The Role of Auxin in Cell Specification during Arabidopsis Embryogenesis

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Contents

Chapter

Introduction

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ABSTRACT

The basic mechanism of auxin as a modulator of gene expression is now well understood. Interactions among three components are required for this process. Auxin is first perceived by its receptor, which then promotes degradation of inhibitors of auxin response transcription factors. These in turn are released from inhibition and modify expression of target genes. How this simple signaling pathway is able to regulate a diverse range of auxin responses is not as well understood, but a clue lies in the existence of large gene families for all components. Recent data indicates that diversification of gene expression patterns, protein activity and protein-protein interactions among components generates a matrix of response machineries that directs specific outputs to the generic auxin signal.

INTRODUCTION

The plant hormone auxin plays an important regulatory role in virtually every aspect of plant growth and development. At the core of the auxin response is the auxin receptor that interacts with and promotes the degradation of one of two antagonistically acting transcription factors. Global analyses of gene expression following the application of exogenous auxin, as well as in auxin signaling mutants, has identified hundreds of genes as being regulated both positively and negatively, e.g. (Tian et al., 2002; Okushima et al., 2005b; Bargmann and Birnbaum, 2009). This indicates that dynamic control of both gene activation and repression are integral to auxin function.

A plethora of research over the past decades has provided us with an inherently simple model of the mechanism of auxin action (Figure 1a). Auxin is now commonly referred to as 'molecular glue' as it enhances interactions between an auxin receptor and a family of transcriptional inhibitors known as Auxin/Indole-3-Acetic Acids (Aux/IAAs) (Tan et al., 2007). The TRANSPORT INHIBITOR RESISTANT1/AUXIN F-BOX (TIR1/AFB) auxin receptor is a subunit of the SKP1 - CULLIN1 - F-BOX $(SCF)^{TIR1/AFB}$ ubiquitin ligase complex and binding of Aux/IAAs triggers their ubiquitin-mediated degradation (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Aux/IAAs themselves bind and inhibit AUXIN RESPONSE FACTORS (ARFs) which are the DNA-binding transcription factors capable of directing the expression levels of auxin-responsive genes. As the auxin concentration in a cell directly determines the Aux/IAA protein abundance and resulting ARF activity, the pathway from auxin to changes in transcription is quite short.

Auxin controls inherently different cellular responses, including cell expansion and division, as well as changes in the developmental fate of the cell. Particularly in the latter case, auxin can promote different cell-fate specification events, depending on where the cell happens to be. For example lateral root initiation is activated in pericycle cells (Dubrovsky et al., 2008), while root formation is

promoted in basal embryo cells (Weijers et al., 2006), and cotyledon initiation is triggered in apical embryo cells (Hardtke et al., 2004). Yet, all these responses are disrupted in *tir1/afb* auxin receptor mutants (Dharmasiri et al., 2005b). Hence a major outstanding question is how specificity is generated in such a brief, simple pathway. Here we review recent literature that sheds light on how specific gene expression responses are elicited by auxin. Even though the pathway is brief, each of the three major components (TIR/AFB receptor, Aux/IAA and ARF) is represented by a sizable gene family (Figure 1b,c). Specificity can therefore be generated by regulation of gene expression patterns, variations in component activity and in interactions between components. We will consider each of these in the following sections.

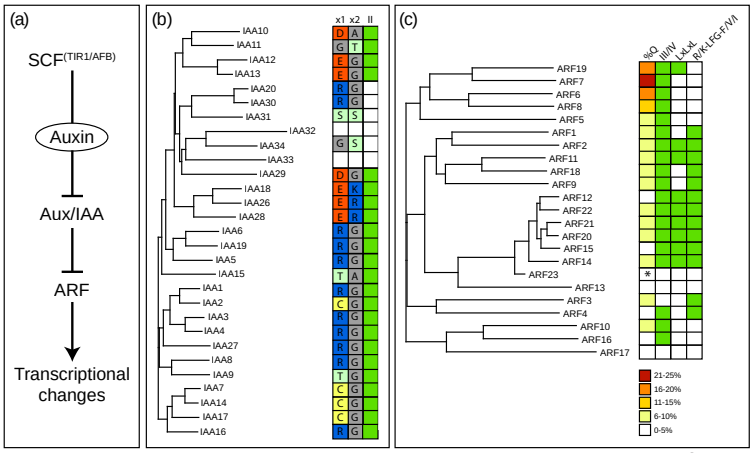

Figure 1. Auxin-dependent gene regulation and annotation of transcription components involved.

(a) Auxin enhances the interaction between the SCF$^{(TIR1/AFB)}$ E3 ubiquitin ligase and domain II of Aux/IAA proteins, making Aux/IAA levels responsive to auxin concentration inside the cell. Aux/IAAs function to inhibit ARFs, DNA binding transcription factors that directly modify the transcription of auxin responsive genes. Hence, an increase in auxin concentration will release ARFs from Aux/IAA inhibition. (b) Phylogenetic relationship and annotation of the 29 *Arabidopsis* Aux/IAA proteins. The first two columns indicate amino acids found at positions 2 and 4 of the LxLxL motif in domain I (x1 and x2 respectively). R = arginine, C = cysteine , T = threonine, E = glutamic acid, D = aspartic acid, G = glycine, S = serine, K = lysine, A = alanine, empty boxes = no motif present. Basic amino acids are highlighted blue, cysteine yellow, acidic amino acids red, neutral amino acids gray, and polar amino acids green. A presence/absence score (green/white) for the domain II degron is shown in the third column (II). (c) Phylogenetic relationship and annotation of the 23 *Arabidopsis* ARF proteins. The percentage of glutamine (%Q) in the middle region (defined as the region between the last conserved amino acid in the DNA-binding domain and the first conserved position of domains III/IV) is depicted in the first column (see color legend) followed by a presence/absence score (green/white) for domains III/IV, an LxLxL motif, and the R/K-LFG-V/I/F motif. * = no middle region. Note that we scored for R/K-LFG-V/I/F, where the last amino acid is hydrophobic, rather than the more stringent R/K-LFG-V (Ikeda and Ohme-Takagi, 2009).

Auxin prepatterns - Regulation of component gene expression

The TIR1/AFB auxin receptors form a small subclade of six genes (TIR1 and AFB1-5) in the *Arabidopsis thaliana* (*Arabidopsis*) F-box gene family. Of these, four (TIR1 and AFB1-3) have been shown to act as auxin receptors (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Dharmasiri et al., 2005b), and do so redundantly *in planta* (Dharmasiri et al., 2005b). There is limited diversification of the gene expression patterns and it is therefore likely that the receptor component is rather generic (Dharmasiri et al., 2005b). Despite this, the level of receptors still offers a node for globally modulating auxin sensitivity. For example, microRNA393 is induced by bacterial infection, and negatively regulates receptor abundance and hence auxin signaling, contributing to antibacterial resistance (Navarro et al., 2006). Conversely, limiting concentrations of inorganic phosphate induce *TIR1* gene expression and enhance Aux/IAA degradation, releasing ARF19 and ultimately modifying the expression of genes involved in lateral root initiation (Pérez-Torres et al., 2008).

With 29 and 23 respective members in *Arabidopsis*, the Aux/IAA and ARF components offer by far the most opportunities to generate diversity, provided that the different proteins encoded by the gene families have unique or at least quantitatively distinct functions (discussed below). No systematic analysis of gene expression patterns has been reported for either of these families in *Arabidopsis*, but a relatively comprehensive survey of rice Aux/IAA and ARF expression at tissue resolution suggest fairly diverse patterns of expression for both families (Song et al., 2009). This in turn means that different sets of Aux/IAAs and ARFs are to be expected in different tissues. Such tissue-specificity is supported by the analysis of *aux/iaa* gain-of-function, and *arf* knockout mutants in *Arabidopsis*. Mutations in *Aux/IAA* genes stabilize the protein by preventing interactions with the receptor (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Several such mutants were recovered in genetic screens and show very diverse phenotypes (Reed, 2001). For example, the bodenlos (*bdl*)*/iaa12* mutation interferes with primary root formation (Hamann et al., 2002), while the solitary root (*slr*)*/iaa14* mutation disrupts lateral root formation (Fukaki et al., 2002), and the short hypocotyl2 (*shy2*)*/iaa3* mutation interferes with hypocotyl elongation (Tian and Reed, 1999). In these cases, the differences between mutant phenotypes correspond to different expression patterns of the *Aux/IAA* genes.

Likewise, even though most *arf* single mutants do not have a phenotype (Okushima et al., 2005b), those that do are quite distinct (e.g. monopteros (*mp*)*/arf5* (Hardtke and Berleth, 1998); *arf8* (Goetz et al., 2006); *arf2* (Okushima et al., 2005a). In each case, this is also accompanied by differing expression patterns. Patterns of ARF accumulation are further elaborated through post-transcriptional mechanisms. It was recently shown that a trans-acting small interfering RNAs (tasiRNA) that specifically directs *ARF2-4* mRNAs acts to limit ARF3 activity to the abaxial side of the leaf (Chitwood et al., 2009). Several other *ARF* transcripts

(*ARF6, 8, 10, 16* and *17*) are targets of microRNA regulation (Wang et al., 2005; Wu et al., 2006).

The cellular complexity of auxin response transcription factors can now only be inferred from various sources of data; appreciation of the complete picture awaits systematic gene expression analysis. Nevertheless, available evidence suggests that diversification of *Aux/IAA* and *ARF* gene expression patterns contributes significantly to generating specificity in auxin-dependent gene regulation.

Are all Aux/IAAs created equal?

Both Aux/IAA and ARF protein families share conserved domains. Particularly for the Aux/IAAs, these conserved domains constitute a large part of the proteins, leaving less opportunity for qualitative diversification. Three functions have been assigned to conserved Aux/IAA domains. Domain I is necessary and sufficient for transcriptional repression (Tiwari et al., 2004), and appears to be important *in vivo* since mutations in domain I of *axr3/iaa17* were recovered as suppressors of the gain-of-function *axr3* phenotype (Rouse et al., 1998). Recently, it was shown that domain I of a number of Aux/IAAs recruits the TOPLESS (TPL) co-repressor. Closer investigation revealed that TPL binds the repression motif (LxLxL) in BDL/IAA12 and is required for BDL/IAA12 to function in hypophysis specification during embryogenesis (Szemenyei et al., 2008). This work suggests that the severe *tpl-1* phenotype, in which the shoot pole is replaced by a second root pole, is the result of effectively removing all Aux/IAAs and that Aux/IAAs function through recruiting TPL. Conserved domain II functions as a 'degron' and confers auxin-dependent instability upon the protein by mediating interactions with TIR1/AFB receptors (Tan et al., 2007). Finally, Aux/IAAs have a C-terminal region called domains III/IV that serve as an interaction domain. These domains mediate homotypic and heterotypic interactions between Aux/IAAs and ARFs.

Interestingly, there are reported cases where expression patterns alone do not account for differences in Aux/IAA function. For example when stabilized versions of *iaa7* or *iaa14* are driven from the *IAA19* promoter they not only confer the same phenotypes as seen in the *iaa19* mutant but result in additional, completely novel developmental phenotypes (Muto et al., 2007). Conversely, when *iaa12* is expressed from the *IAA3* promoter, this protein confers only part of the phenotypes that are conferred by *iaa3* (Weijers et al., 2005). This shows that there must be intrinsic differences between Aux/IAA proteins. These could cause different affinities for TPL, difference in (residual) degradation rate or different affinities for other Aux/IAAs or ARFs, but it remains to be addressed which of these factors contribute to Aux/IAA diversification. At least in principle, each could contribute: variations at the two 'x' positions in the TPL-interaction motif LxLxL (Szemenyei et al., 2008; Figure 1b) could certainly cause differences in affinity; the half-lives of Aux/IAAs are 8-80 min (reviewed in Dreher et al., 2006);

and there are many non-conserved positions in domains III/IV.

Strikingly, Aux/IAAs exist that lack one of the conserved domains (Remington et al., 2004; Figure 1b), and therefore cannot act in a canonical auxin-signaling pathway. The function of these is currently unknown, but non-canonical Aux/IAAs also exist in other genomes, including rice and poplar (Jain et al., 2006; Kalluri et al., 2007). The role of a subclade of three Aux/IAAs comprised of IAA20, IAA30 and IAA31, which do not contain the domain II degron has been investigated recently. As expected over-expression of these Aux/IAAs lead to auxin-related phenotypes (Sato et al., 2008), which suggests that when overexpressed and/or misexpressed these aberrant Aux/IAAs interfere with endogenous ARF-Aux/IAA interactions. One can as yet only speculate on the biological relevance of these permutations and what role if any they play in auxin-mediated processes. With regards to non-degradable versions of Aux/IAAs this may be an additional means of tempering auxin responses by setting a threshold for the amount of auxin required for ARF activation.

Output control – ARF divergence and activity

Being larger than the Aux/IAAs and having only two conserved domains that make up less than half of the protein, the ARFs are inherently more diverse. ARFs have a B3-type DNA-binding domain at their N-terminus that binds TGTCn(C) Auxin Response Elements (AuxRE) *in vitro* (Ulmasov et al., 1999). To date, only direct target genes have been identified for ARF7 and 19 (Okushima et al., 2005b; Okushima et al., 2007). A completely open question is whether ARFs have overlapping sets of target genes. At least to some extent this must be the case, since closely related ARFs have redundant functions that can only be uncovered in double mutants, e.g. (Kalluri et al., 2007; Ellis et al., 2005). Also, replacing ARF5/MP by the distantly related ARF16 partially complements the *mp* mutant defect, implying that ARF16 can bind to (some) MP targets (Weijers et al., 2005). With the identification of more ARF target genes, e.g. (Cole et al., 2009), we will be able to determine how different/similar ARF DNA-binding domains really are.

At the C-terminus most ARFs also have domains III/IV (Figure 1c) and these domains mediate ARF-ARF and ARF-Aux/IAA interactions. It has been proposed that ARF-ARF dimerization increases DNA-binding affinity, and as a result enhances the amplitude of auxin-dependent gene regulation (Ulmasov et al., 1999). This implicates combinatorial possibilities by interactions among co-expressed ARFs, but also suggests that Aux/IAAs might inhibit ARFs by interfering with ARF-ARF interactions (Figure 2), a model that has not received much attention so far.

The region between the conserved N-terminal and C-terminal domains has been termed 'middle region' and is extremely divergent. It is this region that determines the activity of the ARF. In protoplast assays, those ARFs that have a

relatively glutamine-rich middle region (ARF5-8 and 19; Figure 1c) were able to activate synthetic auxin promoters resulting in their classification as activators. Correspondingly, five ARFs with less pronounced Q-enrichment in their middle regions (ARF1-4 and 9) repressed transcription (Tiwari et al., 2003). Based on amino acid composition the remaining ARFs were somewhat arbitrarily classified as repressors despite a lack of experimental evidence. Clearly there is diversification in the middle regions of ARFs, and since these define the activity of the transcription factor, this is where the auxin signaling output is controlled. However, there are a few difficulties with the simplified classification of ARFs as activators or repressors. Global transcriptome profiling of *arf7 arf19* double mutants showed that auxin-induced changes in expression were lost for most auxin-activated (85% of 203) and auxin-repressed genes (65% of 68). This suggests that ARF7 and ARF19 have the capacity to act as both activators and repressors (Okushima et al., 2005b). In fact, in addition to eight other ARFs, ARF19 contains an LxLxL motif (Figure 1c), that would potentially enable TPL recruitment. This suggests that the convenient binary classification of ARFs as either activator or repressor is oversimplified. Recently another repression motif (R/K-LFGV) was identified in several transcriptional repressors (Ikeda and Ohme-Takagi, 2009) and also in several ARFs. Upon closer inspection of the ARF family we found that R/K-LFG-V/I/F (where the last valine is replaced by another hydrophobic amino acid) was present in all ARFs with the exception of ARF5-8, 10, 16, 17 and 19 (Figure 1c). This motif might enable a more robust classification and may also be a recognition motif for a co-repressor, potentially the SEUSS/LEUNIG complex (Pfluger and Zambryski, 2004). It is notable that both repression motifs identified so far are only five amino acids long. Depending on location in the sequence and the tertiary and quaternary structure of the ARF, one could expect that these motifs can be hidden or exposed, which could explain how ARFs could act as both activators and repressors.

Given the size and heterogeneity of ARFs, there is ample opportunity for diversification of ARF regulation by post-translational modification as well as by selective interactions with other proteins (other than Aux/IAAs and ARFs). Hardly anything is known about either of these phenomena. However, database searches reveal several consensus sites for kinases and predicted SMALL UBIQUITIN-RELATED MODIFIER (SUMO)-ylation motifs in ARFs and it has recently been shown that ARF2 is a substrate of the BRASSINOSTEROID INSENSITIVE2 (BIN2) kinase (Vert et al., 2008). By phosphorylating ARF2, BIN2 inhibits both DNA binding and repression activity. Also the transcription factor MYB77 was found to interact *in vitro* with a number of ARFs via their C-terminal domain and was subsequently confirmed to bind ARF7 *in planta*. The promoter of *IAA19* whose expression is down-regulated in arf7/*nph4-1* and *myb77-1*, contains MYB factor-binding motifs and multiple AuxREs in close proximity suggesting that both proteins bind DNA (Shin et al., 2007).

Synthesis – Updated models of auxin-dependent transcription

In regard to the three major components in auxin-dependent transcription, most heterogeneity and hence specificity is among the Aux/IAA and ARF transcriptional regulators. Here, at the business end of auxin signaling, one could envisage a highly dynamic interplay of Aux/IAAs, the degradation machinery, TPL and other co-repressors, ARFs, modifying enzymes, transcriptional co-factors and the target sites in the DNA. It will be challenging to synthesize a model that encapsulates all these components, and mathematical modeling might be required to grasp the dynamic behavior of this system. Nonetheless, even though few core components are involved in the brief auxin signaling pathway, sufficient divergence must exist to accommodate all different auxin responses seen throughout plant life.

Two issues emerge from this discussion. First, it appears that auxin response is conditioned by a pre-pattern of *Aux/IAA* and *ARF* genes, which considering the intrinsic differences between family members will lead to at least quantitatively distinct auxin responses between different cells. Secondly, we lack models that explain how auxin can promote repression of gene expression. The TPL-based co-repression that is brought upon ARFs when in complex with an Aux/IAA predicts that genes will be actively repressed in the absence of auxin but fails to explain how an Aux/IAA-ARF based system could operate in auxin-induced gene repression. As mentioned, auxin-dependent repression of a number of genes is lost in the *arf7 arf19* mutant; however, from this it cannot be deduced that auxin-dependent repression involves Aux/IAA proteins. We investigated publicly available microarray datasets (Bargmann and Birnbaum, 2009) for examples of Aux/IAA-dependent downregulation of auxin-responsive genes and found a subset of genes whose repression is lost upon stabilization of IAA7 or IAA17. It is therefore likely that an Aux/IAA-ARF module operates in transcriptional repression. The most likely scenario to explain auxin-dependent repression through Aux/IAAs would involve Aux/IAAs interfering with repressor ARF dimerization or higher-order complex formation (Figure 2). In any event *in vivo* studies of ARF-ARF and Aux/IAA-ARF interactions, as well as the identification of physiological targets of a number of ARFs, should help clarify this issue.

Conclusions and perspectives

Auxin is a structurally simple molecule, yet it elicits many different responses in plants. The signal transduction pathway has three major components, a ubiquitin ligase/receptor, ARF transcription factors and their Aux/IAA inhibitors. Here we have reviewed how specificity in the output of auxin signaling can be generated by distinct regulation and the unique properties of the members of the Aux/IAA and ARF transcription factor families. Even though we are only scratching the surface of the potential complexity encoded within these families, substantial specialization is already apparent. Multiple layers of regulation include gene

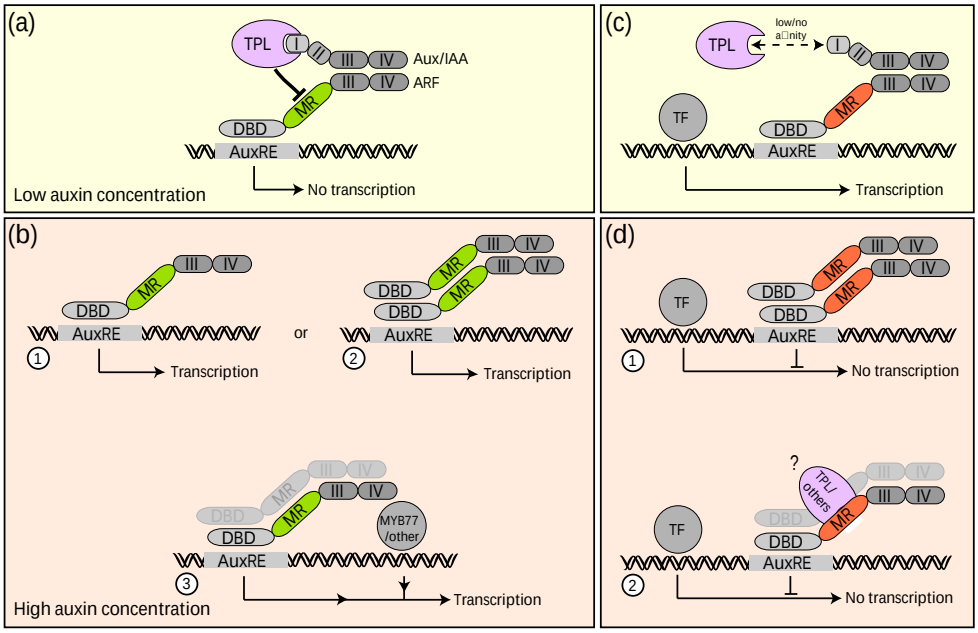

Figure 2. Working models for auxin-induced changes in transcription.
Different models apply for auxin-activated (a,b) or auxin-repressed (c,d) transcription. (a) Under low auxin concentrations, Aux/IAAs are abundant, bind to activator ARFs (note: green middle region) through shared domains III/IV and recruit co-repressor TPL through domain I, hereby repressing the transcription of a subset of auxin responsive genes that contain AuxRE sequences in their promoters. (b) When auxin concentrations increase, Aux/IAAs are rapidly degraded and ARFs are free to activate transcription either as monomers (1) or dimers (2). Release of inhibition could also allow the formation of higher-order complexes with other transcription factors including MYB77 (3). (c) Under low auxin concentrations Aux/IAAs with reduced or no affinity for TPL also inhibit ARFs with repressor activity (note: red middle region), allowing a subset of genes to be activated by other, auxin-independent transcription factors (TF). (d) Auxin-mediated degradation of Aux/IAAs enables repressor ARFs to dimerize (1) and/or complex with unknown co-repressors (2; possibly TPL), ultimately repressing the transcription of these auxin-responsive genes.

expression patterns, post-translational protein modifications and protein-protein interactions, and all contribute to the generation of specificity in auxin response. It is evident that further dissection requires the isolation of physiological target genes whose function is required for the auxin-controlled processes. These, rather than synthetic promoters, should serve as biological models in which the activity and unique properties of Aux/IAAs, ARFs and their domains can be rigorously tested.

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Chapter

A cellular expression map of the Arabidopsis AUXIN RESPONSE FACTOR gene family

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ABSTRACT

The plant hormone auxin triggers a wide range of developmental and growth responses throughout plant life. Most well-known auxin responses involve changes in gene expression that are mediated by a short pathway involving an auxin-receptor/ubiquitin-ligase, DNA-binding ARF transcription factors and their interacting Aux/IAA transcriptional inhibitors. Auxin promotes degradation of Aux/IAA proteins through the auxin receptor and hence releases the inhibition of ARF transcription factors. Although this generic mechanism is now well-understood, it is still unclear how developmental specificity is generated and how individual gene family members of response components contribute to local auxin responses. We have established a collection of transcriptional reporters for the ARF gene family and used these to generate a map of expression during embryogenesis and in the primary root meristem.. Our results demonstrate that transcriptional regulation of ARF genes generates a complex pattern of overlapping activities. Genetic analysis shows that functions of co-expressed ARFs converge on the same biological processes, but can act either antagonistically or synergistically. Importantly, the existence of an "ARF pre-pattern" could explain how cell type-specific auxin responses are generated. Furthermore, this resource can now be used to probe ARF functions in other auxin-dependent processes.

INTRODUCTION

Plant growth and development is profoundly regulated by the plant hormone auxin. External application of auxin alters developmental patterns and growth rate, while decreased auxin levels, such as for example in biosynthesis mutants, impair many developmental processes (Zhao, 2010). Although non-genomic pathways have been identified (Paciorek et al., 2005; Perrot-Rechenmann, 2010), most developmental activities of auxin appear to be mediated through changes in gene expression (Dharmasiri et al., 2005). A complete pathway leading from auxin perception to gene expression has been identified in the last decades (reviewed in Chapman & Estelle, 2009). Auxin binds to the TIR1/AFB subunit of the $SCF^{TIR1/AFB}$ ubiquitin ligase, and increases the affinity of this enzyme for its substrates, the Aux/IAA proteins. The latter become ubiquitinated and degraded by the 26S proteasome (dos Santos Maraschin et al., 2009). Aux/IAA proteins are unstable nuclear proteins (Abel et al., 1994) that bind to and inhibit transcription factors belonging to the AUXIN RESPONSE FACTOR (ARF) family (Kim et al., 1997; Tiwari et al., 2003). Upon Aux/IAA protein degradation in the presence of auxin, ARFs are released from inhibition and can then alter expression of their target genes whose promoters they bind to (reviewed in Chapman & Estelle, 2009). All components (TIR/AFB, Aux/IAA and ARF) of this auxin response machinery are represented by large gene families (Dharmasiri et al., 2005; Remington et al., 2004), whose functional divergence is not yet well-understood.

Auxin action in plants is highly context-dependent. Depending on where auxin accumulates, different cellular responses are elicited, ranging from the specification of various cell identities to altered cell expansion- and/or division rate (reviewed in Perrot-Rechenmann, 2010; Möller & Weijers, 2009; Kieffer et al., 2009). Hence, one of the major remaining questions in auxin biology is how specificity of its response is generated. A plausible scenario would be that members of response component families have diversified and are combinatorially used (Weijers et al., 2005). Examples of this were recently described in lateral root formation, where different Aux/IAA-ARF partners are active at successive steps during the development of a lateral root primordium (De Smet et al., 2010; De Rybel et al., 2010). Combinations between the 6 TIR1/AFB1-5 and 29 Aux/IAA proteins would allow for an enormous array of auxin co-receptor complexes. The many possible Aux/IAA-ARF interactions further elaborate the combinatorial complexity in auxin response while unique properties of the 23 ARFs could allow the precise definition of auxin-dependent gene responses. Previous work has shown that the TIR1/AFB proteins act redundantly in many processes (Dharmasiri et al., 2005), but may have different affinities for Aux/IAA proteins (Prigge et al., 2010). However, if and what role such differential interactions play in defining response specificity has not yet been established. Furthermore, it is conceivable that auxin output specificity is determined by divergence of Aux/IAA and ARF activities, and by differential interactions between and among these families. Here, we have studied the diversification within the ARF family of DNA-binding transcription factors. Several ARF functions have been defined through forward or reverse genetic approaches (reviewed in Guilfoyle & Hagen, 2007). Yet the functions of most ARFs may be masked by redundancy, since single mutants generally do not show phenotypes (Okushima et al., 2005). The potential number of redundant pairs among the 23-member family is vast, and higher-order redundancy would allow enormous numbers of combinations. We reasoned that in order to perform redundant functions, ARFs should at least be co-expressed. However, the availability of resources describing expression of ARF genes at single cell level are very limited. To provide a resource for expression-based reverse-genetic studies on ARFs, we describe here an expression map of the complete Arabidopsis ARF gene family at cellular resolution throughout embryogenesis and in the primary root tip. We find that ARF gene expression is highly diverse, and that phylogenetically distant ARF genes are often co-expressed. Genetic analysis demonstrates that co-expressed ARFs converge on the same developmental processes. Our results suggest the existence of a “pre-pattern” of ARF transcription factors that may underlie the context-dependence of auxin responses observed in plants.

