Regulation of shoot apical meristem activity

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Abstract

All above ground plant organs initiate or derive from stem cells at the shoot apical meristem. The activity of the shoot apical meristem determines the rate of leaf initiation, which is repressed by the ACTIN RELATED PROTEIN2/3 (ARP2/3) complex in the dark. The ARP2/3 complex is an ancient nucleator of actin filament branches, with roles in a variety of subcellular processes. However, the mechanism by which the ARP2/3 complex regulates shoot apical meristem activity is unknown.

In this thesis I show that the increased shoot apical meristem activity of *arp3* in prolonged darkness required the polar auxin efflux carrier PIN-FORMED1 (PIN1). Wild-type shoot apical meristem activity was largely unaffected by inhibitors of polar auxin transport in the dark, and a *pin1* mutant had similar shoot apical meristem activity to wild-type. By stark contrast, the increased shoot apical meristem activity of *arp3* was hypersensitive to inhibitors of polar auxin transport, and abolished in an *arp3pin1* double mutant. Furthermore, multiple phenotypes of a brassinosteroid biosynthesis mutant *det2*, reported to have reduced *PIN1* expression and polar auxin transport, were rescued in an *arp3det2* double mutant grown in the light. These results indicate that the ARP2/3 complex regulates the activity of PIN1, possibly by facilitating PIN1 endocytosis, and suggest that the ARP2/3 complex is a repressor of brassinosteroid responses. The auxin response factors ARF4 and ARF5 were found to be repressors of shoot apical meristem activity in the same pathway as the ARP2/3 complex. This result led to the proposal of a model where auxin minima, rather than auxin maxima (where ARF4 and ARF5 are active) are required to initiate new leaves at the shoot apical meristem.

The increased shoot apical meristem activity of *arp3* required sugar, the glucose sensor TOR kinase, and the initial steps of glycolysis which generate precursors for cell wall biosynthesis.

In a candidate approach to identify novel transcriptional regulators of dark development, the IND transcription factor was found to repress shoot apical meristem activity redundantly with its homologue HEC2. IND was also found to interact genetically with the phytochrome interacting factors PIF3 and PIF4 to differentially regulate shoot apical meristem activity. Microarray analysis revealed that the primary target of IND is the sugar transporter *SWEET15* (upregulated), which promoted shoot apical meristem activity.

This research identifies potential avenues for generating crop varieties with increased shoot apical meristem activity in the dark, which might be advantageous in a mulched system, and for generating semi-dwarf crop varieties, by activating a subset of brassinosteroid responses in brassinosteroid deficient crops.

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Chapter 1 - Introduction

1.1 The shoot apical meristem in darkness

1.1.1 Agronomic relevance of increased shoot apical meristem activity in the dark

Within a growing season, the number of crop cycles achievable is limited by the length of the growing phase. The number of growing days in the crop cycle could be reduced by increasing the rate of shoot apical meristem (SAM) activity. Many significant crop plants germinate in darkness under organic, rubber or plastic mulches, which are advantageous in that they increase crop productivity by preserving soil moisture content, fertility and quality, regulating soil bed temperature, suppressing weed growth, and increasing the overall rate of development (Angima, 2009; Kumar et al., 2005; Van der Westhuizen, 2017). However, initial SAM activity is repressed in the absence of light (Roldan et al., 1999). For *Arabidopsis thaliana* (Arabidopsis), a member of the *Brassicaceae* family, this delay amounts to about one week, even though plants are supplied with a sugar source (Roldan et al., 1999). If the initial stages of seedling development are also reduced when grown under mulch, or dense canopies etc. then modifying SAM development in the dark could expedite the growing phase of the crop cycle.

1.1.2 The shoot apical meristem

The SAM is the primary source of stem cells for all above-ground organs of the plant. A niche of approximately 35 stem cells is maintained in the central zone of the Arabidopsis SAM (Dodsworth, 2009) by homeodomain transcription factors including *WUSCHEL* (*WUS*) (Sarkar et al., 2007). *WUS* is expressed in the organising centre, buried between the stem cell niche and the rib zone (Sarkar et al., 2007). The rib zone provides multipotent cells for differentiating organ primordia (Barton, 2010). Lateral organs initiate from regions of auxin maxima at the peripheral zone (Benkova et al., 2003).

Cells in the SAM are relatively small with dense cytoplasms and small vacuoles (Wyrzykowska et al., 2006). Proplastids in the SAM do not differentiate into chloroplasts, so are unable to photosynthesise (Wyrzykowska et al., 2006). Therefore, the SAM relies on sugar transport for carbon and energy.

1.1.3 Shoot apical meristem activity

The initiation of new organs from the SAM is a direct output of SAM activity. Organs initiate from the SAM following a transition from cell proliferation for stem cell maintenance to differentiation. *WUS* expression has been used as an early marker of shoot apical meristem

activity (Pfeiffer et al., 2016). In this thesis, *SAM activity* is used synonymously with *rate of leaf initiation*, which is a function of leaf appearance rate (Padilla and Otegui, 2005). And *SAM activity in the dark* is used synonymously with *dark development*.

Light is a potent activator of SAM activity, activating photosynthesis to increase carbon availability for growing tissues, and increasing cytokinin levels in the SAM (Yoshida et al., 2011). In a diurnal cycle, starch synthesised during the day is metabolised to generate sugars for respiration at night (Lu et al., 2005). In prolonged darkness, after the depletion of starch reserves, the SAM becomes quiescent and leaf initiation is transiently arrested (Yoshida et al., 2011). SAM activity can be restored by light or by direct contact with exogenous sugars (Roldan et al., 1999; Yoshida et al., 2011). Roldan and colleagues found that 1% sucrose was sufficient to induce the same rate of leaf initiation in dark-grown Arabidopsis as light-grown Arabidopsis, although the onset of SAM activity was delayed by about 1 week. Yoshida and colleagues (2011) found that exogenous cytokinin was also sufficient to activate dark-adapted shoot apices, and required polar auxin transport. In a study by Richard and colleagues (2002), both sugar and cytokinin were shown to have similar effects on stimulating the expression of cell cycle genes, whereas auxin treatment alone had a limited effect. Combinations of sugar and cytokinin treatment typically did not further increase the expression of cell cycle genes; however, either treatment in combination with auxin led to additive or synergistic increases of cell cycle gene expression. A combination of sugar, cytokinin and auxin led to synergistic regulation of certain cell cycle genes. These observations were subsequently linked to SAM activity (Hartig and Beck, 2006). In contrast to light and cytokinins, which initiate photomorphogenic development (Chory et al., 1991a), sugars in the absence of light initiate skotomorphogenic development (Roldan et al., 1999).

1.1.4 Skotomorphogenesis and photomorphogenesis

Dark development is skotomorphogenic rather than photomorphogenic (Roldan et al., 1999), with the exception of constitutively photomorphogenic/de-etiolated mutants, which typically cause *PIF1*/*3*/*4*/*5*-dependent signalling to be repressed in some way. Such mutants are typically gain-of-function photoreceptor mutants (Hu et al., 2009; Yang et al., 2000) which repress protein turnover (Hofmann, 2015; Yang et al., 2001), mutants of protein turnover (Castle and Meinke, 1994; Chory et al., 1989a; Deng et al., 1991; Kwok et al., 1996; Laubinger et al., 2004; Osterlund et al., 1999; Wei et al., 1994; Yanagawa et al., 2005) which represses DELLAs (Dohmann et al., 2010), gain-of-function mutants of *DELLAs* (Chory et al., 1991a; Li et al., 2015b) which repress PIF activity (de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolome et al., 2011), mutants of brassinosteroid biosynthesis/signalling (Azpiroz et al., 1998; Chory, 1992; Clouse et al., 1996; Kim et al., 2009; Szekeres et al., 1996), which promote PIF activity by dephosphorylation

(Bernardo-Garcia et al., 2014), gain-of-function mutants in brassinosteroid signalling repressors (Li et al., 2001b; Ryu et al., 2010) which repress PIF activity by phosphorylation (Bernardo-Garcia et al., 2014), mutants of downstream PIF signalling (Cheng et al., 2000; Rohde et al., 2000; Rohde et al., 1999; Wang et al., 2015), or mutants of *PIF1*/*3*/*4*/*5* themselves (Shin et al., 2009). Distinctive features of skotomorphogenesis include elongated hypocotyls and petioles, smaller slender leaves, and no chloroplast development, whilst photomorphogenesis is characterised by partial or full chloroplast development (observed as greening), shorter hypocotyls and petioles, and broader leaves.

1.2 Genetic regulation of dark development

1.2.1 Mutants with increased dark development (skotomorphogenic)

Various mutants have been described that have increased dark development, but are distinctly etiolated and develop etioplasts, rather than chloroplasts, when dark-grown. Mutants with increased dark development are reviewed.

1.2.1.1 ARP2/3 complex related

Increased dark development has been reported for mutants of the *ACTIN RELATED PROTEIN2/3* (*ARP2*/*3*) complex subunits *high sugar response4* (*arp3*/*hsr4*) and *arpc2a*/*hsr3*/*dis2* (Baier et al., 2004; Zhang et al., 2008), and upstream activators of the *ARP2*/*3* complex *scar2* (*dis3*), *scar1scar3* (Zhang et al., 2008), *nap*, *pir* (Li et al., 2004b), and constitutively active ROP2 lines (Li et al., 2001a; Li et al., 2017). The *ARP2*/*3* complex nucleates filamentous actin (F-actin) branching (see 1.5.3). As yet, there have not been any follow-up studies to show how these mutations lead to increased dark development, except that the high sugar response of *arp3* and *arpc2a* was suppressed by a mutant of the Mediator complex, which is deficient in transcriptional responses to sugars (Seguela-Arnaud et al., 2015). The role of the ARP2/3 complex in shoot apical meristem activity is explored in this thesis.

1.2.1.2 Cell wall related

Increased dark development is also a trait of a cell wall integrity mutant with decreased cell wall fucose *mur1*, mutants deficient in arabinose synthesis *mur3*, *mur4* (*hsr8*), and a cytochrome P450 mutant involved in suberin biosynthesis, *hsr2* (Baier et al., 2004; Li et al., 2007). However, other cell wall integrity mutants developed normally in the dark, including weaker mutants of fucose and arabinose synthesis *mur2* and *mur5*, *mur6*, *mur7*, a mutant with reduced cell wall rhamnose

mur8, a mutant with reduced xylose and fucose *mur9*, mutants with reduced cellulose synthesis *rsw1*, *ixr2*, and a mutant with reduced lignin *irx4* (Li et al., 2007). The increased dark development of *mur4* could be rescued with exogenous L-arabinose. Whereas boric acid, which increases cell wall integrity, rescued the dark development of *mur1*, *mur3* and *mur4*. Mutations in the Mediator complex subunits *med25* and *med8*, which are required for transcriptional responses to sugar and changes in cell wall arabinose composition, also rescued the dark development of *mur4*. (Li et al., 2007; Seguela-Arnaud et al., 2015). Therefore, cell wall integrity and downstream transcriptional responses, as well as transcriptional responses to sugar status, are important for increased dark development.

1.2.1.3 Related to the repression of auxin signalling

The increased dark development of *hls1* (*cop3*), a mutant of the N-acetyltransferase HOOKLESS1, was attributed to ethylene insensitivity and altered auxin homeostasis (Hou et al., 1993), with reduced levels of IAA in leaves (Ohto et al., 2006) and increased expression of auxin response genes (including the repressor of auxin signalling *IAA5* and *SAUR15*) at the shoot apical hook (Lehman et al., 1996). A later study revealed that the increased dark development of *hls1* was caused by the stabilization of auxin response factor ARF2 protein levels which led to repression of auxin signalling (Li et al., 2004a). Mutations in *arf2* suppressed the increased dark development of *hls1*. Repression of auxin signalling by gain of function mutations in other *Aux*/*IAA* auxin response genes, *iaa3* (*shy2-2*), *iaa7* (*axr2*) and *iaa17* (*axr3*), also increased dark development (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000). Additionally a gain-of-function mutant of *LEAFY COTYLEDON1* (*lec1-d*^{tnp}) had increased dark development and increased expression of *IAA2* (Casson and Lindsey, 2006). The *Aux*/*IAA* gain of function mutants were shown to have increased activity from increased Aux/IAA protein stability (Colon-Carmona et al., 2000). These studies indicate that auxin signalling represses dark development.

1.2.1.4 Related to the repression of sugar starvation responses

The *exordium-like1* (*exl1*) mutant had increased dark development in response to low levels of sugars (0.15% glucose or 0.2% sucrose, but not 1% sucrose) and had increased sensitivity to brassinosteroid-induced growth (Schroeder et al., 2011). *EXL1* expression is induced during extended night and low carbon availability but its function remains unclear.

1.2.2 Mutants with increased dark development (constitutively photomorphogenic)

Other mutants with increased development when dark-grown are described as de-etiolated or as constitutive photomorphogenic, and develop functional chloroplasts or intermediates between etioplasts and chloroplasts, as well as other traits resembling light-grown plants.

1.2.2.1 Related to increased cytokinin

The increased dark development of *amp1* (*cop2*/*hls2*), a mutant of the glutamate carboxypeptidase *ALTERED MERISTEM PROGRAM1*, which also had an increased rate of leaf initiation in the light, was attributed to greatly elevated cytokinin biosynthesis in the light and dark (Chin-Atkins et al., 1996; Hou et al., 1993). Treatments with exogenous cytokinin also induced photomorphogenesis in the dark, indicating that cytokinin promotes dark development (Chory et al., 1991a).

1.2.2.2 Related to abscisic acid insensitivity

Dark grown mutants of *ABSCISIC ACID INSENSITIVE3* (*abi3*) have increased leaf initiation rate in the dark (Rohde et al., 2000; Rohde et al., 1999) and around 10% of *abi3* mutants develop leaf primordia in the seed (Nambara et al., 1995). *abi3* mutants partially develop chloroplasts in dark-grown cotyledons and leaves, and develop etioplasts in the shoot apical meristem where plastids are normally undifferentiated, indicating that ABI3 is a repressor of plastid differentiation (Rohde et al., 2000).

abi8 (*eld1*/*kob1*), a putative glycosyltransferase mutant, is constitutively photomorphogenic and has rapid leaf production in the dark (Cheng et al., 2000). Its constitutive photomorphogenesis was shown to be caused by reduced cellulose (Wang et al., 2015); treatment with the cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile phenocopied the photomorphogenesis of dark-grown *abi8* mutants. *abi8* mutants also accumulated ectopic cell wall components including suberin and lignin (Cheng et al., 2000; Pagant et al., 2002). In the light, ABI8 is targeted for degradation by the proteasome, placing it downstream of light signalling (Wang et al., 2015). The expression of *HLS1*/*COP3* was 'drastically reduced' in *abi8* mutants (Wang et al., 2015), suggesting that the increased dark development is partially due to stabilization of ARF2 (Li et al., 2004a). Unlike the cell wall biosynthesis mutant *high sugar response8* (see 1.2.1.2), *abi8* has a low sugar response, and is photomorphogenic in the dark. *abi8* has severely stunted growth, resulting from reduced cell elongation that is not rescued by exogenous hormones, but is partially alleviated by high concentrations (>1%) of glucose (Brocard-Gifford et al., 2004). *abi8* is an

example of a cell wall biosynthesis mutant that also represses auxin signalling. The phenotypes of *abi3* and *abi8* potentially indicate that abscisic acid negatively regulates dark development.

1.3 Regulation of SAM activity by sugars

The SAM is not able to photosynthesise, so it requires sugars to be transported to the stem cells for activation (Roldan et al., 1999; Wyrzykowska et al., 2006). Little is known about the specific sugar transporters or downstream sugar responses required for SAM activity. Plants perceive sugars by glycolysis-dependent and independent sugar signalling pathways (Moore et al., 2003).

Glycolysis is the first stage of respiration and produces energy in the form of ATP and NADP, as well as metabolites required for synthesising amino acids and other molecules necessary for cellular growth and maintenance. Glycolysis has been shown to activate the cell cycle in meristematic root and shoot tissues via the kinase TARGET OF RAPAMYCIN (TOR), which was important for root and shoot apical meristem activity (Pfeiffer et al., 2016; Xiong et al., 2013).

The non-metabolizable glucose analog 2-Deoxy-D-glucose (2-DG) is a commonly used inhibitor of glycolysis and acts by inhibiting hexokinase (Klein and Stitt, 1998). 2-DG competes for the same binding site as glucose (Nakada and Wick, 1956).

Glycolysis-independent sugar signalling has been shown to be important for promoting growth, inhibiting growth and greening on high concentrations of glucose, regulating sensitivity to sugars and phytohormones (Chen et al., 2006; Moore et al., 2003).

In this section, the general and SAM-specific roles (if characterised) of glycolysis and sugar signalling pathways are introduced, with relevance to chapter 4. Two glycolytic enzymes were selected: aldolases because of their relationship with F-actin, and hexokinases for their dual roles in glycolysis and glycolysis-independent sugar signalling.

1.3.1 Aldolases

Fructose bisphosphate aldolases (aldolases / FBA) are glycolytic enzymes that catalyse the reversible conversion of fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, and also the reversible conversion of sedoheptulose 1,7-bisphosphate into dihydroxyacetone phosphate and erythrose 4-phosphate (Mininno et al., 2012). In addition to their roles in glycolysis and the Calvin Cycle, glyceraldehyde 3-phosphate and erythrose 4-phosphate are precursors for isoprenoid biosynthesis, and aromatic amino acid biosynthesis, respectively (Flechner et al., 1999; Herrmann, 1995; Rohmer et al., 1996).

Arabidopsis has eight aldolases, FBA1-8 (Lu et al., 2012). FBA1-3 localise to plastids (Lu et al., 2012; Mininno et al., 2012), and FBA4-8 localise to the cytosol (Lu et al., 2012). FBA6 and FBA8 were also identified in association with mitochondria, and FBA8 participated in the glycolytic pathway associated with the outer or inner mitochondrial membrane (Giege et al., 2003). Plastidic aldolases are important for starch biosynthesis, but do not contribute to sucrose levels (Sonnewald et al., 1994). By contrast, cytosolic aldolases are important for sucrose biosynthesis (Barry et al., 1998). Sucrose biosynthesis is activated by a threshold level of the FBA product/substrate dihydroxyacetone phosphate (Stitt et al., 1987), which is believed to stimulate the reverse (aldolase) reaction and gluconeogenesis, leading to an increase in sucrose and starch precursors. Aldolases in plants are also involved in abiotic stress responses, including responses to salinity, drought, ABA, gibberellic acid, and high sugar concentrations (Lu et al., 2012; Zhang et al., 2003). The SAM has undifferentiated plastids known as proplastids. Whether or not FBA1-3 localise to proplastids when expressed in the SAM is not known, however, glycolytic enzymes including aldolase have previously been identified in proplastids of castorbean (Simcox et al., 1977).

Aldolases behave as tetramers and their activity is dependent on their association with the actin cytoskeleton (Arnold and Pette, 1970; Persson, 1988). Actin-association reduced the affinity of aldolase for fructose 1,6-bisphosphate by about an order of magnitude (Arnold and Pette, 1970). Thus in the literature, actin-bound aldolase is often considered inactive, whereas free aldolase is considered active. Aldolases associate with actin via conserved actin-binding sites, which resemble actin sequence (O'Reilly and Clarke, 1993).

1.3.2 Hexokinases

The hexose sugar glucose feeds directly into glycolysis through the catalytic activity of the receptor hexokinase, which converts glucose into glucose-6-phosphate. Arabidopsis has three HEXOKINASE (HXK) and HEXOKINASE-LIKE (HKL) proteins, the best studied HXK being HXK1, which also has glycolysis-independent sugar signalling activity, although HXK2 has equal catalytic activity (Jang et al., 1997; Karve et al., 2008; Moore et al., 2003). HXK1-3 and HXL1-2 are predicted or demonstrated to have glucose binding properties, but only HXK1-3 are catalytically active and involved in glycolysis (Karve et al., 2008). HXK1 and HXK2 localise to mitochondria, whereas HXK3 localises to the chloroplast (Balasubramanian et al., 2007; Karve et al., 2008). The sugar signalling activity of HXK1 has been shown to be important for a variety of responses including sensitivity to high concentrations of glucose, sensitivity to auxin and reducing sensitivity to cytokinin, promoting hypocotyl, leaf and root growth, and inhibiting growth in response to high concentrations of glucose (Moore et al., 2003).

1.3.3 RGS1

REGULATOR OF G-PROTEIN SIGNALING1 (RGS1) is a glucose responsive G-protein-coupled putative glucose receptor associated with the plasma membrane (Grigston et al., 2008). Glucose perception promoted RGS1 phosphorylation by WITH NO LYSINE (WNK) kinases, leading to disassociation from and activation of G-PROTEIN ALPHA SUBUNIT1 (GPA1) which promotes the RGS1-dependent removal of RGS1 from the plasma membrane by endocytosis (Grigston et al., 2008; Urano et al., 2012). RGS1 directly or indirectly promotes the expression of genes involved in autophagy, which reduces its recovery to the plasma membrane (Yan et al., 2017). Endocytosis of RGS1 increases growth by activating cell division (Urano et al., 2012). Overexpression of RGS1 caused high sugar responses (Urano et al., 2012).

RGS1 signalling is involved in responses to high concentrations of glucose, including the repression of germination, repression of root and hypocotyl growth, repression of greening, and sensitivity to ABA (Chen et al., 2006; Tunc-Ozdemir and Jones, 2017).

1.3.4 TOR

TOR kinase mediates sugar and auxin signalling downstream of primary metabolism and ROP2, respectively, to activate stem cells at the root and shoot apical meristems (Fritzsche et al., 2017; Pfeiffer et al., 2016; Schepetilnikov et al., 2017; Xiong et al., 2013). TOR promotes the cell cycle by directly phosphorylating and activating the E2Fa and E2Fb transcription factors (Li et al., 2017; Xiong et al., 2013), activates translation by upregulating the expression of ribosomal proteins and phosphorylation of the RIBOSOMAL PROTEIN S6 kinases S6K1 and S6K2 (Dobrenel et al., 2016; Xiong et al., 2017; Xiong and Sheen, 2012), and represses autophagy (Zhang et al., 2016b). TOR is also an essential component of long term auxin signalling responses (Deng et al., 2016; Schepetilnikov et al., 2013; Schepetilnikov et al., 2017), and regulates brassinosteroid signalling (Xiong et al., 2017; Zhang et al., 2016b). In Arabidopsis, *tor* mutants are embryonic lethal (Menand et al., 2002), but *TOR* is not required for post-embryonic meristem development in roots (Xiong et al., 2013).

1.4 Regulation of SAM activity by phytohormones

A number of phytohormones are reported to affect SAM activity. In general, phytohormones are small molecule ligands that are differentially synthesised, transported and turned over to regulate a variety of signalling pathways and responses to stimuli. Stimuli can be environmental, such as light or drought perception, or developmental. Hormone responses are often context specific and

can lead to different outcomes in different or cells or tissues. Additionally, the precise outputs of a so-called hormone signalling pathway are complicated by the interaction and interdependency of different hormone signalling pathways.

SAM activity is increased by cytokinin and requires polar auxin transport. Auxin signalling, on the other hand, has both positive and negative roles in SAM activity, while some reports indicate that ethylene and abscisic acid signalling repress SAM activity. These hormone signalling pathways interact with sugar signalling and are considered below, alongside gibberellic acid and brassinosteroid signalling, which also interact with sugar signalling.

1.4.1 Cytokinin

Cytokinin is required for normal shoot growth and meristem functions (Jasinski et al., 2005; Ko et al., 2014; Matsumoto-Kitano et al., 2008; Zhang et al., 2014b). Cytokinin deficiency or insensitivity leads to reduced SAM activity and reduced growth (Holst et al., 2011; Matsumoto-Kitano et al., 2008; Riefler et al., 2006), whilst increased cytokinin levels and signalling causes constitutive photomorphogenesis, delayed senescence, and promotes rapid leaf initiation (Bartrina et al., 2017; Chin-Atkins et al., 1996; Hou et al., 1993; Yoshida et al., 2011), partially mediated by repression of *CLV1* (Gordon et al., 2009; Lindsay et al., 2006; Yoshida et al., 2011).

There has not been convincing evidence that cytokinin is synthesised in the SAM. The *IPT* genes, which catalyse the rate limiting step of cytokinin biosynthesis (Miyawaki et al., 2004), were not expressed in the SAM in *GUS* reporter assays, or microarray analysis of protoplasted SAM cell types (Miyawaki et al., 2004; Yadav et al., 2009). The main evidence suggesting that cytokinin is synthesised in the SAM comes from overexpression of the STM homeobox transcription factor, which is enriched in meristematic tissues. Induction of *35S::*STM-GR led to the upregulation of *IPT7* and increased cytokinin levels (Jasinski et al., 2005; Yanai et al., 2005). Moreover, expression of bacterial *IPT* from the *STM* promoter rescued *stm* shoot meristem defects (Yanai et al., 2005). However, *STM* is also expressed in procambial cells and is essential for xylem development (Liebsch et al., 2014), as is cytokinin biosynthesis (Matsumoto-Kitano et al., 2008). Therefore, *pSTM::IPT* might have rescued the phenotype of *stm* by restoring xylem development, since the SAM has been shown to receive root-derived cytokinins from the xylem (Aloni et al., 2005).

The root is the primary site of cytokinin biosynthesis (Matsumoto-Kitano et al., 2008). A cytokinin transporter *ABCG14* was shown to be important for the root to shoot translocation of cytokinin (Ko et al., 2014; Zhang et al., 2014b). The cytokinin concentration in the xylem of *abcg14* plants was reduced by around 90%, and resulted in significantly reduced shoot

development, similar to cytokinin biosynthesis mutants (Matsumoto-Kitano et al., 2008). Conversely, *abcg14* roots had increased development caused by an accumulation of cytokinin. Grafting *abcg14* shoots onto wild-type roots restored the *abcg14* shoot growth, whereas wild-type shoots grafted onto *abcg14* root stocks phenocopied the *abcg14* mutant. Exogenous application of cytokinin to the shoot was also sufficient to restore *abcg14* shoot growth. These results demonstrate that the primary source of cytokinin in the shoot is delivered from the root through the xylem.

Light or darkness had no significant effect on the levels of cytokinins (zeatin, dihydrozeatin, zeatin riboside, or isopentenyladenosine) in whole seedlings (Chin-Atkins et al., 1996; Chory et al., 1994). However, another study showed that *total* cytokinin levels are similar in light and dark grown plants (pea), but dark-grown plants had greater levels of cytokinin in the roots, and light-grown plants had greater levels of cytokinin in the shoots (Kefeli and Kalevitch, 2013); light triggered the redistribution of cytokinin from roots to shoots within 4 hours of illumination. The light-dependent root shoot distribution of cytokinin is supported by gene expression data of cytokinin reporter genes (Brenner et al., 2005) in the SAM (Lopez-Juez et al., 2008) and expression of the cytokinin reporter *TCS::GFP* in the inflorescence SAM (Yoshida et al., 2011). The previously mentioned cytokinin transporter *ABCG14* does not appear to be regulated by light (eFP Browser; Winter et al., 2007), however, grafting experiments have demonstrated that cytokinin uptake from the root is dependent on the shoot, not the root (Beveridge et al., 1997). Other studies have shown that transpiration rate controls the import of cytokinins from root to shoot (Aloni et al., 2005; Beck and Wagner, 1994), which is reduced in shaded leaves, so that senescence is promoted by lower cytokinin levels (Boonman et al., 2007). Therefore, in a dark development setting, where transpiration rates are minimal, the level of cytokinin in the shoot is much lower than in light-grown plants.

1.4.2 Polar auxin transport

The efficiency of auxin passage across plasma membranes is greatly increased by uptake and efflux carriers (Delbarre et al., 1996). The asymmetric localisation of auxin uptake and polar auxin efflux carriers enables directional auxin flux between cells, termed polar auxin transport. Arabidopsis has four known polar auxin uptake carriers, AUXIN RESISTANT1 (AUX1) and LIKE-AUX1 (LAX)1/2/3, and five known polar auxin efflux carriers, PIN-FORMED (PIN)1/2/3/4/7, involved in polar auxin transport across plasma membranes (Carrier et al., 2008; Peret et al., 2012; Yang et al., 2006; Zourelidou et al., 2014). The concerted action of these polar auxin transporters has been shown to be important for establishing auxin minima and maxima which regulate developmental events including root and shoot meristem activity, organ boundary

formation, phyllotaxis, and fruit dehiscence (Bilsborough et al., 2011; Dubrovsky et al., 2008; Friml et al., 2004; Hofmann, 2014; Sabatini et al., 1999; Sorefan et al., 2009; Vernoux et al., 2010) through the action of downstream transcription factors (Aida et al., 2004; Mahonen et al., 2014; Wu et al., 2015).

PIN polarity is achieved in part by continuous polar endocytosis and recycling to the plasma membrane by a clathrin-dependent endocytosis pathway (Dhonukshe et al., 2007), whereas AUX1/LAX proteins are endocytosed by a separate and as yet undetermined pathway (Kleine-Vehn et al., 2006). Polar localisation of PIN proteins is directed by phosphorylation signals from kinases including PINOID (PID), WAG1, and WAG2, which promote the endocytosis of PINs (Dhonukshe et al., 2010), and phosphatases including the SERINE/THREONINE PROTEIN PHOSPHATASE2A (PP2A) A complex, which stabilize the plasma membrane localisation of PINs (Michniewicz et al., 2007). Five conserved phosphorylated residues at the M3 site of the long hydrophilic loop of PINs are important for polar PIN localisation (Ganguly et al., 2012; Ki et al., 2016; Sasayama et al., 2013). Specific phosphorylation signals are also required for PIN efflux activity (Weller et al., 2017; Zourelidou et al., 2014).

Polar auxin transport in the SAM is primarily mediated by *AUX1*, *LAX1*, and *PIN1* (Bainbridge et al., 2008; Guenot et al., 2012). AUX1 and PIN1 oppositely polarly localise to generate a directional flow of auxin (Swarup et al., 2001). Defects in *AUX1/LAX* or *PIN1* lead to altered phyllotaxis (Bainbridge et al., 2008; Deb et al., 2015; Guenot et al., 2012; Reinhardt et al., 2003), and decreased leaf initiation rate has been reported for a light-grown *pin1* allele (Guenot et al., 2012), while temporary periods of arrested leaf development were reported in the *aux1lax1lax2lax3* quadruple mutant growing in short but not long days (Bainbridge et al., 2008). Additionally, *pin1* mutants develop naked pin-like inflorescences, devoid of lateral organs, which gave rise to their name (Okada et al., 1991; Vernoux et al., 2000). Organ initiation can be rescued by microapplication of auxin to the meristem (Reinhardt et al., 2000), which also requires sufficient endogenous or exogenous cytokinin (Yoshida et al., 2011). A triple mutant of the SAM-expressed polar auxin transporters *pin1aux1lax1* had reduced rosette leaf initiation, but still produced leaves, indicating that polar auxin transport is important but not essential for leaf initiation, at least, in the light (Guenot et al., 2012). On the other hand, the *yuc1yuc4pin1* triple mutant, which has severely impaired auxin biosynthesis in the SAM, as well defective polar auxin transport, completely abolished post-embryonic leaf initiation (Cheng et al., 2007). PIN1 is removed from the plasma membrane in response to prolonged darkness in the vegetative and inflorescence SAMs of Arabidopsis, and the vegetative SAM of tomato (Lauxmann et al., 2016; Sassi et al., 2013; Yoshida et al., 2011).

Several inhibitors of polar auxin transport have been identified, although the precise modes of action haven't been fully characterised. The most widely used inhibitors of auxin efflux are 1-N-naphthylphthalamic acid (NPA), 2,3,5-triiodobenzoic acid (TIBA), and brefeldin A (BFA). NPA affects protein trafficking at concentrations above 50μM (Geldner et al., 2001; Gil et al., 2001; Peer et al., 2009) through direct interaction with the trafficking chaperone TWISTED DWARF1 (Zhu et al., 2016a) and is thought to inhibit PIN activity as well, as it is effectively inhibits polar auxin transport at 1-5μM (Casimiro et al., 2001; Teale and Palme, 2017; Zhu et al., 2016a). TIBA inhibits vesicle motility by indirectly stabilizing actin (Dhonukshe et al., 2008) and might also inhibit PIN activity (Teale and Palme, 2017). BFA is used as an inhibitor of vesicle trafficking to the plasma membrane by blocking the activity of ARF GEFs (Geldner et al., 2003; Geldner et al., 2001; Steinmann et al., 1999). The effects of BFA are rapidly reversed upon removal (Geldner et al., 2001). TIBA and BFA treatment caused PIN1 to accumulate in endosomes (Geldner et al., 2001), similar to the effect of prolonged darkness on PIN1 localisation (Lauxmann et al., 2016; Sassi et al., 2013; Yoshida et al., 2011).

1.4.3 Auxin

Auxin plays a role in almost every developmental process in plants (Leyser, 2017). Changes in auxin levels trigger responses that are context-specific and primarily determined by differential expression of the six *TRANSPORT INHIBITOR RESPONSE1*/*AUXIN SIGNALING F-BOX* (*TIR1*/*AFB*) auxin receptors, twenty-nine *Aux*/*IAA* repressors, and twenty-three *AUXIN RESPONSE FACTORS* (*ARFs*) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Leyser, 2017; Parry et al., 2009; Remington et al., 2004; Vernoux et al., 2011; Walsh et al., 2006). Auxin levels are regulated through biosynthesis, catabolism and through differential polar and non-polar auxin transport. In the SAM, auxin biosynthesis is primarily mediated by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TAA-RELATED2 (TAA1/TAR2) and downstream YUCCA (YUC1/4) (Cheng et al., 2007; Lopez-Juez et al., 2008; Stepanova et al., 2008; Stepanova et al., 2011). The GRETCHEN HAGEN3 (GH3) IAA-amido synthases are the main auxin catabolic enzymes, but DIOXYGENASE FOR AUXIN OXIDATION1/2 (DAO1/2) also mediate auxin catabolism (Mellor et al., 2016; Porco et al., 2016; Zhang et al., 2016a). The role of polar auxin transport in the SAM is discussed separately (1.4.2).

ARFs are auxin-regulated transcription factors that positively or negatively regulate auxin signalling. Aux/IAAs bind and repress ARFs with different affinities (Piya et al., 2014; Vernoux et al., 2011). TIR1/AFBs target Aux/IAAs for proteolytic degradation upon binding auxin, relieving the repression of ARFs (Gray et al., 2001; Kepinski and Leyser, 2005; Weijers and Jurgens, 2004). ARF3 and ARF13 are exceptional in that they lack the C-terminal Phox/Bem1p

(PB1) domain required for interacting with Aux/IAAs (Piya et al., 2014). ARFs form homodimers and interact with each other and other transcription factors to regulate gene expression (Boer et al., 2014; Simonini et al., 2016). Recently it was shown that ARF3 responds to auxin levels directly, negatively affecting its ability to interact with a variety of transcription factors, and affecting its regulatory activity (Simonini et al., 2017; Simonini et al., 2016).

A cascade of ARF expression in the SAM occurs upon transfer of dark-developed plants to the light, preceding the initiation of leaves (Lopez-Juez et al., 2008). One of the roles of ARFs is thought to be the regulation of class I KNOX transcription factors, including *SHOOT MERISTEMLESS* (*STM*), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA* (*KNAT1*/*BP*, *KNAT2*, *KNAT6*) and *REPLUMLESS* (*RPL*/*BLR*/*PNY*/*VAN*) (Schuetz et al., 2008; Tabata et al., 2010), which repress SAM activity (Smith and Hake, 2003) and are locally downregulated preceding the initiation of new lateral organs in vegetative and inflorescence SAMs (Byrne et al., 2003; Jackson et al., 1994; Lincoln et al., 1994). An *ARF5*/*MP* gain-of-function allele reduced the rate of leaf initiation (Garrett et al., 2012), indicating that ARF5 represses leaf initiation. However, a light-grown *arf5* mutant did not significantly affect leaf initiation rate (Schuetz et al., 2008), indicating that ARF5 acts redundantly.

Auxin is required but not sufficient for the initiation of leaves (Reinhardt et al., 2000; Yoshida et al., 2011). Additionally, light or sugar and/or cytokinin and polar auxin transport are required for leaf initiation (Roldan et al., 1999; Yoshida et al., 2011). Auxin-responsive gene expression is particularly high in three-day old dark-developed shoot apices, and was repressed by light, indicating that auxin might repress leaf initiation in the dark (Lopez-Juez et al., 2008), although this was challenged by Yoshida and colleagues, who observed decreased expression of the *DR5::GFP* auxin reporter in dark adapted shoot apices after six days (Yoshida et al., 2011). However, since neither study applied exogenous sugars to the shoot apices, the longer-term downregulation of auxin signalling observed by Yoshida *et al.* (2011) could instead be a starvation response mediated by TOR kinase (Deng et al., 2016), whereas the three day old seedlings examined by Lopez-Juez *et al.* (2008) could still be utilising embryonic sugar reserves. Therefore, it is more likely, in a dark development scenario where exogenous sugars are also supplied, that auxin signalling, mediated by TOR, is maintained in the SAM, supporting the theory that auxin might repress leaf initiation in the dark (Lopez-Juez et al., 2008). Consistent with the hypothesis that auxin represses SAM activity in the dark, treatments with the auxins IAA (0.75 or 1.5µM) or 2,4-D (1 or 2µM) inhibited the leaf initiation of an orchid (Novak and Whitehouse, 2013) and the auxins NAA (1µM) or 2,4-D (10µM) inhibited the leaf initiation of moss (Bennett et al., 2014). Moreover, gain-of-function alleles of Arabidopsis *Aux*/*IAAs* that stabilized Aux/IAA protein in the presence of auxin (Colon-Carmona et al., 2000) increased dark development (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000), indicating that auxin signalling represses dark development.

1.4.4 Ethylene

Studies indicate that ethylene represses leaf initiation. Ethylene biosynthesis and signalling is increased in dark-developed shoot apices, downstream of auxin signalling (Klee and Romano, 1994; Lopez-Juez et al., 2008). Increased ethylene levels correlated with reduced rate of leaf initiation in response to NaCl (Albacete et al., 2008). Whereas the ethylene insensitive mutant *ein2-1* increased leaf initiation under low light intensity (15µM m^{-2} s^{-1}) (Vandenbussche et al., 2003). Another ethylene-insensitive mutant *hookless1* (*hls1*) increased dark development by stabilizing the auxin and brassinosteroid responsive transcription factor ARF2 (Hou et al., 1993; Lehman et al., 1996; Li et al., 2004a; Vert et al., 2008), which negatively regulates auxin and ABA signalling (Lim et al., 2010; Promchuea et al., 2017). Ethylene insensitive mutants have reduced apical hook curvature in the dark (Raz and Ecker, 1999), which is an indicator of increased dark development, whilst ethylene potentiated the formation of an apical hook (Guzman and Ecker, 1990).

1.4.5 Abscisic acid

ABA has been demonstrated to negatively regulate SAM activity. Extended periods of darkness caused substantial increases in endogenous ABA levels (Weatherwax et al., 1996), which correlated with a reduced rate of leaf initiation in tomato plants, where ABA levels were increased in response to NaCl treatment (Albacete et al., 2008). Dark development was increased by stabilization of ARF2 which negatively regulates ABA signalling (Li et al., 2004a; Promchuea et al., 2017); and two ABA-insensitive mutants, *abi3* and *abi8*, had increased dark development (see 1.2.2.2; Cheng et al., 2000; Rohde et al., 2000; Rohde et al., 1999). Furthermore, treatments with ABA biosynthesis inhibitors increased dark development, and exogenous ABA repressed dark development (Rohde et al., 1999). ABA response genes have been shown to be present in all cell types of the SAM (Malhan et al., 2015).

1.4.6 Gibberellic acid

Gibberellic acid (GA) is required for growth of Arabidopsis in the light and the dark (Griffiths et al., 2006; Roldan et al., 1999; Zeevaart and Talon, 1992). The expression of the GA receptor *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*) is regulated by the circadian clock and upregulated at night (Achard et al., 2007; Arana et al., 2011; Ueguchi-Tanaka et al., 2005). *GID1* expression was also increased in dark-developed shoot apices (Lopez-Juez et al., 2008). Consequently, GA responses are upregulated in the dark, promoting elongation of the hypocotyl and skotomorphogenesis (Achard et al., 2007; Roldan et al., 1999). A variety of basic helix-loop-

helix (bHLH) transcription factors are inhibited by interaction with DELLA proteins (Arnaud et al., 2010; de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolome et al., 2011; Hong et al., 2012), notably the PIFs, which repress photomorphogenesis (Leivar et al., 2008). GA-bound GID1 relieves the inhibition of DELLA-repressed transcription factors by binding DELLAs and promoting their degradation (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2005).

Microtubule dynamics are an important component of GA responses (Sambade et al., 2012). GA treatment promotes the formation of transverse arrangements microtubules in light-grown hypocotyls, which was accompanied by rapid growth (Lloyd, 2011; Sambade et al., 2012). Additionally, GA inhibited the highly ordered arrangement of cortical microtubules dynamics in root meristem cells (Ishida and Katsumi, 1991). The effect of GA on microtubule dynamics was shown to be downstream of DELLAs (Locascio et al., 2013).

GA levels in the SAM are suppressed by KNOX homeobox transcription factors by inducing the GA catabolic genes *GA2OX4*/*GA2OX6* in a cytokinin-dependent manner (Jasinski et al., 2005). Constitutive GA signalling coupled with low cytokinin levels was shown to be detrimental to SAM function, with the SAM being consumed by initiating primordia, unable to replenish its stem cell niche (Jasinski et al., 2005).

1.4.7 Brassinosteroid

Brassinosteroids promote cell growth through expansion and proliferation, accelerate senescence, and repress photomorphogenesis (Clouse et al., 1996; Mandava et al., 1981; Mitchell et al., 1970; Worley and Mitchell, 1971; Yopp et al., 1981). The brassinosteroids brassinolide and castasterone bind to the receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) (Wang et al., 2001), which phosphorylates the kinases BR-SIGNALLING KINASE (BSK) (Sreeramulu et al., 2013; Tang et al., 2008) and CONSTITUTIVE DIFFERENTIAL GROWTH1 CDG1 (Kim et al., 2011). CDG in turn phosphorylates and activates the phosphatase BRI1 SUPPRESSOR1 (BSU1) (Kim et al., 2011), which dephosphorylates and inactivates GSK3-like kinase repressors, notably BRASSINOSTEROID INSENSITIVE2 (BIN2) (Kim et al., 2009), named after the phenotype of a gain-of-function allele (Li et al., 2001b), and relieves the repression of the transcription factors including BRASSINAZOLE RESISTANT1 (BZR1), BES1, and PIF4 (Bai et al., 2012; Gampala et al., 2007; Kim et al., 2009), which promote brassinosteroid signal transduction (Oh et al., 2012; Wang et al., 2002; Yin et al., 2002). Dephosphorylation of BZR1 and BES1 is mediated by PROTEIN PHOSPHATASE2A (PP2A) (Tang et al., 2011). The phosphorylated BZR1 and BES1 are stabilized in the inactive state by 14-3-3 proteins (Gampala et al., 2007). Once dephosphorylated, BZR1 and BES1 rapidly translocate to the nucleus (Gampala et al., 2007).

Dephosphorylated BZR1, BES1 and PIF4 are subject to repression by DELLA proteins, attenuating brassinosteroid responses in the light (Bernardo-Garcia et al., 2014; Li et al., 2012).

In the SAM, nuclear localisation of the brassinosteroid-responsive transcription factor BZR1 occurs in areas of high auxin levels (Gendron et al., 2012). Brassinosteroids are excluded from organ boundaries, where it is important for cells not to grow, to support growing organs (Gendron et al., 2012). Overexpression of brassinosteroid biosynthesis or signalling genes occasionally results in fused leaves, similar to mutants with defective polar auxin transport (Gendron et al., 2012). Brassinosteroid-insensitive and deficient mutants are dwarfed and constitutively photomorphogenic (Chory et al., 1991b; Clouse et al., 1996). Brassinosteroid signalling is enhanced in the dark, as the repression of BZR1, BES1 and PIF4 by DELLA proteins is alleviated (Bai et al., 2012; Bernardo-Garcia et al., 2014; Li et al., 2012).

