light in vertical plates where the SAM is in direct contact with sucrose for 7 days. The surface area of the first leaf primordia measured at various time points was recorded. The seedlings in dark with no sucrose show small difference in their leaf area size compared to WT (Fig. 4.12 A and C). However, the presence of sucrose in dark resulted in growth initiation in both, with a larger leaf growth observed in mutant seedlings compared to the WT (Fig. 4.11A) (Fig. 4.12 B and D). The average area of leaf primordia of 7-day old light grown *raptor1*-1 seedlings in presence or absence of

sucrose were similar in size (Fig. 4.11B), therefore presence or absence of sugar in continuous light did not impact leaf growth and development (Fig. 4.13). However, compared to the WT they show a significant reduction in size (Fig. 4.13 C and D).

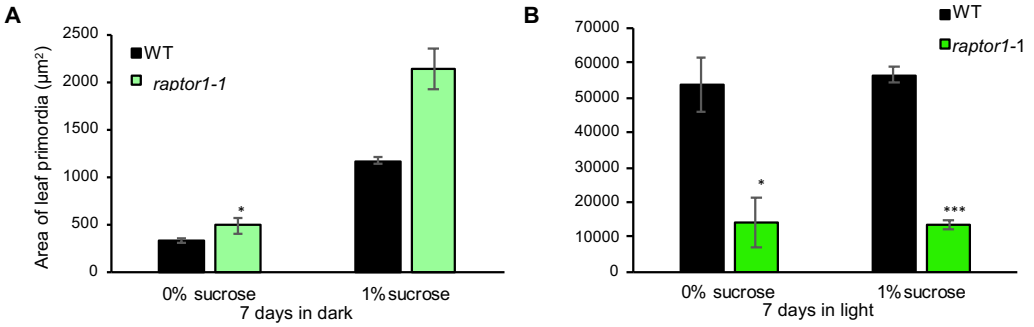

Figure 4. 11 Investigating the Leaf initiation phenotype of the raptor1-1 mutation. Seedlings were grown on Suc or no Suc-containing vertical plates placed in continuous dark or light for 7 days. Area of developing first leaf primordium of WT and *raptor1*-1 seedlings were measured. **A**, 7 days old seedlings in the Dark in presence or absence of Suc (Number of samples: 20-25). **B**, 7 days old seedlings in the light in presence or absence of Suc. The error bars represent SEM. Asterisks represent the significance of differences between the WT and *raptor1*-1: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

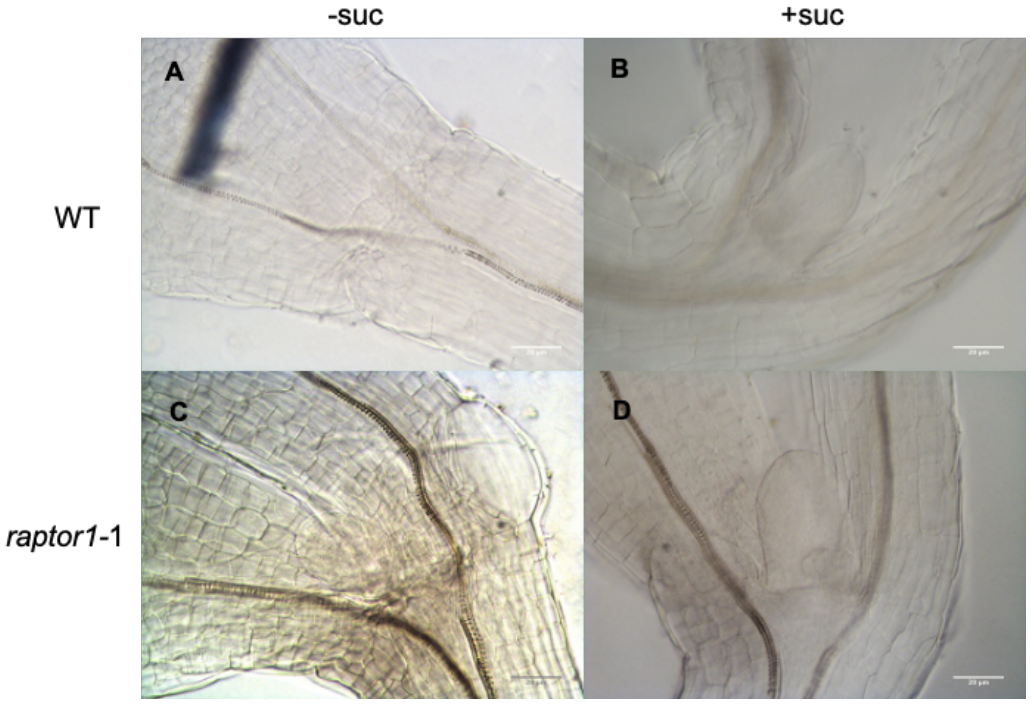

Figure 4. 12 Investigating the leaf initiation phenotype of the raptor1-1 mutation in the dark. The seedlings of WT and *raptor1*-1 were grown on Suc or no Suc-containing vertical plates for 7-days in the dark. **A**, WT, Suc-free plate. **B**, WT, Suc-containing plate. **C**, *raptor1*-1, Suc-free plate. **D**, *raptor1*-1, Suc-containing plate. Scale bar: 20 µm.

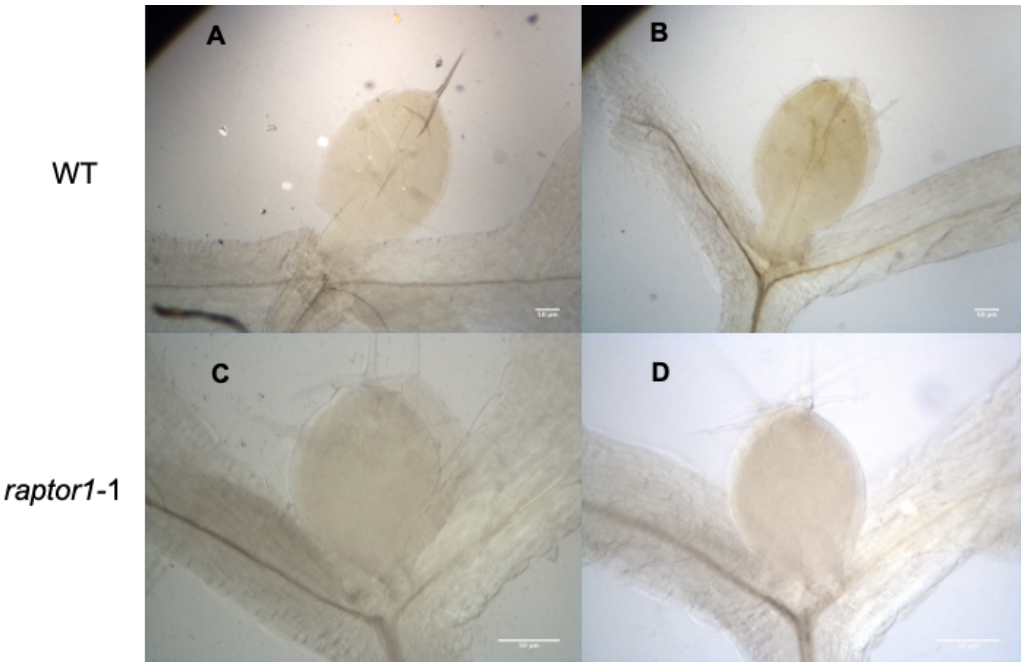

Figure 4. 13 Investigating the leaf initiation phenotype of the raptor1-1 mutation in the continuous light. The seedlings of WT, *raptor1*-1 were grown on Suc-containing horizontal plate for 7-days in the light. **A**, WT, Suc-free plate. **B**, WT, Suc-containing plate. **C**, *raptor1*-1, Suc-free plate. **D**, *raptor1*-1, Suc-containing plate. Scale bar: 50 μm.

4.3.11 S6K disruption impact on growth

The impact of *s6k1* and *s6k2* mutation on leaf primordia development was investigated. Both mutant seedlings were germinated in the dark and then transferred to light to investigate the functional significance of *S6K1* and *S6K2* in regulating meristem and leaf primordia early in growth. Seedlings were collected at different time points and the average area of primordia was measured. Dark grown seedlings show no difference in their leaf size compared to WT (Fig. 4.14). Transfer of 3d dark grown seedlings to light shows an early growth boost during the first day in *s6k1* and *s6k2* seedlings but not much change in both genotypes in the second day in light compared to control (Fig. 4.14).

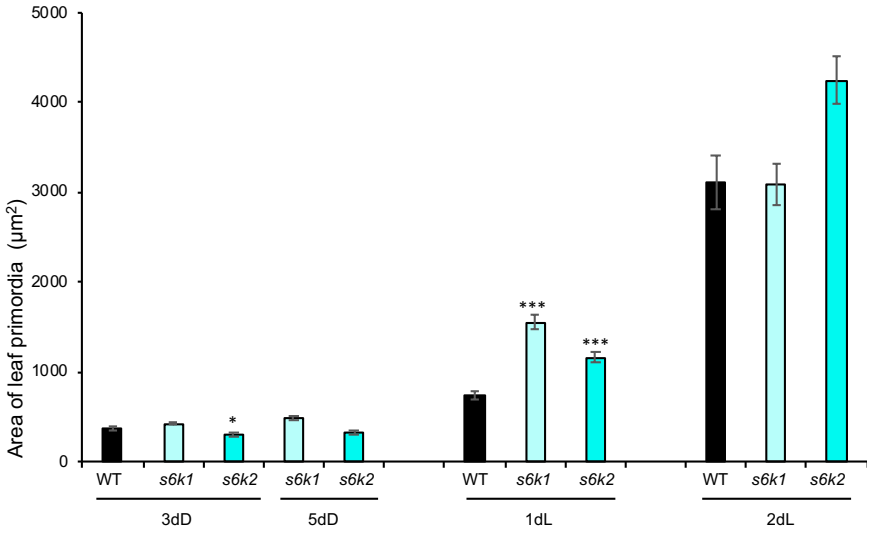

Figure 4. 14 Investigating the leaf initiation phenotype of the s6k1 and s6k2 mutation in the dark and light. The seedlings were grown and germinated in dark for 3 and 5 days, the seedlings were germinated 3-days in dark and then transferred to light for 1 and 2-days (Number of samples: 20-30). The area of first leaf primordia were measured. The error bars represent SEM. Asterisks represent the significance of differences between the WT, *s6k1* and *s6k2*: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

To illustrate the effect of carbohydrate availability (sucrose) and light on the mutant seedlings, they were exposed for a longer period. 7-day old dark grown seedlings of *s6k1* and *s6k2* in direct contact with sucrose on vertical plates showed a smaller boost in the mutant seedlings than in the WT (Fig 4.16 B, D, F). The WT in the presence or absence of sucrose shows a large difference in the primordia growth in the dark (Fig 4.15 A, B). The WT, *s6k1* and *s6k2* seedlings show arrested leaf growth in absence of sucrose in the dark (Fig 4.15A) (Fig 4.16 A, C, E). Both mutants have smaller leaf primordia in the light in presence and absence of sucrose in the medium (Fig 4.17 C, D, E, F). However, *s6k2* seedlings displayed smallest leaf primordia in absence of sucrose in the light (Fig 4.15B). This may indicate that both genes are necessary for a normal sized leaf primordium.

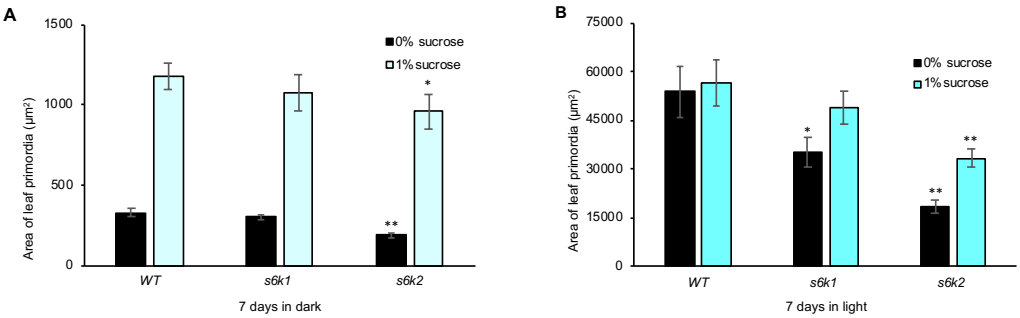

Figure 4. 15 Investigating the leaf initiation phenotype of the *s6k1* and *s6k2* mutant in the presence and absence of light and sucrose. The seedlings were grown in continuous light on horizontal plates or continuous dark in the presence or absence of Suc on vertical plates. The area of the first leaf primordia were measured. **A**, 7 days old seedlings in the Dark in presence or absence of Suc. **B**, 7 days old seedlings in the light in presence or absence of Suc (Number of samples: 20-30). The error bars represent SEM. Asterisks represent the significance of differences between WT and *s6k1* or *s6k2*: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

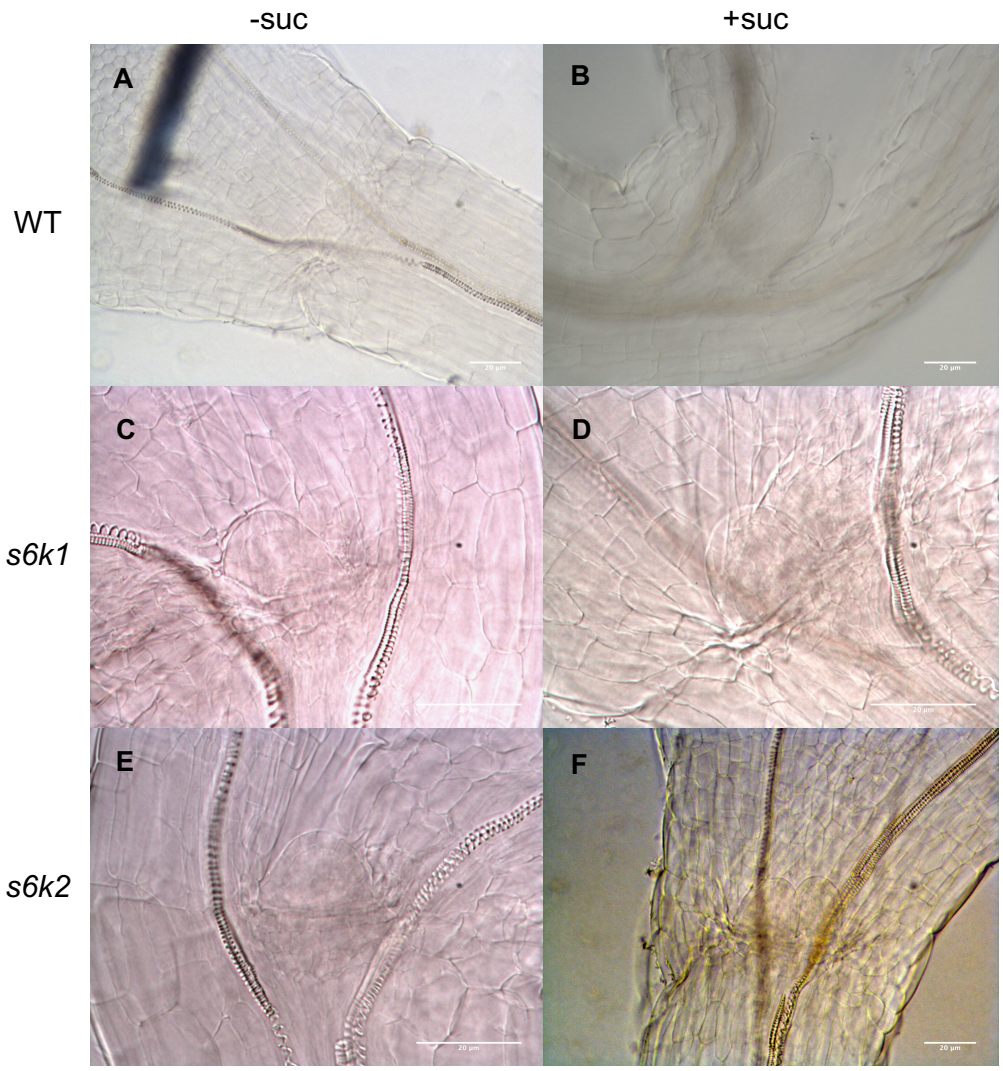

Figure 4. 16 Investigating the leaf initiation phenotype of the *s6k1* and *s6k2* mutation in the dark. WT, *s6k1* and *s6k2* were grown on vertical plates and after 3d cold treatment were transferred to continuous dark incubator for 7d. **A**, WT, Suc-free plate. **B**, WT, Suc-containing plate. **C**, *s6k1*, Suc-free plate. **D**, *s6k1*, Suc-containing plate **E**, *s6k2*, Suc-free plate. **F**, *s6k2*, Suc-containing plate. Previously the experiments confirmed the growth in the dark in presence of sucrose. Scale bar: 20 µm.

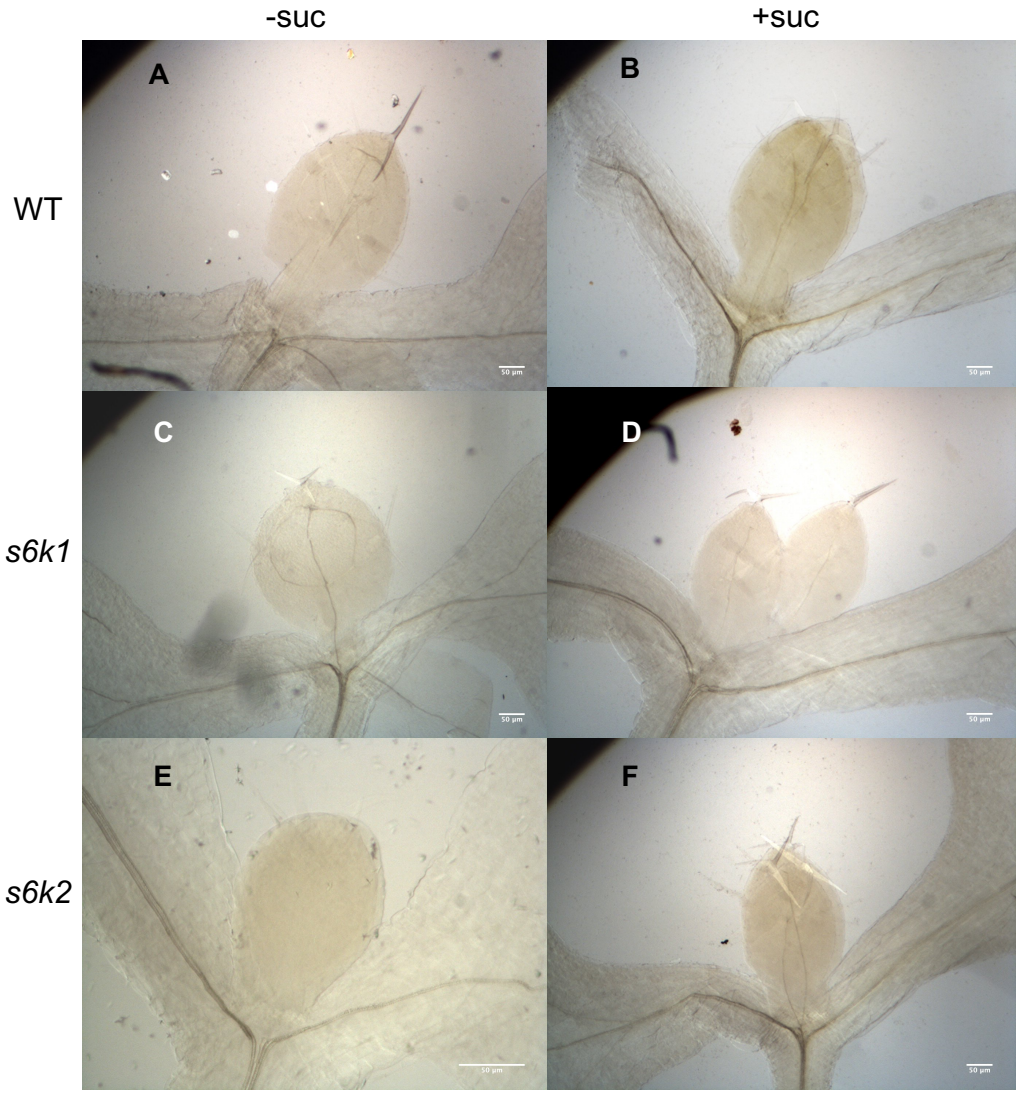

Figure 4. 17 Investigating the leaf initiation phenotype of the *s6k1* and *s6k2* mutation in the light. WT, *s6k1* and *s6k2* were grown on horizontal plate and after 3-days cold treatment was transferred to continuous light for 7-days. **A**, WT, Suc-free plate. **B**, WT, Suc-containing plate. **C**, *s6k1*, Suc-free plate. **D**, *s6k1*, Suc-containing plate **E**, *s6k2*, Suc-free plate. **F**, *s6k2*, Suc-containing plate. Scale bar: 50 µm.

4.3.12 bZIP Transcription factors

As previously observed bZIPs are involved in the starvation responses. I aimed to observe the effect of mutation of *bzip* genes on leaf development under continuous light where nutrient (reduced carbon) is available. The *bzip* transcription factor mutants were grown in continuous light for 7 days. The area of first true leaf was measured on day 7. *bzip63* and the double mutant *bzip1 bzip63* (hereafter referred to as *bzip1-63*) showed a reduction in the leaf size, although the triple mutant carrying in addition the *bzip10* mutation (hereafter referred to as *bzip1-10-63*) showed a small reduction compared to

WT. *bzip63-10* and *bzip1* did not show obvious phenotype difference in development (Fig. 4.18). Observing the seedlings for phenotype I realised that the seedling's first pair of leaf primordia are different in size, one is smaller than the other one, except *bzip1-10-63* (Fig. 4.19). To follow the effect of dark on starvation transcription factors in mutant seedlings, 7-day light grown seedlings were transferred to dark for 9 days. The *bzip* seedlings that fell on medium and whose shoot meristem was in direct contact with sucrose developed pale primordia with a long stem, however the seedlings which remained upright remained green, but their growth was arrested. Among the seedlings with one leaf primordium in contact with sucrose containing-medium, the one primordium in contact with media is elongated in comparison to the one not contacting media (Fig. 4.20).

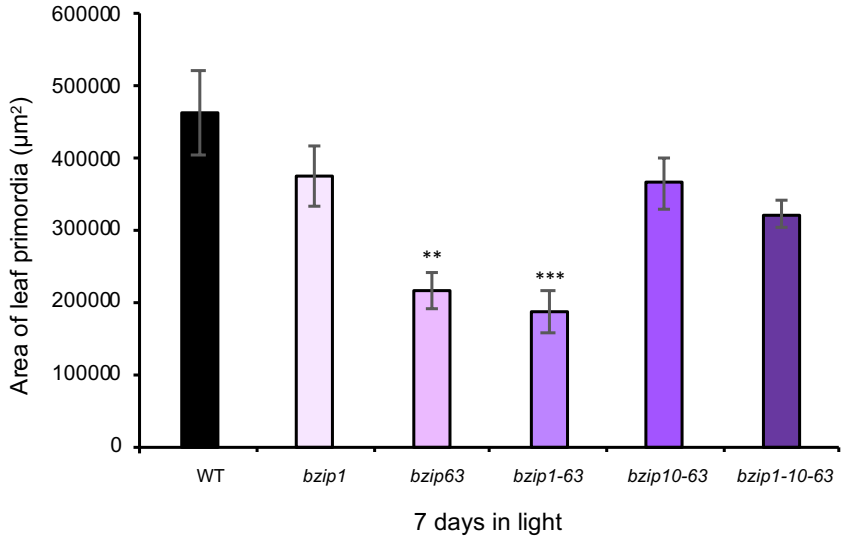

Figure 4. 18 Investigating the leaf initiation phenotype of the *bzip* transcription factors mutation in the continuous light. Seedlings were grown in the light for 7 days and the average area of the first leaf primordium was measured (Number of samples: 20-25). The error bars represent SEM. Asterisks represent the significance of differences between WT and mutant seedlings: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

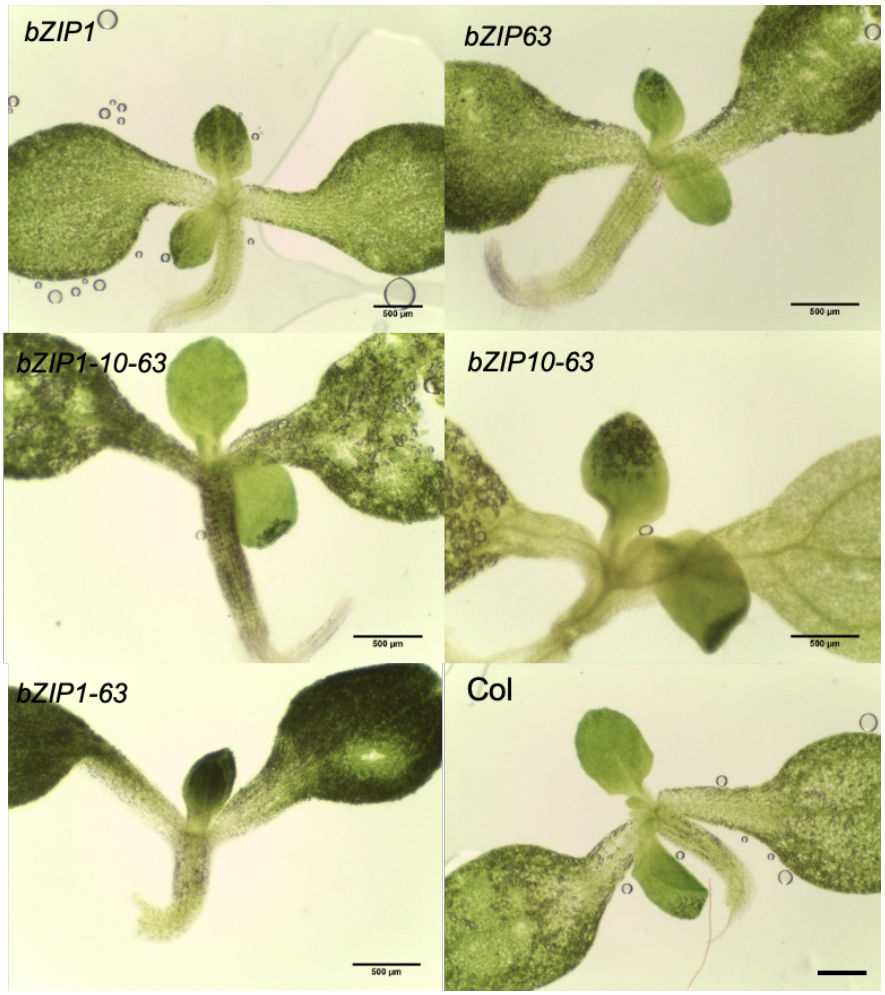

Figure 4. 19 The above images show the 7-day old light grown *bzip* mutated seedlings. The leaf size of *bzip63* and *bzip1-63* is almost half of the size of WT. Observing the seedlings for phenotype shows that the seedlings first pair of primordia have different size and one is smaller than the other, except for *bzip1-10-63*. Scale bar: 500 µm.

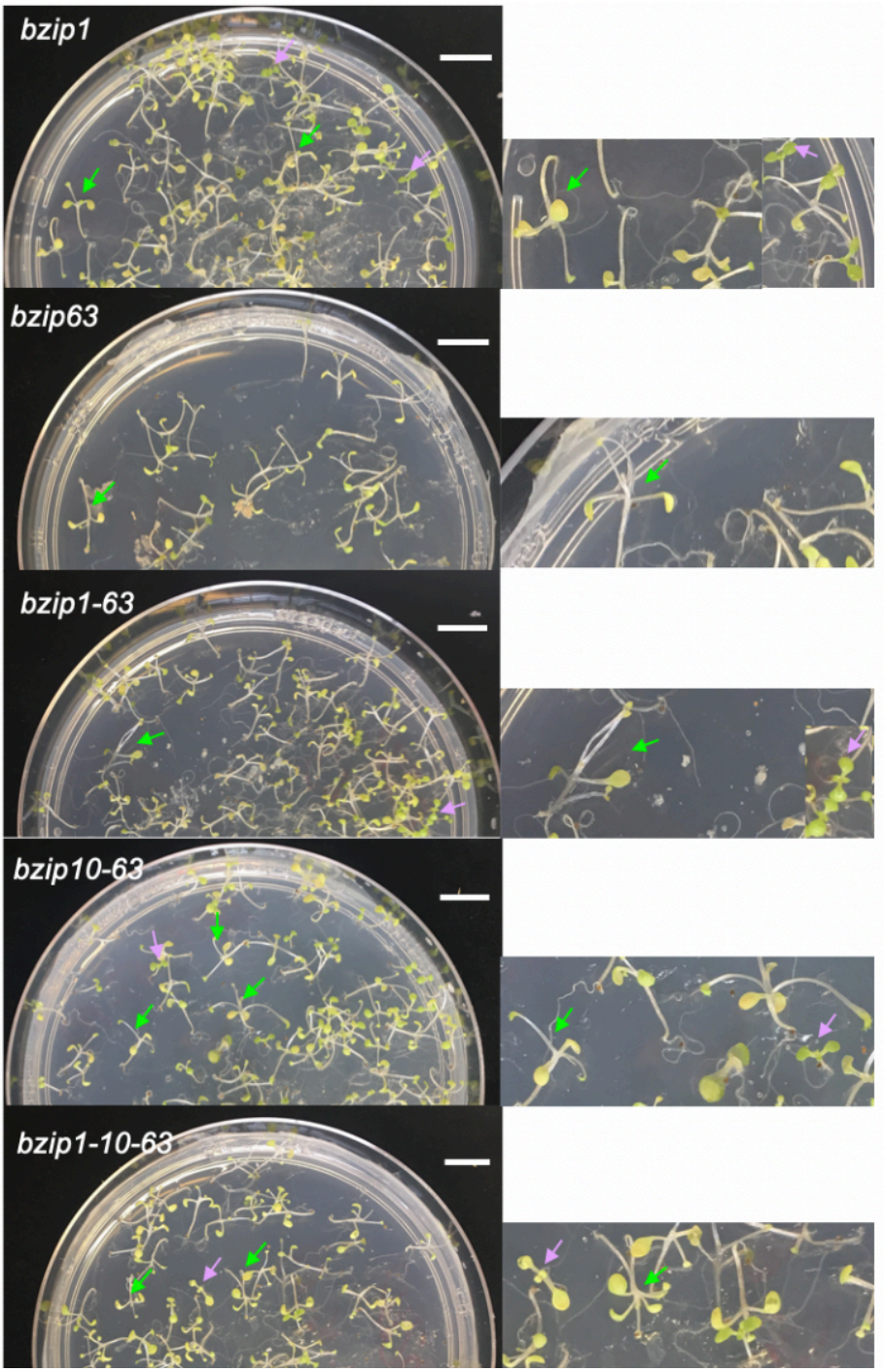

Figure 4. 20 The leaf primordium in contact with Suc-containing medium is elongated in comparison to the one not in contact medium. The above images show the 7-day light grown *bzip* mutated seedlings transferred to continuous dark for 9 days. Observing the seedlings for phenotype shows that the seedlings contacting medium (green arrow) are growing long petioles and pale yellow in colour, but the upright seedlings are green (purple arrow) and with no change in size. Scale bar: 10 mm.