RESULTS

Generating a collection of ARF reporter lines

In order to generate an expression map for all 23 ARF genes at cellular resolution,

we amplified 2 kb of sequence upstream of each ARF start codon, and fused these promoters to SV40-3xeGFP reporter (n3GFP). Due to the SV40 nuclear localization signals, this 3x concatamerized GFP protein localizes exclusively to the nucleus, and generally gives strong fluorescent signals that can easily be assigned to single cells (Takada & Jurgens, 2007). First-generation transgenic plants were selected and the presence of the correct transgene, and integrity of the n3GFP reporter were verified by PCR. Between 5 and 25 independent lines per ARF promoter construct were analyzed by fluorescence microscopy of developing T2 generation embryos and root tips. Two lines were selected that report the representative pattern for each ARF. In some cases, weak expression was found in a single line and no expression in others. In these cases, anti-GFP immunostaining was performed to increase sensitivity of detection. Only those expression patterns that are supported by GFP fluorescence and/or immunostaining signals in several independent lines are represented here. Homozygous lines were generated for each of the selected lines, and the expression patterns were confirmed in subsequent generations. The patterns described in the following are stable over at least two successive generations. This set of 46 ARF promoter-GFP lines (2 lines x 23 ARFs) was used to determine the expression of each ARF during several stages of Arabidopsis development.

Diversity of ARF expression patterns during embryogenesis

The first plant organs and tissue types are established soon after fertilization in the developing embryo. Auxin-dependent transcription has been demonstrated throughout embryogenesis (reviewed in Möller & Weijers, 2009), and several developmental processes during early embryogenesis depend on correct ARF activity (Friml et al., 2003; Hardtke et al., 2004; Schlereth et al., 2010). As the embryo is small, yet features different auxin-dependent processes, we initially focused our analysis on this phase of development. Only a few ARF genes (*ARF8*,*16*,*19*) did not show detectable expression at any stage in embryo or endosperm development. As nuclear GFP expression was found during post-embryonic development in the same lines (see below; Fig. 3F,K,M), the lack of signals in the seed is not due to a problem with these reporter lines. A number of closely related ARFs (*ARF12*,*14*,*15*,*17*,*20-23*; Suppl. Fig. 1) were expressed exclusively in the endosperm. Among these, a cluster of ARFs located near the centromere of chromosome 1 (*ARF12*,*14*,*15*,*20-23*; Class I'; (Remington et al., 2004)) marked the endosperm directly surrounding the embryo (Embryo Surrounding Region; ESR; Fig. 1B; Suppl. Fig. 2). In contrast, ARF17 expression was found throughout the endosperm (Suppl. Fig 2). All remaining ARFs showed expression in the developing embryo some time during development. We decided to focus on three key stages of development for comparing the patterns. A complete overview of all embryo-expressed ARFs is shown for the octant stage, the early globular stage and the heart stage in Figures 1 and 2.

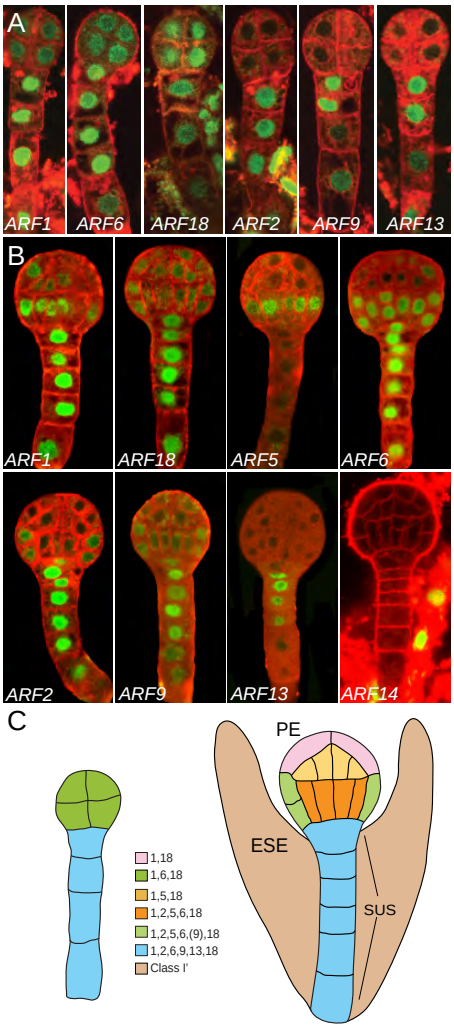

Figure 1. Expression patterns of ARF genes in octant and globular stage embryo. Octant stage (A) and globular stage (B) embryos expressing nuclear SV40-3xGFP (n3GFP; green) under control of *ARF1,2,5,6,9,13* and *18* promoters. Membranes were counterstained with FM4-64. (A) *ARF1,6* and *18* are ubiquitously expressed in octant stage embryos, while expression of *ARF2,9* and *13* is restricted to suspensor cells at this stage of development. (B) While *ARF1* and *18* are ubiquitous at globular stage, *ARF5/MP* marks the lower tier of the embryo and ARF6 is expressed in lower tier and all suspensor cells. *ARF2* and *9* are expressed in all suspensor cells and the lower tier protoderm cells, while *ARF13* marks suspensor cells. *ARF14* is expressed in the endosperm surrounding the embryo. (C) Schematic drawings representing the combined ARF expression patterns in octant (left) and globular (right) stage. Each unique combination of ARFs is highlighted by a different color code (see key in the center). PE, pro-embryo; sus, suspensor; ESE, Embryo-surrounding endosperm.

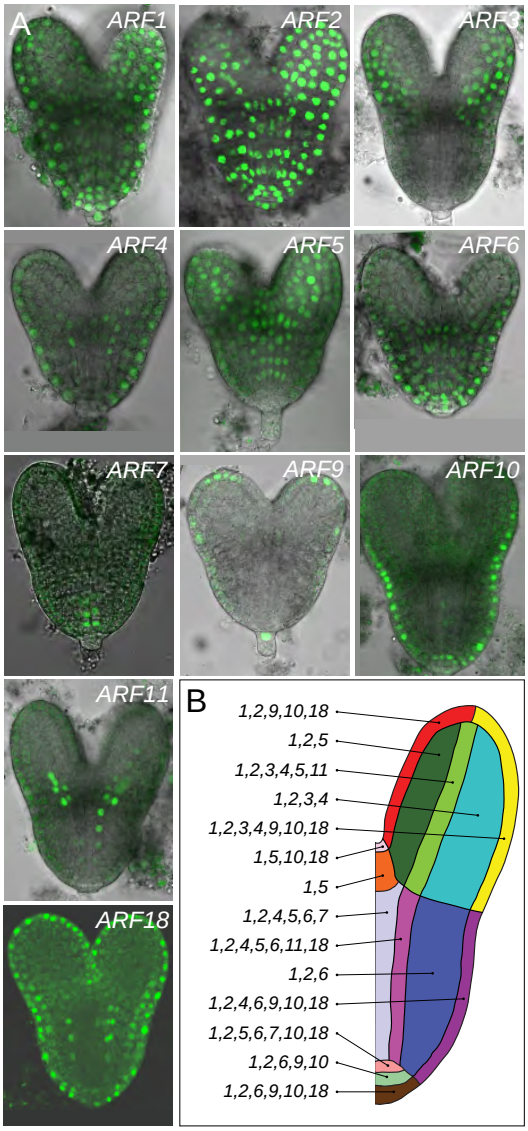

Figure 2. ARF expression patterns in heart-stage embryos.
(A) Expression of n3GFP, driven from *ARF1-7, 9-11,18* promoters in heart-stage embryos. *ARF1* and *2* are ubiquitously expressed, *ARF3* marks the abaxial cotyledon domain and *ARF4,5,11* and *18* promoters are active in subdomains of the vascular tissue. *ARF6,7* and *9* are expressed in the presumptive root meristem and ARF10 marks the protoderm. n3GFP signals (green) are combined with a transmitted light image of the embryo (grey), except for *ARF18*, where red autofluorescence is used to determine the outline of the embryo. (B) Schematic drawing representing domains of combined ARF expression. Each unique combination is given a different color code.

At the octant stage of embryo development, two patterns of ARF expression can be distinguished (Fig. 1A). *ARF1, 6* and *18* are expressed in all cells of the pro-embryo and suspensor. In contrast, *ARF2, 9* and *13* are only detected in the suspensor (Fig. 1A,C). Expression of other ARFs was either absent, or too weak to be detected.

During the globular stage of development, several patterning processes occur and specify protoderm and inner cells, as well as lower tier and upper tier cells, which at this time are morphologically distinct. We found a total of 7 ARFs to be expressed in the globular stage embryo, in partially overlapping patterns (Fig. 1B,C). *ARF1* and *ARF18* are expressed ubiquitously, with slightly higher levels in suspensor cells. It should be noted that such an asymmetry could also follow from a uniform expression level and limited divisions in the suspensor as compared to the pro-embryo. *ARF6* is not detected in the upper tier of the embryo, but expressed in the entire basal tier and suspensor. As reported previously (Hardtke et al., 2004; Schlereth et al., 2010; Hamann et al., 2002), *ARF5/MP* shows strongest expression in the lower tier of the pro-embryo. *ARF13* is expressed in the suspensor and surrounding endosperm (Fig. 1B), while *ARF9* and *ARF2* are expressed in the suspensor, as well as in the protoderm of the lower tier in the pro-embryo. Taken together, it can be concluded that at this stage of development each cell type expresses at least one ARF gene, while most cell types express a combination of ARFs (Fig. 1C).

The number of cell types increases in the heart stage embryo when cotyledons and meristems are established and vascular tissue complexity is elaborated. This increase in tissue complexity is paralleled by a greater number of ARFs that are expressed (Fig. 2). As in earlier stages, the patterns are partially overlapping (Fig. 2B). Again, all cells express at least one ARF gene. Notably, very few ARFs are expressed in the shoot apical meristem region (Fig. 2A,B). Furthermore, 9 ARFs (*ARF1,2,4,5,6,7,10,11,18*) are expressed in the vascular cylinder, although these may differ in the subset of vascular cells that they label. Interestingly, the abaxial and adaxial sides of cotyledons appear to express distinct sets of ARFs at the transcriptional level as *ARF3* expression is restricted to the adaxial domain (Fig. 2A). The protoderm is distinguished by the expression of *ARF9* and *10*, which appear to be specific to this cell type at this stage (Fig. 2A). Finally, the distal root meristem also shows distinct expression of ARFs in the different cell types. While *ARF1,2,6* and *18* are expressed both in QC and columella cells, *ARF5* and *7* are expressed in the QC but not columella and *ARF9* is restricted to the columella cells.

The expression patterns of the ARFs are not static. Rather, several ARFs undergo dynamic changes in their expression pattern throughout embryonic development. Two extreme examples are *ARF6* and *ARF2*. *ARF6* is initially expressed ubiquitously, then restricted to suspensor and lower tier, and ultimately marks the lower half of the embryo (Suppl. Fig. 3). In contrast, *ARF2*

is initially expressed only in the suspensor, then expands to the lower tier protoderm, and is ultimately ubiquitously expressed (Suppl. Fig. 3). This level of dynamics in ARF transcriptional regulation during embryo development emphasizes the need for studying ARF expression at multiple stages of development.

Complexity of ARF expression patterns in the primary root tip

Growth and patterning of the primary root depends heavily on auxin response (reviewed in Overvoorde et al., 2010). It has been demonstrated that auxin accumulation as determined by the PIN efflux network can in principle account for the low cell division rate in the QC area, the division in the proximal meristem and the cell elongation in the elongation zone (Grieneisen et al., 2007). It is however not clear which ARF factors mediate auxin responses in these zones of the root. To generate a first map of ARF expression in the primary root tip, we analyzed the expression of all ARF promoters in 3-4 day-old seedling roots. We found expression of 13 ARF genes in the root meristem (Fig. 3A-M). Among these, only *ARF1,2* and *6* are expressed in all cell types (Fig. 3A,B,D). Other ARFs show more restricted or specific patterns of expression. Notably, the patterns of ARF expression mark two complementary areas with different ARF sets. The QC and the 5-10 cells above the QC, representing the fast-dividing cells express *ARF1,2,5,6,7* and *8* (Fig. 3A-E); while, *ARF11, 18* and *19* are activated after cells leave this zone of fast divisions (Fig. 3I,L,M). Hence, the two auxin activities in the meristem, that of promoting cell division and cell elongation, are correlated with different sets of ARFs being expressed. Therefore, the same cell will have a different set of ARFs as it is being displaced from the QC area (Fig. 3N,O).

Validation of promoter expression patterns

Accumulation of ARF protein in cells depends, in addition to promoter-mediated transcription, on post-transcriptional mRNA processing (Wang et al., 2005; Nogueira et al., 2007; Wu et al., 2006; Mallory et al., 2005) and post-translational (Salmon et al., 2008) control. Furthermore, cis-elements may reside in coding sequences, introns or further upstream or downstream regions. Therefore our expression data should be considered an estimation of the complexity of ARF protein accumulation. Further refinements will certainly occur, and hence our analysis will include a proportion of false positive, and false negative expression signals. However, visualization of protein-reporter fusions can often be challenging due to low levels of protein accumulation, while mRNA in-situ hybridization can be problematic when genes are highly homologous, or when signals are weak and ubiquitous. These considerations warrant the use of this transcriptional reporter collection to first identify ARFs of interest for a cell type or biological process. We initially compared the ARF transcription patterns in root tips as observed using transcriptional fusions (Fig. 3) to those derived from transcript profiling of cell types and developmental zones in the root (Birnbaum

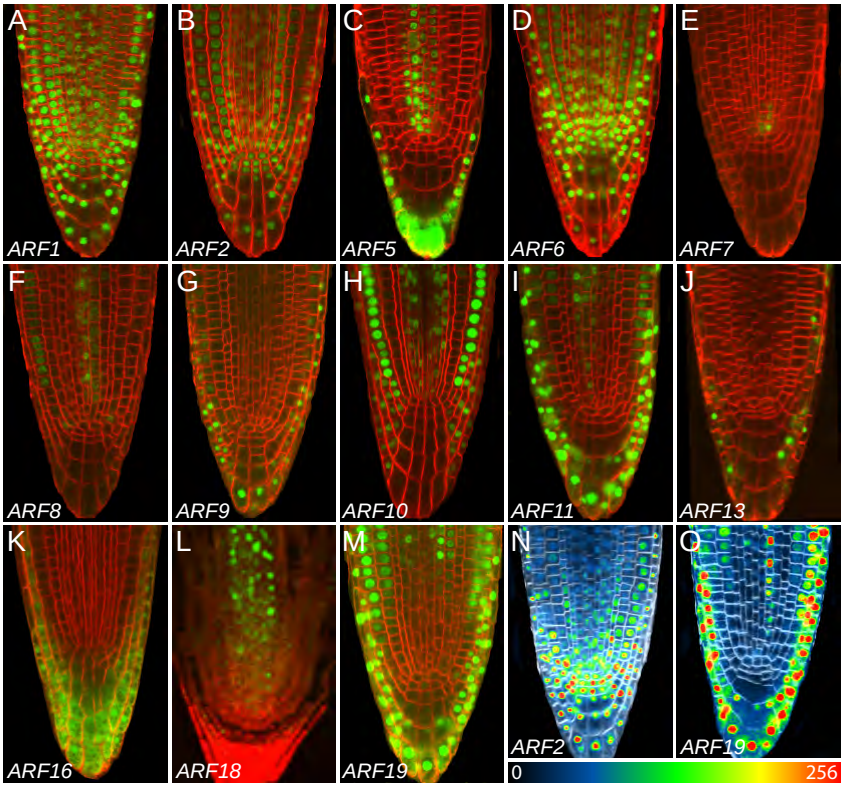

Figure 3: ARF expression in the primary root tip.
Expression of n3GFP under control of *ARF1,2,5-11,13,16,18* and *19* promoters. GFP fluorescence is represented by a green signal in (A-M), while the intensity of the GFP signal in pARF2-n3GFP and pARF19-n3GFP roots is represented by a false color scale in (N) and (O). Roots are counterstained with the membrane dye FM4-64 (red in [A-M], white in [N,O]). ARF18 expression is very weak, and in this case the signal was enhanced by indirect immunofluorescence with an anti-GFP antiserum (L).

et al., 2003). As the patterns generated from this dataset are computationally derived from two independent sets of experiments: one using 5 different sorted cell types, another using 3 developmental zones, we depicted the expression levels for both experiments separately (Suppl. Fig. 4). In general, the patterns observed using both methods agree to the degree allowed by the vastly different methodology, and to the extent in which the two types of patterns can be compared. The most striking exception is the high expression of *ARF10* in columella cells in the transcriptome data (Suppl. Fig. 4), and its complete absence in the pARF10-n3GFP lines (Fig. 3H). In this case, perhaps the length of the pARF10 promoter is insufficient to recapitulate the complete expression pattern.

To investigate the extent to which ARF promoters report the domain of protein accumulation, we initially compared the expression patterns of the *ARF5/MP* promoter with the accumulation pattern of a functional MP-GFP fusion (Schlereth et al., 2010). The MP-GFP protein accumulates in a slightly larger domain than pMP-GFP, presumably because of relatively high stability of the fusion protein compared to n3GFP. However, the patterns in both embryo and root are largely similar (Fig. 2A, 3C; compare with Schlereth et al., 2010). We generated a fusion of a genomic fragment of the *ARF15* gene including its coding region and introns to the GUS enzyme to determine the validity of the unique expression pattern that was found in the embryo-surrounding endosperm for *ARF15* and *7* other ARFs (Fig. 1B). ARF15-GUS plants showed accumulation of GUS enzyme in precisely the same region of the endosperm (Fig. 4A,B). Furthermore, we generated genomic constructs with GFP C-terminally fused to ARF2 and ARF6, and found nuclear GFP fluorescence in root tips in a pattern similar to that observed in the pARF2-n3GFP and pARF6-n3GFP lines (Fig. 3F). Finally, the root cap-specific expression of the *ARF16* promoter (Fig. 3K) faithfully recapitulates the expression of an ARF16-GFP fusion protein (Wang et al., 2005).

Based on the recapitulation of 5 ARF expression patterns representing various cell types and a wide sampling of the phylogenetic tree, we conclude that the expression pattern driven by 2 kb of ARF promoter is informative in predicting the accumulation of the protein.

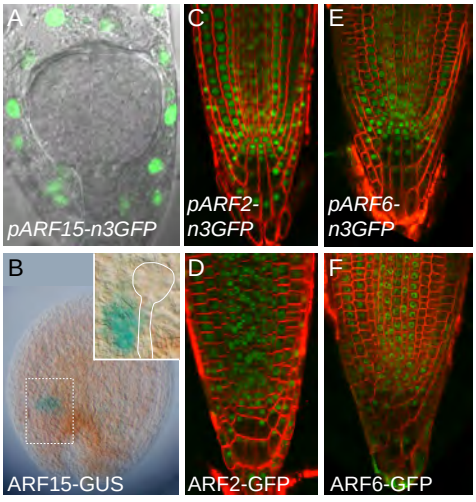

Figure 4. Validation of ARF promoter activity
Comparison of transcriptional (A,C,E) and translational (B,D,F) reporters of *ARF15* (A,B), *ARF2* (C,D) and *ARF6* (E,F). The n3GFP expression in the embryo-surrounding endosperm of a pARF15-n3GFP seed (A) is recapitulated by the ARF15-GUS fusion protein (blue) in a pARF15-ARF15-GUS seed (B). The outlines of the embryo are indicated by a red line in the inset in (B). The accumulation patterns of n3GFP in pARF2-n3GFP (C) and pARF6-n3GFP (E) are faithfully reflected by ARF2-GFP (D) and ARF6-GFP (F) patterns in pARF2-ARF2-GFP (D) and pARF6-ARF6-GFP (F) roots. Membranes in C-F are counterstained with FM4-64 (red). Note that ARF2-GFP and ARF6-GFP localize to nuclei (D,F).

Co-expressed ARFs act on the same developmental processes during embryo development

The expression patterns of only few ARF genes had previously been described at cellular resolution, particularly in the embryo (Hardtke & Berleth, 1998; Hardtke et al., 2004; Wang et al., 2005). Our analysis can now guide the selection of novel mutant combinations to investigate ARF functions masked by redundancy. To determine if co-expression in a given cell type implies convergence upon the same biological process, double mutants between arf5/mp and arf1 or arf6 were generated. These two ARFs are co-expressed with *MP* in the cells that are relevant to embryonic root formation (Fig. 1B; [Weijers et al., 2006]). As the fully penetrant nature of strong mp alleles precludes enhancement of the root defect, we used a weak allele of *MP*, mp-S319, that shows a reduced penetrance of the rootless defect (Schlereth et al., 2010; Cole et al., 2009; Donner et al., 2009). In addition, we introduced a partially rescuing pMP-TMO5 transgene (Schlereth et al., 2010) to further reduce the penetrance of the mp mutant defect and to increase the resolution of genetic interaction tests. While heterozygous mp-S319 plants carrying the pMP-TMO5 transgene segregated 6.1% rootless seedlings (24% penetrance; N=3235 seedlings; Fig. 5A), mp-S319 +/- arf6-1 pMP-TMO5 plants showed 19% rootless defects (N=1668; Fig. 5A), corresponding to 76% penetrance of the mp defect. Conversely, mp-S319 +/- arf1-5 pMP-TMO5 plants segregated only 2.8% rootless seedlings (11% penetrance; N=1148 seedlings; Fig. 5A). These results indicate that ARF6 acts redundantly with MP in embryonic root initiation while the reduction of rootless defects in the mp-S319 arf1-5 double mutant can only be explained as antagonism between MP and ARF1. Hence, co-expression in both cases implies convergence on the root initiation process.

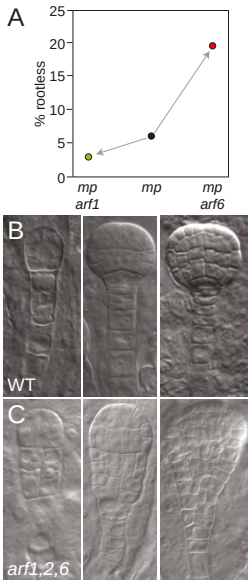

Figure 5. Genetic interactions between co-expressed ARF genes.
(A) Percentage of rootless seedlings in progeny of plants heterozygous for the mp-S319 mutation (black circle), and in progeny of mp-S319 heterozygotes that are homozygous for the arf1-5 (green circle) or the arf6-1 (red circle) mutation. All plants carried a partially complementing pMP-TMO5 transgene. (B,C) Phenotype of wild-type (B) and arf1-5 arf2-8 arf6-1 (C) embryos at 2-cell (left), globular (center) and transition (right) stage. Note that cells divide abnormally at the embryo-suspensor junction of arf1 arf2 arf6 mutant embryos.

Among the embryo-expressed ARF genes, *ARF1, 2* and *6* show striking overlap in their expression patterns (Fig. 1A,B, 2A), and this overlap is also seen in root tips (Fig. 3A,B,D). However, the ARF1, 2 and 6 proteins are distantly related (Suppl. Fig. 1), and while ARF1 and 2 have been shown to act as repressors in a transient assay, ARF6 activates transcription from the same synthetic promoter (Ulmasov et al., 1999). To determine the genetic interaction between ARF1, 2 and 6, we generated double and triple mutant combinations. None of the single or double mutant combinations displayed significant embryo defects (<1% defective embryos in >100 individuals). Since the triple homozygote is completely male sterile (Suppl. Fig. 4), embryo defects were scored in progeny of manually pollinated arf1-5 arf2-8 +/- arf6-1 plants. A striking embryo defect was observed in 15% of the progeny (n=132). Given the heterozygosity of arf2-8, this frequency corresponds to a 60% penetrance in the triple mutant. Triple mutant embryos showed erroneous divisions at the embryo-suspensor boundary (Fig. 5B,C), an area where all three genes are expressed (Fig. 1,2). Hence, the three co-expressed but phylogenetically distant ARFs (1,2,6) share redundant functions. The precise nature of the embryo defect in the arf1 arf2 arf6 mutant, and its relation to the mp defect remains to be investigated in detail. Interestingly, redundant functions of ARF1, 2 and 6 are further supported by enhancement of flower development and male fertility defects (Nagpal et al., 2005; Ellis et al., 2005) in double and triple mutant combinations (Suppl. Fig. 4).

DISCUSSION

Auxin is a plant signaling molecule with a wide range of functions, controlling virtually all aspects of growth and development. Most activities are mediated by changes in gene expression, involving TIR/AFB auxin receptors, Aux/IAA inhibitors and ARF transcription factors (Chapman & Estelle, 2009). Although the generic properties of the signaling mechanism are by now well-understood, the contribution of individual members of the gene families (5 TIR/AFB; 29 Aux/IAA; 23 ARF) to individual responses is not known. Nor is it clear to what extent the diversification of function within these families contributes to generating cell-specific auxin responses. Likewise, many auxin-dependent processes have not yet been associated with Aux/IAA and ARF transcriptional regulators controlling them. As a first step in gaining insight in the extent of diversification within these families, we have determined gene expression patterns of the entire ARF family at cellular resolution using a newly generated collection of transcriptional reporters. We describe gene expression patterns throughout embryogenesis and in the primary root tip and show that there is extensive transcriptional control of ARF genes. This information can now be used to i) determine redundant ARF activities, ii) dissect individual contributions of ARFs in single cell types and iii) infer general properties of the cell type-specific auxin response components.