The loss of BZR1/PIF stabilization in mutants of brassinosteroid biosynthesis *det2* (*cop7*), *dwf4*, *cpd* (Azpiroz et al., 1998; Chory, 1992; Szekeres et al., 1996), perception *bri1* (Clouse et al., 1996; Kim et al., 2009), signalling *bsu-quad* (Kim et al., 2009), gain of function mutants of brassinosteroid signalling repressors *bes1*, *bin2* (Li et al., 2001b; Ryu et al., 2010), and an integrator of light and brassinosteroid signalling *35S::GATA2* (Luo et al., 2010), results in de-etiolation in the dark.

1.5 The Arp2/3 complex

The actin-related protein2/3 (Arp2/3) complex is a protein complex composed of Arp2, Arp3, and five core Arp2/3 complex (ARPC) subunits, named 1-5 in decreasing order of size (Machesky et al., 1994; Machesky et al., 1997; Robinson et al., 2001). As their names suggest, Arp2 and Arp3 are related to actin, which is in terms of sequence similarity (Frankel et al., 1994; Kelleher et al., 1995; Leesmiller et al., 1992; Schwob and Martin, 1992), and belong to an ancient actin superfamily that also includes hexokinase, and evolutionarily precedes the divergence of eukaryotes from prokaryotes (Fyrberg et al., 1994; Kabsch and Holmes, 1995). Arabidopsis has orthologs of each ARP2/3 complex subunit, including two homologs of *ARPC1* and *ARPC2*, each named *A* and *B* (McKinney et al., 2002; Szymanski, 2005).

1.5.1 Upstream regulation of Arp2/3 complex activity

The Arp2/3 complex is activated by direct phosphorylation of Arp2/3 subunits (LeClaire et al., 2015; Serrels et al., 2007; Tunduguru et al., 2017), and by a nucleation promoting complex WAVE, comprising Wiskott-Aldrich syndrome protein (WASP, N-WASP) (Carlier et al., 1999;

Rohatgi et al., 1999), Suppressor of Cyclic AMP Receptor/WASP family verprolin-homologous (Scar/WAVE) (Machesky and Insall, 1998; Stovold et al., 2005), Abl-interactor (Abi) (Eden et al., 2002; Innocenti et al., 2004; Soderling et al., 2002), Nck-associated protein (Nap1/NAP125/kette) (Eden et al., 2002), Specifically Rac1-associated (Sra1/PIR121/CYFIP1) (Kobayashi et al., 1998), and HSPC300 (Eden et al., 2002). Arabidopsis lacks *WASP* and *N-WASP* (Uhrig et al., 2007), and several WAVE complex orthologs have alternative names in Arabidopsis, for instance, HSPC300 is *BRICK1* (*BRK1*) (Le et al., 2006), Abi is *ABI-1-LIKE* (*ABIL*) (Basu et al., 2005), Sra1/PIR121/CYFIP1 is *PIROGI* (*PIR*) (Basu et al., 2004; Li et al., 2004b). ARP2/3 activity in Arabidopsis requires at least one subunit of each WAVE component, as single mutants typically display phenotypes akin to *ARP2/3* mutant phenotypes (Szymanski, 2005). However, Arabidopsis has four SCAR proteins, one SCAR-like protein (SCARL), and four ABIL proteins (Uhrig et al., 2007) with differing levels of functional redundancy within each family (Uhrig et al., 2007; Zhang et al., 2008). WAVE activates Arp2/3 complex activity by inducing conformational changes that relieve Arp2/3 autoinhibition by a conserved region at the Arp3 C-terminus (Rodnick-Smith et al., 2016).

WAVE activity is regulated by a variety of kinases including Rho GTPases (Miki et al., 1998), tyrosine kinases (Ardern et al., 2006; Leng et al., 2005; Sossey-Alaoui et al., 2007; Stuart et al., 2006), cyclin-dependent kinases (Miyamoto et al., 2008), and MAP kinases (Danson et al., 2007). The phosphorylation of WAVE components in general has been demonstrated to activate WAVE and the Arp2/3 complex (Ardern et al., 2006; Leng et al., 2005; Miki et al., 1998; Sossey-Alaoui et al., 2007; Stuart et al., 2006). In Arabidopsis, the RHO-RELATED PROTEIN FROM PLANTS2 (ROP2) is believed to activate the ARP2/3 complex (Yanagisawa et al., 2013) through phosphorylation of PIR (Basu et al., 2005), and/or by interaction and presumed phosphorylation of SCAR2 (Uhrig et al., 2007), resulting in increased cortical fine F-actin (Fu et al., 2002). Additional ROPs are thought to regulate ARP2/3 complex activity, notably, ROP5/7/8/11 were shown to interact with WAVE proteins (Uhrig et al., 2007). Additionally, SCAR1 protein levels are regulated by COP1, which targets SCAR1 for proteolytic degradation (Dyachok et al., 2011). A receptor-like kinase *CURVY1* (*CVY1*) was identified as a putative upstream regulator of the ARP2/3 complex (Gachomo et al., 2014). A *cvy1* mutant had several phenotypes in common with *ARP2/3* complex mutants including distorted trichomes, reduced pavement cell size and cell shape complexity, and reduced stomate area, indicating that CVY1 might positively regulate ARP2/3 complex activity, although *cvy1* and *ARP2/3* mutants also had several conflicting phenotypes (Gachomo et al., 2014).

1.5.2 Expression and localisation of the ARP2/3 complex of plants

In plants, the *ARP2/3* and *WAVE* complexes are ubiquitously expressed in various tissues (Li et al., 2003; Li et al., 2004b). ARP3 is polarly localised in Tobacco cells (Maisch et al., 2009), and ARPC4:HA and endogenous ARP3 were strongly associated with membranes in Arabidopsis (Kotchoni et al., 2009), whereas, upstream WAVE components are specifically localised to cell corners in root cells, and to punctate structures in leaf pavement cells, at the plasma membrane and double membrane structures associated with the endoplasmic reticulum (Dyachok et al., 2008; Wang et al., 2016).

1.5.3 Arp2/3 complex activities

1.5.3.1 Actin branching mediated by the Arp2/3 complex

The Arp2/3 complex binds to the sides of actin filaments with a regular periodicity of approximately 37nm and nucleates F-actin branching in association with active WAVE (Machesky et al., 1999; Mullins et al., 1998a; Mullins et al., 1998b; Mullins et al., 1997). Nascent actin branches extend at a fixed angle of 70° ± 7° and can form dense clouds of fine cortical actin (Mullins et al., 1998a), with regulatory activities, reviewed below. ARP3 has also been shown to associate with actin filaments and localise at the site of actin nucleation in plant cells (Maisch et al., 2009).

1.5.3.2 Clathrin-mediated endocytosis mediated by the Arp2/3 complex

The Arp2/3 complex plays a major role in the internalization step of endocytosis in some organisms (Moreau et al., 1997; Moreau et al., 1996), but in many organisms, the requirement for actin and the Arp2/3 complex for endocytosis is not obligatory and varies even between cell types (Epp et al., 2010; Fujimoto et al., 2000; Zou et al., 2016). The Arp2/3 complex is generally considered to promote clathrin-mediated endocytosis, although Arp2/3-independent endocytosis exists, which occasionally leads to confusion in the literature. For instance, endocytic uptake of the dye FM4-64 to the vacuole, was significantly delayed and 'weaker' in Arp2/3 mutants of *Candida albicans*, taking up to four times as long (Epp et al., 2010), but it was later shown that FM4-64 was also endocytosed by an Arp2/3 and clathrin-independent pathway (Epp et al., 2013). Several studies have also shown that certain plasma membrane proteins, including receptors and permeases, required Arp2/3, and were not endocytosed by Arp2/3-independent pathways (Epp et al., 2013; Leyton-Puig et al., 2017). In Arabidopsis, the endocytic uptake of FM4-64 and the endocytosis of PIN2, PIN3, and PIN7 auxin efflux carriers was significantly delayed in an *arp3* mutant (Zou et al., 2016).

Clathrin-mediated endocytosis occurs from the plasma membrane *de novo* (Henne et al., 2010; Stimpson et al., 2009), and from sustained clathrin plaques, which serve as dynamic hubs of actin polymerisation for the endocytosis of specific cell surface proteins (Grove et al., 2014; Leyton-Puig et al., 2017). Association of cell surface proteins with clathrin plaques can be regulated through external cues such as ligand binding (Leyton-Puig et al., 2017). Actin promotes the formation and constriction of clathrin-coated pits for vesicle internalisation, as well as the budding and merging of clathrin-coated structures (Yarar et al., 2005), although, actin is not obligatory for endocytosis (Fujimoto et al., 2000). Treatments with the actin polymerisation inhibitor Latrunculin A (LatA) stabilized the plasma-membrane association of clathrin and associated proteins, and blocked the internalisation of clathrin-coated vesicles in the yeast *S. cerevisiae* (Kaksonen et al., 2003; Kaksonen et al., 2005; Newpher et al., 2005), and increased the formation of clathrin plaques in HeLa cells (Leyton-Puig et al., 2017), recapitulating phenotypes of Arp2/3 complex mutants (Leyton-Puig et al., 2017; Martin et al., 2005). Therefore, in some cell types and scenarios, the maturation of clathrin-coated pits and the endocytosis of specific receptors is Arp2/3 dependent (Leyton-Puig et al., 2017; Martin et al., 2005). It has been suggested that the actin filament network drives the mechanical force behind the budding and scission of clathrin-coated vesicles (Martin et al., 2005), however, actin might only accelerate endocytosis (Li et al., 2015a).

1.5.3.3 Phenotypes of *ARP2/3* and *WAVE* mutants in plants

In Arabidopsis, the ARP2/3 complex is famously involved in regulating the later stages of trichome development (Mathur et al., 1999). Several ARP2/3 complex components and activators of Arabidopsis were first identified and named based on their distorted trichomes (Hulskamp et al., 1994). These include *DISTORTED TRICHOMES1-3* (*ARP3*, *ARPC2A*, *SCAR2*, respectively), *WURM* (*ARP2*), *CROOKED* (*ARPC5*), *GNARLED* (*NAP1*), *KLUNKER*/*PIROGI* (*PIR*), *SPIKE1* (*SPK1*) (Basu et al., 2005; El-Assal et al., 2004a; El-Assal et al., 2004b; Mathur et al., 2003a; Mathur et al., 2003b; Qiu et al., 2002; Saedler et al., 2004b).

Intracellular phenotypes of Arabidopsis *ARP2/3* and *WAVE* mutants include increased F-actin bundling (Li et al., 2004b; Mathur et al., 2003b; Mathur et al., 1999), delayed endocytosis (Zou et al., 2016), altered vacuole integrity, where instead of a single large vacuole; cells have multiple smaller vacuoles surrounding a larger vacuole (Mathur et al., 2003a), reduced cytoplasmic streaming (Mathur et al., 2003b), decreased chlorophyll levels (Li et al., 2004b), defective autophagosome formation and autophagy (Wang et al., 2016), and altered cell wall composition at cell corners, specifically, absent antibody labelling of de-arabinosylated rhamnogalacturonan and fucosylated xyloglucans (Dyachok et al., 2008).

Root and hypocotyl phenotypes include decreased root length in the light (Dyachok et al., 2008; Dyachok et al., 2011), increased root length in the dark (Dyachok et al., 2011; Li et al., 2004b), and reduced penetration strength resulting from altered cell wall composition at the root tip (Dyachok et al., 2008); reduced hypocotyl length (El-Assal et al., 2004b; Le et al., 2006; Mathur et al., 2003b; Zhang et al., 2008), and increased radial cell expansion of hypocotyls (Fu et al., 2002; Li et al., 2004b).

Stomatal phenotypes include increased stomatal density (Jiang et al., 2012), reduced stomatal development on the hypocotyl (Mathur et al., 2003b), reduced stomate area (Gachomo et al., 2014), and insensitivity to ABA, $CaCl_2$ and dark-induced stomatal closure, with constitutively intermediate stomatal aperture (Isner et al., 2017; Jiang et al., 2012).

Other shoot phenotypes include, defective epidermal cell development, with curling of the ends of rapidly expanding hypocotyl epidermal cells (El-Assal et al., 2004a; Mathur et al., 2003a), and gaps between pavement cells of the leaf epidermis (Li et al., 2004b), as well as reduced pavement cell size and cell shape complexity (Basu et al., 2005; Gachomo et al., 2014; Li et al., 2003; Zhang et al., 2008), increased petiole elongation in the dark (Baier et al., 2004; Li et al., 2004b; Zhang et al., 2008), reduced leaf epinasty (Li et al., 2004b), and increased shoot apical meristem activity in the dark (Baier et al., 2004; Li et al., 2004b; Zhang et al., 2008).

Additionally, *ARP2/3* complex and *WAVE* mutants have enhanced responses to sugars (Baier et al., 2004; Li et al., 2004b), increased susceptibility to nitrogen starvation and decreased salt tolerance from defective autophagosome formation (Wang et al., 2016), reduced gravitropic responses caused by inhibition of amyloplast sedimentation by increased F-actin bundling in roots (Reboulet et al., 2010; Zou et al., 2016), and increased phototropic curvature responses of dark-grown but not light-grown seedlings to blue and red light (Reboulet et al., 2010).

1.5.3.4 Additional functions of the Arp2/3 complex

Additionally, the Arp2/3 complex has been reported to promote phagocytosis (Linder, 2017; May et al., 2000), proteasome mobility (Cabrera et al., 2011), asymmetric cell division (Sun et al., 2011), cell migration (Linder, 2017; Suraneni et al., 2012), cell membrane architecture, notably lamellipodia and filopodia (Suraneni et al., 2012; Wu et al., 2012), and the formation of neural dendrites (Zhang et al., 2017).

1.5.4 Inhibitors of the Arp2/3 complex and chemicals that phenocopy *ARP2/3* mutants

Two Arp2/3 complex inhibitors have been described, CK-666 and CK-869. CK-666 and CK-869 are reported to inhibit the Arp2/3 complex in a wide range of organisms (Avasthi et al., 2014; Davison et al., 2016; Ilatovskaya et al., 2013; Nolen et al., 2009; Sun et al., 2011; Yang et al., 2012), although CK-869 did not inhibit the Arp2/3 complex of fission yeast, while CK-666 did (Nolen et al., 2009). CK-869 also caused off-target effects including membrane blebbing (Rotty et al., 2013). CK-666 binds between the Arp2 and Arp3 subunits and CK-869 binds to a site within Arp3 (Hetrick et al., 2013; Nolen et al., 2009). Both chemicals block a conformational change required for Arp2/3 complex activity (Hetrick et al., 2013). It is not known whether CK-666 or CK-869 inhibit the ARP2/3 complex of plants.

Plants treated with either actin stabilizing (Phalloidin, Jasplakinolide) or destabilizing (Cytochalasin D and Latrunculin B (LatB)) chemicals phenocopied the distorted trichomes of *ARP2/3* and *WAVE* mutants (Mathur et al., 1999; Szymanski et al., 1999), indicating that dynamic actin filamentation is required for wild-type trichome development. LatB treatment led to the obliteration of F-actin, unlike *ARP2/3* mutants (Mathur et al., 1999).

1.6 Chapter summary

The aim of this study was to investigate how ARP2/3 represses SAM activity in the dark (Baier et al., 2004). In the first results chapter (chapter 3) I describe the development of an assay for quantitatively measuring dark development. The dark development assay was then used to demonstrate the specificity of the ARP2/3 complex in regulation of dark development, investigated using a range of actin-related mutants and actin-disrupting chemicals. The role of the ARP2/3 complex in regulating SAM activity in the shade was also investigated, utilising the constitutive shade avoidance phenotype of *phyB*, since the regulation of SAM activity by the ARP2/3 complex is light-sensitive. Genetics and chemical genetics approaches were taken to assess the effect of sugar signalling mutants (chapter 4), and exogenous phytohormones (chapter 5) on the dark development of *arp3* mutants, as *arp3* increased sugar responses (Baier et al., 2004), and several phytohormones are known to regulate SAM activity. A concurrent study utilised the dark development assay to screen for novel transcriptional regulators of SAM activity (chapter 6). A candidate approach was taken, using mutants of transcription factors related to known regulators of SAM activity, in particular, the role of the *HECATE*-like transcription factor *INDEHISCENT* (*IND*) was examined, as it has previously been the subject of study in our lab (Arnaud et al., 2010; Girin et al., 2011; Simonini et al., 2016; Sorefan et al., 2009). Additionally, the potential role of ARF transcription factors in regulating SAM activity downstream of the

ARP2/3 complex was investigated (chapter 6), as polar auxin transport was found to be upregulated in *arp3* (chapter 5).

The following hypotheses were tested:

Chapter 3

The ARP2/3 complex specifically regulates dark development

The ARP2/3 complex regulates dark development by reducing actin bundling

CK-666 is an inhibitor of the Arabidopsis ARP2/3 complex

The ARP2/3 complex increases SAM activity in a constitutive shade response

Chapter 4

The increased dark development of *arp3* is caused by increased sugar signalling

The ARP2/3 complex regulates glycolysis directly by repressing FBA activity

Chapter 5

The increased dark development of *arp3* is caused by altered phytohormone signalling

Brassinosteroids and auxins regulate dark development in the same pathway as ARP2/3

The ARP2/3 complex is a negative regulator of brassinosteroid signalling

The increased dark development of *arp3* requires *TIR1*

The ARP2/3 complex regulates polar auxin transport

The increased dark development of *arp3* requires *PIN1*

Chapter 6

The HECATE-like transcription factor IND regulates SAM activity

IND regulates SAM activity through its primary target *SWEET15*

IND regulates SAM activity redundantly with HECATEs

IND regulates SAM activity with its interaction partners

ARFs regulate SAM activity in the same pathway as ARP2/3

The putative ARF-target *RPL* regulates SAM activity downstream of ARP2/3

Chapter 2 - Materials and methods

2.1 Plant material and growth conditions

2.1.1 Plant lines used:

All plant lines were in the Columbia (Col) ecotype, unless specified Landsberg *erecta* (L*er*). Details of the mutant and transgenic lines used in this study are presented in Tables 2.1 and 2.3; see acknowledgements for stock donors. Uncharacterised T-DNA insertion lines were obtained from the SALK (Alonso et al., 2003) and SAIL (Sessions et al., 2002) seed pools. With the exception of the gain-of-function allele *axr5-1*, the published T-DNA, ethylmethane sulfonate (ems), and X-ray induced mutants used in this study have been described as knockouts, loss-of-function, or null/strong alleles.

Table 2.1A Plant lines relating to multiple chapters

Line	Allele	Gene name	Gene ID	Mutation	Reference
arp3	*dis1-2* SALK_010045	distorted trichomes1	AT1G13180	T-DNA insertion	(Le et al., 2003; Mathur et al., 2003a)
L*er arpc2a*	*dis2-1*	distorted trichomes2	AT1G30825	ems	(Saedler et al., 2004a)

Table 2.1B Plant lines relating to chapter 3

Line	Allele	Gene name	Gene ID	Mutation	Reference
scar2	*scar2-2* SALK_036419	scar homolog2	AT2G38440	T-DNA insertion	(Uhrig et al., 2007)
vln1	*vln1-4* SALK_133579	villin1	AT2G29890	T-DNA insertion	(Khurana et al., 2010)
fh1	*fh1-1* SALK_032981	formin homology1	AT3G25500	T-DNA insertion	(Rosero et al., 2013)
prf1	*prf1-2* SALK_057718	profilin1	AT2G19760	T-DNA insertion	(Cao et al., 2016)
prf2	*prf2-1* SALK_129071	profilin2	AT4G29350	T-DNA insertion	(Mussar et al., 2015)
35S::mTalin	*35S::GFP-mTalin*	mouse talin	#N/A	Transgene	(Kost et al., 1998)
phyB	*phyB-9* *phyB-EMS142*	phytochrome B	AT2G18790	ems	(Neff et al., 1998)

Table 2.1C Plant lines relating to chapter 4

Line	Allele	Gene name	Gene ID	Mutation	Reference
hxk1	*hxk1-3* SALK_070739	hexokinase1	AT4G29130	T-DNA insertion	(Aki et al., 2007; Huang et al., 2015a)
L*er hxk1*	*gin2-1*			ems	(Moore et al., 2003)
L*er hxk1 35S::HXK1*	*gin2-1 HXK1*			Transgene	(Moore et al., 2003)
L*er hxk1 35S::HXK1*^{S117A}	*gin2-1 HXK1*^{S117A}	(catalytic mutant)		Transgene	(Moore et al., 2003)
L*er arpc2a hxk1*	*dis2-1gin2-1*	distorted trichomes2, hexokinase1	AT1G30825 AT4G29130	ems	(Karim Sorefan, unpublished)
rgs1	*rgs1-1*	regulator of g-protein signaling1	AT3G26090	T-DNA insertion	(Chen et al., 2003)
35S::RGS1	*35S::AtRGS1-YFP-HA*			Transgene	(Urano et al., 2012)
XVE-TOR-RNAi	*tor-es1*	target of rapamycin	AT1G50030	Estradiol-inducible RNAi	(Xiong and Sheen, 2012)
fba1	*fba1-1* SALK_063223	fructose-bisphosphate aldolase1	AT2G21330	T-DNA insertion	(Lu et al., 2012)
fba2-1	*fba2-1* SALK_073444	fructose-bisphosphate aldolase2	AT4G38970	T-DNA insertion	(Lu et al., 2012)
fba2	*fba2-2* SALK_000898			T-DNA insertion	This study
fba4	*fba4-1* SALK_124050	fructose-bisphosphate aldolase4	AT5G03690	T-DNA insertion	(Lu et al., 2012)
fba5	*fba5-1* SALK_080758	fructose-bisphosphate aldolase5	AT4G26530	T-DNA insertion	(Lu et al., 2012)
fba6	*fba6-1* SALK_014964	fructose-bisphosphate aldolase6	AT2G36460	T-DNA insertion	(Lu et al., 2012; Tang, 2013)
fba7	*fba7-1* SAIL_870_A09	fructose-bisphosphate aldolase7	AT4G26520	T-DNA insertion	This study
fba8-1	*fba8-1* SALK_124383	fructose-bisphosphate aldolase8	AT3G52930	T-DNA insertion	(Lu et al., 2012; Tang, 2013)
fba8-2	*fba8-2* SALK_007216			T-DNA insertion	(Tang, 2013)

fba8-3	*fba8-3* SAIL_1244_A08			T-DNA insertion	This study

Table 2.1D Plant lines relating to chapter 5

Line	Allele	Gene name	Gene ID	Mutation	Reference
det2	*det2-1*	de-etiolated2	AT2G38050	ems	(Chory et al., 1991b)
bin2bil1bil2	*bin2-3bil1bil2*/ Triple GSK3 mutant FLAG_593C09, SALK_062147, SALK_060220	brassinosteroid insensitive2, bin2-like1, bin2-like2	AT4G18710 AT1G06390 AT2G30980	T-DNA insertions	(Vert and Chory, 2006)
sob7	*cyp72c1-1/ sob7-1* SALK_120416	cyp72c1	AT1G17060	T-DNA insertion	(Takahashi et al., 2005; Turk et al., 2005)
bas1	*bas1-2* SALK_006781	cyp734a1	AT2G26710	T-DNA insertion	(Turk et al., 2005)
taa1tar1	*wei8-1tar1-1*	tryptophan aminotransferase of Arabidopsis1, tryptophan aminotransferase related1	AT1G70560 AT1G23320	T-DNA insertions	(Stepanova et al., 2008)
tir1	*tir1-1*	transport inhibitor response1	AT3G62980	ems	(Ruegger et al., 1998)
dao1	*dao1-1* SALK_093162	dioxygenase for auxin oxidation1	AT1G14130	T-DNA insertion	(Porco et al., 2016)
aux1lax1lax2 lax3	*aux1 lax quad*	auxin resistant1, like aux1, like aux2, like aux3	AT2G38120 AT5G01240 AT2G21050 AT1G77690	X-ray mutagenesis; dSpm insertions	(Bainbridge et al., 2008)
pin1	*pin1-613* SALK_047613	pin-formed1	AT1G73590	T-DNA insertion	(Bennett et al., 2006)
35S::PIN1	*35S::PIN1-GFP*			Transgene	(Benkova et al., 2003)
pin1 pPIN1::PIN1	*pin1-613 ProPIN1::PIN1-GFP*			T-DNA insertion; Transgene	(Ki et al., 2016)

pin1 pPIN1::PIN1 3m1A	*pin1-613 ProPIN1::3m1A PIN1-GFP*	(3m1 phospho-mutant)		T-DNA insertion; Transgene	(Ki et al., 2016)
pin1 pPIN1::PIN1 M3A	*pin1-613 ProPIN1::M3PIN1-GFP*	(M3 phospho-mutant)		T-DNA insertion; Transgene	(Ki et al., 2016)
pid	*pid-14* SALK_049736	pinoid	AT2G34650	T-DNA insertion	(Huang et al., 2010)
35S::PID	*35S:PID-GFP*			Transgene	(Henrichs et al., 2012)

Table 2.1E Plant lines relating to chapter 6

Line	Allele	Gene name	Gene ID	Mutation	Reference
ind	*ind-2*	indehiscent	AT4G00120	ems	(Liljegren et al., 2004)
L*er ind*	*ind-6*			Ds gene trap insertion	(Samuneva et al., 2008)
35S::IND-GR	*35S::IND:GR*			Transgene	(Sorefan et al., 2009)
swt15-1	*sag29-2* SALK_031720	sweet15	AT5G13170	T-DNA insertion	(Seo et al., 2011)
swt15-2	*sweet15* SM_3_14944			T-DNA insertion	(Chen et al., 2015)
ago10	*ago10-4* SALK_138011	argonaute10	AT5G43810	T-DNA insertion	(Zhu et al., 2011)
L*er ago10*	*zll-3*			ems	(Endrizzi et al., 1996)
L*er ind ago10*	*ind-6 zll-3*	indehiscent, argonaute10	AT4G00120 AT5G43810	ems	(Karim Sorefan, unpublished)
hec1	*hec1-2* SALK_045764	hecate1	AT5G67060	T-DNA insertion	This study
hec2	*hec2-2* SALK_071800	hecate2	AT3G50330	T-DNA insertion	This study
hec3	*hec3-1* SALK_005294	hecate3	AT5G09750	T-DNA insertion	(Gremski et al., 2007)
bhlh087	*bhlh087-1* SALK_066339	basic helix loop helix087	AT3G21330	T-DNA insertion	(Khanna et al., 2006)
par1	*par1-1* SALK_022002	phy rapidly regulated1	AT2G42870	T-DNA insertion	This study
alc	*alc-3* SALK_103763	alcatraz	AT5G67110	T-DNA insertion	This study
spt	*spt-12* WISCDSLOX386E06	spatula	AT4G36930	T-DNA insertion	(Ichihashi et al., 2010)

pif3	*pif3* SALK_081927	phytochrome interacting factor3	AT1G09530	T-DNA insertion	(Zhong et al., 2012)
pif4	*pif4-101* Garlic_114_G06	phytochrome interacting factor4	AT2G43010	T-DNA insertion	(Lorrain et al., 2008)
myc2	*myc2-1*/*jin1-7* SALK_040500	jasmonate insensitive1	AT1G32640	T-DNA insertion	(Boter et al., 2004)
arf2	*arf2-12* SALK_035537	auxin response factor2	AT5G62000	T-DNA insertion	This study
arf3	*ett-13* SALK_040513	auxin response factor3/ettin	AT2G33860	T-DNA insertion	(Pekker et al., 2005)
arf4	*arf4-2* SALK_070506	auxin response factor4	AT5G60450	T-DNA insertion	(Pekker et al., 2005)
arf5	*arf5-1/mp* SALK_023812	auxin response factor5	AT1G19850	T-DNA insertion	(Okushima et al., 2005)
arf7	*arf7-1* SALK_040394	auxin response factor7	AT5G20730	T-DNA insertion	(Okushima et al., 2005)
iaa1-D	*axr5-1*	indole-3-acetic acid inducible1	AT4G14560	not reported (SNP)	(Yang et al., 2004)
rpl	*rpl-2* SALK_040126	replumless	AT5G02030	T-DNA insertion	(Roeder et al., 2003)

2.1.2 Standard growth conditions and treatments

Unless otherwise stated, seeds were sown on Levington® Advance Seed and Modular F2+S compost plus horticultural grade sand mixture, pH 5.3-6.0 (ICL, Ipswich, UK), and stratified at 4°C for three days. Square pots measuring 60mm top width by 80mm depth by 47mm bottom width were typically used. Plants were illuminated for 16 hours with 120µmol m^{-2} sec^{-1} light at 23°C, followed by 8 hours darkness at 18°C in a Versatile Environmental Test Chamber MLR 350-HT (Sanyo, Japan). Plants were watered with distilled water.

For growth on agar, seeds were surface-sterilized with 70% ethanol for 10mins and 5% bleach, 0.05% (v/v) Tween™20 for 20mins, then washed three times with autoclaved distilled water. Following stratification at 4°C for three days, sterile seeds were sown on 0.8% (w/v) agar supplemented with ½ Murashige and Skoog salts (Murashige and Skoog, 1962) plus vitamins (MS; Duchefa Biochemie, The Netherlands #M0222) and 0.2% (w/v) glucose (D-(+)-Glucose, Sigma Aldrich #G7021) (or another sugar supplement, as specified) in sterile plates. Plates were sealed with micropore tape to maintain sterility whilst allowing gas exchange.

For dark development assays, sterilized and stratified seeds were sown in two rows of around 25-30, on 10cm square plates containing 35mL of 0.8% (w/v) plant agar supplemented with ½ MS salts and vitamins (Duchefa Biochemie) and treatment (see Tables 2.2A, B), as specified. 0.2% (w/v) glucose was added unless specified otherwise. All genotypes (per experiment) were planted on the same plate; the position on the plate where each genotype was sown was randomised, and at least two plates were scored per treatment. For experiments analysing eight or more genotypes at a time, eight or nine replicate plates were typically used, whereas an experiment comparing two genotypes would use two or three plates per treatment. Plates were sealed with micropore tape for sterility, stacked vertically so that the developing shoot meristem maintained contact with the agar (Roldan et al., 1999), and triple wrapped in aluminium foil to exclude light (Fig. 2.1). Plants were incubated for 28 days at 23°C and then scored.

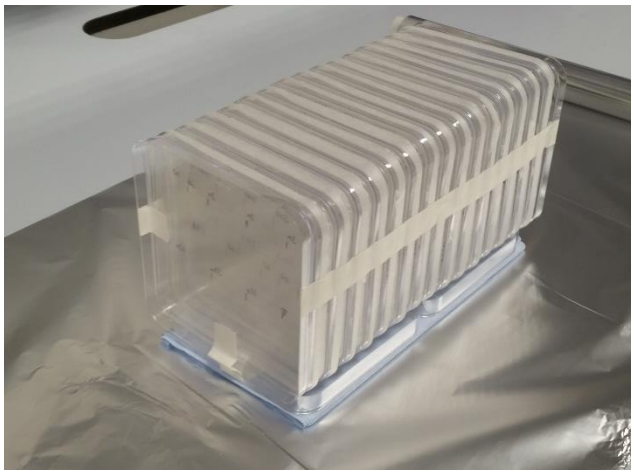


Fig. 2.1. The dark development assay setup. Sterile seeds were sown in two rows of about 30, randomizing the positioning of the genotypes on each plate. Plates were sealed with micropore tape and stacked as shown. Stacked plates were rested on top of horizontal open plates to create a 1cm gap for drainage. The horizontal plates were placed onto a stack of absorbent tissue to soak up additional moisture arising from condensation. Plates were triple-wrapped in aluminium foil and incubated at 23°C for 28 days.

For growth in liquid culture, 30 sterile seeds were sown per biological replicate in 50mL Falcon tubes containing 10mL ½ MS medium. Tubes were constantly illuminated at 120µmol m^{-2} sec^{-1} light and a constant temperature of 23°C, and aerated by shaking upright at 60 rotations per minute (rpm).

2.2 Chemical stocks and storage

General chemical stocks were ordered from Sigma Aldrich and stored as directed by the manufacturer. Additional chemicals were ordered and stored according to Table 2.2. DNA oligonucleotides were ordered from Sigma Aldrich, with the exception of oligos >40bp which were ordered from IDT Integrated DNA Technologies. All oligos were desalted, resuspended to 100µM in ddH_2O, and stored at -20°C; 10µM working stocks were made to reduce freeze-thaw related degradation.

Table 2.2A Chemical treatments

Abbreviation	Chemical name	Company name and product code	Solvent	Storage conditions
Basta	Glufosinate-ammonium	PureAmenity, U.K. (KURTAIL)	dH_2O	4°C (120mg/L w/v)
CK-666	CK-666	Insight Biotechnology, U.K. (361151)	DMSO	4°C
Chx	Cycloheximide	Acros Organics, China (AC35742)	DMSO	-20°C
Dex	Dexamethasone	Alfa Aesar, U.K. (A17590)	DMSO	RT
Est	β-Estradiol	Sigma Aldrich (E8875)	DMSO	-20°C (10mM stock)
LatB	Latrunculin B	Fluorochem, U.K. (M02063)	10% DMSO (v/v)	-20°C (2mM stock)
2-DG	2-Deoxy-D-glucose	Sigma (D-8375)	N/A	RT

RT = room temperature.

Table 2.2B Hormones and related chemicals

Abbreviation	Chemical name	Company name and product code	Solvent	Storage conditions
ABA	Abscisic acid	Scientific Laboratory Supplies (A1049)	EtOH 70% (v/v)	-20°C (1mM stock)
ACC	1-Aminocyclopropane carboxylic acid	Sigma Aldrich, China (A3903)	dH_2O	4°C
BAP	6-Benzylaminopurine	Duchefa, The Netherlands (B0904)	DMSO	RT
eBL	Epibrassinolide	Sigma Aldrich (E1641)	DMSO	-20°C (10mM stock)

GA_3	Gibberellic acid	Sigma, Switzerland (G7645)	dH_2O	RT
IAA	Indole-3-acetic acid	Duchefa, The Netherlands (I0901)	DMSO	RT
NAA	1-Naphthaleneacetic acid	Sigma Aldrich, China (N0240)	DMSO	RT
NPA	Naphthylphthalamic acid	Duchefa, The Netherlands (N0926)	DMSO	-20°C
TIBA	2,3,5-Triiodobenzoic acid	Alfa Aesar, U.K. (L02679)	DMSO	4°C
2,4-D	2,4-Dichlorophenoxyacetic acid	Acros Organics (10617452)	DMSO	RT

RT = room temperature.

2.3 Creating new genetic tools

2.3.1 Crossing of Arabidopsis accessions

Single mutants were crossed to generate double mutants for addressing specific hypotheses. Stamens were stripped from the maternal flower using fine forceps, and the stigma allowed to mature for up to 24 hours. Pollen from the paternal flower was dusted onto the mature stigma to allow fertilization. Resulting F1 progeny were selected phenotypically or genotypically (see Table 2.3), and double or triple homozygous mutants were isolated similarly in the F2-F4 generations. A *sob7bas1* double mutant has been generated and described previously using the same alleles (Turk et al., 2005).

In this study, the naming of double mutants follows the format of maternal genotype followed by paternal genotype. For instance, the double mutant *arp3fba1* was generated from an *arp3* mother fertilized with pollen carrying the *fba1* allele.

Table 2.3 Double mutants generated by crossing

Line	Selection of maternal allele	Selection of paternal allele(s)
arp3fba1	phenotype	genotype
arp3fba2	phenotype	genotype
arp3fba4	phenotype	genotype
arp3fba6	phenotype	genotype
arp3det2	phenotype	phenotypic segregation
sob7bas1	genotype	genotype
arp3tir1	phenotype	sequencing
arp3pin1	phenotype	genotype or phenotypic segregation
arp3pid1	phenotype	genotype

arp3 35S::PIN1	phenotype	GFP fluorescence
arp3 35S::PID	phenotype	GFP fluorescence
arp3ind	phenotype	phenotype
arp3swt15-1	phenotype	genotype
ind ago10	phenotype	genotype
ind hec2	phenotype	genotype
ind hec3	phenotype	genotype
ind pif3	phenotype	genotype
pif4ind	Basta resistance and genotype	phenotype
ind myc2	phenotype	genotype
arp3phyB	phenotype	phenotype

2.3.2 DNA extraction

DNA was extracted from young leaves or flowers according to a published method (Edwards et al., 1991) with modifications. Plant material was ground in DNA extraction buffer (200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) using a pestle and centrifuged at 16,000×*g* for 5mins to pellet plant debris. The supernatant was transferred into a fresh tube containing an equal volume of isopropanol and 0.125% (v/v) GlycoBlue™ (Ambion®, Thermo Fisher Scientific, U.S.). After 5mins at room temperature, tubes were centrifuged for 5mins at 16,000×*g*. The DNA pellet was washed briefly with 70% ethanol to remove residual isopropanol and allowed to air dry at room temperature. The dry DNA pellet was dissolved in TE buffer (10mM Tris, 1mM EDTA, pH8) by vortexing.

2.3.3 Selection by genotyping

Genotyping primers (Table 2.4) were designed either side of the predicted mutation site (https://seqviewer.arabidopsis.org/) using the SIGnAL T-DNA Express primer design tool (http://signal.salk.edu/tdnaprimers.2.html). Where suitable primers could not be generated using this tool, primers were designed using Primer3web (http://primer3.ut.ee/) (Koressaar and Remm, 2007; Untergasser et al., 2012) and BLASTed against the Arabidopsis thaliana genome (taxid:3702) using BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to test for specificity.

To identify homozygous insertion lines, DNA was extracted from individual plants (chapter 2.3.2) and added to three PCR reactions. The first reaction used the Forward (F) and Reverse (R) primers to amplify the wild-type gene; the second and third reactions utilized the F and R primers

respectively, together with the insert primer. Products were visualised by agarose gel electrophoresis.

10µL PCR reactions consisting of 5µL 2×REDTaq® readymix, 0.3µL 10µM Primer 1, 0.3µL 10µM Primer 2, 0.8µL DNA, 3.6µL ddH_2O, were setup on ice and subsequently incubated in a T100™ Thermal Cycler (BIO-RAD, Singapore) programmed accordingly:

94°C 3min
94°C 30sec
52-58°C 30sec } 30 cycles
68-72°C 1min/kb

68-72°C 3min

Table 2.4A Insert primers used for genotyping

Insert	Primer name	Sequence	Reference
SALK	LBb1.3	ATTTTGCCGATTTCGGAAC	http://signal.salk.edu/tdnaprimers.2.html
Garlic/SAIL	LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	(Sessions et al., 2002)
SM	Spm32	TACGAATAAGAGCGTCCATTTTAGAGTGA	http://signal.salk.edu/database/T-DNA/SM.435.pdf

Table 2.4B Genomic primers used for genotyping

Allele name	F Primer Sequence	R Primer Sequence
arp3	AATTGCTGGCAAAGATGTCAC	AGCTCTTCGTGTGTCAATTGG
fh1	GTCTCCGTCACTGTCGTTAGC	TTGTTGTTTAACGACTTCGCC
prf1	ACAAATGATGTTGCCTTCTGG	GAAGAAGACCTTGCATCGATG
prf2	GCAATTAGCTTCAACCGACTG	GGCCATACTTCGATCTCTTTTC
hxk1	TTGTTTTTGATTCCAAATCGG	TCATCAAATGAGGAGGAATCG
rgs1	TTCAGTGTGGATTGAAGGACC	ATCTTCCGGGATTTTACCATG
fba1	TTGTTGGGAATTGTCGATTTC	CTTGTTGGTAGTAAGCAGCGG
fba2	TCCATCCAACAAGATCTCTGG	TGTTCTGTTTTGCCCTGTTTC
fba4	TTTTCGAAAAAGGTGAAATGG	TTACGAAACGTTTTCCGATTG
fba5	AGTCCATGGCTTCAACACATC	AACTATTGGGAAACGATTCGC
fba6	CCATCAACAAGAATCTCAGGC	GTAGTGAGGCCGAAGTCACAC
fba7	TTCTTTGTTCAATCAGGCACC	GTTTCTGTGCTCTCATCTGCC
fba8-2/-3	AACCTCCGTGAGCTTCTCTTC	TCCAGGAAGATGACAAACGTC
tir1	GGAGGTTCCCGAAAGTGAGA	CAGGAACAACGCAGCAAAAC
pid	CAGTCGGGAAACTCAACTGTC	ATTTTGCGATGAAAGTTGTGG
pin1	CAAAAACACCCCCAAAATTTC	AATCATCACAGCCACTGATCC

dao1	TTCCCCACGGAATTAAGGTAC	CTATGGGGAAAAAGGTTCCTG
sob7	CCGACATGTGAAGTAAGCTGG	AACAGAAAAAGCCAAAAAGGC
bas1	CCGACAAGAGGGAATTTGAAG	ACATTTTTCTTCCAAGTCCGG
ago10	TTCTGGGTATTCCCAATTTCC	ATCCTTGGTAGGCAAATCAGC
swt15-1	CACCATGGGAGTCATGATCAATCACCATTTC	TCAAACGGTTTCAGGACGAGTAGCC
swt15-2	CGTTATCTAACTGACGGCGAC	CAAGTCTCTGTACTCGGCTGG
hec1	ACTCAATGACCAACGAACCAG	TGTTACACAAAAGCAAAGGCC
hec2	TTTGGTCAAATGGGAGATGAG	ATATTTCATGTGGATGTGGGG
hec3	CAAGTCTCAATGTGGGAGAGG	TTCTCCTACTCCTCTTCCCCC
bhlh087	TCCGGTTTCACTTTTTCATGTCCTG	ACACTCTGGATGATGGATTTGATTAGG
par1	AAGAAACTCTAGCCACTCCCG	ATAGCTGCATTGTTGTGGGTC
pif3	AGTCTGTTGCTTCTGCTACGC	AAGAACCGGCAAAGATACCAC
pif4	CGACGGTTGTTGACTTTGCTG	GCTTCAAGTGATGTGGATGG
myc2	CACTTGCATTTCACTCTCTTGC	AAAAACCATTCCGTATCCGTC

2.3.4 Selection by phenotype

Several homozygous lines were selected by their recessive phenotypes. The *arp3*, L*er arpc2a*, and *scar2* mutants have distorted trichomes (Le et al., 2003; Mathur et al., 2003a; Saedler et al., 2004a; Uhrig et al., 2007); the mature siliques of *ind* and *alc* mutants do not release their seeds (Liljegren et al., 2004); light-grown *phyB* mutants (originally annotated *hy3*) have elongated petioles, resembling etiolated plants (Chory et al., 1989b); *det2* is dwarfed (Chory et al., 1991b).

2.3.5 Selection by antibiotic resistance

Arabidopsis lines carrying the Basta-resistance bar gene were selected as compost-grown seedlings by spraying with 120mg/L (w/v) Basta and placing under high light. Basta is a herbicide that inhibits the enzyme glutamine synthetase, preventing the production of the amino acid glutamine. Susceptible seedlings show chlorosis and necrosis a few days after application.

2.4 Measurement of gene expression

2.4.1 RNA extraction and cDNA preparation

Total nucleic acid (TNA) extraction was performed using a method adapted from White and Kaper (White and Kaper, 1989). Plant tissue was collected into 2mL tubes containing a 4mm steel ball bearing, and rapidly frozen in liquid nitrogen. Whilst keeping the samples frozen, tubes were vortexed in short bursts until the plant tissue was ground into a powder. Tubes were then

transferred to ice and to each sample, 600µL of extraction buffer (100mM Glycine, 10mM EDTA, 100mM NaCl, 2% SDS, pH 9.5), freshly made from a 10× stock, was added. Homogenization was achieved by periodically vortexing each tube as the plant tissue thawed on ice.

The homogenized material was transferred to a chilled microcentrifuge tube containing 600µL phenol (pH 4) and mixed immediately by vortexing for 10sec. Tubes were then centrifuged for 10min at 16,000 × *g*, 4°C to separate plant debris from the supernatant. The upper phase was transferred to a fresh tube on ice, containing 600µL of 25:24:1 phenol:chloroform:isoamyl alcohol and centrifuged as before. The upper phase was transferred to a fresh tube containing 500µL chloroform:isoamyl alcohol, on ice. Tubes were vortexed for 10sec to mix and centrifuged for 5 min 16,000 × *g*, 4°C. The upper phase was transferred to a sterile tube, where the total nucleic acid fraction was precipitated by the addition of 40µL 4M sodium acetate pH 5.2, 800µL absolute ethanol, and 1µL GlycoBlue™. This was mixed by inversion and incubated for 15 min on ice or stored overnight at -20°C. The TNA was recovered from solution by centrifugation for 15 min at 16,000 × *g*, 4°C. The supernatant was removed by aspiration. To remove residual salts, the pellet was rinsed with 80% ethanol and immediately centrifuged for 5min at 16,000 × *g*, 4°C. The ethanol was removed by aspiration and the pellet was allowed to dry at room temperature for 10min. The TNA pellet was resuspended in 30-50µL RNase-free water, on ice, and stored at -80°C.