4.3.13 A boost in cell proliferation upon transferring seedlings to HL

The intensity and quality of light are significant for plant growth and development (Kozuka et al., 2011; Tan et al., 2008; Weston et al., 2000). The ability of leaf to respond to light intensity shows that plants have evolved various mechanisms to adapt to changes in the environment (Fukuda et al., 2008; Long and Bernacchi, 2003). Plant growing under HL (300 $\mu mol.m^2s^1$) condition, develop leaves with several layers of palisade mesophyll to facilitate efficient photosynthesis performance (Gotoh et al., 2018; Kalve et al., 2014). To examine the effect of light quantity on cell proliferation activity of leaves, CYCB1;1::DB-GUS-expressing seedlings were grown in light (100 $\mu mol.m^2s^1$), acclimated to LL (40 $\mu mol.m^{-2}s^1$) and then divided into two groups, one placed in HL (300 $\mu mol.m^2s^1$) and the other kept in LL. The HL-grown seedlings had a slight increase in cell proliferation compared to the seedlings in LL (Fig 4.21A). Comparing the GUS stained area of HL-exposed leaves to LL, seedlings in HL showed more cells undergo proliferation (Fig 4.21C). As leaf primordia develop, the proportion of the laminae containing cells undergoing proliferation will gradually decline, and this was observed in LL but in this time window under HL the proportion remained constant. The point where the two groups differ the most in the graph is 8 hours after transfer to either HL or LL. This could be observed clearly from the microscopic images took 8 hours after the transfer (Fig 4.21B).

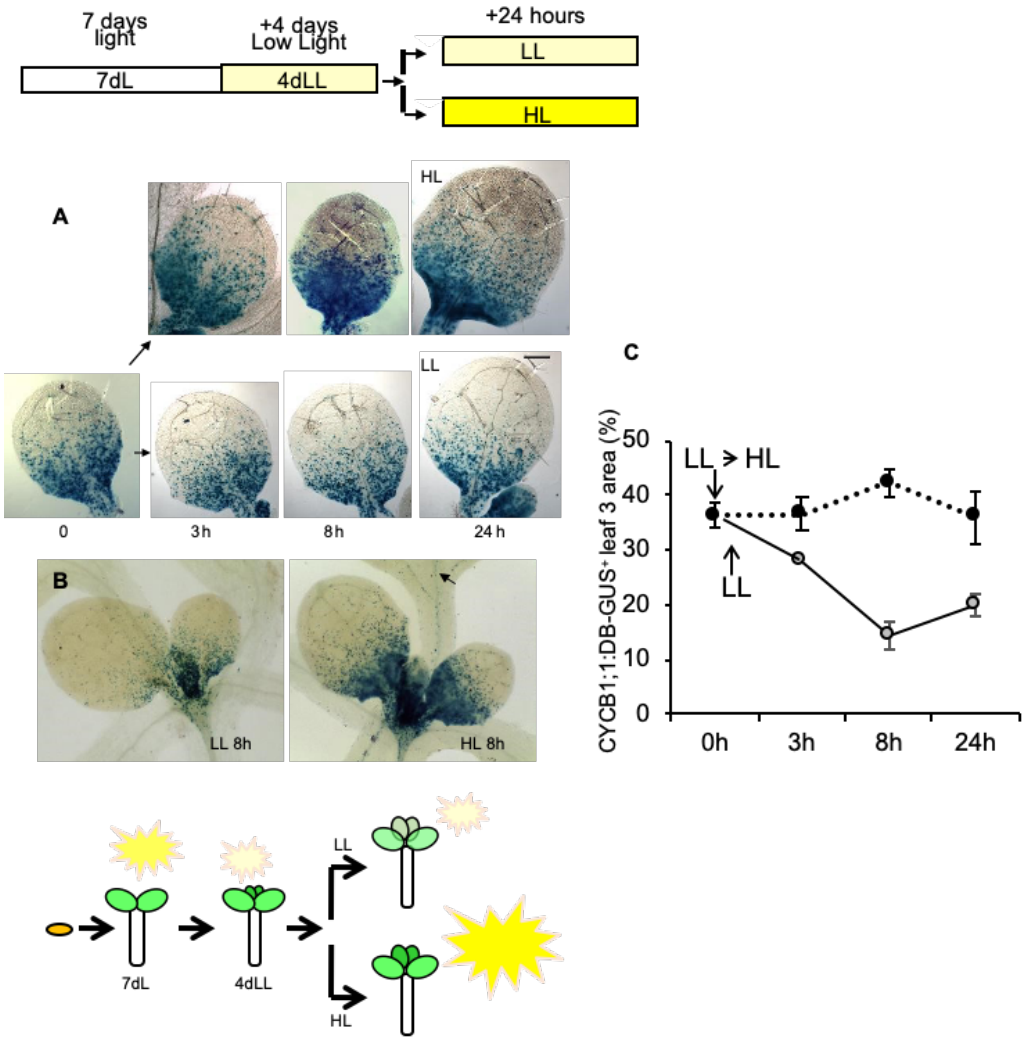

Figure 4. 21 Transferring seedlings to HL rapidly promotes the proliferation of competent cells. The CYCB1;1:DB-GUS-expressing seedlings were grown under continuous light on plates for 7 days (100 µmol.m^{2}s^{1}). At 7 days after germination, seedlings were transferred to soil and exposed to LL (40 µmol.m^{-2}s^{1}). After 4 days, seedlings were divided into two groups. One group was transferred to HL (300 µmol.m^{2}s^{1}) and the other group kept in LL. **A**, Leaf primordium 3 was harvested immediately at the time points correspond to 3, 8, and 24 hours in HL or LL. The area of proliferating cells visualized for GUS reporter was measured. The reference point of 0 hours corresponds to the point before transferring. **B**, displays leaves 3 and 4, 8 hours after transfer to LL or HL. **C**, the area of proliferating cells visualized for GUS reporter were quantified. The reference point of 0 hours corresponds to the point before transferring. The diagrams show the pattern of growth. Error bars indicate SEM. Scale bar: 200 µm.

4.3.14 HL exposure later during development activates cell proliferation in vascular cells

The question raised at this stage was whatever degree of exposure to HL quantity later during development is able to facilitate more mitotic activity in the leaves. In order to observe the effect of HL exposure later in development, 10 day continuous light-grown CYCB1;1::DB-GUS expressing seedlings were exposed to HL for 48h and visualized for GUS reporter activity. A young leaf primordium from seedlings grown under continuous light for 7 days exhibited a vast number of cells going through proliferation**.** After 3 more days in continuous light, leaf primordium mitotic activity was highly reduced and almost disappeared in the seedlings transferred to dark for 3 days (Fig. 4.22A). 10-day old continuous light-grown seedlings exposed to HL led to a few more mitotic events in the leaves, but these were minimal compared to the primordia of seedlings dark-arrested for 3 days then re-expos to light (compare Fig. 4.22A with Fig. 3.7 of chapter 3). The mitotic activity induced in these leaves by HL took place in the vascular cells located throughout the leaf laminae (Fig. 4.22A). The LL and HL grown seedlings later during development when transferred under identical irradiance and photoperiod yet still preserve the light quantity phenotype they showed before their transfer to photoperiodic growth light after 3 days.

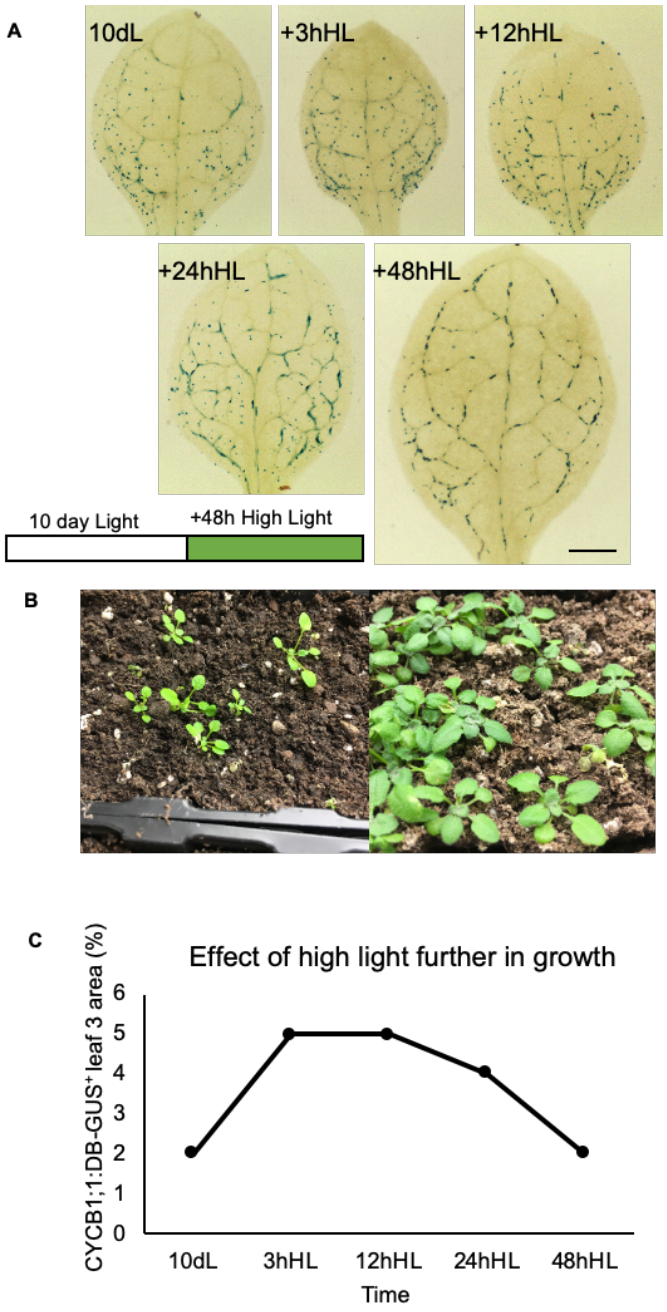

Figure 4. 22 The effect of HL quantity was followed further in growth. **A**, 10 day continuous light-grown CYCB1;1::DB-GUS expressing seedlings were exposed to HL for 48h. The seedlings were harvested at the times of 3, 12, 24, and 48 hours in the HL and visualized and quantified GUS reporter activity. **B**, The percentage of cells going through cell proliferation. Scale bar: 500 µm. **B**, WT seedlings grown under continuous light on plates for 7 days. At 7 days after germination, seedlings were transferred to soil and exposed to LL. After 4 days, seedlings were divided into two groups. One group was transferred to HL and the other group kept in LL for further 3 days, then placed under identical irradiance and photoperiod for another 3 days. The LL seedlings (image A on left) and HL grown seedlings (image B on right) later during development when transferred under identical irradiance and photoperiod.

4.3.15 HL upregulates the growth gene programme and stimulates energy signalling

The results have shown two mechanisms are involved in the leaf initiation control by light, through energy signalling and hormonal mechanisms. The findings from experiments in the dark with supply of sucrose showed energy signalling is capable of re-initiating mitotic activity. Energy signalling control over meristematic cellular activity could adjust the length of organ growth to available resources, which is constantly changing. An experiment equivalent to that in (Fig. 4.3) was carried out to assess, based on gene-expression signatures, the changes in energy signalling and hormonal reshuffle in response to LL and HL exposure. Seedlings were cultured on sucrose-containing medium (horizontal plates) for 7 days under standard light (100 μmol m^{-2} s^{-1}). Then seedlings were transferred to soil and exposed to LL. After 4 days, some of the seedlings were transferred to HL and some maintained in LL. Leaf primordia 3 and 4 were harvested immediately over a time course in HL and LL. The markers of cell proliferation activity *RNR2A* and *CYCB1;1* were used to follow the cell proliferation activity in the seedlings. *RNR2A* showed a gradual increase with the highest peak at 8 hours HL and *CYCB1;1* was slightly increased in 1 and 3 hours and slight decreased after 24 hours in HL (faster growth would involve earlier exit from the phase of cell proliferation competence). This is consistent with the early increase in mitotic activity observed in the CYCB1;1::DB-GUS expressing seedlings. In LL, *RNR2A* and *CYCB1;1* expression remained unchanged compared to the time point before transfer. The ribosomal biogenesis and translation-associated gene *RPS6* expression was up-regulated in HL and showed a slight decrease in LL (Fig. 4.23).

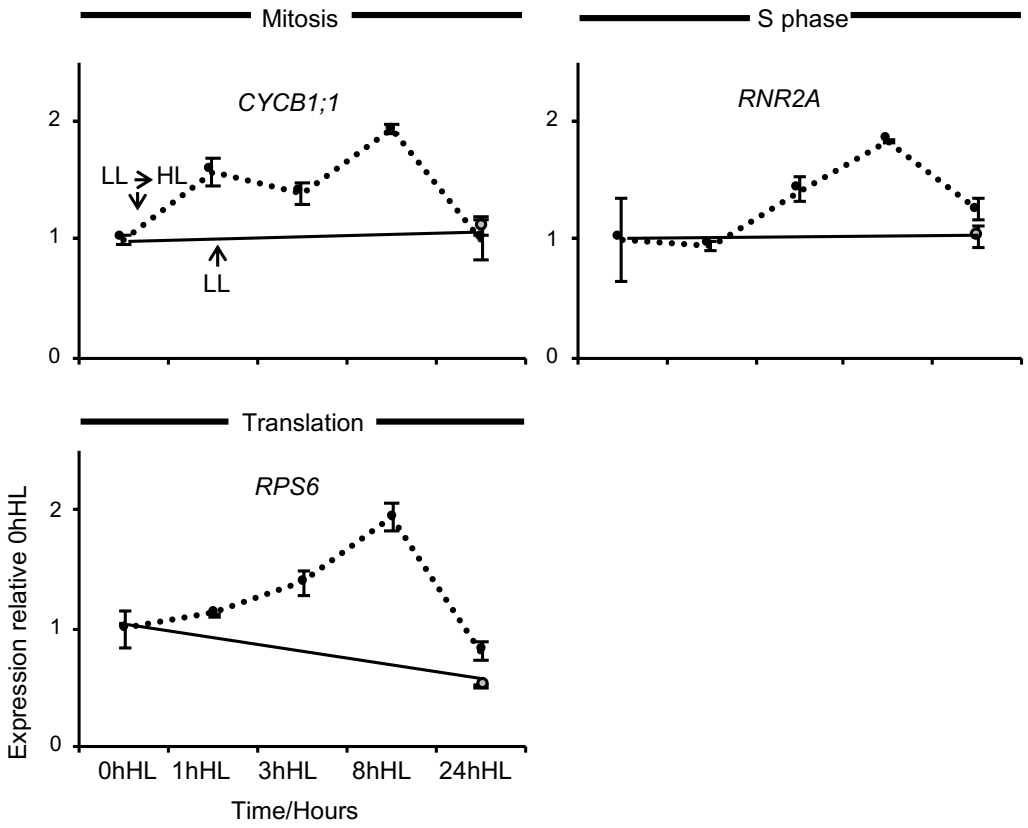

Figure 4. 23 HL exposure increases cell cycle and translation gene expression responses in the emerging leaf primordia. WT seedlings were grown in continuous light on plates for 7 days. At 7 days after germination, seedlings were transferred to soil and adopted to continuous LL for 4 more days. After 4 days, seedlings were divided in two groups. One group were transferred to HL and one maintained in LL. The new leaves were harvested at time points 3, 8, and 24 hours in HL or LL. The reference point of 0 hours in high or low light corresponds to the point before transferring to HL. The gene expression quantifications were produced using qRT-PCR. Note only the HL produced a burst in growth. The values of each time point in the graphs represent averages of 3 biological replicates (n=100 seedlings per biological replicates), each quantified through 2 technical replicates. Error bars indicate SEM (between biological replicates).

4.3.16 Hormonal responses in different light quantity

The markers of the response to hormones, auxin (*AUX1*), cytokinin (*ARR5*), and ethylene (*EIN3*), were used to understand whether they participate following exposure to HL. Auxin-responsive gene *AUX1* showed a gradual increase with the highest expression in 8 hours but transcript levels decreased under LL. Cytokinin responsive gene *ARR5* was unchanged in HL and showed a slight increase in transcript levels under LL. Ethylene-responsive gene *EIN3* was unchanged in both LL and HL (Fig. 4.24).

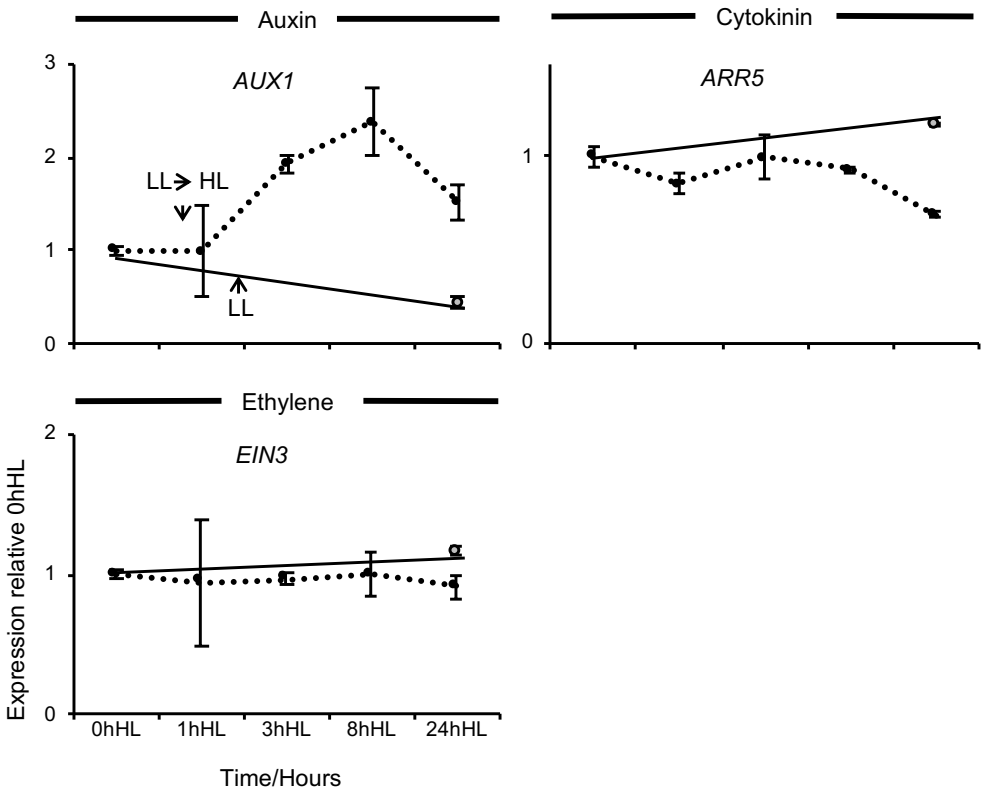

Figure 4. 24 The hormonal gene expression changes upon transferring to HL in the leaf primordia. WT seedlings were grown in continuous light on plates for 7 days. At 7 days after germination, seedlings were transferred to soil in continuous LL for 4 more days. After 4 days, seedlings were divided in two groups. One group were transferred to HL and one maintained in LL. The new leaves were harvested at time points 3, 8, and 24 hours in HL or LL. The reference point of 0 hours in high or low light corresponds to the point before transferring to HL. The gene expression quantifications were produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates (n=100 seedlings per biological replicates), each quantified through 2 technical replicates. Error bars indicate SEM (between biological replicates).

4.3.17 Light quantity sensitively regulates energy signalling in young developing leaves

As it was expected, the transcript levels of carbohydrate starvation response genes *TPS9* and *bZIP1* were decreased in HL and remained unchanged in LL (Fig. 4.25). The decreased transcript level of starvation genes under LL conditions illustrated that light quantity sensitively regulates their expression. These genes do not "turn on" when available sugar is very low, below some threshold, their expression level correlates inversely with sugar level, and while low under LL relative to dark, it becomes further reduced under HL.

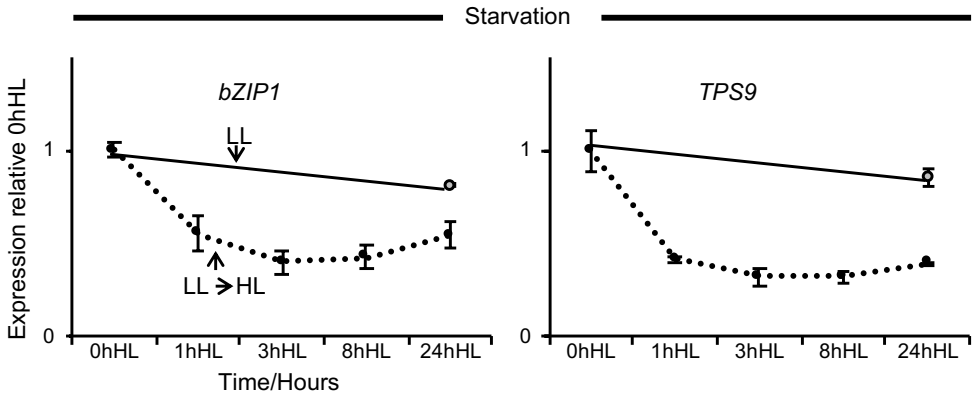

Figure 4. 25 The starvation gene expression changes upon transferring to HL in the leaf primordia. WT seedlings were grown in continuous light on plates for 7 days. At 7 days after germination, seedlings were transferred to soil in continuous LL for 4 more days. After 4 days, seedlings were divided in two groups. One group were transferred to HL and one maintained in LL. The new leaves were harvested at time points 3, 8, and 24 hours in HL or LL. The reference point of 0 hours in high or low light corresponds to the point before transferring to HL. The gene expression quantifications were produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates (n=100 seedlings per biological replicates), each quantified through 2 technical replicates. Error bars indicate SEM (between biological replicates).

4.3.18 Organ-representative genes transcript levels in different light quantity

The vascular differentiation genes *VND6* and *ATHB8* transcript levels were elevated slightly within 8 hours of HL exposure. They showed no change in transcript levels in continuous LL. The early chloroplast biogenesis markers *GC1* and *ARC5* were also elevated slightly after 8 hours in HL and unchanged in LL (Fig. 4.26).

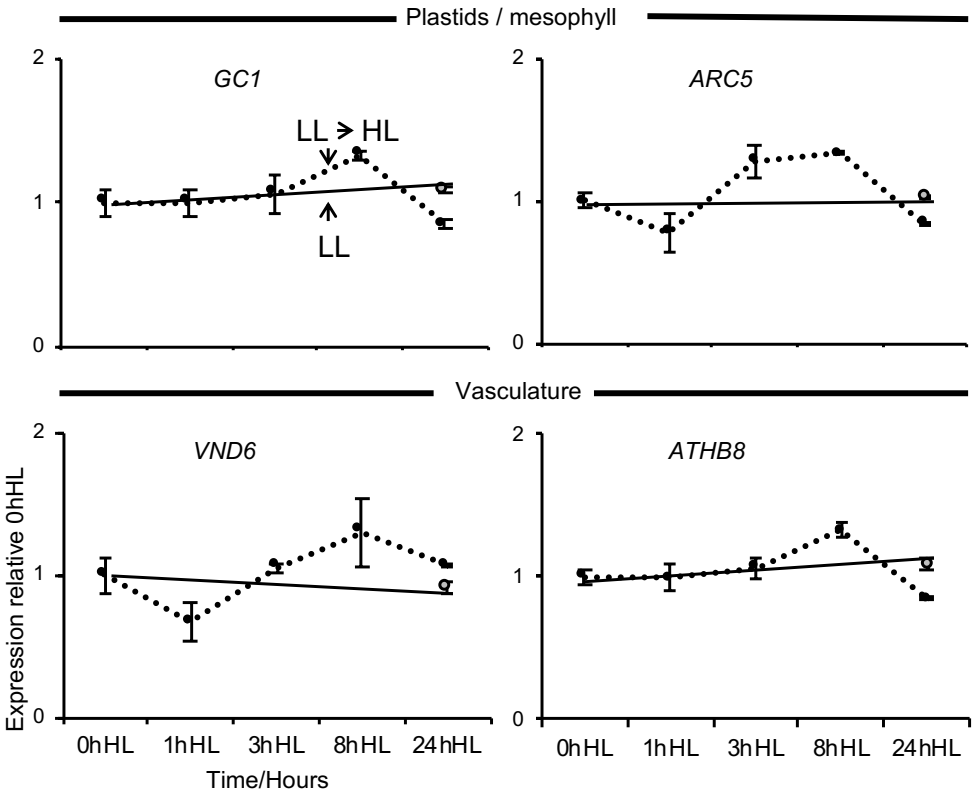

Figure 4. 26 The vascular differentiation and chloroplast biogenesis gene expression changes upon transferring to HL in the leaf primordia. WT seedlings were grown in continuous light on plates for 7 days. At 7 days after germination, seedlings were transferred to soil in continuous LL for 4 more days. After 4 days, seedlings were divided in two groups. One group were transferred to HL and one maintained in LL. The new leaves were harvested at time points 3, 8, and 24 hours in HL or LL. The reference point of 0 hours in high or low light corresponds to the point before transferring to HL. The gene expression quantifications were produced using qRT-PCR. The values of each time point in the graphs represent averages of 3 biological replicates (n=100 seedlings per biological replicates), each quantified through 2 technical replicates. Error bars indicate SEM (between biological replicates).

4.3.19 The dark arrested meristem is activated with direct access to sucrose and initiates leaf development by manipulating hormone homeostasis

Following the extended darkness and sucrose supply to the seedlings for 6 days, an internode between the young leaf primordia and cotyledons and cell proliferation activity was observed (Fig. 4.2C). To further establish the prolonged exposure of the SAM to sucrose in the dark and observe the development of organ in darkness, seedlings were placed in dark for 6 weeks. Sucrose was supplied in the solid medium in the vertical plates to avoid the possibility that hypoxia takes place in liquid medium. The seedlings cultured in the horizontal sucrose-containing plates showed leaf growth

and initiation only in the presence of light, and remained arrested in the dark (Fig. 4.27, A and B). The direct access of the SAM to sucrose in the liquid medium is the reason for the observed leaf initiation in the dark, as the seedlings cultured in sucrose-free medium showed no leaf initiation (Fig. 4.27, C and D). *Arabidopsis* seedlings grown in the dark for 42 days demonstrated that sucrose can promote hypocotyl elongation, and also causes the appearance of internodes (Fig. 4.27, E and F) (Zhang & He, 2015). The growth observed after prolonged exposure of the SAM to sucrose led to growth of extremely elongated seedlings, with very long petioles, new leaves and internodes (Fig. 4.27, E-H). The rib meristem mediates stem elongation in *Arabidopsis* and gives rise to elongation of a miniature internode during vegetative growth (Ruonala et al., 2008). The huge stem internodes' elongation here indicates that the rib meristem is activated prematurely. Meanwhile an underdeveloped leaf lamina can barely be observed but interestingly, seedlings developed normal flower buds (Fig. 4.27H).

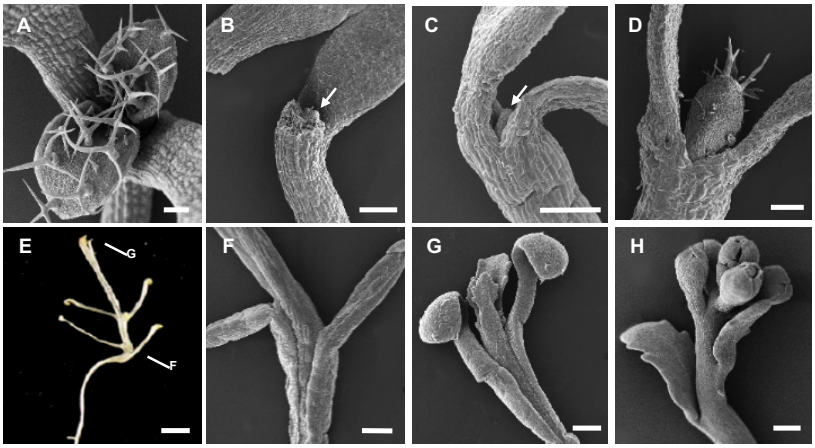

Figure 4. 27 The dark arrested meristem is activated with direct access to sucrose and initiated leaf development or by manipulating hormone homeostasis. All seedlings were grown in continuous dark except for A. **A**, WT, continuous light, 7-days light, horizontal Suc-containing medium plate. **B**, WT, 7 days, as A but continuous dark, the arrow points to the arrested leaf primordia. **C**, WT, 17 days Suc-free liquid medium, the arrow points to the arrested leaf primordia**. D**, WT, 7 days in the dark, Suc-containing liquid medium to put the SAM in the direct contact to sucrose, as a result a grown leaf primordium could be observed. **E**, WT 28 days in the dark with direct contact with sucrose, the arrows point to the different type of growth takes place in the absence of light. **F**, **G**, and **H**, are the enlarged part of seedlings in E, WT, 42 days, vertical Suc-containing plate. The arrows point to the primordia Scale bar: 100 μm (A-D and I-L), 200 μm (F-H) AND 2mm (E).

4.4. Discussion

Through the experiments carried out in this chapter, I aimed to understand the role of energy signalling in activating the shoot apex and regulation of early leaf growth in *Arabidopsis.* One way to address this was to replace the action of light with sucrose and follow the effect on organ growth. Secondly, having observed important genetic programme in the dark in response to sucrose supply, a pharmacological approach carried out to observe which of those genetic aspects were dependent on the TOR pathway. Seedlings grown in the light undergo photomorphogenesis, where their germination is initiated and followed by developing leaf primordia from the SAM (Arsovski et al., 2012). The emergence of leaves requires many intrinsic responses, some of which occur rapidly at the transcript levels. The process of growth is using most of energy resource and it is vital to have mechanisms to sense and report the availability of nutrient and energy. Sugars act as metabolic signals and energy source to regulate plant growth. Access of seedlings to sucrose in the dark led into the stimulation of cell proliferation, a boost of meristematic activity delaying cells going through expansion (Li et al., 2017; Matsoukas, 2017; Pfeiffer et al., 2016; Roldán et al., 1999; Van Dingenen et al., 2016). The results of my observations clearly showed direct access of the SAM to sucrose removed the dark arrest of seedlings completely (Fig. 4.1). The plants developed in the dark produce petioles and internodes, but leaves' shape is different from photomorphogenic-like rosette leaves (Fig. 4.27G). As a result, sugar on its own is not able to produce leaves in the absence of light similar to the ones developing under light exposure. The developmental behaviour of seedlings in the dark is similar to the seedlings over-producing auxin and an extreme form of shade avoidance (Boerjan et al., 1995; Chen et al., 2014).