It should be noted that, being a transcriptional reporter collection, the patterns may not in all cases mirror mRNA and protein accumulation patterns. We have

validated several patterns using complete genomic fusions, but as some ARFs are known to be regulated at post-transcriptional (Wang et al., 2005; Nogueira et al., 2007; Wu et al., 2006; Mallory et al., 2005) and post-translational (Salmon et al., 2008) levels, we expect that conformity between promoter and protein patterns will not be the rule. Furthermore, since the stability of the GFP mRNA or protein may not be identical in all cells, in our analysis we decided to define expression as a qualitative property, rather than a quantitative one. Hence we consider the information gathered using our resource a first step in limiting the number of ARFs potentially involved in a biological process.

Many auxin responses that are inferred from either physiological assays or gene expression reporters such as DR5 (Ulmasov et al., 1997) have not yet been connected to specific ARFs. Since none but a few of the arf mutants shows a single mutant phenotype (Okushima et al., 2005), this is likely due to extensive functional redundancy among ARFs. Based on co-expression, we have generated a double mutant between mp and arf6. These two ARFs are closely related and indeed show a genetic interaction expected for redundant genes. Since arf6 does not show root initiation defects as a single mutant, it appears that ARF6 only becomes important to root formation once MP levels are limiting.
A striking finding is the redundancy among ARF1, 2 and 6 in embryo and flower development. As these ARFs have been shown to have opposite biochemical activities (ARF1 and 2 repress and ARF6 activates) in the same transient assay system (Ulmasov et al., 1999), this suggests that the classification of ARFs as activators or repressors in this assay does not necessarily inform about functional overlap. While many ARF functions will depend on whether the ARF activates or represses transcription, other functions may be independent of this property. For example ARF1, 2 and 6 could recruit the same co-regulator to overlapping sets of target gene promoters via their protein interaction domains III/IV. Interactions with other transcription factors has been shown for ARF7 and MYB77 (Shin et al., 2007) and between ARF6 and the bHLH factor BIGPETALp (Varaud et al., 2011), and similar interactions with other proteins can be envisaged. It will be interesting to see to what extent ARFs interact redundantly with other co-regulators in specific auxin responses. For this the definition of ARF co-expression domains will be a stepping stone linking ARFs to co-factors and subsequent auxin responses.

In addition to uncovering redundant activities, we have also found one case of potential antagonism between two co-expressed ARFs. *ARF1* and *6* are co-expressed with *ARF5/MP* in the lower tier inner cells of the embryo. While the closely related *ARF5/MP* and *ARF6* share redundant functions as evidenced by enhancement of the incompletely penetrant mp-S319 allele, ARF1 and ARF5/MP appear to antagonize each other. The penetrance of the rootless mp phenotype in the weak allele is reduced by removing ARF1. This could mean that ARF1 represses *ARF5/MP* expression, and removal of ARF1 allows more expression of the partially functional MP protein, hence partial rescue. However, such

regulation of ARF transcription by ARFs has not yet been reported, and based on transcriptomics, it appears that the expression of ARF genes is relatively inert to inhibition of ARF protein activity (Suppl. Fig. 5). Alternatively, MP and ARF1 might compete for the same binding sites, with ARF1 opposing the activity of MP. The ARF expression patterns reveal that most cell types express both "repressing" and "activating" ARFs, which could allow for a scenario where antagonism between oppositely acting ARFs set a metastable equilibrium that can be tipped in one direction. Identification of the precise binding motifs and target genes for co-expressed ARFs such as *MP* and *ARF1* should resolve this issue. The recent identification of a first set of MP target genes that mediate its function in root initiation (Schlereth et al., 2010) will allow dissecting this type of functional convergence between ARFs mechanistically.

In addition to serving as an inventory of which ARF genes are expressed in what cells, our data provide a family-wide view on overlapping expression patterns. Interestingly, at all stages of embryogenesis analyzed, and to a lesser extent in the primary root tip, we observed that there appears to be a striking correlation between cell type and the set of ARFs expressed. As such, the transcriptional regulation of ARF genes seems to generate a "pre-pattern" of unique ARF combinations that may act as an underlying template that defines different transcriptional cellular responses to auxin. Such a pre-pattern could provide a plausible molecular explanation for the different responses that various cells display to the plant hormone auxin. Even at the scale of a few cell diameters in the embryo, cells can respond to auxin by inducing vascular tissue formation, cotyledon initiation or hypophysis cell fate specification, depending on their position (reviewed in Kieffer et al., 2009). The ARF expression map implies that each of these cells expresses a different set of ARFs. It will be interesting to determine to what extent ARF sets instruct the specification of a cell type, or result from it, and if other mechanisms such as interactions with other transcription factors further define qualitatively how cells respond to auxin. Nonetheless, provided that ARF proteins are functionally distinct, auxin will trigger different responses in e.g. apical and basal cells of the embryo. This would either cause different sets of genes to be regulated, or the same genes to be regulated quantitatively differently in both cell types. Available literature suggests that ARF proteins are indeed intrinsically different, as suggested by the large degree of primary sequence divergence (Suppl. Fig. 1; Guilfoyle & Hagen, 2007; Okushima et al., 2005; Lokerse & Weijers, 2009). For example, *MP* overexpression causes distinctive phenotypes that can not be induced by overexpression of the related *ARF6, 7, 8* or *19* proteins (Hardtke et al., 2004; Ploense et al., 2009). Furthermore, when expressed in the embryo, ARF16 is much less capable than MP in promoting root meristem initiation (Weijers et al., 2005). Taken together this suggests that the different sets of ARFs found in different cell types will indeed translate to unique auxin responses. It will be interesting to see whether indeed this model can explain context-dependence of auxin action in diverse developmental processes.

MATERIALS AND METHODS

Plant materials and growth conditions

All transgenic lines and mutants are of the Columbia ecotype. The mp-S319, arf1-5, arf2-8 and arf6-1 mutations have been described elsewhere (Schlereth et al., 2010; Cole et al., 2009; Donner et al., 2009; Nagpal et al., 2005; Ellis et al., 2005). For mp-S319 arf1-5 and mp-S319 arf6-1 double mutants, plants were crossed and genotyped for wild-type and mutant alleles in F1 and F2 generations by PCR (primer sequences in Table S1). F2 plants were selected that are heterozygous for mp-S319 and either wild-type for ARF1 or ARF6, or homozygous for arf1-5 or arf6-1. The mp-S319 plant that was used for crosses also carried a homozygous pMP-TMO5 transgene (Schlereth et al., 2010) that partially suppresses the mp rootless defect. The arf1-5 arf2-8 arf6-1 triple mutant was constructed by crossing an arf1-5 arf2-8 double mutant (a gift from Jason Reed) with the arf6-1 mutant. Plants homozygous for arf1-5 and arf6-1 and heterozygous for arf2-8 were selected in the F2 generation and manually self-pollinated to obtain progeny.

Plants were grown under standard conditions at 23^{o}C in a 16H light / 8H dark cycle. Selection for transgenes was performed on solid MS medium supplemented with 25 mg/L kanamycin or 15 mg/L phosphinotricin.

Construction and selection of ARF promoter-GFP lines

All oligonucleotides used in this study are listed in Supplemental Table 1. All PCR-amplified fragments were completely sequenced after subcloning and only clones without PCR-induced errors were used for subsequent cloning steps. For ARF promoter-GFP fusion constructs, approximately 2 kb (for precise length and primer sequences see Suppl Table S1) of each promoter was amplified from the appropriate BAC (Suppl Table S1) or from genomic DNA using Phusion Flash Polymerase (Finnzyme) and cloned into pGEM-T. Each promoter was then cloned directly upstream of SV40-3xGFP in pGreenIIKAN (Takada & Jurgens, 2007). The resulting plasmids were introduced into Agrobacterium strain GV3101 carrying the pSoup plasmid (Hellens et al., 2000), and Columbia wild-type was transformed using floral dip (Clough & Bent, 1998). T1 transformants were selected using kanamycin and genotyped by PCR for the presence of the appropriate transgene and the integrity of the 3xGFP reporter. Between 7 and 27 lines were analyzed for GFP fluorescence in T2 embryos (Numbers in Suppl. Table S1; only 3 and 4 lines were checked for ARF21 and 22) and lines were categorized as weak, intermediate or strong. Those showing patterns deviating from the dominant pattern were eliminated, and those showing representative patterns were used for subsequent analysis. Selected lines were tested for their expression in the root tip. For those ARFs that were not expressed in the embryo, all available lines were included for root GFP analysis. Based on embryo and/or root expression, 2-4 representative lines were selected and homozygotes were identified in the T3 generation. Expression patterns were verified in T3 (root) or T4 (embryo) individuals. The complete set of lines consists of 2 homozygous lines

for each ARF that show robust and representative patterns in embryo and/or root tip.

The ARF15-GUS fusion construct was generated by first cloning a PCR-amplified genomic PstI/SpeI fragment containing the *ARF15* coding sequence minus ATG but including stop codon in a pGreenIIBAR tNOS vector. Next, a 1.8 kb KpnI/PstI *ARF15* promoter fragment (up to and excluding the ATG) was introduced upstream of the *ARF15* coding sequence. Finally, the GUS gene was N-terminally fused to *ARF15* by introducing a PstI-digested PCR fragment representing GUS without a stop codon into this construct.

C-terminal fusions of GFP to genomic fragments of *ARF2* and *ARF6* were generated by ligating PCR-amplified fragments (approximately 2 kb upstream of ATG, and the coding region excluding stop codon; source: wild-type genomic DNA) upstream of eGFP in a pGreenIIBAR eGFP-tNOS vector using ApaI en MluI restriction sites that were introduced in the PCR primers. The pGreenIIBAR eGFP-tNOS vector was constructed by ligating a PCR-amplified eGFP coding sequence into pGreenIIBAR tNOS using EcoRI and BamHI sites introduced in the PCR primers.

Microscopy
Imaging of GFP fluorescence in embryos and root tips was done using a Zeiss LSM510 confocal microscope as described (Schlereth et al., 2010). Embryo phenotypes were analyzed using chloral hydrate cleared preparations on a Leica DMR microscope equipped with DIC optics. Histochemical staining of GUS activity was performed as described (D Weijers et al., 2001). Immunofluorescence staining of GFP was done according to (Lauber et al., 1997) using a custom-made anti-YFP rabbit polyclonal antiserum and an Alexa-488-coupled anti-rabbit antibody (Molecular Probes). False-color intensity images of GFP fluorescence in root tips were generated as in (Donner et al., 2009).

Availability of materials
The complete set of 46 lines (2 lines x 23 ARFs) has been deposited at the Arabidopsis Biological Resource Center.

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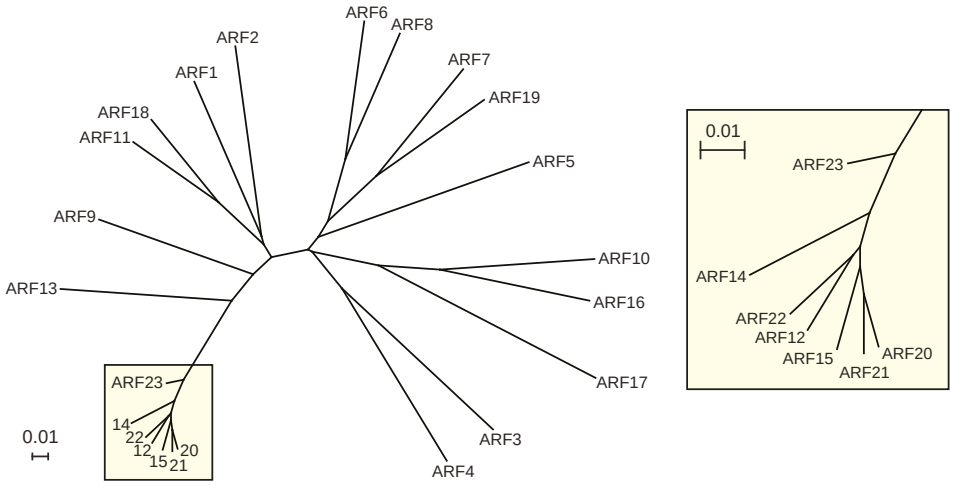
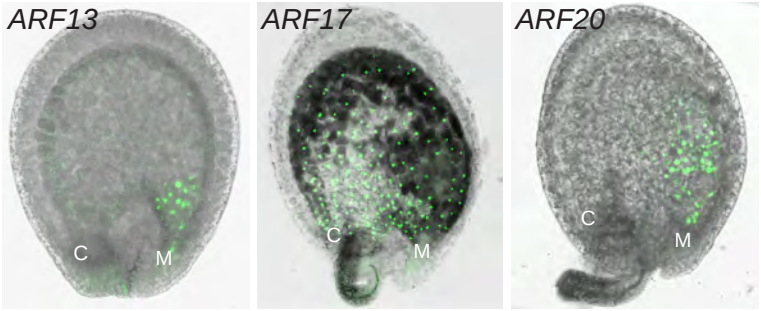
SUPPLEMENTARY INFORMATION

Supplemental Table 1: Oligonucleotides used in this study

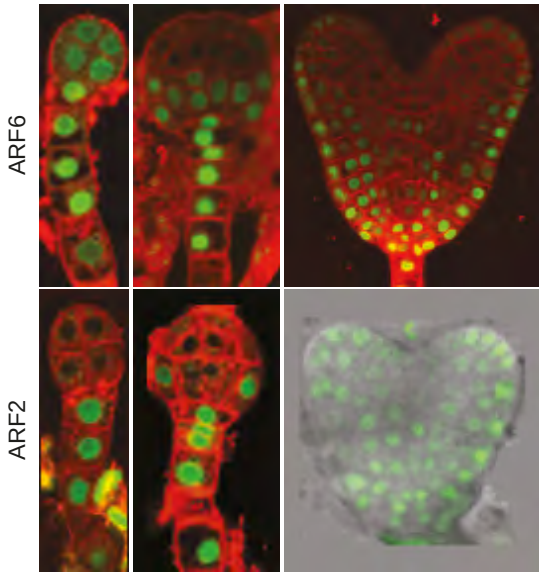
Primer pairs for promoter amplification (5'extension in *italics*, restriction sites in ***bold***)			
Gene	**Forward primer**	**Reverse primer**	**Position**
ARF1	*C**AGGTACC***TTAAAGCTAACTCGC	*C**AGGGCCC***TAGGAATCTACTTAC	-2088
ARF2	*C**AGGGCCC***TGAATGAAAGAGTCG	*C**ACTGCAG***ACCTTCCGAAGCTCAGATC	-2084
ARF3	*C**AGGTACC***ATCTCTCGTTTACTTTGATGC	*C**AGGGCCC***TAAAGAGAGAGAAACAG	-2056
ARF4	*C**AGGTACC***TTTTAGTCGTTGCGGG	*C**AGGGCCC***TGAAAAAGCTTCTCTTTTAAG	-2099
ARF5	*C**AGGTACC***ATATATCTTAGTGACAAACGCG	*C**AGGGCCC***ACAGAGAGATTTTTCAATG	-2071
ARF6	*C**AGGGCCCC***TCTGTATATAATAAACAAGC	*C**ACTGCAG***TTTTTATTCTAACTTAAAAAGC	-2132
ARF7	*C**AGGTACC***TTTAAACGTTAGCTAGGCC	*C**AGGGCCC***GATCACTCAACTTTACTTTC	-2044
ARF8	*C**AGGTACC***TCCATATGGAGTACAGTGAGG	*C**AGGGCCC***GTCTAATTCAACTTCAAGAAACC	-2084
ARF9	*C**GGTACC***TGGTGGTGGGTTTTAAGG	***TGGGCCC***CAGCTGATTAAATCTTCTATCAGTCACACC	-2295
ARF10	*C**AGGTACC***AAAACTTAGGCCTTAGATGG	*C**AGGGCCC***CTAGACGAAGTTGTGTAAC	-2078
ARF11	*C**GGTACC***GATAATCATATTATAACT	***TGGGCCC***CGAAGAAACCAAAAAAAATCC	-2075
ARF12	*A**GGTACC***AATCTTATAAGAAATTAAGAAGAGAATAGA	*A**GGGCCC***AAGCTCGTATCTAAA	-2012
ARF13	*A**GGTACC***AGAGTCAAAAAAATGATTTTGTTTGTGATTA	*C**GGGCCC***GAGCTCTTATTGCAA	-1802
ARF14	*C**GGTACC***AACAAAAACAAAATC	*A**GGGCCC***AAGCTCGTACCTAAA	-2017
ARF15	***GGTACC***GATGGGTTAATTGGGTAAACCATTGAAACC	***CTGCAG***CATAACCTCTTATCCAAAGATCGGTATACC	-1744
ARF16	*C**AGGTACC***TTGGATTTTTGTTTACGTATGG	*C**AGGGCCC***ATTTTTTGTGACCGTT	-2045
ARF17	*C**AGGTACC***TAACCATGATGTGATAGTGGG	*C**AGGGCCC***CAGGTATTTGTTTTCAGTG	-1795
ARF18	*C**GGTACC***CCGTCTCCGCTTTGCAAGG	***TGGGCCC***TGAAGAACCCAGATGAGAACTG	-2265
ARF19	*C**AGGTACC***GTTAATTCGTTTTGATGAGG	*C**AGGGCCC***CGGTTTATAGAAAGAACG	-2063
ARF20	*C**GGTACC***GATATCCGCTCTATG	*A**GGGCCC***AACCTCGTATCCAAA	-2004
ARF21	*C**GGTACC***GCTTAATCTAACAAC	*A**GGGCCC***AAGCTCGTATCTAAA	-2006
ARF22	***AGGTACC***CCGTCGTCAACCTCTG	*A**GGGCCC***AAGCTCGTATCTAAA	-1711
ARF23	*C**GGTACC***GGAAATTCACATCAT	*A**GGGCCC***AAGCTCGTATCCAAA	-805
Primer pairs for translational fusions (5'extension in *italics*, restriction sites in ***bold***)			
Gene	**Forward primer**	**Reverse primer**	**Position**
ARF15 pro	***GGTACC***GATGGGTTAATTGGGTAAACCATTGAAACC	***CTGCAG***CATAACCTCTTATCCAAAGATCGGTATACC	-1744
ARF15 CDS	*GAAATT**CTGCAG***GAAACTGGCAACGTTGTGAATGCACAAC C	*CCATGG**ACTAGT***CTATACTCAAATGTTTAGAATGTAGAT GTGATC	-
GUS	***GAATTC***TTACGTCCTGTAGAAACCCCAACCC	***GAATTC***CTATTGTTTGCCTCCCTGCTGCGGTTTTTC	-
ARF2	*TCAA**GGGCCC***AGTTTACGTGTTGTTAA	*AAGT**ACGCGT***AGAGTTCCCAGCGCTGGACA	-2084
ARF6	*TCAA**GGGCCC***TCACTAAATTCACCCAT	*AAGT**ACGCGT***GTAGTTGAATGAACCCCCAA	-2132
eGFP	*CTA**GAATTCACGCGT***GTGAGCAAGGGCGAGGA	*ATC**GGATCC***TCACTTGTACAGCTCGTCCATGC	-



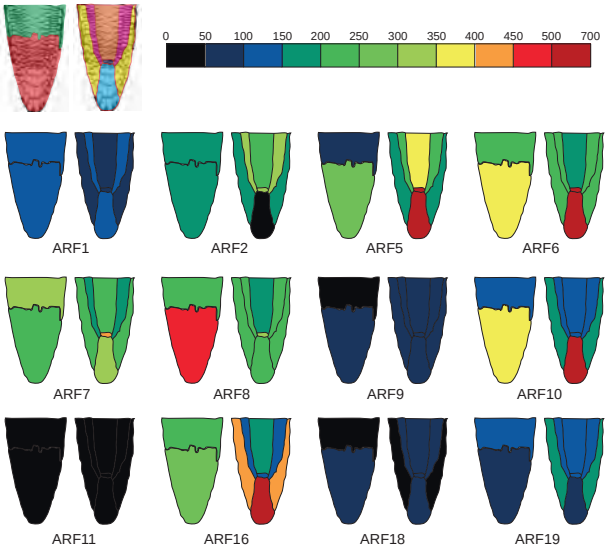
Supplemental Figure 1. Phylogenetic relationship between Arabidopsis ARF proteins.
Unrooted phylogenetic tree of all 23 predicted ARF proteins encoded in the Arabidopsis genome. If multiple splice annotations are available, the most complete (including most domains) was used, and entire proteins were used in the phylogenetic analysis using ClustalX. The tree was drawn using Archaeopterix software. Distance scale (0.01) shows the branch length corresponding to 1% amino acid changes. The cluster I' ARFs (12,14,15,20,21,22,23) form a closely related subgroup, a higher magnification of which is shown in the yellow box.



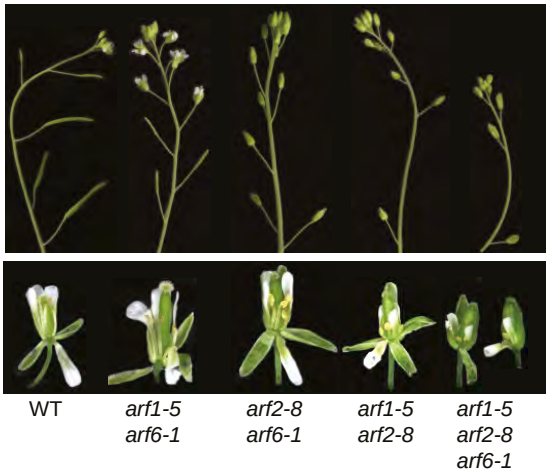
Supplemental Figure 2. Expression of ARFs in the endosperm.
Nuclear GFP fluorescence in developing seeds of pARF13-n3GFP, pARF17-n3GFP and pARF20-n3GFP lines. Note that expression of *ARF13* and *ARF20* is detected only at the micropylar (M) pole of the endosperm, while *ARF17* is detected in both the micropylar and chalazal (C) pole.



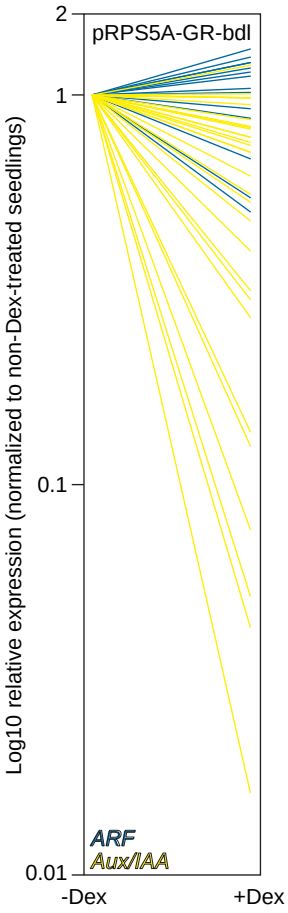
Supplemental Figure 3. Temporal dynamics of ARF expression in the embryo.
GFP fluorescence in octant (left), globular (center) and heart (right) stage embryos carrying pARF6-n3GFP (top row) or pARF2-N3GFP (bottom row) transgenes. Note that *ARF6* is initially expressed in all cells of the proembryo and suspensor, and becomes restricted first to the suspensor and lower embryo tier, and subsequently to the lower half of the embryo. *ARF2* is initially specific for suspensor cells, than expands into the lower embryo tier and eventually marks the entire heart-stage embryo.



Supplemental Figure 4. Expression of ARF genes in root developmental zones and cell types.
Average expression values for 2 developmental zones (top left cartoon: red, distal zone; green, elongation zone) and 5 non-overlapping cell types (top left: yellow, epidermis/lateral root cap; pink, cortex/endodermis; orange, stele; dark blue, quiescent center; light blue, columella root cap) after [Birnbaum et al., Science (2003) 302, 1956-1960]. Color legend (top right) shows colors corresponding to expression values. All ARFs for which uniquely matching probesets are available on the Affymetrix ATH1 array, and whose expression could be detected in pARF-n3GFP lines are depicted in two cartoons displaying expression in zones (left) or cell types (right).



Supplemental Figure 5. Floral phenotypes in arf multiple mutants. Inflorescences (upper panel) and dissected flowers (lower panel) of wild-type (WT), arf1-5 arf6-1, arf2-8 arf6-1, arf1-5 arf2-8 and arf1-5 arf2-8 arf6-1 mutant plants. All double and triple mutant plants suffer from decreased fertility. All combinations that include arf2-8 show a failure in opening of the flower. Note that the reduced stamen size in mutants carrying the arf2-8 allele is enhanced in the triple mutant flower. Also, petal size appears strongly reduced in the triple mutant.



Supplemental Figure 6. Effect of GR-bdl induction on ARF and Aux/IAA gene expression.
Relative expression of ARF (blue) and Aux/IAA (yellow) genes in DEX/IAA-treated RPS5A-GR-bdl seedlings, compared to IAA-treated controls (set to 1). Microarray data were from (Schlereth et al., 2010). Note that while most Aux/IAA genes are strongly downregulated by DEX-treatment, most ARF genes are unaffected.

Chapter

Different auxin response machineries control distinct cell fates in the early plant embryo

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ABSTRACT

The cell types of the plant root are first specified early during embryogenesis and maintained throughout plant life. Auxin plays an essential role in embryonic root initiation, in part through the action of the ARF5/MP transcription factor and its auxin-labile inhibitor IAA12/BDL. MP and BDL function in embryonic cells, but promote auxin transport to adjacent extra-embryonic suspensor cells, including the quiescent center precursor (hypophysis). Here we show that a cell-autonomous auxin response within this cell is required for root meristem initiation. ARF9 and redundant ARFs, and their inhibitor IAA10, act in suspensor cells to mediate hypophysis specification, and surprisingly also to prevent transformation to embryo identity. ARF misexpression, and analysis of the *short suspensor* mutant demonstrates that lineage-specific expression of these ARFs is required for normal embryo development. These results imply the existence of a pre-pattern for cell type-specific auxin response that underlies the auxin-dependent specification of embryonic cell types.

INTRODUCTION

Early embryogenesis in plants generates the primary organs that support further post-embryonic development. Importantly, stem cell niches (meristems) for the shoot and root systems are initiated early during embryogenesis (Weigel and Jürgens 2002). These meristems contain stem cells (SC; also called initial cells) and organizer cells (OC; quiescent center [QC] cells in the root) that prevent differentiation of the adjoining stem cells (van den Berg et al., 1997). After their establishment in the embryo, shoot and root meristems are maintained throughout the life of the organism, and produce most of the plant body post-embryonically. Despite their fundamental importance for plant development, the mechanisms underlying the initiation of meristems in the early embryo are not yet well-understood.

Currently most known factors that control root meristem initiation in *Arabidopsis thaliana* converge on the activity of the transcription factor MONOPTEROS/AUXIN RESPONSE FACTOR5 (MP/ARF5; reviewed in (Möller and Weijers 2009). MP accumulates in embryonic cells (Weijers et al., 2006), and mutations in the *MP* gene prevent specification of the hypophysis, leading to rootless seedlings (Berleth and Jürgens 1993). MP is regulated by the plant hormone auxin through association with the interacting BODENLOS/AUX/IAA12 (BDL) inhibitor protein (Hamann et al. 2002), that is degraded in response to auxin (Dharmasiri et al. 2005). A mutation in BDL that prevents degradation leads to phenotypes that are identical to those in mp loss of function mutant embryos (Hamann et al., 1999).