Total RNA yield was estimated by nanodrop. RNA quality was checked on a 1% TBE agarose gel. The gel was loaded with approximately 1µg TNA extract denatured for 5min at 65°C with an equal volume of 2× RNA loading buffer (formamide, 10mM EDTA, pH 8).

To remove contaminating genomic DNA, TNA was treated with DNase using the Ambion DNA-free™ kit. 1-2µg TNA was incubated at 37°C for 30 min with 2U rDNaseI in DNase I Buffer. The DNase was inactivated using a 1:5 volume of DNase Inactivation Reagent and incubating for 2min at room temperature, mixing 2-3 times. To remove the DNase enzyme, the tubes were centrifuged at 10,000 × *g* for 1.5min. The supernatant containing the RNA was transferred to a fresh tube.

cDNA was synthesised from RNA using the High Capacity cDNA Synthesis kit (Invitrogen). Kit components were thawed on ice. <2µg RNA was added to a PCR tube containing 1×RT buffer, 4mM dNTPs, 1×Random primers, and 5U MultiScribe Reverse Transcriptase, on ice, and mixed by pipetting. A brief centrifuge collected the contents and then the tubes were transferred to a thermocycler. For reverse transcription, the thermocycler was set to 25°C for 10mins, 37°C for 120mins, 85°C for 5mins. cDNA was diluted 10× in distilled deionised water.

2.4.2 Semi-quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed using SYBR Green Jump-start™ *Taq* Ready-Mix™ (Sigma-Aldrich S5193). Primers were designed to span 80-150bp regions of DNA, preferably spanning intronic sequence (Table 2.5), using AtRTPrimer (http://pombe.kaist.ac.kr/blan/genoPP.pl) or Primer3web (http://primer3.ut.ee/). A 15µL solution of SYBR ready-mix containing 3.3µM forward and reverse primers and 0.01% ROX reference dye was pipetted onto 1µL cDNA or no DNA (control) in wells of a clear 96-well Non-Skirted PCR plate (STARLAB) on ice, and sealed with a Microseal 'B' Adhesive seal (Bio-Rad MSB-1001). 2-3 technical replicates using the same sample of cDNA were used to identify and negate pipetting error. The plate was spun briefly to 2000rpm in a 3K15 centrifuge (Sigma, Germany) and placed inside a Mx3005P™ Multiplex Quantitative PCR System (Stratagene, U.S.A. #401455) qPCR machine running MxPro™ software (Mx3005P v 4.10, Stratagene, U.S.A.). The thermocycler was set to 94°C for 2min, followed by 40 cycles of 94°C for 15sec, and X°C for 1min/kb. The annealing temperature X was 5°C below the lowest primer T_m as calculated using Sigma OligoEvaluator ™ (http://www.oligoevaluator.com).

Table 2.5 Primers used for qRT-PCR

Target	Target ID	F primer sequence	R primer sequence
ACT2	AT3G18780	GGTAACATTGTGCTCAGTGGTGG	GGTGCAACGACCTTAATCTTCAT
UBQ5	AT3G62250	AATCGACGCTTCATCTCGTCC	GCTTGTGCTTGATCTTCTTCGGC
IND	AT4G00120	GAACCGCCGTAACGTAAGGA	AAGCTGTGTCCATCTTCGCA
SWT15	AT5G13170	CGTGGCTCGTGTGATAAAGACAAAG	CCACCACGTTTGGAATCGCTATG
SAG12	AT5G45890	GGCGGCTTGACAACTGAGTCAAA	GCCACTGCCTTCATCAGTGCTT
AtNAC3	AT3G15500	GCCACTGCCTTCATCAGTGCTT	GCCACTGCCTTCATCAGTGCTT
ORE1	AT5G39610	TTGAAAATCTTCCCCAAACAGCT	GAACCTTTGTACCATCGGCAC
AGO10	AT5G43810	CCTTTGTAGCCATGCGGGTATTCA	TGCACCGCGCATAGGTATAACAG

2.4.3 qRT-PCR analysis

Amplification plots and product melting curves were viewed in MxPro to identify and exclude from further analysis any reactions that might have amplified more than one product (confirmed by agarose gel electrophoresis). Threshold fluorescence was computed by the software, and was

consistent for each primer set. Cycles to threshold fluorescence (C_t) values were compared using Excel (Microsoft, U.S.A.). The change in C_t (ΔC_t) was calculated for each biological replicate, averaging 2-3 technical replicates and subtracting the C_t value of reference genes (an average of ACT2 and UBQ5 C_t values) for the same cDNA sample. Statistical analyses were performed on untransformed ΔC_t values by the stated statistical test.

2.5 Light microscopy

To score dark development phenotypes, a STEMI DV4 dissecting microscope (ZEISS, Germany) fitted with a 10W bulb, was used.

Light microscopy for imaging and fluorescence microscopy for phenotypic selection were performed using a M165 FC Stereomicroscope (LEICA, Germany) fitted with a DFC450 C camera (LEICA, Germany), running Leica Application Suite X (LAS X) software version 2.0.0.14332.

2.6 Dataset analysis

Transcriptome datasets were mined for gene expression in the SAM. Yadav et al., (2009) produced a microarray dataset from light-grown seedlings, where cells expressing cell-type specific fluorescent reporters were protoplasted and sorted to measure gene expression by cell type. Lopez-Juez et al., (2008) dissected SAMs grown in darkness as well as SAMs transferred to light, and measured gene expression by microarray. This dataset was used to search for gene expression in dark-grown SAMs.

A phosphorylatome dataset (Hou et al., 2017) was used to identify phosphorylation changes following eBL treatment in rice. Phosphorylation intensities are averages of three biological replicates, as reported by the author.

Where results are derived from published datasets, the author's normalisation and statistical criteria are used, but the presentation of the results is new, and the specific analysis of ARP2/3 and WAVE complex genes is original.

2.7 Graphs and statistical analyses

All data were initially collected in Microsoft Excel. Graphs and statistical analyses were generated using Graphpad Prism v7. All statistical analyses are noted in the figure legends. Where *p* values are given, *<0.05, **<0.01, ***<0.001. Heat maps were generated using Morpheus (Broad

Institute; software.broadinstitute.org/morpheus/). DNA sequencing results were visualised in SnapGene Viewer 4.1.9 (GSL Biotech LLC).

Mutants of the *ARP2/3* complex and upstream *WAVE* complex were shown to have increased SAM activity in the dark (Baier et al., 2004; Li et al., 2004b; Zhang et al., 2008). The ARP2/3 complex is known to nucleate actin branches in plants (Maisch et al., 2009), and has also been shown to mediate the dissipation of F-actin bundles, endocytosis and autophagy (Li et al., 2004b; Zou et al., 2016; Wang et al., 2016). But despite the strong dark development phenotype of *ARP2/3* complex mutants, the role of the ARP2/3 complex in regulating SAM activity has not been investigated. Moreover, the role of other actin modifying genes in regulating dark development is not known, so the specificity of the ARP2/3 complex in regulating SAM activity, over other actin modifying proteins, should be investigated. Various mutants of the *ARP2/3* complex have been described, with characteristic distorted trichomes (El-Assal et al, 2004a; Mathur et al., 2003a; Mathur et al., 2003b). Additionally, chemical inhibitors of Arp2/3 complexes of non-plant species are available, but whether these inhibitors also target plant ARP2/3 complexes remains to be studied. A specific inhibitor of the ARP2/3 complex would greatly facilitate studies of the ARP2/3 complex in plants, as existing genetic resources could be utilised without the timely crossing in of *ARP2/3* complex mutants.

The ARP2/3 complex is one of several actin modifying protein complexes/proteins in Arabidopsis. Formin homology and villin genes are two other classes of F-actin nucleators (Kovar and Pollard, 2004; Zhai et al., 2001). Unlike the ARP2/3 complex which nucleates F-actin branches from existing filaments (Mullins et al., 1998a), formin homology proteins and villins nucleate F-actin *de novo* (Kovar and Pollard, 2004; Zhai et al., 2001). Villins have multiple actin modifying functions besides nucleation, including capping to limit the extension of F-actin (Walsh et al., 1984b), severing (Walsh et al., 1984a; Kumar and Khurana, 2004), and bundling F-actin (George et al., 2007). The activity of villins depends on various factors including the relative concentrations of actin, villin, calcium, phosphatidylinositol 4,5-bisphosphate, and post translational modifications (Walsh et al., 1984a; Walsh et al., 1984b; Kumar and Khurana, 2004; Kumar et al., 2004). Profilins facilitate F-actin nucleation by catalysing the renewal of ATP-bound globular actin monomers (ATP-G-actin) from expended ADP-G-actin for F-actin elongation (Goldschmidt-Clermont et al. 1992). Fimbrins cross-link actin filaments to generate dense actin bundles (Bretscher, 1981). THRUMIN1 is another actin bundling protein required for the light-responsive movement of chloroplasts (Whippo et al., 2011). By contrast, the family of actin depolymerisation factors, as their name suggests, in general promote cytoskeleton disassembly through the severing of actin filaments and bundles (Andrianantoandro and Pollard, 2006). A targetted analysis of the expression of actin modifying genes in the SAM is lacking, although transcriptomic data is available for such an analysis (Yadav et al., 2009). An analysis of actin modifying gene expression in the SAM could provide insight into the actin-related genes

and processes important in SAM tissues. Additionally, the involvement of other actin modifying genes in dark development should be investigated to ascertain the specificity of ARP2/3 complex signalling in regulating leaf initiation.

To study the role of the ARP2/3 complex in dark development ideally requires a robust assay that measures the output of the SAM, i.e. leaf initiation. Most assays of dark development only go as far as to measure the presence or absence of an apical hook (Hou et al., 1993), or first true leaves (Baier et al., 2004; Reed et al., 1998), after a timepoint of interest. This binary scoring system has the advantage of short growth periods (up to two weeks), but provides limited scope for measuring differences in SAM activity under different treatments. Quantitative assays of dark development have also been used to measure the rate of true leaf emergence in the dark between different Arabidopsis accessions (Chin-Atkins et al. 1996; Roldan et al., 1999). Roldan and colleagues averaged and plotted true leaf number to form a sigmoidal curve of development over time, providing a greater depth of resolution for measuring differences in leaf production between timepoints, accessions or treatments. A number of studies have also utilised a dim dark green "safe light" to avoid the activation of photoreceptors during dark development, allowing treatments or transfers to be performed during dark development (Nambara et al., 1995; Peters et al., 1998); however, the specific light filter is often not reported.

Mutants of the ARP2/3 complex have increased development in the dark but not in the light (Baier et al., 2004), however, the reason for this light sensitivity is not known. Furthermore, whether *ARP2/3* mutants have increased development in low light or shade has not been investigated. Dark morphogenesis has previously been likened to the morphogenesis of light-grown double and triple phytochrome mutants (Roldan et al., 1999). Phytochrome PhyB is the dominant red light receptor in Arabidopsis (Chory et al., 1989b; Reed et al., 1993), and *phyB* mutants have been described as having a constitutive shade avoidance response (Devlin et al., 1992), with reduced chlorophyll, elongated petioles, increased hyponastic growth, and early flowering (Chory et al., 1989b; Koornneef et al., 1980; Reed et al., 1993). One study demonstrated that the PhyA and PhyB and the cryptochrome Cry1 regulate the stability of the ARP2/3 activator SCAR1 in response to far red, red, and blue light, respectively (Dyachok et al., 2011). Another study showed that dark-grown *arp3* seedlings had enhanced phototropism responses to blue and red light (Reboulet et al., 2010). This is the extent to the knowledge about the ARP2/3 complex and light signalling, therefore, the relationship between the ARP2/3 complex and light signalling pathways needs further investigation.

This chapter aims to characterise the effect of an *arp3* mutant on SAM activity in the dark utilising a modified dark development assay, and investigate whether the *arp3* mutant also affects SAM activity during a constitutive shade response. The role of the ARP2/3 complex in dark

development over other actin modifying genes is also investigated, and chemical means of inhibiting the ARP2/3 complex are trialled to offer alternative means to study the role of the ARP2/3 complex (than time-consuming classical genetics approaches). In order to achieve these aims, this chapter also decribes the optimization of a quantitative assay of dark development, including a safe light filter for working in the dark (e.g. sterile dark transfers, in cases where seeds cannot be germinated on media containing a particular treatment).

3.1 Measurement of SAM activity

SAM activity can be measured by the developmental output of the SAM, i.e. the rate of organ initiation. Mutants of the ARP2/3 actin nucleating complex have increased SAM activity in the dark (Baier et al., 2004). To study the role of the actin in SAM activity, a dark development assay was developed. The dark development assay measures shoot development after four weeks in the dark, grown upright on media containing nutrients, sugar as a carbon and energy source, and relevant treatment, as detailed in chapter 2.1.2. Shoot development was measured using a quantitative scoring system, based primarily on the number of true leaves developed (Fig. 3.1). It was found that Col-0 plants consistently grew to stage 3-5 on average, whilst *arp3* plants were typically two stages more advanced than Col-0 (see for example Fig. 3.3-3.4). Plants were able to reach developmental stages of 9-11 before clearly bolting (data not shown). To allow for decreases as well as increases in development, growth was factored in the scoring system at earlier stages of development, providing increased resolution on the earlier stages of shoot development (Fig. 3.1).

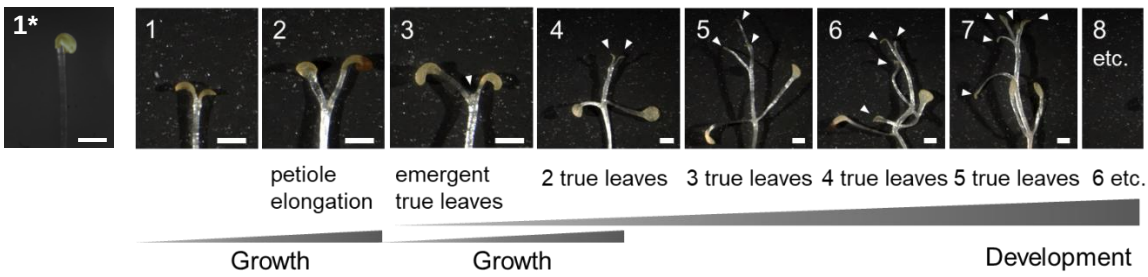


Fig. 3.1. Dark development scoring system. Developmental stage in top left corner of each image. White triangles indicate true leaves. Stage 1: no true leaves, no extension of cotyledon petioles (1* shows a stage 1 plant grown without sugar; an apical hook remains). Stage 2: no true leaves, extension of one or both cotyledon petioles. Stage 3: the emergent SAM and/or first true leaves are visible, but not elongated. Stage 4: 2 true leaves, elongated. Stages 5 onwards: additional true leaves visible. Therefore, the differences observed between stages 1 and 2 and between 3 and 4 are growth-related, but the differences between stages 2 and 3, and between 4 and higher are developmental. Scale bars = 1mm.

3.1.1 Development of a dark development assay

In order to express developmental scores as averages, the developmental scoring system needed to produce data that were consistent with a Gaussian distribution. Of particular concern was the integration of growth and developmental criteria in the scoring system (see 3.1 above, Fig 3.1). To demonstrate that the developmental scoring system did not skew the data, the results of 178 treatments or genotypes (most of the earlier work recorded in this thesis) were analysed for Gaussian distribution using the recommended test in GraphPad Prism v7, the D'Agostino-Pearson omnibus K2 test, which accounts for skewness.

Seventy percent of results showed Gaussian distribution (Fig. 3.2), which was not dependent on genotype, or treatment, or average developmental scores greater than 2, indicating that the developmental scoring system is a valid method of measuring development. Over sixty percent of results that did not show Gaussian distribution were averages of predominantly one or two developmental scores, which was more of an indication that the average developmental scores had low deviation from the mean. Because the developmental scores showed Gaussian distribution, dark development could be represented as averages with standard error, and standard statistical analyses performed (Fig. 3.3).

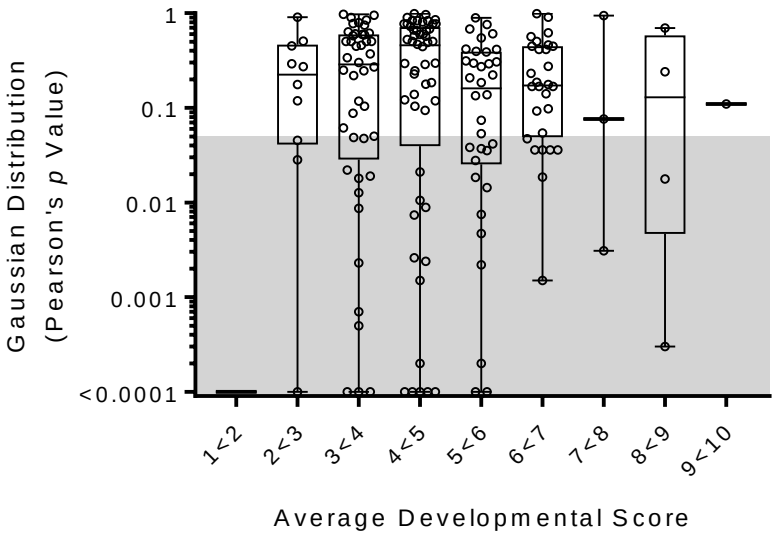


Fig. 3.2. Averaged developmental scores showed Gaussian distribution 70% of the time (unshaded area), except when developmental scores averaged below 2. Dark development data from 178 individual treatments or genotypes were analysed for Gaussian distribution by D'Agostino-Pearson omnibus K2 test; *p* values were plotted against average developmental score (binned) on a scatter and box plot, where whiskers indicate range. Average developmental scores with a Pearson's *p* value <0.05 did not show Gaussian distribution (shaded area).

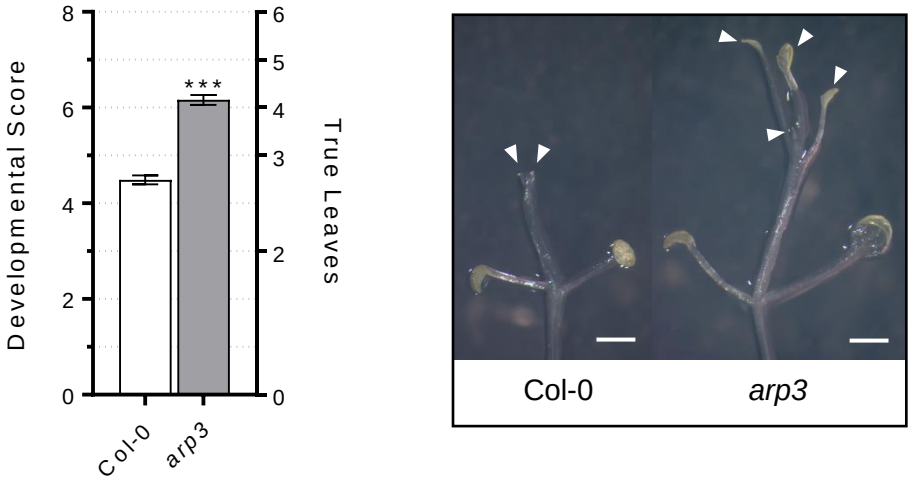


Fig. 3.3. The *arp3* mutant has increased dark development. (**A**) Average developmental scores are shown with standard error. Statistical significance calculated by unpaired two-tailed t-test; $n > 50$. (**B**) Representative photographs of Col-0 and *arp3* seedlings after 28 days dark development on 1/2MS supplemented with 0.8% plant agar and 0.2% glucose. White triangles indicate true leaves corresponding to developmental scores of 4 (Col-0) and 6 (*arp3*). Scale bar = 1mm.

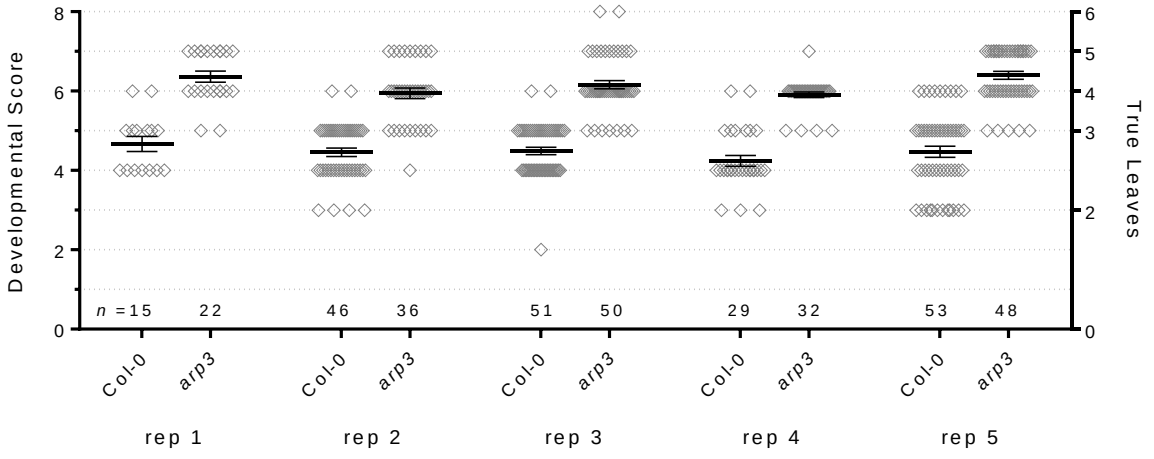


Fig. 3.4. Distribution of data points between 5 independent experiments (reps). *n* is number of data points (diamonds) per experiment, corresponding to individual plants / biological replicates. Average developmental score and standard error are overlaid in black. There is significant variation between experiments (1.5%; $p=0.01$), and between genotypes (47%; $p<0.0001$), but no significant interaction between experimental and genotypic variation by 2way ANOVA; *n* numbers specified in figure.

3.1.2 Optimization of sugar concentration

In line with other literature (Li et al., 2017; Roldan et al., 1999), it was found that dark development required sugar (Fig. 3.5). Glucose or sucrose specifically stimulated true leaf development at concentrations above 0.02%. To control for changes in osmotic pressure, plants were also treated with the sugar analog mannitol, which did not stimulate dark development. In further dark development assays, 0.2% glucose was used to maximise the developmental stages between first true leaf development (developmental scores 3 and 4) and bolting (developmental scores 9-11). Col-0 and L*er* had similar responses to sugars, but the dark development of L*er* was slightly increased compared to Col-0, which was highly significant on 0.2% sucrose (Fig. 3.5).

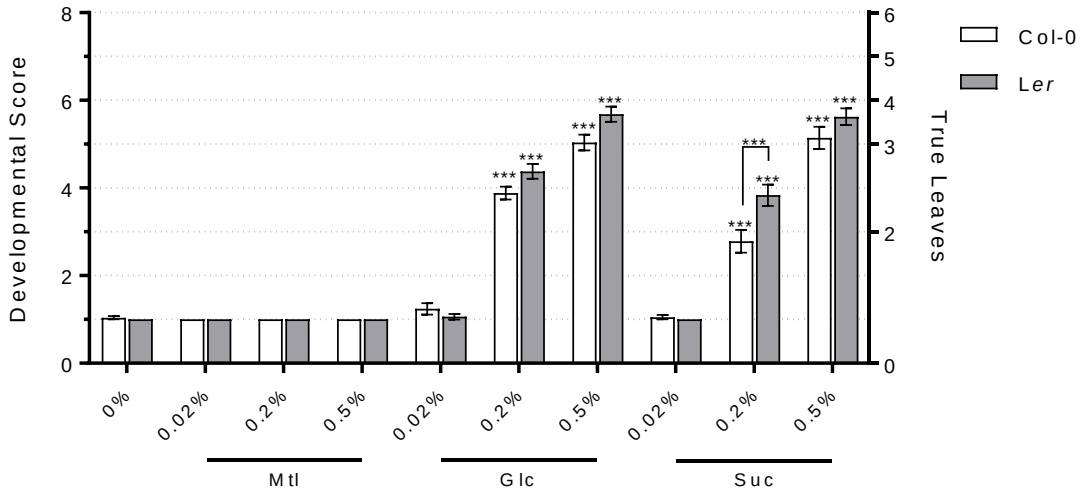


Fig. 3.5. Sugar is required for true leaf development in the dark. Seedlings were germinated on vertical 1/2MS agar plates supplemented with glucose (Glc), sucrose (Suc), or the non-metabolizable sugar analog mannitol (Mtl) in the dark. True leaf development was scored after 28 days at 23°C. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; $n > 14$.

3.1.3 Increasing assay robustness

A germination control plate was included for each experiment, which was unwrapped three days after sowing. Seed stocks that germinated poorly were noted, fresh seed stocks were made, and the experiment repeated. Alternatively, germinated seedlings were marked under a dark green light (see chapter 3.1.4) 3 days post sowing.

An effective drainage system was developed to collect excess liquid, presumed to be water produced by respiration, and prevent it from soaking back through the micropore tape, which often led to infection. Before foil-wrapping, stacks of vertical experimental plates were mounted

onto 1cm deep empty petri dishes on top of a stack of tissue paper. This markedly reduced the incidence of infection.

To address concerns that humidity or the lack of air circulation caused a build-up of the plant hormones ABA and ethylene, plants were treated with ABA and ACC (an ethylene precursor). Plants were responsive to both hormones, suggesting that under these experimental conditions, ABA and ethylene are not saturating (Fig. 5.1A,C of chapter 5).

3.1.4 Dark sterile plate transfers

For certain treatments, to avoid inhibiting germination or early SAM development, or where germination frequency was low, it was necessary to germinate seeds days prior to treatment. Seeds were germinated on untreated media 3 days (in the case of germination frequency defective seed stocks) or 5 days (in the case of inhibiting treatments) prior to dark sterile transfer onto mock or treated media. To maintain darkness during seedling transfer, a light filter was selected, that transmitted a low fluence of light at wavelengths with low perception by plant phytochromes (Fig. 3.6 A, B), and fitted to a laminar flow hood in a dark room. Brief exposure to the light filter (around 30 minutes) during dark development had no apparent effect on the morphology or development of 4 week old dark-grown seedlings (Fig. 3.6 C).

3.2 ARP2/3 complex specific regulation of SAM activity

3.2.1 ARP2/3 complex-related genes are expressed in the SAM

To investigate the tissue specific expression of ARP2/3 related genes in the SAM, microarray data from protoplasted cells was analysed (Fig. 3.7). Expression was strongest in the rib meristem, however, the expression of different components was not equal. *ARP3* and *ARPC2A* had the strongest expression, whilst *ARPC3* and *ARPC5* had relatively low expression. Mutant analyses have shown that ARP2/3 complex components are not redundant, such that a single mutant in any one component results in knockdown of the ARP2/3 complex as a whole (Mathur et al., 2003a). Therefore, ARPC3/ARPC5 expression might be limiting for ARP2/3 complex activity in the SAM, providing potential for dynamic regulation of the whole complex. Moreover, the upstream activators SCAR1-4, BRK1, NAP1, PIR, are also largely non-redundant (Deeks et al., 2004; Le et al., 2006; Li et al., 2004b; Zhang et al., 2008; Zhang et al., 2005). Other than *SCAR1*, each component of the WAVE complex was well expressed in the SAM. The expression of *ARPC2B*, *ARPC4*, *SCAR3*, *ABIL2* was not measured, as the microarray did not have probes for these genes.

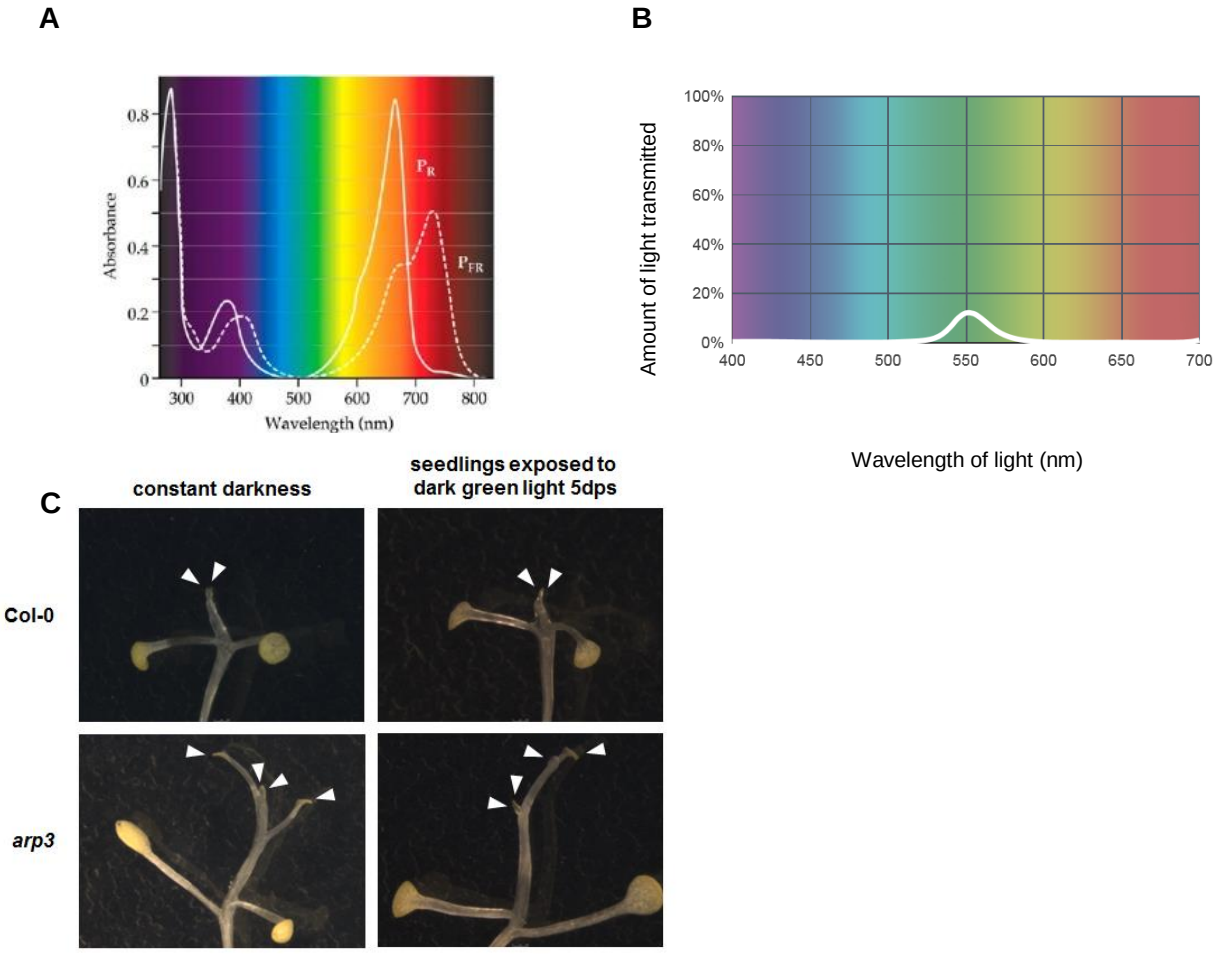


Fig. 3.6. Selection of filter for working by darkness. (**A**) Wavelengths of light absorbed by plant phytochromes (photobiology.info/). (**B**) Light transmitted by the filter 740 Aurora Borealis Green (LEE Filters, U.S.A.). (**C**) Representative images of four week old seedlings maintained in constant darkness, or exposed to approximately 30 minutes of dark green light at 5 days post sowing (dps; using the 740 Aurora Borealis Green filter), and transferred back to constant darkness. White triangles indicate true leaves; images to scale.

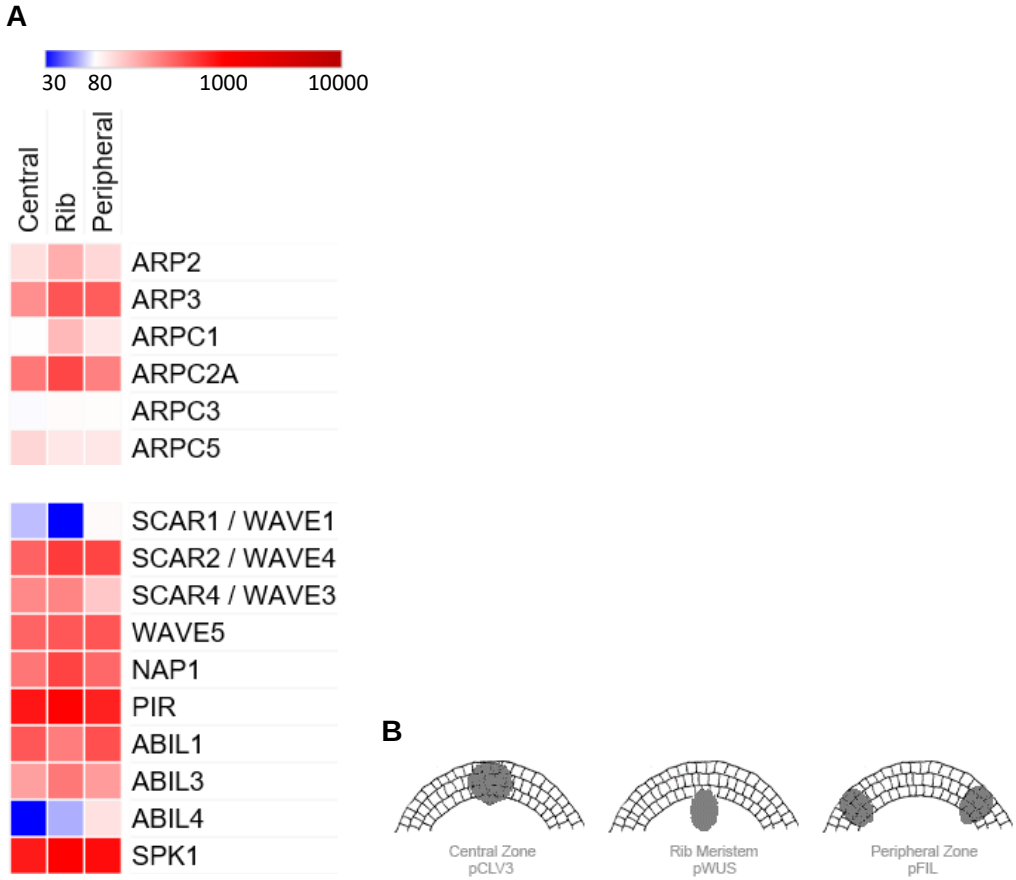


Fig. 3.7. Expression of *ARP2/3* complex genes and upstream regulators from protoplasted cells, sorted by shoot apical meristem-specific cell type, adapted from Yadav et al., 2009. (**A**) Heat map showing gene expression in the central zone, rib meristem and peripheral zone of the shoot apical meristem (**B**). (**A**) The heat map was generated in Morpheus (Broad Institute; software.broadinstitute.org/morpheus/), showing GCOS/MAS5 normalised microarray data (Winter et al., 2007; Yadav et al., 2009) for genes of interest from a large dataset. Cells from the Central, Rib Meristem, and Peripheral Zones were sorted by the expression of fluorescent reporters expressed under the respective *pCLV3*, *pWUS* and *pFIL* cell-type specific promoters (Yadav et al., 2009). Images of corresponding SAM regions (**B**) were downloaded from eFP Browser (Winter et al., 2007).

3.2.2 Increased dark development is a specific trait of ARP2/3 complex-related mutants

To assess the specificity of the ARP2/3 complex in regulating dark development, a screen was performed using a variety of actin-related mutants (Fig. 3.9). Mutants were chosen based on their expression in the SAM (Fig. 3.8) and reported phenotypes. Of the VILLIN family, *VLN1* was the only gene to be expressed highly in the SAM, so the mutant would be unlikely to have redundant function. The *vln1-4* mutant was demonstrated to be null for *VLN1*, but *vln1* phenotypes have not been studied to date (Khurana et al., 2010). Other *VILLIN* mutants have reduced F-actin severing, reduced thick actin bundles, and reduced growth (Huang et al., 2015b). VLN1 protein promotes F-actin bundling and severing *in vitro* (Huang et al., 2005; Khurana et al., 2010). Arabidopsis has over twenty FORMIN HOMOLOGY genes and several were highly expressed in the SAM. The *fh1-1* mutant was shown to have thicker and more frequent actin bundles, increased root diameter, and hypersensitivity to the effects of LatB on root growth (Rosero et al., 2013). Three PROFILIN genes were expressed highly in the SAM. PRF1 and PRF2 have partially redundant activity (Mussar et al., 2015), but the single *prf1-2* mutant reduced actin nucleation, bundling, and rate of filament elongation, and reduced axial cell expansion (Cao et al., 2016), and the single *prf2-1* mutant reduced leaf size and plant height (Mussar et al., 2015). The constitutively expressed *35S::GFP-mTalin* line was included as it has been reported to disrupt the activity of actin depolymerising factors and increase actin bundling (Ketelaar et al., 2004; Sheahan et al., 2004).

Mutants of the ARP2/3 complex, *arp3* and *arpc2a* increased dark development, as did the ARP2/3 complex activator mutant *scar2* (Fig. 3.9), consistent with previous reports (Baier et al., 2004; Zhang et al., 2008). The actin bundling line *35S::GFP-mTalin* also increased dark development, however other actin-related mutants *vln1*, *fh1*, *prf1* and *prf2* did not (Fig. 3.9). Despite previously reported phenotypes for each of these mutants, redundancy within the respective gene families cannot be excluded, except perhaps for *vln1*, as other *VILLIN* genes were not highly expressed in the SAM.

3.3 Identification of Arabidopsis ARP2/3 complex inhibitors

There are many genetic resources for Arabidopsis which could be utilized to study different biological signalling pathways involved in the increased dark development of *ARP2/3* mutants. However, to cross each (or multiple) of these mutants into an *ARP2/3* mutant background would be a time-expensive process. A chemical inhibitor of the ARP2/3 complex would circumvent this problem as it could be applied to existing genetic variants. Whilst a couple of chemical Arp2/3 inhibitors have been described for other organisms, none have yet been described for the Arabidopsis ARP2/3 complex. Plants were treated with the Arp2/3 complex inhibitor CK-666 and

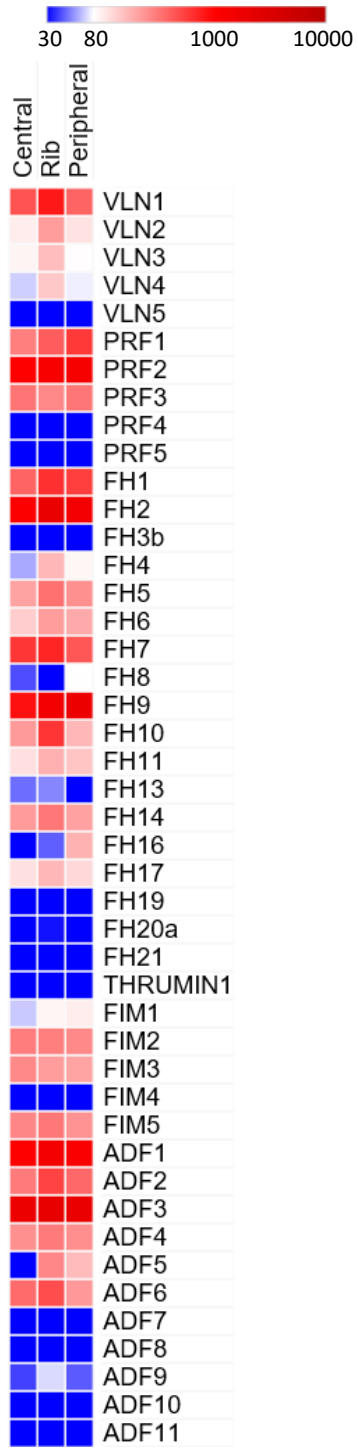


Fig. 3.8. Expression of actin-related genes in the shoot apical meristem (Yadav et al., 2009). *VLN1* was the most highly expressed *VILLIN* gene in the SAM; *FH1*, *FH2*, *FH7* and *FH9* were the most highly expressed *FORMIN HOMOLOGY* genes; *PRF1*, *PRF2* and *PRF3* were the most highly expressed *PROFILIN* genes; *FIM2*, *FIM3* and *FIM5* were the most highly expressed *FIMBRIN* genes, but were not as highly expressed as the other families. *THRUMIN1* was not well expressed in the SAM. *ADF1*, *ADF2*, *ADF3* were the most expressed *ACTIN DEPOLYMERISATION FACTOR* genes. Microarray analysis and heat map generation as Fig 3.7.

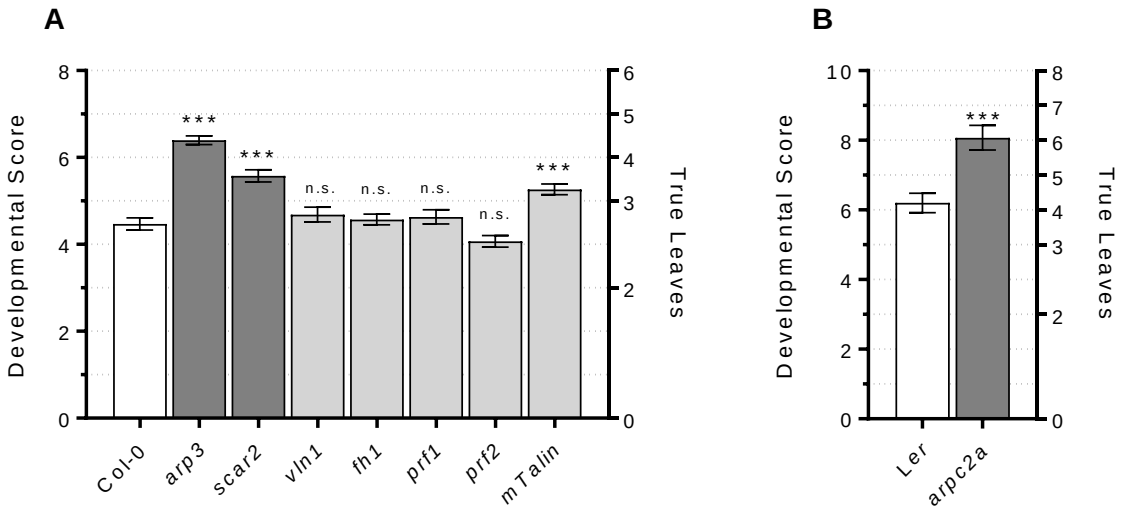
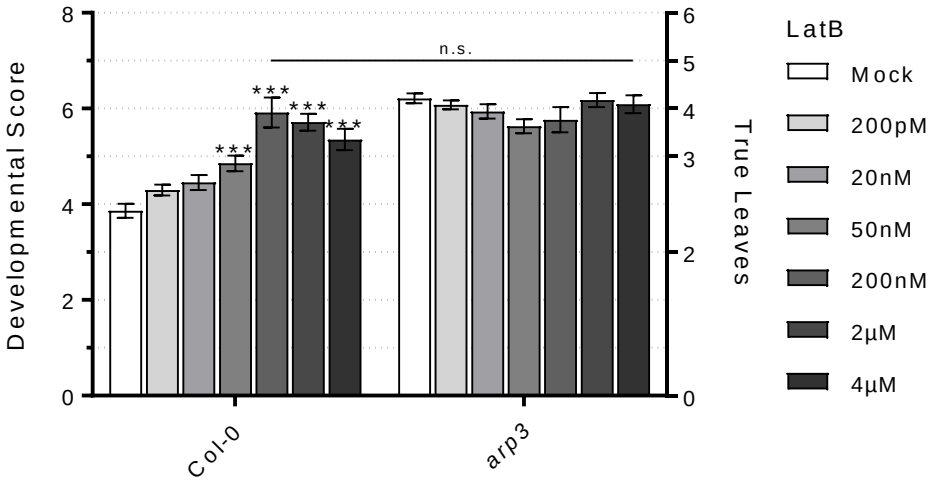


Fig. 3.9. Actin-related mutants have unaffected dark development with the exception of ARP2/3 complex related mutants (dark grey) and *35S::GFP-mTalin*, indicating the specific function of the ARP2/3 complex in regulating dark development. Statistical significance was calculated using 1way ANOVA and Dunnett's multiple comparisons test; $n \geq 45$ (**A**), or by unpaired two-tailed T-Test; $n \geq 15$ (**B**).

the F-actin polymerisation inhibitor Latrunculin B (LatB) to test for *arp3*-like phenotype induction.