The gene expression analysis of dark-grown seedlings with the SAM in direct contact with sucrose suggests sucrose on its own could activate part of the expression response of hormone-related and cell cycle genes (Fig. 4.3, 4.4). The expression of the cell cycle signature genes gradually increased once sucrose was available to seedlings. A surprising finding was the fact that sucrose is able to induce some auxin responses and changes were smaller for cytokinin readout gene *ARR5*. Cytokinin impact on plant growth is affected by both light and sucrose (Guo et al., 2005). This experiment together with monitoring expression response of signature genes of cytokinin in presence or absence of light and sucrose conclude the effect of both on cytokinin but the effect of sucrose on cytokinin in my experiments could be seen more than effect of light (Guo et

al., 2005). Herein, one interesting difference between growth stimulation by sucrose or light is the fact auxin-responsive *AUX1* gene transcript level was reduced in the dark adaptation period and supply of sucrose elevated responses, however in the dark-to-light transition auxin responses transiently decreased but this rapid reduction in the expression was not observed under influence of sucrose. This observation, confirmed by three different signature genes of auxin action, suggests that the transient export of auxin from the SAM under sucrose supply did not take place. The auxin export marker gene transcript level in the sucrose supply in the dark compared to the dark-to-light experiment showed that auxin export is photomorphogenesis dependent. The ethylene responses are strong in the dark and barely effected upon sucrose exposure. The previous chapter dark-to-light gene expression study exhibited that ethylene action quickly stops upon light exposure and therefore responding to photomorphogenesis. The auxin export in the light, perhaps something which quickly stops ethylene action. One important difference observed in the genetic responses to sucrose supply compared to the dark-to-light exposure was slower changes in transcript levels, which became clear after 8 hours. It is known that sugar signalling has a role in triggering signalling pathways involved in regulating plant growth (Lastdrager et al., 2014).

Starvation genes

The starvation genes used as "readouts" in my analysis were *TPS9* and *bZIP1* (Usadel et al., 2008). *TPS9* was suggested to be involved in sugar sensing and *bZIP1* in the plant carbohydrate starvation responses, and is also sugar-sensitive (Kang et al., 2010). In this study, both genes were used, not necessarily because of their individual roles, but because they are strongly energy status-dependent and behave as starvation genes. The microarray analysis by Lopez-Juez et al. (2008) had revealed plants experienced starvation in the dark, this being rapidly lost upon light exposure, specifically in the SAM, such response was utterly absent in the cotyledons (López-Juez et al., 2008). The expression of *bZIP1* and *TPS9* was reduced in response to sucrose supply, indicating the starvation state of the shoot apex was removed and presence of sucrose was visible but not complete within one hour (Fig. 4.5), while in the dark-to-light experiment, it was complete within one hour. However, the response of starvation genes to the sucrose supply in the dark was slower than in the dark-to-light transition. The reduction in expression of starvation genes was transient, as they were enhanced

after 24h in light, which may suggest that sucrose supply could not keep up with rapid growth. The expression of chloroplast biogenesis and vasculature genes in sucrose exposure indicates making leaves require both venation and mesophyll-related gene expression.

TOR

Among the genes used as signatures of the cell cycle, hormone action, and starvation, all responded to sucrose as they did to light. Monitoring this genetic programme TOR dependence showed the ethylene signature gene *EIN3* also was not sensitive to TOR inhibition. This indicates that the ethylene marker gene up-regulation is not mediated by energy signalling or the TOR pathway. Inhabiting the TOR pathway activity clearly reduced the positive effect of exogenous sucrose supply on cell cycle signature genes (*CYCB1;1*, *H2A*, *RPS6*, and *EBP1*). Two out of four auxin-responsive genes (*AUX1* and *HAT2*) were shown to be partially regulated by the TOR pathway (Fig. 4.7). The results obtained following inhibition of TOR indicate this master regulator is involved in mediating the cell proliferation and synthesis of protein in response to sugars (Lastdrager et al., 2014) and also auxin response relays on TOR signalling for regulation of some auxin response factors expression (Schepetilnikov et al., 2017, 2013). A member of family of GTPases Rho-related protein 2 (ROP2) are involved in the activation of TOR through auxin signalling (Schepetilnikov et al., 2017). Thus, TOR kinase may occupy a key role in growth actions involving auxin and energy signalling, therefore clarify some of their partly shared activities. Energy signalling action is key to the regulation of cellular growth and proliferation (Dobrenel et al., 2016; Xiong et al., 2013). The repression of starvation genes expression upon sucrose supply was unexpectedly further reduced in presence of TOR inhibitor suggesting their expression is not TOR-dependent. The meristematic activity could be increased (Li et al., 2017; Pfeiffer et al., 2016) by energy signalling and override the dark repression of the meristem through direct sugar supply. However, the leaves develop through energy signalling alone is not enough to make photomorphogenic-like leaves. The developmental growth observed in the dark through energy signalling is mainly petioles and internodes and leaves with limited leaf laminae. This indicates TOR-dependent sugar signal on its own is able to stimulate cell proliferation, but making a normal leaf requires a photomorphogenesis-like hormonal-responses. The growth in the dark is similar to the auxin overproducing plants (Chen et al., 2014). A hormone

that showed strong response in the dark, was down regulated in the light and was completely unaffected by sucrose and TOR inhibition (Fig. 4.7), is ethylene. This was deduced from expression of a single gene, *EIN3*, and examination of further marker genes could strengthen the case, the result was unambiguous. Ethylene is necessary for hypocotyl hook formation and has a positive effect on cell expansion (Ma et al., 2018). The auxin synthesis genes *TAR2* and *TAA1* were shown to be induced by ethylene, indicating the stimulation of ethylene action can enhance auxin synthesis at the hook (Stepanova et al., 2008; F. Vandenbussche et al., 2010). A low concentration of applied ethylene promoted the rate of leaf elongation in the *Poa alpina* and *Poa compressa* (Fiorani, 2002). However, by increasing the ethylene concentration higher than the growth-stimulating optimum, this hormone was shown to be growth limiting (Dubois et al., 2018). The elongation of organs such as petioles and internodes in the dark at the time when the gene expression results showed ethylene responses were high, suggests ethylene plays a key role in promoting growth of petioles, and reduced expansion of leaf lamina. The concluding mark from these results is that the activation of meristem through sucrose access takes place in a TOR pathway-dependent approach.

RAPTOR

The RAPTOR protein is conserved among all eukaryotes and a fundamental partner of the TOR kinase, to activate the TOR complex (Ryabova et al., 2019). In mammals, RAPTOR is important in stimulating responses to nutrient availability of S6K1, and therefore associate with mTOR to regulate cell size (Fingar et al., 2004) and also mTOR protein expression itself (Kim et al., 2002). The interaction between RAPTOR and mTOR could also modulate the activity of mTOR kinase in a negative way in sensitive conditions. It was proposed that RAPTOR and mTOR are held together in two ways: a constitutive interaction, necessary for mTOR function and a nutrients sensitive interaction (Hara et al., 2002) that forms under poor nutrient condition that repress mTOR kinase activity (Kim et al., 2002; Yadav et al., 2013). The mTOR-RAPTOR complex stability is increased during starvation period in the cell (Yadav et al., 2013). During stress situation ABA leads to dissociation of RAPTOR1 by phosphorylation from TOR by activation of SnRK2. Thus, ABA represses TOR through the SnRK (which is also how starvation acts), and that the SnRK action

happens by phosphorylating and dissociating an active TOR partner RAPTOR1 (Shi et al., 2018). To better understand the interaction of RAPTOR with TOR, the *raptor1*-1 mutation was transferred to dark in presence and absence of sucrose. *raptor1*-1 mutant growth in presence of sucrose in dark shows increase in primordia growth compared to WT, and also a small increase in the dark grown seedling in the absence of sucrose. Previous studies have shown both *raptor1-1* and *raptor2-1* have no phenotype when grown under non-limiting light condition (Deprost et al., 2005; Moreau et al., 2012). These results suggest providing exogenous sucrose to the SAM will compensate for the reduction of functional RAPTOR1. Based on the information from mammalian studies, we can also suggest that under nutrient poor conditions in the dark, the stability of RAPTOR interaction with TOR increases and inhibits growth but in the condition where functional *raptor* is reduced this probably leads to a reduction in RAPTOR-TOR complex, thus inhibition of growth happens less pronounced. The larger primordia size of *raptor1*-1 in presence of sucrose in comparison to WT may also suggest an inhibitory role for this protein aside from its growth promoting function. However, the presence of functional RAPTOR1 is important for a normal life cycle and development of *Arabidopsis* plant in continuous light. The reduction in size in continuous light may indicate *raptor 1-1* affects the leaf growth later in development and this effect cannot be observed early in growth.

S6K

RAPTOR interacts and forms a complex with S6K (a putative substrate of TOR), as well as with TOR, to regulate the responses to osmotic stress: the TOR pathway seems to be responsible for adjusting cell growth through controlling S6K activity (Mahfouz, 2006). The data provided by microscopic study to illustrate the physiological role of S6K1 and S6K2 in regulation of meristem, and therefore leaf development, shows *s6k2* seedlings in dark develop smaller primordia compared to WT but not *s6k1* seedlings. Mutation in a single *S6K* gene shows no phenotype. A study by Henriques et al (2010) indicated that S6K is required to repress cell proliferation in conditions where the seedling is starving. To follow the effect of nutrient availability (sugars) and light on growth of seedlings carrying a single mutation, they were exposed for longer period of time (7 days). The pervious experiments have shown the meristem could be activated in presence of direct sucrose access. Both *s6k1* and *s6k2* have slightly smaller primordia compared to WT but comparing their growth on sucrose-containing medium

to their own growth in absence of sucrose indicates the meristem is also activated in these mutant lines, but not as much as in the WT. S6K1 has been previously shown to be in an antagonistic relationship with E2FB in their activity and protein abundance (Henriques et al., 2013). RPS6 is the substrate of S6K and the process of phosphorylation could be initiated by nutrients and growth stimuli. Phosphorylation of RPS6 is highly reliant on light and auxin pathway but independent of photosynthetic activity (G. H. Chen et al., 2018). The intensity of RPS6 phosphorylation depends on the length of time in light exposure. Therefore TOR-S6K-RPS6 pathway is fundamental for protein synthesis in light for *Arabidopsis* seedlings (G. H. Chen et al., 2018). As ribosomal biogenesis is very important for growth of seedlings, the involvement of S6K in phosphorylation of RPS6 shows the importance of S6K and TOR in growth. The loss-of-function *rps6* mutant showed reduction in cell size, smaller leaf and slowed flowering and growth (Ren et al., 2012). The smaller primordia in the mutated seedlings may be a result of less protein synthesis. However, a single mutation of S6Ks does not show a substantial phenotype, which may reflect the compensation by other factors in the pathway. The double mutant of *s6k1s6k2 is* gametophytic lethal (Henriques et al., 2014) and hemizygous *s6k1s6k2/++* mutant contained smaller cells, however the transgenic plants overexpressing *S6K1* contained larger cells (Nukarinen et al., 2016).

bZIP transcription factors

The transcription factor bZIP63 has appeared to be crucial for primordia growth. The mutation present in *bzip63*, singly or in combination with *bzip1*, causes development of smaller primordia thus a delay in development. bZIP63 activity in response to starvation is regulated by KIN10, an α subunit of SnRK1 (a sugar sensing kinase). bZIP63 helps circadian clock genes to change the clock phase in response to sugars (Frank et al., 2018). Therefore, bZIP63 can partially activate the transcriptional responses necessary for energy relocation (Usadel et al., 2008). The effect of direct sucrose supply to the shoot meristem has shown that the growth observed in the dark in these seedlings is caused by sucrose, however the seedlings with sucrose access through their root (indirect access) in the dark cannot replicate the same effect. These seedlings remained green the hypocotyl elongation observed in the dark was not observed in these mutants. Another way to examine the effect of these seedlings would be to grow them on vertical plates for shorter time course and follow the changes. The

transgenic plants with a promoter-reporter fusion gene P_{bZIP}*:GUS* will give insight into the sucrose effect on proliferation in the bZIP genes of interest. A recent study showed *bzip63* mutation is insensitive to extended sucrose stimulation. The regulation of bZIP63 by glucose, ABA, and mannitol, suggest the role of this gene in mediating the cross-talk between carbohydrates and ABA responses (Matiolli et al., 2011). These studies indicate the importance of bZIP63 in sucrose-induced changes. It would be interesting to determine if activity of bZIP63 is controlled by energy level. One unexpected observation from our data is the fact that loss of bZIP10 appears to suppress the effects of loss of *bzip63*, given the WT behavior of seedlings carrying both mutations. Thus, loss of *bzip10* is able to correct the effect of loss of *bzip1* and *bzip63*. This is a surprising finding which could reflect opposing functions of these two transcription factors. Understanding the nature of the relationship between these two transcription factors would require further studies.

Light quantity influences growth

Upon transfer from LL to HL, expression of starvation genes, which was already low relative to dark, rapidly decreased further and remained relatively low during the remaining time course of the HL (Fig. 4.25). *bZIP1* has been shown to have a dramatic sugar response. Data from a previous study confirms that sugar signalling, and not the absence of light, regulates the transcript accumulation of *bZIP1* (Dietrich et al., 2011). Accordingly, down-regulation of transcripts level of starvation genes in these experiments indicates that the SAM is not starving and, consequently, there is sugar access to the SAM and also shows that HL sensitively regulates the energy signalling. Starvation in the dark seems a natural to take place. The earlier results showed it is not as simple, only the shoot apex is starving, and facilitating the access is sufficient to restart growth, therefore access is the likely control point by light. It is possible in the case of HL exposure, either this sugar access or the amount of sugar available are increased. It is becoming more obvious that energy signalling is the underlying mechanism regulating the cellular makeup of leaves under light intensity. Energy signalling allows this by regulating cell division and growth pathways, highly dependent on the TOR pathway. The young leaf primordia are dependent on the supply of sugar from photosynthetically active source leaves to go through cellular decisions (Andriankaja et al., 2012). The developing cellular anatomy of young leaves are determined by mature leaves, not the young primordia (Yano and Terashima, 2001).

Once a leaf is ready to photosynthesise, the cell proliferation transition to cell differentiation and expansion go hand-in-hand with the initiation of chloroplast differentiation (Andriankaja et al., 2012). However, by providing exogenous sugar to the young seedlings, chloroplast development is delayed, and cell proliferation remains active for longer and will increase the final size of the leaf (Dingenen et al., 2016). The HL-grown *Hopea odorata* showed to possess leaves with increased volume of palisade cells (Lee et al., 2000). Also, the size of HL palisade mesophyll cells is almost twice the size of LL grown equivalent cells of *Arabidopsis* seedlings (Davis et al., 2011). Thus, both the duration of the cell proliferation and cell size are important in determining the final size of the leaf (Gonzalez et al., 2009; Horiguchi and Tsukaya, 2011).

By exposing the 10 days old constant light grown seedlings to HL for 48h showed most primordia cell proliferation activity occurred early during development and was restricted to a critical period earlier in development. Later during development, when most cells had exited the cell cycle, only vascular cells were prone to respond to HL exposure by cell proliferation, thus HL is unable to re-activate mitotic activity in most cells in the leaf. The mesophyll biogenesis and vasculature responsive genes *ARC5* and *VND6* showed a transient reduction in the first 3 hours followed by an increase after HL exposure. The unaffected transcript levels of these organ-representative genes in the initial hours of HL and LL exposure illustrate the fact that cell cycle genes are accompanied with an early arrest in cellular differentiation. Later, the transcript elevation co-occurs with the up-regulation in the cell cycle genes' expression, indicating the transient burst in mitotic events goes along with cellular differentiation transcript changes.

The cell proliferation activity is higher in the primordia of seedlings grown under HL. In contrast, a consistent level of expression in LL may suggest that cell proliferation is passing less frequently through the mitotic phase. Translation is also a fundamental aspect of eukaryotic cell growth (Sonenberg and Hinnebusch, 2009). The changes in transcript levels of *RPS6* as translation and *CYCB1;1* mitosis and *RNR2A* as S phase readouts illustrate that the growth of new leaves under HL involves enhancing cell cycle activity as well as translation capacity. Therefore, TOR probably connects the information about the availability of HL to translation capacity. The increase in the cell division rate in the HL leaves contributes to final organ size.

A large number of studies looked at the effect of light irradiance on the phytohormones, but the interpretation of results often has been challenging due to the tissue-specific responses. A study by Lopez-Juez et al. (2008) revealed that some auxin-responsive genes are expressed highly in the shoot apex during dark but decreased transiently in light. Auxin mostly moves from leaves to the hypocotyl and root. In this study LL decreased the level of the auxin marker gene *AUX1* while a substantial increase was observed in HL (Fig. 4.24). Furthermore, the young leaves growing will synthesize auxin (Ljung et al., 2001; Overvoorde et al., 2010). The observation in my study indicates that the increase in the auxin response accompanies closely the increase in cell proliferation and leaf development. Cytokinin promotes cell division and influences numerous developmental programs. The role cytokinin plays in mediating the light quantity on plant growth is complex and requires cross talk with other hormones (Kurepin et al., 2007; Thomas et al., 1997). The changes in the gene representing ethylene response (*EIN3*) to the light quantity (Fig. 4.24) may suggests that ethylene activity was decreased to keep the hypocotyl short under HL to keep the plant safe from light stress (Yoshida et al., 2011). The up-regulation in the ethylene by growing leaves and stems are frequently linked to growth inhibition (Kurepin and Pharis, 2014). In the sunflower, the mild increase in the ethylene production is associated to increases in the leaf area, however reduction in the growth of leaf area was observed in presence of large production of ethylene (Lee and Reid, 1997). Canola seedlings in LL showed enhanced ethylene production in the leaf, and as a result inhibited leaf area growth (Kurepin et al., 2007; Kurepin and Pharis, 2014). The results from the light intensity experiments indicate that, like the dark-to-light transition, the cell proliferation, hormonal, differentiation programme adjust to the changes to light intensity. one similarity between the growth in the dark in presence of sucrose and the increase in the cell proliferation in the HL compared to LL is the presence of available sugar that suggests, sugar leads the extent of growth. The photosynthate mediated sugar is only in the access of meristem when light is available. Thus, it is likely that light has a permissive role regarding the access of meristem to sugar and regulating the extent of meristem activity by energy signalling. The available sugar for de-etiolation experiment probably comes from cotyledon reserve which, only becomes available in the presence of light.

The action of light is relying on photomorphogenic pathways, since it depends on photoreceptors (López-Juez et al., 2008) and COP1. One hypothesis on how light

permissive role works, could be in the way that auxin export takes place, sugar transport to the meristem is photoreceptor dependent in a COP1 dependent approach. This hypothesis may be able to explain the dramatic growth observed upon direct sugar access to meristem and as a result activation of meristem in the absence of light which, was not observed if the sugar in medium was not in direct contact with meristem. If sugar is the likely key to the growth observed in the light re-exposure and HL transfer experiments, then the sugar probably is provided by photosynthesis. Thus, meristematic activity is dependent on sugar provided by photosynthesis in presence of light or if TOR-mediated sugar is directly available to the meristem in absence of sugar. This was confirmed by CO_2 deprivation experiment in which by inhibiting photosynthesis, the cell proliferation activity was repressed. These results, together with previous studies, have started to untangle the role of interactions and cross-talks between hormonal and energy signalling to establish meristematic activity and early organ cells upon light exposure.

Chapter 5

Signalling and cell cycle targets in meristem control by light

5. Chapter 5: Signalling and cell cycle targets in meristem control by light

5.1. Introduction

5.1.1 The MAPK cascades

Mitogen-activated protein kinases (MAPKs) are conserved signal transduction pathways involved in diverse aspects of development and physiological processes in eukaryotes. In plants, they are involved in connecting the environmental stimuli and signals into adaptive responses (Bigeard and Hirt, 2018; Taj et al., 2010). To understand the connected signal transduction system in plants it is critical to reveal the specificity of MAPK components (Dóczi et al., 2019; Huck et al., 2017; Jonak et al., 2002). MAPKs regulate their substrates' stability, activity, and subcellular localization by post-translational phosphorylation. Once an environmental stress is detected, MAPKs contribute in the transfer of signals to the nucleus, leading to reprogramming at various levels such as transcriptional, post-transcriptional, translational, post-translational (Bigeard and Hirt, 2018; Lee et al., 2015). However, MAPKs also have targets outside the nucleus and can mediate responses which are not directly gene expression-based. Among others, MKK7 and MKK9 are kinases of interest for their involvement in the cross-talk between development and stress regulation (Dóczi et al., 2019; Zhang et al., 2007). MKK7 negatively regulates PAT (Dai et al., 2006), and promotes pathogen defence in tobacco (*Nicotiana benthamiana*) (Popescu et al., 2009). The exact role of MKK7 in different processes within the cell remains elusive. MPK6 and MPK3 were both revealed as downstream targets of MKK7 (Jia et al., 2016), furthermore, MKK7/MPK3 and MPK6 are involved in localization of PIN proteins to the plasma membrane (in order to modulate shoot branching) and are thus important for PIN's polar localisation and direction of auxin flow (Huck et al., 2017; Jagodzik et al., 2018; Jia et al., 2016). To assess the functional importance of MAP kinase in development and meristematic activity, a *mkk7* mutated line was used.

5.1.2 Transcriptional activation and repression by MYB 3R proteins

The oscillated transcription of genes is vital for normal progress through the cell cycle (Nayeri, 2014). There are three crucial phases of gene transcription during the cell cycle process, they take place during different transition points: G_1-to-S phase is regulated mainly by E2F and DP family proteins, and the others occurs during the G_2-to-M and M-to-G_1. The E2Fa level increases during S phase but E2Fb level stays uniform along

the cell cycle. E2F family transcription factors positively regulate the cell cycle but are not essential to drive cell proliferation (Magyar et al., 2016). The late cell cycle genes (G_2-to-M specific genes) are under the control of R1R2R3-type Myb transcription factors (MYB3Rs) (Ito et al., 1998). *Arabidopsis* contains 5 MYB3R genes, MYB3R1 and MYB3R4 are transcriptional activators and have a partial role in positive regulation of mitosis and cytokinesis (Haga et al., 2007). MYB3R3 and MYB3R5 of *Arabidopsis* act as transcriptional repressors of G_2-to-M specific genes. MYB3R1 can also act as a repressor with MYB3R3 and MYB3R5 to repress the transcription of specific genes of G_2-to-M phase of the cell cycle (Kobayashi et al., 2015). All the MYB3Rs are present in complexes that contain proteins such as RBR and E2F isoforms (Fischer and Decaprio, 2015). The study by Kobayashi et al. (2015) proposed that MYB3Rs act in a coordinated way for the regulation of the mitosis and post mitosis genes. This may be because MYB3Rs act in different stages and times during organ development. The knockout mutation of activators *myb3r1/4* led to downregulation of small numbers of late cell cycle genes, while these same genes were up-regulated in the *myb3r1/3/5* (repressors) mutant. The plants with loss of function of *myb3r1/4* and *myb3r1/3/5* showed abnormalities in the cells.

5.1.3 The transcript level changes of E2F transcription factors

The results obtained by Lopez-Juez et al (2008) demonstrated a rapid and synchronous increase in the transcript level of growth promoting genes in the SAM upon transfer of seedlings to light. This method offers a great experimental system to study underlying transcript responses of growth in the light. E2Fa and E2Fb transcription factors are the activators of S-phase specific genes and E2Fc is repressive of cell cycle activity. The expression level of this gene is high and decreases upon light exposure. The microarray data showed a rapid change in the expression. The transcript level of *E2Fb* was higher in the dark during the early stages of growth and rapidly dropped upon light induction, however after 6 hours, it increased and reached the highest peak in 24 hours. The presence of light led to a drop in *E2Fa* transcript level of about half-fold within 6 hours (Fig. 5.1).

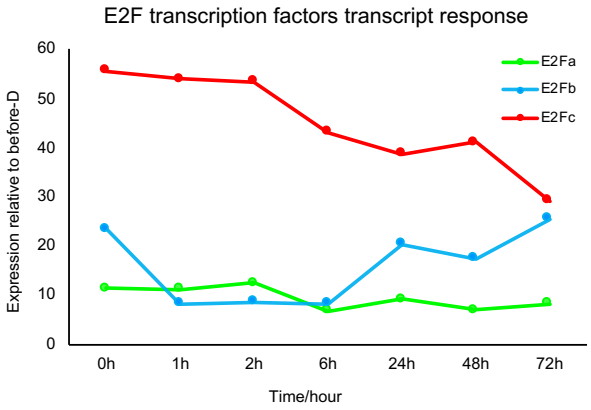

Figure 5. 1 The transcript level of E2F transcription factors, key genes of G1/S cell cycle transition, in the SAM in dark and upon transition to light. The 3-day dark germinated seedlings of Arabidopsis were transferred to light to follow growth. The SAM and leaf primordia were harvested at 0, 1, 2, 6, 24, 48, and 72 hours upon transition to light. The values at each time point in the graph represent averages of 2 replicates, each containing material from over 1000 seedlings. The graph replots microarray gene expression data from Lopez-Juez et al. (2008). The Y axis represents the ratio of each sample relative to the average of all samples (equal 1).

5.2. Aims and objectives

Hypothesis 1:

Expression of several MAPKs genes such as MPK6 were high in the dark and showed a rapid down regulation of transcript levels in the light (López-Juez et al., 2008). I hypothesise that MKK7-MPK6 module has a role in suppressing meristematic activity. Thus *mkk7*/*mpk6* knockouts should enhance the leaf primordia growth and organ size in unfavourable conditions such as dark. This hypothesis is based on the previously known role of MKK7 and MPK6 role in negative regulation of PAT and on both kinases being involved in plant growth regulation.

Hypothesis 2:

MYB3Rs are involved in cell cycle regulation, the hypothesis here is that the knockout of repressors MYBs will result in an enhancement in leaf primordia development in presence and absence of light. The knockout of activator MYBs will negatively affect leaf primordia growth.

Hypothesis 3:

The E2F transcription factors are involved in the cell progression through proliferation or differentiation. The hypothesis is that the plants with knockout of E2Fs will respond partly to sucrose in the dark.

5.3. Results

5.3.1 The *mkk7* mutant has a development effect on leaf primordia

MKK7 overexpressing seedlings have a malformed shoot and root meristem and could be lethal in constitutively active MKK7 type of seedlings. *mkk7* mutant seedlings have shown no phenotype in a standard constant growth condition (Dóczi et al., 2019). The transcript levels of the *MPK6* gene in the dark are high in the SAM and they are quickly down regulated upon light exposure (Fig. 5.2), but the *MKK7* expression showed no change (López-Juez et al., 2008). It was hypothesised that this gene played a role in the dark repression, therefore *mkk7* and *mpk6* seedlings would show enhance in primordia growth compared to WT. To test this hypothesis, the physiological role of these genes in early leaf development was examined. Seeds carrying either *mkk7* or *mpk6* mutation and WT were germinated and grown in dark, and after 3 days they were transferred to continuous light. Seedlings were harvested on day 3 in the dark and over 1 and 2 further days in light to stimulate growth (Fig. 5.2). The surface areas of the first leaf primordium of all genotypes was measured. The harvest of seedlings after 3 days in dark indicates no difference in the primordia size. However, the *mkk7* and *mpk6* primordia became larger than WT during the early days of development in the light. The primordia size showed *mpk6* was larger than *mkk7* and WT in light or after 5 days in continuous dark (Fig. 5.4). This indicates that the *mpk6* mutation has a higher impact on organ growth than *mkk7*.

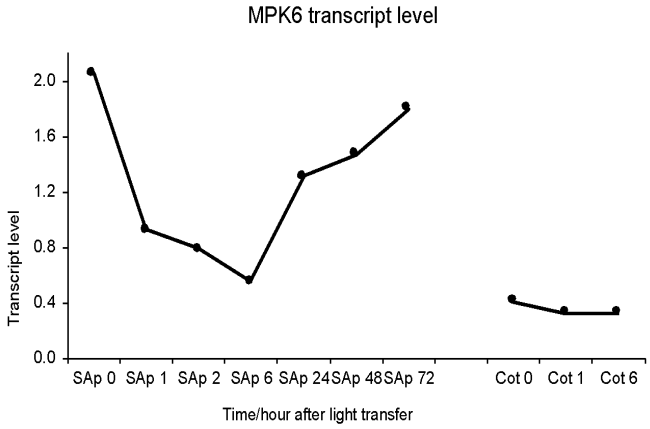

Figure 5. 2 The transcript levels of *MPK6* in the shoot apex and cotyledons of seedlings in dark and upon transition to light. The 3-day dark germinated seedlings of Arabidopsis were transferred to light to follow growth. The SAM and leaf primordia were harvested at 0, 1, 2, 6, 24, 48, and 72 hours upon transition to light. Cotyledons were also harvested at 0, 1 and 6 hours. The values at 0, 1 and 6 hours in the graph represent averages of 2 replicates, each containing material from over 1000 seedlings. The graph replots microarray gene expression data from Lopez-Juez et al. (2008). The Y axis represents the ratio of each sample relative to the average of all samples (equal 1).

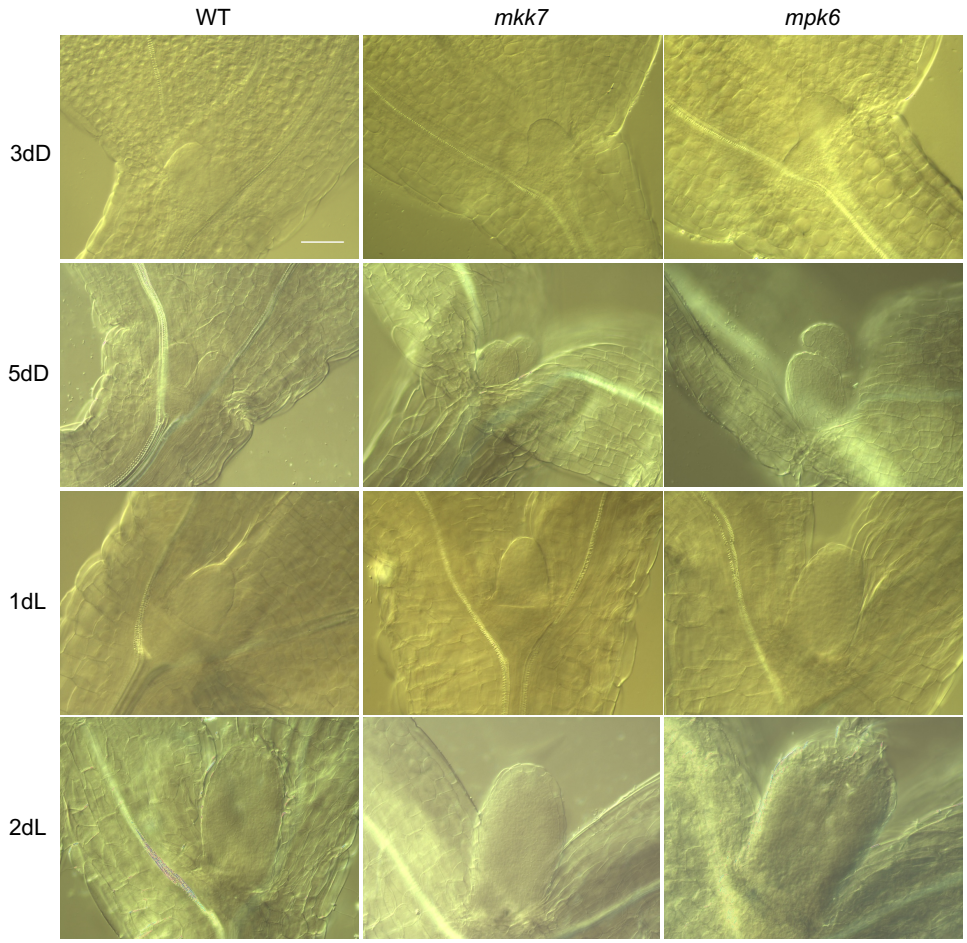

Figure 5. 3 The above images show the development of the leaf in the seedlings of WT, *mkk7* and *mpk6*. 3-day old dark-grown seedlings were transferred to continuous light for 1 and 2 days and some seedlings remained in continuous dark for 5 days to monitor leaf development. The surface area of leaf primordia was established by investigating microscopic images. Scale bar: 50 µm (all images at the same scale).