MP accumulates in embryonic cells and controls hypophysis specification non cell-autonomously. In part, auxin appears to mediate MP-dependent hypophysis specification: the auxin efflux carrier PIN1 becomes polarly localized

in embryonic cells, pointing toward the suspensor, and the auxin-responsive DR5 reporter gene is activated in the hypophysis around the time of its specification as evidenced by hypophysis-specific gene expression reporters (Weijers et al., 2006; Haecker et al., 2004). Both *PIN1* expression and DR5 activation are strongly downregulated in mp mutant embryos (Weijers et al., 2006). Although activation of auxin response in the uppermost suspensor cell is probably not sufficient for hypophysis specification, but may also need other factors, such as the TARGET OF MONOPTEROS7 (TMO7) protein (Schlereth et al., 2010), loss of DR5 activity in the hypophysis is strongly correlated with a failure to initiate the root meristem in several other mutants (Friml et al., 2003). However, a major unresolved question is whether auxin response in the future hypophysis is actually required for root meristem initiation, and if so, which ARF and Aux/IAA transcription factors mediate this response.

ARFs and Aux/IAAs are encoded by large families with 23 and 29 members, respectively, in Arabidopsis (Remington et al., 2004). As the expression pattern and mutant phenotype in the embryo has not been explored but for a few of these genes, in theory any or all could be involved in mediating auxin-dependent hypophysis specification. Importantly, auxin response is activated both in embryonic cells and in suspensor cells, but the cellular processes that are associated with this response are vastly different. Another major question is how accumulation of auxin in these two cell types can lead to different developmental responses.

Only the uppermost suspensor cell is respecified as hypophysis, and this local specification may rely on transport of the TARGET OF MP7 (TMO7) transcription factor to this cell (Schlereth et al., 2010). The other suspensor cells do not contribute to the mature embryo. Interestingly however, these cells do have a wider developmental potential, and can develop into embryo cells. Excessive division of normally quiescent suspensor cells has previously been observed in the abnormal suspensor (sus) and twin (twn) classes of mutants (Schwartz et al., 1994; Vernon and Meinke 1994; Zhang and Somerville 1997). Based on the expression of embryo-specific genes or morphological properties, suspensor cells in these mutants develop embryo characteristics. In all but one of these mutants, the suspensor proliferation is preceded by arrest or strong impairment of pro-embryo cells as is the case after induced ablation of the primary embryo (Haccius 1955; Weijers et al., 2003). Therefore, no cell-autonomous regulators that control suspensor versus embryo identity have been identified. Importantly, it is currently unknown whether this developmental response in suspensor cells is mechanistically related to the specification of hypophysis identity in the uppermost cell.

Here we investigate the role and nature of the auxin response machinery in the suspensor. We find that a cell-autonomous auxin response is required for hypophysis specification and root meristem initiation, and identify Aux/IAA

and ARF transcription factors that mediate this response. Surprisingly, we find that, in addition to mediating hypophysis specification, auxin response also acts to maintain suspensor cell identity. Finally, we find that the auxin response components in the pro-embryo and the suspensor are intrinsically different, and that their regulated, lineage-specific expression creates a prepattern enabling different developmental auxin responses.

RESULTS

Auxin cell-autonomously controls hypophysis and suspensor cell fate

The auxin-responsive DR5 reporter is activated in the hypophysis around the time of its specification (Fig. 1A), and mutations that impair auxin biosynthesis (Cheng et al., 2007; Stepanova et al., 2008) or transport (Friml et al., 2003; Weijers et al., 2006) in the pro-embryo lead to a loss of its expression, associated with a failure to initiate the root meristem. This correlation suggests a requirement for auxin response in hypophysis specification, but direct evidence is lacking.

Previously we demonstrated that auxin response can be blocked in discrete domains of the pro-embryo by locally expressing a non-degradable bdl protein using the GAL4-UAS two component system (Weijers et al., 2006). Given the limited specificity of stabilized aux/iaa proteins for ARF proteins (Weijers et al., 2005; Muto et al., 2007), bdl misexpression should in principle inhibit most ARFs expressed in a cell. After prescreening an embryo-expressed subset of GAL4 driver lines (Table S1), we selected four drivers that express GAL4 in suspensor cells. Two of these, RPS5A and J3281 (Fig. 1B,C) are ubiquitously expressed and two M0171 and KS068 (Fig. 1D,E) are restricted to suspensor cells until the globular stage. When crossed with the GAL4-dependent UAS-bdl line we observed erroneous hypophysis divisions (e.g. RPS5A>>bdl: 15% at 8-cell stage [n=46]; 100% at globular stage [n=73]; M0171>>bdl: 31% at globular stage [n=149]; 83% at early heart stage [n=126]) and subsequently rootless seedlings (Fig. 1G-J, L-O) with all four GAL4 lines, while control crosses with UAS-BDL showed normal development (e.g. RPS5A>>BDL: 100% at 8-cell stage [n>100]; 100% at globular stage [n>100]). These results indicate that auxin response in the suspensor is required for hypophysis specification.

Interestingly, in addition to the hypophysis defects, additional phenotypes were observed upon misexpression of bdl in suspensor cells. In wild-type embryos, suspensor cells undergo few rounds of anticlinal cell division to yield a filamentous structure of one cell diameter before cell division stops. Inhibition of auxin response in the suspensor caused excessive divisions in suspensor cells. Extra divisions could either be horizontal, or along aberrant planes (Fig. 1 H-J). Defects were found shortly after the onset of bdl misexpression (Fig. S1), and continued until late stages. These, however, developed complex phenotypes that include pro-embryo defects (e.g. Fig. 1J,N,O) presumably as secondary

consequence of the initial suspensor defects, or later expression of bdl in the pro-embryo (Fig. S1; Table S1).

Importantly, bdl-induced suspensor proliferation in M0171>>bdl and KS068>>bdl embryos was not preceded by pro-embryo defects (Fig. 1I; Fig. S1), unlike in most sus (Schwartz et al., 1994) and twin (Vernon and Meinke 1994; Zhang and Somerville 1997) mutants that also show excessive suspensor proliferation. Furthermore, expression of bdl using the pro-embryo-specific driver line Q0990 did lead to hypophysis defects (Weijers et al., 2006) but not to proliferation of suspensor cells (not shown). Hence, we conclude that auxin response is cell-autonomously required to control suspensor proliferation in addition to specification of the hypophysis.

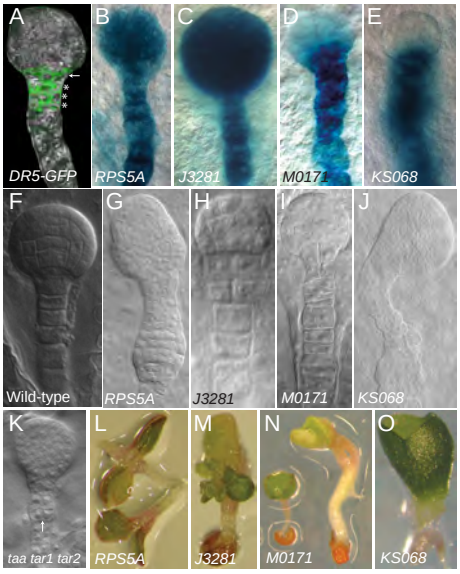

Figure 1. Reduced auxin activity in suspensor cells causes defective hypophysis and root formation, and leads to enhanced proliferation.
(A) DR5-GFP expression (green signal) in a globular-stage wild-type embryo. Note that in addition to the hypophysis (arrow), three subtending suspensor cells also express the marker (asterisks). (B-E) Patterns of GUS activity (blue staining) directed by RPS5A (B), J3281 (C), M0171 (D) and KS068 (E) GAL4 driver lines in F1 embryos resulting from crosses between each driver and the UAS-bdl;UAS-GUS line.
(F-J) Phenotypes of wild-type (F), RPS5A>>bdl (G), J3281>>bdl (H), M0171>>bdl (I) and KS068>>bdl (J) F1 embryos. Cells in suspensors of bdl-expressing embryos divide excessively, and along aberrant planes, in contrast to the single-file wild-type suspensor.
(K) taa tar1 tar2 triple mutant embryo displaying altered cell division planes in suspensor cells (arrow). (L-O) Phenotypes of F1 seedlings derived from RPS5A>>bdl (L), J3281>>bdl (M), M0171>>bdl (N) and KS068>>bdl (O) crosses. In each case, the root is completely absent, while in RPS5A>>bdl, twin seedlings develop (L), and in J3281>>bdl and M0171>>bdl, lateral or apical outgrowths are found. (See also Figure S1).

Accumulation of a stabilized Aux/IAA protein should render a cell insensitive to auxin by constitutively inhibiting available ARF proteins. Similar phenotypes should thus arise when auxin itself is absent and Aux/IAA proteins are not degraded. To determine if this is indeed the case for the suspensor proliferation defects observed upon suspensor-specific bdl expression, we analyzed embryo phenotypes of a mutant deficient in the redundant auxin biosynthesis genes *TAA1*, *TAR1* and *TAR2* (Stepanova et al., 2008). This triple mutant has previously been shown to display hypophysis division defects leading to rootless seedlings (Stepanova et al., 2008). Closer inspection of early embryos in this mutant showed suspensor defects (Fig. 1K) similar to those found in M0171>>bdl or KS068>>bdl the taa1 tar1 tar2 phenotypes in the suspensor are generally weaker than those induced by bdl misexpression, presumably due to residual auxin biosynthesis, the finding that auxin deficiency causes suspensor division defects suggests that an endogenous auxin response pathway controls suspensor development. Consistent with this finding, misexpression of wild-type BDL from the same set of promoters did not cause any defects in suspensor development (not shown), which demonstrates that auxin-dependent degradation of BDL protein is operational in suspensor cells.

An auxin response maintains suspensor cell identity

The excessive divisions of suspensor cells upon inhibition of auxin response could be the result of these cells losing quiescence, and additionally or alternatively of a transformation of their extra-embryonic cell fate towards embryonic identity. Interestingly, we occasionally found true twin embryos and seedlings upon suspensor-specific bdl expression (Fig. 1L), favouring the latter interpretation. To determine the identity of excessively dividing suspensor cells, we used molecular markers. Throughout embryogenesis up to the heart stage, expression of the M0171>>GFP reporter is normally confined to suspensor cells (Fig 1D; Fig. 2A; Fig. S1). In M0171>>bdl embryos however, GFP expression was strongly reduced after the initial expression (Fig. 2B), which indicates that there is at least a partial loss of suspensor identity.

We next analyzed the expression patterns of three genes, whose transcripts are only found in the pro-embryo of wild-type embryos, and which can be considered embryonic markers in this context. Strikingly, the expression of all three genes - *MP* (Hardtke and Berleth 1998), *SHOOT MERISTEMLESS* (STM; Long et al. 1996) and *WUSCHEL* (WUS; Mayer et al., 1998) - was detected in excessively dividing suspensor cells of RPS5A>>bdl embryos (Fig. 2C-F). The disorganized expression domains of these three genes in suspensor-derived embryo structures are consistent with the later defects in seedling organization (Fig. 1L-O). Prolonged RPS5A>>bdl expression led to secondary defects at later stages of development (Fig. 1G), and these are accompanied by loss of *MP* and *WUS* expression (Fig. 2D,E). This phenotype however is not due to impaired viability of the pro-embryo, as the cell division marker *KNOLLE* (Lukowitz et al., 1996) is

normally expressed in defective RPS5A>>bdl embryos (Fig. 2G,H). This result strongly suggests that upon inhibition of auxin response, suspensor cells lose their extra-embryonic identity and at least partially gain embryonic cell fate. By inference, this means that auxin response is required in these cells to maintain extra-embryonic cell fate.

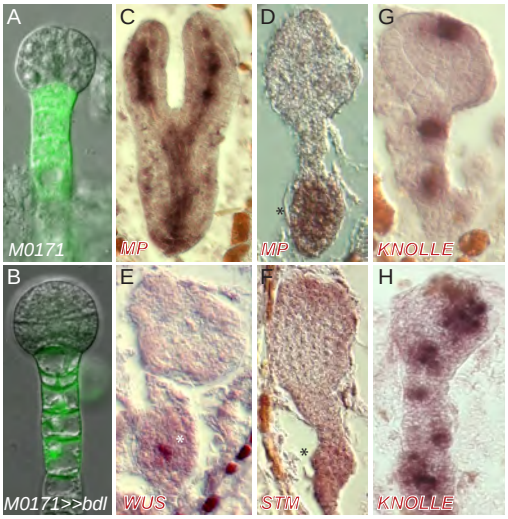

Figure 2. Inhibition of auxin response causes a transformation from suspensor to embryo identity.
(A,B) Expression of the suspensor-specific M0171>>GFP marker (green signal) in M0171>>COL (A, control) and M0171>>bdl (B) embryos. Expression is strongly reduced in suspensor cells expressing bdl. (C,D) In-situ hybridization of MP transcript (brown-red staining) in wild-type (C) and RPS5A>>bdl (D) embryos. The embryo-specific MP transcript is activated in proliferating suspensor cells and simultaneously lost in the pro-embryo in RPS5A>>bdl embryos. (E) Expression of the shoot meristem-specific WUS transcript in the center of proliferating RPS5A>>bdl suspensor cells. (F) Expression of shoot meristem-specific STM transcript in proliferating RPS5A>>bdl suspensor cells. The suspensor-derived embryo-like structure is marked with an asterisk in (D-F).

Identification of IAA10 as a component of the suspensor-specific auxin response machinery

The phenotypic defects caused by misexpression of bdl in the suspensor reveal the existence of an auxin response with an unknown auxin response machinery at its base. The core of canonical auxin response machineries is formed by pairs of interacting Aux/IAA and ARF factors (reviewed in Lokerse and Weijers 2009). Whereas gene expression changes are effected by the DNA-binding ARF, auxin-dependence is conferred by the inhibitory Aux/IAA protein.

To identify which of the 29 Aux/IAA genes are expressed in suspensor cells, we first analyzed publicly available microarray data sets for embryos (Le et al., 2010),

but found these inconclusive as expression data at early stages of embryogenesis is only available for one fifth of the Aux/IAA genes (not shown), and mRNA abundance as predicted by these arrays did not match in-situ hybridization patterns in several cases (not shown).

Subsequently we examined datasets (Birnbaum et al., 2003; Brady et al., 2007) for cells that originate from suspensor cells. In the seedling root, these are the columella root cap and QC cells, both derived from the hypophysis. Arguing that suspensor-specific Aux/IAA genes may also be enriched in suspensor-descendants, we focused on six Aux/IAAs (IAA7/AXR2, IAA10, IAA11, IAA17/AXR3, IAA20 and IAA33) that showed preferential expression in columella cells (Fig. S2A). Of these, IAA20 and IAA33 are non-canonical Aux/IAAs lacking essential domains for auxin-dependent inhibition of ARFs (Dreher et al., 2006; reviewed in Lokerse and Weijers 2009). The expression patterns of the remaining IAA7, IAA10, IAA11 and IAA17 were determined by generating fusions of each promoter (1-2 kb upstream of ATG) to nuclear-localized 3xGFP (n3GFP; (Takada and Jürgens 2007)). While QC/columella expression could not be confirmed for IAA7 and IAA17, pIAA10 and pIAA11 drove GFP expression in these cell types (Fig. S2C,E,G,I) as predicted by the microarray data (Fig. S2B,D,F,H). We next investigated GFP fluorescence during embryogenesis and found that only the IAA10 promoter drove expression in all suspensor cells and before hypophysis specification (Fig. 3C,D). In contrast, the IAA11 promoter only became active in the embryonic root after hypophysis specification (Fig. 3E,F), IAA7 expression was specific to the pro-embryo, and restricted to the proximal root stem cells (Fig. 3A,B) and IAA17 could not be detected during early embryogenesis (not shown).

We next generated mutants for IAA10 and IAA11 in which the first proline in the GWPP(I/L/V) motif of domain II was replaced by a serine. This conserved motif directly binds to the auxin-TIR1 receptor complex (Tan et al., 2007), and the P>S mutation has been shown to prevent this interaction and stabilize the protein (Gray et al., 2001). As predicted by the expression patterns of transcriptional fusions, pIAA11-iaa11 embryos did not show altered suspensor development, but occasional erroneous hypophysis division (Fig. 3J). In contrast, pIAA10-iaa10 embryos showed excessive and altered suspensor divisions (Fig. 3H,I; Fig. S2K; Table S2). Consistent with the predicted stabilization of the mutant iaa10 protein, no phenotypes were found in pIAA10-IAA10 embryos (not shown). A double mutant pIAA10-iaa10 pIAA11-iaa11 showed the pIAA10-iaa10 phenotype, consistent with IAA10 having an earlier specifying function. IAA11 might be involved in subsequent hypophysis and/or descendent cell fate maintenance (not shown).

Interestingly, pIAA10-iaa10 phenotypes were weaker than those induced by misexpression of bdl (compare Fig. 3H,I with Fig. 1I,J). Several scenarios could explain this result. For example, the protein levels may significantly differ between M0171>>bdl and pIAA10-iaa10, such that a smaller proportion of the

ARF(s) in suspensor cells are inhibited in pIAA10-iaa10 embryos. Alternatively, like the M0171 reporter (Fig. 2B), the suspensor-specific IAA10 promoter may be downregulated as a consequence of (partial) loss of suspensor fate in pIAA10-iaa10 embryos. Consistent with the latter interpretation, pIAA10-GFP is detectable throughout embryogenesis, but a functional (phenotype-inducing) iaa10-GUS protein fusion could not be detected during embryogenesis. Yet, this protein was detected in the egg cell or zygote and in the post-embryonic root tip (Fig. S2N-P). To directly test whether iaa10 activity suppresses IAA10 gene expression, the ARF-interacting domains III/IV were deleted from a stabilized iaa10-3xGFP protein. This pIAA10-iaa10NT-3xGFP fusion protein was detected throughout embryogenesis in a pattern indistinguishable from the pIAA10-n3GFP reporter (Fig. 3K) but did not induce defects.

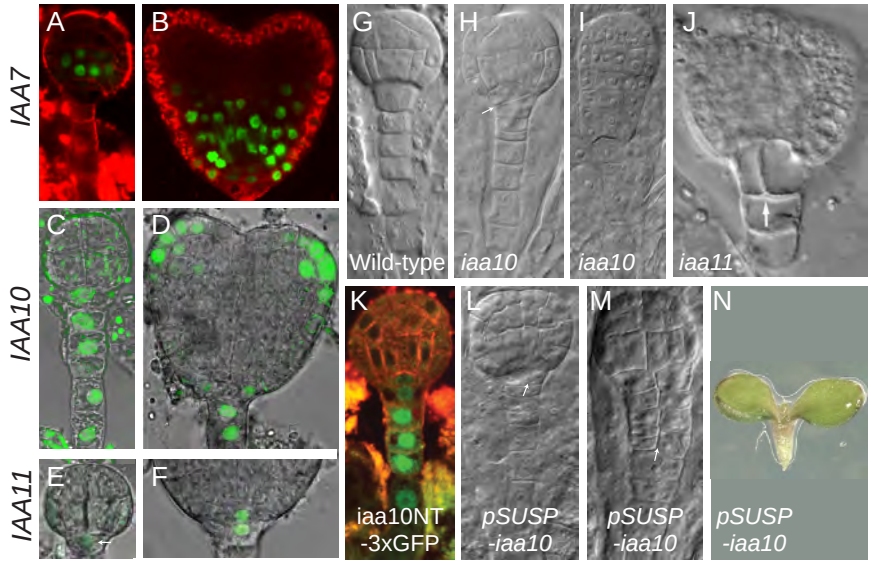

Figure 3. IAA10 cell-autonomously regulates hypophysis division and suspensor development.
(A-F) Expression of a nuclear GFP reporter (SV40-3xGFP; n3GFP) driven by IAA7 (A,B), IAA10 (C,D) and IAA11 (E,F) promoters in globular (A,C,E) and heart (B,D,F) stage embryos. IAA7 expression is specific for root stem cells in the pro-embryo, while IAA10 expression is initially specific to suspensor cells, and IAA11 marks the hypophysis and its descendants. Note that IAA10 is activated in cotyledon primordia at heart stage (D). (G-J) Phenotypes of wild-type (G), pIAA10-iaa10 (H,I) and pIAA11-iaa11 (J) globular-transition stage embryos. pIAA10-iaa10 mutant embryos display altered divisions in hypophysis (H, arrow) and suspensor (I), while in pIAA11-iaa11 mutants the hypophysis occasionally divides aberrantly (J). (K) Expression of the N-terminal region of iaa10, fused to 3xGFP from an IAA10 genomic fragment reproduces the suspensor-specific expression pattern. (L-N) Expression of iaa10 from a suspensor-specific promoter (pARF13) induces hypophysis division defects (L, arrow), stronger suspensor division defects (M) and results in rootless seedlings (N). (See also Figure S2).

Finally, we used another suspensor-specific promoter (pARF13; see below; also named pSUSP [Schlereth et al., 2010]) of comparable strength (as judged by similar levels of pIAA10-n3GFP and pARF13-n3GFP fluorescence) to express iaa10. In contrast to the IAA10 promoter, pARF13 does not appear to be affected by impairment of suspensor identity (see below; Fig. 7F,H). Indeed, we found that this induced increased phenotypic severity and persistence of defects as compared to expression from the IAA10 promoter. pARF13-iaa10 embryos displayed aberrant hypophysis division (Fig. 3L), excessive suspensor divisions (Fig. 3M; Fig. S2L) and seedlings from these lines were often rootless (Fig. 3N; Table S2). In summary, IAA10 represents a component of an endogenous auxin response machinery that cell autonomously controls suspensor fate maintenance and root initiation.

Even though Aux/IAA proteins are thought to act by binding to other Aux/IAAs or to ARFs, it is possible that the effects caused by bdl or iaa10 accumulation in suspensor cells are unrelated to auxin-dependent gene regulation. To determine whether this is the case, we examined the expression of an endogenous reporter for auxin responsive gene expression in the embryo, *IAA30*. *IAA30* is induced by auxin (Sato and Yamamoto. 2008), and according to microarray data (Birnbaum et al., 2003; Fig. S2A) mildly enriched in columella cells. Since IAA30 is a non-canonical Aux/IAA that lacks the auxin-dependent degradation domain II, we did not include this gene in our functional analysis. Nonetheless, a pIAA30-n3GFP reporter showed auxin-inducible expression in the root tip (Fig. S3C,D). During embryogenesis, *IAA30* expression marked all known sites of auxin response in both pro-embryo and suspensor (Fig. 4A-C; Fig. S3A,B) and can therefore be regarded a natural indicator of auxin response. We examined pIAA30-n3GFP expression in pARF13-iaa10 embryos. Strikingly, while activation of *IAA30* in pro-embryo cells was unaffected (Fig. 4F), the activity in suspensor cells was completely lost (Fig. 4D,E). We therefore conclude that iaa10 accumulation in suspensor cells cell-autonomously inhibits auxin-dependent gene expression.

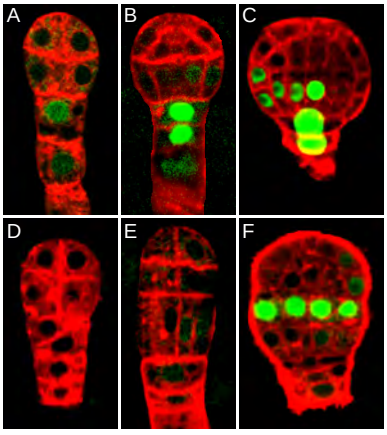

Figure 4. Auxin-responsive gene expression is cell-autonomously blocked in pARF13-iaa10 suspensor cells.
(A-C) Expression of pIAA30-n3GFP in octant (A), dermatogen (B) and globular (C) stage wild-type embryos. Expression of IAA30 is first observed in the uppermost suspensor cell at the octant stage (A), remains in the daughters of this cell and is activated in lower tier pro-embryo cells (B,C).
(D-F) pIAA30-n3GFP expression in octant (D), dermatogen (E) and globular (F) stage pARF13-iaa10 embryos. Note that expression of pIAA30 is lost in suspensor cells, but still activated in lower tier pro-embryo cells (F). (See also Figure S3).

ARF control of suspensor development

To reveal which ARF(s) may act in suspensor cells, we determined the expression pattern of each ARF using a collection of transcriptional reporters (Rademacher et al., 2011). We found 6 ARFs to be expressed in the suspensor, of which 3 (*ARF2,9,13*) are initially restricted to suspensor cells and are mostly specific to the suspensor during the stage at which iaa10-induced phenotypes first appear (Fig. 5A-C; Rademacher et al., 2011). These ARFs are therefore good candidates for being interactors of IAA10 and effectors of auxin response in the suspensor. To determine whether these and other ARFs contribute to normal suspensor development, we analyzed embryo development in individual mutants for each ARF gene. One reference allele was used for most ARFs, and additional alleles were included for the poorly described suspensor-specific *ARF9* and *ARF13* genes. Apart from the well-documented arf5/mp defects (Berleth and Jürgens 1993), we did not find robust embryo phenotypes in any of the arf mutants (Table S3). Different arf9 insertion lines did show defects in the upper half of the suspensor, similar to those induced by bdl or iaa10 (not shown), but these phenotypes were highly stochastic, and varied between plants and growth conditions. It could therefore not be unequivocally demonstrated that the arf9 mutation causes these defects. An arf9 arf13 double mutant was generated, and this showed the same variable defects (not shown).

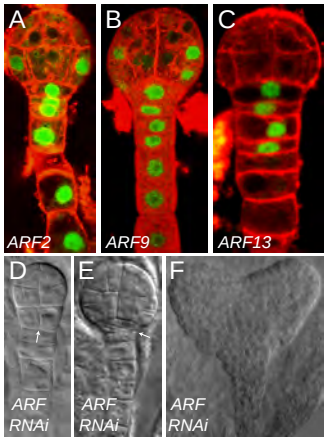

Figure 5. Suspensor-expressed ARF genes contribute to hypophysis and suspensor development.
(A-C) Expression of a nuclear GFP reporter (n3GFP) from ARF2 (A), ARF9 (B) and ARF13 (C) promoters in globular stage embryos. While ARF13 is only expressed in suspensor cells, ARF2 and ARF9 are additionally expressed in the lower tier protoderm of the pro-embryo. (D-F) Phenotypes in embryos expressing an RNAi fragment directed against ARF9 and ARF13 show defects in hypophysis (D,E; arrows) and suspensor (F) development. (See also Figure S4).