3.3.1 Treatment of Arabidopsis with LatB increased dark development

Latrunculin B treatment increased dark development at concentrations above 50nM (Fig. 3.10). Other studies have shown that 50nM LatB is sufficient to cause F-actin fragmentation in pollen tubes, reducing the F-actin levels in different regions of pollen tubes by 30-60% after 30 minutes (Chang and Huang, 2015), and is 25% effective at inhibiting root hair elongation (Bibikova et al., 1999). Concentrations of LatB above 100nM increased the dark development of Col-0 plants to the same level as *arp3* mutants, whereas *arp3* was insensitive to LatB-induced dark development, suggesting that LatB and *arp3* increase dark development via the same mechanism. Both Col-0 and *arp3* were sensitive to LatB-induced dwarfism, forming compact rosettes in the dark (Fig. 3.10 B), a cell expansion defect resulting from the loss of F-actin (Baluska et al., 2001).



B

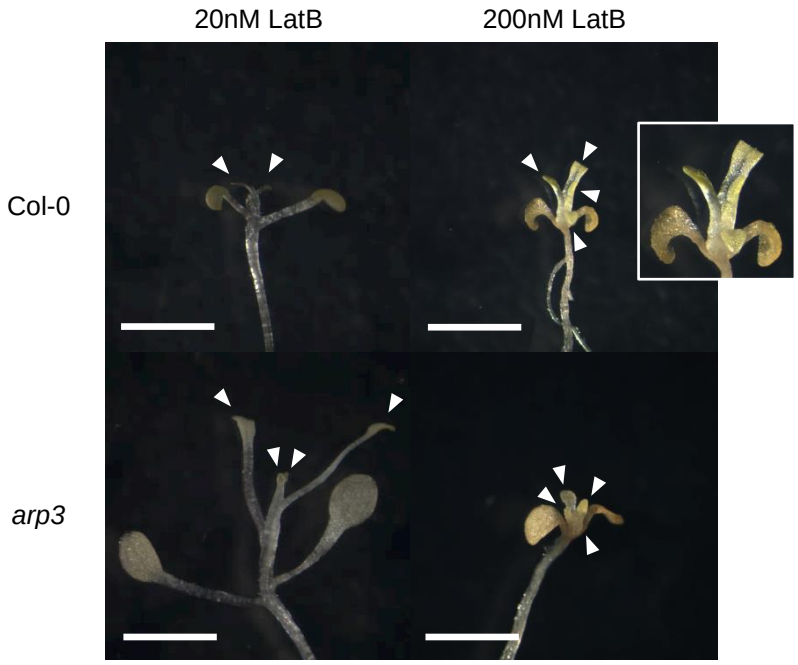


Fig. 3.10. The dark development of Col-0 plants was increased to the same level as *arp3* plants by inhibiting actin polymerisation with >50nM LatB; *arp3* dark development was insensitive to LatB treatment (**A**) but sensitive to LatB-induced dwarfism (**B**). A representative mock treatment is shown (0.001% v/v DMSO) which is the mock treatment for 200pM-200nM LatB. Mock treatments for 2µM (0.01% v/v DMSO) and 4µM (0.02% v/v DMSO) were not statistically different to the mock shown and do not affect the interpretation of the result (not shown). Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; $n > 12$. (**B**) Images were taken at different focal planes and rendered using Helicon Focus 6.1 (Helicon Soft Ltd.). Triangles indicate true leaves. Inset - enlarged image of Col-0 grown on 200nM LatB. Scale bar = 2mm.

3.3.2 Treatment of Arabidopsis with CK-666

CK-666 was shown to bind a pocket between Arp2 and Arp3 in the bovine Arp2/3 complex, blocking a conformational change required to activate the Arp2/3 complex (Baggett et al., 2012; Hetrick et al., 2013). To predict whether CK-666 could bind the equivalent region in the Arabidopsis ARP2/3 complex would require structural knowledge. Whilst the complete bovine ARP2/3 complex has been crystallised and its structure resolved (Baggett et al., 2012; Jurgenson and Pollard, 2015; Robinson et al., 2001), there is no crystal structure of the Arabidopsis ARP2/3 complex, and the nature of the binding pocket for CK-666, between Arp2 and Arp3 subunits, makes homology modelling particularly difficult. Programs such as SwissDock (swissdock.ch) that predict the energetic favourability of a ligand binding to a site within a protein are not currently designed to also predict the interactions between two proteins (i.e. Arp2 and Arp3 homology models) and give an accurate prediction of a ligand interaction at a binding site between them. Knockout mutants of the Arabidopsis ARP2/3 complex have distorted trichomes and increased dark development (as reviewed in chapter 1.5.3.3). Therefore, to test whether CK-666 is an effective inhibitor of the Arabidopsis ARP2/3 complex, the effects of CK-666 on trichome development and dark development were analysed.

To investigate whether CK-666 treatment distorted trichome development, Col-0 seedlings were grown on soil and treated by drop application onto the centre of the rosette with 20mM CK-666 (in 100% DMSO), 2mM CK-666 (in 10% DMSO), 200µM CK-666 (in 1% DMSO), or relevant mock treatment. 0.1% Tween20 was used as a surfactant to spread the treatment across a leaf. Existing and developing trichomes were examined morning and evening over the next few days, and compared with untreated Col-0 and *arp3* controls. Since no difference between mock and treated plants was seen, the experiment was repeated in liquid culture to increase the surface contact of the treatment. Liquid culture grown seedlings also failed to show distorted trichome phenotypes in response to 200µM CK-666 (1% DMSO), 20µM CK-666 (0.1% DMSO), 2µM CK-666 (0.01% DMSO) or 200nM CK-666 (0.001% DMSO).

CK-666 treatments also did not increase dark development. Neither Col-0 or L*er* plants had increased dark development when treated with CK-666 (Fig. 3.11). *arp3* and LatB treatment were included as positive controls. Therefore CK-666 was not suitable as an ARP2/3 complex inhibitor for Arabidopsis studies.

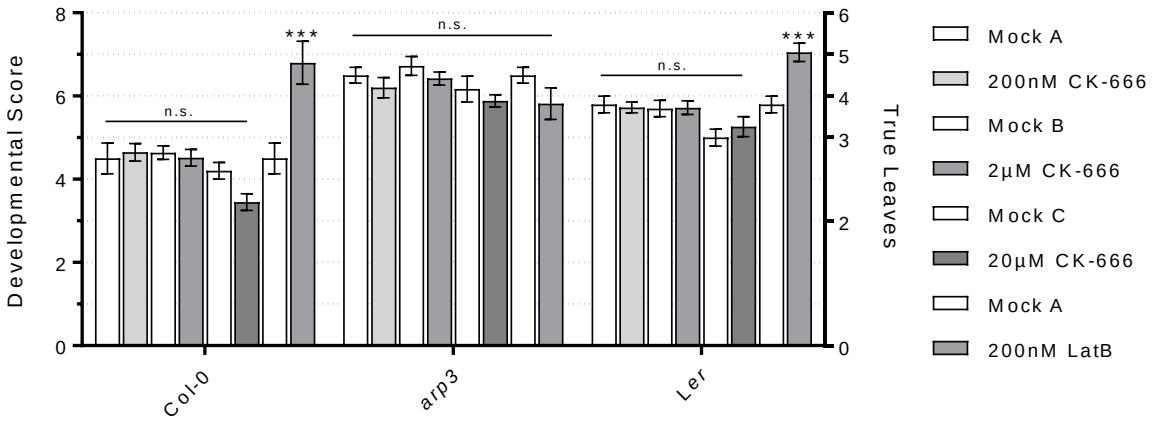


Fig. 3.11. The yeast and mammalian Arp2/3 complex inhibitor CK-666 did not increase or affect the dark development of Arabidopsis, indicating that CK-666 does not inhibit the Arabidopsis ARP2/3 complex. LatB treatment was included as a positive control for increased dark development. Mock treatments A = 0.001% DMSO; B = 0.01% DMSO; C = 0.1% DMSO. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; $n > 6$.

3.4 ARP2/3 signalling in constitutive shade reponses

To investigate the light sensitivity of the increased development of *ARP2/3* mutants, *arp3* was crossed with the photoreceptor mutant *phyB* to examine the effect of *arp3* on the constitutive shade avoidance response of *phyB*.

3.4.1 The *arp3* mutant accelerated the early flowering of *phyB* in long days

In long days, *arp3* and *phyB* mutants bolted after producing a similar number of rosette leaves to wild type (Fig. 3.12), although *phyB* plants trended towards bolting after producing fewer leaves, in line with other reports (Blazquez and Weigel, 1999; Goto et al., 1991). Col-0 produced on average 8.8 ± 0.4 (standard error) leaves, *arp3* produced 8.8 ± 0.3 leaves and *phyB* produced 7.8 ± 0.2 leaves. Strikingly, the *arp3phyB* double mutant bolted after producing only 4 ± 0.2 leaves. This was highly significant ($p<0.001$) and also observed in a second independent repeat.

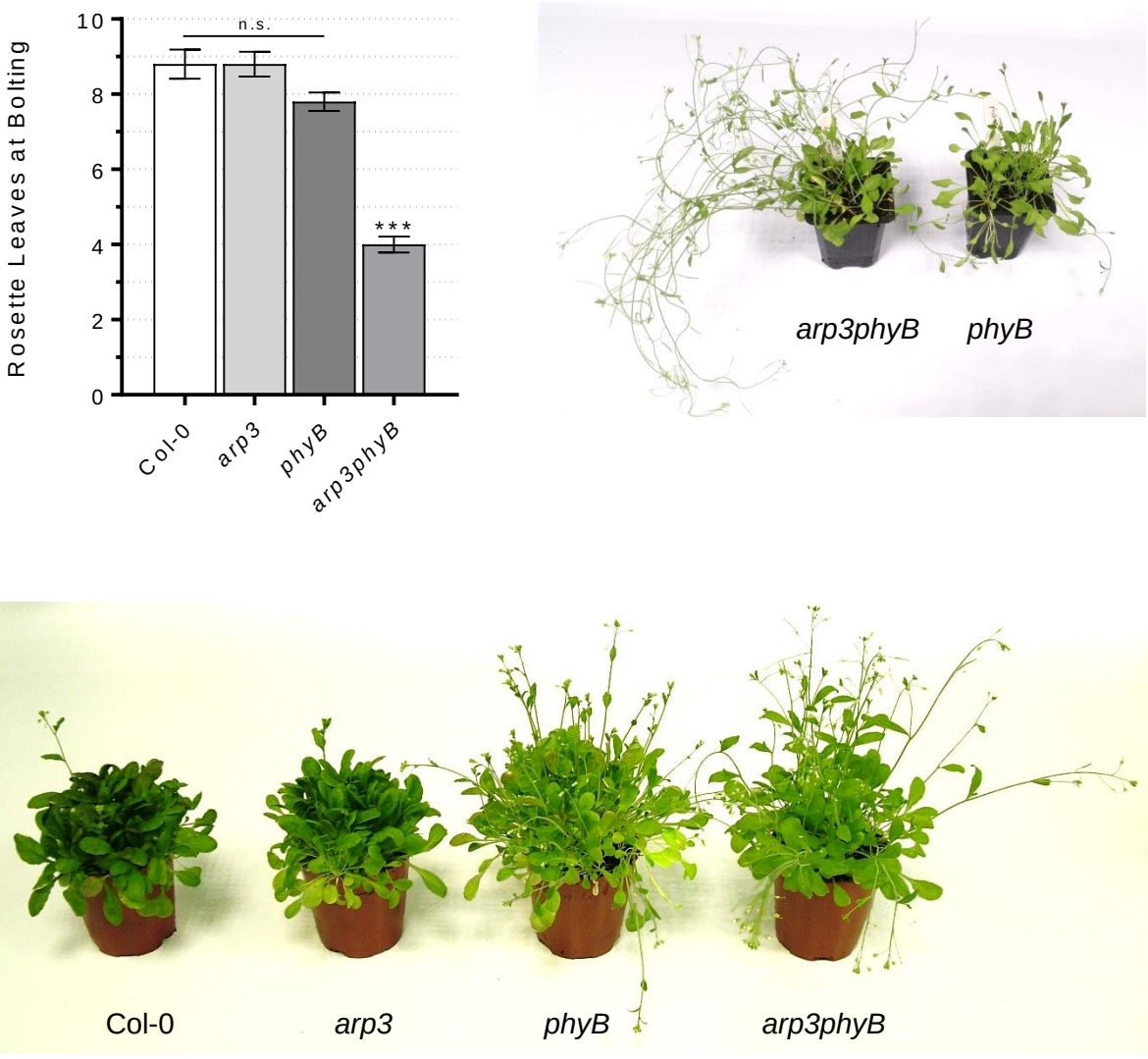


Fig. 3.12. *arp3* increased development in the light when *phyB* was also mutated. *arp3* development was indistinguishable from Col-0 when grown in long days; however, *arp3phyB* double mutants bolted after producing much fewer leaves. The *phyB* mutant trended towards bolting after fewer leaf production, consistent with other literature, but not to the extent of the double mutant. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; the sample size for each genotype $n = 10$.

3.5 Discussion

3.5.1 A dark development assay was developed to study dark-dependent regulators of shoot apical meristem activity. A scoring system based primarily on true leaf development (Fig. 3.1) gave a highly reproducible representation of dark development with Gaussian distribution (Fig. 3.2-3.4), allowing data to be represented as mean developmental stage and standard statistical analyses to

be used. Differences between average developmental stages between experiments might result from differences in incubation temperature; since the incubator lacked a cooling system, it was not possible to maintain a constant temperature of 23°C when the ambient room temperature increased above this level. Previously reported scoring systems for dark development have focussed more on growth and/or the presence or absence of true leaves (Baier et al., 2004; Reed et al., 1998), which provides less resolution of SAM activity, or measured the number of rosette leaves produced at bolting (Roldan et al., 1999), which is an indicator of a different kind of SAM activity. The scoring system used is most similar to a scoring system used by Roldan and colleagues (1999), who measured average true leaf number and standard error.

SAM activity in the dark required a sugar source, and was markedly increased by mutations of the ARP2/3 complex, as previously reported (Baier et al., 2004; Roldan et al., 1999). ARP2/3 genes as well as other actin-related genes are expressed in the SAM, but only mutants relating to ARP2/3 activity caused increased dark development (Fig. 3.7-3.9). An *mTalin* overexpressor was an exception, also causing increased dark development. Both *35S::GFP-mTalin* and mutants of the ARP2/3 complex are reported to increase actin bundling (Mathur et al., 2003a; Sheahan et al., 2004), whereas actin bundling was decreased in the *prf1* mutant (Cao et al., 2016) and might be expected to decrease in the *prf2* and *vln1* mutants. Therefore, the increased dark development could be attributed to increased actin bundling. However, the *fh1* mutant was also reported to have increased actin bundling (Rosero et al., 2013) but did not have increased dark development, and treatment with the actin-depolymerising drug LatB did not complement *arp3* dark development, as would be expected if actin bundling led to increased development. Instead, LatB increased the dark development of Col-0 to the same level as *arp3*. Moreover, the *arp3* mutant was insensitive to LatB-induced increased dark development, suggesting that *arp3* and LatB increase dark development by a common mode of action or pathway. It therefore seems likely that the observed increases in dark development are because of a loss of or interference with F-actin. Given that mutants of other actin nucleators (*fh1, vln1*) and nucleation facilitators (*prf1, prf2*) did not increase dark development, these data indicate that a specific subset of F-actin regulates dark development, mediated by the ARP2/3 complex.

3.5.2 Further study of the role of the ARP2/3 complex in SAM development would be greatly facilitated by a chemical inhibitor of the ARP2/3 complex. LatB treatment phenocopied the dark development of *arp3* but also caused unrelated effects owing to general loss of cellular F-actin, limiting its usefulness. Treatment with CK-666, reported to specifically inhibit the Arp2/3 complex in diverse organisms including yeast, algae, mammals, amphibians, and molluscs (Avasthi et al., 2014; Davison et al., 2016; Nolen et al., 2009; Sun et al., 2011; Yang et al., 2012),

did not induce phenotypes akin to *ARP2/3* mutants in Arabidopsis, suggesting that it does not inhibit the Arabidopsis ARP2/3 complex. Alternatively, CK-666 might be metabolised by the plant, or is not taken up to be sufficiently bioavailable. Therefore, in further analyses, a genetic approach was taken, where possible, to derive ARP2/3-specific functions.

3.5.3.1 Regulation of the ARP2/3 complex by light

Very little is known about the regulation of the ARP2/3 complex by light signalling. The stomatal aperture of *arp3* and related mutants is unresponsive to light/darkness, although this phenotype was shown to be caused by increased actin bundling, as it was rescued by actin polymerisation inhibitors (Isner et al., 2017; Jiang et al., 2012). SCAR1 was shown to be degraded by the proteasome in the dark through direct interaction with COP1, which was disrupted by phytochrome and cryptochrome signalling (Dyachok et al., 2011). Dyachok and colleagues proposed a simple model whereby light promotes ARP2/3 complex activity by inhibiting the COP1-mediated degradation of SCAR through the photoreceptors PhyA, PhyB and Cry1, while in the dark, SCAR is degraded by the proteasome, and the ARP2/3 complex is inactive (Dyachok et al., 2011). This model fitted their observation that the ARP2/3 complex promoted root development in the light, but did not explain their observation that the ARP2/3 complex repressed root development in the dark, as it assumed that the ARP2/3 complex was inactive in the dark. For the same reason, it does not explain why the *arp3* mutant has any phenotype in the dark. In their study, Dyachok and colleagues only tested the activity and COP1 association of SCAR1 in relation to light, therefore, since the ARP2/3 complex must be active in the dark, other SCARs must be able to compensate for the loss of SCAR1. Indeed SCAR1 was shown to have functional redundancy with SCAR3 (Zhang et al., 2008). Moreover, the *scar1scar3* double mutant, but not the *scar1* or *scar3* single mutants had increased dark development (Zhang et al., 2008), demonstrating not only that SCAR3 can compensate for the loss of SCAR1 in the dark, but that SCAR1 is not completely degraded in the dark. Therefore, the regulation of ARP2/3 complex activity by light is clearly more complex than off or on.

3.5.3.2 Co-regulation of flowering time by ARP3 and PHYB

To investigate whether light signalling through PhyB interfered with *arp3* developmental phenotypes, and to identify whether the ARP2/3 complex regulated development in the shade, *arp3* was crossed with a mutant of PhyB, which is the dominant red-light receptor of Arabidopsis (Chory et al., 1989b; Reed et al., 1993). Strikingly, the *arp3phyB* double mutant bolted after producing half as many leaves at wild-type (Fig. 3.12), making it a class I early flowering line (Glover, 2014), although chronologically, *arp3phyB* did not flower much earlier than the *phyB*

single mutant. Class I early flowering lines are distinct from class II early flowering lines, as they bolt after producing an average of 4 rosette leaves in long days, rather than 6 rosette leaves, and retain photoperiod-sensitivity (Glover, 2014). The requirement for both *arp3* and *phyB* mutations to increase the rate of development indicates that PhyB and ARP3 dependent signalling interact to regulate rosette leaf number and bolting. This is important for allowing the plant to develop enough photosynthetic tissue before expending its resources on reproductive development (Glover, 2014).

The *arp3phyB* double mutant resembled *35S::PIF4* lines, which also flower after producing just four rosette leaves, and have constitutive shade responses (Kumar et al., 2012). PIF4 is repressed by DELLA proteins in the light, but this repression is alleviated in the dark and in the red-light insensitive *phyB* mutant (Nozue et al., 2007). PIF4 is further stabilized by exogenous brassinolide in the dark, but not in the light (Bernardo-Garcia et al., 2014). Remarkably, the stabilization of PIF4 by brassinolide, caused by reduced phosphorylation, was shown to be mediated by the actin cytoskeleton (Bernardo-Garcia et al., 2014). It is conceivable then, that the combination of *phyB*, which alleviates repression by DELLAs, and *arp3*, which exhibits constitutive brassinosteroid responses (see chapter 5) could lead to synergistic stabilization of PIF4 in the *arp3phyB* double mutant, resulting in early flowering.

The *arp3phyB* double mutation appeared to reduce the frequency of rosette leaf initiation, opposite to the phenotype of *arp3* in the dark. A recent study showed that the rate of true leaf development stimulated by red light was compromised in the *phyAphyB* double mutant, while blue light-stimulated true leaf development was compromised in the *cry1cry2* double mutant (Li et al., 2017). Therefore, to investigate the signalling pathways which antagonise the positive effect of *arp3* on leaf initiation rate, it might be necessary to make *arp3phyAphyB* and *arp3cry1cry2* triple mutants.

Mutants of the ARP2/3 complex were identified in a high sugar response (hsr) screen (Baier et al., 2004). Mutants of *ARP3* and *ARPC2A* had increased sugar sensitivity compared to wild-type plants grown on low concentrations of sugars. Phenotypes included increased sugar-induced gene expression, and enhanced growth and development responses. For instance, *ARP2/3* mutants had elevated expression of the sugar-induced genes *ApL3* and *β-Amy*, reduced chlorophyll levels, increased anthocyanin accumulation, and increased dark development. Biochemical measurements of sugar levels showed that *ARP2/3* mutants had comparable levels of glucose, fructose, sucrose, total sugar and starch to wild type plants. Moreover, the uptake of C^{14} labelled glucose was comparable between wildtype and *ARP2/3* mutants, indicating that *ARP2/3* mutants do not have elevated sugar uptake.

Since the increased dark development of *ARP2/3* mutants is an elevated sugar response, seemingly independent of endogenous sugar levels and uptake (Baier et al., 2004), it is possible that the increased dark development results from increased sugar signalling. Sugars are perceived through glycolysis-dependent and independent sugar-signalling pathways (hereafter referred to as glycolysis and sugar signalling). Various sugar signalling pathways exist in plants, including TOR, HXK, and RGS1 signalling. The relationship between the ARP2/3 complex and glycolysis or sugar signalling pathways has not been explored, so an investigation targetting each sugar signalling pathway is required.

Glycolysis is the pathway in which sugars (glucose, and/or fructose) are converted into precursors for the biosynthesis of cell building materials, including polysaccharide, phospholipid and amino acid precursors, and provides pyruvate for downstream respiration, the primary energy production pathway in plants. Both the biosynthesis of cell wall building materials and the production of energy are important requirements for growth. Glycolysis has also been shown to activate plant meristems via the downstream kinase TARGET OF RAPAMYCIN (TOR) (Pfeiffer et al., 2016; Xiong et al., 2013; Xiong and Sheen, 2012), see chapter 1.3.4. Leaf initiation in the dark was blocked by estradiol (Est)-induced *TOR* knock-down using *XVE-TOR*-RNAi lines, and repression of TOR with the specific inhibitors rapamycin or torin2 (Li et al., 2017). Additionally leaf initiation was compromised in a double mutant of the TOR-targets *E2Fa*/*E2Fb* (Li et al., 2017). It is not known if the ARP2/3 complex regulates glycolysis or TOR kinase signalling, but the high sugar response and increased leaf initiation of *ARP2/3* complex mutants (Baier et al., 2004) indicate that glycolysis and/or TOR might be upregulated in an *ARP2/3* background.

It is possible that the ARP2/3 complex regulates glycolysis directly, by inhibiting the activity of aldolases. Fructose bisphosphate aldolases (FBA) are essential glycolytic enzymes that are inhibited in association with F-actin (Arnold and Pette, 1970). Release from F-actin promotes

aldolase activity and glycolysis (Arnold and Pette, 1970; Pagliaro and Taylor, 1992). The actin polymerisation inhibitor cytochalasin D also promoted free aldolase (Pagliaro and Taylor, 1992). Therefore, an *ARP2/3* mutant, which has reduced F-actin, could have increased aldolase activity and glycolysis.

Analysis of gene expression data (Yadav et al., 2014) shows that *FBA1/2/3/6/8* are highly expressed in the SAM, with *FBA8* the most highly expressed by almost an order of magnitude. However, in dark developed shoot apices (Lopez-Juez et al., 2008), *FBA2* had the highest expression, followed by *FBA8* (half as expressed), and *FBA1/3/6* were expressed at a sixth of the expression of *FBA2*. Therefore, if aldolases are important for leaf initiation, a *fba2* mutant might be expected to have the most significant dark development phenotype.

The best characterised sugar signalling pathway in Arabidopsis is mediated by HEXOKINASE1 (HXK1), which contributes to glycolysis by its catalytic activity, and sugar signalling by an independent and separable pathway (Moore et al., 2003). Serine117 was shown to be important for the catalytic activity of HXK1 but not its sugar signalling activity (Moore et al., 2003). Consequently, a *hxk1* mutant expressing $HXK1^{S117A}$ has been used to study the role of HXK1-mediated sugar signalling uncoupled from its catalytic activity (Moore et al., 2003). Arabidopsis has three hexokinases (Jang et al, 1997). Gene expression data (Lopez-Juez et al., 2008; Yadav et al., 2009) show that *HXK1* is the most highly expressed *HXK* in the SAM, although *HXK2* is also highly expressed. The glycolysis inhibitor 2-DG is a hexokinase inhibitor and can be used to overcome redundancy between hexokinases in functional studies (Klein and Stitt, 1998; Nakada and Wick, 1956). HXK1 has been demonstrated to bind F-actin (Balasubramanian et al., 2007). Whilst the functional significance of this interaction is not clear, the F-actin binding of HXK1 could provide a mechanism for interaction with ARP2/3 signalling.

RGS1 is another regulator of sugar signalling and a putative glucose receptor (Grigston et al., 2008), described in chapter 1.3.3. RGS1 activity is regulated by glucose and endocytosis (Grigston et al., 2008; Urano et al., 2012), and transcriptome data (Yadav et al., 2009) show that *RGS1* is well expressed in all SAM tissues. Whether the ARP2/3 complex interacts with RGS1 is not known; however, there is potential for the ARP2/3 complex to regulate RGS1 activity by mediating its subcellular localisation, since the ARP2/3 complex has been implicated in regulating endocytosis (Zou et al., 2016). Alternatively, the ARP2/3 complex could interact with RGS1 through potential undiscovered roles in sugar uptake, the implication being that RGS1 is a downstream regulator of SAM activity in ARP2/3 signalling.

This chapter aims to identify glycolysis or sugar signalling pathways involved in the high sugar response phenotype of *arp3*, which includes the increased dark development phenotype (Baier et al., 2004). Mutants in a broad range of known glycolysis and sugar signalling pathways were used

in conjunction with the *arp3* mutation (classical genetics) or LatB treatment (chemical genetics) to screen for mutations that complement the associated increase in dark development. Complementation of the *arp3* dark development response (relative to the control), could indicate that a particular sugar signalling pathway is upregulated in the *arp3* background, causing the high sugar response (Baier et al., 2004). Dependence on the particular sugar signalling pathway could be direct or indirect, so follow-up studies would be required to investigate the precise relationship between the sugar signalling pathway and ARP2/3 complex signalling.

4.1 The role of HXK1

The role of *HXK1* in dark development was tested using *hxk1* mutants in the Col-0 and L*er* ecotypes. *hxk1* did not affect dark development in the Col-0 ecotype (Figure 4.1A), but was required for dark development in the L*er* ecotype (Fig 4.1 B, C). Moreover, the L*er hxk1* mutant had a similar level of dark development to Col-0 (compare Fig. 4.1 A, C). *hxk1* mutants reduced the LatB-induced dark development of both Col-0 and L*er* (Fig. 4.1 A, D), and partially complemented the dark development of *arcp2a* (Fig. 4.1 B). To test whether the catalytic or sugar signalling function of HXK1 was required for its activity in dark development, transgenic lines expressing the wild-type or catalytic-inactive forms of *HXK1* in the *hxk1* background (Moore et al., 2003) were obtained. Both lines complemented mock-treated *hxk1* in the L*er* background (Figure 4.1 C), indicating that the sugar signalling function of HXK1 is important for the increased dark development of L*er*, and potentially important for the increased dark development of L*er* relative to Col-0. Interestingly, neither the wild-type or S117A catalytic mutant of HXK1 complemented the dark development of LatB-treated *hxk1* (Figure 4.1 D). Moreover, *hxk1* plants overexpressing wild-type *HXK1* were insensitive to the development-promoting effects of LatB, suggesting a balance of HXK1 activity is required for optimal dark development.

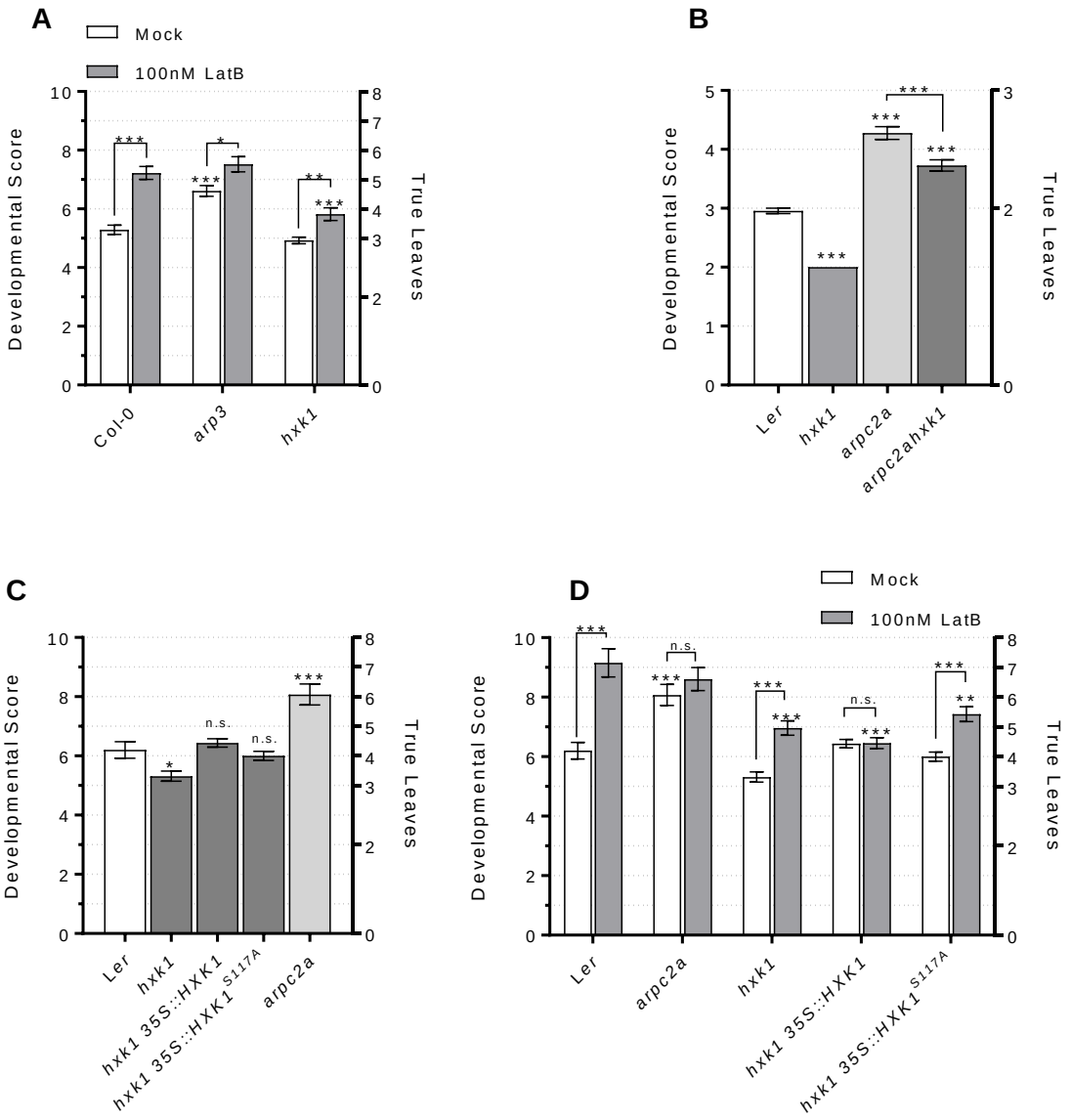


Fig. 4.1. The glycolysis and sugar signalling mutant *hxk1* partially complemented LatB-induced (**A**, **D**) and *arpc2a* (**B**) dark development. Untreated mutants of *hxk1* had reduced dark development in L*er* (**A**, **C**) but not Col-0 (**B**). *arpc2ahxk1* double mutants and LatB-treated *hxk1* plants were unable to increase dark development to the extent of LatB-treated plants (**A**, **D**), or *arpc2a* single mutants (**B**). Overexpression of *HXK1* and the catalytic mutant *HXK1*^{S117A} complemented the dark development of untreated *hxk1* mutants (**C**) but not LatB-treated *hxk1* (**D**). Fig. 4.1C and 4.1D shows the same mock treatment data. (**B**) Data was collected by Karim Sorefan (unpublished) and the scale adapted to be consistent with this thesis. (**B**) Plants were grown on media supplemented with 0.25% glucose for 21 days instead of 0.2% glucose for 28 days. Statistical analysis was calculated by 2way ANOVA and Tukey multiple comparisons test (**A**, **B**, **D**), or by 1way ANOVA and Dunnett's multiple comparisons test (**C**). (A) $n > 18$; (B) $n > 16$; (C) $n > 15$; (D) $n > 13$.

4.2 The role of RGS1

The role of the sugar-responsive sugar signalling protein RGS1 in dark development was also investigated. To investigate the role of RGS1 in ARP2/3-mediated dark development, the LatB responses of *RGS1* mutant and overexpressor lines was tested in a dark development assay. Like the *HXK1* overexpressor, *RGS1* mutant and overexpressor lines were insensitive to LatB treatment (Fig. 4.2.). *rgs1* and *35S::RGS1* both showed phenotypes that were intermediate between mock and LatB-treated Col-0. Apart from mock treated *35S::RGS1* (p=0.02), the dark development of *rgs1* and *35S::RGS1* was also intermediate between mock treated Col-0 and *arp3*. This further supports a role for sugar signalling in dark development downstream of F-actin dynamics.

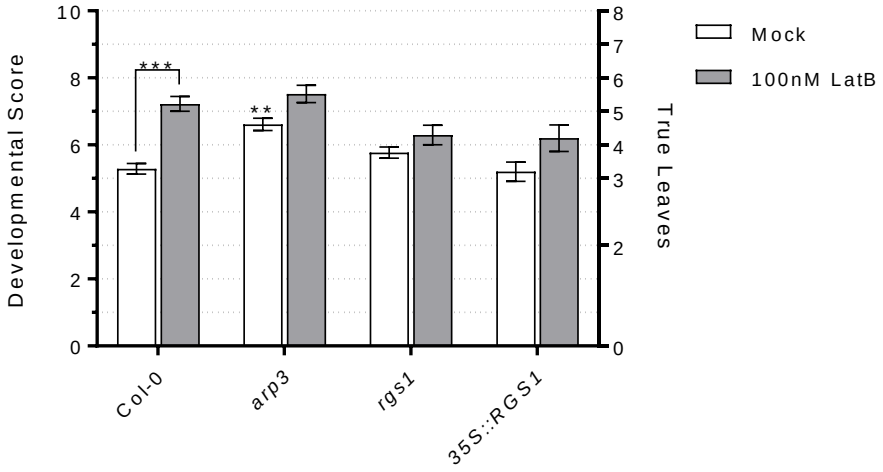


Fig. 4.2. Mutation and overexpression of *RGS1* led to LatB-insensitive, intermediate dark development. Statistical analysis was calculated by 2way ANOVA and Tukey multiple comparisons test; $n > 10$.

4.3 The role of glycolysis

To clarify whether glycolysis is important for dark development, Col-0 and *arp3* plants were treated with the glycolysis inhibitor 2-deoxyglucose (2-DG) in a dark development assay. 0.5mM 2-DG reduced the dark development of *arp3* but not Col-0, indicating that glycolysis is required for the increased dark development of *arp3* mutants (Fig. 4.3). The effect of 5mM 2-DG was also tested, but post-germination growth and development was strongly inhibited (data not shown).

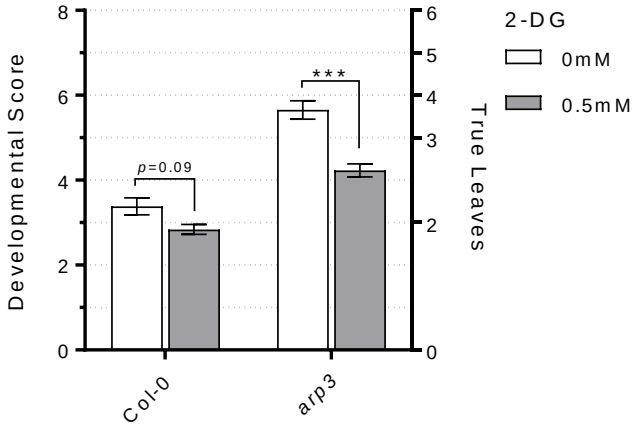


Fig. 4.3. Glycolysis is required for the increased dark development of *arp3* mutants. The glycolysis inhibitor 2-DG partially suppressed the dark development phenotype of *arp3*, but had no significant effect on Col-0 dark development at 0.5mM. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; $n > 26$.

4.4 The role of TOR

To investigate the relationship between ARP2/3 and TOR signalling in regulating leaf initiation in the dark, the dark development of Est-induced *TOR*-RNAi was investigated with and without LatB. Leaf initiation in the dark was severely compromised in *XVE-TOR*-RNAi seedlings treated with Est (Fig. 4.4). By contrast, mock-treated *XVE-TOR*-RNAi seedlings resembled mock or Est-treated Col-0 (Fig. 4.4 B), indicating that the *XVE-TOR*-RNAi phenotype was specifically caused by RNAi. Furthermore, there was no difference in the dark development of *XVE-TOR*-RNAi seedlings germinated on treated plates or transferred to treated media five days after sowing (not shown), indicating that TOR regulates shoot apical meristem activity post meristem establishment, consistent with the role of TOR in regulating root apical meristem activity post root apical meristem establishment (Xiong et al., 2013). LatB treatment was not able to rescue the loss of leaf initiation of Est-treated *XVE-TOR*-RNAi plants, indicating that F-actin-mediated dark development requires TOR. However, Est treatment interfered with the effect of LatB on Col-0 dark development (Fig. 4.4 A and Suppl. Fig. S.1), so may have interfered with the effect of LatB on *XVE-TOR*-RNAi development; *arp3* plants did not show Est sensitivity.

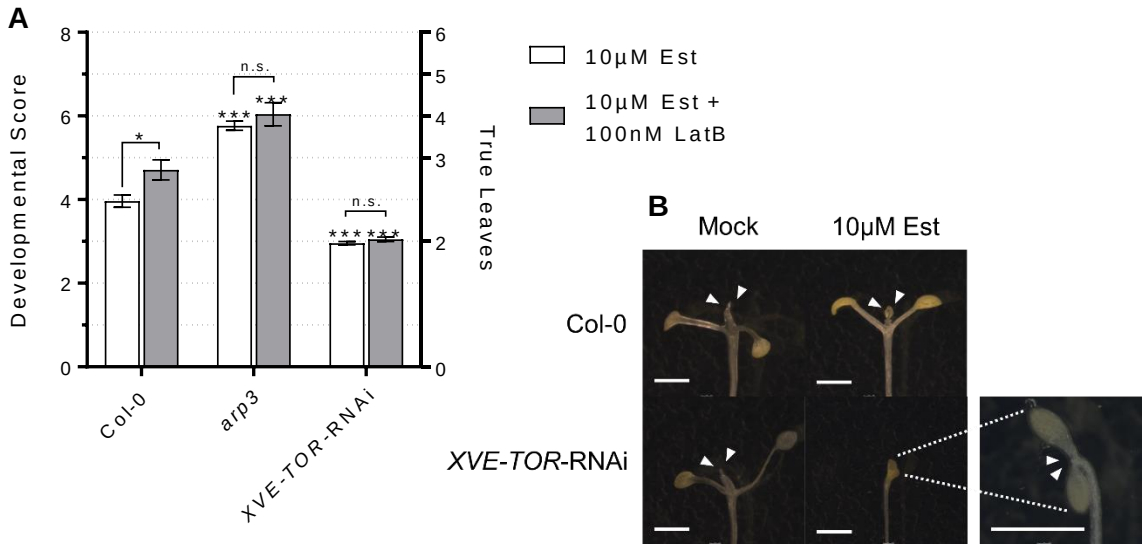


Fig. 4.4. *TOR*-dependent dark development. (A) Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; $n > 25$. (B) *XVE-TOR*-RNAi was specifically inhibited by Est, however, true leaves still initiated at the SAM. Inset shows magnified seedling with cotyledons manually separated. Scale bar = 1mm.

4.5 The role of aldolases

To investigate whether aldolases are important for dark development, aldolase mutants were screened for leaf initiation phenotypes in the dark, and crossed into the *arp3* background to test for hypersensitivity to the loss of aldolase.

4.5.1 Obtaining mutants of aldolases

Mutants of *FBA1,2,4,5,6,8*, but not *FBA3,7* have been described (Lu et al., 2012). Homozygous *fba1-1*, *fba5-1*, *fba6-1*, *fba8-1* were ordered from NASC, and homozygous *fba4-1* was isolated from a segregating population.

A segregating stock of *fba2-1* was ordered, but it was not possible to identify the T-DNA insert at the predicted insertion site by PCR. TAIR SeqViewer (seqviewer.arabidopsis.org) was used to identify a new allele of *fba2*. The mutant, named *fba2-2*, was ordered and isolated from a segregating population. *fba2-2* plants grew slowly and flowered late.

A putative *fba7* allele was identified using TAIR SeqViewer, with a T-DNA insertion in the 5′ UTR. A segregating stock was ordered and screened for resistance to BASTA. All plants showed BASTA resistance but only one in eleven seedlings harboured the T-DNA insertion of interest, indicating that the line had a secondary T-DNA insertion. Most progeny of the *fba7* heterozygote did not contain the T-DNA insertion of interest, so it was not possible to isolate the homozygous line. Therefore, the *fba7* allele was not used in dark development assays.

The *fba8-1* mutant did not germinate on soil or on agar supplemented with 1/2MS, vitamins and 0.5% glucose. A second stock of *fba8-1*, requested from ABRC by NASC, also failed to germinate. Two other *fba8* T-DNA insertion lines were ordered: *fba8-2*, described in a thesis as a strong allele (Tang, 2013), and a new allele, named *fba8-3*, which has a T-DNA insertion at a similar position (Fig. 4.5). A single homozygous *fba8-3* line was obtained from over 30 seedlings from a heterozygous parent; no homozygous *fba8-2* lines were obtained from a similar number of seedlings from a segregating population. The homozygous *fba8-3* line produced a few hundred seeds, but these seeds did not germinate. Since it was not possible to work with either homozygote, segregating *fba8-2* and *fba8-3* populations were screened for dark development phenotypes associated with the heterozygotes.

The T-DNA insertions of the newly described alleles were sequence-verified using primers that anneal the left T-DNA border and adjacent sequence (Figure 4.5). *fba2-2* (SALK_000898) has a T-DNA insertion in the second exon 603bp downstream of the start codon. *fba7-1* (SAIL_870_A09) has a T-DNA insertion in the 5′ UTR, 288bp upstream of the start codon. *fba8-*

2 (SALK_007216) and *fba8-3* (SAIL_1244_A08) have T-DNA insertions in the third exon, located 921bp and 1181bp downstream of the start codon, respectively. *fba8-2* also contains a duplication of 15bp (ATTAGCTAGATACGC) either side of the T-DNA insert, which presumably arose from the T-DNA insertion; *fba8-3* has a 35bp deletion (CAGCTGTTC CAGCCATTGTCTTCTTATCTGGAGGA) associated with the T-DNA insertion site.