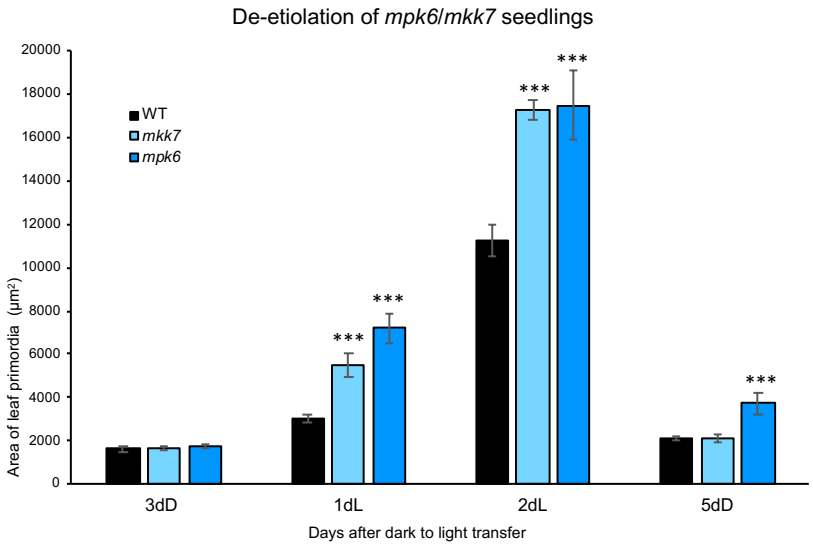

Figure 5. 4 The *mkk7* and *mpk6* mutant have larger leaf primordia compared to WT. 3-day old dark grown seedlings of *mkk7*, *mpk6* and WT were transferred to continuous light for 1 and 2 days, and some seedlings remained in continuous dark for 5 days to follow growth. The area of first leaf primordium was measured using ImageJ software. The error bar represents SEM. Asterisks represent the significance of differences between the WT and *mkk7* or *mpk6*: t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

5.3.2 MKK7 is part of the negative regulators causing shoot meristem arrest in the dark

To gain insight into the role MKK7 plays in the dark-induced growth repression and light-induced release of growth at the molecular level, the relative expression of genes associated with mitosis (*CYCB1;1*), DNA synthesis (S phase) (*H2A*), and translation capacity (*RPS6*) were observed. My previously examined gene expression programme seen during de-etiolation, after the dark arrest and light reactivation of growth in the first leaf pair (chapter 3) and the same setup was used to monitor gene expression in the repression/de-repression process. WT and *mkk7* seedlings were cultured in constant light on sucrose-containing plates for eight days, transferred to dark for three days to arrest growth, and returned to light after three subsequent days. All three genes were down-regulated during the three-day dark period and were up-regulated within 8h following re-exposure to light. The dark transfer led to arrest of meristematic and proliferation activity. Mitosis, S-phase and translation gene markers decreased in the dark and increased upon light re-exposure (Fig. 5.5A). However, a marked difference observed in the transcript level of *mkk7* mutant compared to WT was the kinetics of the changes during the dark repression period. Repression of tested cell cycle genes

was delayed in *mkk7* compared to WT in the dark, although the transcript level has shown to be higher in the light after 8 and 24 hours (Fig. 5.5A). Indeed, during light re-exposure the up-regulation of *CYCB1;1*, but not *H2A* and *RPS6*, was accelerated at 8 hours (Fig. 5.5B).

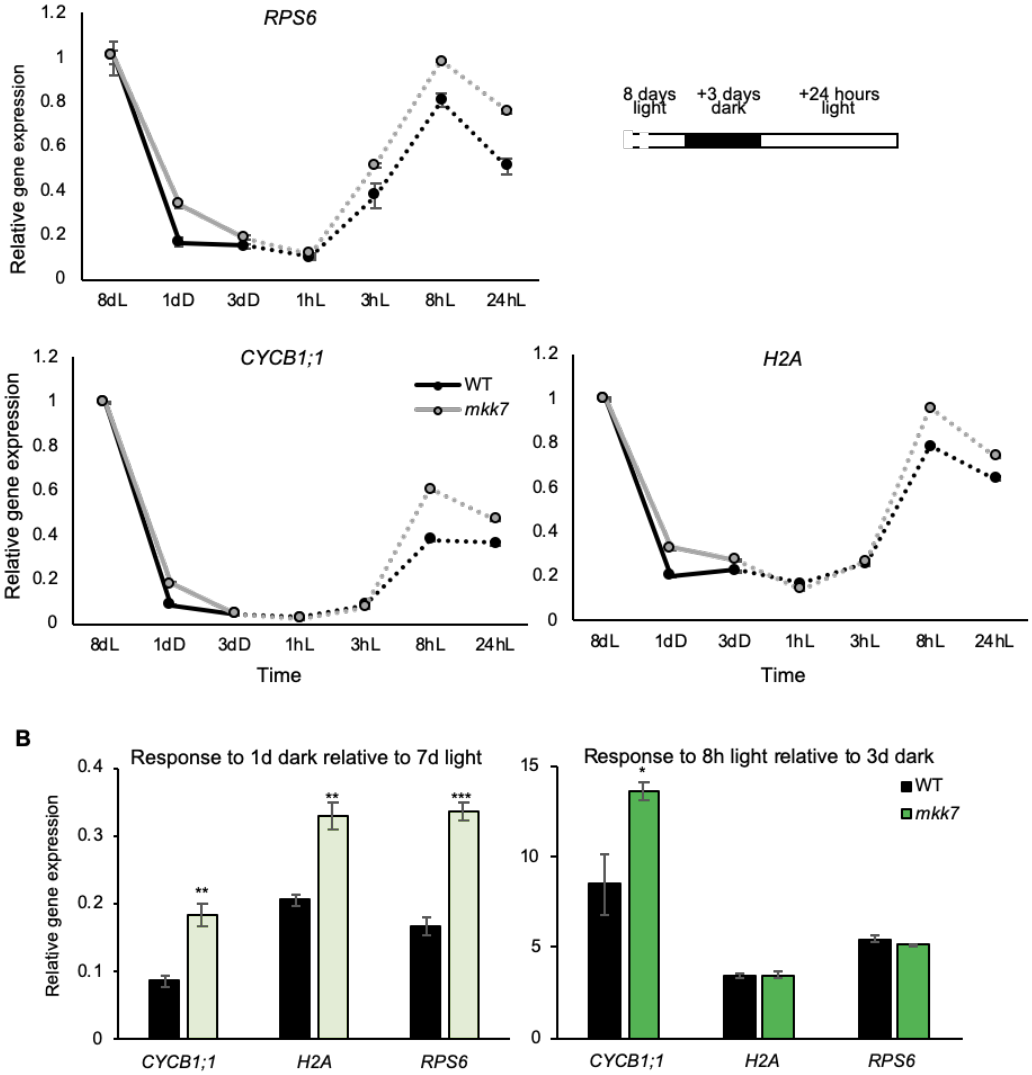

Figure 5. 5 The reversible dark arrest of cell proliferation in young leaf primordia was attenuated in *mkk7* seedlings. The gene expression programme changes were monitored in 8-day old light grown WT seedling in Suc-containing medium, transferred to the dark for 3 days, and re-transferred to light for 24 hours. 200 seedlings were harvested for each biological replicate and had the primordia of leaves 1 and 2 dissected on 1 and 3 days in the dark and 1, 3, 8, and 24 h in the light re-exposure. Gene expression quantification was produced using qRT-PCR. Expression of each gene was measured relative to expression of a constitutive gene (*ACT2*). **A**, the gene expression changes in cell cycle signature genes. **B**, the expression responses of cell cycle genes at 1 day in dark and 8 hours in light re-exposure transfer. Error bars indicate SEM (between biological replicates n=3). Asterisks represent the significance of differences between the WT and *mkk7*: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

5.3.3 The shoot meristem responds better to sucrose in dark in absence of E2Fa

The E2F transcription factors are involved in the cell progression through proliferation or differentiation. To elucidate the functional significance of E2F transcription factors in the repression of growth in the absence of light, their physiological role in leaf development was addressed using seedlings with loss-of-function of *e2fa* and *e2fb*. Both *e2fa* and *e2fb* seedlings were grown in continuous dark for 3 and 5 days. The area of first leaf primordium was measured. Both mutants did not show any distinctive phenotype compared to WT in continuous dark. To pursue the influence of these transcription factors early in growth, 3-days dark germinated seedlings were transferred to light for 2 days. The *e2fa* and *e2fb* mutated seedlings developed larger leaf primordia upon transfer to light, however the size difference became larger in the second day compared to WT in the light (Fig. 5.6).

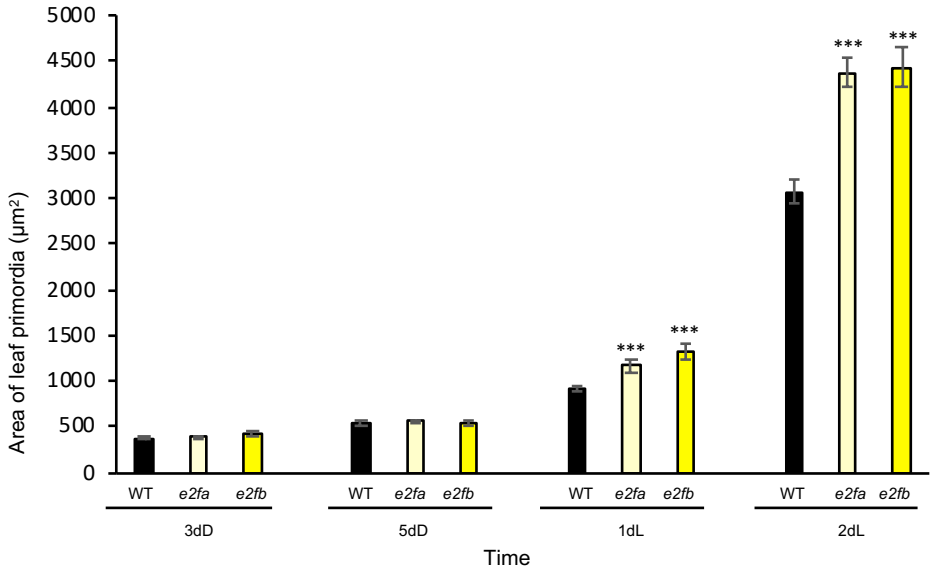

Figure 5. 6 Investigating the leaf initiation phenotype of the *e2fa* and *e2fb* mutant in the light. Seedlings were grown and germinated in dark for 3 and 5 days, the light seedlings being germinated for 3 days in dark and then transferred to light for 1 and 2 days. The area of first leaf primordia were measured using ImageJ software (Number of samples: 20-30). The error bar represents SEM. Asterisks represent the significance of differences between the WT and *e2fa* and *e2fb*: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

To assess whether the possible effect of E2Fa and E2Fb in the dark-induced growth repression, where an unfavourable environment occurs for the plant, relates to the hormonal or energy-dependent mechanisms. Hence, seedlings were grown for 7 days

on vertical plates in presence or absence of exogenous sucrose in presence and absence of the light. The WT and *e2fb* seedlings, with the absence of sucrose in the medium, showed arrest in true leaf growth (Fig. 5.7A) with only a mild increase in the *e2fa* seedlings was apparent in the dark (Fig. 5.7A). This may indicate their functional redundancy when the SAM is repressed, and that no cell proliferation takes place. Activating the SAM by providing direct access to sucrose in the dark promotes primordia growth as it was observed in previous experiments (Fig. 5.8 B,D,F). *e2fb* and WT seedlings showed similar size primordia (Fig. 5.7A). Unexpectedly, *e2fa* seedlings exhibited substantially larger primordia, about twice as large compared to WT in the dark (Fig 5.8 A-F). The growth of seedlings in the light showed presence and absence of sucrose in the medium had no effect on *e2fa* seedlings, however, *e2fb* showed a greater response and growth in sucrose-containing medium (Fig 5.9 A-F). A mild decrease was observed in the average primordia size of *e2fa* in the presence and absence of sucrose and in the *e2fb* seedlings in the absence of sucrose in the light compared to WT (Fig. 5.7B). The WT seedlings exhibited no difference in the presence or absence of sucrose in the medium in the light (Fig. 5.7B). The knockout seedlings of *e2fa* showed smaller leaf primordia compared to WT and sucrose exerted no effect on the size of leaf primordia in the light. The *e2fb* mutant seedlings showed mild differences in relation to WT, of which only a small increase in presence of sucrose was significant (Fig. 5.7B).

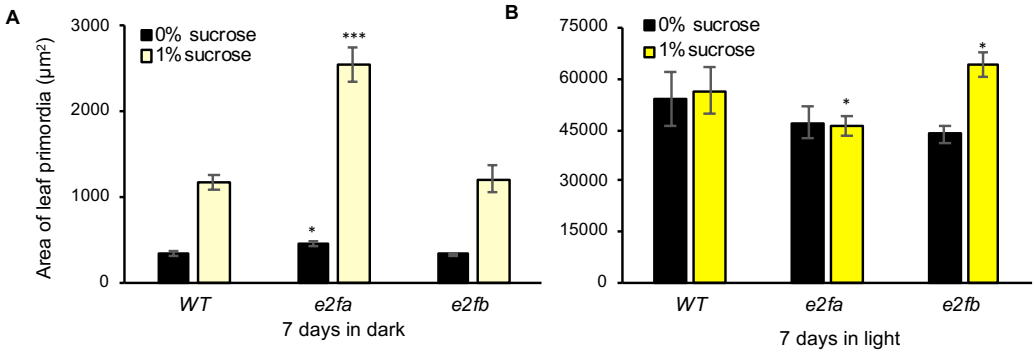

Figure 5. 7 Investigating the leaf initiation phenotype of the e2fa and e2fb mutants in the presence and absence of light. The seedlings were grown in continuous dark in vertical plates (A) or continuous dark in presence or absence of sucrose in horizontal plates (B). The area of first leaf primordia were measured. The error bar represents SEM. Number of samples (n=25-30). Asterisks represent the significance of differences between the WT and *e2fa* and *e2fb*: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

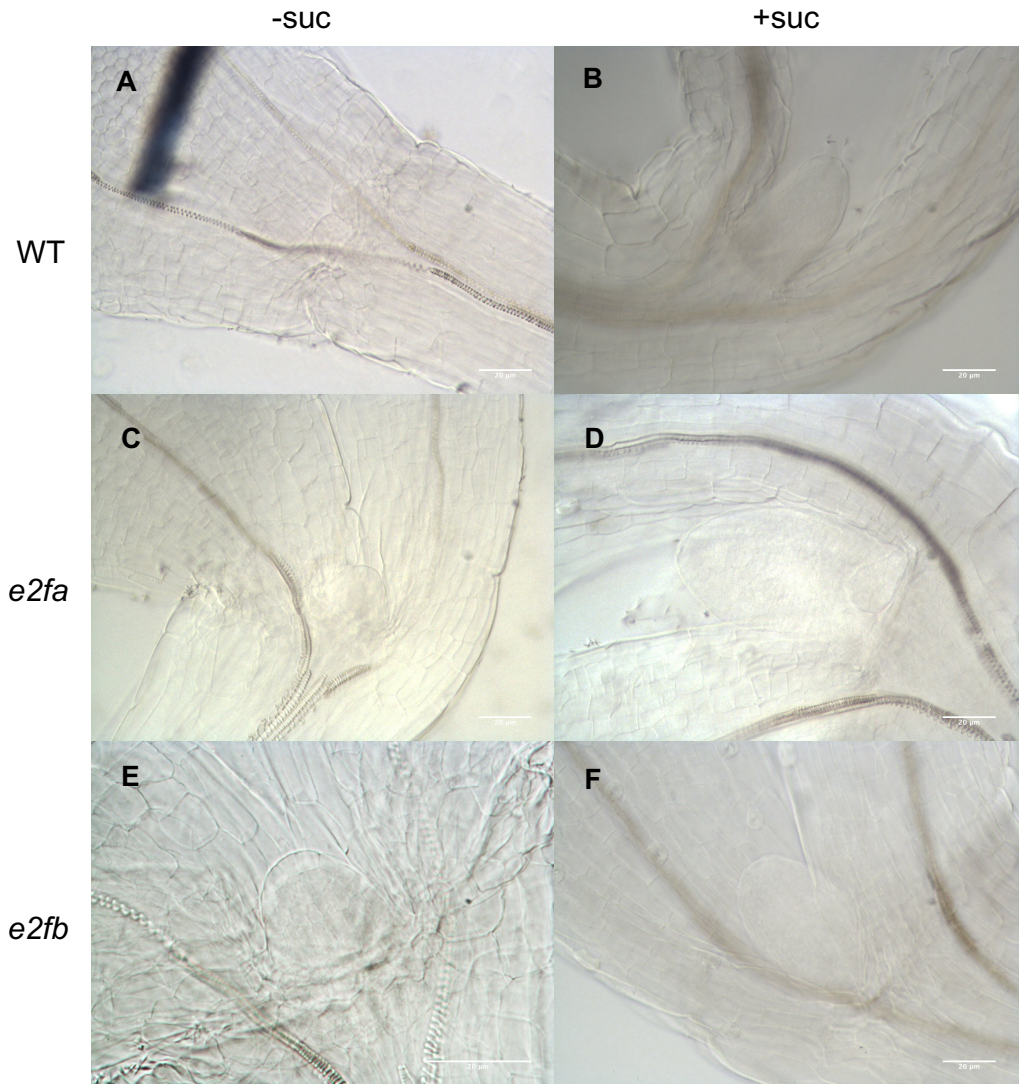

Figure 5. 8 Investigating the leaf initiation phenotype of the *e2fa* and *e2fb* mutants in the dark. Previously, experiments confirmed the growth in dark in presence of sucrose. WT, *e2fa* and *e2fb* were sown on vertical plates and after 3 days cold treatment were transferred to continuous dark for 7 days. **A**, WT, Suc-free plate. **B**, WT, Suc-containing plate. **C**, *e2fa*, Suc-free plate. **D**, *e2fa*, Suc-containing plate **E**, *e2fb*, Suc-free plate. **F**, *e2fb*, Suc-containing plate. Scale bar: 20 µm.

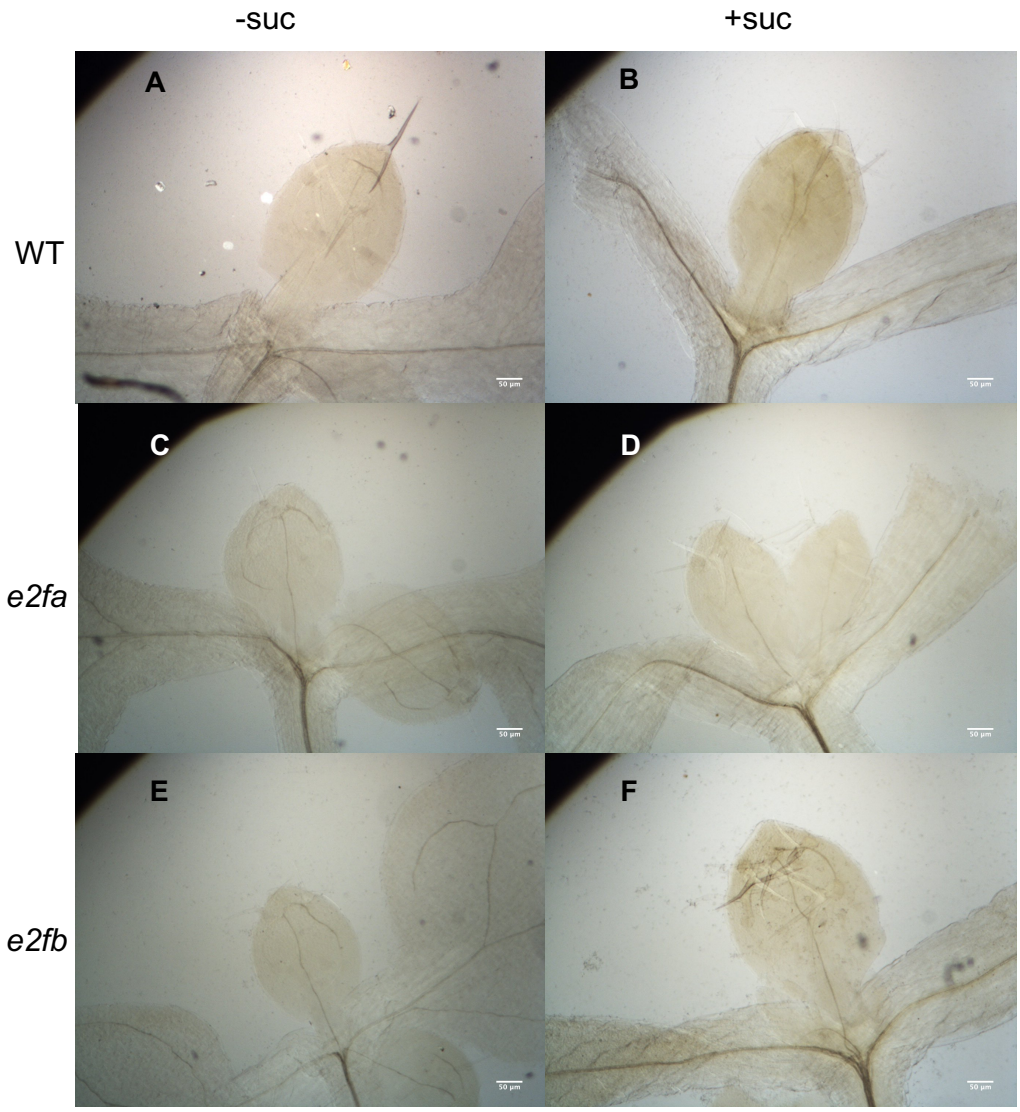

Figure 5. 9 Investigating the leaf initiation phenotype of the *e2fa* and *e2fb* mutants in the light. WT, *e2fa* and *e2fb* were grown on horizontal plates and after 3d cold treatment were transferred to continuous light incubator for 7 days. **A**, WT, Suc-free plate. **B**, WT, Suc-containing plate. **C**, *e2fa*, Suc-free plate. **D**, *e2fa*, Suc-containing plate **E**, *e2fb*, Suc-free plate. **F**, *e2fb*, Suc-containing plate. Scale bar: 50 µm.

5.3.4 MYB 3R transcript levels

Expression levels of genes encoding *MYB3Rs* in the SAM during dark and upon transition to light were obtained through microarray profiling of differential gene expression (Fig. 5.10). MYB3R4 and MYB3R1 are shown to activate transcription of genes of G_2/M phase (Haga et al., 2011; Menges et al., 2005). *MYB3R1* behaves, according to expression profile, more like an activator than a typical repressor. *MYB3R3* and *MYB3R5* transcript levels are up-regulated slightly in the dark and behave more

like repressors. Note the difference in the expression pattern of *MYB3R3* and the level of transcription in the SAM is different from cotyledon expression, indicating the importance of study the specific tissue in the study rather than using whole seedlings. *MYB3R3* expression pattern is quite similar to *E2Fb* expression and *MYB3R1* to *E2Fa*. *MYB3R1* transcription level is mirroring the expression of *E2Fa* (Fig. 5.10).

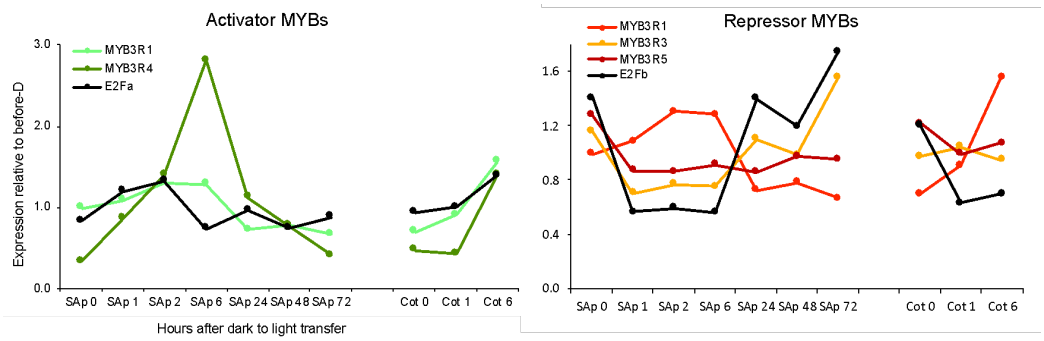

Figure 5. 10 The transcriptional responses of MYB3Rs in the SAM plus leaf primordia (SAp) and cotyledon (Cot) changes upon first light exposure after 3 days in the dark. The x axis shows the time, 0 represent the expression in the seedlings germinated in dark for 3 days and further hours show the time course when seedlings were transferred to the light (SAp 0, 1, 2, 6, 24, 48, 72 hours). The values of each time point represent averages of 2 technical replicates. The graph is created using microarray gene expression data from Lopez-Juez et al. (2008).

5.3.5 Removing MYB3R repressors removes some degree of growth repression in the dark

To illustrate the role of MYB3R1/3/5 (transcriptional repressors ΔRep) and MYB3R4 (transcriptional activators ΔAct) in the leaf primordia development, the respective combined mutants, ΔRep and ΔAct were monitored. One set of seedlings was grown in continuous dark, and one set of seedlings placed in light for 24 hours after 4 days in the dark on sucrose-containing medium. To examine the action of these transcription factors in the light, seedlings were harvested 5 days post-germination in the light. The area of first leaf primordium was then measured (Fig. 5.11). The ΔRep seedlings revealed larger primordia compared to WT in continuous dark and upon light transfer (after 1d), indicating their involvement in repressing meristematic activity in the dark. ΔAct displayed a minor effect on the leaf primordia size in continuous light (Fig. 5.12).

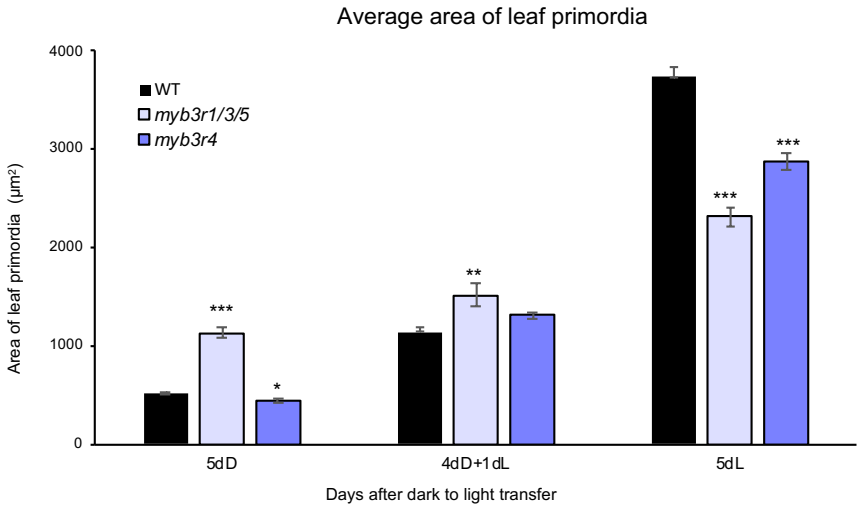

Figure 5. 11 Repressor MYB3Rs are essential for growth arrest in the dark. *myb3r1/3/5* and *myb3r4* mutant seedlings were grown in three conditions, in dark or light for 5 days, and 4 days dark growth then transferred to light. The area of first leaf primordia were measured. The error bar represents SEM. Asterisks represent the significance of differences between the WT and *myb3r4* and *myb3r1/3/5*: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

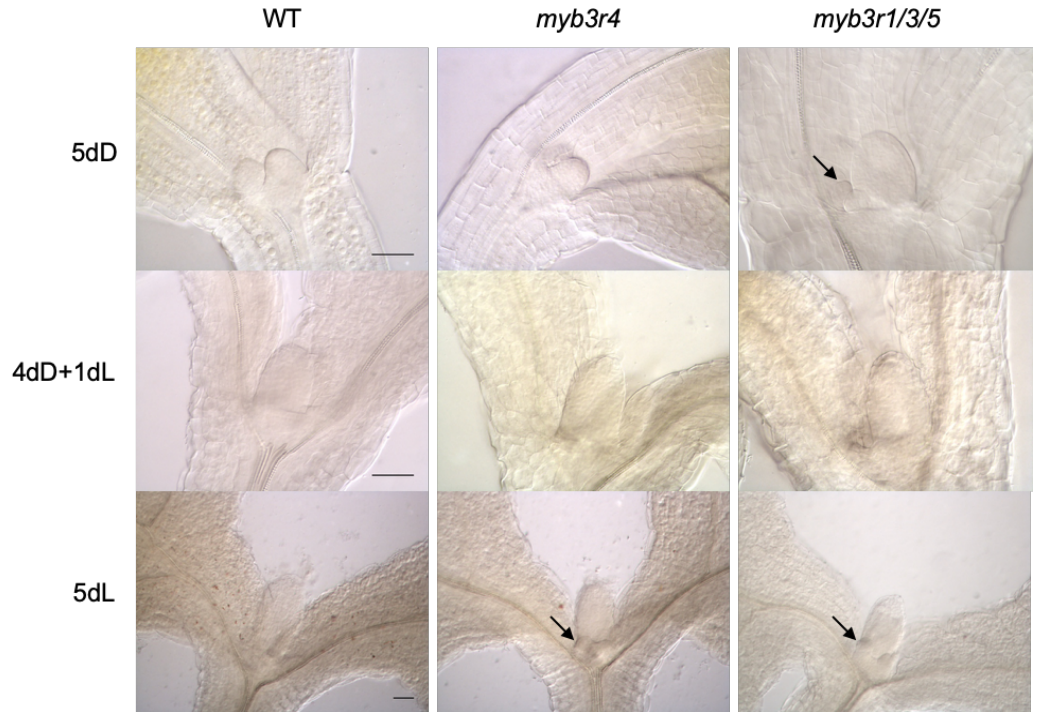

Figure 5. 12 Repressor MYB3Rs are essential for full growth arrest in the dark. *myb3r1/3/5* and *myb3r4* mutant seedling grown in three conditions, in dark or light for 5 days, and 4 days dark growth then transferred to light for 1 day. The arrow shows the emergence of new primordia (of leaves 3, 40. Scale bar: 50 μm (same scale for all samples collected at the same time point).