To provide independent evidence for a potential role of ARF9 and related ARFs in suspensor development, we generated lines expressing an RNAi fragment directed against ARF9 and several related ARFs from the 35S and RPS5A promoters. A phenotype very similar to the one seen in pIAA10-iaa10, M0171>>bdl or pARF13-iaa10 transgenic lines was found in these RNAi lines (Fig. 5D-F; Fig. S2M; Table S4). However, due to high sequence similarity between ARF9 and a cluster of closely related ARFs (ARF12-15,20-23; 55-61% at transcript level, 56-60% at protein level), no ARF9-specific RNAi could be generated, and for the same reason, RNAi-induced transcript downregulation could not

be unequivocally determined (not shown). Expression analysis of these genes revealed that in wild-type, *ARF12,14,15,20,21* and *22* are all expressed in the endosperm surrounding the embryo (Fig. S4). We tested whether ARF activity in the endosperm may contribute to suspensor development, but found that expression of three different stabilized aux/iaa proteins (shy2/iaa3, bd/iaa12 and axr3/iaa17) in the endosperm using 3 independent GAL4 driver lines (KS117, J2641, M0186; Ingouff et al., 2005) did not affect suspensor development (no phenotypes in >100 embryos examined for each cross). Therefore, only the downregulation of *ARF9* and *13* in the embryo by this RNAi fragment is expected to be relevant to suspensor development. Therefore, although it is possible that co-expressed ARFs (*ARF1,2,6,18*) also contribute, or that in the absence of ARF9 (and 13) the closely related endosperm-specific ARFs are upregulated, we conclude that ARF9 and/or ARF13 contribute to auxin-dependent suspensor and hypophysis development.

ARF9 and ARF13 have not been characterized in detail, and a critical question is if these proteins are part of an auxin-regulated network. It has previously been reported that only ARFs with a Q-rich middle region (MR) interact with Aux/IAA proteins (Shen et al., 2010). Neither ARF9 nor ARF13 has a Q-rich MR, which prompted the question whether they can interact with iaa10 protein. Although peptides corresponding to the C-terminus of ARF13 have been found in shotgun proteomics experiments (Castellana et al., 2008), this seems to be derived from a non-canonical splice form that we have not been able to isolate (not shown). Therefore, we restricted our analysis to ARF9, for which a full-length transcript could be isolated. To determine ARF9-iaa10 interactions, we optimized a live protoplast Förster Resonance Energy Transfer (FRET) assay that is based on detection of the fluorescence lifetime (FLIM) of the CFP protein in a CFP-YFP FRET pair (Kremers et al., 2006). Direct interaction of CFP- and YFP-tagged proteins leads to a decrease in the fluorescence lifetime of CFP (e.g. (Russinova et al., 2004; Kremers et al., 2006)). The advantage of this assay over other in-vivo methods to detect protein-protein interactions, such as bi-molecular fluorescence complementation (BiFC; Hu et al., 2002), is that the method detects dynamic interactions and results can be quantified, and hence statistically evaluated. In this assay, the well-elucidated MP-BDL interaction (Hamann et al., 2002; Weijers et al., 2006) was confirmed (MP-CFP with bdl-YFP Fig. 6B). In contrast, MP-CFP did not interact with ARF3-YFP (Fig. 6B) that lacks the domains III/IV that mediate ARF-ARF and ARF-Aux/IAA interactions (reviewed in Lokerse and Weijers, 2009). ARF9 and iaa10 proteins both localized to the nucleus of protoplasts (Fig. 6A), and FLIM analysis showed a decrease in ARF9-CFP fluorescence lifetime when co-expressed with iaa10-YFP, indicating that these proteins indeed interact (Fig. 6B).

The co-expression of *ARF9* and *IAA10*, comparable phenotypes of arf RNAi and iaa10 gain-of-function mutations and the ability of the two proteins to physically interact strongly suggest that these proteins form the core of a suspensor-specific auxin response machinery.

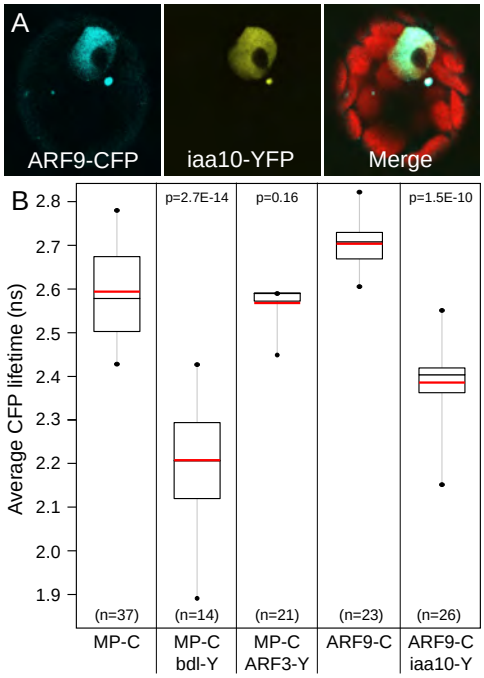

Figure 6. ARF9 and iaa10 proteins interact in planta.
(A) Localization of ARF9-CFP (left, cyan) and iaa10-YFP (middle, yellow) proteins in Arabidopsis leaf mesophyll protoplasts. Right panel shows an overlay of both signals and the red fluorecence of chloroplasts.
(B) Förster Resonance Energy Transfer (FRET), as measured by Fluorescence Lifetime Imaging (FLIM) of ARF and Aux/IAA proteins in mesophyll protoplasts. The fluorescence lifetime (ns, nanoseconds) of the CFP-tagged protein (MP-C, ARF9-C) is represented in boxplots. The box represents the first to third quartile of measured values (n is given in each column), average and median values are depicted as red and black lines. Extremes are indicated by a black dot. Co-expression of bdl-YFP (bdl-Y) with MP-CFP (MP-C) leads to a decrease of fluorescence lifetime (p-value for student's t-test given in top of column). In contrast, a protein lacking interaction domains (ARF3-YFP; ARF3-Y) does not induce this decrease in lifetime. Co-expression of iaa10-YFP (iaa10-Y) with ARF9-CFP (ARF9-C) also induces a strong decrease in fluorescence lifetime, indicating physical interaction between ARF9 and iaa10.

A prepattern for cell type-specific auxin response

The biological processes controlled by the suspensor-specific auxin response machinery - maintenance of suspensor identity and promotion of hypophysis identity - most likely differ from the ARF5/MP-BDL machinery that acts in the pro-embryo to promote cell-cell communication. Conceivably, these two machineries constitute a prepattern that allows cell-specific responses to the same hormonal trigger, provided that the ARFs involved are functionally divergent. *ARF5/MP* is

exclusively expressed in embryonic cells (Hardtke and Berleth 1998; Hamann et al., 2002; Weijers et al., 2006; Schlereth et al., 2010), and its function is required for normal pro-embryo development (Berleth and Jürgens 1993; Friml et al., 2003). In contrast, *ARF9* and *ARF13* are exclusively expressed in the extra-embryonic suspensor at early stages with *ARF9* expression expanding to the pro-embryo at later stages. To determine if lineage-specific ARF expression is required for normal development, we swapped promoters between *MP* and *ARF9/ARF13*.

To test whether ARF9 is interchangeable with MP, we expressed a pMP-ARF9 transgene in the weak mp-S319 allele. This allele shows approximately 40% penetrance of the rootless phenotype, corresponding to approximately 10% rootless seedlings among the progeny of a heterozygous mutant (Cole et al. 2009; Donner et al. 2009; Schlereth et al. 2010). We have previously shown that the phenotypic penetrance can be quantitatively suppressed by pMP-driven expression of its targets *TMO5* or *TMO7*, while it can be aggravated by RNAi suppression of *TMO7* expression (Schlereth et al., 2010). In 4 out of 5 pMP-ARF9 lines, the penetrance of the mp-S319 mutation was increased from 40% to 64-78% (Table S5). Therefore we conclude that ARF9 cannot replace MP during root initiation, but potentially antagonizes MP when expressed in its expression domain.

In a reciprocal experiment, *MP* was misexpressed from the *ARF13* promoter in an otherwise wild-type background. *pARF13* is expressed in the suspensor as well as the surrounding endosperm (Fig. 5C; Fig. S4). pARF13-MP embryos developed suspensors of about half the size of wild-type (Fig. 7A-D; Table S6), suggesting that MP interferes with the normal developmental program of suspensor cells. To test whether this phenotype results from an extra ARF dose irrespective of the identity of the ARF, we generated lines that carry an additional ARF13 or ARF9 genomic fragment (pARF13-ARF13; pARF9-ARF9). These lines developed normal embryos (not shown), suggesting that ectopic MP activity interferes with normal suspensor development.

We conclude that the lineage-specific expression of functionally distinct ARF transcription factors (MP and ARF9/13) allows for the different auxin-dependent development of both lineages.

Regulation of the ARF prepattern by a zygotic patterning factor

The suspensor defect induced by ectopic MP activity closely resembles the phenotype of the short suspensor (ssp) mutant (Fig. 7E; (Lukowitz et al., 2004; Bayer et al., 2009b)). *SSP* encodes a receptor-like kinase whose RNA is paternally delivered to the zygote, and that controls zygote elongation and suspensor development (Bayer et al., 2009b). To test whether the defect in ssp mutant embryos is related to altered expression of ARFs, we analyzed expression patterns of all ARFs that could be detected by promoter-GFP fusions at globular

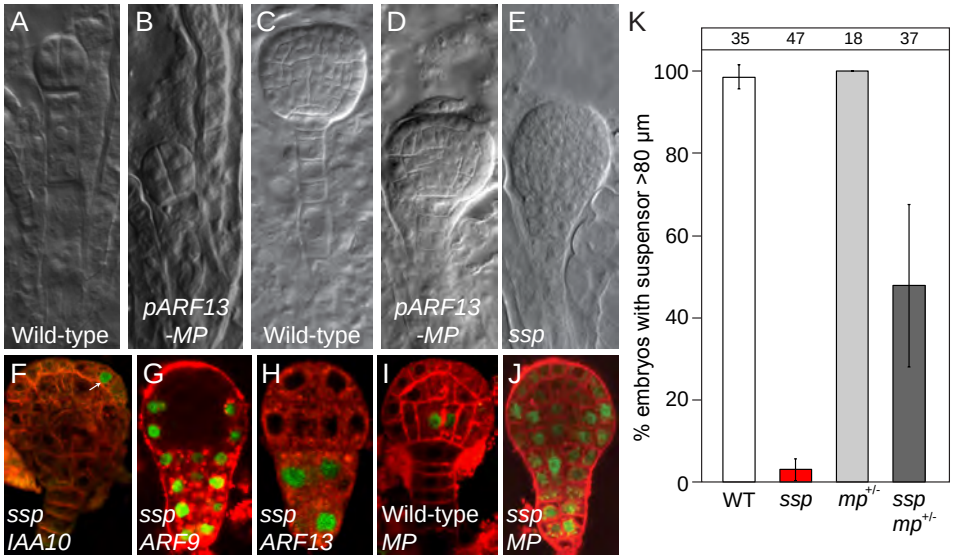

Figure 7. Regulation of embryo-specific MP expression is required for normal suspensor development.

(A-D) Phenotypes of wild-type (A,C) and pARF13-MP (B,D) embryos at 4-cell (A,B) and transition (C,D) stage. Note that suspensors in pARF13-MP embryos are strongly reduced in size. (E) ssp mutant embryo at late globular stage shows strongly reduced suspensor size and aberrant divisions. (F) Suspensor-specific expression of pIAA10-n3GFP is lost in ssp mutant embryos, while cotyledon primordium expression is maintained (arrow). (G,H) Expression patterns of pARF9-n3GFP (G) and pARF13-n3GFP (H) are unaltered in ssp mutant embryos. (I,J) Expression of pMP-n3GFP in wild-type (I) and ssp (J) embryos. Expression is ectopically activated in ssp mutant suspensors.

(K) Genetic interaction between ssp and mp. The percentage of embryos of which the pro-embryo is at least 40 µm in size (globular stage), with suspensor length greater than 80 µm is plotted for wild-type (WT), ssp-2 -/-, mp-B4149 +/- and ssp-2 -/- mp-B4149 +/-. The number of embryos measured nis shown on the top of the graph. Note that suspensor length in mp is indistinguishable form WT, and that ssp mp double mutants areintermediate between the ssp and mp single mutants. Error bars indicate the standard deviation in three independent experiments. (See also Figure S5).

stage (*ARF1,2,5,6,9,13,18*; Rademacher et al., 2011) in the ssp mutant. Expression of *ARF1,2,6,9,13* and *18* was unchanged even in phenotypically severely affected mutant embryos (Fig. 7G,H; Fig. S5A), despite loss of suspensor-specific *IAA10* expression (Fig. 7F). This result confirms the independent regulation of pIAA10 and pARF13 as suggested by the different phenotypic severity of pIAA10-iaa10 and pARF13-iaa10 lines (Fig. 3H,I,L,M).In contrast however, *MP* expression was expanded into ssp mutant suspensor cells (Fig 7I,J). Hence, the phenotypic resemblance of pARF13-MP to ssp mutant embryos is consistent with ectopic *MP* expression in the ssp mutant suspensor. To test whether ectopic activation of MP functionally contributes to the short suspensor phenotype in ssp, we generated

an ssp mp double mutant and analyzed suspensor length. At late globular stage (pro-embryo ≥40µm), wild-type suspensors reach their final length of 80-120 µm. In ssp mutants less than 5% of suspensors reach or exceed 80µm (Fig. 7K; Fig. S5B) the majority being in the range of 50-70 µm (Fig. S5). While the mp mutation itself did not change suspensor length (Fig. 7K; Fig. S5B), ssp mutants segregating the mp mutation (ssp/ssp mp/+) showed a partial restoration of suspensor length (Fig. 7K; Fig. S5B). This result suggests that eliminating MP activity in the ssp mutant partially suppresses the short-suspensor phenotype and is consistent with a contribution of ectopic MP expression to the short suspensor defect. These findings suggest that SSP in part acts through preventing MP expression in suspensor cells.

DISCUSSION

The root meristem is established early during embryogenesis, and involves the specification of embryonic and extra-embryonic cells towards stem cell and organizer identities (Scheres et al., 1994; Weigel and Jürgens 2002). Previously, the critical role of the auxin-dependent transcription factor ARF5/MP in promoting the specification of the QC precursor (hypophysis) has been established (Weijers et al., 2006; reviewed in Möller and Weijers 2009). MP activity in hypophysis specification is non cell-autonomous and is relayed by the transport of auxin and other signals from the pro-embryo to the adjacent extra-embryonic cell (Weijers et al., 2006). The role of auxin response in the incipient hypophysis, and the nature of the response mechanism in this cell had thus far remained elusive. We demonstrate here that cell-autonomous auxin response is required for hypophysis division and root meristem formation. Furthermore, we have identified components of the transcriptional response to auxin in the hypophysis and show that their activity differs from that of the embryonic ARF5/MP transcription factor. Finally, we demonstrate that the lineage-specific expression of ARFs generates a prepattern for cell type-specific auxin response in development.

Context dependence of auxin response, i.e. distinct developmental output of auxin activity in different cells, is further highlighted by the role of auxin in suspensor cells. While in the uppermost suspensor cell, auxin promotes the specification and asymmetric division of the hypophysis, in more basally located suspensor cells, auxin prevents proliferation and transformation to embryo identity. Inhibition of auxin response in these cells leads to enhanced division along aberrant planes. These excessively dividing cells acquire embryo identity as judged by the loss of a suspensor marker and gain of expression of several embryo-specific genes.

Transformation of suspensor cells towards embryo identity has been described for several mutants of the abnormal suspensor (sus), raspberry (rsp) and twin (twn) classes in Arabidopsis (Schwartz et al., 1994; Vernon and Meinke 1994;

Yadegari et al., 1994). So far, analysis of *SUS* and *TWN* genes has not revealed mechanisms of suspensor identity maintenance or suspensor-embryo transformation (Zhang and Somerville 1997). However, based on mutant phenotypes a model has been proposed where the pro-embryo actively suppresses embryo identity in the suspensor, possibly through chemical signals (Schwartz et al., 1994). This hypothesis is attractive as genetic ablation (Weijers et al., 2003) or X-ray irradiation (Haccius 1955) of the embryo also induces proliferation and embryogenesis in the suspensor. We have now identified an auxin response pathway and its molecular components that cell-autonomously regulate suspensor identity and embryonic transformation. Whether auxin itself is the hypothesized embryo-derived signal that suppresses proliferation in the suspensor (Schwartz et al., 1994), or merely a component of suspensor identity regulation remains to be seen. However, with IAA10, ARF9, and potentially redundant suspensor-expressed ARFs, we now have the tools to start identifying the mechanisms of quiescence and proliferation in extra-embryonic cells in plants.

Based on our findings, there appears to be strong cell type-dependence of auxin response in the embryo. Auxin activates cell-cell communication in pro-embryo cells, hypophysis specification and division in the future hypophysis and suppression of proliferation or maintenance of identity in suspensor cells. Our expression analysis of the ARF family (this Chapter; Rademacher et al., 2011) has shown that while some ARFs (*ARF1,6,18*) are ubiquitously expressed, others (*ARF2, 9, 13*) are initially specific for the suspensor. Therefore, at least the pro-embryo and suspensor have different ARF transcription factors to mediate cell type-specific gene expression responses. The different developmental output in these cell types would most readily be explained by different gene repertoires being regulated by the lineage-specific ARFs. Our promoter-swap experiments between ARF9 and MP suggest that these two transcription factors are not interchangeable, rather to some extent antagonistic. An attractive hypothesis as to the mechanistic basis for this intrinsic difference between MP and ARF9 would be that ARF9 and MP have opposite biochemical activities. Indeed MP has been shown to activate a synthetic promoter, while ARF9 can repress the same promoter (Ulmasov et al., 1999).

Furthermore, lineage-specific expression of *MP* depends on the early patterning factor SSP (Bayer et al., 2009b), which demonstrates that the separation of ARF expression domains is part of the developmental program that establishes embryonic and extra-embryonic cell fates. The existence of a prepattern of intrinsically different, lineage-specific ARF proteins suggests that auxin accumulation in embryonic and extra-embryonic cells triggers distinct transcriptional changes. Identification of target genes for suspensor-specific ARFs will allow comparison to the recently identified genes regulated by MP (Schlereth et al., 2010).

Different scenarios could explain the differences in auxin response between the future hypophysis and the remaining suspensor cells. Based on expression analysis, all suspensor cells contain the same ARFs throughout development (this study; Rademacher et al., 2011). A means by which uniform ARF expression can lead to different protein levels among suspensor cells would be differential stability of proteins between cells. Detailed analysis of ARF protein levels in the suspensor should resolve this question. Similarly, there may be subtle differences in the expression and/or stability of IAA10 between the uppermost and more basal suspensor cells prior to hypophysis specification. Furthermore, a large proportion of the Aux/IAA gene family has not been investigated during embryogenesis. Members, other than IAA10 and IAA12/BDL, may also play a role in auxin responses in the embryo, and add additional spatiotemporal diversification.

An alternative explanation for the distinct auxin-dependent processes in the uppermost versus the remaining suspensor cells is that the uppermost cell receives more auxin from the pro-embryo than other suspensor cells. This would be consistent with the higher DR5-GFP expression in this cell (Friml et al., 2003). However, even when high levels of auxin are externally applied to embryos, hypophysis identity and its characteristic asymmetric division remains restricted to the uppermost cell (Weijers et al., 2006), rendering this interpretation unlikely. Finally, it is well possible that the upper cell, in addition to auxin, receives other signals from the pro-embryo that modify its auxin-dependent gene expression program. The recent identification of the TMO7 transcription factor as an MP-dependent mobile signal that moves to the uppermost suspensor cell only (Schlereth et al., 2010) will now enable us to answer whether mobile signals do indeed modify the auxin response properties of the uppermost suspensor cell to allow hypophysis specification.

MATERIALS AND METHODS

Plant Material

Plants used in all experiments were Columbia (Col-0) ecotype except for GAL4-GFP enhancer trap lines, which were in the C24 background. The UAS-BDL and UAS-bdl lines (Weijers et al., 2006), DR5-GFP (Friml et al., 2003), the pMP-n3XGFP line, mp-S319 (Schlereth et al., 2010) and the ssp-2 (SALK_051462) line (Bayer et al., 2009b) have been described previously. The seeds of the taa1 tar1 tar2 triple mutant (Stepanova et al., 2008) were a kind gift from J. Alonso (Raleigh, NC, USA). GAL4 driver lines and T-DNA insertion lines for the ARFs have been obtained from various sources as listed in supplemental tables 1 and 5. The arf9-1 and arf13-2 alleles were used to generate a double mutant. pARF1,2,6,12,14,15,18,20,21,22,23-n3GFP reporters have been described in detail elsewhere (Rademacher et al., 2011).

After sterilization, seeds were typically plated on ½ strength MS plates containing 0.8% Daishin agar (Duchefa), 1% sucrose and the appropriate

antibiotic for selection of transgenic seeds. The concentrations of the antibiotics in these plates were 50 mg/l kanamycin or 15 mg/l phosphinotricin. After two weeks of growth on these selection plates resistant seedlings were transferred to soil and further grown under a long day light regime at 22°C. Plant transformation was carried out by floral dipping (Clough and Bent 1998). Embryos were harvested for microscopy between 3 and 6 days after pollination.

Microscopy

For differential interference contrast (DIC) microscopy, ovules were dissected from siliques and mounted in clearing solution of chloral hydrate, water and glycerol (ratio w/v/v: 8:3:1). After incubation at room temperature (for several hours to over-night) cleared samples were investigated with a Leica DMR microscope equipped with DIC optics.

For determining expression patterns in the ssp mutant, ssp -/- plants were crossed as male parent with pIAA10-n3GFP, pARF1-n3GFP, pARF2-n3GFP, pARF5/MP-n3GFP, pARF6-n3GFP, pARF9-n3GFP, pARF13-n3GFP and pARF18-n3GFP plants. F1 embryos were analysed 4-6 days after manual pollination.

The suspensor length analysis in ssp-2 mp-B4149 double mutant embryos was done on DIC images of cleared ovules using a Zeiss Axio Imager Z1 microscope, equipped with an AxioCam HRc camera. Measurements were taken with a dedicated tool of the AxioVision software.

For fluorescence microscopy, ovules were first transferred from the silique to a drop of PBS buffer containing 4% (w/v) PFA, 5% glycerol (v/v) and 1 µM FM4-64 on a microscope slide. After applying the cover slip, embryos were squeezed out of the ovules by applying mild pressure on the cover slip with a pencil tip. Embryos were investigated for GFP signals by using a Carl Zeiss LSM510 confocal scanning laser microscope and exiting GFP and FM4-64 with an Argon laser line at 488 nm. GFP signals were recorded by using a bandpass filter ranging from 505 to 535 nm, while FM4-64 signals were taken after passing a longpass filter of 650 nm.

GUS staining and in-situ hybridization

GUS staining and in-situ hybridization were performed as described (Schlereth et al. 2010). The in-situ probes have been previously described in (Hamann et al. 2002) for MP, (Mayer et al., 1998) for WUS and (Long et al., 1996) for STM .

FRET-FLIM

For determining molecular interactions among ARF and Aux/IAA proteins in plant protoplasts, cDNAs were cloned into pMON999 sCFP3A and pMON999 sYFP2. These are modified versions of pMON999 that carry CFP and YFP versions optimized for FRET studies (Kremers et al., 2006). Transfections of Arabidopsis (Columbia wild-type) mesophyll protoplasts, harvested with a tape sandwich (Wu et al. 2009), were performed as described (Russinova et al., 2004).

FRET-FLIM measurements were performed on a Biorad Radiance 2100 MP system (Hercules CA) combined with a Nikon TE 300 inverted microscope (Tokyo, Japan)

as described (Russinova et al., 2004). For the FLIM experiments, a Hamamatsu R3809U MCP PMT (Hamamatsu city, Japan) was used, which has a time resolution of 50 ps. FRET between sCFP3A and sYFP2 was detected by monitoring donor emission using a 470-500 nm band pass filter. Images with a frame size of 64 x 64 pixels were acquired and the average count rate was around 10^4 photons per second for an acquisition time of ± 90 sec. Donor fluorescence lifetimes (sCFP3A) were analyzed with SPCImage 3.10 software (Becker&Hickl) using a two-component decay model. Several cells (n>14) were analyzed and average fluorescence lifetimes of different combinations were exported for generating a boxplot. Statistical significance of differences between samples was determined using a two-tailed Student's T-test.

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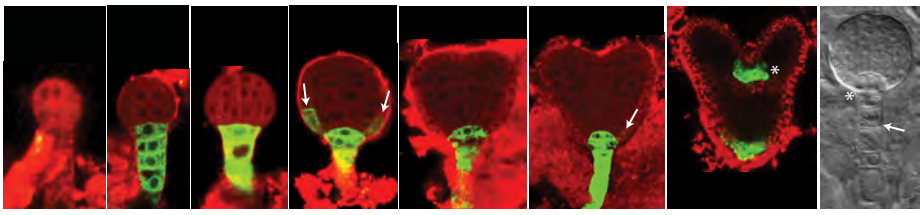
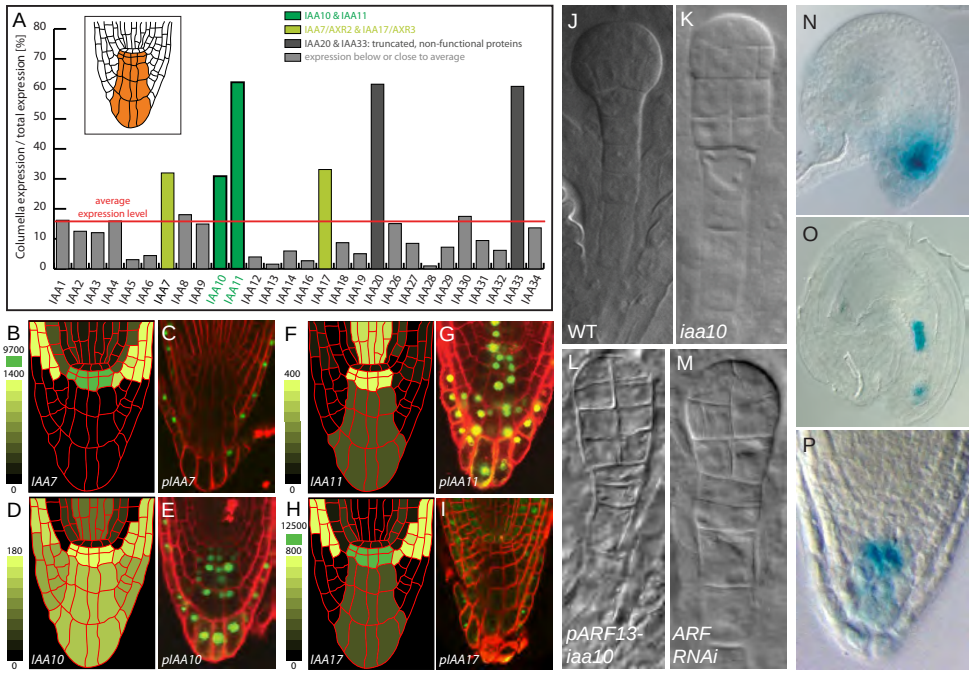
SUPPLEMENTARY INFORMATION



Supplemental Figure S1 (related to Figure 1). Expression of the M0171 GAL4 driver line as visualised by the linked UAS-GFP reporter (ER-localized green signal).
Expression is not yet observed in 8-cell embryos, but becomes detectable in all suspensor cells at the 16-cell stage. From then on, expression can be observed in all suspensor cells up to the late heart stage. Additional expression foci are found at late globular stage in single epidermal cells adjacent to the suspensor (arrows) and in the cotyledon boundaries at late heart stage (asterisk). Note that these additional expression domains are established well after hypophysis division. Membranes are counterstained with the red FM4-64 dye.
The right panel shows an M0171>>bdl F1 embryo in which bdl is expressed from a UAS-bdl gene, driven by the M0171 line, highlighting the first observable defects. The hypophysis does not divide (asterisk) and suspensor cells start to divide along a non-horizontal plane (arrow). Note that these defects are found shortly after the onset of M0171 expression and that no defects in the proembryo can be detected.