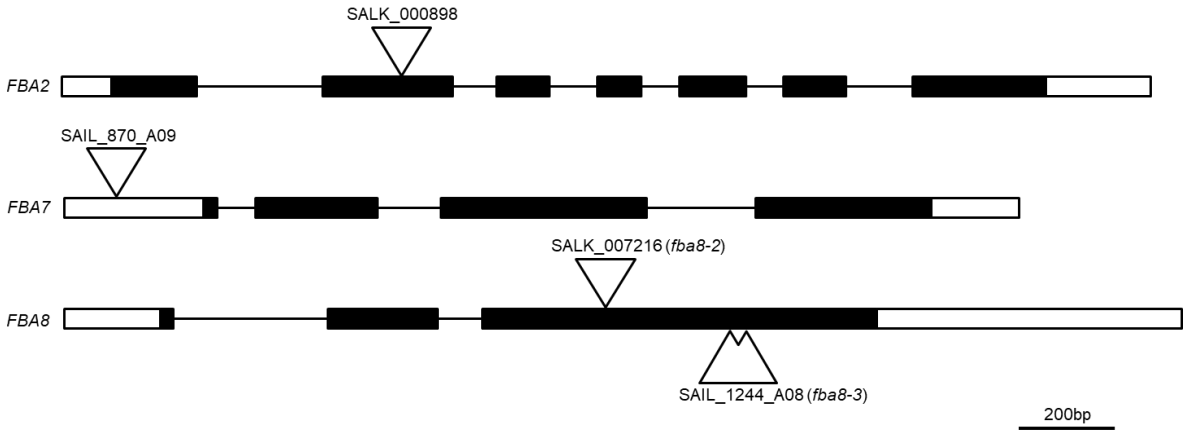


Fig. 4.5. Sequence characterization of unpublished T-DNA insertion lines. All sequences are to scale and oriented 5′ to 3′. Exonic sequences are depicted as black boxes, intronic sequences as lines, 5′ and 3′ UTR sequences as white boxes. Triangles indicate the sequence-verified T-DNA insertion site; where a triangle is divided, the T-DNA has two left borders and the sites indicate the sequences flanking each left border.

4.5.2 Dark development of aldolase mutants

The aldolase mutant *fba2* had decreased dark development which was complemented by *arp3* (Fig. 4.6). Other *FBA* mutants did not affect dark development in the Col-0 or *arp3* background, which could be a result of redundancy within the FBA family. It was not possible to screen homozygous *fba7* or *fba8* mutants (for reasons described above), however, the distributions of the segregating *fba8-2* and *fba8-3* developmental scores were not different to wild type (1way ANOVA), indicating that there was no effect of the heterozygous mutations on dark development.

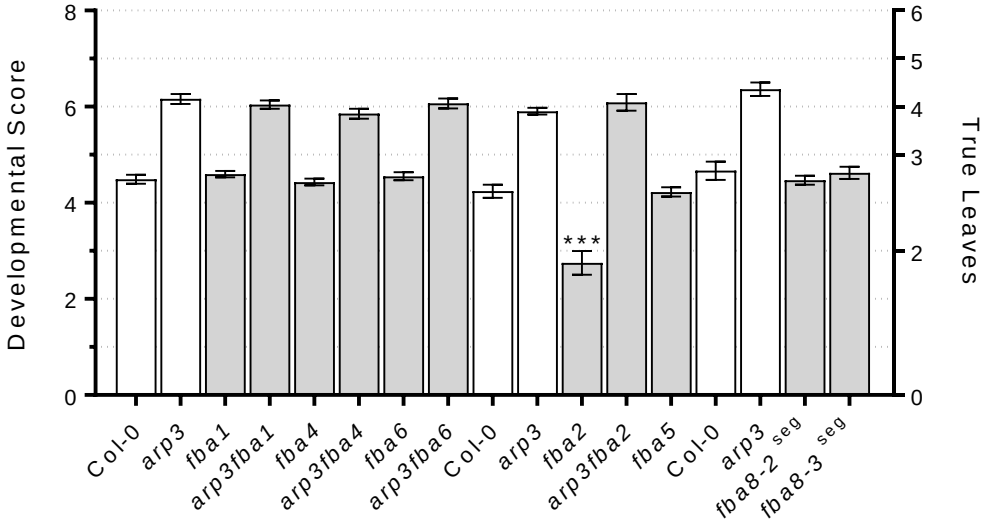


Fig. 4.6. Dark development of aldolase mutants. Where a double mutant was generated, statistics were calculated by 2way ANOVA and Tukey multiple comparisons test, otherwise a 1way ANOVA and Dunnett's multiple comparisons test were used against the relevant Col-0 control. n >15; *fba8-2*^{seg} n = 71; *fba8-3*^{seg} n = 40.

4.6 Discussion

ARP2/3 mutants are hypersensitive to sugars (Baier et al., 2004), so the roles of sugar signalling pathways were investigated in dark development. Both glycolysis and sugar signalling were important for promoting dark development in response to LatB and the *arp3* mutant. Interestingly, a balance of *HXK1* and *RGS1* expression was required for increased dark development (Fig. 4.1, 4.2). Both mutants and overexpression of *HXK1* and *RGS1* conferred decreased sensitivity or insensitivity to LatB (Fig. 4.1D, 4.2).

The dark development of *hxk1* overexpressing the wild-type or catalytically inactive (S117A) forms of HXK1 was tested to differentiate between the dual activities of HXK1 in sugar signalling and glycolysis (Fig 4.1D). Both transgenes complemented the reduced dark development of the *hxk1* mutant on mock-treated plates, indicating that the reduced dark development of *hxk1* was caused by reduced sugar signalling, not glycolysis. Interestingly, the *hxk1* mutant had a stronger phenotype in L*er* than in Col-0, suggesting that L*er* has increased sugar signalling, potentially explaining its increased dark development relative to Col-0.

However, when treated with LatB, neither the wild-type or catalytically inactive HXK1 fully complemented *hxk1* dark development, and overexpression of wild-type *HXK1* in fact caused apparent insensitivity to LatB. The interpretation of this result should be treated with caution as Moore and colleagues showed that *hxk1 35S::HXK1* had greatly increased HXK1 protein levels,

whereas *hxk1 35S::HXK1*^{S117A} had comparable HXK1 levels to wild type (Moore et al., 2003). In light of the differences in protein expression, these data indicate that the catalytic activity but not the sugar signalling activity of HXK1 is required for the increased dark development of *arp3*, as the dark development of *hxk1 35S::HXK1*^{S117A} was reduced compared to wild-type, to the same level as *hxk1*. But the catalytic activity of HXK1 was not in itself sufficient to increase dark development, as mock-treated *hxk1 35S::HXK1* did not have increased dark development, unless the concurrent increase in sugar signalling suppressed the positive effect of increased catalytic activity.

These results indicate that the sugar signalling activity, not the catalytic activity of HXK1 is important for wild-type dark development. However, the reverse was true for the increased dark development of *arp3*/*acpc2a*, since the catalytic activity of HXK1 was required for the increased dark development induced by LatB, indicating that the ARP2/3 complex upregulates glycolysis/glycolysis-dependent signalling downstream of HXK1.

The glycolysis inhibitor 2-DG was used to clarify the role of glycolysis in dark development. Col-0 was insensitive to 0.5mM 2-DG, but the *arp3* mutant was hypersensitive, having an inhibitory effect on dark development (Fig. 4.3). Since glycolysis was important for the increased dark development of *arp3*, the effect of *TOR*-RNAi on LatB-induced dark development was also examined, as TOR is an important integrator of signalling from glycolysis to the cell cycle. Previous studies have shown that *TOR*-RNAi blocks leaf initiation (Li et al., 2017; Xiong and Sheen, 2012), but these were short term studies of up to nine days. In this 28-day assay, *TOR*-RNAi also blocked development, and this could not be rescued by LatB treatment (Fig. 4.4), indicating that TOR is essential for shoot development, and may act downstream of F-actin dynamics. Interestingly, Est-treated *XVE-TOR*-RNAi resembled stage 1 developed plants (Fig. 4.4 B), but manual opening of the cotyledons consistently revealed that the first true leaf primordia were apparent, but these did not grow (stage 3), indicating either that *TOR* was not entirely suppressed by the RNAi, or that *TOR* is not required for the initiation of the first true leaf primordia, but is required for growth. Nevertheless, *XVE-TOR*-RNAi showed insensitivity to LatB (Fig. 4.4 A), indicating that *TOR* is required for the promotion of development in response to LatB.

The potential for ARP2/3 to regulate glycolysis directly, by promoting the F-actin-dependent inhibition of aldolase activity, was explored. Mutants of five out of eight Arabidopsis aldolases were screened for dark development phenotypes and complementation of *arp3*. Mutants of *FBA8* could not be used, as homozygosity conferred sterility (Lu et al., 2012; Tang, 2013). An allele of the plastidic aldolase *FBA2*, the most highly expressed aldolase in dark developed shoot apices (Lopez-Juez et al., 2008), was the only aldolase mutant with defective dark development (Fig.

4.6). *fba2* had significantly decreased dark development, but did not complement *arp3* dark development. This could indicate that FBA2 acts upstream of ARP2/3, leading to its repression and the promotion of leaf initiation. However, this is unlikely since the glycolysis inhibitor reduced *arp3* development, indicating that glycolysis acts downstream of or in concert with ARP2/3 activity to regulate leaf initiation in the dark. Another possibility is that increased mobilization and activity of other aldolases compensated for the loss of FBA2 in the *arp3* mutant, which supports the hypothesis that ARP2/3-nucleated F-actin represses aldolase activity.

It was recently reported that inhibition of the Arp2/3 complex reduced aldolase mobilization and glycolysis in human breast cancer cell lines, which was thought to be a result of reduced F-actin turnover (Hu et al., 2016). If the ARP2/3 complex of Arabidopsis was also a positive regulator of aldolase mobilization, the *arp3* mutant could be expected to have reduced development. Therefore, the role of the Arabidopsis ARP2/3 complex in promoting FBA activity is not consistent with phenotypic data.

Setting aside a potential role of ARP2/3 in regulating FBA activity, these data also suggest that the increased dark development of *arp3* requires factors downstream of HXK/*HXK1* catalytic activity but not downstream of *FBA2* (Fig. 4.7). In the glycolysis pathway, just two glycolytic enzymes, PHOSPHOGLUCOSE ISOMERASE (PGI) and PHOSPHOFRUCTOKINASE (PFK), and three metabolites (glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate) separate HXK and FBA. In plastids, where *FBA2* is expressed (Lu et al., 2012; Mininno et al., 2012), glucose 6-phosphate is also a substrate for the biosynthesis of starch, amylopectin, and precursors of cell wall polymers including rhamnose, galactose, xylose, apiose and arabinose (Caspar et al., 1985; Reiter and Vanzin, 2001), while fructose 6-phosphate is also a substrate for the biosynthesis of the cell wall polymer precursors mannose, fucose and galactose (Yin et al., 2011). Notably, levels of glucose 6-phosphate and fructose 6-phosphate were not affected by inhibition of downstream glycolysis in Arabidopsis in the dark (Draborg et al., 2001). It is tempting to speculate that these metabolites, rather than the glycolytic pathway as a whole, are required for the increased dark development of *arp3*. Rhamnose, galactose, xylose, apiose, arabinose, mannose, and fucose are important substrates of cell wall modifying enzymes (Gille et al., 2013; Madson et al., 2003; O'Neill et al., 2004; Perrin et al., 2003; Strasser et al., 2006). Moreover, alterations of cell wall composition are a prerequisite and sufficient for promoting lateral organ initiation (Fleming et al., 1997; Li et al., 2007; Peaucelle et al., 2011). Furthermore, *brk1* and *arp2* mutants had wild-type levels of cell-wall associated rhamnose, galactose, xylose, arabinose, mannose, fucose and glucose, indicating that the biosynthesis of these cell wall components is not regulated by the ARP2/3 complex (Dyachok et al., 2008). Although, the *brk1* mutant had altered cell wall composition specifically at the cell corners of root cells where the ARP2/3 complex is considered active (Dyachok et al., 2008), indicating that the ARP2/3 complex

might regulate other processes during cell-wall assembly. In alignment, *ARP2/3* and *WAVE* mutants have phenotypes associated with polar cell wall defects (Jarvis, 2011), including separation of elongating hypocotyl and leaf pavement cells (El-Assal et al., 2004a; Li et al., 2004b; Mathur et al., 2003a), and reduced root penetrance strength (Dyachok et al., 2008). Also, the F-actin inhibitors LatB and cytochalasin D disrupted the intracellular trafficking of cell wall components (Chen et al., 2007; Geitmann et al., 1996). To summarise, this hypothesis suggests not only that the biosynthesis of substrates for cell wall modification is required for the increased dark development of *arp3*, but also that the ARP2/3 complex might regulate dark development by affecting cell wall composition.

To explore the possibility that ARP2/3-dependent changes in cell wall composition regulate dark development, existing knowledge about the cell wall composition of *ARP2/3* mutants and the effects of such changes on dark development were compared. Only one study on ARP2/3-dependent changes in cell wall composition was found. The study, using a limited array of antibodies, showed that an *arp2* mutant is deficient in de-arabinosylated rhamnogalacturonan and fucosylated xyloglucan sugars in the cell wall, proximal to sites of WAVE localisation (Dyachok et al., 2008). Since areas of the cell wall where the WAVE complex was not localised had uncompromised biosynthesis and incorporation of these cell wall sugars, the indication is that their trafficking at sites of WAVE localisation is dependent on ARP2/3 complex activity (Dyachok et al., 2008). A deficiency in de-arabinosylated rhamnogalacturonan might be expected to reduce dark development rather than increase dark development, since mutants of arabinose synthesis have increased dark development (Li et al., 2007). Therefore the increased dark development of *arp3* is not likely to be caused by a deficiency in de-arabinosylated rhamnogalacturonan. A reduction in fucosylated xyloglucans might be associated with the increased dark development of the *mur1* mutant, which is deficient in fucose biosynthesis (Freshour et al., 2003; Li et al., 2007). However the *mur2* mutant, which specifically lacks fucosylated xyloglucans, owing to a mutation in the SAM-rich xyloglucan-specific fucosyl transferase *FUT1* gene (Perrin et al., 2003; Vanzin et al., 2002; Yang et al., 2016), did not have increased dark development (Li et al., 2007), indicating that loss of fucosylated xyloglucans alone cannot account for the dark development phenotype of *arp3*. It is worth noting that the *mur2* mutant also had wild-type cell wall strength (Vanzin et al., 2002), whilst the increased dark development of *mur1* and *mur4*, which are deficient in fucose and arabinose biosynthesis, respectively, was related to reduced cell wall integrity (Li et al., 2007). Given that various phenotypes of *ARP2/3* mutants indicate reduced cell wall integrity (see above paragraph), and the current only analysis of cell wall composition of *ARP2/3* mutants (Dyachok et al., 2008) was not comprehensive, it is possible that the increased dark development of *arp3* results from reduced cell wall integrity through a reduction of other cell wall composites.

In summary, sugar signalling and glycolysis were shown to be important for the dark development response to LatB. Genetic analysis indicated that low level sugar signalling promoted dark development, whilst high level glycolysis and/or sugar signalling was repressive, consistent with the developmental effect of low and high levels of sugar on development (Jang et al., 1997). Glycolysis and downstream TOR signalling were required for leaf initiation, but the *arp3* mutant showed greater sensitivity than Col-0 to the glycolysis inhibitor 2-DG. The mechanism by which the ARP2/3 complex regulates responses to glucose remains to be determined.

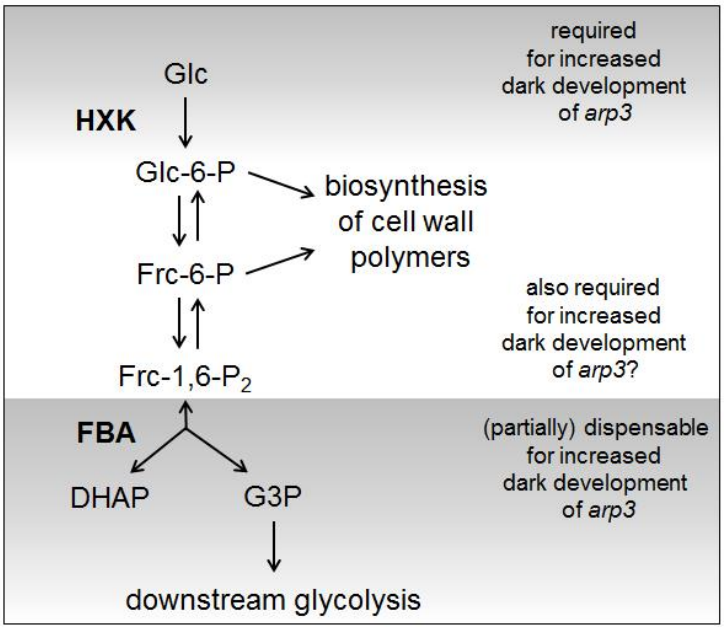


Fig. 4.7. The increased dark development of *arp3* was found to be dependent on glucose and the glycolytic activity of hexokinase (HXK), but independent of *FBA2*. These findings indicate that glycolysis downstream of FBA is (at least partially) dispensable for the increased dark development of *arp3*, but the question remains whether the intermediary steps between HXK and FBA, which are important steps for cell wall biosynthesis, are also required for the increased dark development of *arp3*. Further work is required to establish whether the ARP2/3 complex promotes the rate of cell wall biosynthesis by inactivating FBA, activating HXK, regulating sugar bioavailability, and/or acting on the cell wall biosynthesis pathway to inhibit dark development.

Chapter 5 - Phytohormone signalling in dark development

Sugar and phytohormone signalling pathways interact to regulate plant development. For instance, mutants of positive effectors of the ethylene signalling pathway show glucose hypersensitivity (Rolland et al., 2006; Yanagisawa et al., 2003). The stability of the ethylene signalling transcription factor EIN3 was shown to be negatively regulated by glucose downstream of HXK1 to promote developmental arrest (Yanagisawa et al., 2003). Sensitivity to high concentrations of glucose, including developmental arrest, involves a number of ABA biosynthesis and signalling genes (Dekkers 2006; Nambara et al., 2002; Rolland et al., 2006). Several ABA biosynthesis genes were shown to be upregulated by glucose (Cheng et al., 2002; Gonzalez-Guzman et al., 2002). The auxin receptor TIR1 was also shown to be important for glucose-induced developmental arrest (Moore et al., 2003). Moreover, the *hxk1* mutant showed insensitivity to auxin-induced root development, and hypersensitivity to cytokinin induced senescence and shoot development from hypocotyl explants (Moore et al., 2003). HXK1 is involved in the glucose-induced upregulation of auxin biosynthesis (Sairanen et al., 2012). Cytokinin treatment antagonised the developmental arrest induced by high concentrations of glucose (Zhou et al. 1998). Cytokinin and glucose activate a similar, broad, subset of cell cycle regulators (Richard et al., 2002). Brassinosteroid responses, potentially upstream of auxin signalling, increased the glucose-sensitive emergence of lateral roots in a pathway requiring *HXK1* but not *RGS1* (Gupta et al., 2015). The interactions between sugar and phytohormone signalling are complex and not fully understood, but the potential for crosstalk between the high sugar response mutant *arp3* and hormonal signalling should be investigated.

Hormones are also involved in regulating dark development, for instance, a number of studies have shown that cytokinin promotes leaf initiation and dark development. The *amp1* mutant, which has increased endogenous cytokinin from elevated cytokinin biosynthesis, produced leaves more rapidly in the light and in the dark (Chin-Atkins et al., 1996; Hou et al., 1993). Additionally, exogenous cytokinin increased the rate of leaf initiation in dark development assays (Chin-Atkins et al., 1996; Chory et al., 1991a; Yoshida et al., 2011). The effect of cytokinin on leaf initiation in the dark was shown to require polar auxin transport (Yoshida et al., 2011), and in contrast to *arp3*, the increased dark development induced by cytokinin did not require exogenous sugar (Yoshida et al., 2011), and also promoted photomorphogenic development (Chin-Atkins et al., 1996; Chory et al., 1991a). If the effect of cytokinins on leaf initiation is separable to its effect on photomorphogenesis, it could be possible that the ARP2/3 complex represses this part of the cytokinin response.

In contrast to cytokinin, ABA has been shown to repress dark development (Rohde et al., 1999). Exogenous ABA repressed leaf initiation in a dark development assay, while treatments with

ABA biosynthesis inhibitors increased dark development (Rohde et al., 1999). Moreover, several ABA signalling mutants were shown to have increased dark development (Cheng et al., 2000; Rohde et al., 2000; Rohde et al., 1999). Therefore, the ARP2/3 complex could promote or mediate ABA signalling to repress leaf initiation in the dark.

Limited evidence also points towards auxin and ethylene as negative regulators of dark development (see chapter 1.4). For instance, mutations that increase the stability or levels of Aux/IAAs or ARF2 (negative regulators of auxin signalling) in the presence of auxin increased dark development (Kim et al., 1998; Kim et al., 1996; Li et al., 2004a; Nagpal et al., 2000). Ethylene treatment potentiated the formation of an apical hook, indicative of reduced dark development (Guzman and Ecker, 1990), whilst ethylene insensitivity had the opposite effect, indicating increased dark development (Raz and Ecker, 1999). It is difficult to conclude the effect of ethylene on dark development since most studies have examined development prior to true leaf emergence. Further studies are needed to establish the effect of auxin and ethylene on dark development, as well as the effects of other hormones, which are lacking any reports.

Hormone signalling pathways are also known to interact with actin dynamics. Auxins and brassinosteroids promote the formation of F-actin to facilitate growth (Holweg et al., 2004; Lanza et al., 2012), and brassinosteroids partially mediate their signalling through actin (Lanza et al., 2012). Polar auxin transport was affected by inhibitors of actin polymerisation, which reduced the cycling of PIN proteins from the plasma membrane (Geldner et al., 2001). ABA treatment has been shown to rapidly and reversibly modulate actin dynamics, from a radial to fragmented and randomized arrangement in guard cells, accompanied by stomatal closure (Eun et al., 2001).

Not a lot is known about the interactions between hormone signalling and the ARP2/3 complex specifically. Prior to the initial dark development screen (Fig. 5.1), mutants of the ARP2/3 complex had been shown to have unaltered ethylene precursor-induced reduction of dark grown hypocotyl length (Baier et al., 2004), unaltered ABA-induced root elongation response (Baier et al., 2004), and insensitivity to ABA-induced stomatal closure (Jiang et al., 2012). Additionally, the localisation of ARP3 was shown to be a more persistent marker of cell polarity than the localisation of the PIN1 auxin efflux carrier, using a fluorescent reporter assay in tobacco BY-2 cells (Maisch et al., 2009). Maisch and colleagues hypothesised that the ARP2/3 complex might be involved in the upstream regulation of PIN1 polarity. Further information about the relationship between the ARP2/3 complex and hormones such as auxin, cytokinin, gibberellic acid, or brassinosteroid signalling was lacking, warranting further investigation into the relationship between ARP2/3 complex and hormonal signalling.

To investigate whether phytohormone responses are important for the *arp3* dark development phenotype, the effects of exogenous phytohormones on *arp3* dark development were tested.

Ethylene, ABA, cytokinin, auxin, and brassinosteroid were chosen based on their interactions with sugar signalling, the roles of ABA in repression of dark development (Rohde et al., 1999), and cytokinin in promoting dark development (Chory et al., 1991a; Yoshida et al., 2011), and the putative roles of ethylene and auxin as negative regulators of dark development (see chapter 1.4). Gibberellic acid was also included as a negative control, as GA is not known to play a role in shoot development. Instead, GA is involved in coordinating growth with carbon availability (Ribeiro et al., 2012). It is not expected, therefore, that GA would have an effect on dark development.

5.1 Effects of exogenous phytohormones on *arp3* dark development

The dark development of Col-0 and *arp3* responded similarly to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Fig. 5.1 A) and abscisic acid (ABA; Fig. 5.1 C), which repressed dark development, and also to the cytokinin 6-benzylaminopurine (BAP; Fig. 5.1 B), which promoted dark development (Fig 5.1 A-C). Importantly to note, the response to cytokinin demonstrated that *arp3* dark development can be increased, which has implications for other mutants or treatments that compare to *arp3* levels of dark development. Cytokinin treated plants also had altered leaf morphology, with jagged leaf edges, affecting both Col-0 and *arp3* leaves (not shown).

Col-0 dark development was also repressed by the auxin indole-3-acetic acid (IAA; Fig. 5.1 F) above 1µM, but was not significantly affected by gibberellic acid (GA_3; Fig. 5.1 D) or brassinolide (eBL; Fig. 5.1 E, Suppl. Fig. S.3) by 2way ANOVA, although Col-0 showed a trend towards increased dark development between 10-20nM eBL, seen in two independent repeats, which at 20nM was significant by 1way ANOVA (p=0.03). In contrast, *arp3* mutants showed hypersensitivity to auxin, gibberellic acid, and brassinosteroid, which reduced dark development (Fig. 5.1 D-F). *arp3* dark development was reduced to wild-type levels by 20nM eBL or 0.1µM IAA, and showed a trend towards decreased dark development at eBL concentrations as low as 0.1nM (Suppl. Fig. S.3). This could suggest that *arp3* mutants are deficient in or suppress aspects of auxin, gibberellic acid or brassinosteroid signalling.

The phenotype of the auxin receptor mutant *tir1* (Fig. 5.1 F) indicated that disruption of auxin signalling at the level of perception does not cause increased dark development. Because of the considerable cross-talk between the brassinosteroid and auxin signalling pathways, and the availability of genetic resources in our lab, further experiments focussed on the roles of auxin and brassinosteroid in *arp3* dark development, although the potential roles of gibberellic acid signalling are discussed.

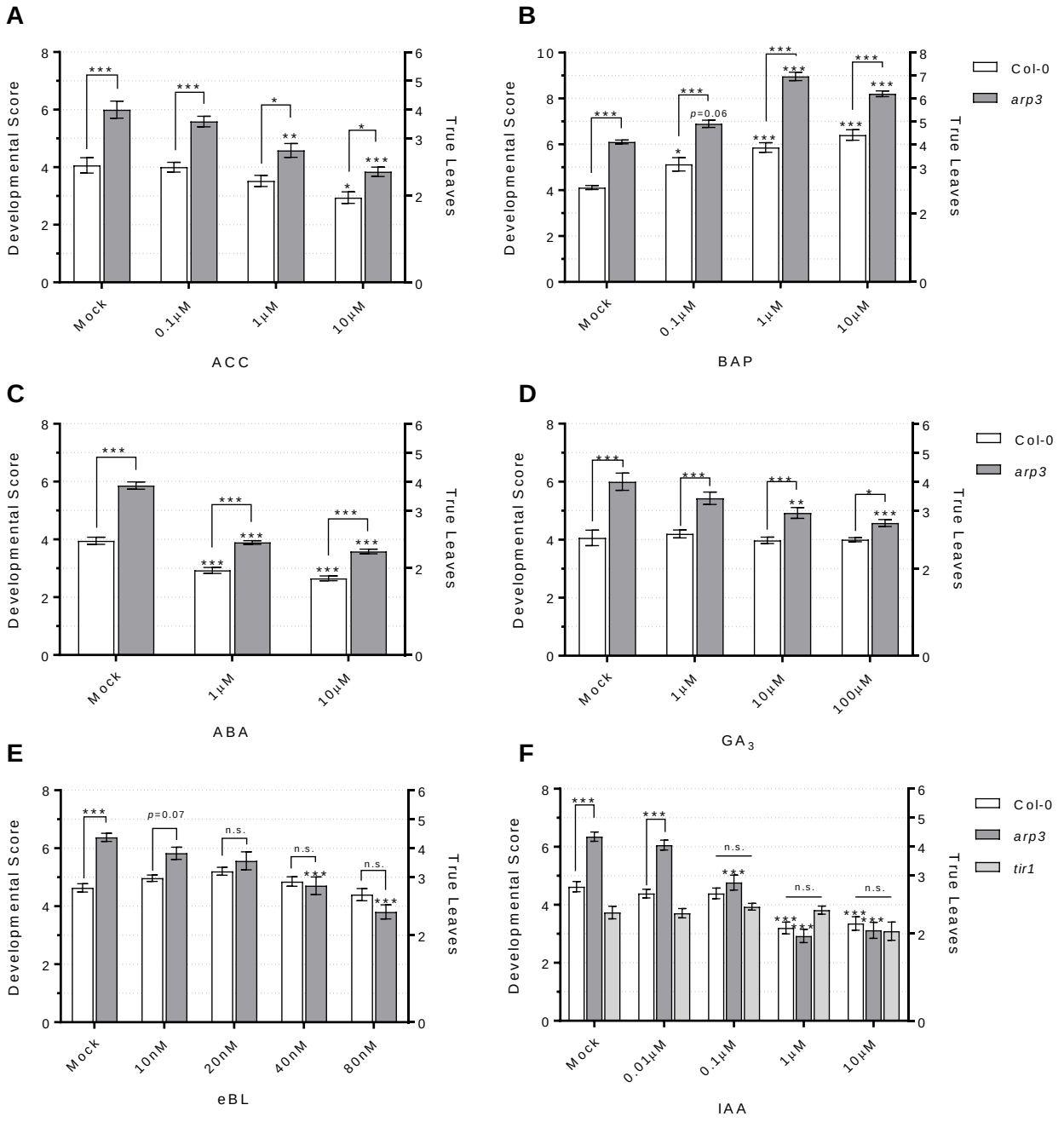


Fig. 5.1. Effects of phytohormones on *arp3* dark development. (**A**) The ethylene precursor ACC had a similar inhibitory effect on the dark development of Col-0 and *arp3*. There was no significant interaction between the variation caused by ACC treatment and genotype, when compared by 2way ANOVA. (**B**) Treatment with the cytokinin 6-benzylaminopurine (BAP) had a similar promotory effect on the dark development of Col-0 and *arp3*. *arp3* showed hypersensitivity to 1µM BAP but otherwise development increased proportionally to Col-0, as confirmed by 2way ANOVA. (**C**) ABA had a similar inhibitory effect on the dark development of Col-0 and *arp3*. As ABA is an inhibitor of germination, seeds were germinated without the hormone for 5 days before dark sterile transfer to plates containing ABA or mock, as indicated. (**D**) GA_3 specifically reduced the dark development of *arp3*. (**E**) The synthetic brassinosteroid epibrassinolide (eBL) reduced *arp3* dark development to wild-type levels. *arp3* mutants had significantly reduced dark development in response to 10nM eBL. (**F**) 0.1µM IAA reduced *arp3* development to wild-type levels, indicating that the dark development of *arp3* is hypersensitive

to auxin. The auxin signalling mutant *tir1* was insensitive to IAA across the range of concentrations tested, indicating that the reduction in Col-0 dark development caused by 1-10µM IAA was an effect of auxin signalling. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test. Asterisks above individual bars denote statistical significance compared to the relevant mock treatment, whilst brackets indicate statistical significance between genotypes. (**A**) n >13, (**B**) n >49, 0.1µM n > 21, (**C**) n >41, (**D**) n >13, (**E**) n >24, (**F**) n >22.

5.2 Effects of endogenous brassinosteroid and auxin on LatB-induced dark development

To test whether altering endogenous auxin or brassinosteroid levels or signalling influenced dark development and LatB responses, a range of biosynthesis, signalling and catabolism mutants were screened for dark development phenotypes in the presence and absence of LatB. Dark development was affected by endogenous brassinosteroid levels, but not brassinosteroid signalling mediated by the *BIN2*/*BIL1*/*BIL2* repressors (Fig. 5.2). The *det2* mutant, deficient in brassinosteroid biosynthesis (Fujioka et al., 1997), had decreased dark development (p=0.02), which was complemented by LatB. The *sob7bas1* double mutant, deficient in brassinosteroid catabolism and shown to have increased endogenous brassinosteroids (Turk et al., 2005), had increased dark development (mock p=0.01; LatB p<0.001), similarly to treatment with 20nM eBL (Fig. 5.1 E). Furthermore, LatB-treated *sob7bas1* showed the same dark development as LatB-treated *arp3*, indicating that the increased endogenous brassinosteroids and the *arp3* mutation affect a common signalling pathway. Consistent with the role of auxin in repressing dark development (Fig. 5.1 F), the auxin biosynthesis mutant *taa1tar1* (Stepanova et al., 2008) had increased dark development compared to Col-0, which was statistically significant between LatB treated Col-0 and *taa1tar1* (p=0.04). However, the auxin catabolism mutant *dao1* (Porco et al., 2016) showed a wild-type response, although it had noticeably increased lateral root length as previously described (Porco et al., 2016; not shown). Surprisingly, given the auxin sensitivity of *arp3* (Fig. 5.1 F), the auxin signalling mutant *tir1* (Ruegger et al., 1998) did not affect the dark development of mock or LatB treated plants (Fig 5.1 F, 5.2).

5.3 Effect of *tir1* on *arp3* dark development

To confirm that *TIR1* is not required for the increased dark development and auxin responsiveness of *arp3* mutants, an *arp3tir1* double mutant was made (Suppl. Fig. S.2B). In a dark development assay, mock-treated *tir1* had slightly reduced dark development (p=0.04), but the dark development of *arp3tir1* was the same as *arp3* (Fig. 5.3). Moreover, the *arp3tir1* double mutant showed the same level of auxin sensitivity as *arp3*, and had increased auxin sensitivity compared to the *tir1* single mutant (p=0.03), which was insensitive to auxin. The auxin sensitivity of *arp3* is therefore not *TIR1*-dependent.

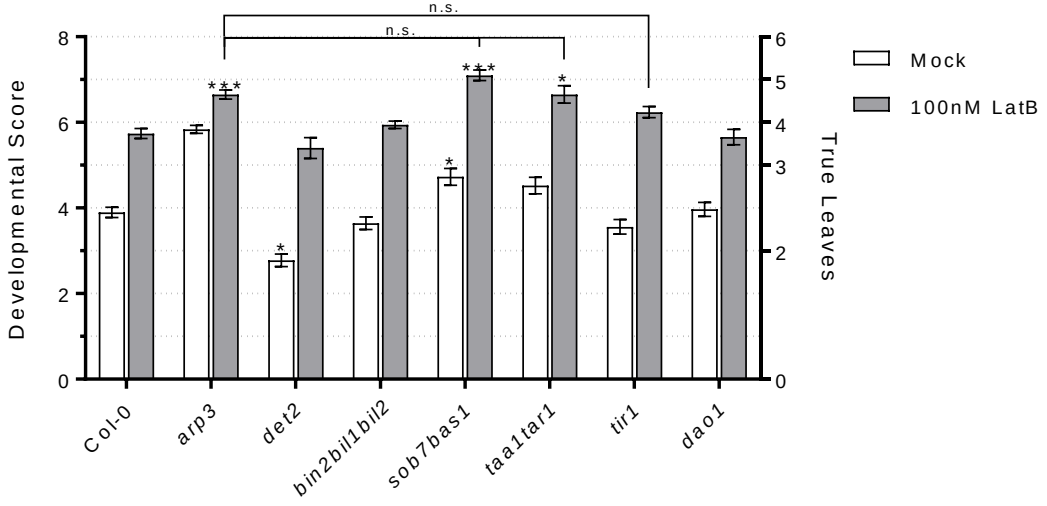


Fig. 5.2. Dark development of brassinosteroid and auxin-related mutants. *det2* is deficient in brassinosteroid biosynthesis (Fujioka et al., 1997), *bin2bil1bil2* has increased brassinosteroid signalling (Yan et al., 2009), *sob7bas1* is deficient in brassinosteroid catabolism (Turk et al., 2005), *taa1tar1* is deficient in auxin biosynthesis (Stepanova et al., 2008), *tir1* is deficient in auxin signalling (Ruegger et al., 1998), *dao1* is deficient in auxin catabolism (Porco et al., 2016). Statistical significance was calculated by 1way ANOVA and Tukey multiple comparisons test on mock or LatB-treated plants. Asterisks indicate statistically significant differences to the respective Col-0 control. Germination was checked under a safe light 3dps; *n* >31, *det2 n* >5 (but repeated with similar results), *taa1tar1 n* >21.

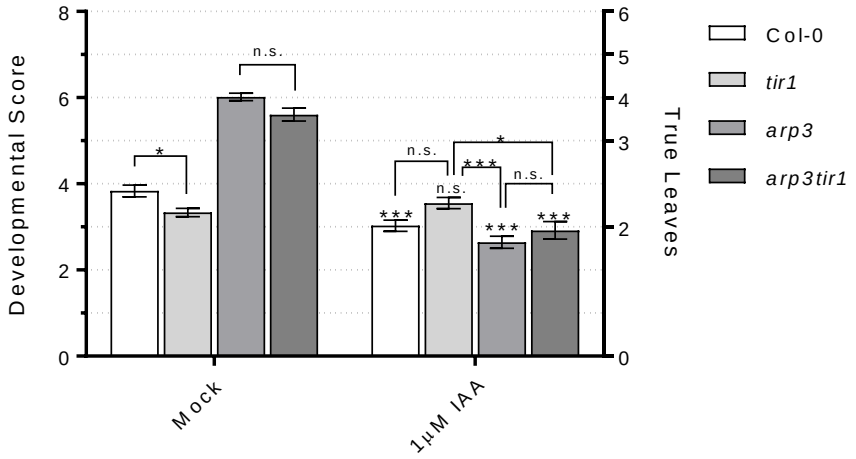


Fig. 5.3. The increased dark development and auxin sensitivity of *arp3* is independent of *TIR1*-mediated auxin signalling. *tir1* did not affect *arp3* development or sensitivity to IAA. Mutation of *arp3* even caused a reduction in *tir1* development in response to IAA, indicating that the IAA-sensitivity of *arp3* is independent of TIR1-mediated auxin signalling. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test; *n* >33.

5.4 Effects of polar auxin transport inhibitors on *arp3* dark development

Auxin and brassinosteroid can affect and act via polar auxin transport (Bao et al., 2004; Nick et al., 2009). To test whether *arp3* increased dark development by modulating polar auxin transport, the effects of two polar auxin transport inhibitors, 1-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), on dark development were analysed. NPA and TIBA block auxin efflux by different modes of action affecting the actin cytoskeleton, vesicle trafficking, and auxin transport, and causing auxin to accumulate within cells (Dhonukshe et al., 2008; Maisch and Nick, 2007; Zhu et al., 2016a).

Polar auxin transport is primarily mediated by the asymmetrically localised PIN polar auxin efflux carriers and AUX1/LAX auxin influx carriers (Swarup and Peret, 2012). In the SAM, AUX1 and LAX1 are the primary auxin importers (Bainbridge et al., 2008), and PIN1 is the only expressed PIN protein (Guenot et al., 2012). In wild-type vegetative and floral SAMs, prolonged darkness stimulates the endocytosis and complete removal of PIN1 from the plasma membrane, causing PIN1 to localise to endosomal compartments, and inhibiting intercellular polar auxin transport (Lauxmann et al., 2016; Sassi et al., 2013; Yoshida et al., 2011). Consistent with the internalisation (and inactivity) of PINs in the dark, Col-0 dark development was insensitive to inhibitors of polar auxin transport (10µM NPA/10µM TIBA; Fig. 5.4) although dark development was reduced by 100µM NPA. In contrast, *arp3* development showed hypersensitivity to inhibitors of PIN-mediated auxin efflux (Fig. 5.4) indicating that PIN-mediated auxin efflux is functional in dark grown *arp3* mutants, and moreover, is important for the increased dark development of *arp3*. 100µM NPA or 25µM TIBA reduced *arp3* dark development to wild-type levels.

5.5 Effects of different auxins on *arp3* dark development

To dissect the roles of AUX1/LAX auxin influx and PIN auxin efflux in *arp3* dark development, the effect of different auxins, which differentially utilise the influx and efflux transporters, was examined. IAA and 2,4-D, which require AUX1/LAX proteins for cell entry, both reduced the dark development of *arp3* to wild-type levels (Fig. 5.5 A, B), whilst only a high concentration of IAA (100µM) also inhibited Col-0 dark development. By contrast, NAA, which diffuses into cells independently of AUX1/LAX import proteins, did not affect *arp3* or wild-type dark development (Fig. 5.5 C). NAA was active, however, as antigravitropic growth was disrupted (Fig. 5.5 D).

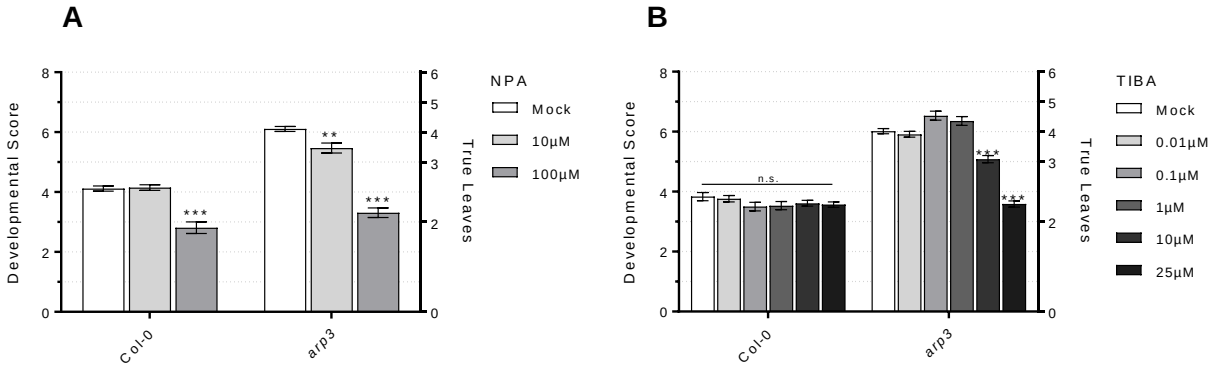


Fig. 5.4. *arp3* showed hypersensitivity to the polar auxin transport inhibitors NPA and TIBA. (**A**) 10µM NPA had no effect on Col-0 dark development, but significantly reduced *arp3* dark development. (**B**) TIBA reduced *arp3* dark development to wild-type level, but had no effect on Col-0 dark development. In contrast to Col-0, *arp3* showed a trend of increased development with concentrations of TIBA <1µM. 25µM TIBA or 100µM NPA reduced *arp3* dark development to wild-type level. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test. (**A**) *n* >32, (**B**) *n* >37.

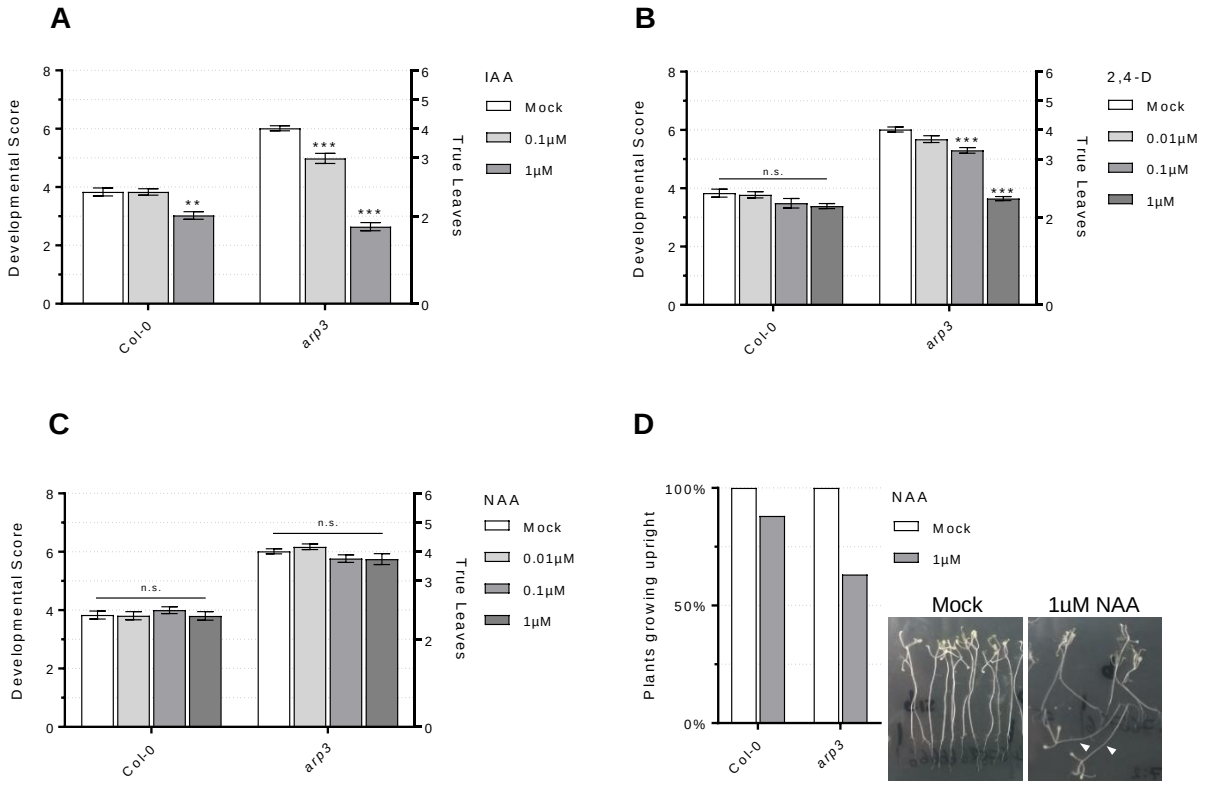


Fig. 5.5. The auxins IAA and 2,4-D reduced *arp3* dark development to wild-type level (**A**, **B**). NAA had no effect on dark development (**C**), although NAA destabilized antigravitropic growth (**D**). White arrows indicate plants not showing upright / antigravitropic growth. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test. (**A**) *n* >37, (**B**) *n* >47, (**C**, **D**) *n* >36.