5.4. Discussion

Light is an important environmental factor for plant growth and development and thus an easy factor to manipulate in order to understand the state of meristematic activity and its function in growth. The de-etiolation experimental strategy can be a tool to analyse the mechanisms involved in the regulation of leaf initiation, development, and expansion. Unlike in animals, plant development is tremendously plastic, and plants can optimise their growth according to the perception and integration of environment around them (De Jong and Leyser, 2012). Results have shown that MKK7-MPK6 cascade is involved in the regulation of meristematic activity in the changing environment. A line overexpressing *MKK7* showed that constitutive activity of the MKK7-MPK6 pathway leads to a distorted SAM and arrest of growth. The *mkk7* knockout seedlings in standard conditions show no visible phenotype compared to WT (Dóczi et al., 2019). However, the microscopic, physiological study of leaf development in this thesis to observe if there is any effect on early stages of primordia development in the light showed *mkk7* and *mpk6* mutant produce larger leaf primordia. This illustrates the negative role of both genes in the regulation of meristematic activity in the dark. Thus, MAPK signalling participates in the suppression of leaf growth from the SAM under adverse environmental conditions (absence of light). The relative expression of genes of cell cycle in the *mkk7* mutant compared to WT showed a kinetic difference during the dark repression period. The repression delay observed in *mkk7* seedlings indicates that this gene is involved in the suppression of leaf development through inhibition of meristematic activity when the conditions are not favourable for the plant. Seedlings of the single *mkk7* and *mpk6* mutants showed no phenotype under standard growth conditions, thus looking closely and quantitatively at leaf primordia at early stages of growth was able to reveal a function, which would have otherwise remained hidden. Also, the transcript level of *mpk6* in the whole seedling studies showed no difference in the responses in the dark and light (Menges et al., 2008), however in the Lopez-Juez et al. (2008) study specifically of the SAM and young leaf primordia showed otherwise (Fig. 5.1). The *MPK6* transcript levels shown to be down regulated very rapidly within one hour of transfer to light in the SAM but not in the cotyledon (López-Juez et al., 2008). This illustrate the importance of tissue specific analysis rather than more superficial analyses of whole seedlings on growth activators and inhibitors. Moreover, my results further confirm that de-etiolation can be used as

synchronized system to study meristematic activity and to understand the mechanisms underlying leaf growth and development.

The CLAVATA signalling pathway is involved in the regulation of the stem cell maintenance in the SAM. The phosphorylated residue of kinase domains involved in the pathway act as binding sites for the molecules such as ROP. This model is similar to the mammals' response to growth factors by activating of ERK MAP kinase pathway. Indeed, MPK6 activity is modulated by CLV receptors in the plant. The leaf primordium initiation site is marked by auxin maxima, formation of leaf and vascular cells are coupled with presence of auxin efflux transporter protein PIN1. The DR5:GUS reporter showed a reduction in auxin activity upon light exposure accompanied by the appearance of auxin maxima highlighting the importance of PAT in the leaf primordia growth. The reduction in the auxin activity in the new leaf primordia is a fundamental part of leaf growth. The MPK6 and MKK7 act as a PAT repressor and the phosphorylation of PIN1 mediated by MPK6 regulates localization of PIN1 patterning in the cell (Jia et al., 2016). Light is involved in the activation of the MPK signalling pathway, and MKK3/MPK6 is being modulated by blue light at some levels (Sethi et al., 2014). Therefore, by removal of MKK7 or MPK6 the leaf initiation process could be enhanced, illustrating that auxin removal could also act as a regulatory mechanism involved in accelerating leaf primordia growth in absence of *mpk6* or *mkk7*. Taken together, the regulation of meristem through MAPK is probably complex and carried out by post-translational modification of several substrates.

Transcription of genes is vital for normal progression through the cell cycle. The E2Fs transcription factors are involved in the cell cycle progression and have a key role in cell progression through proliferation or differentiation (Attwooll et al., 2004). The leaf primordia area of *e2fa* mutant seedlings are larger than that of WT in the dark. The response of *e2fa* mutant seedlings to sucrose in dark may suggests that E2Fa has a key role in inhibition of meristematic activity by energy signalling in the absence of light. Thus, E2Fa may be one of the factors involved in cessation of cell proliferation activity in the dark. Previous work showed glucose or sucrose could activate E2Fa target genes and suggested that the glucose-TOR cascade may partly regulate cell proliferation through E2Fa transcription factor in the root meristem; the study's gene expression analysis of *e2fa* showed reduced glucose sensitivity in activating S-phase genes through TOR in the root meristem (Xiong et al., 2013). This would be consistent

with a positive role of E2Fa in the transition of cells into S phase. However, the results obtained from the microscopic analysis of *e2fa* mutant leaf development towards sensitivity to sucrose showed otherwise, suggesting negative role of E2Fa in response to sucrose in dark. In animals, it has become clear that E2Fs have effects on various biological processes such as upregulation of cell proliferation genes, but they also inhibit genes other than cell proliferation ones, regulated by development processes (Magyar et al., 2012). In plants, the rapidly dividing cells are located in the SAM and leaf primordia tissues (Kobayashi et al., 2015). The cell proliferation control machinery requires mechanisms to modulate and restrict mitotic genes by repression of genes related to G_2/M phase. The repressors MYB3Rs are required for active repression of mitotic genes, to exit cell proliferation in later stages of tissue growth, by narrowing their expression window outside the mitotic phase (Kobayashi et al., 2015; Magyar et al., 2016). The role of transcriptional repressors and activators of cell division was followed by looking at their effect on primordia growth. It is interesting that predictions as to which genes encoded candidate cell cycle repressors and which one's activators were possible to make based exclusively on transcriptomic data. Such predictions were actually tested in the growth assays. The activator *MYB3R4* transcript responses may indicate this gene is mainly and uniquely transcribed during the mitotic state. *MYB3R1/3/5* are the repressor MYB3Rs, which are expressed more widely during development, but which correlate inversely with the growth transcription peak (6 h after transfer to light, Lopez-Juez et al., 2008). MYB3R3 expression pattern is remarkably similar to E2Fb expression and MYB3R1 to E2Fa. The combined mutation of *myb3r1/3/5* led to the growth of larger primordia in the dark, which is evidence of their involvement in repressing meristematic function. The rise in the size of leaf primordia has been observed by previous researchers in the case of loss of the E2Fc and RBR1 cell cycle repressors (Borghi et al., 2010; Kobayashi et al., 2015), making it likely that here also the increase in the size of leaf primordia is due to an enhanced cell proliferation activity. Moreover, the results obtained from studying de-etiolation can be useful as a synchronized developmental system to assess the meristem activity and also understand the early mechanisms involved in regulation of leaf primordia initiation and growth in plant biology. However, for the findings as in the case of E2Fa, it is important to follow up by further functional testing to confirm or reject those predictions by transcriptomic data. The transcriptomic data are valuable

in making functional predictions. The results demonstrated that MPK6-MKK7 have a regulatory developmental role with a specific function in regulation of the SAM, confirming the role of post-translational modifications in the early responses. The results provided earlier confirmed the role of cell cycle control and of its activator and repressor transcription factors in growth control, for both M and S phase regulators. This study shows the value and importance of transcriptomic data to make functional predictions, but also of the need for functional testing to confirm or reject those predictions, as in the case of the role of E2Fa.

6. General discussion

6.1. What have we learned?

Plant development is regulated by multiple external and internal cues that coordinate the time and extent of growth according to the surrounding environment to meet the requirements to grow. The quantity and quality of light, length of light duration, together with available nutrients adjust the extent of growth. In experiments of growth in tissue culture conditions, the absence of sucrose in the media slows down the growth and it takes longer for the first pair of leaf primordia to emerge and expand compared to the *Arabidopsis* seedlings developing in the sucrose-containing media. However, the leaf primordia growth is depending on regulation of meristematic activity by light. Light signalling and its quality and quantity have been extensively studied. An important question in this PhD research was how leaves form from the SAM. An important and key environmental factor is the natural trigger of growth, light triggers the activation of meristem from a repressed state. This raised the question of how light brings about the activation of meristem and cell proliferation. Understanding this transition can provide information towards meristem activity and leaf initiation in the light exposure. The arrested state of meristem in the natural conditions while in the dark takes place because of central repressors of photomorphogenesis, such as COP1, targeting the degradation of factors involved in light signalling. The activation of meristem upon light exposure initiates the division of cells, where the stem cells with no identity yet acquire an identity and become the first cells to initiate the first leaf. During the dark arrest the meristem displays a strong but delocalised auxin response, limited action of cytokinin perhaps through cytokinin turnover, and a strong ethylene response. Also, a strong starvation state is exhibited by the meristem, which could be due directly to repression of the TOR-mediated energy signalling, or indirectly to deprivation of sugar. The starvation gene *bZIP11* is involved in the activation of genes expressed during the sugar starvation and is inhibited by sucrose (Rook et al., 1998). The expression pattern of this gene specifically in meristematic cells during skotomorphogenesis indicates a starvation state in this area that promptly is released once light is present. These actions all together lead to growth arrest. MAPKs and other pathways active in the dark kept at least some of PIN1 phosphorylated and away from the membrane, preventing it from mediating the relocalisation and export of auxin. Upon light transfer, photomorphogenesis takes place by initiation of transport of auxin towards tip maxima and export through developing vasculature. The cytokinin response

increases, whereas ethylene response is repressed. The starvation state of the meristem is repressed, this caused by unknown mechanisms, and energy signalling is turned on very quickly. These combined actions all take place to initiate cell proliferation under light exposure. The expression of auxin synthesis genes increases, accompanied by the establishment of PAT, which results in localised auxin responses later in the light. Ethylene responses are strongly reduced and cytokinin response remains. The starvation response is repressed within an hour, before any photosynthetic apparatus has had time to assemble. I suggest the quick repression of starvation of the meristem is a result of the fast break down of storage reserve of light exposed cotyledons. Later, the photosynthesis produced glucose further activates the SAM in a TOR-dependent manner called a feast state. At this stage, cell proliferation and growth are proceeding and are later followed by transition to differentiation-associated cellular expansion, endoreduplication, and tissue growth. As a result, normal leaf primordia emerge and develop into normal leaves with fully developed laminae. This occurs due to the presence of reduced carbon supplied, eventually, by photosynthesis. The arrested state of meristem in the dark could be removed by the direct access to sucrose (Roldan et al., 1999). Once in contact with sucrose, energy/TOR signaling becomes highly activated. The auxin response increases back, while the ongoing, simultaneous ethylene response remains. Consequently, strong cell proliferation activity takes place. The meristem is activated, and the emergence of new leaves is observed in the dark. However, development in the dark is different, leaves develop with limited laminae and differentiation is disproportionally directed towards petiole and internode organogenesis. The availability of sucrose to the meristem and activation of cell proliferation indicates sucrose promotes cell proliferation but making of normal leaves demands a coordination of photomorphogenesis-like hormonal responses to direct the cell proliferation towards making leaves. Plants invest biomass in boosting the height-focused growth to reach light and reduce the associated investment in other organs such as in leaf size, complexity, and shape (Vermeulen et al., 2006). However, mechanisms linking the leaf forms with the environmental cue has remained little known. Furthermore, making a leaf is a plastic process, responding to the environment around it and making this more complicated (Chitwood et al., 2012; Kalve et al., 2014). The key aspects of growth were observed through a genetic programme in the sucrose-dependent growth in the dark. However, the full mechanisms controlling the extent of cell proliferation and directing the decision of cells to go through differentiation remain

to be elucidated. This may be due to regulation of access to reduced carbon by the sensors activating the TOR pathway. As a result, the TOR signaling pathway directly modulates the expression of cell cycle genes and induces cell proliferation as its output. There is emerging evidence indicating the direct involvement of TOR in regulation of translation, therefore also regulating meristem activity, cell proliferation, and differentiation (Schepetilnikov & Ryabova, 2018). The TOR signaling also regulates the extent of cell proliferation activity in the young leaves under different irradiances of light. The cell cycle and cell growth gene transcription programmes are to a large extent mediated by TOR pathway; and sucrose acts on the meristem through them, in a TOR pathway-dependent manner. The growth observed in the *raptor1-2* mutant in the sucrose-induced growth in the dark was unexpected. However, in mammals it was suggested that SnRK1 inhibits TOR by interaction and phosphorylation of RAPTOR and upstream components in the pathway. Accordingly, SnRK1 interacts and phosphorylates RAPTOR in plants. It is interesting to suggest RAPTOR phosphorylation cannot happen in the *raptor* mutant and therefore TOR is not inhibited, and therefore could regulate the growth and cell proliferation. However, the growth of *raptor* mutant compared to WT in sucrose-induced dark growth is larger, which indicates this regulatory subunit of TOR may be involved in the regulation of TOR in the extent of growth. Understanding the role of RAPTOR in the TOR pathway further would help us in the future to be able to regulate the extent of growth in the plants, and more importantly perhaps improving the yield of crops.

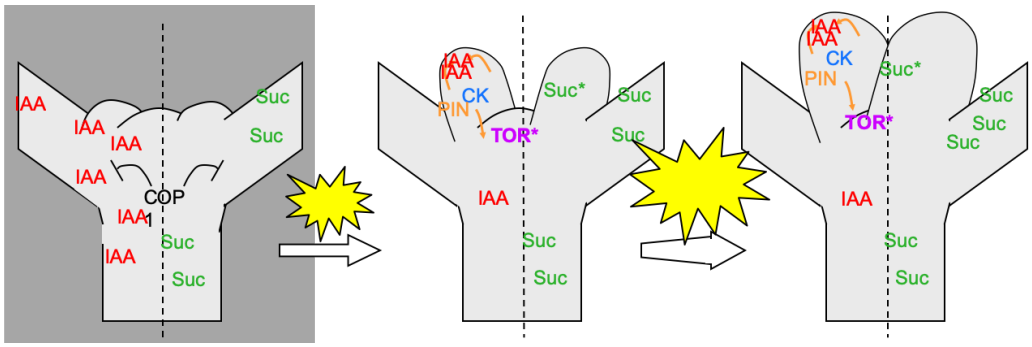

Figure 6. 1 The scheme shows the impact of photoreceptor activation, or exposure to high or low light, and the occurrence or magnitude of leaf organ growth. The SAM in all the seedlings above show hormone-related responses (Left) and energy-related response (Right). COP1 is active in the dark (Left) , auxin (IAA) action is delocalised and new primordia experience starvation state. Light initiates transport of auxin by PIN1 localisation to membrane, results in localised maxima at the tip of primordia and rib meristem, and cytokinin action takes place at the primordia and SAM. The starvation state of both organs is terminated by presence of Suc. TOR action is initiated once in the light and drives cellular growth. High light quantity increases available sucrose and elevates cellular proliferation and growth responses.

6.2. Was it necessary to do tissue-specific analysis to understand growth?

Eukaryotic complex organisms, including plants, consist of several types of organ and tissue. The different organs are developed during the life cycle by modulation of expression of a particular part of the same genome in different cells, both temporally and spatially. Light displays a tissue-specific genome expression profile, for example; carbon metabolism pathway is stimulated in the hypocotyl and cotyledon but not in the root (Ma et al., 2005). Studies on the SAM has been restricted mostly to the whole seedling dissection, this is due to the small size of the SAM and new leaf primordia, which is very laborious and time consuming to dissect. However, there are new methods to isolate small population of cells to be able to study tissue specific responses such as purification of nuclear RNA from embryo using fluorescence-activated nuclear sorting (FANS), the results of these studies illustrates the importance of tissue specific study (Marion-Poll et al., 2014; Slane et al., 2014). To study the difference between the three layers of meristem, a tissue specific study of only 10 cells carried out and shows that different layers of meristem have great diversity in gene expression (Yadav et al., 2014).

However, the difference in the transcript levels confirmed that the results were quite “diluted” compared to the microarray-based data from dissected tissue provided by Lopez Juez, (2008) that was carried out in the shoot apex (SAM, emerging primordia

and minimal surrounding tissue) and, separately, cotyledons. The work carried out in that study is very laborious, it requires lots of time and patience to collect tissue of the shoot apex in 3 days dark-germinated seedlings. The transcript levels of genes expressed in the SAM and cotyledon illustrated that there are differences in the expression level of genes of growth processes between both tissues (Lopez-Juez et al., 2008). It was hugely revealing but also impossibly laborious to dissect the SAM of 3 days dark-germinated seedlings, and I worked that away and made it possible through dark arrest of light grown seedlings. My work on monitoring whole seedlings in the dark indicated that absence of light acts as a brake on growth. To take advantage of this process, I established a system in which the seedlings went through leaf initiation in the light and were then transferred to dark to arrest them. This system made it possible to study seedlings during meristematic growth arrest and re-exposure to light and to be able to collect the SAM and the new leaf primordia, or just the actively-growing, new leaf primordia. I speculate that the tissue-specific approach offers a very important and more timely and strategic approach to understand how the meristem works and leaves are initiated. I could not possibly learn and been able to do the analysis of MAPK function through mutants the old way by studying the whole seedlings (or, equally, by dissecting etiolated shoot apices at multiple time points from multiple genotypes). One important example of the importance of tissue specific analysis is the targeting of T6P to a specific tissue and cells had positively affected the crop yield and resistance to stress (Paul et al., 2018). Feeding sucrose directly to the stem of abiotic stressed maize plants was effective in reducing the detrimental effects, maintaining grain filling and yield (Hiyane et al., 2010). These two research examples on a specific tissue of the plant illustrate that choosing the right tissue to experiment on could give invaluable knowledge and opportunities towards better understanding and increasing crop yield.

6.3. Why two regulators? Why the need for such a complexity?

The role of light in the control of leaf initiation and development involves a combination of hormonal responses and TOR mediated energy signalling. This master regulator is also involved in the expression of several ARFs induced in response to auxin (Schepetilnikov et al., 2013). The action of ROPs and auxin in a coordinated manner activate TOR signalling to modulate translation of specific mRNAs (Schepetilnikov et al., 2017). Thus, this kinase could explain some of the shared action

of auxin and energy signalling during growth. Light carries a lot of information for example exercising a permissive role for growth initiation or arrest, extent of growth, sensing the environmental irradiance, and patterning growth. The shape of growth that a plant chooses to go through is under the control of hormones, but growth initiation or arrest is where energy signalling comes to place. Therefore, the role of light goes beyond the regulation of initiation of growth. The 3 days dark arrest was characterised by low cytokinin, high auxin, and high ethylene responses. This suggests that SAM activity and leaf initiation are arrested as a result of high auxin response and low cytokinin in the dark, and upon light exposure, increasing the cytokinin response and changes in auxin flow induce leaf initiation. Also, my study illustrated that one reason for arrest of meristematic activity in the dark is energy deprivation or starvation and probably inhibition of sugars access to meristem tissue. Therefore, the right balance and cross talk of hormone signalling and presence of energy signalling are part of the story for meristem arrest in the dark.

The decision plants make between growth patterns, for example growth as in shade or growth as in sun or no growth at all in dark, is regulated by energy signalling and auxin flux but the decision to go through which type of growth depends on and is regulated by hormones. This takes place by the cross talk and interplay between five major hormones, auxin, cytokinin, ethylene, gibberellin, and abscisic acid. The sun-grown plant is short, and its leaves present a larger lamina, which indicates more cell proliferation and translation activity has taken place. The shade-grown plants grow smaller leaves with reduced leaf lamina and lots of vascular and elongated organs. The shade leaves therefore differ in two aspects of regulation of growth pattern, the decrease in growth such as leaf size is regulated by energy signalling but the increase in elongation of stem and petiole to reach light is under the control of hormones (auxin and ethylene). The role of energy signalling in the cellular make-up of the leaf occurs through regulation of cell growth and cell proliferation pathways, critically, dependent on TOR. Both of these regulatory mechanisms are necessary for plant growth as they are involved in different roles. The hormonal and energy signalling are both regulated by light, because light impacts the action of both mechanisms. Phytochromes do more than sensing the presence and absence of light, they are able to detect the colour of light and may also be involved in sensing the quantity of the light. The quantity of light determines the extent of growth. Energy signaling is, in a way, “regional”, it can turn growth on or off, but it cannot easily provide directionality to growth. In a way the same

happens with cytokinin signaling. However, auxin in particular has directional flow. It also has actions on the cell wall, which allow directional differences (directional cell expansion, elongate narrow) (Biedroń and Banasiak, 2018; Majda and Robert, 2018). In a way auxin in particular is much more suitable to provide patterning information. Ethylene, for some reason, often work in concordance with auxin, ethylene tissue specific effects are connected to local auxin production, TAA1 (auxin biosynthesis gene) expression is induced by ethylene(Stepanova et al., 2008). Therefore, it is fundamental to separate systems of regulation, because one, at least for TOR signaling, cannot control both aspects of growth.

6.4. Can any of my findings can be turned to immediate use?

The information, which was learned about meristem biology, positively to activate the meristem or negatively to stop it, can be made use of. Learning about the mechanisms by which the meristem is arrested in dark (hormones profile in dark and probable lack of energy access to the meristem) could help in crops such as potato and onion to devise strategies to inhibit their sprouting during storage and maintain a good quality. One strategy would be to screen for novel TOR inhibitors which are plant specific. By gaining knowledge on meristematic activity and understanding the role of sugar signalling in development, one would be able to use it towards greater yield in crops for food security. This may could be approached by genetic modification of Snrk1 and TOR activity, this approach could allow more supplement of resources to harvested tissues during the time of sugar abundance. Thus, it is important to understand the TOR signaling pathway and the molecular interactions involved in growth to be able to manipulate TOR. *Arabidopsis* plants overexpressing TOR are larger (Deprost et al., 2007). The overexpression of TOR targets, such as EBP1s, results in growth of larger organs in *Arabidopsis* and potato (Horváth et al., 2006). Expression of *Arabidopsis* TOR in rice growing in drought environment increases the yield and resilience (Bakshi et al., 2017). Increasing the sucrose content of the kernel by genetic manipulation was used as a strategy to increase the yield in maize (Xie et al., 2018). By targeting carbon allocation, rather than targeting water intake, the drought tolerance improved in field conditions. *Arabidopsis* as a plant model has provided an excellent framework for understanding the role of sucrose and energy signalling play and will continue to be a precious tool. *Arabidopsis* is very important in the improvement of crops, as it has

genetic resources available and is easy to manipulate and cultivate. Many of discoveries gained in this species about function of T6P will also hold true in crops because of the conserved role of signalling pathways in eukaryotes (Paul et al., 2018). Manipulating sugar signaling through T6P has also been shown able to increase the yield and resilience of wheat grain development (Griffiths et al., 2016).

6.5. What else needs to be learned?

This study, together with previous ones, contributes to our understanding of the role and importance of cross talk between hormones and energy signaling, through the TOR kinase. The results also opened several new questions. The sucrose-induced developmentally different growth in the dark through exclusive activation of energy signalling illustrates that light may play a role as gate, giving permission to energy signaling to access the meristem, and lead to activation of growth. The role of light in activation of the meristem is dependent on photomorphogenesis. Light is dependent on photoreceptors (Lopez-Juez et al., 2008) and repressors including COP1. One hypothesis towards how this light-gating may work is that sugar import to the meristem is regulated by photoreceptors in a COP1-dependent way. The auxin export from cell to cell is through PIN1, could sugar also be transported through a PIN1-like protein (in functional, rather than physical terms) to the meristem. The inhibition of sugar transporter proteins by pharmacological agents may help towards understanding how light releases the access of meristem to sugar.

One important area of research in sugar signalling is how the photosynthetic apparatus sends sugar to the rest of the plant. The sugar efflux transporters SWEETs (sugars will eventually be exported by transporters) are involved in sugar loading (Chen, 2014). The *DsSWEET12* (of *Dianthus spiculifolius*) transcript level was induced by sugar deprivation, the overexpression of *Arabidopsis DsSWEET12* promoted growth depending on sucrose content of seedlings (Zhou et al., 2018). The importance of sugar transport in this case is how sugar offload occurs, understanding this process will help towards the mechanism of sugar access to meristem. Thus, it may assist to reveal what determines sugar access to meristem and by knowing this, we may be able to manipulate plant on how and where to grow, as this could be a critical key for increasing the crop yield. The same wheat plant could produce more grain by stimulating energy signalling through T6P derivative (Griffiths et al., 2016). There is evidence that T6P acts as a

signal of abundance of sucrose in *Arabidopsis* and wheat (Lunn et al., 2006; Martínez-Barajas et al., 2011). Research on T6P shows to inhibit SnRK1 in several crops such as potato (Debast et al., 2011) and wheat (Martínez-Barajas et al., 2011).

The first place to start is by identifying which genes are involved in sugar offload and transport, and these may be thus far uncharacterized genes. It is likely such genes will not be identified through transcript-monitoring approaches, that their control is carried out at post-translational level, like the phosphorylation by MAPKs of PIN1, therefore it could be possible it is a protein that has transport properties which is present but is inactive in the dark.

We observed, and it is universally experienced, that growth quantity is increased in HL. Would this also be the action of phytochrome? This could be addressed by using the phyA through an experiment testing activation of phyA without photosynthetic impacts such as far red irradiation , to observe if seedlings respond in the short term to HL. How important is ethylene in growth, and could this be a crucial hormone in the response to shade avoidance? One small experiment to get this started would be growing the *ein3* mutant in vertical plates containing sugar. The result could give some insight to observe if ethylene has a central role in shade avoidance responses. The hypothesis would be that, the seedlings will produce WT leave phenotype with laminae.

What controls auxin transport? MKK7 proved to be part of the “brake” mechanism of growth in dark as seen by the acceleration of growth in light in *mkk7* mutants. However, MKK7 is only part of the story, as the brake and growth reinitiation still occurred in the mutant. What are the other regulatory mechanisms that are involved in the auxin transport inhibition in the dark?

One question about auxin is how the auxin control happens, and whether the fundamental issue in photomorphogenic control of meristematic activity is PAT. To address this a pharmacological agent named 2,3,5-TRI-IODOBENZOIC ACID (TIBA), which inhibits PAT through PIN1 could be used (Michniewicz and Brewer, 2007). COP1 coordinates shoot and root meristem by light partially through regulation of transcription of PIN1 (Sassi et al., 2012), but the localization (and therefore activity) of PIN1 is also directly dependent on light. Examining the *cop1* mutant in the dark in TIBA-containing medium, could address the question. Responses occur qualitatively in the dark as do in the WT in the light, except for auxin export control which still requires an independent, direct light action, without which photomorphogenesis is incomplete.

Overall, I clearly cannot claim to have shown a full mechanistic scenario and learned everything about “light control of leaf growth and meristematic activity” chapter, however, I believe this thesis makes a significant contribution to the understanding of that question.

7. Appendices

7.1. Primers used for gene expression analysis are as follow

GENE	AGI code	Primer set
KRP4	At2g32710	5'-TCGTGGTGATGGGTCTAGGT-3'
		5'-GCCAAAGGTTGGATCTTTATTG-3'
RNR2A	At3g23580	5'-TGTCTGCAAGGCTCTTCCATGTG-3'
		5'-CCTTTCACATCCCAAGGTAACCAGGTAACCAG-3'
H2A	At1g51060	5'-ATTCAGCTTGCAGTGAGGAACG-3'
		5'-CATTCGCAATCGTCACACTTCCC-3'
CYCB1;1	At4g37490	5'-ACCTCGCAGCTGTGGAATATGTG-3'
		5'-ATCTCGTGGCCTCCATTCACTCTC-3'
RPS6	At4g31700	5'-TTGAAGGAACAGCGTGACAG-3'
		5'-GGTGACATCTTTGATTTGATTCTC-3'
EBP1	At3g51800	5'-GCCTGGCTCATGTCGTTTTG-3'
		5'-TTCCTGAAGTGTATGTGAAGTGA-3'
AUX1	At2g38120	5'-TGCGTTTGTGGAGGGTTCTT-3'
		5'-AGCTTAGCACGCATTTAAAGGG-3'
IAA1	At4g14560	5'-TTGGGATTACCCGGAGCACAAG-3'
		5'-GCGCTTGTTGTTGCTTCTGACG-3'
HAT2	ATt5g47370	5'-AACGTCGAGGAAGAAGCTCAGG-3'
		5'-AGCTAGCTTCTGTTTGGGATTGAG-3'
TAA1	At1g70560	5'-TTCGTGGTCAATCTGGATCATGG-3'
		5'-ACCACGTATCGTCACCGTACAC-3'
TAR2	At4g24670	5'-GCTCTTCACTGCTTCAAAGAGCAC-3'
		5'-TCTGTCTTTCACCAAAGCCCATCC-3'
ARR5	At3g48100	5'-AGTTCGGTTGGATTTGAGGATCTG-3'
		5'-TCCAGTCATCCCAGGCATAGAG-3'
EIN3	At3g20770	5'-AACTGGCATGTCCACATCGAGAC-3'
		5'-ATGAAACCTGGATGGTGCTGCTC-3'
EBP	At3g16770	5'-AACTCACGGCTGAGGAACTCTG-3'
		5'-ACGTTAACTTGGTTGGTGGGATGG-3'
bZIP1	At5g49450	5'-CAGTGAGAGATGTCGAGCTGTAA-3'
		5'-ATCTAAGACCCGCGTTCTCC-3'
TPS9	At1g23870	5'-CGCAAATGAGCCTGTAGTCGTC-3'
		5'-GACCTTTGCTTACTCCCTGTGG-3'
GC1	At2g21280	5'-GCCACGGCTGTTGGTTACTATG-3'
		5'-TCCTTCCCATTCTCTGCAAACCTC-3'
ARC5	At3g19720	5'-AGGTGTTTCTCAAGCGGGTTGC-3'
		5'-ACGTAGACCAGCTCGGTTCTTG-3'
VND6	At5g62380	5'-GCCATGGGACATCCAAGAGTTATG-3'

		5’-CCCGTTGCTCTATTGGTTCGTG-3’
ATHB8	At4g32880	5’-TGTCATGTGTTGCTCACTCAAGGC-3’
		5’-TTGAAGTGCCACCAACGTCGTC-3’
UBQ10	At4g05320	5'-GGAGGATGGTCGTACTTTGG-3'
		5'-TCCACTTCAAGGGTGATGGT-3'
GL2	At1g79840	5'-GCAACTCAGTGGCAATCCAGAC-3'
		5'-TGTCTTGCAGCACCCATATGCTC-3'
LHW	At2g27230	5'-TCGCAGATTGGATCAGAAGCT-3'
		5'-TTTCCATCCTCGTCACGTCG-3'

8. Publications:

8.1. Converging Light, Energy and Hormonal Signaling Control Meristem Activity, Leaf Initiation, and Growth

Binish Mohammed[1], Sara Farahi Bilooei[1], Róbert Dóczi, Elliot Grove, Saana Railo, Klaus Palme, Franck Anicet Ditengou, László Bögre, and Enrique López-Juez

1. Joint first authors

Published in **Plant Physiology**

My contribution:

- Microscopy
- Gene expression analyses using qPCR
- Computer analysis
- Contribution to design and organisation of the experiments
- Contribution to preparation and write up of the manuscript

Converging Light, Energy and Hormonal Signaling Control Meristem Activity, Leaf Initiation, and Growth[1][CC-BY]

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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 Target of Rapamycin (TOR) kinase in meristematic activity, yet a picture of how these two regulatory mechanisms depend on light perception and interact with each other has yet to emerge. Their relevance beyond leaf initiation also is unclear. Here, we report the discovery that the dark-arrested meristematic region of Arabidopsis (*Arabidopsis thaliana*) experiences a local energy deprivation state and confirm previous findings that the PIN1 auxin transporter is diffusely localized in the dark. Light triggers a rapid removal of the starvation state and the establishment of PIN1 polar membrane localization 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 the manipulation of auxin and cytokinin activity as well as through the activation of energy signaling, both targets of photomorphogenesis action, but the organ developmental outcomes differ: while TOR-dependent energy signals alone stimulate cell proliferation, the development of a normal leaf lamina requires photomorphogenesis-like hormonal responses. We further show that energy signaling adjusts the extent of cell cycle activity and growth of young leaves non-cellautonomously to available photosynthates and leads to organs constituted of a greater number of cells developing under higher irradiance. This makes energy signaling 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., 2014a). This meristem consists of a pool of stem cells and their close descendants, is organized 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 program, which helps young seedlings to emerge through the ground, before the photomorphogenesis program commences aboveground. 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 program 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,

[1] Work funded in part by NSF/BBSRC bilateral grant BB/M025047 to L.B.