Supplemental Figure S2 (related to Figure 3).

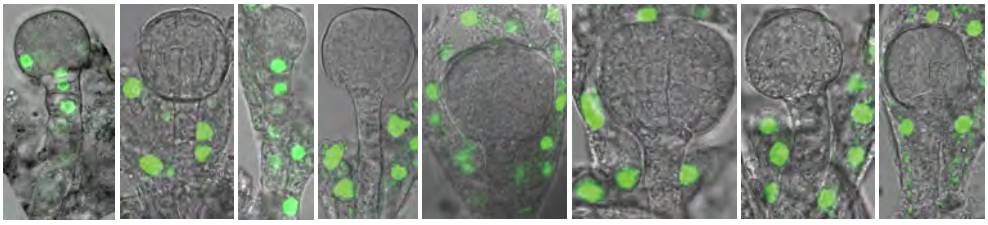
(A) Relative expression levels of all Aux/IAA genes were calculated by dividing the normalized average expression in the columella through the summed normalized and averaged expression levels in each root cell type of root zones 1 and 2 (Birnbaum et al. 2005). The red line indicates the relative expression level of a gene that is expressed equally in all 8 cell types. For IAA15 (At1g80390) no probes are present on the ATH1 GeneChip.

(B-I) Expression of IAA7, 10, 11 and 17 in seedling root tips. (B,D,F,H) Representation of microarray-derived expression levels according to (Brady et al., 2007). Expression levels are shown as values on a linear colour scale. For IAA7 (B) and IAA17 (H), an extra colour (green) and value is added due to the strong deviation from the remaining expression values. (C,E,G,I) Expression of pIAA7 (C), pIAA10 (E), pIAA11 (G) and pIAA17 (I) fused to nuclear 3xGFP in primary root tips. Note that expression patterns match the microarray-based pattern for IAA10 and IAA11, but differ for IAA7 and IAA17. The exceptionally high values in columella cells (green) is not reproduced in the pIAA7-n3GFP and pIAA17-n3GFP lines. Roots are counterstained with FM4-64 (membranes, red signal).

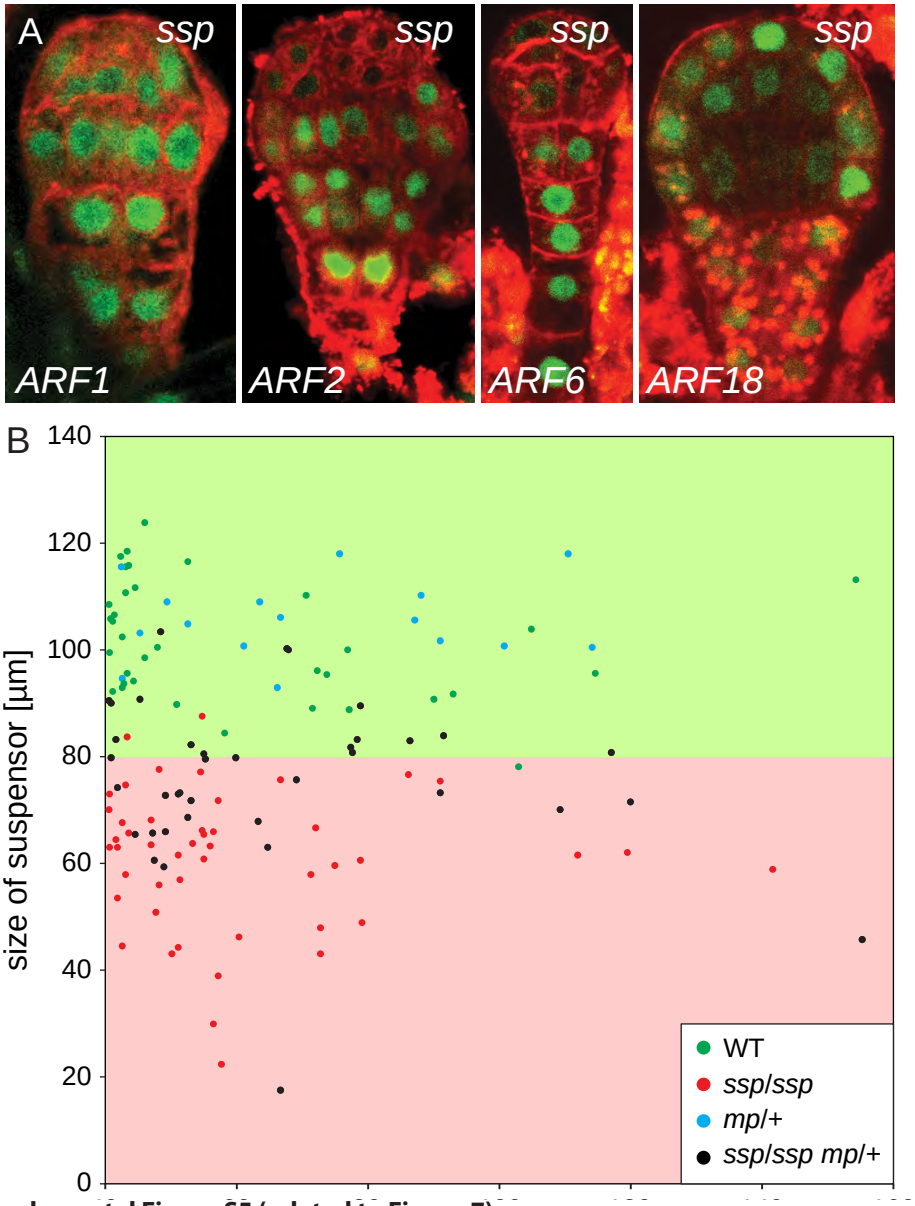
(J-M) Early embryo phenotypes resulting from interfering with suspensor-expressed Aux/IAA and ARF genes. Shown are 16-cell stage embryos of wild-type (J; WT), pIAA10-iaa10 (K; iaa10), pARF13-iaa10 (L) and p35S-ARF-RNAi (M; ARF-RNAi). Note that the uppermost suspensor cell has divided along an incorrect plane, giving rise to a duplicated embryo structure.

(N-P) iaa10-GUS expression in the embryo sac, zygote and root tip. (N,O) Expression of iaa10-GUS protein in the zygote as detected by GUS staining (blue). In the ovule shown in (O), faint staining is observed also in a degenerated synergid and antipodal cell. (P) While no expression can be detected during embryogenesis, iaa10-GUS fusion protein is found in columella cells of the post-embryonic root tip.

Supplemental Figure S3 (related to Figure 4). *IAA30* expression in post-globular stages and in the primary root tip.
(A,B) pIAA30-n3GFP expression in transition (A) and heart (B) stage embryos. Note intense fluorescence in incipient root and cotyledon primordia and the vascular tissue, sites of auxin activity. (C,D) pIAA30-n3GFP expression in primary root tips treated with mock medium (C), or with medium containing 1 µM 2,4-D (D) for 24 hours.



Supplemental Figure S4 (related to Figure 5). ARF expression in the embryo-surrounding endosperm (ESE).
Transcriptional fusions of n3xGFP to 2 kb long fragments of the promoters of ARF9, ARF12, ARF13, ARF14, ARF15, ARF20, ARF21 and ARF22 allow for detection of GFP signals (green) in the ESE.



Supplemental Figure S5 (related to Figure 7).
(**A**) Expression of pARF1, pARF2, pARF6 and pARF18, as determined by a nuclear 3xGFP reporter in ssp mutant embryos. Note that in each embryo, GFP signal is detected in suspensor cells despite their ssp-induced abnormal divisions.
(**B**) Suspensor length in wild-type (green dots), mp-B4149 (blue), ssp-2 (red) and ssp mp (black) embryos. Only embryos of which the pro-embryo size exceeds 40 µm were included. Each dot represents a single embryo. The range of suspensor length in wild-type embryos is indicated by a light-green box, and sizes below this in a light-red box.

Supplemental Table S1. GAL4 driver lines used in this study (related to Figure 1).

Driver line	ABRC Seed Stock #	Expression pattern		Phenotype of driver X UAS-bdl	Reference
		at globular stage	at torpedo stage		
J0571	N9094	n.d.[a]	Cortex + endodermis with initials	-[b]	1
J1092	N9147	n.d.	QC + “inner” root cap	-	1
J3281	N9128	Ubiquitous	Vasculature + central root cap	+[b]	1
KS068	N9256	Suspensor	Suspensor, shoot apex	+	1
M0028	N9274	n.d.	Root cap in embryo and seedling	-	1
M0136	N9302	n.d.	Basal half of embryo	-	1
M0148	N9303	n.d.	Cotyledon junction	-	1
M0164	N9307	n.d.	Cotyledon junction and cotyledons	-	1
M0167	N9308	n.d.	Cotyledon junction	-	1
M0171	N9312	Suspensor	SAM, cotyledon junction, RAM, suspensor	+	1
M0223	N9336	n.d.	SAM	-	1
Q0680	N9209	n.d.	Central root cap + vasculature	-	1
Q0990	N9217	Inner cells of the proembryo	Central cell and stele initials, vasculature	+	1
Q1630	N9227	n.d.	Columella, vasculature	-	1
RPS5A	n.a.[a]	Ubiquitous	Ubiquitous	+	2

a: “n.d”, not determined; “n.a.”, not applicable.
b: Each line was crossed with the same UAS-bdl line. “-“ means that no abnormal phenotypes were observed in F1 embryos, “+” indicates that abnormal phenotypes were observed.
References for GAL4 driver lines: (1) http://www.plantsci.cam.ac.uk/Haseloff/, (2) Weijers et al. (2003)

Supplemental Table S2. Percentage of abnormal embryos and rootless seedlings in *iaa10*-expressing plant lines (related to Figure 3).

Genotype	Preglobular		Globular		Post-globular		Rootless seedlings	
	% phen	N	% phen	N	% phen	N	%	N
pIAA10-iaa10								
line 1	16	100	17	47	nd[a]	nd		
line 2	7.0	57	12	50	nd	nd		
line 3	1.7	58	4.1	148	2.0	50		
pARF13-iaa10								
line 1	28	96	26	246	48	115		
line 2	31	49	17	156	9.6	73		
line 3	22	85	16	196	22	100		
line A							45	64
line B							34	67
line C							29	21
line D							24	58
line E							16	67
line F							9.2	327
line G							9.0	155
line H							6.3	64
line I							4.3	161

Embryos and seedlings of *pIAA10::iaa10* (3 independent transgenic lines) or *pARF13::iaa10* (12 independent lines) lines were scored for phenotypic aberrations at 3 different stages of embryo development as well as after germination. The percentage of embryos showing abnormal cell division patterns or seedlings lacking a root is shown (% phen) as is the number of individuals counted at each stage for each plant line (N).

a: "nd", not determined

Supplemental Table S3. ARF T-DNA lines used in this study (related to Figure 5).

Gene	Locus	Allele	Stock #	Reference
ARF1	At1g59750	arf1-5	SALK_079046	1
ARF2	At5g62000	arf2-8	SALK_108995	1
ARF3	At2g33860	arf3-3	SALK_031544	n.a.[b]
ARF4	At5g60450	arf4-2	SALK_070506	2
ARF5/ MP	At1g19850	mp-B4149	n.a.[b]	3
ARF6	At1g30330	arf6-1	CS24606	4
ARF7	At5g20730	arf7-1	SALK_040394	4
ARF8	At5g37020	arf8-4	fwf	5
ARF9	At4g23980	arf9-1	CS24609 / SAIL_881_H05	4
		arf9-2	CS24610 / SAIL_1207_ H04	4
		arf9-3	SALK_019903	n.a.[b]
		arf9-4	SALK_032472	n.a.[b]
		arf9-5	SALK_005473	n.a.[b]
		arf9-6	SALK_032480	n.a.[b]
		arf9-7	SALK_060870	n.a.[b]
		arf9-8	SALK_063144	n.a.[b]
ARF10	At2g28350	arf10-1	SALK_143232	4
ARF11	At2g46530	arf11-2	SALK_063778	n.a.[b]
ARF12	At1g34310	arf12-2	SAIL_1161_E12	4
ARF13	At1g34170	arf13-1	SALK_005960	4
		arf13-2	SALK_138188	n.a.[b]
ARF14	At1g35540	arf14-1	FLAG_497G08	n.a.[b]
ARF15	At1g35520	arf15-101	SALK_121828	n.a.[b]
ARF16	At4g30080	arf16-1	SALK_021448	3
		arf16-2	SALK_021432	3
ARF17	At1g77850	arf17-1	SALK_062511	n.a.[b]
ARF18	At3g61830	arf18-2	GABI_513E07	n.a.[b]
ARF19	At1g19220	arf19-1	CS24617	4
		arf19-2	CS24618	4
ARF20	At1g35240	arf20-1	SALK_019051	4
ARF21	At1g34410	arf21-101	SALK_020702	n.a.[b]
ARF22	At1g34390	arf22-101	SALK_123566	n.a.[b]
ARF23	At1g43950	n.a.[c]	n.a.[c]	n.a.[c]

a: "n.a.", not applicable; *mp*^{B4149} is an EMS-induced allele that is not available from the Stock Centre; No insertion lines or mutants are available for *ARF23*.
b: "n.a.", not applicable"; To our knowledge, these ARF T-DNA insertions have not been used or described previously. We have given these conventional allele numbers.
c: "n.a.", not applicable"; No insertion lines are available for ARF23.
References: (1) Ellis et al. (2005) (2) Pekker et al. (2005) (3) Weijers et al. (2005) (4) Okushima et al. (2005) (5) Vivian-Smith et al. (2001).

Supplemental Table S4. Embryonic aberrations caused by ARF-RNAi (related to Figure 5).

Genotype	Defective embryos	
	%	N
35S-1	7.3	205
35S-2	14	100
RPS5A-1	7.2	131
RPS5A-2	3.0	52

Overexpression of an RNAi fragment targeted against a group of highly similar ARFs (ARF12-15 and ARF20-23) caused embryonic defects. The frequencies of defective embryos (%) and the total numbers of embryos analyzed (N) are given. Two independent lines were analyzed for p35S-RNAi and pRPS5A-RNAi constructs.

Supplemental Table S5. pMP-ARF9 rootless frequencies.

Line #	Rootless seedlings	
	%	N
3	16	730
4	19	513
7	7.1	560
9	16	1125
10	19	549

The percentage of rootless seedlings (%) and total number of seedlings counted (N) are given for 5 independent pMP-ARF9 lines in the mp-S319 background.

Supplemental Table S6. Suspensor defects in pARF13-MP lines (related to Figure 7).

Line #	Defective embryos	
	%	N
1	10	30
2	8.3	60
3	17	80
4	18	95
5	11	105

Embryo defects were scored in embryos derived from 5 independent primary transgenics. The frequencies of defective embryos (%) and the total numbers of embryos analyzed (N) are given.

Supplemental Table S7. Primers used for cloning.

Gene	Primer orientation	Sequence (5' extension in bold)	Position relative to ATG	Template
Primer pairs for promoter amplification (5'extension in bold letters)				
IAA7	Sense	TAGTTGGAATGGGTTCGAATATTTAAGTTTCAAAAGC	-2082	genomic
	Antisense	**TTATGGAGTTGGGTTCGAA**GTTACTTGTAATAGATTAG	-1	
IAA10	Sense	**CTCGAG**AGTGGATCGAAGCATACGACCTTG	-1014	genomic
	Antisense	**AGCTGCAG**TATCCCAAATTGCCAAACGAATAC	-1	
IAA11	Sense	**CTCGAG**AGTGGATCGAAGCATACGACCTTG	-1442	genomic
	Antisense	**CTTACCTA**CTCCAGCTCCAATTGATG	-1	
IAA17	Sense	TAGTTGGAATGGGTTCGAATTTTTGTAATCATGTAGG	-2152	genomic
	Antisense	TTATGGAGTTGGGTTCGAATATTAACCTTTCTTCTTC	-1	
IAA30	Sense	TAGTTGGAATGGGTTCGAATATTTAAGTTTCAAAAGC	-2082	genomic
	Antisense	**TTATGGAGTTGGGTTCGAA**GTTACTTGTAATAGATTAG	-1	
ARF9	Sense	**CGGTACC**TGGTGGTGGGTTTTAAGG	-2295	T32A16
	Antisense	**TGGGCCC**CAGCTGATTAAATCTTCTATCAGTCACACC	-1	
ARF13	Sense	**AGGTACC**AGAGTCAAAAAAATGATTTTGTTTGTGATTA	-1802	F12G12
	Antisense	**CGGGCCC**GAGCTCTTATTGCAA	-1	
Primer pairs for amplification of genomic fragments and cDNAs				
IAA11 locus	Sense	AGTTGAAGTTGAGAAGAAG	1	genomic
	Antisense	TAATATCATCTGAGCTGTAA	2222	
IAA10 locus	Sense	AGTGGATCGAAGCATACGACCTTG	1	genomic
	Antisense	CTTACCTACTCCAGCTCCAATTGATG	3687	
IAA10NT	Sense	**CTCGAG**AGTGGATCGAAGCATACGACCTTG	-1014	genomic
	Antisense	**AGCTGCAG**AAGCATAGAAGTCCTGGACG	702	
ARFRNAi	Sense	ATAGATCCAAGAGCTATATG		genomic
	Antisense	CACGACATTGAAGCTTAGAAGGAAG		
ARF5/MP	Sense	GACTCGAGGCTTCATTGTCTTGTGTTG	1	cDNA
	Antisense	CCACTAGTTGAAACAGAAGTCTTAAGATCG	2709	
ARF9	Sense	TAGTTGGAATAGGTTCATGGCAAATCGCGGAGGTGA	1	cDNA
	Antisense	AGTATGGAGTTGGGTTCTTAGTTGGAATGATTATCTG	1917	
Primer pairs for FRET-FLIM cDNA cloning				
MP	Sense	**ACGGTACC**ATGATGGCTTCATTGTCT		cDNA
	Antisense	**AGGGCCC**TGAAACAGAAGTCTTAAG		
ARF3	Sense	**GGTACC**ATGGGTGGTTTAATCGAT		cDNA
	Antisense	**GGGCCC**GAGAGCAATGTCTAGCAA		
ARF9	Sense	**ACGGTACC**ATGGCAAATCGCGGAGGT		cDNA
	Antisense	**AGGGCCC**GTTGGAATGATTATCTGT		
bdl	Sense	**ACGGTACC**ATGCGTGGTGTGTCAGAATTGG		cDNA
	Antisense	**AGGGCCC**AACAGGGTTGTTTCTTTG		
iaa10	Sense	**ACGGTACC**ATGAATGGTTTGCAAGAAG		cDNA
	Antisense	**AGGGCCC**CTTACCTACTCCAGCTCC		
5' phosphorylated primers for site-directed mutagenesis (exchanged nucleotide in bold)				
iaa10		CTGTAGGTTGG**A**CGCCTCTACGG		
iaa11		GTCCTTATTGGTGACCATCCCAC		

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Chapter

Identification of putative cell identity determinants through targeted and specific inhibition of auxin response in the Arabidopsis suspensor

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ABSTRACT

During early Arabidopsis embryo development, two distinct cell lineages are formed following the initial zygote division. The apical cell gives rise to cells with embryonic identity and the basal cell gives rise to a single file of cells with extra-embryonic identity. Little is known about what distinguishes these pools of cells at molecular level. Here, we exploited the finding that inhibition of auxin responses in the extra-embryonic lineage causes these cells to adopt embryo-like identity. We inhibited auxin response in these cells and used a novel technique to dissect intact embryos. Coupled to a microarray-based experiment, we generated a unique dataset that includes transcriptional changes in genes that are involved in extra-embryonic cell identity and genes involved in acquisition of embryonic identity. The number of misregulated genes is large and includes secondary transcriptional changes in embryo-expressed genes due to the interconnectedness between the cell lineages. Therefore global analysis guided the selection of seventy genes, for which transcriptional reporters were made and expression in early embryogenesis was documented. Subsequently functional characterization of correctly expressed genes was conducted in an attempt to place these genes into a developmental identity context. While no non-redundant pro-embryo or suspensor regulators were identified, this study defines a novel set of genes whose expression is regulated during suspensor to embryo transformation.

INTRODUCTION

In *Arabidopsis thaliana* (Arabidopsis), zygotic embryogenesis begins with the hallmark asymmetric cell division of the zygote, generating a smaller apical and a larger basal cell. This polarity is further elaborated upon with the apical cell dividing horizontally and vertically to form a spherical pro-embryo and the basal cell dividing only horizontally to give rise to an extra-embryonic file of cells called the suspensor (reviewed by Peris et al., 2010). Throughout these early events, the pro-embryo and suspensor represent distinct cell pools with different identities and fates, which we here refer to as embryonic and extra-embryonic, respectively. Our understanding of the factors that control early embryogenesis and play a role in the formation of apical (embryonic) and basal (suspensor/extra-embryonic) cell lineages is limited to a few pathways.

The SSP-YDA pathway includes a mitogen-activated protein kinase (MAPK) signaling cascade, the temporal activation of which is initiated by zygotic translation of pollen-derived *SHORT SUSPENSOR* receptor-like kinase (*SSP*) transcripts (Bayer et al., 2009). Sequential function of the MAPKKK YODA (YDA) and MPKs (MPK3 and MPK6) control elongation and asymmetric division of the zygote. These events are essential for subsequent suspensor development, and disruptions in this pathway lead to small cells that do not fully differentiate as suspensor and sometimes undergo aberrant cell divisions (Lukowitz et al., 2004).

No transcription factors that are controlled by this MAPK cascade in the embryo have been identified. However, mutations in the RWP-RK transcription factor GROUNDED/RKD4 cause phenotypes similar to those in *yda* and *ssp* (Waki et al., 2011; Jeong et al., 2011).

Another pathway involves several WOX homeobox transcription factors, which are co-expressed in the zygote and then, following the asymmetric cell division, become restricted to apical (WOX2) and basal (WOX8 and WOX9) cells (Haecker et al., 2004). Here, they activate distinct transcriptional programs, which are important for the patterning and specification of their descendants (Breuninger et al., 2008). Both *wox2* single and *wox8 wox9* double mutants display aberrant cell divisions throughout early embryogenesis, including infrequent extra cell divisions in the suspensor cell lineage in *wox8 wox9* (Haecker et al., 2004; Breuninger et al., 2008). Interestingly, the WRKY2 transcription factor was recently identified as a direct regulator of *WOX8/9* transcription (Ueda et al., 2011).

Besides these pathways, the hormone auxin plays a prominent role throughout embryo development. Mutations in auxin biosynthesis, transport and response induce apical embryo phenotypes, some as early as the aberrant division of the apical daughter cell just after zygote division (reviewed by Möller and Weijers, 2009). Auxin is mostly known to act when the uppermost suspensor cell undergoes an identity change, is specified as hypophysis and incorporated into the embryo where it undergoes a hallmark asymmetric division to give rise to the quiescent centre and columella stem cells. This identity change requires both cell autonomous and non-cell autonomous auxin responses (Chapter 3 and Schlereth et al., 2010).

When investigating the cell autonomous transcriptional auxin response required for hypophysis specification, we found that this response is also needed to maintain extra-embryonic, suspensor cell identity (Chapter 3). Previously it was demonstrated that auxin response could be blocked in discrete domains of the pro-embryo by locally expressing a non-degradable (auxin resistant) Aux/IAA protein using the GAL4-*UAS* based two component gene expression system (Weijers et al. 2006). The same approach was used to block auxin response in suspensor cells, which responded by undergoing proliferative cell divisions. Although reminiscent of *ssp* and *wox8 wox9* suspensor phenotypes, there is little evidence functionally connecting these pathways. *WOX* genes do not appear to mediate the auxin response inhibition phenotype, as *WOX2/8/9* expression patterns are unchanged even in phenotypically affected *iaa10* mutant embryos (Rademacher, 2009). However, the *ARF5* transcriptional reporter expression pattern is expanded from the apical domain into phenotypic suspensor cells in *ssp* mutant embryos, opening up the possibility that this misregulation is casual to the suspensor defect in *ssp* (Chapter 3).

The auxin response inhibition phenotype distinguishes itself by forming pro-embryo-like structures from proliferated suspensor cells. Indeed, through the analysis of several apical embryo markers in this background, it appeared that suspensor cell identity was compromised and cells had undergone a transition to embryo identity. Stabilization (against auxin mediated degradation) of an endogenous Aux/IAA (iaa10) in the suspensor also led to suspensor proliferation and postembryonic defects including seedling rootlessness (Chapter 3).

Suspensor to embryo transformation demonstrates the inherent embryonic potential of the suspensor. Suspensor proliferation phenotypes are often preceded by defects in the pro-embryo, as is the case for the *twn2*, *abnormal suspensor* and *raspberry* mutants (Schwartz et al., 1994; Yadegari et al., 1994; Vernon and Meinke, 1994; Zhang and Somerville, 1997). These observations imply the presence of apical cell/embryo derived signals that normally inhibit embryonic development in suspensor cells (Vernon and Meinke, 1994). Nevertheless, viable suspensor derived secondary embryos can be formed without defects in early pro-embryo development in the *twn1* and *amp1* mutants (Vernon et al., 2001; Vernon and Meinke, 1994). Also, disruption of any member of two pairs of closely related genes, *LEAFY COTYLEDON* (*LEC1*) and *LEAFY COTYLEDON LIKE* (*L1L*) or *LEAFY COTYLEDON2* (*LEC2*) and *FUSCA3* (*FUS3*) cause suspensor proliferation phenotypes, in addition to their better characterized roles in the temporal regulation of embryo development (Lotan et al., 1998; Stone et al., 2001; Kwong et al., 2003). Of these only *FUS3* is expressed in the suspensor at early stages of embryo development (Kroj et al., 2003) and may therefore be directly involved in suppression of early embryonic development in the suspensor.