5.6 Effects of polar auxin transport mutants on *arp3* dark development

To test the hypothesis that PIN1 is required for the dark development of *arp3* mutants, and not AUX1/LAX proteins, the dark development of *pin1* and an *aux1lax1lax2lax3* quadruple mutant was tested. The homozygous *pin1* allele used was infertile, so a segregating line was used (*pin1*^{seg}); specifically, *pin1*^{seg} seed were collected from a plant that was confirmed to be heterozygous for the *pin1* allele by PCR (Suppl. Fig. S.2). The distribution of developmental scores of the mock-treated *pin1*^{seg} population was not significantly different to the wild-type developmental distribution (p=0.84 by χ^2 analysis; Suppl. Fig. S.4 C). However, the *pin1*^{seg} population segregated phenotypically in the presence of 100nM LatB, with bimodally distributed developmental scores consistent with a 3:1 ratio by χ^2 analysis (Suppl. Fig. S.4 C, E). To calculate the 3:1 ratio, the seventyfive percent of plants which best fit the wild-type developmental distribution were separated from the remaining twentyfive percent. The distributions of these observed values were compared to the distributions of LatB and mock treated Col-0 (expected values) by χ^2 analysis and were found to be not significantly different from each other (p>0.19; Suppl. Fig. S.4 C, E). Therefore, it became apparent that the *pin1* mutant was completely insensitive to the dark development-promoting effects of 100nM LatB (Fig. 5.6 A). By contrast, the dark development of the *aux1lax1lax2lax3* quadruple mutant resembled wild type, and did not affect the dark development response to LatB (Fig. 5.6 A). The mock-treated *pin1* line also showed wild-type development (Fig. 5.6 A), indicating that *PIN1* is not required for the dark development of wild-type seedlings.

To test whether *PIN1* was required for the increased dark development of *arp3*, the *pin1* allele was crossed into the *arp3* background. *arp3pin1* double mutants were also infertile, so an *arp3* line segregating for *pin1* was used (*arp3pin1*^{seg}). Similarly, *arp3pin1*^{seg} seed were obtained from plants that were phenotypically homozygous for *arp3*, and heterozygous for *pin1* by PCR (Suppl. Fig. S.2). The dark development of the segregating *pin1* line was not significantly different to wild type (p=0.11 by χ^2 analysis; Suppl. Fig. S.4 B, D), whilst the *arp3pin1*^{seg} population showed a 3:1 phenotypic segregation, supported by χ^2 analysis of difference (p>0.34; Suppl. Fig. S.4 D, F). *pin1* completely suppressed the increased dark development of *arp3*, but did not affect Col-0 dark development (Fig. 5.6 B), indicating that PIN1 is essential for the increased dark development of *arp3* mutants, and supporting the hypothesis that PIN1 is not required in the dark developed wild type.

5.7 Role of PIN1 expression and phosphorylation in *arp3* dark development

The targeting of PIN1 to the plasma membrane is directed by phosphorylation of residues on its hydrophilic loop at the M3 site, by kinases including PID (Ki et al., 2016). Several

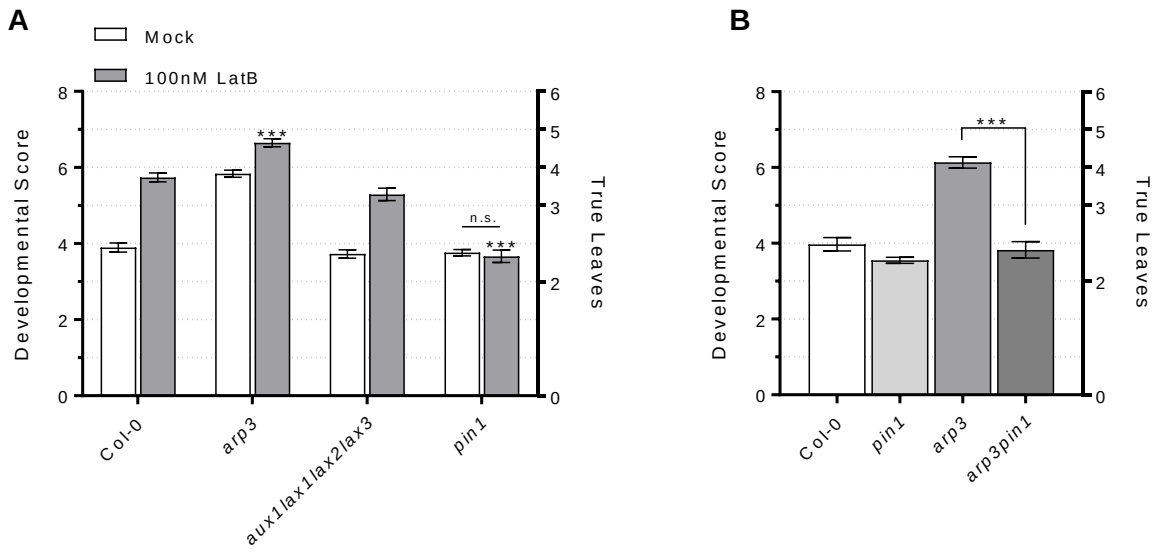


Fig. 5.6. The increased dark development of *arp3* requires *PIN1*. Homozygous *pin1* and *arp3pin1* were identified by phenotypic segregation (Suppl. Fig. S.4). Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test. (**A**) *n* >27, (**B**) *n* >23.

phosphomutants of PIN1 that are unable to be phosphorylated at these sites have reduced apical/basal plasma membrane targeting (Ki et al., 2016). To test whether PIN phosphorylation is required for increased dark development, *pid*, *pin1* and *pin1* mutants complemented with wild-type *pPIN1::PIN1* or PIN1 phosphomutants *pPIN1::PIN1*^{3M1A} or *pPIN1::PIN1*^{M3A}, which have three and five alanine-substituted phosphorylatable residues sites at the M3 site, respectively, were assayed with and without LatB. Each construct complemented the *pin1* mutant phenotype, and had wild-type dark development responses to LatB, as did the *pid* mutant (Fig. 5.7 A). Different lines carrying the same transgene had slightly different average developmental scores, indicating that dark development is sensitive to subtle changes in PIN1 expression.

To test whether PIN1 overexpression increased dark development, *arp3* was crossed with *35S::PIN1*, and dark development was assayed. Additionally, *arp3* was crossed with *35S::PID* to indicate whether potential increases in PIN1 phosphorylation affected dark development. Surprisingly, *35S::PIN1* did not increase wild-type dark development, and partially suppressed the increased dark development of *arp3* (p=0.002), whilst *35S::PID* did not affect dark development (Fig. 5.7 B).

Interestingly, when an *arp3pid* double mutant was made, the *arp3* mutation increased the severity of the *pid* mutant, such that it resembled a strong *pin1* mutant, producing very few flowers, and in many cases, no seed, although three seeds were eventually produced by one plant, indicating that *ARP2/3* and *PID* interact genetically in the light to promote organ initiation from the floral SAM (data not shown).

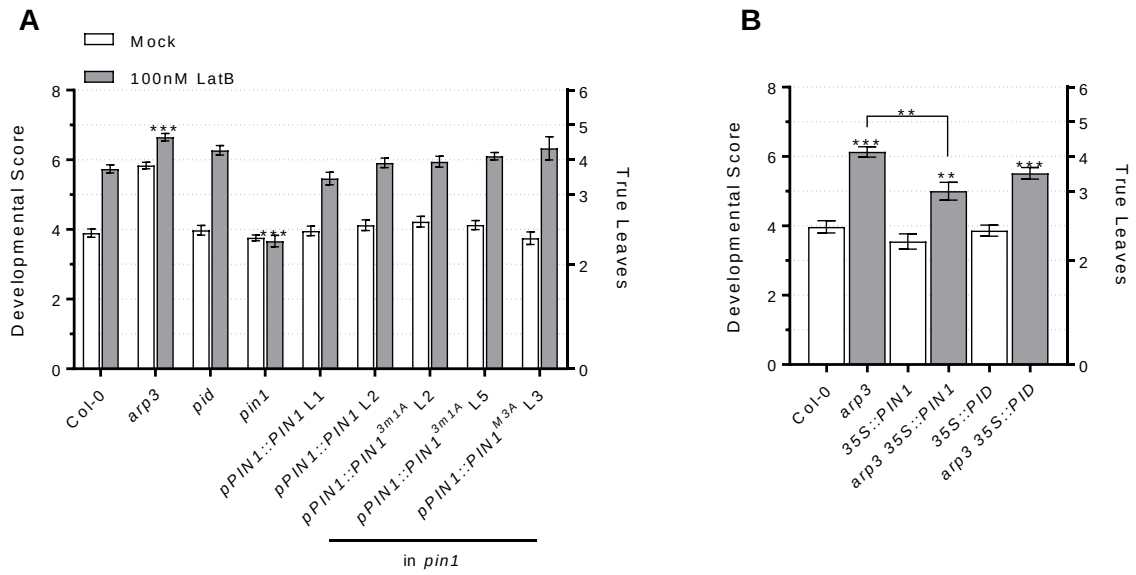


Fig. 5.7. The role of PIN1 phosphorylation and expression in dark development. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test. (**A**) $n > 40$, *pPIN1::PIN1*^{M3A} L3 $n = 12$, (**B**) $n > 17$.

5.8 Role of the ARP2/3 complex in brassinosteroid signalling

The data thus far suggest that polar auxin transport through PIN1 mediates its increased dark development, perhaps by stabilization of PIN1 plasma membrane localisation. Brassinosteroid also promotes polar auxin transport (Bao et al., 2004; Li et al., 2005), and both polar auxin transport and PIN1 expression are reduced in brassinosteroid biosynthesis mutants (Li et al., 2005). The results shown in Fig. 5.2 indicate that brassinosteroids promote dark development in the same pathway as *arp3*, and LatB treatment rescued the decreased dark development of the brassinosteroid biosynthesis mutant *det2*. Therefore, brassinosteroids might increase dark development through polar auxin transport. *det2* has strong growth and developmental phenotypes in the light, although reduced leaf initiation is not one of them (Chory et al., 1991b). Since *arp3* might increase polar auxin transport, *arp3* was crossed with *det2* to test whether any *det2* phenotypes were mediated by the ARP2/3 complex. Strikingly, *arp3* suppressed many of the developmental phenotypes of *det2*, including reduced petiole length, altered leaf morphology, delayed flowering time, decreased silique length, and delayed senescence, but did not complement the reduced leaf size of *det2* (Fig. 5.8.). This suggests that much of brassinosteroid signalling acts by downregulating the action of the ARP2/3 complex to promote polar auxin transport.

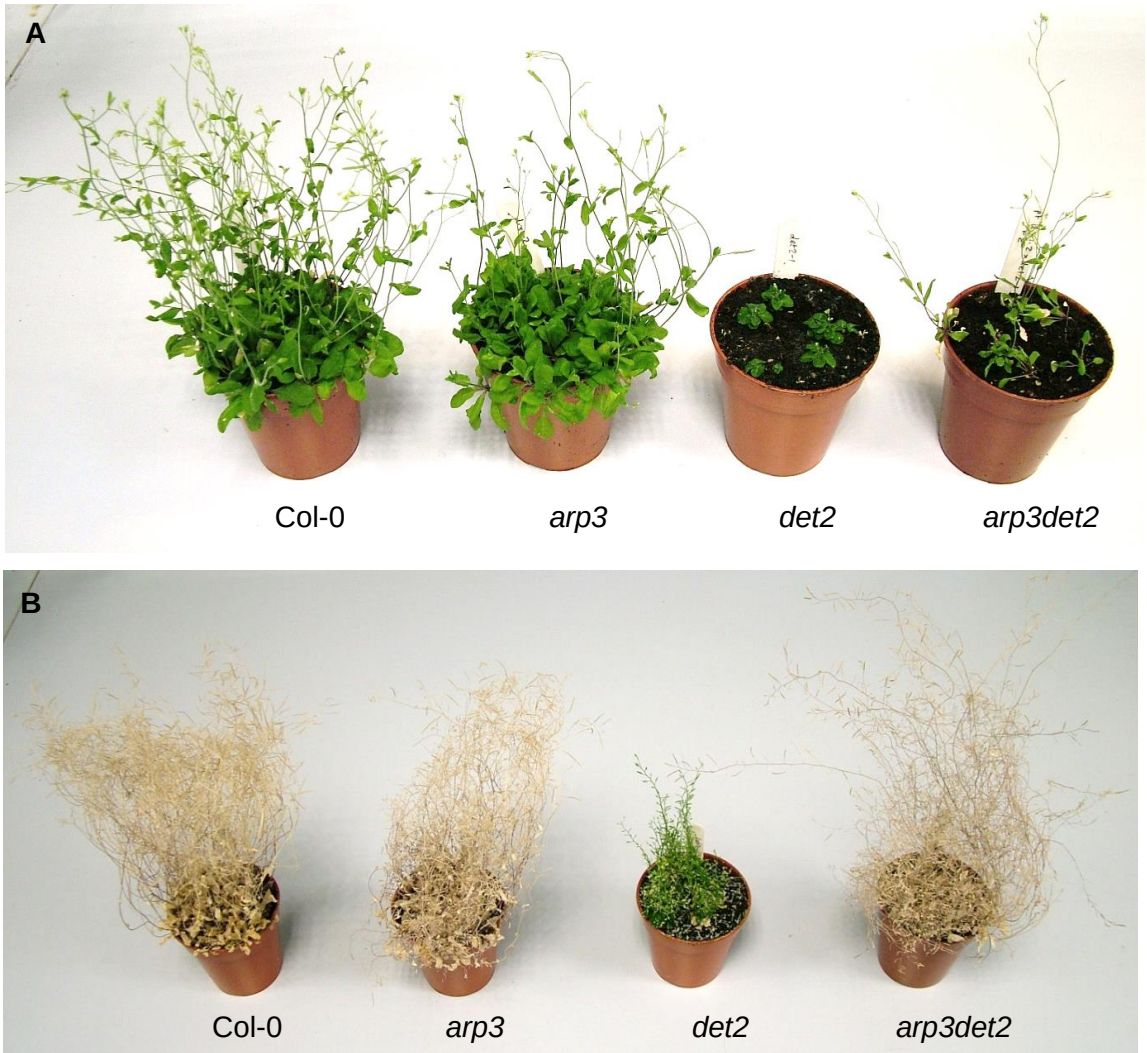
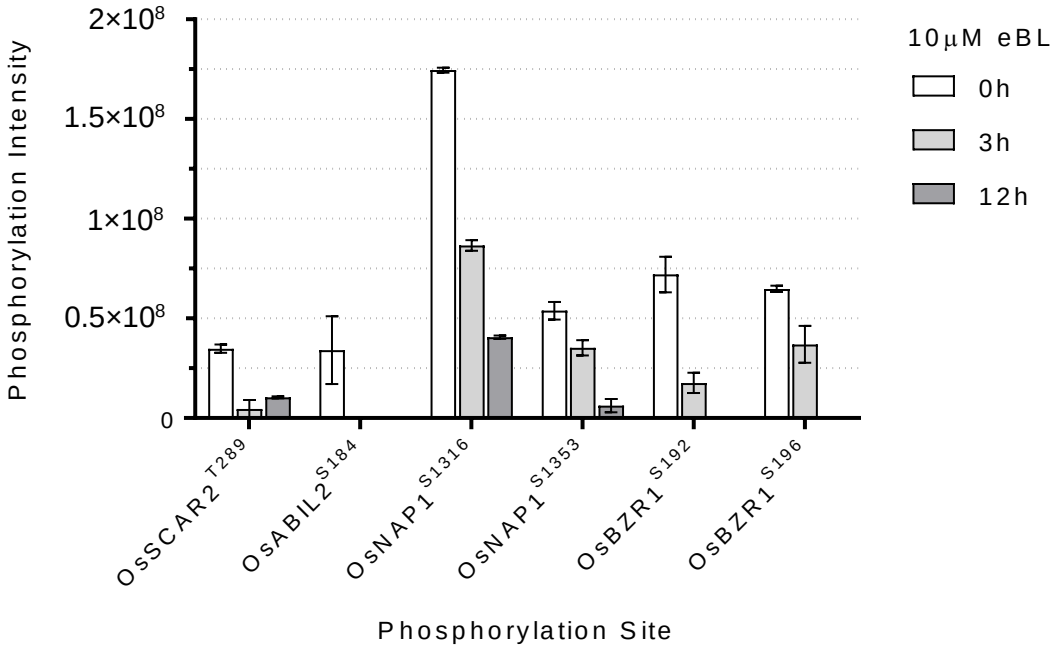


Fig. 5.8. *arp3* partially complements *det2* developmental phenotypes. The brassinosteroid biosynthetic mutant *det2* has reduced growth and development in the light (Chory et al., 1991b). (**A**, **B**) *arp3* partially complemented the delayed flowering of *det2* as well as the decreased petiole length, inflorescence height, silique length, altered leaf morphology, and delayed senescence. (**C**) The *arp3det2* double mutant had more erect leaves compared to Col-0, *det2*, or *arp3* (*arp3* shown for comparison).

5.9 Regulation of the ARP2/3 complex by brassinosteroids

To test whether the ARP2/3 complex is repressed by brassinosteroid signalling, published proteomic and transcriptomic data were analysed. A recent study reported the changes in phosphorylation of the rice proteome following brassinolide treatment (Hou et al., 2017). Analysis of this dataset revealed that several orthologs of ARP2/3 activating proteins are differentially phosphorylated in response to eBL at early and late timepoints (Fig 5.9 A). Treatment with 10µM eBL significantly reduced the phosphorylation of OsSCAR2 (Os01g11040), OsABIL2, (Os01g13530) and OsNAP1 (Os08g43130) after three hours, similar to the response of the positive control OsBZR1 (Os07g39220). Sequence alignment of OsSCAR2 and OsABIL2 to the orthologs in Arabidopsis revealed that the phosphorylated residues in rice were not conserved in Arabidopsis (not shown). By contrast, the two phosphorylated serines of NAP1 were conserved (Fig 5.9 B). S1316 and S1353 of OsNAP1 correspond to S1385 and S1416 of AtNAP1, respectively. According to the Arabidopsis protein phosphorylation database PhosPhAt (Durek et al., 2010), S1385 and S1416 of AtNAP1 are predicted phosphorylation sites within a phosphorylation hotspot. Furthermore, S1385 was demonstrated to be phosphorylated in Arabidopsis (Wang et al., 2013), but upstream regulators of AtNAP1 phosphorylation remain to be determined. These results indicate that brassinosteroid signalling represses ARP2/3 activity by reducing the phosphorylation of ARP2/3 activating factors.

Analysis of ChIP and transcriptome datasets revealed that *ROP11*, *CVY1*, *SCAR4*, *ABIL1*, and *ARPC5* are high stringency BZR1 targets, while *ROP5*, *ROP10*, and *ARPC2A* are potential BZR1 targets (Sun et al., 2010). *ABIL1* expression was downregulated following BL treatment (Nemhauser et al., 2006; Nemhauser et al., 2004); *ROP5*, *ROP10*, *ARPC2A* and *ARPC5* were downregulated by the constitutively active *BZR1* mutant *bzr1-1D*, and upregulated in the *BRI1* mutant *bri1-116* (Sun et al., 2010), indicating that these genes are repressed by BZR1. By contrast, expression of the putative ARP2/3 regulator *CVY1* was upregulated in *bzr1-1D* and downregulated in *bri1-116*, indicating that BZR1 promotes *CVY1* expression. The expression of *ROP11* and *SCAR4* was not affected by BL treatment or the *bzr1-1D* and *bri1-116* mutations. Therefore, brassinosteroid signalling might repress the activity of the ARP2/3 complex by transcriptional repression of upstream activators and transcriptional repression of the *ARP2/3* complex itself.



B

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OsNAP1    ERQLRSMRRSGPLDYTGTRKVKFVEGSSSGSHGAGSGSLQRFAVSRSGPLSYK---  1359
AtNAP1    NKQRGSSRRSGPLDYSSSHK------GGSGSNSTGPSPLPRFAVSRSGPISYKQHN  1425
          ::*  * ********:.::*      ..***:.:* . * *********:***
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Fig. 5.9. (**A**) Phosphorylation intensities of proteins in two week old rice seedlings following treatment with eBL (Hou et al., 2017). Average intensity and standard error of three biological replicates are shown for proteins of interest from a large phosphorylatome dataset. Proteins corresponding to the rice ARP2/3 and WAVE complexes were selected for study, and the positive control OsBZR1 is shown for comparison. All known ARP2/3 and WAVE complex genes that met the stastistical criteria (Hou et al., 2017) are shown. (**B**) Sequence alignment of OsNAP1 and AtNAP1 was performed in Clustal ω (Goujon et al., 2010). The two differentially phosphorylated serine residues in the OsNAP1 C-terminus are conserved in Arabidopsis. Blue boxes indicate differentially phosphorylated serine residues, `*` (asterisk) indicates conserved residue, `:` (colon) indicates conservation of strongly similar properties, `.` (period) indicates conservation of weakly similar properties. Biological replicates $n = 3$.

5.10 Discussion

5.10.1 Responses to ethylene, ABA and cytokinin

The dark development of the *arp3* mutant showed wild-type responses to exogenous ABA and ethylene precursor (Fig. 5.1 A, C), consistent with a report showing that *arp3* and *arpc2a* mutants have wild-type responses to ethylene repression of hypocotyl elongation and abscisic acid

repression of root growth (Baier et al., 2004). The *arp3* mutant also had a wild-type dark development response to cytokinin (Fig. 5.1 B), indicating that the increased dark development of *arp3* does not result from increased cytokinin signalling.

5.10.2 Interactions between ARP2/3 and brassinosteroid signalling

Exogenous brassinosteroid inhibited the dark development of the *arp3* mutant, but up to 20nM eBL slightly increased Col-0 dark development (Fig. 5.1 E). Consistently, the *sob7bas1* double mutant, which has increased endogenous brassinosteroids (Turk et al., 2005), also had increased dark development, while the brassinosteroid biosynthesis mutant *det2* had reduced dark development (Fig. 5.2). Interestingly, the *bin2bil1bil2* triple mutant did not have increased dark development, indicating that these repressors of brassinosteroid signalling are not involved in regulating dark development. However, additional kinases are able to repress brassinosteroid signalling through phosphorylation in the *bin2bil1bil2* background (Yan et al., 2009), so it is still possible that brassinosteroids promote dark development through the canonical brassinosteroid signalling pathway.

A similar relationship between brassinosteroid levels and signalling with polar auxin transport and *PIN1* expression has been demonstrated (Li et al., 2005). Li and colleagues measured basipetal and acropetal polar auxin transport in roots, finding that polar auxin transport was decreased in the brassinosteroid biosynthesis mutant *dim1*, and increased by eBL treatment, but only slightly reduced in the *bri1* brassinosteroid receptor mutant (Li et al., 2005). Moreover, *PIN1* expression in shoots was reduced in *dim1*, *det2*, and *bri1*, and increased by eBL treatment (Li et al., 2005). These observations are consistent with the hypothesis that brassinosteroids regulate dark development through modulating polar auxin transport. Brassinosteroid signalling is not generally associated with regulating leaf initiation, rather, ectopic brassinosteroid signalling in rice occasionally arrested shoot meristem activity (Tsuda et al., 2014). It is possible that, like *arp3*, the effects of brassinosteroids on leaf initiation rate are only evident in the dark.

Brassinosteroids and ARP2/3 regulate dark development in a common pathway (Fig. 5.10). The dark development of LatB-treated *sob7bas1* was not significantly different to LatB-treated *arp3*. Moreover, LatB and *arp3* complemented a variety of *det2* developmental phenotypes, including altered leaf morphology, reduced petiole elongation, delayed flowering and delayed senescence (Fig. 5.2, 5.8), indicating that the repression of the ARP2/3 complex is important for brassinosteroid responses. Examination of phosphorylatome and transcriptome datasets identified a variety of mechanisms in which brassinosteroids and brassinosteroid signalling potentially repress the ARP2/3 complex (Fig. 5.9, and chapter 5.9). Interestingly, concentrations of eBL greater than 20nM did not promote dark development (Fig. 5.1 E), and 20nM eBL or increased

endogenous brassinosteroids in *sob7bas1* were not sufficient to increase dark development to the levels achieved by the *arp3* mutant (Fig. 5.1 E, 5.2), suggesting that brassinosteroids only cause partial repression of the ARP2/3 complex.

The *arp3det2* mutant had a semi-dwarf phenotype with erect leaves (Fig 5.8 C), recapitulating the phenotype of quadruple and quintuple *BSK* mutants (Sreeramulu et al., 2013). This phenotype is unusual among brassinosteroid related mutants in Arabidopsis (Sreeramulu et al., 2013), although more common in rice, where erect leaves are a feature of weak *OsBRI1* alleles and several brassinosteroid biosynthesis mutants (Hong et al., 2002; Hong et al., 2003; Sakamoto et al., 2006; Yamamuro et al., 2000). However, it should be noted that rice lacks core components of the Arabidopsis brassinosteroid signalling pathway, including *BSKs*, *BSU* and *PP2A* (Zhang et al., 2014a). The common phenotype between the quadruple *BSK* mutant and *arp3det2*, suggests that both are deficient in the same aspects of brassinosteroid signalling, apart from those downstream of ARP2/3 repression. The *BSK* quad showed reduced brassinosteroid sensitivity (Sreeramulu et al., 2013), and *det2* and *arp3* showed brassinosteroid hypersensitivity (Fujioka et al., 1997; Fig. 5.1 E; Suppl. Fig. S.3). Altogether, these observations suggest that brassinosteroid signalling can be divided into two major pathways; the first, which is positively regulated by *BSK3*/*4*/*6*/*7*/*8*, primarily regulates growth, in particular, rosette size and hypocotyl elongation (Sreeramulu et al., 2013), and the second, which is negatively regulated by the ARP2/3 complex, primarily regulates development, including the rate of leaf initiation, leaf morphology, flowering time, senescence, and seed production (Fig. 5.2, 5.8). It would be of great interest to identify the components of brassinosteroid signalling that negatively regulate the ARP2/3 complex.

Brassinosteroids are known to regulate cytoskeletal dynamics, moreover, brassinosteroid and auxin have similar effects on F-actin configuration, which is important for aspects of their signalling (Lanza et al., 2012). Both high concentrations of brassinolide (200nM eBL for 2 hours) and low and high concentrations of auxin (100nM NAA for 24 hours; 5µM NAA/IAA/2,4-D for 2 hours) have been reported to promote ROP2 expression and/or ROP2 activity, which stabilized the plasma membrane localisation of PIN1 (Li et al., 2005; Nagawa et al., 2012; Paciorek et al., 2005), which is inconsistent with the proposed role of ROP2 as an upstream activator of the ARP2/3 complex (Yanagisawa et al., 2013), if the ARP2/3 complex is required for endocytosis. The complementation of *det2* by *arp3* (Fig. 5.8) indicates that *arp3* shows constitutive brassinosteroid responses, albeit not all brassinosteroid responses, as *arp3* did not complement certain phenotypes such as leaf size (Fig. 5.8). A gain-of-function allele of *ACTIN2* (*act2-5*), which has a point mutation leading to the amino acid substitution R179C, also showed constitutive brassinosteroid responses, including wavy roots (Lanza et al., 2012), which is not a phenotype of *arp3*. The *act2*-5 mutation also eliminated the phosphorylation (and inactivation) of the

brassinosteroid-regulated transcription factor BZR1 (Lanza et al., 2012). It would be interesting to investigate whether the phosphorylation of BZR1 is also affected in *arp3*.

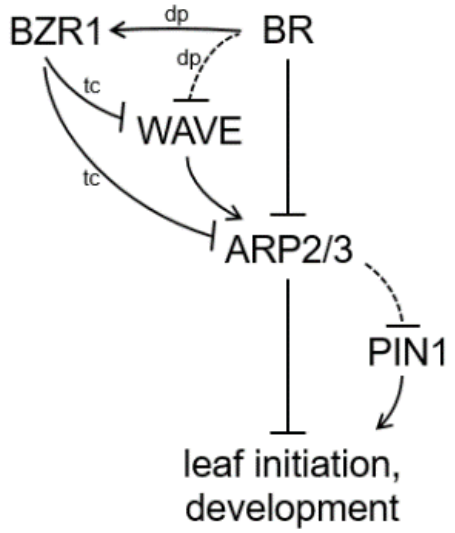


Fig. 5.10. Model of the interactions between the ARP2/3 complex and hormone signalling to regulate BR-dependent development, and leaf initiation in the dark. Brassinosteroid signalling increases leaf initiation by repression of ARP2/3 complex activity through dephosphorylation (dp) of WAVE, and repression of transcription (tc).

5.10.3 Interactions between ARP2/3 and auxin signalling

Brassinosteroid and auxin signalling is also mediated by polar auxin transport (Friml, 2003; Li et al., 2005). To dissect the role of auxin transporters in *arp3* dark development, Col-0 and *arp3* were treated with the auxins NAA, IAA and 2,4-D and inhibitors of polar auxin efflux NPA and TIBA. Col-0 was insensitive to all treatments except for concentrations of IAA above 1µM (Fig. 5.5 A), and 100µM NPA (Fig. 5.4 A). In contrast, the *arp3* mutant was hypersensitive to two inhibitors of polar auxin efflux (Fig. 5.4), and responded to the auxins IAA, 2,4-D, but not NAA (Fig. 5.5). Although the concentration of NAA used was sufficient to disturb gravitropism (Fig. 5.5 D), it is possible that higher concentrations are required to affect dark development.

To explain the responses to different auxins, it was assumed that NAA and IAA require PIN proteins for efflux, whereas 2,4-D is unable to be effluxed via PIN proteins, supported by measurements in tobacco cell suspensions (Delbarre et al., 1996). Supposing AUX1/LAX import was functional, 2,4-D, IAA and NAA would all be able to enter cells. In this scenario, all three auxins would accumulate in the absence of PIN auxin efflux, otherwise IAA and NAA would be effluxed if PIN auxin efflux was also functional. Therefore, if AUX1/LAX import was functional, it would be expected that the three auxins have a common inhibitory effect on *arp3* dark

development, or that IAA and NAA, but not 2,4-D would have a common effect. Since this was not the case phenotypically (Fig. 5.5), it can be assumed that AUX1/LAX proteins are not functional in the dark-grown *arp3* mutant. In this scenario, where AUX1/LAX influx is inactive, NAA, but not IAA or 2,4-D, is able to enter cells efficiently. Therefore, IAA and 2,4-D have a common effect, and NAA has a different effect (Fig. 5.11), as seen phenotypically (Fig. 5.5). This suggests that the increased dark development of *arp3* mutants is mediated either by increased auxin biosynthesis, which might not require AUX1/LAX influx proteins, or by increased PIN auxin efflux. This analysis, together with the increased dark development of the *taa1tar1* auxin biosynthesis mutant (Fig. 5.2) and the inhibition of dark development by inhibitors of polar auxin transport (Fig. 5.4) indicate that PIN1-mediated polar auxin efflux is mediating the increased dark development of *arp3*. Therefore, the dark development of a *pin1* mutant was tested.

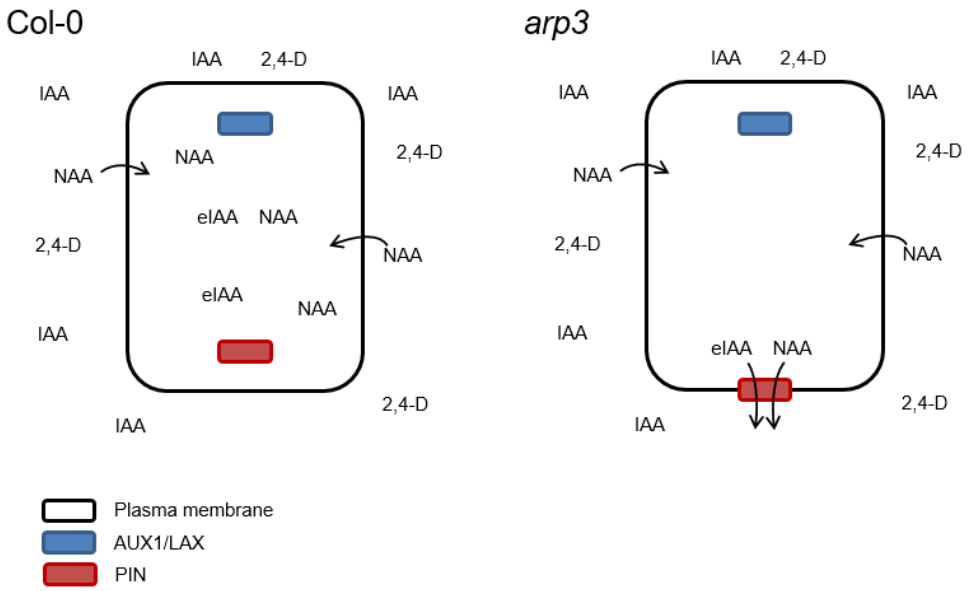


Fig. 5.11. Model of the flow of auxins in Col-0 and *arp3* based on differential phenotypic responses to exogenously supplied auxins in the dark. In Col-0, AUX1/LAX and PIN are internalized; exogenous IAA, 2,4-D and NAA accumulate outside the cell, and endogenous IAA (eIAA) and NAA also accumulate inside the cell. In the *arp3* mutant, polar plasma membrane localisation of PIN proteins facilitates the directional transport of NAA (and eIAA), but exogenous IAA and 2,4-D accumulate outside the cell.

Remarkably, *pin1* completely suppressed the dark development of *arp3* (Fig. 5.6), while a quadruple mutant of the auxin influx carriers AUX1/LAX1/2/3 had no significant effect on dark development (Fig. 5.6 A). In the wild-type SAM, PIN1 is internalised in prolonged darkness (Lauxmann et al., 2016; Sassi et al., 2013), and in line with this, the *pin1* mutation had no effect on dark development (Fig. 5.6), indicating that PIN1-dependent polar auxin transport is not

functional in wild-type dark grown shoot apices. In contrast, polar auxin transport was required for the increased shoot apical meristem activity of *arp3* in the dark, suggesting that the ARP2/3 complex represses PIN1 activity, perhaps by mediating the internalisation of PIN1 in response to prolonged darkness, and maybe also in response to brassinosteroid deficiency.

PIN1 expression in the SAM directs the efflux of auxin towards the site of incipient leaf primordia, forming a local auxin maximum (Benkova et al., 2003; Reinhardt et al., 2003). Localised auxin maxima are important for directing and initiating organ development from the floral meristem, although, disruption of *PIN1* also occasionally leads to arrested organ initiation from the vegetative meristem (Guenot et al., 2012). Organ initiation defects from the *pin1* vegetative SAM were enhanced in a *pin1aux1lax1* triple mutant (Guenot et al., 2012), and an auxin-deficient *yuc1yuc4pin1* triple mutant, which failed to produce true leaves altogether (Cheng et al., 2007). In the dark development assay, arrested meristem phenotypes were scored 2, and would be expected to significantly reduce the average developmental score compared to Col-0 if they were the main cause of reduced leaf initiation. However, the majority of *pin1* and *arp3pin1* mutants initiated two true leaves, resulting in wild type-like development. To my knowledge, only one other study has shown that PIN1 promotes the rate of leaf initiation from the vegetative SAM of Arabidopsis (Guenot et al., 2012). PIN1-directed auxin gradients in the vegetative SAM are mainly associated with the regulation of phyllotaxis (Deb et al., 2015; Reinhardt et al., 2003). However, in maize and tomato, the polar auxin transport inhibitor NPA blocked leaf initiation from the vegetative SAM (Reinhardt et al., 2000; Scanlon, 2003), suggesting that PIN-mediated polar auxin transport is more important for the development of these significant crop plants.

PIN1-mediated polar auxin transport is important for the basipetal transport of auxin from the shoot to the root to promote root growth in the light (Sassi et al., 2013). Consequently, dark grown plants and light-grown *pin1* mutants have reduced primary root length (Sassi et al., 2013). Consistent with the increased activity of PIN1 in the *arp3* mutant, *ARP2/3* mutants, including *arp3,* have increased root length in the dark (Dyachok et al., 2011). Interestingly, *ARP2/3* mutants have decreased root length in the light (Dyachok et al., 2011), indicating that PIN1 activity, though increased in the dark, might be compromised in the light.

The ARP2/3 complex might directly regulate the internalisation of PIN1 in response to prolonged darkness. The hypothesis that ARP2/3 might act upstream of PIN auxin efflux carriers was proposed by Maisch and colleagues in 2009, who observed that ARP3 was polarly localised at the plasma membrane at mutually exclusive sites to PIN1 accumulation, and that ARP3 polarity was more persistent than PIN1 polarity during cell division (Maisch et al., 2009). Previously, it had been shown that LatB inhibited the endocytosis of PIN1 induced by brefeldin A (BFA), and also inhibited the relocalisation of PIN1 to the plasma membrane following washout of BFA, and

the same effect was observed using the actin polymerisation inhibitor cytochalasin D (Geldner et al., 2001). PIN1-GFP colocalises with clathrin (Dhonukshe et al., 2007), and specific inhibition of clathrin-mediated endocytosis greatly reduced the internalisation of PIN1-GFP, suppressing the effect of dominant negative ROP2 which promoted PIN1 internalisation (Nagawa et al., 2012). Studies in other organisms have demonstrated that the ARP2/3 complex is a facilitator of clathrin-mediated endocytosis (Martin et al., 2005; Moreau et al., 1997; Moreau et al., 1996) (chapter 1.5.4). Therefore, it is highly conceivable that the *arp3* mutation directly stabilizes the plasma membrane localisation of PIN1 in the SAM by inhibiting PIN1 endocytosis, promoting the formation of local auxin maxima required for leaf initiation in the dark.

Recently, a study showed that the translocation of PIN2, PIN3 and PIN7 to and from the plasma membrane was decelerated in an *arp3* mutant (Zou et al., 2016). *arp3* affected the rate of PIN endocytosis and recycling to the plasma membrane, but did not affect PIN polarity. Zou and colleagues used BFA to block the recycling of PIN2, PIN3 and PIN7 to the plasma membrane of Col-0 and *arp3.* Because of the toxicity of BFA, the studies by Zou and colleagues were limited to two-hour treatments. Within two hours of BFA treatment, PIN2-GFP was substantially internalised in Col-0 and *arp3*, but the membrane localisation of PIN3-GFP and PIN7-GFP was barely affected, indicating that different PIN proteins have different responses to BFA. Zou and colleagues proposed that the delayed gravitropic root curvature response of *arp3* and *arpc2a* resulted from their effects on polar auxin transport. However, in contrast to the PIN1-dependent phenotype presented in this thesis, the gravitropism phenotype was rescued rather than mimicked by LatB, indicating that *ARP2/3* might regulate PIN1 activity by a different mechanism to these polar auxin transporters. Additionally, the *ARPC2A* allele *dis2-1* did not affect the rate of endocytosis and PIN2 internalisation, although the same allele increased dark development (Fig. 3.9 B). Although the authors were not able to identify a phenotype that was consistent with the role of ARP2/3 complex-mediated F-actin nucleation in endocytosis, their results did demonstrate that the ARP2/3 complex facilitates endocytosis in Arabidopsis, and to my knowledge, are the first to demonstrate ARP2/3 complex-mediated endocytosis in any plant.

The effect of *35S::PIN1* on dark development was examined to test if dark development could be increased by overexpressing *PIN1*. *PIN1* overexpression did not increase dark development in Col-0 or *arp3* (Fig. 5.7 B), and even caused a significant reduction in *arp3* dark development, consistent with previous reports that *35S::PIN1*, like the *pin1* mutant, was defective in establishing well-defined auxin maxima, and had reduced organ initiation rate (Benkova et al., 2003). However, subtle differences in dark development were observed between *pin1* lines complemented with *PIN1* expressed under its own promoter (Fig. 5.7 A), indicating that subtle changes in *PIN1* expression affect dark development. This observation also indicates that the dark

development assay is sensitive to differences in transgene expression, as hypothesised in chapter 4.6, relating to Fig. 4.1 D.

The effect of PIN1 phosphorylation on dark development was also investigated (Fig. 5.7), as PIN1 phosphorylation is important for its polar localisation (Ki et al., 2016). Specifically, phosphorylation of the hydrophilic loop of PIN1, which is partially mediated by PID, promotes plasma membrane localisation (Sasayama et al., 2013) and apical/basal polarity (Ki et al., 2016). Substitution of 3m1 or M3 phosphorylation sites on the hydrophilic loop with alanine decreased the polarisation of PIN1 (Ki et al., 2016), but did not affect dark development (Fig. 5.7 A). Moreover, the *pid* mutation and *35S::PID*, which respectively cause decreased or increased phosphorylation and apical targeting of PIN1 (Friml et al., 2004), did not affect dark development (Fig. 5.7 B). These results indicate that the phosphorylation status of PIN1 is not important for dark development. Whether the polar localisation of PIN1 is affected in these mutants under the conditions of the dark development assay remains to be determined. Interestingly, the *arp3pid* double mutant revealed a strong genetic interaction between *ARP3* and *PID* in the light. *arp3* increased the severity of the *pid-14* allele, which led to 'pin-like' inflorescences, and severely reduced floral development and seed production, as seen in strong *pin1* alleles (Okada et al., 1991; Vernoux et al., 2000) and to a lesser extent in some *pid* alleles (Bennett et al., 1995). This indicates that PID-directed PIN1 localisation is required in light-grown *arp3* floral meristems, but not in the dark developed *arp3* vegetative SAM.

Downstream of polar auxin transport, auxin maxima are believed to trigger canonical auxin signalling through the auxin receptor TIR1 (Kepinski and Leyser, 2005; Leyser, 2006). It was surprising then, that the increased dark development of *arp3*, and even the auxin sensitivity of *arp3* dark development was not affected by the *tir1* mutant (Fig. 5.3). TIR1 is one of six TIR1/AFB proteins, which have partially redundant activities (Dharmasiri et al., 2005; Vernoux and Robert, 2017), although, of the single mutants, *tir1* has the strongest phenotypes (Dharmasiri et al., 2005). Because of the potential for functional redundancy, it cannot be concluded based on the *tir1* phenotype alone, that other AFB proteins are not important for the increased dark development of *arp3* mutants. Analysis of *TIR1*/*AFB* gene expression in the dataset from Lopez-Juez *et al*. (2008) showed that *TIR1*/*AFB1*/*2*/*3*/*5* were all highly expressed in dark developed shoot apices.

5.10.4 Potential interactions between ARP2/3 and gibberellic acid signalling

Exogenous GA_3 suppressed the dark development of *arp3*, while Col-0 was unaffected (Fig. 5.1 D). This result could suggest that GA inhibits factors causing the increased development of *arp3* in the dark, or promotes the activity of factors that inhibit dark development, but this would not

explain why Col-0 was not also affected. Perhaps the factors regulating *arp3* dark development are not normally present in Col-0, such as PIN1. Exogenous GA_3 has previously been shown to both stabilize the expression of *pPIN1::PIN1-GFP* and repress the expression of the *pPIN1::GUS* promoter reporter in the root apical meristem, whilst *PIN1* promoter activity increased in response to genetic and chemical disruption of GA biosynthesis (Willige et al., 2011), although the transcriptional effect was not observed in microarray studies of the effect of GA biosynthesis inhibition and DELLA repression on whole seedlings (Cheminant et al., 2011; Gallego-Bartolome et al., 2012). Given that GA signalling is already very pronounced in the dark (Achard et al., 2007; Arana et al., 2011; Cowling and Harberd, 1999; Lopez-Juez et al., 2008; Roldan et al., 1999), and the concentrations of exogenous GA_3 used in the dark development assay were high (for instance, the gibberellin biosynthesis mutant *ga1-2* was sensitive to the effect of PIN1 GA_3 on hypocotyl elongation in the range of 0.1-30µM (Jacobsen and Olszewski, 1993)), it is possible that the negative effect of exogenous GA_3 on *PIN1* expression outweighs the stabilization of PIN1 protein, resulting in reduced polar auxin transport and reduced dark development.