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B.M. and S.F.B. performed the majority of experiments; R.D., E.G., S.R., F.A.D., and E.L.-J. performed essential experiments; B.M., S.F.B., R.D., F.A.D., L.B., and E.L.-J. analyzed and discussed data; K.P., L.B., and E.L.-J. supervised work; E.L.-J. wrote the article; all authors contributed to the final article.

www.plantphysiol.org/cgi/doi/10.1104/pp.17.01730

the 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 signaling 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 postgermination 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 in understanding 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 24 h after light exposure. Gene expression signatures associated with cell proliferation and cytoplasmic growth (protein translation) peaked at 6 h and were followed by expansion growth-associated signatures, including cell wall remodeling 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 down-regulation of auxin and ethylene signaling at the apex was postulated, one that preceded an up-regulation of cytokinin responses. The latter coincided with the peak in the 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 relocalization of the PIN1 auxin transport protein. Cells at this position then enter rapid cell proliferation, but leaf emergence requires a second relocalization 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, 2010). Thus, auxin plays fundamental and separate roles in the positioning and early development of leaves (Capua and Eshed, 2017).

An elegant study carried out in tomato (*Solanum lycopersicum*) shoot meristems showed that the auxin efflux transporter, PIN1, became internalized when light-grown shoot apices were transferred to the dark, while in the light, the auxin maxima established by plasma membrane-localized PIN1 determined the positions for cytokinin action to drive leaf initiation (Yoshida et al., 2011). A subsequent study (Pfeiffer et al., 2016) demonstrated that sugars acting through the Target of Rapamycin (TOR) kinase pathway, together with cytokinin activity, lead to the induction of *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 roles as reduced carbon sources for oxidative phosphorylation, both Glc and Suc 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 medium 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 a 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 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-signaling 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., 2016a) and to auxin (Schepetilnikov et al., 2013, 2017). It was shown recently that, in shoot meristems, light stimulates the TOR activity via two parallel pathways, through photosynthates and through light signaling 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 a state. We show that, upon light exposure of dark-arrested leaf primordia, PIN becomes rapidly polarized and that 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 also can occur in the dark by direct access of the meristem to sugar, in a TOR-dependent manner (i.e. through the activation of energy signaling, the second target of photomorphogenesis action), but with differing results: energy signals stimulate cell proliferation, but the development of a normal leaf lamina requires photomorphogenesis-like hormonal responses. Lastly, we show that available photosynthates impact energy signaling and adjust the extent of cell cycle activity in meristematic cells in a non-cellautonomous 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 6,000 differentially expressed genes (López-Juez et al., 2008). Among these genes, we identified one cluster that was composed of hundreds that responded rapidly, within 1 h, 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 reexamined the expression of all such genes in our transcriptome data. The resulting expression plot of the complete set of starvation-defined genes shows a generalized, rapid down-regulation of transcript levels (Fig. 1). More than 50% of starvation genes were expressed 2-fold or higher in the dark than after 1 h 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 h, we postulate that the rapid repression of starvation genes in the shoot apex is a consequence of the rapid mobilization of reserves stored in the cotyledons upon light exposure. This is in contrast to the growth of the hypocotyl, which occurs rapidly at the etiolated stage, demonstrating that resources do not limit the growth of another organ in the dark. Interestingly, the down-regulation of starvation genes in the shoot apex was transient in most cases, expression becoming high again 24 h 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 6 h after light exposure (Fig. 1). We conclude that skotomorphogenesis in the dark imposes a starvation state specifically on cells within the shoot apex and that this state is released rapidly upon light exposure.

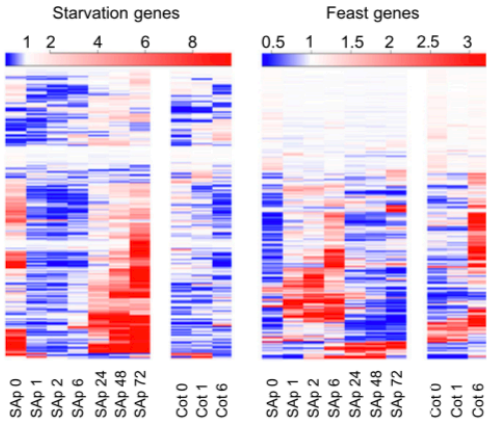

Figure 1. Shoot apices of 3-d-old seedlings exhibit in the dark a starvation response, which disappears within 1 h of light exposure. The expression levels of genes defined as carbon-repressed starvation and carbon-induced feast (Usadel et al., 2008) were plotted using data from a previous microarray experiment (López-Juez et al., 2008). Heat maps 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. The number after each sample type indicates hours after light exposure. Color scales are shown above each plot.

Light Triggers the Polar Localization 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 localization of the PIN1 protein in the arrested meristems of dark-germinated seedlings, before and after their first exposure to light, using immunofluorescence labeling. Confirming a previous report (Yoshida et al., 2011), the PIN1 signal was weak, with limited membrane localization, 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 localization became polar on the plasma membrane within 2 h, in a pattern pointing toward the tips of emerging leaf primordia at the epidermal cell layer and away from the tips of primordia in a cell file at the center of the leaf lamina. This pattern was particularly evident 24 h after exposure to light, the position of the PIN1 signal indicating auxin transport toward the primordia tips and export toward the rib meristem (Fig. 2A).

Consistent with the diffuse PIN1 localization pattern in the dark, the *DR5:GUS* auxin activity reporter

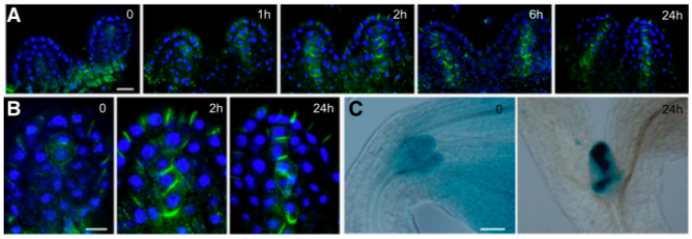

Figure 2. Light exposure of dark-grown seedlings triggers a rapid PIN1 polarization at the leaf primordia and the establishment of localized auxin maxima. A, PIN1 immunofluorescence localization in the first two leaf primordia of wild-type seedlings: PIN1 (green) and 4′,6-diamino-2-phenylindole (DAPI; blue). Seedlings were germinated in the dark for 3 d, 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 localization images in A after 0, 2, and 24 h. C, *DR5:GUS* reporter activity of seedlings in the dark and exposed to white light for 24 h. Bars = 10 μm (A), 5 μm (B), and 50 μm (C).

(Ulmasov et al., 1997) revealed a relatively high but delocalized 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 localization. However, 24 h 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 (Fig. 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 deetiolated state in the dark, manifest as short hypocotyl and open cotyledons. It also has been shown that exposure of wild-type Arabidopsis (*Arabidopsis thaliana*) 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 and low cytokinin activity) into the one normally found after light exposure (low auxin and 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 Suc-containing plates in the dark. Without BAP, the leaf primordia remained arrested in the dark in the wild type, while a substantial increase in leaf primordia size was observed in the *axr1-12* mutant grown in the same conditions (Fig. 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 increased further in the *axr1* mutant. After 5 d in the dark, the leaf primordium size of BAP-treated *axr1* seedlings reached about one-third 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 the removal of auxin and the activation of the 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). Seven-day-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 (Fig. 4A; Supplemental Fig. S1). Flow cytometric ploidy analysis of these leaf primordia showed that around 60% of cells had 2N and 40% had 4N nuclear DNA content (Fig. 4C). Cell cycle analysis of the flow cytometry data revealed that a high proportion of nuclei were undergoing DNA synthesis (Fig. 4B; for extended data, see Supplemental Fig. S2A), indicating that these cells are very actively proliferating. A further 3 d 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 that entered endoreduplication (with 8N nuclei; Fig. 4C;

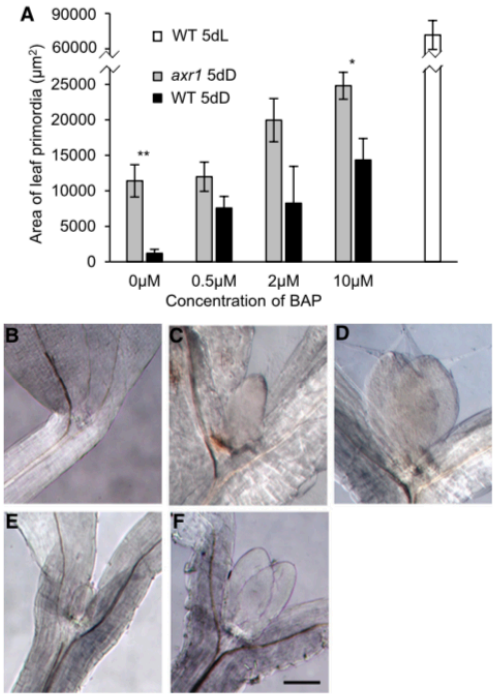

Figure 3. In the *axr1-12* mutant, leaves initiate in the dark, this being enhanced by the addition of cytokinin. A, Seedlings of the Columbia wild type (WT) and *axr1-12* were germinated and grown in the dark for 5 d, on 1% Suc-containing medium with or without BAP at the concentrations indicated, or for the wild type in the light for 5 d on medium without BAP. The area of one of the first two leaf primordia is indicated. Error bars represent SE. Asterisks reflect the significance of differences between *axr1* and the wild type. B to F, Images of leaf primordia of representative shoot apical regions of seedlings as in A. B to D, The wild type. E and F, The *axr1-12* mutant. B and E, Dark, no BAP. C and F, Dark, 10 μM BAP. D, Light. Bar = 200 μm.

Supplemental Fig. S2B). In contrast, transfer to dark for 3 d led to the almost complete losses of mitotic activity, organ expansion, and endoreduplication; instead, an increase in the proportion of 2N nuclei occurred, indicating a widespread G1 arrest in the dark (Fig. 4; Supplemental Fig. S2). Reexposure to light triggered a reinitiation of cell proliferation, as indicated at 12 h by the increased mitotic activity (note the GUS mitotic signal in Fig. 4A), increased number of cells undergoing DNA synthesis as measured by flow cytometry (note the increase of S-phase nuclei in Fig. 4B; Supplemental Fig. S2), and an increased percentage of 4N nuclei, indicating cells that had passed through DNA synthesis (Fig. 4C). At the later time point of 48 h, cells with 8N nuclei also appeared, indicating the start of endoreduplication-associated cell expansion (Supplemental Fig. S2B).

The above observations were made on seedlings grown on Suc-containing plates, but similar phenomena took place in the absence of exogenous Suc 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 Fig. S2), others, like the reinitiation of mitotic events, were even more so (Supplemental Fig. 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 Suc-free or Suc-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 Program

We previously observed a program 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 buildup (Table I). We assessed whether a comparable gene expression program 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 Suc-containing medium.

Genes associated with mitosis (*CYCB1;1*), DNA synthesis (*RNR2A* and *H2A*), and translation (*RPS6* and *EBP1*) were all repressed during the 3-d dark period and were up-regulated in the first leaf pair within 8 h following reexposure to light; in several cases, up-regulation could be detected already at 3 h after reexposure (Fig. 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 1 h a transient, substantial drop, which preceded a mild up-regulation of cytokinin-responsive gene expression (*ARR5*). At later time points during light-reinitiated leaf growth, between 3 and 24 h, both the expression of auxin biosynthesis genes (*TAR2* and *TAA1*) and that of auxin-responsive *AUX1* and *HAT2* increased. In contrast, the expression of two genes representing ethylene response (*EIN3* and *EBP*) was elevated consistently in the dark and reduced in the light.

As expected, the expression of starvation genes became up-regulated in the dark, reflecting the establishment of a starvation state, and rapidly dropped upon transfer to light, within 1 h (Fig. 5). Since this

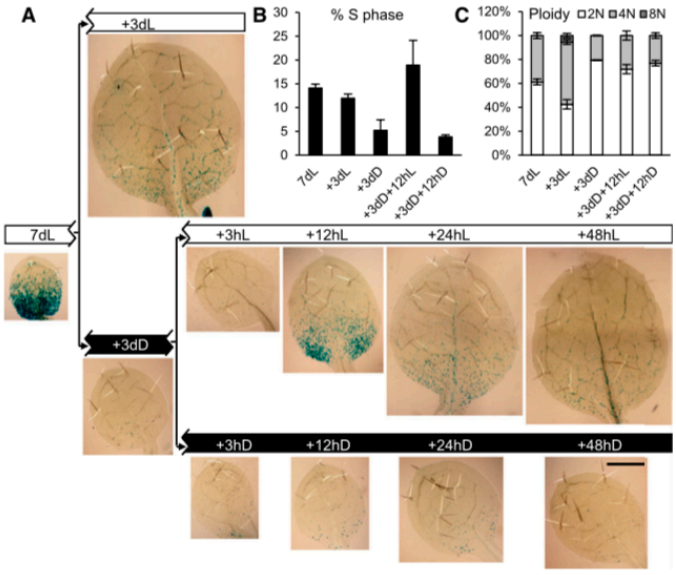

Figure 4. Proliferation activity arrest following transfer to dark, and reinitiation of mitotic activity in the light in proliferation-competent cells at the leaf base. A, *CYCB1;1::DB-GUS*-expressing seedlings were grown for 7 d in continuous light (7dL), harvested immediately or transferred to 3 d of 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 visualizing the GUS reporter, is shown. Blue GUS stain indicates cells undergoing mitosis in an acropetal gradient. 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 with different ploidy levels under the conditions indicated. Error bars represent SD (n = 3, with each sample containing a pool of at least five leaves).

happened in spite of the fact that the seedlings were grown on Suc-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 reexposure on plates with Suc also were largely replicated when seedlings were grown in the absence of exogenous Suc (Supplemental Fig. S4). A notable difference between the experiments on Suc-containing and Suc-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

Table I. *Genes monitored as representatives of biological growth processes, and products they encode*

Process	Gene	Arabidopsis Genome Initiative Code	Encoded Product
Cell cycle entry block	*KRP4*	At2g32710	Kip-related protein4
DNA synthesis (S phase)	*RNR2A*	At3g23580	Ribonucleoside-diphosphate reductase small chain A
	H2A	At1g51060	Histone 2A
Mitosis (M phase)	*CYCB1;1*	At4g37490	Cyclin B1;1
Ribosome biosynthesis	*RPS6*	At4g31700	40S ribosomal protein S6-1
	EBP1	At3g51800	ERBB-3 binding protein 1
Auxin response	*AUX1*	At2g38120	Auxin resistant1
	IAA1	At4g14560	indole-3-acetic acid inducible1
	HAT2	At5g47370	Homeobox-Leu zipper protein2
Auxin synthesis	*TAA1*	At1g70560	Trp aminotransferase1
	TAR2	At4g24670	Trp aminotransferase-related protein2
Cytokinin response	*ARR5*	At3g48100	Arabidopsis two-component response regulator5
Ethylene response	*EIN3*	At3g20770	Ethylene insensitive3
	EBP	At3g16770	Ethylene-responsive element binding protein
Starvation of reduced carbon	*bZIP1*	At5g49450	Basic Leu zipper1
	TPS9	At1g23870	Trehalose-6-phosphatase/synthase9
Mesophyll cell (chloroplast) development	*GC1*	At2g21280	Giant chloroplast1
	ARC5	At3g19720	Accumulation and replication of chloroplasts5
Vascular/vein development	*VND6*	At5g62380	Vein deficient6
	ATHB8	At4g32880	Homeobox-Leu zipper protein8

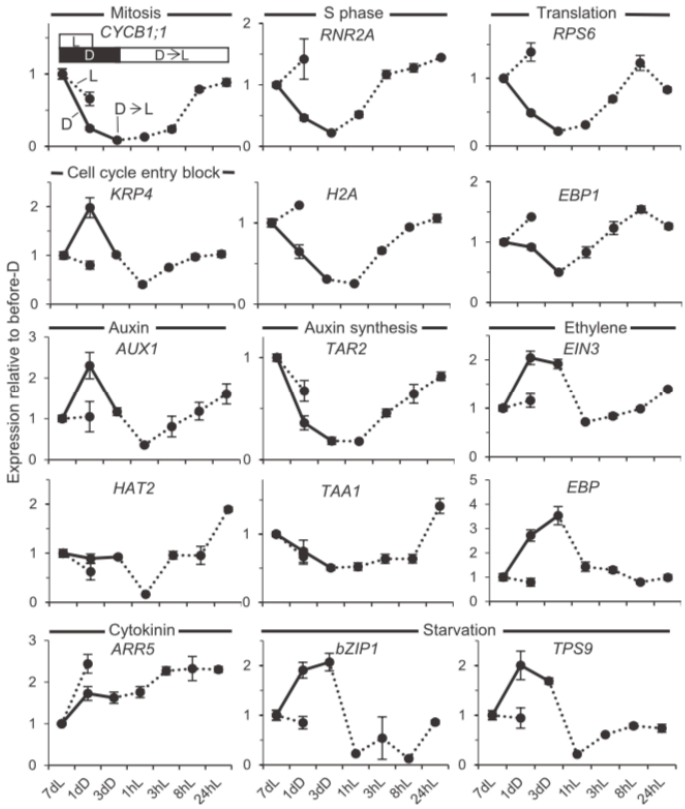

Figure 5. Expression of signature genes during dark arrest and subsequent light exposure in young leaf primordia. The dark arrest blocks the cell proliferation and growth genetic program and activates starvation genetic responses. Light reverses these and brings about hormonal resetting. Wild-type seedlings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

conditions. These gene expression changes are unlikely to be circadian regulated. Although eight 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*) coincided with the observed pattern in our experiment, an elevation at the start of light exposure (dawn [Zeitgeber 0 h]; Supplemental Table S1). The extended, slightly finer time course examined for seedlings in the absence of Suc 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 reinitiation of leaf development necessitates the differentiation of all cell types that, 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 the expression of marker genes for early chloroplast biogenesis (*GC1* and *ARC5*) and for the initiation of vascular development (*VND6* and *ATHB8*) and that both types of cellular differentiation were promoted by reexposure to light (Supplemental Fig. S5A).

The Starvation/Growth Arrest Gene Expression Program Is Largely under the Control of the COP1-Dependent Photomorphogenic Pathway

To address whether the gene expression program upon dark arrest and light reexposure of young developing leaves is imposed by photosynthetic activity status or light signaling, we performed these experiments using the *cop1-1* mutant. In the dark, this mutant maintains active photomorphogenic signaling 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 (Fig. 6, inset). We then monitored gene expression signatures associated with

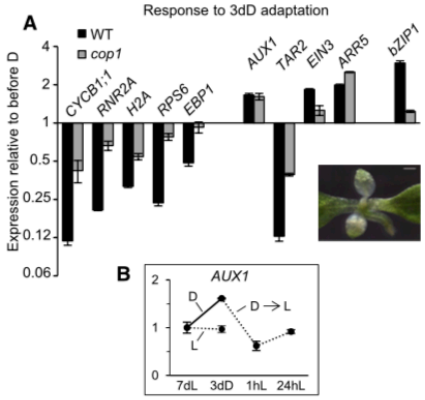

Figure 6. The gene expression program change in the light is brought about to a large extent by COP1-dependent photomorphogenesis pathways. A, Expression in leaf primordia of the genes indicated after 7dL + 3dD is shown, plotted on a $\log_2$ scale relative to the levels after 7dL, in the *cop1* mutant and its wild type (WT) grown on Suc-containing plates. The inset shows leaf primordia of a 3dD-adapted *cop1* seedling. B, Expression of *AUX1* after 7dL, + 3dD, and following transfer back to light (times indicated). Error bars indicate SE.

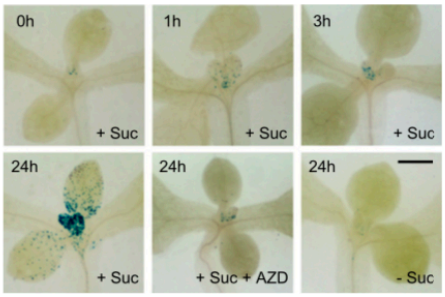

Figure 7. Direct Suc access to the meristem reactivates cell proliferation in the absence of light in a TOR-dependent manner. *CYCB1;1::DB-GUS*-expressing seedlings were grown on solid medium plates in light for 7 d, transferred to Suc-free liquid medium in the dark for 3 d, and visualized for GUS expression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

growth, hormones (auxin, ethylene, and cytokinin), and starvation in *cop1-1* mutant seedlings compared with the wild type upon 3 d in dark (Fig. 6A). Compared with the wild type, cell proliferation and growth gene expression signatures were less impacted by the dark adaptation in *cop1-1* (Fig. 6A). The reduced expression of a gene involved in auxin synthesis, as well as the up-regulation of ethylene action and of the starvation response in the dark, were all attenuated in *cop1* (Fig. 6A). The same difference in expression was observed for the cell type-specific signature genes (Supplemental Fig. S5B). We then examined the kinetics of the auxin response by monitoring *AUX1* gene expression both during dark arrest and light reactivation. The rapid, transient down-regulation of auxin response following reexposure to light was present in the *cop1-1* mutant (Fig. 6B). This implies that a COP1-dependent photomorphogenic pathway is responsible for the bulk of the gene expression program in the dark. However, the transient down-regulation of auxin signaling during the dark-to-light transition appears to be independent of COP1 action.

Direct Suc Access to the Shoot Apex Activates Cell Proliferation and the Growth Gene Expression Program in the Dark

We have shown that the shoot apex in the dark locally experiences a starvation state, which is terminated rapidly by light in a way that cannot be explained by photosynthetic activity. Intriguing observations have shown that exposure of the meristem to Suc or Glc can trigger the further growth of organs in the dark (Roldán et al., 1999; Li et al., 2017). We made Suc available to the shoot apices of seedlings in the dark using the following strategy: 7-d light-grown seedlings, exhibiting active meristematic activity, were arrested by transferring to dark in Suc-free liquid culture, and after 3 d, the culture medium was replaced, under very dim green safelight, with Suc-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 (Fig. 4) all but disappeared during dark adaptation in the absence of Suc, while exposure to Suc resulted in a reemergence of mitotic activity, which was most pronounced after 24 h (Fig. 7; Supplemental Fig. S6A). The most frequent localization of such events was the proximal region of leaf primordia (Fig. 7).

We monitored the gene expression program initiated by direct exposure of the meristem to Suc in the dark (Fig. 8). As expected from the observation of reactivation of mitotic activity visualized by the *CYCB1;1:DB-GUS* reporter, the cell proliferation- and growth-associated gene expression also was strongly stimulated by direct Suc access, with a simultaneous rapid down-regulation of starvation signature genes (Fig. 8). The induction of genes associated with plastid biogenesis and vasculature development also exhibited light-like responses (Supplemental Fig. S5C). Three notable differences, however, could be observed in comparison with the response to light. First, the response of growth-related genes to direct Suc supply was somewhat slower than that to light, generally clear after 8 h rather than 3 h. Second, the rapid, transient down-regulation of auxin responses upon the dark-to-light transition was not seen when dark-adapted seedlings were exposed to Suc; only a strong increase of such responses

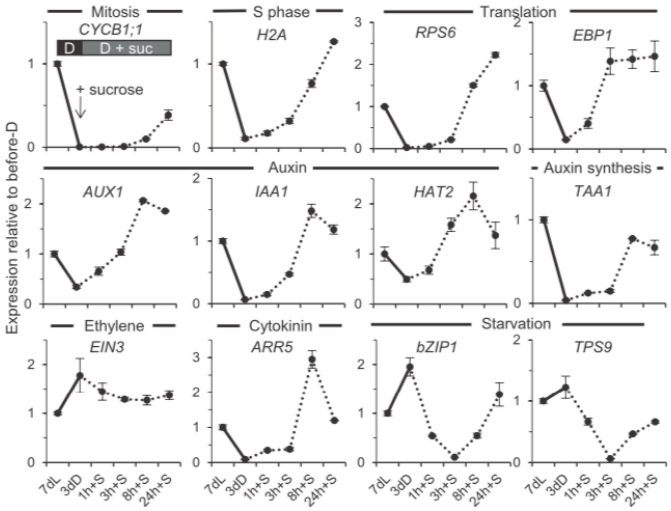

Figure 8. Direct Suc access activates a proliferation and growth gene expression program. Wild-type seedlings grown for 7 d in continuous light on solid medium were transferred to Suc-free liquid medium in darkness for 3 d, then transferred to Suc-containing medium for the times indicated. Seedling shoot apices were dissected, and gene expression was quantified and displayed as in Figure 5.

was observed, as confirmed by three separate signature genes, suggesting that a rapid activation of auxin export had not taken place under Suc influence, only the up-regulation of auxin synthesis had. Third, ethylene responses, which were rapidly down-regulated by light, were reduced only mildly after Suc exposure in the dark (Fig. 8). We conclude that, during leaf development, cell proliferation, cytoplasmic growth, aspects of plastid biogenesis, and vasculature differentiation all are under Suc 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 6-d incubation in Suc-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 Fig. S7). To examine this further, we administered a prolonged exposure of the meristem to Suc in the dark while avoiding the hypoxia that characteristically occurs in liquid culture, by growing seedlings on vertical Suc-containing solid medium, where apices of seedlings contact the medium's surface, as carried out by Roldán et al. (1999). The shoot apex of seedlings grown on horizontal, Suc-containing medium developed leaves only in the light but was completely arrested in the dark (Fig. 9, A and B). The meristem of seedlings grown in the dark in liquid medium without Suc also was arrested, while if the medium contained Suc, the leaf primordia developed (Fig. 9, C and D). This indicated that direct sugar access is required for leaf initiation. Prolonged growth of shoot apices in contact with Suc led to extraordinarily elongated seedlings (Fig. 9E), with unusually long petioles of cotyledons and new leaves as well as internodes (Fig. 9, E–H). Elongation of the internodes reflects premature activation of the rib meristem. Leaf lamina barely developed (Fig. 9G); however, the transition to flowering occurred (Fig. 9H). Addition of BAP to the medium of seedlings whose shoot meristems were not in contact with Suc also initiated leaf development, both in the wild type and in the *axr1* mutant background (Fig. 9, I–J). In addition, we noted in the *axr1* mutant occasional tumor-like growths on some leaf primordia when exposed to cytokinin (Fig. 9K). The *cop1* mutant also developed leaf primordia in the dark without direct contact with Suc-containing medium (Fig. 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 also can be overcome by a light-like shift in hormonal activity or by the removal of COP1, thus activating photomorphogenic signaling.

The strategy of enhancing Suc access through the growth of seedlings on Suc-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 Suc in the light. To this end, we designed an experimental setup that depletes CO_2 in air (see "Materials and Methods"; Supplemental Fig. S8). The transfer of seedlings for 3 d into darkness on vertical plates without Suc led to the almost complete cessation of cell proliferation activity, while on Suc-containing vertical plates, with apices being in

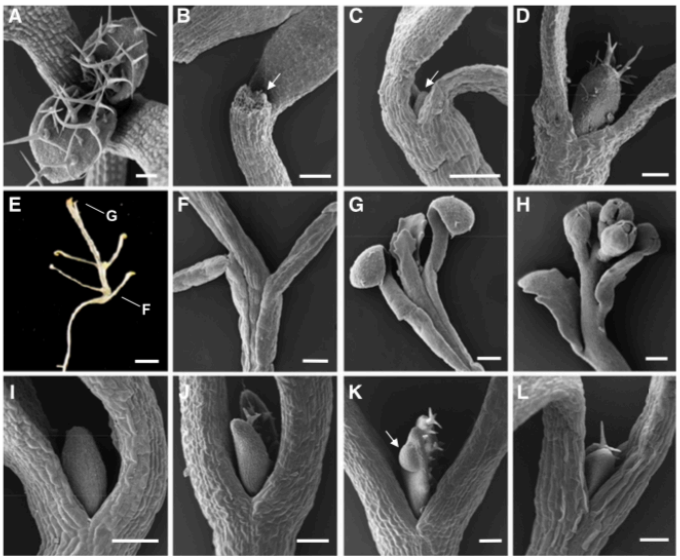

Figure 9. The dark arrest of leaf initiation can be overcome by direct access to Suc, change in hormonal response, or by the loss of COP1. Scanning electron micrographs show shoot apices of seedlings of the wild type (A–I), *axr1-12* mutant (J and K), and *cop1* mutant (L) genotypes. All seedlings except that in A were grown in continuous dark. A, Wild type, continuous light, 7 d, horizontal Suc-containing plate. B, As in A but in continuous dark. C, Wild type, 17 d, Suc-free liquid medium. D, Wild type, 7 d, Suc-containing liquid medium. E, Wild type, 28 d, vertical Suc-containing plate. F, Detail of a seedling equivalent to that in E. G, Detail of a seedling equivalent to that in E. H, Detail of a seedling equivalent to that in E but grown for 42 d. I, Wild type, 7 d, horizontal Suc-containing plates with 10 μM BAP. J, *axr1* mutant, 7 d, horizontal Suc-containing plates with 2 μM BAP. K, As in J. L, *cop1* mutant, 7 d, horizontal Suc-containing plates. Arrows in B and C indicate leaf primordia; the arrow in K indicates a tumor-like growth. Bars = 100 μm (A–D and I–L), 200 μm (F–H), and 2 mm (E).

contact with the plate, cell proliferation remained active. An 8-h light exposure reactivated cell proliferation in the shoot meristem of seedlings grown in Suc-free medium, but this was prevented in CO_2-free air, where photosynthesis cannot take place (Supplemental Fig. S8; for quantitation, see Supplemental Fig. S6B). We conclude that the photomorphogenic response of the meristem and leaf primordia to light requires photosynthesis-generated reduced carbon.