Regardless, auxin response is the first cell autonomous mechanism shown to prevent embryonic transformation of the suspensor. This is rather counterintuitive, as auxin treatment (and response) is also inductive for somatic embryogenesis (reviewed in Karami and Saidi, 2010). These seemingly opposite effects highlight the context-dependent nature of auxin response. Like natural and induced changes in cell identity, auxin response mediated suspensor to embryo transformation is envisioned to involve reprogramming of the transcriptional profile of the cells involved.

This auxin response-mediated cellular reprogramming event now gives us a handle to investigate the role of auxin in the determination of embryonic versus extra-embryonic cell identities. Importantly, a description of suspensor cells at molecular (transcript) level is lacking, and less than a handful of suspensor-specific genes are known to date. Therefore, to understand suspensor identity and its transformation to embryo identity, a genome-wide approach at identifying determinants is warranted.

By specifically inhibiting auxin response in extra-embryonic cells and subsequently investigating early transcriptional changes, we aim to identify key regulators of suspensor cell identity and of the suspensor to embryo transformation. We achieve the targeted and specific inhibition of auxin response by expressing a stabilized bdl protein under the control of the GAL4 driver line M0171 as described in Chapter 3. In this chapter, we couple this two component system with newly developed methods to isolate whole live *Arabidopsis* embryos and perform genome-wide transcriptional profiling to identify differentially expressed genes when auxin response is inhibited in suspensor cells. Following a global analysis of the resulting datasets, a subset of differentially expressed genes was selected and initially screened for expression pattern in wild type embryos. Using this approach, we identify many genes with previously undescribed patterns of expression in both the embryo and suspensor. This wealth of novel expression pattern information provides new leads for investigating the specification of specific embryonic and suspensor derived cell types. We also identify and further investigate several candidate genes that may be involved in maintenance of suspensor cell identity or suspensor to embryo transformation.

RESULTS

Time point for Microarray

In Chapter 3 it was shown that the GAL4 driver line *M0171* specifically activates expression of *UAS-bdl* in suspensor cells up until heart stage of embryogenesis, when expression of GAL4 (as reported by GFP) expands to cells in the pro-embryo (Chapter 3, figure 2). In order to select a time point at which embryos would be dissected and used in microarray experiments, *M0171* was crossed with *UAS-bdl* and ovules were harvested at specific time points following pollination to ascertain when the first aberrant cell divisions were occurring (Fig. 1). Optimally, transcript profiling should be performed just before the first morphological abnormalities are detected. Based on the expression of the M0171 GAL4 driver line as visualized by the linked UAS-GFP which becomes detectable in all suspensor cells at the 16-cell stage (Chapter 2, Suppl. Fig. 1) and the coincident detection of the first aberrant cell division(s) in approximately one third of embryos, the time point 72 hours post pollination was chosen for subsequent embryo dissection, RNA isolation and hybridization.

Generation of the M0171-bdl datasets

Seventy two hours post pollination of M0171-GFP flowers with either *UAS-bdl* or wild type pollen, embryos were isolated from ovules using a newly developed dissection method (see Materials and Methods). RNA was extracted, amplified and labeled, prior to hybridization to the microarray slides as detailed in the Materials and Methods. Following hybridization, image analysis and signal

quantification were performed using the QuantArray program (GSI Lumonics). After initial statistical analysis of the raw array data using Limma Software (Smyth, 2004), two M0171-bdl datasets were generated, containing positively and negatively misregulated genes. The upregulated gene list contains 1366 unique entries and the downregulated gene list contains 1050 unique entries (cut off 1.5 fold misregulated and False Discovery Rate (FDR) < 5.5 %). With a more stringent cut off of at least 2.0 fold misregulated (and FDR of < 5.5 %) the bdl-M0171 datasets included 621 upregulated genes and 349 downregulated genes (Suppl. Table S1).

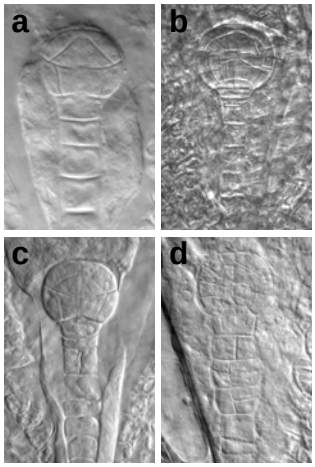

Figure 1. Selection of time point for M0171-bdl microarray experiments.
Embryos from crosses, M0171 X wild type (a,d) and M0171 X UAS-bdl (c,d) prepared 72 hours post pollination (a,c) and 96 hours post pollination (b,d). At 72 h post pollination approximately one third of M0171XUAS-bdl embryos show a suspensor cell division phenotype (c).

Global analysis of M0171>bdl dataset

Given the large number of misregulated genes, unguided data analysis did not reveal obviously enriched functions or known regulators. Hence, to better comprehend qualitative aspects of the transcriptome regulation underlying suspensor to embryo transformation, the M0171-bdl datasets were validated using a global analysis and bioinformatics approach. This approach also facilitated the selection of a subset of genes. These genes were selected not only for validation of the datasets but also as biologically meaningful candidate genes and pathways in suspensor and embryo identity. The global analysis of the M0171-bdl datasets and criteria for selection of genes are detailed in the Supplementary Information to this chapter. The global analysis revealed that suspensor-specific bdl misexpression induces a complex transcriptional response that represents a genome-scale reprogramming, including both primary effects in suspensor cells and secondary effects on pro-embryo cells. Among the transcriptional changes, there is a prevalence of transcription factors and auxin-regulated genes, as well as zygote-enriched genes.

Selection of genes for further investigation

Based on previously published research and the global analysis of the M0171-bdl datasets, seventy genes were selected for further analysis (Table 1). All genes were at least 2.0 fold misregulated and 19 genes were in the top 10% most misregulated genes. Twenty two of the selected genes were identified as auxin responsive in the AHD2.0 (including one Aux/IAA) and 33 were transcription factors as annotated in the Plant Transcription Factor Database v2.0, of which 20 belong to auxin responsive transcription factor families (Suppl. Information). The subset also includes 14 bHLH superfamily genes and 8 genes involved in auxin response/homeostasis. Furthermore, three embryo specific transcription factors (as identified through analysis of LCM microarrays) were included as well as four genes enriched in either the zygote transcriptome or the hypophysis derivatives transcriptome. Finally, 6 genes were included because their expression during embryogenesis has been well documented and therefore they serve as controls for the transcriptional fusion approach adopted below (Table 1).

Generation of transcriptional fusion lines

In order to identity determinants of embryonic and extra-embryonic cell identity it was essential to first investigate the embryonic expression domains of the selected genes in wild type embryos. Specifically, genes upregulated in the M171-bdl dataset should not be expressed in the suspensor (but could be expressed in the apical pro-embryo) whereas downregulated genes should be specifically expressed in the suspensor cells of wild type embryos (at least up until hypophysis specification). As a moderately high-throughput strategy for determining gene expression patterns, we chose to generate transcriptional fusions to a sensitive, nuclear localized triple GFP reporter. These were made by amplifying 2.0 Kb of the upstream regions of the selected genes and cloning these into a binary vector using a ligation-independent cloning strategy (see Materials and Methods). Subsequently, a simplified Arabidopsis transformation procedure was used to introduce the transcriptional fusions into Arabidopsis (see Materials and Methods).

Expression analysis of transcriptional fusion lines

Of the 70 genes selected, we obtained transgenics for 68. As patterns of expression and intensity of GFP signals can vary between individual transgenics, expression was analyzed by epi-fluorescence in embryos prepared from the T2 ovules of up to 8 independent primary transformants. Candidate genes whose transcriptional reporters were not expressed, or expressed in a pattern that conclusively excluded their direct involvement in suspensor cell identity maintenance or suspensor transformation, were not further investigated at this stage. For candidate genes whose transcriptional reporters conformed to expression patterns consistent with putative roles in these processes, two

representative lines were selected and expression patterns were extensively imaged using a confocal microscope. An overview of the results of this expression analysis is given in Table 2.

Table 1. Seventy genes selected for further analysis.
AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. Fold Change = fold change in M0171-bdl dataset.

internal #	AGI	Other Name(s)	Short Gene Model Description	Fold Change	Criteria
1	AT1G22590	AGL87	MADS box transcription factor	3.1 up	Transcription Factor
2	AT1G48910	YUC10	Auxin biosynthesis enzyme	3.5 up	Auxin homeostasis, control, previously published expression pattern
3	AT1G61566	RALFL9	Member of predicted peptide family	2.6 up	Predicted to be zygote enriched
4	AT1G68320	MYB62	R2R3-MYB transcription family	3.0 up	Transcription Factor
5	AT1G68510	LBD42	LBD transcription factor	2.8 up	Auxin responsive TF family
6	AT1G68920		basic helix-loop-helix (bHLH) superfamily protein	2.5 up	Auxin responsive TF family , bHLH, enriched in hypophysis derivates transcriptome
7	AT1G76500	SOB3/AHL29	AT hook domain containing protein	2.3 up	
9	AT2G12900		Basic-leucine zipper (bZIP) transcription factor	2.0 up	Transcription factor, predicted suspensor specific (LCM)
10	AT2G21420		IBR domain containing protein	2.8 up	
11	AT2G26850		F-box family protein	3.4 up	Predicted to be zygote enriched
12	AT2G27250	CLV3	One of the three CLAVATA genes controlling SAM size	44.8 up	Top 10% of misregulated genes
13	AT2G35310		Transcriptional factor B3 family protein	2.4 up	Transcription factor, predicted proembryo specific (LCM)
14	AT3G03770		Leucine-rich repeat protein kinase family protein	2.1 up	
15	AT3G25170	RALFL26	Member of predicted peptide family	4.0 up	Predicted to be zygote enriched
16	AT3G54320	WRI1	Transcription factor of the AP2/ERWEBP class	4.2 up	Transcription factor, control, previously published expression pattern
17	AT3G54800		PH and lipid-binding START domain containing protein	24.4 up	Top 10% of misregulated genes
18	AT3G57800	bHLH060	basic helix-loop-helix (bHLH) superfamily protein	2.4 up	Auxin responsive TF family , bHLH
19	AT3G61150	HDG1	HD-ZIP IV family homeobox-leucine zipper family protein	2.4 up	Auxin responsive TF family, predicted proembryo specific (LCM)
20	AT3G61160		Protein kinase superfamily protein	4.1 up	Predicted to be zygote enriched
21	AT4G28650		Leucine-rich repeat transmembrane protein kinase family	3.3 up	
22	AT4G32540	YUC1	Auxin biosynthesis enzyme	5.9 up	Auxin homeostasis, control, previously published expression pattern, top 10% of misregulated genes
23	AT4G34530	bHLH063/CIB1	basic helix-loop-helix (bHLH) superfamily protein	9.9 up	Auxin responsive TF family , bHLH, top 10% of misregulated genes
24	AT5G06250		AP2/B3-like transcriptional factor family protein	3.8 up	Transcription factor predicted proembryo specific (LCM)
25	AT5G11320	YUC4	Auxin biosynthesis enzyme	7.9 up	Auxin homeostasis, control, previously published expression
26	AT5G11530	EMF1	Involved in regulating reproductive development	2.2 up	Known function
27	AT5G25830	GATA12	GATA factor family of zinc finger transcription factors	6.3 up	Transcription factor, top 10% of misregulated genes
28	AT5G26650	AGL36	DNA binding transcription factor	2.7 up	Transcription Factor
29	AT5G26950	AGL93	DNA binding transcription factor	2.5 up	Transcription Factor
30	AT5G39860	BHLH136/PRE1/BNQ1	basic helix-loop-helix (bHLH) superfamily protein	5.2 up	Auxin responsive TF family , bHLH, top 10% of misregulated genes
31	AT5G47670	LEC1-LIKE	Functions as a regulator of embryo development.	2.5 up	Transcription factor, known regulator of somatic embryogenesis
32	AT5G52600	MYB82	Member of the R2R3 factor gene family.	2.0 up	Transcription factor
33	AT5G56270	WRKY2	WRKY Transcription factor	2.5 up	Auxin responsive TF family
34	AT5G57670		Protein kinase superfamily protein	2.2 up	
91	AT3G05800	bHLH150/AIF1	basic helix-loop-helix (bHLH) superfamily protein	2.9 up	Auxin responsive TF family , bHLH
92	AT3G28857	bHLH164/PRE5	basic helix-loop-helix (bHLH) superfamily protein	2.4 up	Auxin responsive TF family , bHLH
35	AT1G13960	WRKY4	WRKY DNA-binding protein	2.2 down	Auxin responsive TF family
36	AT1G14600		Homeodomain-like superfamily protein	2.5 down	Transcription factor
37	AT1G15670		Galactose oxidase/kelch repeat superfamily protein	3.1 down	Predicted suspensor specific (LCM), top 10% of misregulated genes
38	AT1G31320	LBD4	LBD transcription factor	2.8 down	Auxin responsive TF family
39	AT1G62000		unknown protein	2.4 down	
40	AT1G62220		unknown protein	2.2 down	
41	AT1G80440		Galactose oxidase/kelch repeat superfamily protein	3.5 down	Top 10% of misregulated genes
42	AT1G80640		Protein kinase superfamily protein	2.3 down	
43	AT2G18300	bHLH064	basic helix-loop-helix (bHLH) superfamily protein	2.2 down	Auxin responsive TF family , bHLH
44	AT2G18890		Protein kinase superfamily protein	3.7 down	Top 10% of misregulated genes
45	AT2G23050	NPY4	Involved in auxin-mediated organogenesis	3.2 down	Auxin homeostasis, enriched in hypophysis descendent
46	AT2G41170		F-box family protein	2.3 down	
47	AT2G45430	AHL22	AT hook domain containing protein	2.3 down	
49	AT3G13960	GRF5	Growth regulating factor encoding transcription activator	25.4 down	Trancription factor, top 10% of misregulated genes
50	AT3G54780		Zinc finger (C3HC4-type RING finger) family protein	4.8 down	Top 10% of misregulated genes
51	AT3G62100	IAA30	Encodes a member of the Aux/IAA family	3.4 down	Auxin responsive TF family, enriched in hypophysis
52	AT4G04090		BTB/POZ domain-containing protein	2.8 down	
53	AT4G10270		Wound-responsive family protein	2.2 down	
54	AT4G18740		Rho termination factor	2.1 down	
55	AT4G24390		RNI-like superfamily protein	2.1 down	
56	AT4G31820	ENP/NPY1/MAB4	member of the NPY family genes	5.9 down	Auxin homeostasis, top 10% of misregulated genes
57	AT4G36240	GATA7	GATA factor family of zinc finger transcription factors	2.0 down	Transcription factor
58	AT5G18270	NAC087	Arabidopsis NAC domain containing protein	3.6 down	Trancription factor, top 10% of misregulated genes
59	AT5G48940		Leucine-rich repeat transmembrane protein kinase	3.0 down	Enriched in hypophysis descendent transcriptome, top 10% of misregulated genes
60	AT5G49490	AGL83	MADS-box transcription factor family protein	2.2 down	Transcription factor
61	AT5G50915	bHLH137	basic helix-loop-helix (bHLH) superfamily protein	4.6 down	Auxin responsive TF family , bHLH, top 10% of misregulated genes
62	AT5G66560		Phototropic-responsive NPH3 family protein	2.2 down	
67	AT1G29950	bHLH144	basic helix-loop-helix (bHLH) superfamily protein	3.5 down	Auxin responsive TF family , bHLH, top 10% of misregulated genes
68	AT1G68810	bHLH030	basic helix-loop-helix (bHLH) superfamily protein	2.7 down	Auxin responsive TF family , bHLH
69	AT2G01420	PIN4	Auxin efflux carrier	15.5 down	Auxin homeostasis, control, previously published expression
74	AT1G05710	bHLH153	basic helix-loop-helix (bHLH) superfamily protein	2.2 down	Auxin responsive TF family , bHLH, predicted embryo specific (LCM)
75	AT2G41240	bHLH100	basic helix-loop-helix (bHLH) superfamily protein	2.0 down	Auxin responsive TF family , bHLH
76	AT2G41130	bHLH106	basic helix-loop-helix (bHLH) superfamily protein	2.2 down	Auxin responsive TF family , bHLH
85	AT1G73590	PIN1	Auxin efflux carrier	4.9 down	Auxin homeostasis, control, previously published expression
96	AT2G42870	bHLH165/PAR1	basic helix-loop-helix (bHLH) superfamily protein	4.2 down	Auxin responsive TF family , bHLH, top 10% of misregulated genes

Table 2. Expression Overview

AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. FC = fold change in M0171-bdl dataset.

internal #	AGI	Other Name(s)	FC	Expression pattern during embryogenesis
1	AT1G22590	AGL87	3.1 up	Suspensor and protoderm
2	AT1G48910	YUC10	3.5 up	no expression detected
3	AT1G61566	RALFL9	2.6 up	no expression detected
4	AT1G68320	MYB62	3 up	suspensor and basal tier of proembryo
5	AT1G68510	LBD42	2.8 up	no expression detected
6	AT1G68920	bHLH049	2.5 up	inner cells of proembryo
7	AT1G76500	SOB3/AHL29	2.3 up	no expression detected
9	AT2G12900		2 up	suspensor and protoderm
10	AT2G21420		2.8 up	no expression detected
11	AT2G26850		3.4 up	no expression detected
12	AT2G27250	CLV3	44.8 up	no expression detected
13	AT2G35310		2.4 up	apical tier and SAM area of proembryo
14	AT3G03770		2.1 up	no expression detected
15	AT3G25170	RALFL26	4 up	no expression detected
16	AT3G54320	WRI1	4.2 up	junction apical and basal tier
17	AT3G54800		24.4 up	no expression detected
18	AT3G57800	bHLH060	2.4 up	protoderm of proembryo
19	AT3G61150	HDG1	2.4 up	hypohysis descendents
20	AT3G61160		4.1 up	no expression detected
21	AT4G28650		3.3 up	suspensor and protoderm
22	AT4G32540	YUC1	5.9 up	apical protoderm, SAM area of proembryo
23	AT4G34530	bHLH063/CIB1	9.9 up	junction apical and basal tier
24	AT5G06250		3.8 up	sam area of proembryo
25	AT5G11320	YUC4	7.9 up	suspensor and apical protoderm
26	AT5G11530	EMF1	2.2 up	no expression detected
27	AT5G25830	GATA12	6.3 up	suspensor
28	AT5G26650	AGL36	2.7 up	no expression detected
29	AT5G26950	AGL93	2.5 up	hypohysis descendents and protoderm
30	AT5G39860	BHLH136/PRE1/BNQ1	5.2 up	no expression detected
31	AT5G47670	LEC1-LIKE	2.5 up	no expression detected
32	AT5G52600	MYB82	2 up	inner cells of proembryo
33	AT5G56270	WRKY2	2.5 up	suspensor
34	AT5G57670		2.2 up	no expression detected
91	AT3G05800	bHLH150/AIF1	2.9 up	no expression detected
92	AT3G28857	bHLH164/PRE5	2.4 up	no expression detected
35	AT1G13960	WRKY4	2.2 down	no expression detected
36	AT1G14600		2.5 down	apical protoderm and SAM area of proembryo
37	AT1G15670		3.1 down	suspensor and descendents
38	AT1G31320	LBD4	2.8 down	suspensor and basal tier of proembryo
39	AT1G62000		2.4 down	no expression detected
40	AT1G62220		2.2 down	no expression detected
41	AT1G80440		3.5 down	apical cells following hypophysis division
42	AT1G80640		2.3 down	inner cells of proembryo
43	AT2G18300	bHLH064	2.2 down	no expression detected
44	AT2G18890		3.7 down	inner cells of proembryo
45	AT2G23050	NPY4	3.2 down	dynamic, embryo proper and qc
46	AT2G41170		2.3 down	no expression detected
47	AT2G45430	AHL22	2.3 down	no expression detected
49	AT3G13960	GRF5	25.4 down	proembryo
50	AT3G54780		4.8 down	no expression detected
51	AT3G62100	IAA30	3.4 down	initially suspensor specfic, expanding to prevasculature
52	AT4G04090		2.8 down	suspensor and hypophysis
53	AT4G10270		2.2 down	protoderm
54	AT4G18740		2.1 down	suspensor
55	AT4G24390		2.1 down	initially suspensor specfic, expanding to prevasculature
56	AT4G31820	ENP/NPY1/MAB4	5.9 down	lower tier of proembryo
57	AT4G36240	GATA7	2 down	no expression detected
58	AT5G18270	NAC087	3.6 down	protoderm
59	AT5G48940		3 down	basal tier of proembryo
60	AT5G49490	AGL83	2.2 down	not cloned, no analysis performed.
61	AT5G50915	bHLH137	4.6 down	protoderm of basal tier of proembryo
62	AT5G66560		2.2 down	sam area of proembryo
67	AT1G29950	bHLH144	3.5 down	no expression detected
68	AT1G68810	bHLH030	2.7 down	not analysed
69	AT2G01420	PIN4	15.5 down	Initially suspensor, later also in lower tier of proembryo
74	AT1G05710	bHLH153	2.2 down	inner cells of proembryo
75	AT2G41240	bHLH100	2 down	suspensor
76	AT2G41130	bHLH106	2.2 down	not analysed
85	AT1G73590	PIN1	4.9 down	apical tier of proembryo
96	AT2G42870	bHLH165/PAR1	4.2 down	basal cells following hypophysis division

In summary, GFP expression was detected in the transcriptional reporters of 41 different genes. Of these, 16 candidates were expressed in patterns that were in accordance with a potential role in suspensor/extra-embryonic cell identity or acquisition of embryonic cell identity (discussed below). The remaining 24 had expression patterns that precluded their involvement in these processes, the most common being downregulated genes that were found to be expressed in subdomains of the pro-embryo. Presuming that these transcriptional reporters faithfully report the expression patterns of the selected genes (discussed below), these results could represent secondary effects that are a consequence of the change in suspensor cell identity and their respective functions and the connectivity between the suspensor and pro-embryo, and are therefore hard to mitigate in this experimental approach. Indeed, the control *PIN1* was 4.9 fold downregulated in M0171-bdl dataset and normally expressed in the pro-embryo, not the suspensor (Friml et al., 2003). As expected, the expression as determined using the transcriptional fusion lines generated in this work were identical to those previously described (Fig. 3; Friml et al., 2003). Four of the five remaining controls were also expressed as expected (discussed in turn below).

Following the initial expression analysis, 16 candidates remained, eight potential regulators of suspensor to embryo transformation and eight potentially involved in the maintenance of suspensor/extra-embryonic cell identity.

Candidates for regulators of suspensor-embryo transformation

The eight embryo-expressed candidate genes potentially involved in suspensor to embryo transformation include a YUCCA gene, *YUCCA1* (*YUC1*). The YUCCA gene family consists of 11 members and encode flavin monooxygenase proteins that catalyze a rate limiting step for tryptamine dependent auxin biosynthesis (Zhao et al., 2001). Previously, 4 closely related YUCCAs (1,4,10 and 11) were shown to be expressed in the apical half of the pro-embryo throughout early embryogenesis and the quadruple mutants had defects in cotyledon development as well as basal defects leading to rootless, monocot seedlings lacking hypocotyls (Cheng et al., 2007). *YUC1*, and *YUC4* expression as analyzed by RNA *in situ* hybridization was similar to the apical tier expression patterns as detected by control transcriptional fusions in this study, validating our approach (Fig. 2, Cheng et al., 2007). Expression of *YUC4* was also detected at earlier time points than previously published, in suspensor cells (Fig. 2). Endosperm, but no embryo expression was detected in *YUC10* transcriptional reporter lines (data not shown), in contrast with weak signals previously observed *in situ* hybridization experiments (Cheng et al., 2007). The most likely reason for this is discrepancy is the presence of residual endosperm in the *in situ* hybridization experiments.

Three remaining candidate genes, AT1G68920 (*bHLH049*), AT3G57800 (*bHLH060*) and AT4G34530 (*bHLH063*) belong to the 167 member bHLH transcription factor superfamily (Carretero-Paulet et al., 2010). Interestingly, they all belong

to the same subfamily (of which there are 28 in Arabidopsis), namely subfamily 25, which includes 14 other members. Of the three, only *bHLH063* has been functionally characterized and is also known as *CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1* (*CIB1*). As implied by its name, *CIB1* interacts with crypochrome 2 and promotes CRY2 dependent floral initiation (Liu et al., 2008). These bHLHs have distinct expression patterns in the embryo. *bHLH049* is expressed in the inner cells (prevasculature) of the basal tier of the embryo and in derivatives of these cells at later stages. *bHLH060* is expressed in the outer cells (protoderm) of the basal tier of the embryo (Fig. 2). The expression of *bHLH063* is limited to subprotodermal cells at the apical basal junction from heart stage on.

Two other candidate genes encode members of the plant specific B3 superfamily of transcription factors. AT5G06250 belongs to the 13 member RAV family, and is one of 6 members that contain an AP2 DNA binding domain in addition to their B3 DNA binding domain (Swaminathan et al., 2008). More recently is has been shown that 11 RAV members, including AT5G06250, contain a functional repressor domain (Ikeda and Ohme-Takagi, 2009) inferring that this gene encodes a transcriptional repressor. AT2G35310 belongs to the REM family, subgroup B. This subgroup contains 18 members, none of which have been functionally characterized. AT5G06250 is expressed in the inner cells of the embryo and AT2G35310 expression is restricted to the inner cells of the apical tier of the embryo, including the future SAM (Fig. 2)

AT3G54320/*WRINKLED1* (*WRI1*) encodes an AP2/EREB transcription factor involved in the control of storage compound (oil) biosynthesis during seed maturation in Arabidopsis (Cernac and Benning, 2004). It is activated by LEAFY COTYLEDON2, a master regulator of seed maturation, whose ectopic expression causes cells to accumulate mRNAs associated with seed maturation (Baud et al., 2007; Santos Mendoza et al., 2005). Late embryonic *WRI1* expression has previously been investigated using *in situ* hybridization and transcriptional fusions to GUS, which showed strong expression around the hypocotyl region and abaxial cotyledon region and no expression in the RAM area, consistent with our analysis (Baud et al., 2007 and Fig. 2).

Finally, AT5G52600 or *MYB82*, has not been assigned to a subgroup, but forms a phylogenetic clade with subgroup 15, which includes *MYB0/GL1*, *MYB66/WER* and *MYB23* (Dubos et al., 2010). These MYBs are involved in cell identity specification in trichomes and the root epidermis (Lee and Schiefelbein, 1999; Kirik et al., 2005 and Kang et al., 2009). Like *bHLH049*, *MYB82* is expressed in the inner cells of the basal embryo tier (Fig. 2).

Interestingly, even though all these nine candidate genes are upregulated during suspensor to embryo transformation, their expression patterns in wild type embryos are quite distinct. It can therefore be excluded that a simple distinct cell fate respecification occurs during transformation. Alternatively, these genes

could share a common expression pattern at very early stages. Due to the relatively low expression level of most reporters at the 1-4-cell stage, this remains an open question.