Another possibility is that the *arp3* mutant was hypersensitive to the effects of GA_3 on microtubule dynamics (Ishida and Katsumi, 1991; Locascio et al., 2013; Sambade et al., 2012). Disruption of the microtubule-associated protein ZWICHEL, or treatment with microtubule-disrupting chemicals had a synergistic effect on trichome development in mutants of *arp3* and *arp2* (Mathur et al., 1999; Schwab et al., 2003).

An alternative hypothesis considers the detrimental effects of gibberellin on SAM function (see chapter 1.4.6). It is possible that GA_3 restricts *arp3* leaf initiation by repressing meristem propagation, since cytokinin levels in the SAM are very likely to be low under the conditions of the dark development assay (see chapter 1.4.1 and discussion below). The rapid initiation of leaves in the *arp3* mutant would make it more susceptible to the effects of GA on SAM function, resulting in the loss of stem cells in the SAM and reduced capacity to initiate leaves. In this scenario, the insensitivity of Col-0 dark development to exogenous GA_3 is also accounted for, as its development is not so rapid that it is able to renew its stem cell niche even in relatively high concentrations of GA_3.

For GA_3 to repress *arp3* development by inhibiting meristem propagation, requires cytokinin levels to be low in dark grown *arp3* shoot apical meristems. Whilst this is true for wild-type SAMs grown in the dark (Lopez-Juez et al., 2008; Yoshida et al., 2011), it may not be true in *arp3* plants. Cytokinin translocation to the SAM is dependent on transpiration rate, which is greatly influenced by stomatal aperture (Aloni et al., 2005; Beck and Wagner, 1994; Darwin, 1916). A mutant of the ARP2/3 complex *arpc2a* (*hsr3*) had increased transpiration rates because it was unable to regulate its stomatal aperture in response to light/darkness, with constitutive intermediate stomatal

aperture (Jiang et al., 2012). Mutants of *ARP2*, *ARP3*, *NAP1* and *PIR* also had similar stomatal aperture phenotypes (Isner et al., 2017). Therefore, increased transpiration rates could contribute to the increased dark development of ARP2/3 complex mutants by increasing cytokinin translocation to the SAM. However, the actin polymerisation inhibitor cytochalasin D rescued the stomatal aperture phenotypes of *arpc2a* and *pir*, which was also rescued by LatB, suggesting that the aperture response defects resulted from increased actin bundling, not a loss of F-actin (Isner et al., 2017; Jiang et al., 2012). Since the dark development of *arp3* was insensitive to actin depolymerisation by LatB (Figure 3.10), the increased dark development phenotype is not likely to be caused by increased levels of cytokinin in the shoot resulting from increased transpiration rate. Moreover, *arp3* mutants had a wild-type cytokinin response (Fig. 5.1 B) and lack features of mutants with increased cytokinin levels/signalling in the shoot, which promotes photomorphogenesis (see chapter 1.1.4; 1.2). Therefore, cytokinin levels in the SAM are also likely to be low in dark-grown *arp3* mutants, supporting the theory that GA_3 represses *arp3* dark development by repressing meristem propagation.

Chapter 6 - Transcriptional regulation of dark development

Transcriptional responses are known to be important for the high sugar response (hsr) of *ARP2/3* complex mutants, because a mutation of the transcriptional Mediator complex, *med25* (*pft1*), suppressed the sugar hypersensitivity of *arpc2a* (*hsr3*), *arp3* (*hsr4*) and another high sugar response mutant *hsr8* (Seguela-Arnaud et al., 2015). Two Mediator complex mutants, *med25* and *med8*, also suppressed the increased dark development of *hsr8* (Seguela-Arnaud et al., 2015). It was not tested if the mutations suppressed the increased dark development of *arpc2a* or *arp3*, but this seems likely since the dark development phenotype is a high sugar response phenotype (Baier et al., 2004). Therefore it is likely that transcriptional responses are important for the increased dark development of *arp3*.

In the previous chapter, PIN1-mediated polar auxin transport was shown to be essential for the increased dark development of *arp3*. Polar auxin transport generates areas of auxin maxima and minima at the SAM, triggering a differential auxin response (Heisler et al., 2005; Vernoux et al., 2010; Vernoux et al., 2011). The activity of several of the ARF family of transcription factors are responsive to high and low concentrations of auxin (Piya et al., 2014; Vernoux et al., 2011). In general, local auxin maxima (which are associated with leaf emergence; Benkova et al., 2003) cause the degradation of Aux/IAAs and concurrent derepression of ARFs leading to downstream transcriptional changes associated with the "auxin response" (Parry et al., 2009; Vernoux et al., 2011). Conversely, auxin minima stabilize the repression of ARFs by Aux/IAAs, repressing the "auxin response" (Parry et al., 2009; Vernoux et al., 2011). Mutations stabilizing Aux/IAAs (and the repression of ARFs) in the presence of auxin have been shown to increase dark development (Colon-Carmona et al., 2000; Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000). Given the importance of *PIN1* in the dark development response of *arp3*, it would be interesting to examine whether ARFs are required for the downstream transcriptional responses that lead to leaf initiation.

The inactive SAM is maintained through the transcriptional control of homeobox transcription factors KNAT1, WUS, RPL and others in order to maintain a pool of undifferentiated stem cells. RPL and KNAT1 interact with each other and both repress leaf initiation (Byrne et al., 2003; Smith and Hake, 2003). Mutants of *rpl* have increased numbers of rosette and cauline leaves (Bhatt et al., 2004; Byrne et al., 2003), and *KNAT1* overexpressors have decreased numbers of rosette leaves, while *rpl knat1* double mutants develop ectopic aerial leaf rosettes (Smith and Hake, 2003). Additionally, RPL interacts with STM, a negative regulator of the differentiation of stem cells into organ primordia, and functions to maintain a pool of stem cells in the SAM in the absence of weak alleles of *stm* (Bhatt et al., 2004; Byrne et al., 2003; Smith and Hake, 2003). *RPL* and *KNAT1* mRNA are excluded from sites of organ initiation at the time of, or immediately prior to organ initiation (Byrne et al., 2003; Jackson et al., 1994; Lincoln et al., 1994).

The HECATE (HEC) family of bHLH transcription factors are important for SAM function, antagonistically to SAM maintenance (Schuster et al., 2014), and promote polar auxin transport (Gremski et al., 2007), which is important for dark development (Yoshida et al., 2011; chapter 5). The HECATE transcription factors belong to group VIIIb of bHLH transcription factors (Heim et al., 2003), which includes HEC1, HEC2, HEC3, bHLH087 and INDEHISCENT (IND). Probes for *HEC2*, *HEC3*, *bHLH087* and *IND* are not featured on the ATH1 microarray, so information about the expression of these genes is limited to alternative arrays and targeted qRT-PCR and promoter::reporter analyses.

HEC1 promotes stem cell proliferation and antagonises the expression of *CLV3* to reduce stem cell differentiation (Schuster et al., 2014). HEC1 also dampens cytokinin responses by upregulating the expression of type A *ARR* cytokinin metabolism genes (Schuster et al., 2014). *HEC1* expression is under direct repression by WUS to prevent its expression (and cell proliferation) within the stem cell niche (Schuster et al., 2014).

HEC1, HEC2 and HEC3 likely play redundant roles in promoting stem cell proliferation in the SAM, since a *hec1/2/3* triple mutant had significantly reduced SAM size (Schuster et al., 2014). Furthermore, organ initiation patterns were aberrant in the *hec1/2/3* triple mutant, indicating that SAM development, as well as growth, is regulated by HEC transcription factors (Schuster et al., 2014).

bHLH087 is a PhyA early repressed gene (Khanna et al., 2006). *bHLH087* transcripts are expressed more highly in dark-grown seedlings, and depleted after only 1 hour of monochromatic red light (Khanna et al., 2006). A *bhlh087* line exhibited no detectable hypocotyl length, cotyledon size or de-etiolation phenotype when examined (Khanna et al., 2006).

IND has primarily been studied in the fruit valve margin, where it is an essential regulator of seed pod dehiscence (Liljegren et al., 2004). IND is closely related to HEC3 and regulates polar auxin transport by direct upregulation of *WAG2* and repression of *PID* kinase expression, affecting PIN polarity (Sorefan et al., 2009). Our lab has shown that *IND* is also expressed in the SAM, under the control of AGO10, using *pIND::GUS* and *pIND::IND-YFP* reporter lines, as well as genetic and transcriptional analyses (Manoj Valluru and Karim Sorefan, unpublished). The role of IND in the SAM is not known, but its relatedness to the HEC genes might make IND a novel regulator of SAM functionality.

Like other bHLH transcription factors, IND binds to DNA as a dimer (Girin et al., 2011; Murre et al., 1989). IND acts both as a homodimer and forms various heterodimers to differentially regulate gene expression (Simonini et al., 2016). IND has been shown to interact directly with SPATULA (SPT) to control auxin distribution and gynoecium development (Girin et al., 2011), ALCATRAZ (ALC) to direct valve margin development (Arnaud et al., 2010; Liljegren et al.,

2004), and interacts with AUXIN RESPONSE FACTOR3 (ARF3/ETT) to regulate reproductive development in an auxin-sensitive manner (Simonini et al., 2016). IND has also been shown to interact directly with PHYTOCHROME INTERACTING FACTORs (PIF3, PIF4, PIF5) and PHYTOCHROME RAPIDLY REGULATED1 (PAR1) in a series of yeast two-hybrid screens (Braun et al., 2011; Gremski, 2006), but as yet, no functional information about the interactions between IND and PIF3-5 or PAR1 has been reported.

This chapter aims firstly to identify novel transcriptional regulators of dark development, with particular attention to the bHLH transcription factor INDEHISCENT (IND) and its related transcription factors, and secondly to identify transcription factors acting in the same molecular pathway as the ARP2/3 complex to regulate dark development, with focus on the AUXIN RESPONSE FACTOR family which could act downstream of polar auxin transport.

6.1 Regulation of dark development by IND

6.1.1 IND regulation of *SWT15* and dark development

Microarray data from our lab was examined to identify the downstream targets of the IND. It was found that IND primarily upregulates the expression of a sugar transporter *SWEET15* (*SWT15/SAG29*) (Simonini et al., 2016). The same dexamethasone (DEX)-inducible *35S::IND-GR* system as used in the microarray was used to confirm the regulation of *SWT15* by qRT-PCR. *SWT15* was highly upregulated following DEX-induced translocation of IND-GR to the nucleus, even following co-treatment with cycloheximide (CHX), an inhibitor of protein synthesis (Fig. 6.1 A). This indicates that the upregulation of *SWT15* was not a secondary response of IND-induction, but that *SWT15* is an immediate target of IND. Because sugar is essential for dark development, we tested mutants of *swt15* for dark development phenotypes, reasoning that SWT15 may regulate sugar transport at the SAM. The *ind* and *swt15* mutants were also crossed with *arp3* to identify genetic interactions.

The *ind* mutant did not affect the dark development or Col-0 or *arp3* plants, indicating that IND does not regulate dark development, or that its function is redundant (Fig. 6.1 B). By contrast, mutants of the IND-target *SWT15* had consistently reduced dark development, but did not suppress the dark development of *arp3* (Fig. 6.1 B), suggesting that SWT15 acts upstream of ARP3 to activate the SAM through sugar transport.

6.1.2 Testing the requirement for IND in *SWT15* regulation

To test the hypothesis that IND-regulation of *SWT15* is redundant with other transcription factors, the expression of *SWT15* was analysed in the *ind* mutant under conditions where *SWT15* expression is known to be upregulated, namely, during senescence, ABA or salt treatment, and high osmotic pressure (Goda et al., 2008; Kilian et al., 2007; Lackman et al., 2011; Quirino et al., 1999). eFP Browser (Winter et al., 2007) was used to identify conditions where *SWT15* is upregulated. There is limited knowledge about the expression of *IND*, as there is no *IND* probe on the Affymetrix ATH1 microarrays, so *IND* expression was also analysed under conditions where *SWT15* is upregulated.

One of the conditions in which *SWT15* is upregulated is senescence (Quirino et al., 1999). Senescence assays are often performed over a timeseries, requiring many plants (and growth space) in order to measure gene expression from the same number leaf (e.g. leaf 5) with biological replicates (for example, see Breeze et al., 2011). To test senescence-induced expression, a new assay was developed that negates the need to harvest leaves over a timeseries, by comparing the expression of genes of interest with a well-known marker of senescence (*SAG12* expression), allowing gene expression to be analysed from leaves at different stages of senescence from a single plant, and at a single timepoint (for a full description of the assay and its validation see Fig. 6.2). There was no difference between the expression of SWT15 in Col-0 or the *ind* mutant (Fig. 6.3 B), although both *SWT15* and *IND* expression increased with senescence (Fig. 6.3 A, B).

SWT15 is also strongly upregulated in response to ABA (Goda et al., 2008; Seo et al., 2011), salt stress and osmotic stress (Kilian et al., 2007; Lackman et al., 2011). Under all conditions tested, there were no differences in *SWT15* expression between *ind* mutants and wild-type plants (Fig. 6.3 B, D, F). *IND* expression was consistently low (Fig. 6.3 A, C, E).

SWT15 expression was also analysed in an *ago10* mutant which has defective SAM development caused partly by ectopic *IND* expression (Karim Sorefan, unpublished). *SWT15* expression was not significantly increased in *ago10* seedlings grown in constant light (Fig. 6.1 C), and neither did *ago10* increase leaf initiation in the dark (Fig. 6.1 D). There was a trend towards upregulated *SWT15* expression in *ago10* (p=0.08), which was suppressed in the *ind ago10* double mutant (Fig. 6.1 C), suggesting that any increased *SWT15* expression in *ago10* was caused by upregulation of *IND*. Given that *SWT15* expression was not decreased in the *ind* mutant, *IND* must either not normally be expressed, or its effects are different in the light and the dark. Alternatively, IND acts redundantly with other transcription factors to regulate *SWT15* expression and dark development.

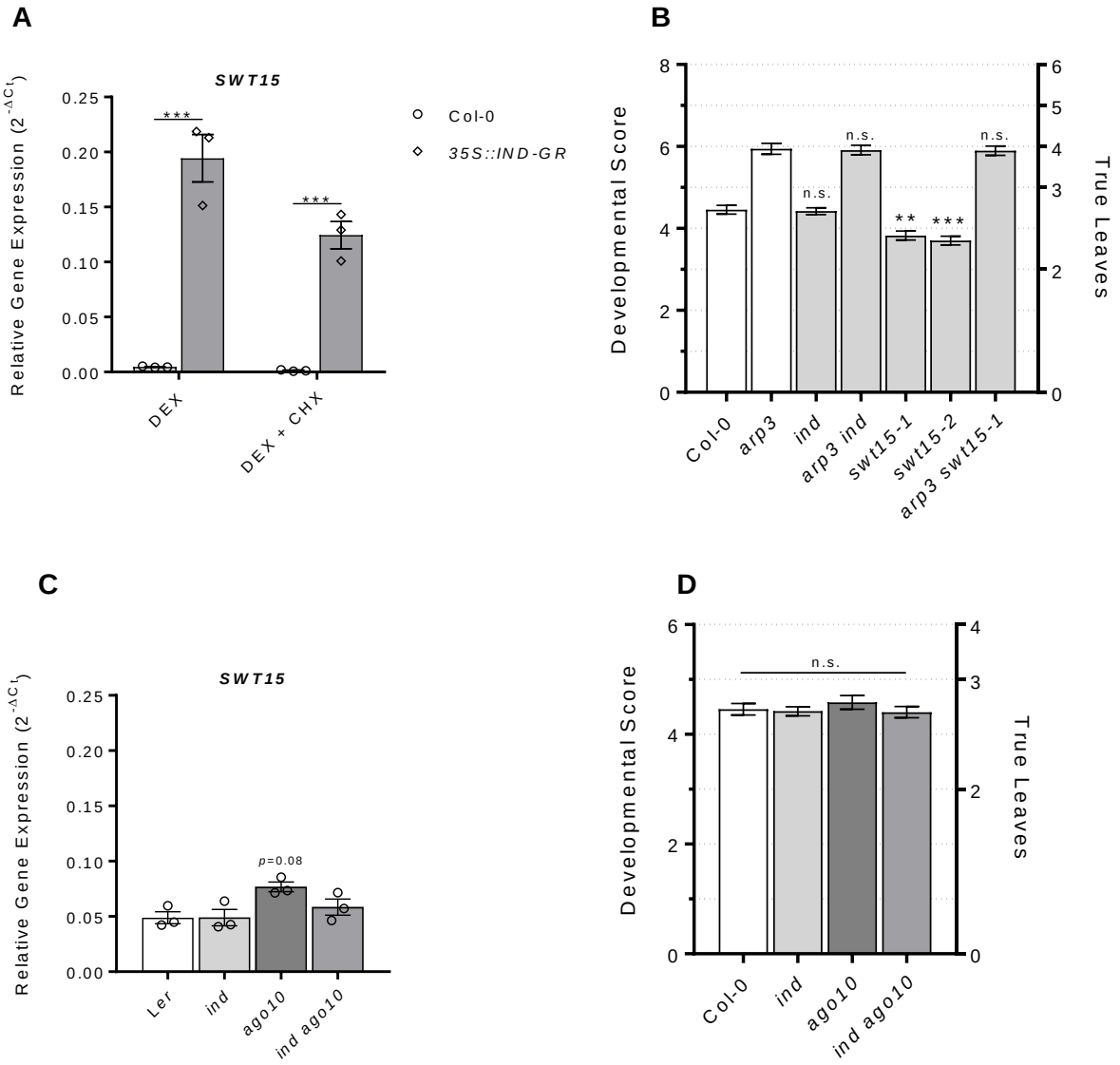
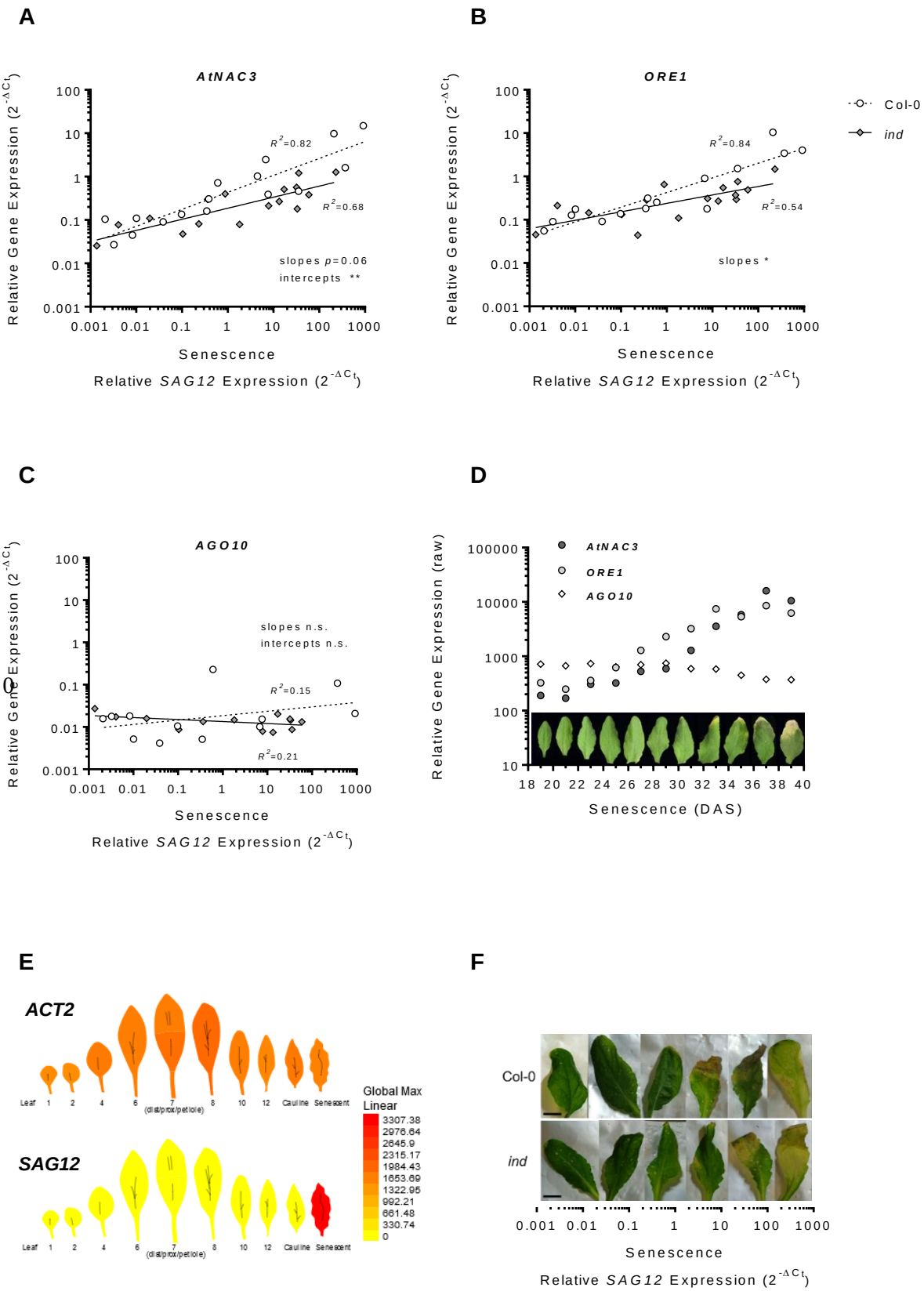


Fig. 6.1. *SWT15* is an immediate target of IND (**A**) and is required for Col-0, but not *arp3* dark development (**B**). *SWT15* expression is not significantly altered in *ind* or *ago10* seedlings (**C**); *ind* and *ago10* mutants have wild-type dark development (**B**, **D**). (**A**) *SWT15* expression following DEX-induced translocation of IND-GR to the nucleus. Col-0 plants with and without the *35S::IND-GR* transgene were treated with DEX, or DEX and CHX for six hours to compare indirect and immediate effects of IND induction. Plants were grown in liquid culture as described previously (Sorefan et al., 2009). (**C**) *SWT15* expression in 8 day old seedlings germinated on ½ MS agar, 0.5% Glc, in constant light; the first true leaves were apparent. 25 seedlings with 'wild type' development (see supplemental Fig. S.5) were pooled per biological replicate. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test (**A**-**D**) on ΔC_t values (**A**, **C**). (**B**) $n > 36$; (**D**) $n > 42$.


A
AtNAC3
Relative Gene Expression ($2^{-\Delta Ct}$)
R^2=0.82
R^2=0.68
slopes p=0.06
intercepts **
Senescence
Relative SAG12 Expression ($2^{-\Delta Ct}$)
B
ORE1
R^2=0.84
R^2=0.54
slopes *
Col-0
ind
C
AGO10
slopes n.s.
intercepts n.s.
R^2=0.15
R^2=0.21
D
AtNAC3
ORE1
AGO10
Relative Gene Expression (raw)
Senescence (DAS)
E
ACT2
SAG12
Leaf
Cauline
Senescent
(dist/prox/petiole)
Global Max
Linear
3307.38
2976.64
2645.9
2315.17
1984.43
1653.69
1322.95
992.21
661.48
330.74
0
F
Col-0
ind
Senescence
Relative SAG12 Expression ($2^{-\Delta Ct}$)

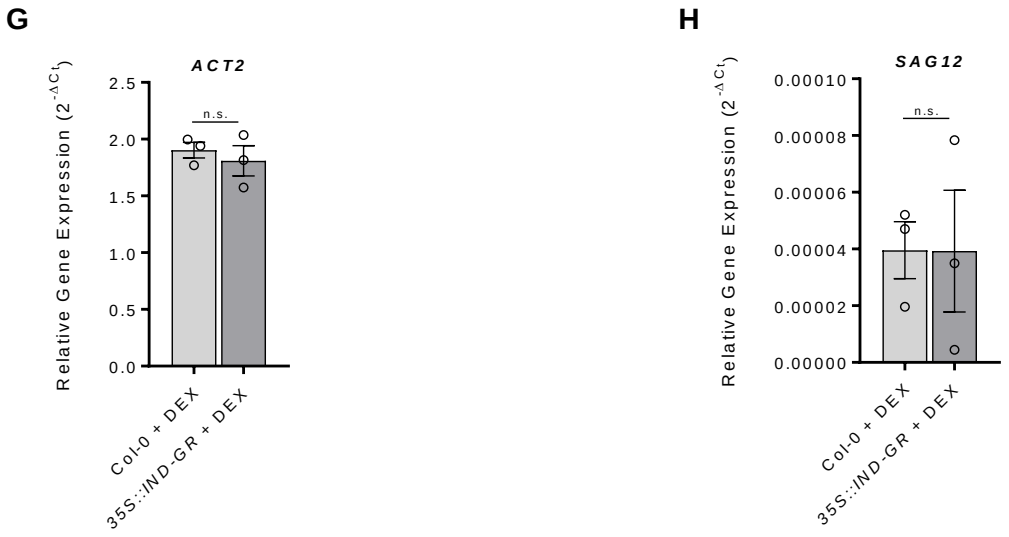


Fig. 6.2. Development of an assay to study the requirement for IND for the induction of *SWT15* expression during senescence. A novel method to analyse gene expression during senescence was developed to allow multiple leaves to be harvested from a single plant at a single timepoint, rather than harvesting the same leaf from multiple plants over a timeseries. In principle, gene expression can be compared between different genotypes at different stages of senescence, assuming that the reference genes (*SAG12* and *ACT2*) are not differentially regulated between the genotypes of interest. The main limitation with this senescence assay over a timeseries, is that it does not provide data on senescence rate.

Individual leaves of a similar size (approximately 3cm x 2.5cm, 170mg) and petioles, from 3 month old (99 day) plants grown in compost in a long day regime, were harvested 5-6 hours after the start of the photoperiod, photographed, and immediately frozen in liquid nitrogen for RNA extraction and cDNA synthesis (see methods). Gene expression was measured by qRT-PCR and normalised to *ACT2* expression (**A-C**) which is not regulated by senescence (**E**) or by IND (**G**), or normalised to *UBQ5* expression (**G**, **H**); *ACT2* has previously been used to normalise gene expression for senescence data (Breeze et al., 2011). Senescence was measured by *SAG12* expression, as *SAG12* is specifically induced by senescence (Lohman et al., 1994), its expression does not vary with leaf number (**E**) (Schmid et al., 2005), visualised using ePlant (Waese et al., 2017), and is not regulated by IND (**H**). Linear regression analysis can be used on $\log_{10}$ transformed data to indicate differences in gene expression with senescence between genotypes. Photographs of representative leaf samples are shown at their approximate position on the X axes (**D**, **F**); scale bar (**F**) = 1cm.

The senescence markers *AtNAC3* and *ORE1*, but not the negative control *AGO10*, were upregulated with senescence (**A-C**), as expected, and the pattern of gene expression in (**A-C**) resembles a microarray dataset (**D**) (Breeze et al., 2011), where senescence was quantified as days after sowing (DAS). p values are given referring to significant differences between the slopes or intercepts, as calculated by linear regression analysis in Graphpad Prism. IND induction experiments (**G**, **H**) were performed as described in Fig. 6.1A, but normalised to *UBQ5* expression, testing the assumption that IND is not a regulator of the control genes *ACT2* or *SAG12*. Statistical analysis was performed using unpaired two-tailed t-test on ΔC_t values.

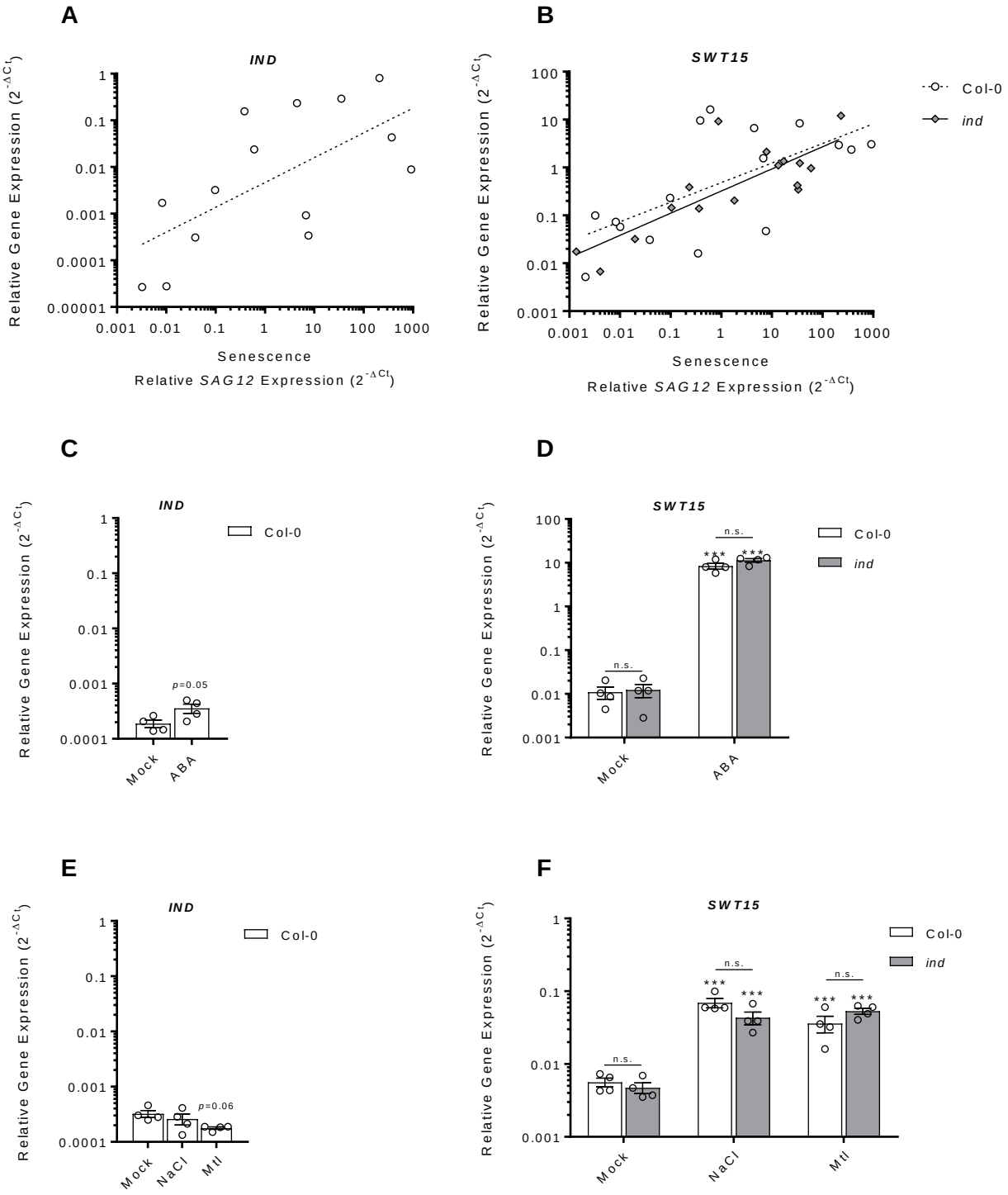


Fig. 6.3. Testing the requirement for IND for *SWT15* expression under conditions where *SWT15* is induced (senescence, ABA, salt and osmotic stress). IND is not required to induce *SWT15* expression in response to (**B**) senescence, (**D**) ABA, or (**E**) osmotic stress. (**A**, **B**) *IND* and *SWT15* are induced by senescence (**A**, **B**), consistent with published data (Breeze et al., 2011). *IND* and *SWT15* expression was measured from mature rosette leaves harvested from three-month old Col-0 and *ind* plants, by qRT-PCR. Relative *SAG12* expression was used as a quantitative measure of senescence as its expression depends on senescence and not leaf number (refer to Fig. 6.2). Linear regression showed that Col-0 (R^2=0.43) and *ind* (R^2=0.66) leaves equally induced *SWT15*

expression in response to senescence (slopes p=0.74; intercepts p=0.51). (**C-F**) SWT15 expression was induced by ABA, NaCl and Mannitol (Mtl) treatment, as previously reported (Goda et al., 2008; Kilian et al., 2007; Lackman et al., 2011). There was no difference between Col-0 and *ind* indicating that IND is not required for the induction of SWT15 under these conditions. Col-0 and *ind* seedlings were grown in liquid culture (1/2 MS, 0.5% Glc, constant light, 100rpm) and treated with 10µM ABA or 0.07% ethanol (mock) for 3 hours (**C**, **D**) or media containing 150mM NaCl, 300mM Mtl or untreated media (mock; by media exchange) for 24 hours (**E**, **F**) and flash frozen 8 days after sowing. Gene expression was normalized to the reference gene *ACT2*. All biological replicates are shown (circles). Statistical significance was calculated on ΔC_t values by unpaired two-tailed t-test (**C**), 1way ANOVA with Dunnett's multiple comparison test against the Col-0 control (**E**), or 2way ANOVA and Tukey multiple comparisons test (**D**, **F**).

6.2 Regulation by IND- and SWT15-related transcription factors

Continuing a candidate approach to identify regulators of dark development, a selection of transcription factors relating to IND, or the regulation of *SWT15* expression, were chosen on the basis that they might regulate a similar subset of genes as IND. These candidates include members of the same clade of bHLH transcription factor as IND (HEC1-3, bHLH087), genes known to interact with IND (ALC, SPT, ARF3, PAR1, PIF3, PIF4) and a related bHLH transcription factor known to regulate *SWT15* expression, since bHLH transcription factors form homo and heterodimers to coordinate gene expression (for more details including references, see Table 6.1). Mutants of these transcription factors were assayed for changes in dark development, along with several double mutants that were made, to identify genetic interactions with *IND*, including redundancy.

Transcription Factor	Transcription Factor Family	Justification	References
HEC1	bHLH group VIIIb	IND-related (bHLH group VIIIb), partially overlapping and redundant functions; HEC1 represses *SWT15* expression; *SWT15* expression is increased in a *hec1/2/3* triple mutant	(Gremski et al., 2007; Heim et al., 2003; Ogawa et al., 2009; Schuster et al., 2014)
HEC2			
HEC3			
bHLH087		IND-related (bHLH group VIIIb), unstudied	(Heim et al., 2003)
ALC	bHLH group VIIb	Interacts directly with IND	(Liljegren et al., 2004)
SPT			(Girin et al., 2011)
ARF3/ETT	B3		(Simonini et al., 2016)
PAR1	atypical bHLH		(Braun et al., 2011)
PIF3	bHLH group VIIa		(Gremski, 2006)
PIF4		Interacts directly with IND, promotes *SWT15* expression	(Gremski, 2006; Oh et al., 2012)
MYC2	bHLH group IIIe	Promotes *SWT15* expression	(Qi et al., 2015)

Table 6.1. Transcription factors selected for targeted mutant screen. Mutants relating to *SWT15* or the *SWT15* and polar auxin transport regulator IND were chosen as candidate regulators of dark development.

6.2.1 Characterization of new alleles

Several alleles not previously published were used for this screen for differing reasons, described below. Predicted T-DNA insertion sites were identified using TAIR SeqViewer (seqviewer.arabidopsis.org) and verified by sequencing the left T-DNA border and adjacent sequence (Fig. 6.4). A *hec1* allele exists from the GABI-Kat collection (Gremski et al., 2007). The putative *hec1* allele used in this study (SALK_045764) has a 3′ UTR T-DNA insertion 178bp downstream of the stop codon. No *hec2* alleles have been described in the literature, although there is a *HEC2*-RNAi line (Gremski et al., 2007). *hec2* (SALK_071800) has a promoter T-DNA insertion 565bp upstream of the start codon. The *bhlh087* (SALK_066339) allele has previously been described to produce a truncated transcript (Khanna et al., 2006), but the location of the T-DNA insertion was not described; *bhlh087* was found to have an exonic T-DNA insertion 482bp downstream of the start codon. The new allele of *alc* (SALK_103763) is the first to be described in Col-0; it has a T-DNA insertion in the first exon, 170bp downstream of the start codon, and produced indehiscent siliques, indicating that it is a strong allele (Liljegren et al., 2004). No mutant of *par1* has been described in the literature. The putative *par1* allele used here (SALK_022002) has a 3′ UTR T-DNA insertion 174bp downstream of the stop codon, associated with a 97bp deletion (TAATCTTTAATTTTAATGTTCTGATTTATTTATTAAGCCGTTTCACGGTTATGAGAAGTGTTCATTAACGAAAATTAGCATTAATTAATGTAGTTAT).

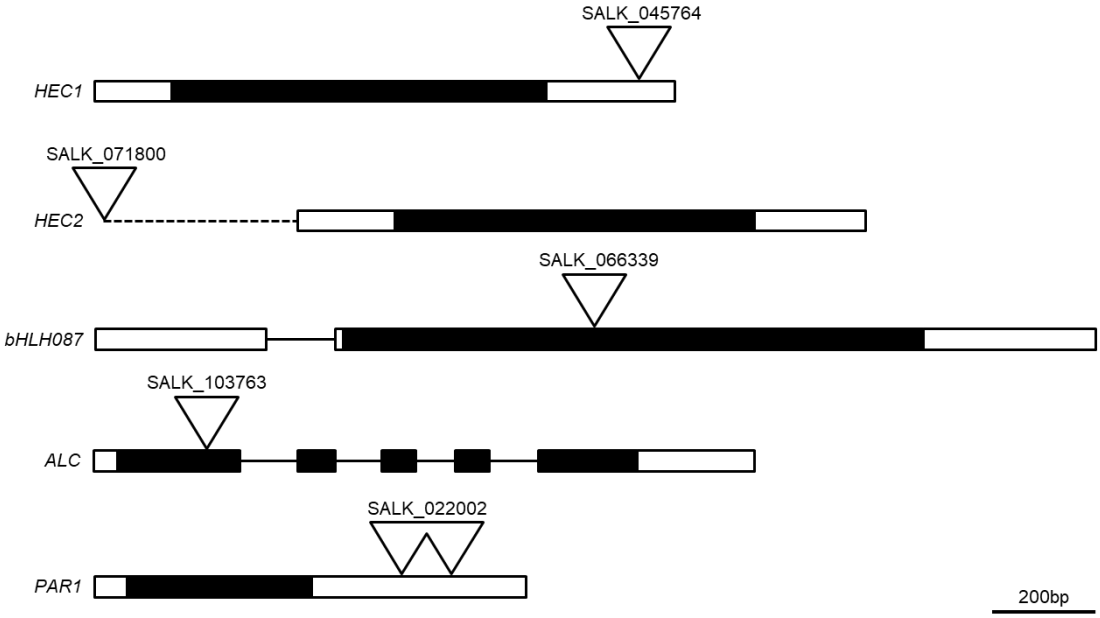


Fig. 6.4. Sequence characterization of unpublished T-DNA insertion lines. All sequences are to scale and oriented 5′ to 3′. Predicted exon sequences are depicted as black boxes, intron sequences as lines, 5′ and 3′ UTR sequences as white boxes, and promoter sequence as dashed lines, based on TAIR10 genome annotation (Berardini et al., 2015). Triangles indicate the sequence-verified T-DNA insertion site; where a triangle is divided, the T-DNA has two left borders and the sites indicate the sequences flanking each left border.

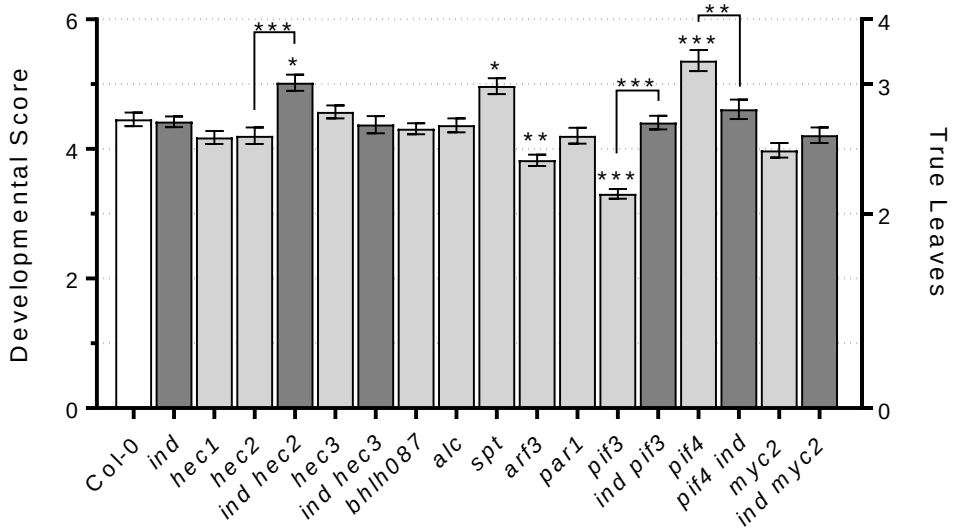


Fig. 6.5. Dark development of IND-related transcription factor mutants (see Table 6.1). Mutants and double mutants of *ind* are shaded dark grey. Where a double mutant was generated, statistics were calculated by 2way ANOVA and Tukey multiple comparisons test, otherwise a 1way ANOVA and Dunnett's multiple comparisons test were used against the Col-0 control. $n > 33$.

6.2.2 The role of *HEC1* and *PAR1*

Even though *hec1* and *par1* lacked dark development phenotypes (Fig. 6.5.), it cannot be assumed that these genes are not involved in regulating dark development because the alleles (both 3′ UTR insertions) are not verified knockouts. It is also possible that these alleles are true knockouts and the phenotype masked by redundancy. The gene expression of *HEC1* and *PAR1* should be measured in these mutants to verify the knockout or knockdown of expression.

6.2.3 Regulation by *IND*, *HEC2* and *SPT*

The single *ind* and *hec2* mutants did not have a dark development phenotype, but in combination, the *ind hec2* mutations increased dark development (Fig. 6.5.), indicating that IND and HEC2 are redundant repressors of leaf initiation in the dark. However, *HEC2* expression in the *hec2* mutant should be analysed before drawing conclusions about the role of HEC2. The *spt* mutant also had increased dark development, and increased dark development to the same extent as the *ind hec2* double.

6.2.4 Regulation by *PIF3*/*4* and *IND*

The phytochrome interacting factor mutants *pif3* and *pif4* had strong and opposite effects on dark development (Fig. 6.5.). The *pif3* mutant had significantly reduced dark development, indicating that PIF3 promotes dark development, while the *pif4* mutant had significantly increased dark development, indicating a repressive role of PIF4 in dark development. Remarkably, *ind* suppressed the phenotypes of *pif3* and *pif4*, suggesting that these genes interact.

6.2.5 Regulation by *ARF3*

The auxin response factor mutant *arf3* also had reduced dark development (Fig. 6.5.), indicating that ARF3 promotes leaf initiation. Since the increased dark development of *arp3* is mediated by polar auxin transport (Fig. 5.4, 5.6), but independent of canonical auxin signalling through TIR1 (Fig. 5.2, 5.3), ARF3 might regulate leaf initiation downstream of ARP2/3, since ARF3 responds to auxin directly (Simonini et al., 2016). Therefore, the involvement of *ARF3* in regulating dark development downstream of ARP2/3 was investigated.

6.3 Regulation by *AUXIN RESPONSE FACTORs*

ARF3 is one of several *ARFs* that are sequentially upregulated in the SAM preceding the development of leaf primordia, including *ARF6*, *ARF8* and *ARF4* (Lopez-Juez et al., 2008). *ARF5* and *ARF10* are highly expressed in the SAM at the time of primordia development (Lopez-Juez et al., 2008). Therefore, ARF transcription factors might coordinately regulate leaf initiation. The activities of ARFs, except for ARF3/11/12/13, are regulated by auxin responsive Aux/IAA repressors (Piya et al., 2014; Weijers et al., 2005). Furthermore, gain of function mutants of Aux/IAAs have increased dark development (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000), suggesting that ARF repression might promote leaf initiation (see chapter 1.2.1.3). Given that the repression of ARFs by Aux/IAAs occurs mostly when auxin levels are minimal (Gray et al., 2001; Kepinski and Leyser, 2005; Weijers and Jurgens, 2004), for Aux/IAA activity to promote leaf initiation through the repression of ARFs would indicate that auxin minima are important for promoting leaf initiation, in contrast to the dogma of auxin maxima promoting leaf initiation from the SAM, as suggested by Benkova and colleagues (2004).