The Growth Response to Suc Is Mediated by the TOR Pathway

Having observed key aspects of the genetic program that are initiated by exposure to Suc, 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 PI3K-like protein kinase 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 to be effective in plants (Montané and Menand, 2013; Dong et al., 2015; Kravchenko et al., 2015; Schepetilnikov et al., 2017). The fact that *AtTOR* heterozygous knockout 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 (Fig. 7; Supplemental Fig. S6C). It also reduced the Suc-induced expression of cell cycle and cell growth signature genes, confirming that these processes are, to a large extent, mediated by the TOR pathway (Fig. 10; note the log scale). Remarkably, the up-regulation of two out of three auxin-response genes also was found to be partially TOR dependent. Interestingly, we found genes involved in plastid biogenesis to be particularly sensitive to TOR inhibition (Supplemental Fig. S5D). In the dark, the addition of Suc repressed the expression of starvation genes in leaf primordia, but only to some extent after 24 h (Fig. 10). Unexpectedly, the addition of AZD-8055 further reduced their expression, indicating that the sugar repression of starvation genes is modulated, but not dependent on TOR signaling. We conclude that Suc 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 utilize energy signaling to determine meristematic activity would be that it would allow them to adjust organ growth to the

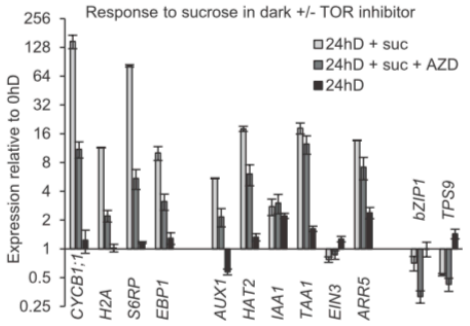

Figure 10. The gene expression program induced by Suc in the dark is largely TOR dependent. Expression in the shoot apex and leaf primordia of the genes indicated is shown following the growth treatment described for Figure 7 (7dL in solid medium followed by 3dD in Suc-free liquid medium), after transfer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

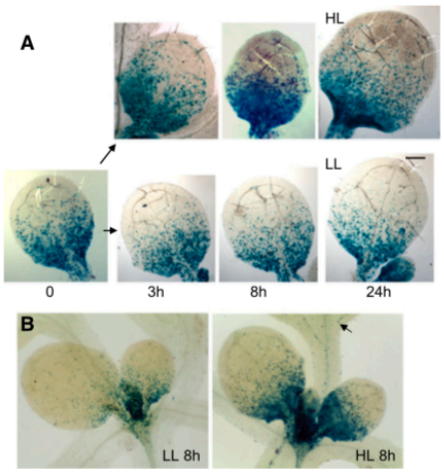

Figure 11. During growth in the light, exposure to HL for 8 or 24 h increases cell proliferation. A, *CYCB1;1*::*DB-GUS*-expressing seedlings were grown for 7dL, transferred to soil, adapted to LL (40 μmol m^{-2} s^{-1}) until day 11 (see "Materials and Methods"), then harvested immediately or after transfer to HL (300 μmol m^{-2} s^{-1}; top row) or maintained at LL (bottom row) for the times indicated, and visualized for GUS reporter activity. Leaf 3 is shown. Bar = 200 μm. B, Apical region, displaying primordia of leaves 3 and 4, 8 h after the light transfer, visualized for the GUS reporter. The arrows indicates mitotic events in the young leaf 2.

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 (López-Juez et al., 2007; Kalve et al., 2014b). Here, we tested how the mitotic activity becomes modulated in response to changing light intensity throughout the leaf by analyzing 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; Fig. 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 (leaf 3 onward), but a few were visible even in leaves 1 and 2 only under HL (Fig. 11B). Cells of leaves 3 and 4 also entered endoreduplication at an accelerated rate in HL (Supplemental Fig. 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 Fig. S10A). The average size of mesophyll cells was much smaller in the proximal than in the middle and distal regions, whether grown under LL or HL, with size increasing as cell expansion took place. Here, we detected a higher mitotic activity in leaves at LL compared with those of the same age at HL, indicating that the entire developmental program is slowed down and that there is a delayed exit from proliferation to differentiation under LL (Supplemental Fig. S10A). In agreement, while after 4 d some mitotic activity remains at the distal region of the leaf in LL, mitotic activity had already ceased in this area in leaves developing in HL (Supplemental Fig. S10B).

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 (Fig. 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 signaling in developing leaves (Fig. 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 Fig. S5E). We conclude that, like the dark-to-light transition, a change in light intensity rapidly alters the energy, hormonal, cell proliferation, and differentiation programs.

The Effect of HL on Cell Proliferation in Young Leaves Is Non-Cellautonomous

If available photosynthates, produced by photosynthetically-competent leaves, are indeed the proliferative

localization of PIN1 toward primordia tip maxima in the epidermis and away in the developing mid vein toward the rib meristem. 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, 2010). As part of the complex action of auxin, we confirmed that a strong, localized 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 demonstrated previously (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 form an intrinsic part of the endogenous, early photomorphogenic program 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 signaling through direct exposure of the meristem to Suc is itself capable of promoting at least some auxin responses, as evidenced by the regulation of signature genes (Fig. 8). This action was, for two out of three genes tested, TOR dependent (Fig. 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 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 signaling and explain some of their partly shared responses.

Energy signaling plays a central role in the control of both cellular growth (Dobrenel et al., 2016a, 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, on its own, it cannot lead to photomorphogenic-like rosette leaves. Instead, the meristem overwhelmingly produces petioles and internodes (Fig. 9; Supplemental Fig. S7). While such developmental behavior 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 Suc exposure. Ethylene signaling is necessary for hypocotyl hook formation, a component of the skotomorphogenic program (Marín-de la Rosa et al., 2014), and pea (*Pisum sativum*) 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 synthesized 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 toward elongating organs, like internodes and petioles in the dark, when ethylene response is high, or toward leaf laminae, with their distinct epidermal and mesophyll cellular makeup in the light, when ethylene responses are repressed. Whether this possible ethylene switch of the proliferative potential acts solely through auxin activities is unknown at present. An elegant genetic screen 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 note, nevertheless, that following a substantially extended period of dark growth on Suc, after the transition to flowering, one could observe comparatively normal cauline leaves as well as floral buds (Fig. 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 signaling, or competence to respond to it, is only a matter of conjecture at present.

Photomorphogenesis acts through a COP1-dependent pathway. Transcription factors that 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 signaling. Light appears to play what could be described as a gating, or permissive, role toward energy signaling in that the extent of meristem activity is dependent on seed reserves or, later, photosynthates, but only when light is present does this reduced carbon become 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 the growth of seedlings on Suc-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 localized in the nucleus, where its targets are light-associated transcription factors. Thus, the transient auxin response drop is most probably the result of a postranslational control of auxin export via PIN1, the control being mediated by PIN1 localization. The nature of this control remains poorly understood, but posttranslational signaling cascades, mediated by protein kinases, control PIN1 localization under other developmental scenarios (Benjamins et al., 2001; Jia et al., 2016; Dory et al., 2017). Transcript levels of PIN1 also are 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 signaling becomes most apparent in the control of the cellular makeup 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 primordium which is physically possible to examine, composed of just a few tens of cells (Kalve et al., 2014b), 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 the light exposure of mature ones (Yano and Terashima, 2001), one can conclude that the recruitment of meristematic cells to primordia is under non-cellautonomous, 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 (Fig. 11; Supplemental Figs. S9 and S10), where high irradiance promoted extra cell proliferation in our leaf primordia of HL-exposed plants. Our data show that this also is a systemic response, dependent on the irradiance received by mature leaves, adding a further dimension to the impact of photosynthate signaling on meristematic activity. It was shown recently (Van Dingenen et al., 2016) that the larger organs under HL are explained to an extent by an increased import capacity of Glc into chloroplasts of young, meristematic, proximal leaf cells. This causes a down-regulation 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 observed is not. How these interorganellar and energy-signaling regulatory mechanisms interact and delay or accelerate the exit into endoreduplication/differentiation remains to be answered.

CONCLUSION

Two stages in which the action of light determines meristematic activity become apparent in this study (for model, see Supplemental Fig. S13). First, the presence of light plays a permissive role (i.e. no cell cycle and growth activities can occur in prolonged dark). This action utilizes photomorphogenic pathways and is photoreceptor and, largely, COP1 dependent and makes use of auxin-, cytokinin-, and ethylene-dependent mechanisms of meristem organization, leaf initiation, and cell fate decision making, together with a photomorphogenic gating control of energy signaling. The latter may be due to the control of access to reduced carbon, activates the TOR signaling pathway, and has cell proliferation and growth as its output. Second, 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 results here, together with previous studies, contribute to untangling the complex role and interactions of hormonal and energy signaling, through the action of the TOR kinase, to determine the activity of meristematic and early-organ cells in the light. They also have opened many new questions. Understanding the means by which TOR action arises from the combined energy and auxin response, uncovering the mechanism of photomorphogenic energy-signaling gating, the way in which the starvation state is imposed in the absence of photoreceptor action, and unraveling the different cellular and organ fates produced by meristematic activity under light or energy-only signaling should be among the matters addressed by further analyses. It is apparent, nevertheless, that energy signals may constitute the most important determinant of plant growth and, therefore, biomass production in nonstressed conditions.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Experimental Treatments

Wild-type Arabidopsis (*Arabidopsis thaliana*) plants of the Columbia (Col) ecotype and the *axr1-12* mutant (Leyser et al., 1993) were obtained from the

Nottingham Arabidopsis Stock Centre. The *cop1-4* mutant (Deng et al., 1991), the *CYCLINB1;1:Dbox-GUS* line (Colón-Carmona et al., 1999), and *DR5:GUS* (Ulmasov et al., 1997) were kind gifts of J. Gray, P. Doerner, and T.J. Guilfoyle 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) or in a dark incubator at 21°C, on horizontal plates containing 0.8% (w/v) agar-solidified MS medium and 1% (w/v) Suc, unless stated otherwise, and when required in the presence of BAP (Duchefa/Melford Laboratories) at the indicated concentration, as described previously (López-Juez et al., 2008). In liquid culture experiments, unless stated, seedlings were grown for 7 d on horizontal plates in the light as above, then transferred to six-well microtiter plates containing liquid MS medium devoid of Suc, for a further 3 d in the dark, with shaking (80 rpm), at which point the medium was replaced under very dim green safelight with fresh Suc-free or 1% Suc-containing medium, with or without the addition of 2 μM AZD-8055 (Sigma-Aldrich). Liquid-cultured seedlings shown in Figure 9 were grown from germination in the 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 Suc-containing vertical plates in the light, and after 7 d, they were transferred to fresh Suc-containing or Suc-free vertical plates and double clear bagged with or without 5 g of indicator-containing soda lime (Fisher Scientific), as described previously (Kircher and Schopfer, 2012). For light quantity experiments, seedlings grown for 7 d on MS horizontal plates were transferred to soil, grown for 2 d at 100 μmol $m^{-2}s^{-1}$, and adapted for a further 2 d to 40 μmol $m^{-2}s^{-1}$ continuous LL, before transfer if required to 300 μmol $m^{-2}s^{-1}$ continuous HL. For the cellular makeup experiment (Supplemental Fig. 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 d. 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 6 d to achieve a sufficient rosette size, before adapting to LL for another 3 d, and then subjected to LL, HL, local HL or systemic HL by exposure to HL and the use of custom-sized neutral density celluloid filters. Leaves were collected 8 h 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 to a final concentration of 0.3 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-glucuronic acid under vacuum with 0.5 atm pressure for 10 min. Seedlings were then kept in the dark at 37°C for 14 h followed by postincubation in 3:1 (v/v) methanol:acetic acid for 2 h and washes in 70% ethanol at 65°C for 10 min. Seedlings were mounted on slides in Hoyer's solution. Digital images were recorded using a Nikon SMZ1500 equipped with a Nikon DXM1200 camera or Leica EZ4HD (Leica Microsystems) stereomicroscopes.

Primordia of BAP-treated *axr1* or Columbia 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, individual cells, or GUS-stained areas were measured using ImageJ software (https://imagej.nih.gov/ij/). Except where indicated, measurements used 10 seedlings. To quantify cellular anatomy, leaves were divided into basal, mid, and distal thirds, average cell areas were measured in each region, the number of cells for each region was estimated as one-third of the leaf area divided by the corresponding average cell area, and the resulting number of cells was added for the three regions.

For immunocytochemistry, samples were fixed and processed as described previously (Gälweiler et al., 1998). PIN1 was detected in permeabilized seedlings incubated with an affinity-purified mouse anti-PIN1 monoclonal antibody (1:100) and monoclonal secondary antibody (Alexa 488-labeled goat anti-mouse at 1:1,000 dilution). Fluorescence was analyzed with a Zeiss LSM 5 DUO scanning microscope. Fluorescence-labeled anti-PIN antibody and DAPI fluorescence were monitored using multitracking in frame mode. Alexa 488 was excited using the 488-nm laser line in conjunction with a 505- to 530-nm band-pass filter. DAPI was excited with the 405-nm laser line and collected using a 420- to 480-nm band-pass filter.

Flow Cytometry Analysis

To determine cell DNA content, leaf primordia of a minimum of five 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), and cells were chopped with a sharp razor blade as described previously (López-Juez et al., 2008). One milliliter of DAPI DNA-staining solution (Partec) was added, the sample was mixed, filtered, and analyzed through a PAS flow cytometer (Partec), and the fluorescence of different ploidy peaks was calibrated using Arabidopsis floral tissue. The proportions of peak areas at different ploidy levels were measured using Flomax software. Where only 2N and 4N peaks were present, cell cycle 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 7 d at 4°C, before dissecting primordia using 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-8055, 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) following the manufacturer's instructions and quality checked by agarose gel electrophoresis. Two-microgram aliquots were reverse transcribed using the Maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific). DNA was used for real-time amplification as described previously (Hills et al., 2015). Three independent biological replicates, each containing 150 to 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 ΔCt 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 are as listed (Supplemental Table S2).

Assessment of the possible circadian behavior 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% [v/v] glutaraldehyde plus 4% formaldehyde in 0.1 M PIPES, pH 7.2) at room temperature and stored at 4°C for 12 h. The primary fixative was removed, and seedlings were 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 CO_2, mounted on an aluminum scanning electron microscope stub with conductive glue, and sputter coated with gold/palladium before observation in an FEI Quanta 200 scanning electron microscope (Biomedical Imaging Unit, Southampton University Hospital).

Accession Numbers

Accession numbers are listed in Table I.

Supplemental Data

The following supplemental data are available.

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

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

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

Supplemental Figure S4. Gene expression analysis showing that dark arrest blocks the cell proliferation and growth genetic program and activates starvation genetic responses at the shoot apex in seedlings on Suc-free plates.

Supplemental Figure S5. Expression of genes associated with plastid biogenesis (primarily leaf mesophyll) and vascular development at the

shoot apex in the dark arrest, *cop1*, Suc supply, TOR inhibitor, and light fluence rate experiments.

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

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

Supplemental Figure S8. *CYCB1;1:DB-GUS* expression showing that access to external Suc maintains cell proliferation in the dark; light activates cell proliferation in the absence of external Suc, but this requires access to CO_2 for photosynthesis.

Supplemental Figure S9. Flow cytometric cell cycle parameters showing that transfer to HL rapidly promotes cell proliferation and subsequently accelerates entry into endoreduplication.

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

Supplemental Figure S11. Experimental setup and *CYCB1;1:DB-GUS* expression quantitation showing that HL acts systemically on cell proliferation in young leaf primordia after perception by mature leaves.

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

Supplemental Figure S13. Model of the impact of photoreceptor activation or exposure to HL and the occurrence or extent of leaf organ growth.

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

Supplemental Table S2. Primers used for gene expression analysis.

ACKNOWLEDGMENTS

We are indebted to John Gray, Peter Doerner, Thomas J. Guilfoyle, and the Nottingham Arabidopsis Stock Centre for the supply of materials; Rajat Yadav, James Hall, and Miranda Burke for skillful help with hormone reporter and deetiolation assays; Anton Page and members of the Southampton Bioimaging Unit for excellent support for scanning electron microscopy; and Czaba Papdi and members of the E.L.-J. and L.B. laboratories for constructive criticisms.

Received December 4, 2017; accepted December 21, 2017; published December 28, 2017.

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8.2. The MKK7-MPK6 MAP Kinase Module Is a Regulator of Meristem Quiescence or Active Growth in Arabidopsis

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Published in **Frontiers in plant science**

My contribution:

- Gene expression analyses using qPCR
- Computer analysis

frontiers in Plant Science

ORIGINAL RESEARCH
published: 05 March 2019
doi: 10.3389/fpls.2019.00202

The MKK7-MPK6 MAP Kinase Module Is a Regulator of Meristem Quiescence or Active Growth in Arabidopsis

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OPEN ACCESS

Edited by:
Michael James Considine, The University of Western Australia, Australia

Reviewed by:
Toshiro Ito, NARA Institute of Science and Technology (NAIST), Japan
Stefan de Folter, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Mexico
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Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *10 September 2018*
Accepted: *06 February 2019*
Published: *05 March 2019*

Citation:
Dóczi R, Hatzimasoura E, Farahi Bilooei S, Ahmad Z, Ditengou FA, López-Juez E, Palme K and Bögre L (2019) The MKK7-MPK6 MAP Kinase Module Is a Regulator of Meristem Quiescence or Active Growth in Arabidopsis. Front. Plant Sci. 10:202. doi: 10.3389/fpls.2019.00202

Plant growth flexibly adapts to environmental conditions. Growth initiation itself may be conditional to a suitable environment, while the most common response of plants to adverse conditions is growth inhibition. Most of our understanding about environmental growth inhibition comes from studies on various plant hormones, while less is known about the signaling mechanisms involved. The mitogen-activated protein kinase (MAPK) cascades are central signal transduction pathways in all eukaryotes and their roles in plant stress responses is well-established, while increasing evidence points to their involvement in hormonal and developmental processes. Here we show that the MKK7-MPK6 module is a suppressor of meristem activity using genetic approaches. Shoot apical meristem activation during light-induced de-etiolation is accelerated in *mpk6* and *mkk7* seedlings, whereas constitutive or induced overexpression of MKK7 results in meristem defects or collapse, both in the shoot and the root apical meristems. These results underscore the role of stress-activated MAPK signaling in regulating growth responses at the whole plant level, which may be an important regulatory mechanism underlying the environmental plasticity of plant development.

Keywords: MAP kinase, meristem, Arabidopsis, signaling, de-etiolation

INTRODUCTION

Organogenesis in plants differs from the process in vertebrates, being mainly postembryonic and continuing throughout the life of the plant. Furthermore, organ growth and morphogenesis in sessile plants show a remarkable plasticity to allow environmental adaptation. "Classical" stress hormones act mainly as growth repressors, while other hormones act as growth promoters. It is increasingly clear that action of the long-established growth factor, auxin, is also strongly influenced by environmental signals (Potters et al., 2007; Kazan and Manners, 2009; Habets and Offringa, 2014). A good example of environmentally induced developmental response is that of the shoot apical meristem (SAM) to light. In flowering plants, etiolated seedlings which germinate in darkness undertake a developmental program called skotomorphogenesis, in which the embryonic stem

elongates but leaf growth at the SAM is arrested, i.e., the meristem is in a state of "quiescence". While de-etiolation of subterranean seedlings emerging into light is a critical event in plant development, the rapid and synchronous induction of growth in shoot apices when dark-grown seedlings are transferred to light (photomorphogenesis) also offers an excellent synchronized experimental system to assess the state of shoot meristem activity. De-etiolation was successfully used to unravel the regulatory program underlying meristem activation in *Arabidopsis thaliana* (López-Juez et al., 2008; Yoshida et al., 2011; Pfeiffer et al., 2016; Mohammed et al., 2018). Remarkably, a number of mitogen-activated protein kinase (MAPK) signaling genes, including MPK6, were identified with high dark expression and rapid light downregulation (López-Juez et al., 2008).

The MAPK phosphorylation cascades are conserved signaling modules in all eukaryotes, consisting of three types of enzymes, which are activated through sequential phosphorylation (Avruch, 2007). In Arabidopsis, genes encoding 20 MPKs and 10 MAPK kinases (MKKs) were identified, and both MPKs and MKKs are classified into four phylogenetic groups, designated A–D (MAPK Group, 2002). Plant MAPKs have been mainly associated with stress signaling, but their role in developmental processes is increasingly evident (Colcombet and Hirt, 2008; Hahn and Harter, 2009; Pitzschke et al., 2009; Rodriguez et al., 2010; Xu and Zhang, 2015).

Although our current knowledge of the intervening MKKs belonging to group D is restricted to two members of this group, MKK7 and MKK9 appear to be of special interest in terms of cross-talk between developmental and stress regulation. MKK9 participates in salt signaling (Alzwiy and Morris, 2007; Xu et al., 2008) and is functionally associated with ethylene biosynthesis and signaling (Xu et al., 2008; Yoo et al., 2008). MKK7 inhibits polar auxin transport (PAT) and promotes pathogen defense and programmed cell death, while expression of the *MKK7* gene is induced by pathogen infection (Dai et al., 2006; Zhang et al., 2007; Popescu et al., 2009; Jia et al., 2016). MKK7 and MKK9 are also involved in stomatal cell fate regulation (Lampard et al., 2009).

Newly formed organs in plants are derived from meristems, the source and organizing tissue of growth. By utilizing light-induced de-repression of etiolated SAMs as a synchronized plant developmental model and using complementary genetic approaches, here we demonstrate a meristem-repressive function of a MAPK pathway, minimally consisting of the MKK7-MPK6 module. Control of meristem activity by environmentally activated, MAPK-mediated signaling represents a novel regulatory mechanism underlying the environmental plasticity of plant development.

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana Col-0 was used as genetic background. Seeds were germinated on 0.5× Murashige and Skoog (MS) medium (Duchefa), and plants were grown at 21–23°C, 60–70% relative humidity and 140 (±20) μmol m^{-2} sec^{-1} cool white light under long-day (16 h of light/8 h of dark) conditions. The T-DNA insertion lines SM_3_21446, SM_3_21961, and SM_3_36605 for *mkk7* and Salk_073907 for *mpk6* were obtained from the Nottingham Arabidopsis Stock Centre. The insertion sites were verified by cloning and sequencing the PCR products of left-border- and a flanking-sequence-specific primer pairs. Transgenic Arabidopsis lines were generated using the floral dipping method (Clough and Bent, 1998). Inducible MKK7 overexpression lines are viable (Huck et al., 2017; Dory et al., 2018), two independent lines were used in the experiments for this study. The experiments reported here were repeated with at least three independent biological replicates; with similar results.

Meristem De-Etiolation Assay

The principle of using de-etiolation for assaying SAM activation is described in López-Juez et al. (2008). Following sterilization and stratification, seeds were exposed to light for 30 min to induce germination, and incubated in the dark for 72 h. The etiolated seedlings were subsequently transferred to continuous light and harvested at various time points. Twenty to forty seedlings were measured for each genotype and time point in all experiments. Seedlings were fixed in 90% acetone on ice and washed and stored in 70% ethanol. For microscopic image capture seedlings were mounted in Hoyer's solution (80 g chloral hydrate, 10 ml glycerol in 30 ml water) before visualization in an Optiphot 2 microscope equipped with a DXM1200 camera (Nikon). For statistical analysis area of emerging leaf primordia were quantified using the ImageJ software (National Institutes of Health, United States). The experiments were repeated three times with *mpk6* and *mkk7* (SM_3_21446) with similar results. In case of *mkk7* the experiment was also carried out with two additional insertion lines (SM_3_21961 and SM_3_36605) with similar results.

Quantitative Real-Time PCR

Total RNA was isolated using the Qiagen RNaesy Plant Mini Kit (Qiagen), according to manufacturers' instructions. The optional DNase treatment was also performed using the Qiagen DNase away (Qiagen).

cDNA was synthesized using the Retroscript kit (Ambion) from RNA extracted from 10-day old Col-0 and pK2GW7::MKK7 seedlings (samples collected and pooled from 20 primary transformants). PCR reactions were performed in a Rotor Gene 2000 Real Time Cycler (Corbett Research, Australia), set up with Quantitect SYBR Green PCR Master Mix (Qiagen). Amplifications were performed in duplicate and a control amplification using primers specific for actin was carried out for each run. Primers used: MKK7 F: CCGGAGAGATTTGACTCTGC, R: TTCACGGAGAAAAGGGTGAC, actin: F: GAAGAACTA TGAATTACCCGATGGGC, R: CCCGGGTTAGAAACATTTT CTGTGAACG. Gene expression data was calculated by the Delta Ct method.

For relative gene expressions of *CYCB1;1*, *H2A*, and *RPS6* (**Figures 1F,G**), the experimental setup, sample fixation and shoot apex dissection, RNA isolation, reverse transcription, quantitative RT-PCR, gene-specific primers and data processing were as previously described (Mohammed et al., 2018). First leaf

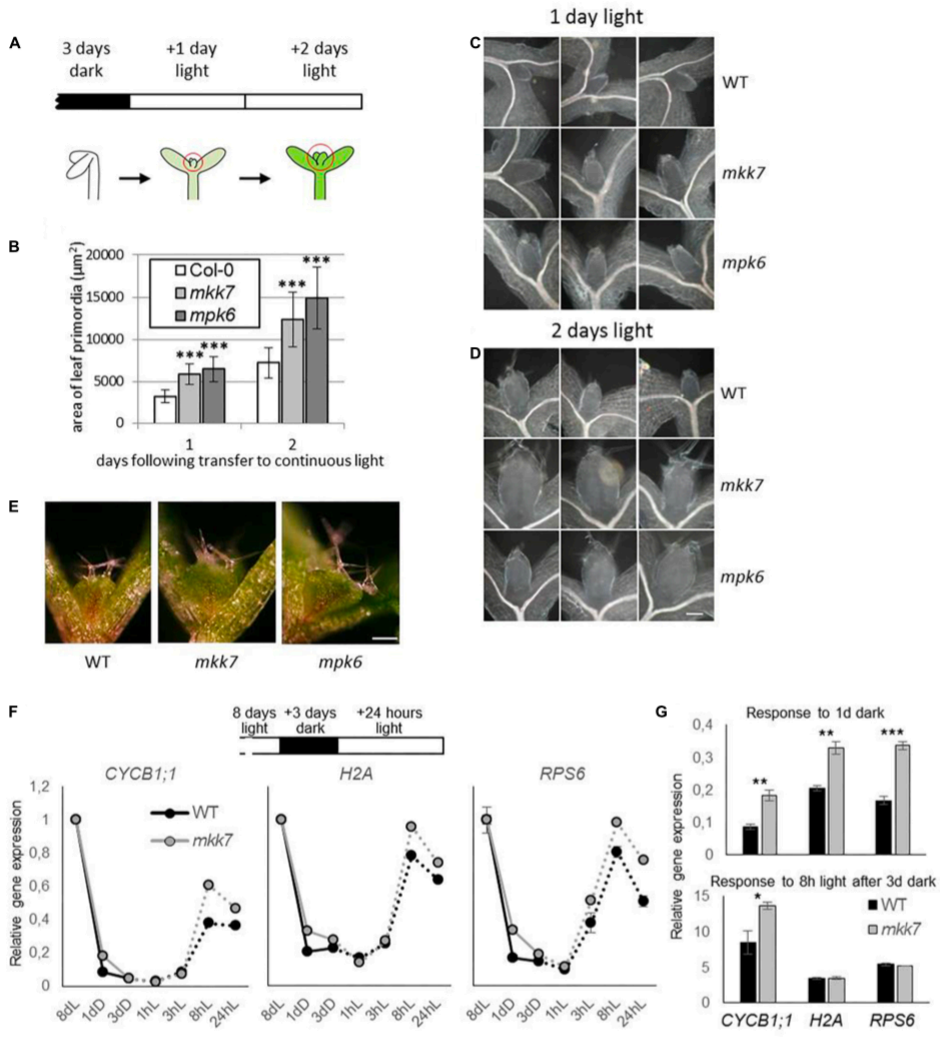

FIGURE 1 | Utilization of seedling de-etiolation as a synchronized developmental model. **(A)** De-etiolation experimental setup. Seeds were germinated for 3 days in darkness then transferred to continuous light. Area of developing leaf primordia were quantified by images of microscopic preparations of seedlings made 1 or 2 days following transfer. **(B)** Area of developing Col-0, *mpk6*, and *mkk7* leaf primordia at 1 and 2 days in continuous light, following 3 days growth in dark. About 30 seedlings were used for each sample. *P*-values: 1.32E-09–2.29E-14, indicated by three asterisks. The error bars represent standard deviation. **(C,D)** Representative images of Col-0, *mkk7*, and *mpk6* leaf primordia at one **(C)** and two **(D)** days following exposure to light. Scale bar: 50 µm. **(E)** Col-0, *mkk7*, and *mpk6* seedlings germinated in darkness for 3 days then exposed to continuous light for further 3 days. Scale bar: 250 µm. The images are representative of at least 12 seedlings. **(F,G)** Relative gene expression of signature genes during dark arrest and subsequent light exposure in emerging leaf primordia. Wild-type seedlings were gown in light on sucrose-containing plates for 8 days, transferred to dark and returned to light after three subsequent days. Two hundred seedlings were harvested for each biological replicate at the corresponding times and had the primordia of leaves 1 and 2 dissected. Gene expression was monitored by quantitative real-time PCR and shown as the entire time course **(F)** and for specific time points relative to that at the onset of dark treatment or at the onset of the light treatment **(G)**. Error bars indicate standard error of the mean (between biological replicates). Asterisks indicate level of significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

pairs of around 200 seedlings were dissected for each sample and time point under a stereomicroscope. qPCR relative values were determined as amplification efficiency (1.7 or above) to the power of the number of critical threshold cycle.

Histology and Microscopy

Plant fixation and embedding was done according to (Begheldo et al., 2013). Briefly, plants were fixed with 4% paraformaldehyde in PBS and vacuum infiltrated for 5 min. After fixation, plants were embedded in Paraplast and thin section microscopy was carried out according to Cnops et al. (2006), using 8 μm sections generated with a RM2245 microtome (Leica). Mounted sections were imaged using a Zeiss inverted microscope, images were processed using AxioVision LE software (Zeiss). For visualization of the plant vasculature, seedlings were cleared with 100% ethanol overnight then gradually rehydrated, and stored and dissected in 50% glycerol. Images were obtained using dark field optics on a Zeiss Stemi SV11 Apo stereomicroscope (Carl Zeiss, Göttingen, Germany).

Ethynyl deoxyuridine (EdU), a thymidine analog, kit (Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit) was used to stain S-phase cells in the root meristem. Arabidopsis seedlings were incubated for 1 h in 10 μM EdU-containing liquid MS media, shoots were excised, and the roots transferred to microcentrifuge tubes. Cut-roots were fixed with 3.7% formaldehyde and 0.1% Triton X100 in microtubule-stabilizing buffer (MTSB) under vacuum for 1 h, and subsequently washed with MTSB (3 × 5 min). Samples were then permeabilised with 0.5% Triton X100 in PBS for 15 min at room temperature, and subsequently washed with PBS (3 × 5 min). Next, samples were incubated in the Click-iT reaction mixture for 40 min at room temperature and protected from light, and washed afterwards with PBS (3 × 5 min). EdU-labeled roots were then incubated in 25% of Sysmex CyStain UV Precise P staining buffer which contains 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 15 min at room temperature and protected from light. Finally, samples were washed with PBS (3 × 5 min).