To investigate the role of ARF signalling in SAM activity, a range of mutants associated with ARF function were assayed for dark development phenotypes. These included a gain-of-function mutant of Aux/IAA1 (*iaa1-D*), the *arf2*, *arf3*, *arf4*, *arf5*, *arf7* mutants, and a mutant of the KNOX transcription factor *RPL*, which together with *KNAT1*, is thought to be repressed by ARFs at sites of incipient lateral organ primordia (Byrne et al., 2003; Jackson et al., 1994; Lincoln et al., 1994; Schuetz et al., 2008; Tabata et al., 2010) and interacts directly with ARF3 (Simonini et al., 2016). Additionally, the LatB responses of these mutants were tested to determine whether these factors might regulate the downstream response of *arp3*-mediated dark development (Fig.6.6). Mock-treated *iaa1-D* had significantly increased dark development compared to Col-0 (p=0.04), as reported for other constitutively active Aux/IAA alleles (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000), but was not significantly different to *arp3* (p=0.83), indicating that these genes act on the same pathway to regulate dark development. *arf4* and *arf5* also showed significantly increased dark development relative to Col-0 (p=0.006; p<0.001), consistent with the repression of ARF4 and ARF5 by IAA1 (Piya et al., 2014; Tiwari et al., 2004). LatB-treated *arf4* and *arf5* developed as LatB-treated Col-0, indicating that these genes also act in the same pathway as LatB/*arp3*. The mutant of *rpl* also had increased dark development (p=0.02) and an enhanced LatB response, which was not significantly different to LatB-treated *arp3* (p=0.88). In contrast to Fig. 6.6, *arf3* did not have significantly reduced dark development in this experiment (p=0.36).

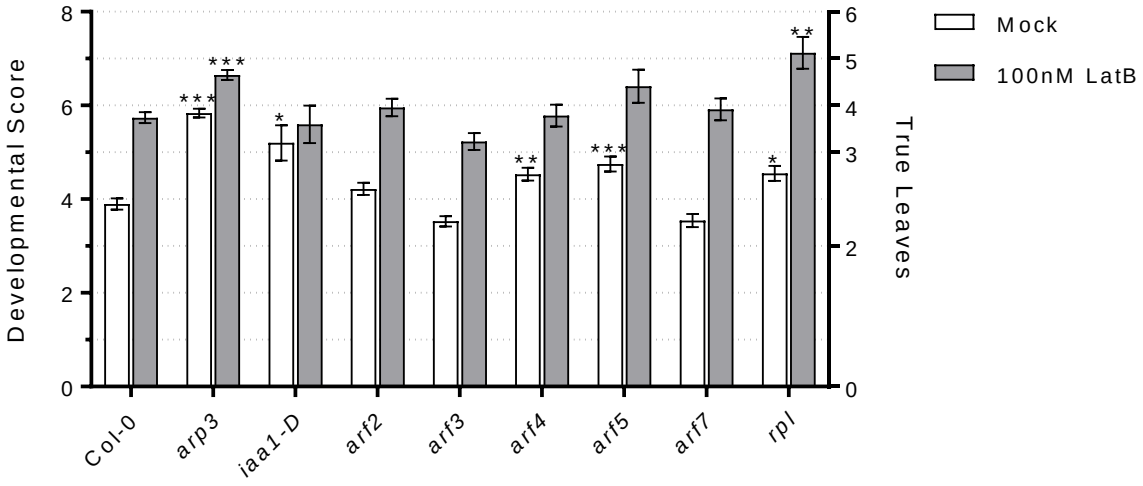


Fig. 6.6. Role of ARF transcription factors in dark development. Statistical analysis was performed by 1way ANOVA and Tukey multiple comparisons test against the relevant Col-0 control. Germination was checked under a safe light 3dps; *n* >16, *iaa1-D n* >5.

6.4 Discussion

The transcription factor IND, which was recently shown to be expressed at the SAM (Valluru and Sorefan, unpublished), was shown to primarily regulate the expression of a sugar transporter *SWEET15*. Single mutant analysis showed *ind* mutants had wild-type dark development, but mutants of *SWT15* had significantly reduced dark development. Analysis of gene expression data showed that *SWT15* is not expressed in the SAM or cotyledons in the dark (Lopez-Juez et al., 2008), so it is unclear how *SWT15* increases SAM activity. While *SWT15* was regulated by IND, IND was not required for *SWT15* expression in seedlings, or under several conditions known to upregulate *SWT15* expression. That the *ind* mutant did not have decreased dark development like mutants of *SWT15*, and *SWT15* expression appeared to be unaltered in *ind* mutants, indicated that *SWT15* is redundantly regulated by IND and other transcription factors.

To identify genes that redundantly regulate *SWT15* expression (and therefore dark development) with IND, crosses were performed to generate double mutants of *ind* and close homologs, as well as known IND interactors. Various new alleles were used in the screen (Fig. 6.4), but, since these alleles were not confirmed to be knockouts by qRT-PCR, it cannot be assumed that the developmental phenotype (null or otherwise), represents a knockout or reduced expression phenotype. However, the *alc* allele had indehiscent seed pods in accordance with other literature (Liljegren et al., 2004), so is likely a null allele.

Potential genetic redundancy was identified between *IND* and *HEC2*, as negative regulators of dark development. Neither the *ind* or *hec2* single mutants affected dark development, but the *ind hec2* double mutant had increased dark development, similar to the *spt* mutant. IND and HEC2 both interact with SPT to regulate gene expression (Girin et al., 2011; Gremski et al., 2007). Therefore, SPT-IND and SPT-HEC2 heterodimers might have redundant activities that repress dark development (Fig. 6.7).

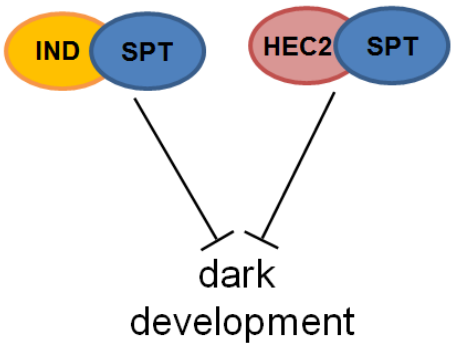


Fig. 6.7. Model of genetic redundancy between IND and HEC2.

IND and *PIF3/4* had striking genetic interaction. The *pif3* mutant had significantly reduced dark development, while the *pif4* mutant had significantly increased dark development (Fig. 6.5), indicating that PIF3 promotes dark development and PIF4 represses dark development. However, the *ind* mutant suppressed the dark development phenotypes of *pif3* and *pif4* (Fig. 6.5). One explanation for this phenomenon is that PIF4-IND act together to repress dark development and PIF3-IND act together to promote dark development. Loss of either PIF enriches the activity of the other; i.e. the repressing activity of PIF4-IND is revealed in the *pif3* mutant background, and the promoting activity of PIF3-IND is revealed in the *pif4* mutant background. In wild type plants, these two activities would balance out, whilst in the *ind* mutant the loss of PIF3-IND and PIF4-IND activities also balance, to appear wild-type (Fig. 6.8). The significance of this interaction would be to differentially regulate leaf initiation based on specific contextual signals relayed by phytochromes.

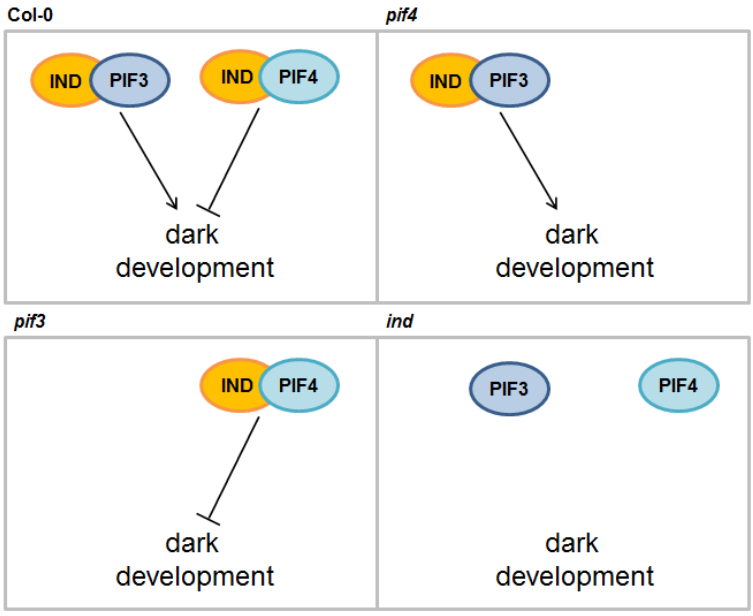


Fig. 6.8. Model of IND interactions with PIF3 and PIF4 to regulate dark development. In Col-0, IND-PIF3 and IND-PIF4 activities cancel out, no net effect on dark development. In *pif4*, dark development is increased through IND-PIF3 activity. In *pif3*, dark development is reduced through IND-PIF4 activity. In *ind*, PIF3 and PIF4 are unable to regulate dark development.

Recently PIFs were shown to form a negative feedback loop with HECs to fine-tune photomorphogenesis (Zhu et al., 2016b). HEC1/2 interacted directly with PIF1/3/4 to repress PIF activity. The dark development data do not support a similar model for PIF-IND interactions. However, HECs and IND have opposite activities on gene expression for genes such as *SWT15*, so might have evolved opposite regulatory roles for PIF interactions. Analysis of gene expression datasets showed that IND and PIF4 positively regulate *SWT15* expression, whereas HEC1 negatively regulates *SWT15* expression (Oh et al., 2012; Schuster et al., 2014; Simonini et al., 2016). Therefore, IND might act antagonistically to HECs in the regulation of PIF activities.

The *arf3* mutant also had decreased dark development (Fig. 6.5), but this was not apparent in a repeat where Col-0 dark development was lower (Fig. 6.6). However, characterisation of other *ARF* mutants revealed that mutants of *ARF4*/*5* which are among the subset of ARFs repressed by Aux/IAAs (Piya et al., 2014), and a constitutively active allele of *IAA1*, which represses ARFs including ARF4 and ARF5 (Piya et al., 2014), increased dark development in the same pathway as LatB-treatment. This indicates that ARF4 and ARF5 repress SAM activity, following the establishment of auxin maxima. The finding that ARF4 and ARF5, which are repressed by Aux/IAAs, repress lateral organ initiation is quite significant, although I am not the first to show that gain-of-function Aux/IAA alleles have increased dark development (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000). It goes against the current widely held view that lateral organ

initiation is triggered by auxin maxima, and indicates that organ initiation precedes the establishment of auxin maxima, suggesting that lateral organ initiation in fact begins by the establishment of auxin minima, which precede auxin maxima, and which cause the repression of ARF4/5 activity.

I propose the following model for the initiation of lateral organs stimulated by light. In darkness, the closure of stomata and consequent reduction in transpiration (Caird et al., 2007), reduces the translocation of cytokinin from the roots to the SAM (Aloni et al., 2005; Beck and Wagner, 1994). The reduction in cytokinin and gradual decline in starch-derived sugars in the SAM reduces cell cycle gene expression throughout the night (Richard et al., 2002). In prolonged darkness without exogenous sugars, starvation responses trigger repression of auxin signalling (Yoshida et al., 2011) by TOR (Deng et al., 2016; Schepetilnikov et al., 2013). PIN1 is internalised leading to an initial accumulation of auxin in the SAM (Lauxmann et al., 2016; Lopez-Juez et al., 2008; Sassi et al., 2013; Yoshida et al., 2011). Auxin accumulation antagonises the establishment of auxin minima in the dark, and ARF4/5 actively repress lateral organ initiation, such that lateral organ initiation effectively ceases (Yoshida et al., 2011; Roldan et al., 1999; Fig. 4.4) and the SAM enters a quiescent state which can be reactivated by perception of light (Yoshida et al., 2011). Light reactivates TOR downstream of photoreceptors, COP1 and ROP2 (Cai et al., 2017; Li et al., 2017), to repress starvation responses and reactivate auxin signalling (Schepetilnikov et al., 2017; Xiong and Sheen, 2012; Yoshida et al., 2011). Additionally, light stimulates transpiration and translocation of cytokinin to the SAM (Aloni et al., 2005; Beck and Wagner, 1994), which together with auxin and photosynthesis-derived sugars, stimulates the cell cycle (Hartig and Beck, 2006; Richard et al., 2002). PIN1 is also targeted to the plasma membrane downstream of COP1, auxin and ROP2 (Cai et al., 2017; Nagawa et al., 2012), and excess PIN1 is targeted for degradation in the vacuole downstream of cytokinin and AHK4 (Marhavy et al., 2011). Note, overexpression of PIN1 prevents the establishment of functional auxin gradients (Benkova et al., 2003). Under these conditions, PIN1 and auxin co-direct auxin gradients, leading to the formation unevenly distributed auxin levels, including auxin minima (Heisler et al., 2005), which cause ARF4/5 to be repressed by Aux/IAAs, promoting leaf initiation. I predict that these are the zones in which the expression of Class I KNOX transcription factors such as *KNAT1* and *RPL* are repressed preceding lateral organ initiation (Byrne et al., 2003; Jackson et al., 1994; Lincoln et al., 1994) downstream of ARFs (Schuetz et al., 2008; Tabata et al., 2010). Auxin maxima, then, regulate later (albeit early) stages of lateral organ initiation, such as phyllotaxis (Reinhardt et al., 2003), and the promotion of new auxin minima (Heisler et al., 2005; Reinhardt et al., 2003).

The difference between this model and the currently established dogma, is that auxin maxima do not promote lateral organ initiation, as suggested by a couple of landmark papers (Benkova et al., 2003; Heisler et al., 2005), and perpetuated on this assumption (Capua and Eshed, 2017; de

Reuille et al., 2006; Griffiths and Halliday, 2011; Vernoux et al., 2010), instead, leaf initiation begins with auxin minima. This model explains why auxin does not promote development in the dark. It also fits with the observation that the auxin-deficient *taa1tar1* mutant more readily formed leaves in the dark, when stimulated with LatB (Fig 5.2). Very recently, a similar conclusion has been made for the transition between cell division and differentiation in root cells, which requires an auxin minimum (Di Mambro et al., 2017), except that ARFs were not studied specifically, whilst the requirement for an auxin minimum in axillary meristem initiation has been known for several years (Wang et al., 2014a; Wang et al., 2014b). Therefore, I propose that, contrary to the current dogma, leaf initiation begins with auxin minima.

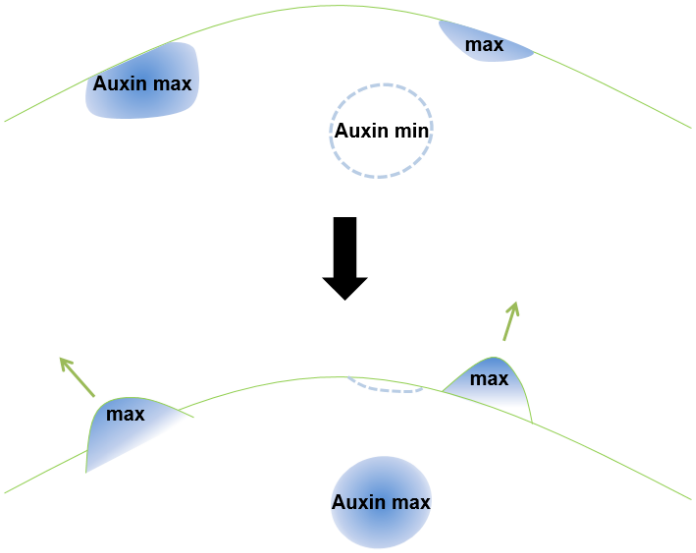


Fig. 6.9. Model of auxin levels and leaf initiation at the SAM. At auxin maxima, ARF4/5 transcription factors are active, the growth of predefined leaf initials is promoted (observed leaf initiation). At auxin minima, ARF4/5 are repressed, the homeobox genes e.g. KNAT1/RPL are locally downregulated, and new leaf initiation sites are defined (leaf initiation).

Classical and chemical genetics approaches were taken to elucidate signalling pathways involved in shoot apical meristem activity in the dark. The dark development assay had the advantage of high sensitivity (see, for instance, Suppl. Fig S.2), and consistency (Fig. 3.4), and facile manipulation of signalling pathways through a combination of classical and chemical genetics. The long-term nature of the assay meant that immediate effects of treatments could not be examined, and interpretation of the data should always consider that downstream effects could be being observed, so a comprehensive understanding of the signalling pathways involved is very beneficial. Shoot apical meristem activity is known to be repressed by the ACTIN RELATED PROTEIN2/3 (ARP2/3) complex in the dark (Baier et al., 2004), but the mechanism and signalling pathway were not known. LatB treatment was found to recapitulate the effect of the *arp3* mutation on true leaf initiation in the dark, but LatB treatment did not affect the dark development of *arp3* plants (Fig 3.10 A). One reason for the LatB insensitivity could be that the dark development of *arp3* had already plateaued at maximum leaf initiation rate, but this was disproven by treatment with cytokinin, which increased the dark development of *arp3* substantially (Fig. 5.1 B). Therefore it was concluded that LatB and *arp3* increase dark development in the same pathway. Subsequently, LatB treatment was often utilized to infer involvement in the ARP2/3 signalling pathway. However, as well as phenocopying *arp3* dark development, LatB treatment had ARP2/3-independent effects on growth (Fig. 3.10 B). Therefore, because of the potential for non-specificity, classical genetics was used (i.e. stably integrating the *arp3* mutation into a mutant background), where possible, to confirm results of the more convenient chemical genetics approach (utilizing LatB, in this case).

LatB and the *arp3* mutation are both known to adversely affect actin filamentation (Mathur et al., 1999; Szymanski et al., 1999). Since the *arp3* mutation was sufficient to mediate the maximal effect of LatB treatment on dark development (Fig 3.10 A), it is likely that ARP2/3-dependent F-actin branching, and not F-actin more broadly, or other potential activities of the ARP2/3 complex, is specifically involved in the repression of dark development. It is assumed that LatB does not interfere with the integrity or action of the ARP2/3 complex or activating WAVE complex directly, but solely interacts with and interferes with actin polymerisation, consistent with gel filtration data indicating the integrity of the bovine Arp2/3 complex in the presence of LatA (Gaucher et al., 2012), which binds the same cleft of actin as LatB (Helal et al., 2013). Further evidence for ARP2/3 specificity came from the lack of dark development phenotypes of a selection of other actin-related mutants, however, redundancy within the *FORMIN HOMOLOGY*, *VILLIN*, and *PROFILIN* gene families could not be excluded. The precise role for ARP2/3-dependent F-actin branching in dark development required further investigation.

This study identified several genetic interactions between the ARP2/3 complex and other signalling genes (*PhyB*, *HXK1*, *FBA2*, *PIN1*, *PID*, *DET2*, and by inference from LatB data, *ARF4*, *ARF5*, *RPL*). Arguably the most striking genetic interaction identified in this work was the complementation of *det2* phenotypes by *arp3* (Fig. 5.8). *det2* is a mutant in the brassinosteroid biosynthesis pathway (Chory et al., 1991b). This strongly indicates that the ARP2/3 complex is repressed by brassinosteroid signalling, perhaps to promote development through polar auxin transport and downstream signalling (chapter 5). That the ARP2/3 complex is repressed by brassinosteroid signalling was partially demonstrated in chapter 5, from transcriptome datasets in Arabidopsis and a phosphorylatome dataset in rice. To provide further evidence that the ARP2/3 complex is repressed by brassinosteroid signalling, a reporter assay that measured the change in transcript or protein levels of ARP2/3 and WAVE components could be utilized. Additionally, it would be useful to design an assay that measured the direct output of ARP2/3 complex activity, such as a BifC assay that revealed the interactions between ARP2/3 and nascent F-actin. It would also be interesting to determine whether the Arabidopsis WAVE complex is dephosphorylated in response to eBL, as the rice WAVE complex is (Fig 5.9; Hou et al., 2017). If so, the next step would be to determine which kinases and phosphatases interact with WAVE in an eBL dependent manner, as reported for other effectors of the brassinosteroid signalling pathway (He et al., 2002; Bernardo-Garcia et al., 2014). Moreover, to determine the effect of phosphorylation on WAVE activity and localisation, phosphomimics and phosphomutants of WAVE components could be introduced into the relevant mutant background, with a fluorescent reporter, and the effects on localisation and interaction with ARP2/3 complex, or simply the effects on dark development, as a measure of ARP2/3 complex activity, could be determined.

Additionally, the interactions between *arp3* and *phyB* (chapter 3) and *arp3* and *det2* (chapter 5) suggest that the ARP2/3 complex represses brassinosteroid signalling, including BZR1 and PIF4, as a *PIF4* overexpression-like response was observed in the *arp3phyB* background, and a brassinosteroid-signalling-like response was observed in the brassinosteroid-deficient *arp3det2* background. Immunoblot analysis could be used to investigate whether the protein levels of BZR1/PIF4 are stabilized in *arp3*; additionally, calf intestinal phosphatase treatments could be used to identify levels of BZR1/PIF4 phosphorylation, which is another indicator of brassinosteroid signalling activity (Bai et al., 2012; Gendron et al., 2012). The phosphorylated (inactive) forms of BZR1 and PIF4 should be excluded from the nucleus (Bai et al., 2012; Gendron et al., 2012), so the nuclear or cytoplasmic localisation of these transcription factors could also be examined in Col-0 and *arp3* using fluorescent reporters. A *pBZR1::BZR1-CFP* line (Wang et al., 2002) is available from NASC (N65991). Additionally, the expression of BZR1 and PIF4 target genes should be investigated in the *arp3* background as another indicator of upregulated BZR1/PIF4 activity.

A couple of lines of evidence indicate that ARP2/3-dependent endocytosis might be mediating the dark development response. The Arp2/3 complex has been shown to be involved in endocytosis of a variety of organisms, including Arabidopsis (Epp et al., 2010; Leyton-Puig et al., 2017; Moreau et al., 1997; Moreau et al., 1996; Zou et al., 2016). In Arabidopsis, an *arp3* mutant was shown to have much delayed endocytosis of PIN2 and the dye FM4-64 (Zou et al., 2016). The requirement of *PIN1* for the dark development response of *arp3* (Fig. 5.6), indicates that PIN1 endocytosis might be defective in *arp3*, since PIN1 at the SAM is predominantly localised to endosomes during prolonged darkness (Lauxmann et al., 2016; Sassi et al., 2013; Yoshida et al., 2011). If this is the case, it would be expected that PIN1 accumulates at the plasma membrane of dark developed *arp3* shoot apices, relative to Col-0. This could be tested using a *pPIN1::PIN1-GFP* reporter (Benkova et al., 2003) in the Col-0 and *arp3* backgrounds.

Another possible role of ARP2/3 complex-mediated endocytosis could be for regulating the assembly of cell walls during leaf initiation. Cell divisions require the polar loosening of cell wall structures (Yang et al., 2016), through auxin-mediated "acid growth" (Perrot-Rechenmann, 2010), the downstream action of wall-loosing factors including expansins (Cosgrove, 2016), and changes in cell wall composition (Yang et al., 2016). Cell wall composition has been shown to be important for leaf initiation, with mutants in fucose, arabinose, suberin and cellulose biosynthesis having reduced cell wall integrity (Li et al., 2007) and increased dark development (Baier et al., 2004; Li et al., 2007; Wang et al., 2015). Cell wall components are transported to the plasma membrane by vesicular trafficking, where they are secreted for subsequent cell wall synthesis (Kim and Brandizzi, 2016). Additionally, cell wall materials including pectins and xyloglucans are recycled by an endocytic pathway (Baluška et al., 2005; Dhonukshe et al., 2006). Furthermore, cell wall modifying enzymes, such as the cellulose synthase complex, are transported to and from the plasma membrane by vesicle trafficking (Bashline et al., 2013). The potential for the ARP2/3 complex to regulate cell wall composition through the trafficking of cell wall sugars or wall-modifying enzymes should be investigated. The *CESA6-YFP* reporter (Ivakov et al., 2017) could be used to visualise *ARP3*-dependent trafficking of vesicles containing cell wall components from the golgi apparatus to the plasma membrane. Such a study is warranted since it has already been shown that an *arp2* mutant has alterations in cell wall composition specifically at sites of WAVE localisation (Dyachok et al., 2008), and phenotypes associated with weakened cell walls (see chapter 4.6).

For the ARP2/3 complex to repress dark development by promoting both cell wall strength and recycling of PIN1 (Fig. 7.1) makes sense biologically, since both cell wall strengthening and PIN1 internalisation are associated with reduced cell division and development (Li et al., 2007; Yang et al., 2016; Yoshida et al., 2011). The endocytosis of PIN1, the cellulose synthase complex, and various cell wall materials is known to be mediated through a clathrin-dependent pathway

involving ARA7 and AP-2 (Dhonukshe et al., 2006; 2007; Fan et al., 2013; Kim and Brandizzi, 2016; Nagawa et al., 2012). Moreover, LatB treatment inhibited the endocytosis of PIN1, causing PIN1 accumulation at the plasma membrane (Geldner et al., 2001), and recapitulated *arp3* dark development in a *PIN1*-dependent manner (chapters 3, 5), indicating that the ARP2/3 complex is required for the endocytosis of PIN1 during prolonged darkness (Lauxmann et al., 2016; Sassi et al., 2013; Yoshida et al., 2011). It would be interesting to test whether mutants specifically defective in clathrin-mediated endocytosis such as *chc2-1* (Kitakura et al., 2011) also have increased dark development. This would support a role of the ARP2/3 complex in clathrin-mediated endocytosis, and the importance of endocytosis for dark development.

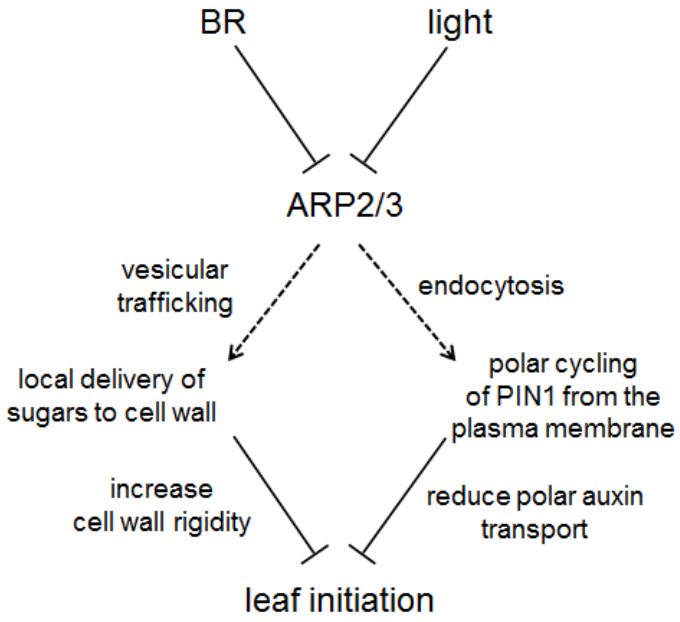


Fig. 7.1. The ARP2/3 complex plays a central role in a pathway in which light and brassinosteroid signalling promote leaf initiation by coordinately increasing local polar auxin transport and cell wall flexibility at the SAM.

ARF4/*5* and *TOR* were also important for dark development (chapters 4, 6). *AUXIN RESPONSE FACTOR* mutants (*arf4*, *arf5*) increased dark development in the same pathway as LatB, indicating that ARF4 and ARF5 repress SAM activity in the ARP2/3 pathway (Fig. 6.6). *TOR* was required for dark development and the *arp3* dark development phenotype (Fig. 4.4). Both ARFs and TOR are important for mediating auxin responses (Deng et al., 2016; Schepetilnikov et al., 2013; Vernoux et al., 2011; Xiong et al., 2013; Xiong and Sheen, 2012), and therefore may act downstream of polar auxin transport in the dark development pathway. However, due to the

long term nature of the dark development assay, it could not be determined whether ARFs or TOR were regulated by *arp3* or whether they were acting in concert to affect dark development. The ability of the *arf4* and *arf5* mutants to increase dark development (Fig. 6.6), demonstrates that auxin signalling is important for regulating dark development. The role of ARF4 and ARF5 in dark development is consistent with previous literature showing that dominant mutants of AuxIAAs have increased dark development (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000). In this study, when treated with LatB, *iaa1-D*, *arf4* and *arf5* all showed a wild-type dark development response (Fig. 6.6), indicating that auxin signalling acts in the same pathway as the ARP2/3 complex to regulate dark development. This is the first time that the two bodies of literature covering the ARP2/3 complex and auxin signalling in dark development have been connected.

That negative regulators of auxin signalling promote dark development (Kim et al., 1998; Kim et al., 1996; Nagpal et al., 2000) contradicts the dogma that auxin maxima promote leaf initiation (Benkova et al., 2004; Reinhardt et al., 2000). Therefore, in chapter 6 I proposed that the auxin minima preceding auxin maxima are required for the initiation of leaves. One of the core concepts of this hypothesis is the transcriptional reprogramming at areas of auxin minima, which precedes the growth that follows subsequent auxin maxima formation. It would be interesting to investigate whether the transient local exclusion of *KNAT1*/*RPL* transcripts (Byrne et al., 2003; Jackson et al., 1994; Lincoln et al., 1994) occurs at auxin minima, rather than auxin maxima. This could be determined using fluorescent reporters to detect *KNAT1*/*RPL* transcripts and concurrently report auxin levels using *DII-VENUS* (Brunoud et al., 2012). This would provide insight into the hypothetical pathway that determines new leaf initials by transcriptional reprogramming at sites of auxin minima at the SAM.

In summary, my data have highlighted several aspects of ARP2/3 signalling in Arabidopsis that have not been demonstrated before, including the regulation of brassinosteroid responses, the requirement for PIN1 activity, TOR kinase, and the initial stages of glycolysis, positioning the ARP2/3 complex as a central node of several pathways for the control of SAM activity. Additionally, I showed that CK-666 is not a useful inhibitor of the Arabidopsis ARP2/3 complex, at least for whole plant studies. My data have also revealed genetic redundancy between *IND* and *HEC2*, genetic interactions between *IND* and *PIF3*/*4*, novel roles of brassinosteroids and *PIFs* in the regulation of SAM activity, and has led to the generation of some interesting hypotheses that can be tested.

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Supplementary Figures

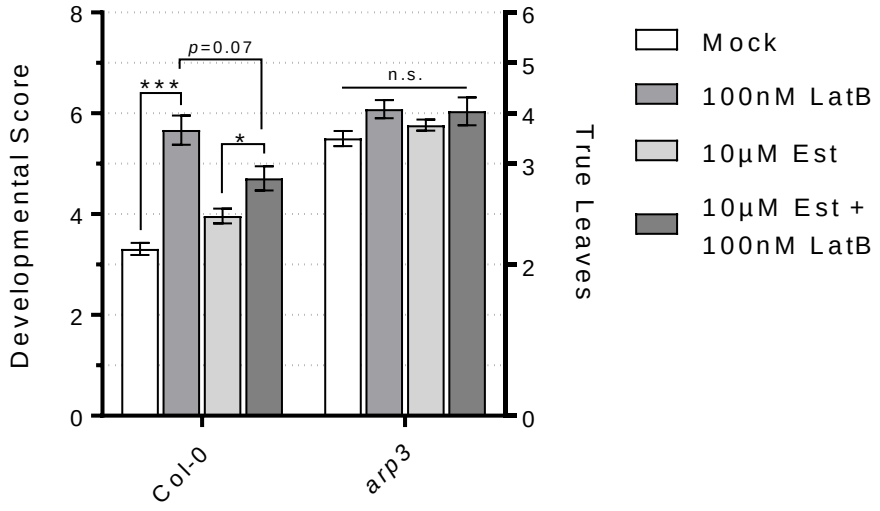

Fig. S.1. Estradiol compromised the effect of LatB on dark development. Statistical significance was calculated using 2way ANOVA and Tukey multiple comparisons test; $n > 15$.

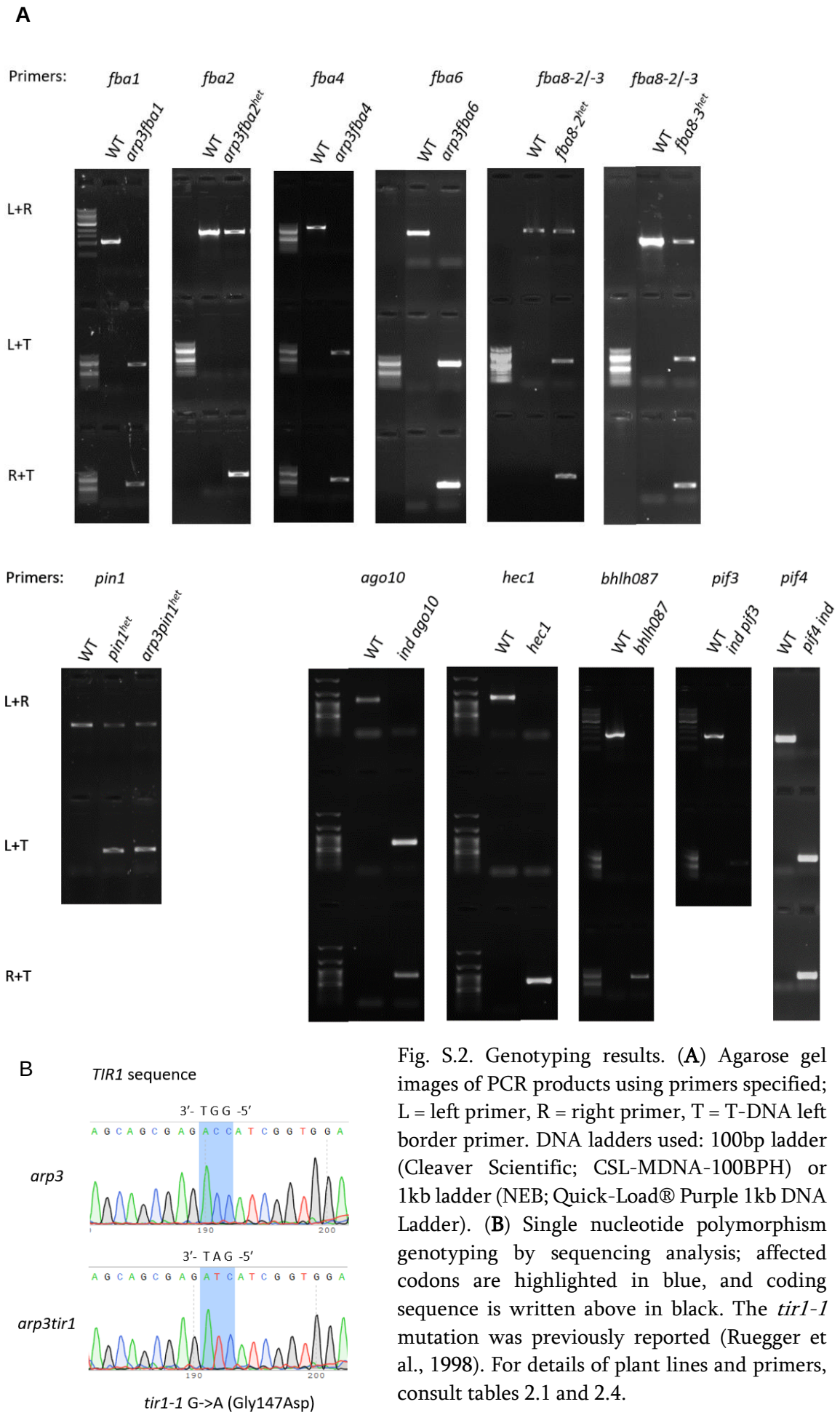

Fig. S.2. Genotyping results. (**A**) Agarose gel images of PCR products using primers specified; L = left primer, R = right primer, T = T-DNA left border primer. DNA ladders used: 100bp ladder (Cleaver Scientific; CSL-MDNA-100BPH) or 1kb ladder (NEB; Quick-Load® Purple 1kb DNA Ladder). (**B**) Single nucleotide polymorphism genotyping by sequencing analysis; affected codons are highlighted in blue, and coding sequence is written above in black. The *tir1-1* mutation was previously reported (Ruegger et al., 1998). For details of plant lines and primers, consult tables 2.1 and 2.4.

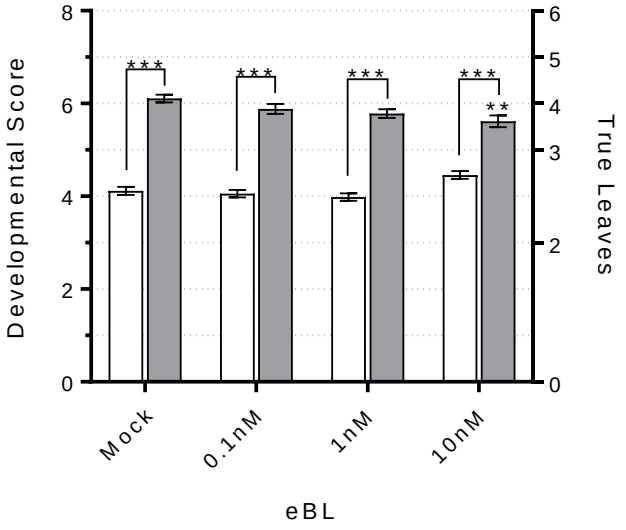

Fig. S.3. *arp3* shows a trend towards eBL sensitivity at 0.1nM. White bars Col-0, grey bars *arp3*. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test.

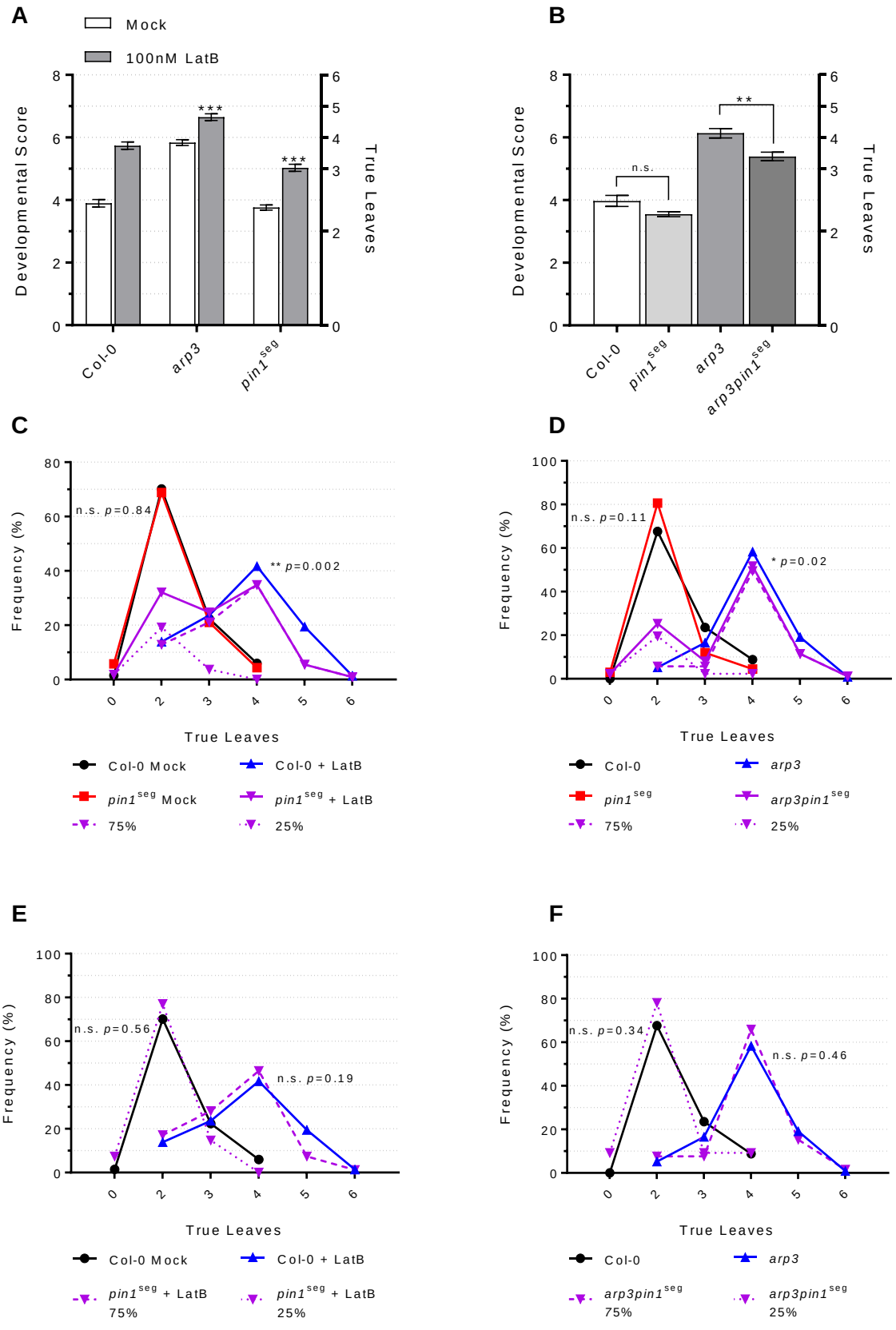
A
Mock
100nM LatB
Developmental Score
True Leaves
***
***
Col-0
arp3
pin1seg
B
Developmental Score
True Leaves
n.s.
**
Col-0
pin1seg
arp3
arp3pin1seg
C
Frequency (%)
n.s. p=0.84
** p=0.002
True Leaves
Col-0 Mock
Col-0 + LatB
pin1seg Mock
pin1seg + LatB
75%
25%
D
Frequency (%)
n.s. p=0.11
* p=0.02
True Leaves
Col-0
arp3
pin1seg
arp3pin1seg
75%
25%
E
Frequency (%)
n.s. p=0.56
n.s. p=0.19
True Leaves
Col-0 Mock
Col-0 + LatB
pin1seg + LatB
75%
pin1seg + LatB
25%
F
Frequency (%)
n.s. p=0.34
n.s. p=0.46
True Leaves
Col-0
arp3
arp3pin1seg
75%
arp3pin1seg
25%

Fig. S.4. Phenotypic segregation of *pin1*. The dark development of mock (**A**, **C**) and untreated (**B**, **D**) *pin1* (seg) was not significantly different to wild type and showed a wild type frequency distribution. LatB-treated *pin1* (seg) and *arp3pin1* (seg) had bimodally distributed developmental scores (**C**, **D**), which was consistent with a 3:1 segregation of the *pin1* allele (**E**, **F**). Purple dashed lines show the 75% of *pin1* (seg) + LatB (**C**, **E**) or *arp3pin1* (seg) (**D**, **F**) developmental scores that most closely match the frequency profile of the Col-0 + LatB/*arp3* controls. The remaining 25% of developmental scores are represented by purple dotted lines; these values were assumed to be representative of homozygous *pin1*/*arp3pin1* development, and were used to generate average developmental scores in Fig. 5.6. Statistical significance was calculated by 2way ANOVA and Tukey multiple comparisons test (**A**, **B**), or χ^2 test (**C**-**F**) comparing observed with observed, or observed with expected numbers of plants with 0-2, 3, 4, 5-6 true leaves. (A, C) $n >$ 67; *pin1*^{seg} $n = 109$; (B, D) $n > 30$; *pin1*^{seg} $n = 134$; *arp3pin1*^{seg} $n = 87$.

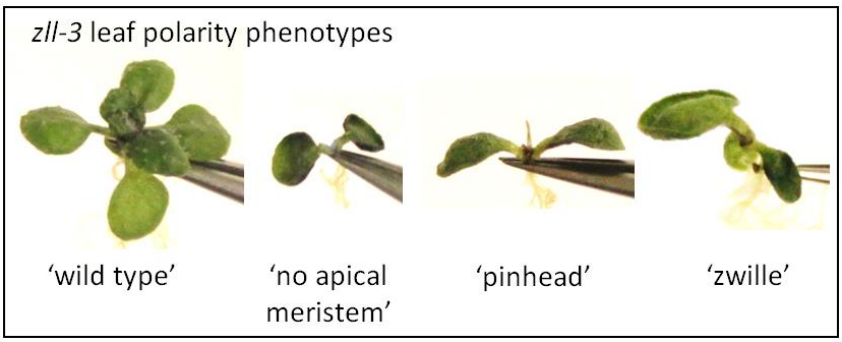

Fig. S.5. Leaf polarity phenotypes of *ago10* (*zll-3*) mutants. Approximately three quarters of *ago10* (*zll-3*) mutants in the L*er* ecotype (much fewer in the Col-0 ecotype) had developmental abnormalities ranging from 'no apical meristem' to a single central first true leaf 'zwille' (zwille is German for slingshot), as previously reported (Moussian et al., 1998). Seedlings were grown for 10 days on ½ MS agar in constant light. The *zll-3* 'wild type' phenotype resembles the L*er* control.