An Olympus IX-81 FV-1000 confocal laser-scanning microscope was used. DAPI, Alexa Fluor 488, and propidium iodide were exited using 405, 488, and 543 nm lasers, respectively, and emitted fluorescence were collected using band pass filters 420–480 for DAPI, 505–530 filter for EdU, and long pass filter 570 for propidium iodide.

Immunofluorescence Analysis

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 goat anti-mouse at 1:1000 dilution). Fluorescent proteins were analyzed with a Zeiss LSM 5 DUO scanning microscope. GFP and DAPI fluorescence were monitored using multi-tracking in frame mode. GFP 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.

Scanning Electron Microscopy

Eight and twelve day old seedlings grown in long-day conditions were used for the scanning electron microscopy (SEM) studies. Seedlings were fixed in 3% glutaraldehyde, 4% formaldehyde, in phosphate buffer (pH 7.2), for at least 2 h and were then washed three times in phosphate buffer (pH 7.2). Samples were gradually dehydrated in 30, 50, 70, 90, and 2 × 100% EtOH. Samples were then critical point dried and then samples were coated with gold by sputter coating. Images were obtained using a Hitachi S-3000N Scanning Electron Microscope.

RESULTS

The MKK7-MPK6 Module Is a Negative Regulator of Shoot Meristem De-Repression

We took advantage of the rapid and synchronous induction of growth in shoot apices when dark-grown seedlings are transferred to light, and our previous observation of the rapid, co-occurring repression of several kinase genes, including *MPK6*, to assess the functional significance of MAP kinase signaling in regulating meristem activity. Three-day old dark-grown seedlings were transferred to continuous light to follow the development of leaf primordia. Seedlings were collected at various time points and the surface area of leaf primordia was determined by analyzing microscopic images (**Figure 1A**). Our results show that the average leaf primordia area in *mpk6* mutants is more than two fold larger than that of control, both 1 and 2 days following exposure to light (**Figures 1B–D**). Since the upstream MKK7 has a PAT inhibitory function (Dai et al., 2006) we also tested leaf primordia development of *mkk7* seedlings in this setup, with similar results to *mpk6* (**Figures 1B–D**). The trend of larger leaf primordia in both mutants could be observed even after 3 days of light exposure (**Figure 1E**).

To gain insight into the role of MKK7 in the dark-induced growth repression at the molecular level we examined the relative expressions of genes associated with mitosis (*CYCB1;1*, encoding Cyclin B1;1), DNA synthesis (S phase) (*H2A*, encoding Histone 2A), and translation capacity (*RPS6*, encoding 40S ribosomal protein S6-1). We have recently demonstrated that a comparable gene expression program to that seen during de-etiolation takes place after an imposed dark arrest, during light reactivation of growth in the emerging leaf primordia (Mohammed et al., 2018). We used this experimental setup to monitor gene expression throughout the dark repression and light de-repression process. Eight-day-old seedlings grown under continuous light were transferred to dark for three days. In line with Mohammed et al. (2018), all three genes were repressed during the 3-day dark period and were up-regulated within 8h following re-exposure to light in both genotypes (**Figure 1F**). However, a quantitative difference was observed in the kinetics of changes in gene expression between wild type and *mkk7* seedlings. Repression of all three genes was delayed in *mkk7* (**Figure 1F**), while upregulation of *CYCB1;1*, but not *H2A* and *RPS6* was accelerated (**Figure 1G**).

These results indicate that MAPK signaling participates in suppression of leaf growth from the SAM under unfavorable environmental conditions (absence of light).

Increased Expression of MKK7 Impairs Meristem Organization and Leaf Development

In order to gain further insight into the role of MKK7 in adaptive growth regulation, we attempted to generate plants that overexpress MKK7 tagged with a c-Myc epitope at the N-terminus (*35S:MKK7*). In line with the reported lethality of overexpression of a constitutively active MKK7 version (Meng et al., 2012), kanamycin-resistant *35S:MKK7* primary transformants were not viable, therefore transgene overexpression was assayed in pooled primary transformant plantlets (**Figure 2**). Intriguingly, we observed severely impaired meristem development of the primary transformant seedlings. The primary transformants failed to initiate true leaves (**Figure 2A**), failure of organ initiation was confirmed by scanning electron microscopy (**Figure 2C**). Longitudinal sections along the apical-basal axis (**Figure 2D**) revealed that *35S:MKK7* seedlings either completely lacked a SAM, or some meristematic-like tissue was observed at the top of the hypocotyl, indicated by small, densely stained cells. Besides the failure to establish normally organized meristems and to properly initiate organs, *35S:MKK7* cotyledons have a simplified vascular pattern missing two of the four loops normally present in the WT cotyledon (**Figure 2E**).

Due to the severity of the phenotypes produced using the constitutive 35S promoter to express *MKK7*, we also generated lines that express *MKK7* under the control of a β-estradiol-inducible promoter system (Huck et al., 2017; Dory et al., 2018). In the absence of β-estradiol all primary transformants developed normally and produced seeds, progeny from two independent homozygous lines was used in subsequent experiments. Transgenic seeds germinated on 0.05 or 0.1 μM β-estradiol gave rise to severely deformed seedlings (**Figure 3A**). In contrast to the accelerated leaf outgrowth of de-etiolated *mkk7* seedlings, and in accordance to the disturbed SAM organization caused by constitutive overexpression of MKK7, induced overexpression of MKK7 resulted in a severe inhibition and eventual arrest of meristem activation by light during seedling de-etiolation (**Figure 3B**).

Induced overexpression of MKK7 also led to the rapid arrest of root growth. Six-day old seedlings grown on vertical plates were transferred to β-estradiol-containing media and subsequent root growth was observed for 3 days. Transferring six-day old Arabidopsis seedlings to inducing media plates inhibited root growth by ∼50% at 0.01 μM β-estradiol and led to a complete inhibition at 0.1 and 1 μM β-estradiol within 24 h (**Figures 3C,D**), strongly suggesting that MKK7 overexpression also affects the root apical meristem (RAM). Induction by 0.1 μM β-estradiol led to meristem shortening by ∼30%, while the number of meristematic cells was reduced by ∼50% (**Figures 3E,F**). This was paralleled by a slight increase of meristematic cell size.

To further demonstrate that MKK7 negatively regulates cell proliferation as indicated by meristem repression, we carried out EdU labeling at the aforementioned estradiol concentrations at 16 h, a known cell cycle length in the root meristem (Hayashi et al., 2013; Yin et al., 2014). EdU incorporation visualizes active DNA replication, and thus can be used as a marker for cell cycle-driven meristem activity. We counted EdU-positive cells in 50 μm sections from the QC cells to capture the cell cycle dynamics across the meristematic zone. There was no inhibitory effect at 0.01 μM, but a dramatic ∼70% inhibition at 0.1 μM and ∼90% at 1 μM was observed (**Figures 3G,H**). This implies that MKK7 over-expression prevents the onset of DNA replication (S phase) and thus entry into the cell cycle.

Polar Auxin Transport Is Established During Shoot De-Etiolation Process

Directional auxin distribution is necessary for the recruitment of stem cells into leaf primordia and for their subsequent development into leaves and depends on the re-distribution of auxin efflux transporters, including PIN1 (Heisler et al., 2005). PIN1 directs auxin flow to converge in the marginal epidermis of developing leaf primordia and PIN1 expression is further detected close to the center of each young primordium, aligned toward the hypocotyl in the emerging provascular cells (Scarpella et al., 2006; Tsugeki et al., 2009). Accordingly, shoot apex de-etiolation is characterized by the transient downregulation of auxin responsive genes (López-Juez et al., 2008). The importance of auxin responses in the arrest/activation of the meristem in the light is also highlighted by observations in tomato (Yoshida et al., 2011). Moreover, we have recently demonstrated the gradual establishment of PAT during the de-etiolation process by using the auxin-responsive promoter, DR5 and by immunolocalization of the auxin transporter, PIN1 (Mohammed et al., 2018).

As MPK6-mediated phosphorylation has been implicated in the regulation of PIN1 cellular patterning (Jia et al., 2016; Dory et al., 2018) we decided to examine the role of MPK6 in the establishment of PIN1 pattern. In line with Mohammed et al. (2018), upon transfer to light there is a gradual accumulation of PIN1 in epidermal cells and in the forming midvein. Intracellular distribution of the accumulating PIN1 proteins is mainly polar: apical in epidermal cells and basal in provascular cells. In comparison to wild type, the establishment of the PIN1 distribution pattern is accelerated in the *mpk6* background (**Figure 4**), implying a negative regulatory involvement of MPK6 in this process.

DISCUSSION

Due to sessile life style plant development is able to flexibly respond to changing environmental conditions. This developmental plasticity is one of the characteristic differences between animal and plant kingdoms, and it must be orchestrated by integrated environmental and developmental signaling mechanisms. Here we present genetic evidence that the MKK7-MPK6 module participates in meristem regulation driven by a naturally occurring environmental variable.

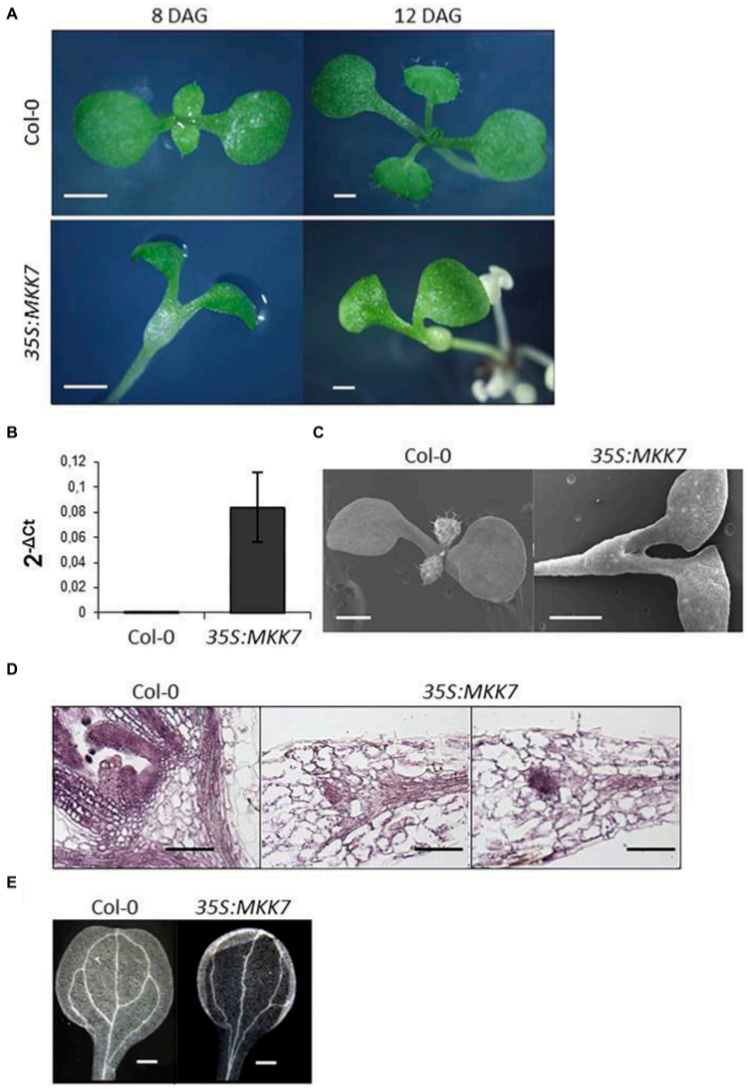

FIGURE 2 | Constitutive overexpression of MKK7 impairs leaf initiation and meristem organization in Arabidopsis. **(A)** Development of Col-0 and *35S:MKK7* seedlings, images are representative of at least 20 seedlings. Images were obtained on a stereomicroscope of Col-0 and *35S:MKK7* seedlings at 8 days and 12 days after germination (DAG) as indicated. Scale bars: 2 mm. **(B)** Detection of transgenic MKK7 expression. qPCR data of MKK7 transcript levels, in Col-0 and *35S:MKK7* seedlings. **(C)** scanning electron microscopic images of 8-day old Col-0 and *35S:MKK7* seedlings, scale bars: 1 mm. **(D)** Longitudinal sections of shoot apices of Col-0 and *35S:MKK7* 8-day old seedlings, scale bars: 200 μm. **(E)** Dark field optics microscopic images showing the vasculature of Col-0 and *35S:MKK7* cotyledons, scale bars: 400 μm. Images are representative of at least five seedlings.

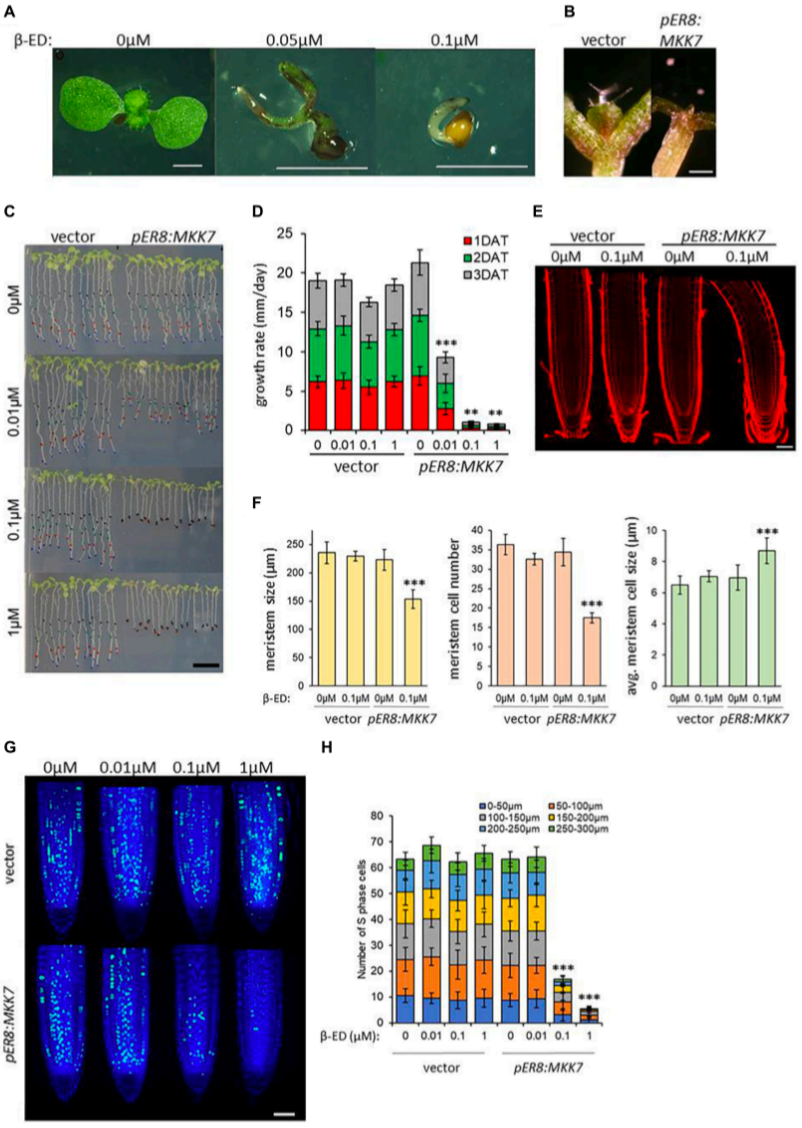

FIGURE 3 | Induced overexpression of MKK7 impairs development and meristem organization in Arabidopsis. **(A)** *pER8:MKK7* (β-estradiol inducible) seeds germinated in the absence or in the presence of 0.05 and 0.1 μM β-estradiol. Scale bars: 2 mm. **(B)** Effect of induced MKK7 overexpression on leaf outgrowth during seedling de-etiolation. Empty-vector transformant and *pER8:MKK7* seedlings were germinated in darkness for three days then transferred to 1 μM β-ED-containing media and exposed to continuous light for further 3 days. Scale bars: 250 μm. **(C,D)** Root growth arrest in response to induced MKK7

(Continued)

FIGURE 3 | Continued
overexpression. Six-day old seedlings of empty vector and inducible MKK7 overexpression lines were transferred to β-estradiol containing media at the indicated concentrations. The positions of the root-tips were marked daily for a three-day period. Scale bar = 10 mm. Root growth rate after transfer to β-estradiol-containing growth medium **(D)**. About 40 seedlings were measured for each sample. Error bars indicate standard deviation, asterisks indicate level of significance. **(E,F)** Root meristems at ∼16 h after transfer to ±0.1 μM β-estradiol. Representative images are shown in panel **(E)**, the roots are aligned by the position of the quiescent center (QC). Scale bar = 50 μm. Cell length was measured longitudinally from the quiescent center cells on left-hand side of the root. Root meristem length, number of meristematic cells and average meristematic cell size of empty vector and *pER8:MKK7* lines were measured at ∼16 h after transfer to ±0.1 μM β-estradiol **(F)**. Seven to ten seedlings were measured for each sample. Error bars indicate standard deviation, asterisks indicate level of significance. **(G)** representative micrographs showing cells in S-phase by EdU labeling (green) in the root meristem ∼16 h after transfer to β-estradiol concentrations indicated. Nuclei are counterstained with DAPI (blue). Scale bar = 50 μm. **(H)** Quantification of S-phase cells in the root-tip. Cell counting was performed in 50 μm sections in 300 μm regions upwards from the QC. About 20 roots were quantified for each sample. *T*-tests were performed on the averages of the 50 μm root sections. Asterisks indicate level of significance. Error bars represent standard deviation.

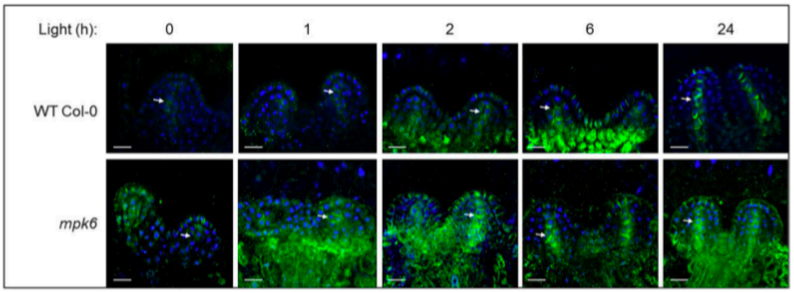

FIGURE 4 | PIN1 expression and localization in developing leaf primordia, in wild type and *mpk6* mutant seedlings, as detected by immunostaining of PIN1. PIN1 (green) and DAPI (blue). Images of the first two leaf primordia of the wild type (top panel) and *mpk6* (bottom panel) during light-induced meristem activation. Seedlings were germinated in the dark for three days, then exposed to continuous white light for the times indicated above (in hours). Arrows indicate midvein. Scale bars: 10 μm. The images are representative of 10 seedlings.

Light provides an easy-to-manipulate environmental switch for the state of SAM activity and can help to address fundamental questions in meristem function (López-Juez et al., 2008; Mohammed et al., 2018). Along with various other MAPK signaling genes, *MPK6* transcript levels were revealed to be down-regulated within an hour during meristem de-repression, this taking place specifically in the seedling shoot apex, but not in the cotyledons (López-Juez et al., 2008), an especially intriguing finding considering that *MPK6* transcript levels do not vary significantly under most conditions when quantified in whole seedlings (Menges et al., 2008). Therefore, we utilized the light-induced de-repression of the SAM as a tool to survey the activity of the meristem by measuring the expansion rate of developing leaf primordia, and found that MPK6 and the upstream MKK7 function as repressive modulators of organ development from the SAM. Moreover, our results further demonstrate that de-etiolation can be utilized as a synchronized developmental system in plant biology to assess meristem activity, and to analyze the mechanisms controlling leaf initiation and development. In contrast to the accelerated meristem activation in null mutants, constitutive or induced overexpression of MKK7 results in the collapse of meristem organization and a subsequent growth retardation or even full arrest, inducible overexpression demonstrating this to occur in a dose-dependent manner.

Growth of *mkk7* and *mpk6* mutants under standard conditions is wild-type like, single mutants develop normally. In this light, we consider our findings of accelerated de-etiolation remarkable, as in this developmental setting we were able to assign a developmental phenotype to *mkk7* and *mpk6*, which implies their meristem-regulatory functions.

Furthermore, our findings not only demonstrate a developmental impact of this signaling module, they also reveal a biological role, and potentially a fitness value, for this action, in the regulation of quiescence or active growth of the meristem under the control of a natural environmental signal (the first exposure of dark-grown seedlings to light).

These results complement previous findings revealing aspects of developmental regulatory functions of MKK7-MPK6 with a specific role in regulating apical meristems, the central organizing tissues of plant growth.

The CLAVATA (CLV) pathway operates in the regulation of the stem cell population size in the SAM (Dodsworth, 2009). According to the established model a CLV1–CLV2 receptor heterodimer binds the CLV3 peptide ligand. This ligand–receptor interaction leads to the transphosphorylation of the CLV1 kinase domains. Phosphorylated residues of the kinase domain act as binding sites for downstream effector molecules such as kinase-associated protein phosphatase (KAPP) and a Rho GTPase-related protein (ROP). This model is remarkably analogous to

the animal growth factor recognition that activates the ERK MAP kinase pathway in response to growth factors and it has been long proposed that the CLAVATA signal transduction could conceivably involve a MAP kinase cascade (Clark, 2001). Indeed, MPK6 activity is controlled by CLV receptors (Betsuyaku et al., 2011), while MAPK-mediated phosphorylation of meristem-regulatory transcription factors has been also demonstrated (Popescu et al., 2009; Dory et al., 2016).

Polar auxin transport and the resulting local auxin maxima sites are important in establishing developmental patterns in plants. PINs determine the direction of PAT through their asymmetric subcellular localization and thus signaling pathways regulating PIN localization can modulate developmental programs in response to triggering stimuli (Robert and Offringa, 2008; Sassi et al., 2012). During leaf initiation auxin maxima mark sites of incipient primordia (Heisler et al., 2005) and leaf venation (Mattsson et al., 2003; Scarpella et al., 2006). The formation of leaf primordia and the emerging vascular cells within are accompanied by the appearance and polar localization of the auxin efflux carrier protein PIN (Tsugeki et al., 2009). In agreement with these findings, a decrease in overall auxin activity during de-etiolation, coupled with the emergence of auxin maxima sites using the *DR5:GUS* reporter line, was found (Mohammed et al., 2018), underscoring the importance of establishing PAT in de-repressed leaf primordia. Leaf primordia tips and pro-vascular cells display particularly strong or emerging auxin response, consistent with auxin drainage being an integral element of the phenomenon of leaf primordia growth (Deb et al., 2015). The MKK7-MPK6 pathway has been demonstrated as a PAT repressor (Dai et al., 2006; Jia et al., 2016) and MPK6-mediated phosphorylation modulates PIN1 cellular localization (Jia et al., 2016; Dory et al., 2018). Therefore we compared the establishment of PIN1 pattern in the emerging leaf primordia in wild type and *mpk6* seedlings and found that this process is accelerated in the absence of MPK6, implying that a difference in auxin drainage can be another regulatory layer underlying the observed acceleration of leaf emergence in this genetic background.

Plants exposed to either abiotic or biotic stress conditions respond by actively altering their growth pattern as part of the overall defense response, which serves to minimize exposure (stress avoidance) and to divert limited resources to defense mechanisms at the expense of growth (Potters et al., 2007). It has been suggested that there is a generic 'stress-induced morphogenic response' common to most stresses, which comprises the inhibition of cell elongation, localized stimulation of cell division and alterations in cell differentiation status. This response is regulated by increased reactive oxygen species (ROS) production and altered phytohormone transport and metabolism (Potters et al., 2007). ROS are not only commonly formed during most stresses, they are also well-known MAPK activators. Moreover, redox-regulatory mechanisms are also involved in meristem regulation (Schippers et al., 2016). Remarkably, redox imbalance due to glutathione depletion results in growth reduction and perturbations in both SAM and RAM through inhibition of auxin transport, implying that PIN function is dependent on a post-translational redox regulation (Bashandy et al., 2010; Koprivova et al., 2010).

Taken together, MAPK-mediated meristem regulation is probably highly complex, exerted through the phosphorylation of several substrates. A recent study identified a number of differentially phosphorylated proteins downstream of MKK7-MPK6/3 (Huck et al., 2017), although most of these proteins are defense related, in line with the positive regulatory role of MKK7 in pathogen response (Zhang et al., 2007). However, meristem-derived materials are underrepresented in whole-plant samples and thus are rarely detected by most high-throughput approaches. Detailed characterization of the regulatory network underlying the meristem-regulatory role of stress-activated MAP kinase signaling requires further studies and may unveil an important regulatory mechanism of the environmental plasticity of plant development.

AUTHOR CONTRIBUTIONS

EH, EL-J, KP, LB, FD, and RD designed the research. RD, EH, SFB, ZA, EL-J, LB, and FD performed the research. EH, EL-J, KP, LB, FD, and RD analyzed and discussed the data. RD wrote the manuscript with input from all authors.

FUNDING

This work was supported by the EC Marie Curie Reintegration Grant, ERG 256554, the Hungarian Research Fund (OTKA K101250 and NN114511), GENPROF IF-18/2012 Research Infrastructure Grant of the Hungarian Academy of Sciences, the UK BBSRC Grant BB/M025047/1, the Deutsche Forschungsgemeinschaft (SFB 746), the Excellence Initiative of the German Federal and State Governments (EXC 294), the Bundesministerium für Forschung und Technik (BMBF SYSTEC, PROBIOPA), the Deutsches Zentrum für Luft- und Raumfahrt (DLR 50WB1022), the Freiburg Initiative for Systems Biology and the European Union Framework 6 Program (AUTOSCREEN, LSHG-CT-2007-037897). RD was a recipient of the EC Marie Curie Fellowship grant (IEF 041909) and the Bolyai Fellowship of the Hungarian Academy of Sciences. EH was a recipient of the Royal Holloway Ph.D. studentship. ZA was a recipient of the BBSRC-DTP studentship (BB/M011178/1).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Abbreviation list

%: Percent
°C: Degree celcius
ABA: Abscisic acid
AGPase: ADP-glucose pyrophosphorylase
AHK: Arabidopsis histidine kinase
AHP6: Arabidopsis histidine phosphotransfer protein 6
ARC5: Accumulation and replication of chloroplast 5
ARF: Auxin response factor
ARP: Asymmetric leaf1/rough shea th2/phantastica
ARR: Arabidopsis response regulator
AS1: Asymmetric leaf1
At: Arabidopsis thaliana
ATHB8: Homeobox-leu zipper protein 8
ATM: Atmospheric pressure
AUX1: Auxin resistant
LAX: Like aux1
BAP: Synthetic cytokinin 6-benzylaminopurine
bHLH: basic-helix-loop-helix
bZIP: Basic leucine zipper
CDK: Cyclin-dependent kinase
CHASE: Cyclase histidine kinase associated sensory extracellular
CLV: clavata
COP/DET/FUS: Constitutive photomorphogenic/ de-etiolated/fusca
COP1: Constitutive photomorphogenic 1
COT: Cotyledon
CRY-DASH: Drosophila – Arabidopsis–Synechocystis–Human
Cry: Cryptochrome
CZ: Central zone
DAG: Day after germination
DET1: De-etiolated1
DP: Dimerization partner
E2F-DP: E2F transcription factor dimerization partner
EBP: Ethylene responsive element binding protein
EBP1: ERBB-3 binding protein 1
EIL1: Ethylene-insensitive3-like1
EIN3: Ethylene insensitive 3
ER: Endoplasmic reticulum
ERF1: Ethylene response factor 1
FBP/FIS1: Fructose 1-6-bisphosphatase/ fructose-insensitive 1
GC1: Giant chloroplast 1
GL2: Glabra 2
H2A: Histone-2a
H2O: Hydrogen-2 Oxygen-1 bond/Water
HAT2: Homeobox-lucine zipper protein 2
HFR1: Long hypocotyl in far red
HL: High light
HXK: hexokinase
HY5: Elongated hypocotyl 5

IAA: Indole-3-acetic acid
IPyA: Indole-3-pyruvic acid
KIN10: SNF1 kinase homolog 10
KNOX1: Knotted 1- like homeobox
KOH: Potassium hydroxide
KRP4: Kip-related protein 4
L1: Epidermal layer
LAF1: Long after far-red light 1
LL: Low light
LOG: Lonely guy
MDR: Multidrug resistance
MES: 2-(N-morpholino)ethanesulfonic acid
mg: Milligram
MgCl2: Magnesium chloride
ml: Millilitre
mm: Millimietres
mM: Millimolar
mRNA: MessengerRNA
MS: Murashige and Skoog
N2 : Liquid nitrogen
NaCl: Sodium chloride
OC: Organisating centre
PAT: Polar auxin transport
PGP: P-glycoprotein
PHOT: Phototropin
Phy: Phytochrome
PIF: Phytochrome interacting factor
PIKK: Phosphatidylinositol kinase-related kinases
PIN: Pin formed
PORA: Protochlorophyllide oxidoreductase a
PRR7: Pseudo-response regulator7
PSII: Photosystem II
PZ: Peripheral zone
Q-RT-PCR: Quantitative real time PCR (RNA)
RAPTOR: Regulatory associate protein of tor ,
RB: Retinoblastoma
RBR: Retinoblastoma-related protein
RBR1: Retinoblastoma related 1
RGS1: G-protein signalling 1
RNR2A: Ribonucleotide reductase 2a
RP: Ribosomal protein
RPS6: Ribosomal protein s6
rRNA: Ribosomal RNA
RZ: Rib zone
S6K: Ribosomal s6 protein kinase1
SAM: Shoot meristem
SAP: SAM plus leaf primordia
SEM: Standard error of the mean
SnRK1: SNF1-related kinase 1
SPL: Squamosa promoter binding protein-like

SPS: Sucrose phosphate synthase
STM: Shootmeristemless
Suc: Sucrose
SUT: Sucrose transporter
T6P: Trehalose 6-phosphate
TAA1: Tryptophan aminotransferase of arabidopsis 1
TAR: TAA1 related
TAR2: Tryptophan aminotransferase related 2
TB: Transfer buffer
TBE: Tris boric-acid EDTA
TIR1/AFB: Transport inhibitor response1/auxin signaling f-box
TOR: Target of rapmaycin
TORC: TOR complex
TPS: Trehalose phosphate synthase
TPS9: Trehalose-6-phosphatase9
tRNA: Transfer RNA
TRP: Tryptophan
UVR8: UB-B resistance 8
VND6: Vascular-related nac-domain 6
WOX: Wuschel-like homeobox
WT: Wild type
WUS: Wuschel
X-gluc: 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YHB: Phytochrome B-Y276H
α: Alpha
β: Beta
γ: Gamma
µg: Micrograms
µl: Microlitres

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