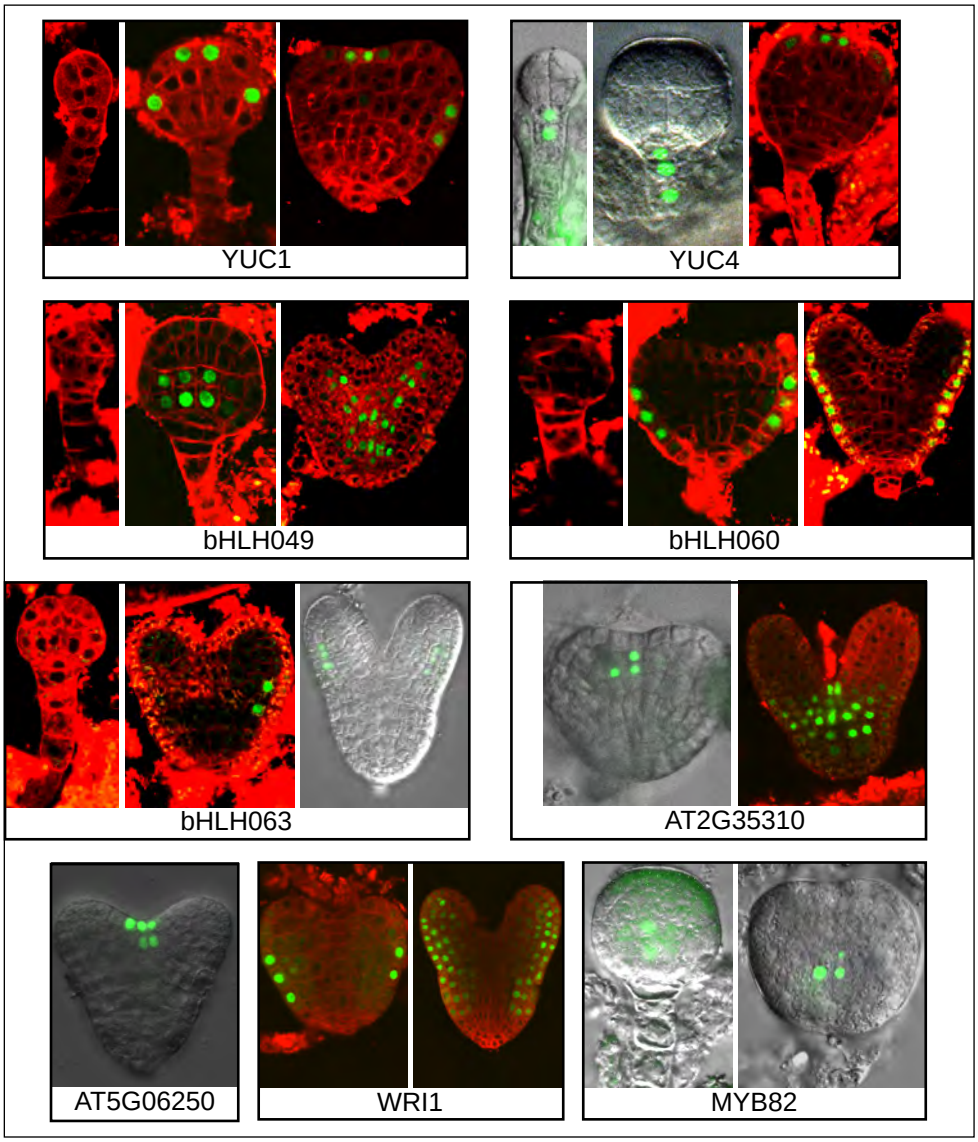

Figure 2. Expression of selected genes upregulated in the M0171-bdl dataset. Transcriptional fusions of 2.0 Kb promoter of 9 upregulated genes to nuclear-localized triple GFP (green signal). Red signal from membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

Candidates for regulators of suspensor cell identity

Strictly suspensor-specific expression requirement was only fulfilled by 2 of the selected genes. Firstly, AT4G04090, which is one of 80 Arabidopsis genes that contains a BTB/POZ (Bric-a-brac, Tramtrack, and broad complex/Pox virus and zinc finger) domain, which is a widely conserved protein-protein interaction domain. BTB/POZ domain containing proteins are known to interact with Cullins (CULs) which act as scaffolds to form multisubunit ubiquitin-protein ligase (E3) complexes, that can ubiquitinate and thereby target specific proteins for degradation (Gingerich et al., 2005). Several BTB/POZ domain proteins have been characterized, including ETO1, which interacts with a rate limiting enzyme for ethylene biosynthesis, specifically targeting it for degradation (Christians et al., 2008). Interestingly, two genes that are closely related to AT4G04090 (in the 8 member subfamily D1) are also misregulated in the M0171-bdl dataset, AT3G29740 is 1.8 fold downregulated and AT5G48510, is 2.4 fold upregulated. The second suspensor specific gene identified is AT4G18740, which is annotated in TAIR as a Rho transcriptional termination factor, but has not been characterized to date. Both AT4G04090 and AT4G18740 are exclusively expressed in the suspensor lineage during embryogenesis, and may therefore be determinants or at least markers of this cell identity.

All other downregulated, suspensor-expressed genes either display dynamic expression patterns, which are initially suspensor specific and later expand to include pro-embryo cells, or are expressed in only a subdomain of the suspensor. These patterns are consistent with being part of a cellular state in suspensor cells at a given stage, but cannot be considered determinants or markers of suspensor identity.

One gene with a dynamic pattern was *IAA30*, which encodes an unusual, noncanonical Aux/IAA in that it does not contain the domain required for auxin-mediated degradation. As described in Chapter 3, expression is initially suspensor-specific, later relatively weaker expression is observed in the basal tier cells and eventually, expression is also detected in cotyledon primordia (see Chapter 3 Fig. 4A-C and Suppl. Fig. 6A,B).

AT1G15670 encodes one of approximately 100 F-box proteins with a C-terminal kelch repeat (Schumann et al., 2011). These, and other F-box proteins, are (similar to BTB/POZ domain proteins) thought to be the specificity generating subunits of SCF E3 ubiquitin ligases, selectively recruiting target proteins via their protein-protein interaction domain, in this case the kelch domain (reviewed by Schumann et al., 2011). This gene was predominantly expressed in the suspensor and suspensor derivatives, later expression expanded to the basal tier of the pro-embryo (Fig. 3). The most closely related gene, AT1G80440, was also included in the subset for transcriptional analysis and was found to be very specifically expressed in the apical cell following hypophysis division (Fig. 3) and

its subsequent descendants, the quiescent centre. As such, this gene represents an example of a gene marking a suspensor subdomain.

Another F-box protein initially expressed in the hypophysis and all of its descendants is AT4G24390/*AUXIN SIGNALING F-BOX protein 4* (*AFB4)* (Fig. 3), a member of the auxin receptor family (Gagne et al., 2002). Later, expression is expanded to include prevasculature (Fig. 3). Unlike *TIR1* and the other AFBs, *AFB4* appears to be expressed at very low levels and translational fusions to GUS give weak staining at the root-shoot junction and in central, perhaps QC cells of the root meristem (Greenham et al., 2011).

The dynamic embryonic localization of the auxin efflux carrier PIN4 has been well elucidated. Initially at late globular stage, *PIN4* is localized primarily in the hypophysis (and adjacent suspensor cell). Later at transition stage, its expression domain is expanded to include vascular precursor cells and at heart stage also include the endodermis initials (Friml et al., 2002). The transcriptional fusion made in this research closely resembles the previously documented expression, but is expanded to include suspensor cells at early globular stage, probably reflecting enhanced sensitivity of the nuclear localized triple GFP reporter (Fig. 3).

AT2G41240/*bHLH100*, also a member of the bHLH transcription factor superfamily, shows strong suspensor specific expression up until hypophysis specification (as detected by a change in cell morphology), after which weak expression in the basal tier protoderm cells was occasionally observed (Fig. 3).

AT2G42870/*bHLH165*/*PHY RAPIDLY REGULATED 1* (*PAR1*) encodes a novel atypical bHLH sequence recently accepted into the bHLH superfamily (Carretero-Paulet et al., 2010). Atypical bHLHs are predicted to lack DNA-binding activity but have retained protein-protein interaction capability and therefore thought to function as transcriptional coregulators. A role for PAR1 in shade avoidance response has been demonstrated, in which PAR1 acts as a transcriptional corepressor (Roig-Villanova et al., 2007). Initially, *PAR1* is specifically expressed in the basal cell generated following hypophysis division and its descendents, the columella initials (Fig. 3). At late heart stage expression is also detected in future ground tissue initials (Fig. 3).

In summary, the subset of genes downregulated during suspensor to embryo transformation and normally expressed in the suspensor lineage represent a diversity of patterns. These include a few that are strictly suspensor-specific, expressed in a subset of suspensor cells, and others that are also expressed in other cell types. This set of gene expression patterns reveals molecular differences between the suspensor cells, and also molecular similarities with non-suspensor cells.

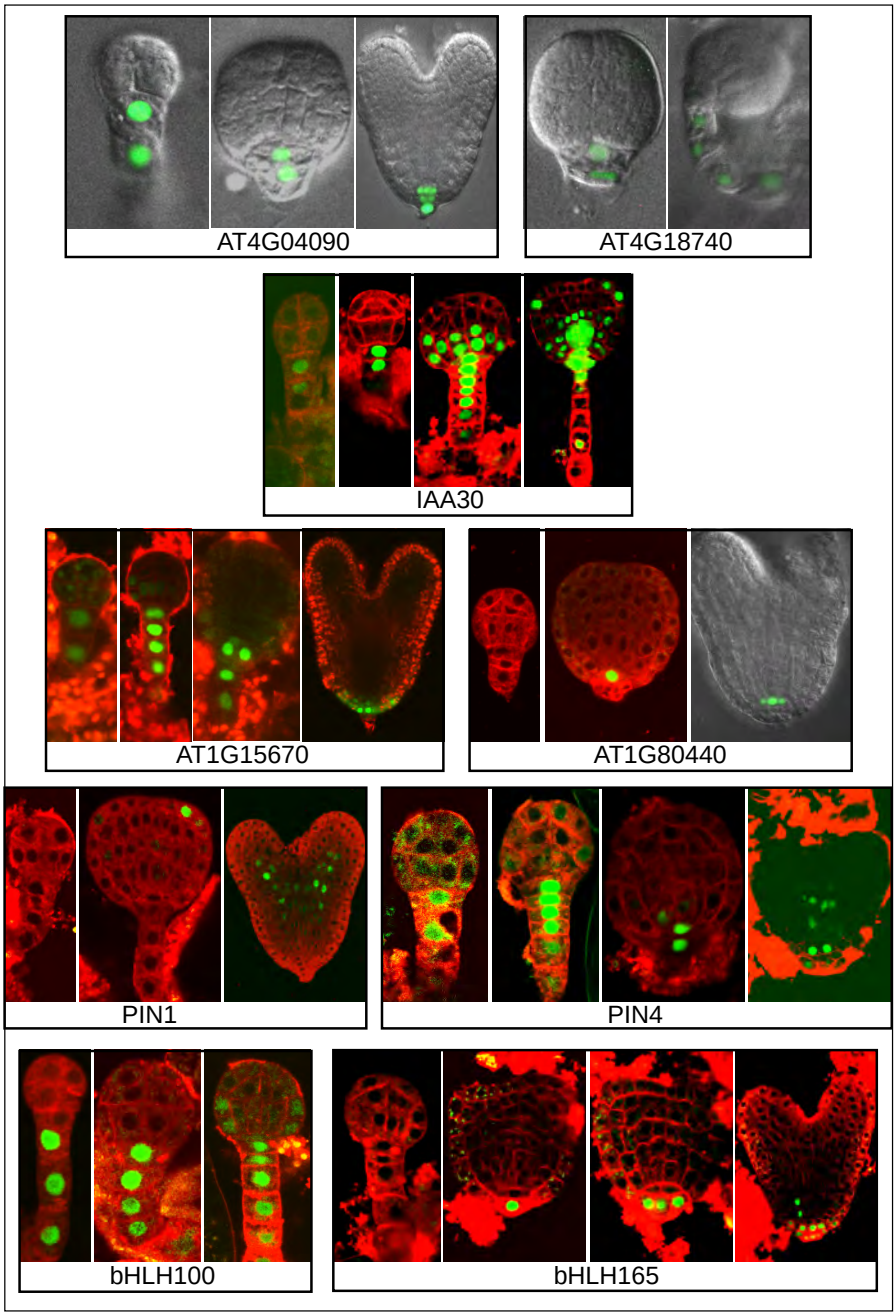

Figure 3. Expression of selected genes downregulated in the M0171-bdl dataset. Transcriptional fusions of 2.0 Kb promoter of 9 downregulated genes to nuclear-localized triple GFP (green signal). Red signal from membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

Insertion line analysis

Next, we investigated the function of the remaining 16 genes one by one. We reasoned that key factors involved in suspensor cell maintenance may have suspensor cell proliferation phenotypes when knocked down. Conversely, key effectors of the suspensor to embryo transformation could be important for normal development and therefore may display aberrant phenotypes in the pro-embryo when compromised.

We excluded YUC1 and PIN4 as previous research has investigated the embryonic phenotypes resulting from loss of function alleles. The *yuc1 yuc4 yuc10 yuc11* mutant displays aberrant divisions in both the pro-embryo and embryo-suspensor junction, including hypophysis division defects, which lead to seedlings lacking hypocotyls and roots (Cheng et al., 2007). Similar functional redundancy was found in the PIN family, with the *pin4 pin7* double mutants displaying embryo defects which were further enhanced by mutations in *PIN1* and *PIN3* (Friml et al., 2003).

We made use of publically available T-DNA insertion line collections. Specifically, we initially used the SALK homozygote mutant collection (http://signal.salk.edu/cgi-bin/homozygotes.cgi), which currently contains two independent homozygous T-DNA insertion lines for 9276 genes (O'Malley and Ecker, 2010). This collection aims to prevent false positives as observed phenotypes can be almost conclusively assigned to a specific gene if seen in two independent lines.

Of the remaining 14 candidates, only five had two homozygous lines and four had one homozygous line each (Table 4 in Materials and Methods). From these 14 lines, eight plants were sown and ovules from three siliques per plant were prepared and scored for embryo phenotypes. No aberrant phenotypes were detected in any of the plants analyzed. In addition to the SALK homozygous collection lines, several other lines were investigated (Table 4 in Materials and Methods). No insertion lines were available for bHLH063/CIB1 or bHLH165/PAR1. Of the available lines, aberrant phenotypes, which increased in penetrance throughout embryonic development (from 5% at preglobular stage to 26% at heart stage), were only found in *afb4-2* (Table 3). The regular pattern of cell divisions was disrupted at the apical-basal junction, leading to a proliferation of cells (Fig. 4). Interestingly, the *afb4-2* mutant was previously shown to have both shorter roots and also produce more lateral/adventitious roots than wild type seedlings (Greenham et al., 2011).

In summary, without further characterization of the aphenotypic loss of function lines investigated in this chapter, we cannot rule out the possibility that the corresponding candidate genes encode regulators of suspensor/embryo identity. The aberrant embryo phenotype in *afb4-2* line suggests that the specific expression of *AFB4* in the hypophysis, its descendents and the prevasculature is

important for the correct specification of cells at the apical-basal junction of the embryo. However, it is unclear if this function is related to the maintenance of extra-embryonic identity.

Table 3. Penetrance of the afb4-2 mutant phenotype.

Developmental stage	Aberrant Phenotype %	N
pre eight-cell	0	15
eight- /16- cell	5	64
early globular	7	73
late globular	11	148
transition	22	98
heart	26	101

Embryo phenotypes were scored in preparations made from afb4-2 homozygous plants The frequencies of phenotypcially aberrant embryos (%) and the total numbers of embryos analyzed (N) are given.

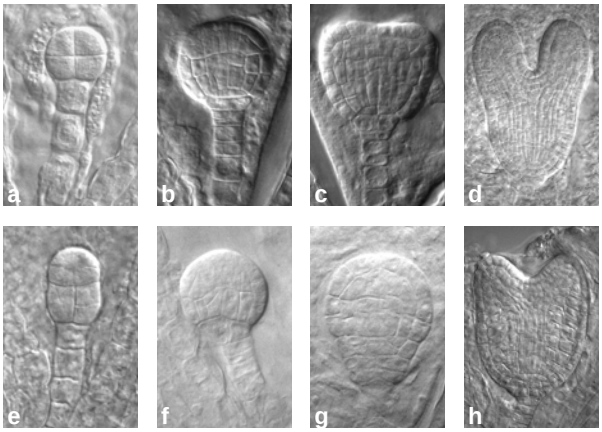

Figure 4. The AFB4 mutant *afb4-2* displays diorganised cell divisions at the embryo-suspensor boundary. Developmental series (preglobular, globular, transition and heart stage embryos) of a wild type control displaying normal embryo patterning (a-d), and *afb4-2* mutant embryos with aberrant patterning (e-f).

Suspensor specific misexpression of candidate genes involved in suspensor transformation

For those genes that are upregulated during suspensor to embryo transformation, M0171>>bdl-like phenotypes could be expected when genes are misexpressed in the suspensor. The eight candidate genes involved in suspensor to embryo transformation were subsequently driven from the suspensor specific *ARF13* promoter to determine the effect of their individual and specific misexpression. Suspensor to embryo transformation can yield a low frequency of twin embryo seedlings. Furthermore, suspensor proliferation

can also be expected to interfere with root meristem development, resulting in rootless seedlings as is the case for p*ARF13*:iaa10 (Chapter 3). Unfortunately, despite screening all T1 seed harvested following the transformation of five Arabidopsis plants per construct, no twin or rootless seedlings were found. Embryos from T2 ovules dissected from genotyped T1 plants were also screened (estimate numbers), however suspensor proliferation (or other defects) was not found. Hence, the suspensor to embryo transformation seen in M0171>>bdl embryos does not appear to result from misexpression of any of these 8 genes individually, and perhaps is a cumulative effect of multiple genes being ectopically activated, and others being repressed.

DISCUSSION

Division of the zygote results in the generation of two developmentally distinct domains, the pro-embryo and suspensor. Intriguingly, the suspensor is maintained as an extra-embryonic entity, in part by repressing its embryonic potential. Auxin response is integral to the maintenance of the extra-embryonic state of the suspensor. We have combined several techniques to generate unique datasets that function as a starting point to identify key regulatory components downstream of this autonomous auxin response.

The binary GAL4-*UAS* system was used to specifically inhibit auxin response in suspensor cells and through crossing lines carrying the GAL4 driver and *UAS-bdl* constructs, temporal specificity could be generated, enabling us to target the earliest changes in the process of suspensor identity loss/embryonic identity acquisition. This was coupled to embryo dissection to further enrich for suspensor responses and a transcriptomics approach was used to identify components downstream of the primary effectors of auxin response, the ARFs.

As expected *BDL* is 10.6-fold upregulated in the M0171-bdl datasets. Another important embryo marker, *STM*, which was shown to be expressed in proliferating suspensor cells with *in situ* hybridization in Chapter 3, is 2.3 fold upregulated in the M0171-bdl dataset. Conversely, IAA30, having suspensor specific expression at early stages of embryogenesis, which is lost in p*ARF13*:IAA10 background (in which auxin response is also inhibited in the suspensor; see Chapter 3), is 3.5 fold downregulated in the M0171-bdl dataset. These corroborating findings validate the microarray datasets generated in this Chapter.

Despite our attempt to generate an experimental system that will identify high confidence candidates involved in embryonic or extra-embryonic identity determination, we are aware of several unavoidable caveats. The most important is that although the embryo and suspensor domains are well defined, there is no complete physical barrier separating them. In fact, the suspensor is characterized as a conduit of nutrients and growth regulators from surrounding tissues to

the pro-embryo (reviewed in Kawashima and Goldberg, 2009). Also, defects in the pro-embryo are known to have an effect on developmental status of the suspensor, as discussed in the introduction. An example of communication between the embryo and suspensor is demonstrated by the recent identification of a mobile transcriptional regulator that is transported from the embryo cells adjacent to the suspensor to the uppermost suspensor cell and is involved in hypophysis identity specification (Schlereth et al., 2010). Therefore, changes to the suspensor can be expected to induce secondary consequences in the embryo, which would result in transcriptional changes that are incorporated into the datasets generated in this study. Many genes were misregulated in M0171-bdl embryos despite the limited complexity of the tissue and the relatively few rounds of cell division in which auxin response was inhibited. This reflects the prominent role of auxin in these cells and complexity of the auxin response, in that it involves both positive and negative regulation of genes.

Because of these caveats and because relatively large numbers of genes were misregulated and included in the M0171-bdl datasets, it was essential to further mine the datasets by global and specific comparisons to previously generated datasets, to make an informed subselection for further investigation. Enrichment for both auxin responsiveness and transcription factors were the most important leads for selection. Perhaps more importantly, although a subselection was necessary, it was essential to cast as wide a possible net. This was achieved by opting for a transcriptional fusion approach, with the selection of 70 genes for analysis.

Of the 70 genes selected, 40 were expressed in the embryo and/or suspensor. Although only 16 of these expression patterns are relevant for our research question, the remaining expression patterns could provide leads to study specification of other cells types such as the hypophysis and its descendants. These promoters are important resources and could be used, together with the newly developed INTACT method (Deal and Henikoff, 2010), to enable isolation of nuclei from specific cell types/tissues of the embryo/suspensor for subsequent expression profiling, or for example as unique novel markers for these cell types. Of the 40 expressed genes, 35 were on the arrays used to generate the globular stage embryo and globular stage suspensor enriched datasets using laser capture microdissection (LCM). Of these, the LCM predictions only matched the expression patterns (assigned as embryo, suspensor, or embryo and suspensor) of 14 genes (not shown). This highlights the need for more accurate expression profiling of these cell types.

Regardless the transcriptional fusion approach chosen to validate the microarray and further implicate selected genes as candidates for regulators of suspensor and embryo identity proved to be both efficient and importantly appears to faithfully report gene expression patterns. This latter statement is based on the finding that the expression patterns of five control genes overlap with previously

published expression and/or protein data. One observed difference is in the expansion of PIN4 expression from the two uppermost suspensor cells at late globular stage to suspensor cells at earlier stages. As previously mentioned this perhaps reflects the enhanced sensitivity of the nuclear localized triple GFP reporter relative to whole-mount *in situ* immunolocalization experiments using an AtPIN4-specific antiserum (Friml et al., 2002).

No embryo expression was detected in transcriptional reporter lines representing the 29 remaining genes. There are several explanations for this, firstly that these genes are indeed not expressed in the embryo/suspensor. However there is also the possibility that these are false negative results. These could arise from several scenarios, including that these genes have a very low level of expression that is below the detection limit of the microscopes used. Alternatively, the constructs could have been inserted into genome locations that prevent the transgene from being properly expressed, also known as the position effect. Screening more lines per construct could resolve this issue. Another possibility is that not all regulatory sequences necessary for expression are contained within the 2 kb upstream sequences used, extra information could reside further upstream, in UTRs, in introns or even in the coding sequences. These two latter considerations, as well as the existence of post-transcriptional mechanisms that effect expression patterns, mean that our approach could also lead to false positives. Translational fusions could have been used to incorporate more of the native context of the gene, however these are in practice more difficult to both generate and detect, which would have prevented us from being able to screen as large a number of genes.

Two immediately emerging properties of the microarray and expression analysis is the involvement of auxin homeostasis components and bHLH transcription factors in the suspensor auxin response. These form the basis of subsequent chapters in this thesis and will therefore not be further discussed here.

Unfortunately subsequent analyses of loss of function and misexpression approaches were less revealing than the expression analysis. The only insertion mutant found to have an embryonic phenotype was *afb4-2*. AFB4 is one of six members of the TIR1/AFB family, and phylogenetic analysis of this family in land plants places TIR1/AFB1-3 and AFB4/AFB5 in distinct, evolutionarily conserved lineages inferring distinct functions for these clades (Parry et al., 2009). Indeed, unlike TIR1/AFB1-3, which are auxin receptors and subunits of SCF complexes that specifically target Aux/IAAs and thereby positively regulate auxin signaling, AFB4 was recently shown to act as an auxin receptor that negatively regulates auxin signaling, through an as yet unknown mechanism (Greenham et al., 2011). The canonical TIR1/AFB1-3 clade has been the more comprehensively investigated of the two clades. Members do not appear to be regulated by auxin but some are under significant posttranscriptional regulation and therefore may not be as ubiquitous as previously thought. Furthermore comprehensive analysis has

revealed that despite a degree of functional redundancy each member has differences in expression and specificity (Parry et al., 2009; Dharmasiri et al., 2005). It will be interesting to investigate the expression patterns of the TIR/AFB family during embryogenesis. Further analysis will be required to assess the relative contribution of suspensor and embryo expressed *AFB4* (and potentially other members) to embryo patterning. Several explanations can be envisioned for the lack of phenotype in other lines. Firstly, the T-DNA insertion point is a major factor in determining the effect of the insertion on the transcription of a targeted gene (reviewed by Wang, 2008). The insertion points of the mutants were investigated and in six cases were upstream of the start codon. Wang et al., (2008) noted that less than half of T-DNA insertions upstream of the start codon investigated result in a full knock down. Full or partial genetic redundancy resulting from genomic duplication (and gene duplication) is another common reason for the lack of phenotype in single mutants in Arabidopsis (reviewed by Briggs et al., 2006). The generation of double/higher order mutants should resolve this issue.

Suspensor misexpression of any of the nine candidate genes did not result in suspensor proliferation phenotypes, inferring that none of these genes is sufficient to act as trigger for an extra-embryonic to embryonic identity transformation. Indeed, only a handful of genes have previously been shown to have this capacity (as discussed in the introduction). However, we can also not rule out that the *ARF13* promoter used is not strong enough to drive sufficient expression of the candidate genes to induce phenotypic changes. An alternative strategy could be to use the GAL4/UAS two component system for misexpression. Nonetheless, as the transcriptional changes are complex, it is unlikely that a single gene would be sufficient to trigger the same response as induced by bdl misexpression. More detailed analysis of suspensor cells in p*ARF13-geneX* lines by either subcellular structure analysis or by analyzing the expression of suspensor or embryo marker genes should help defining the contribution of individual genes to this complex phenotype.

Thus, in the context of the Arabidopsis suspensor, it appears that the response that occurs during cell identity transformation is complex in that it involves a large scale reprogramming of the suspensor transcriptome prior to visible morphological aberrations. This suggests that these cells have a high degree of transcriptional plasticity, perhaps reflecting the dynamic nature of suspensor cell identity in which the uppermost cell later undergoes an identity transformation into hypophysis and the remaining cells eventually undergo programmed cell death. However it is also important to take into account that secondary transcriptional changes in the embryo occurred due to embryo-suspensor continuity, also complicating the dataset and subsequent identification of any potential regulators of cell identity. Further research is clearly required to confirm the role of selected candidate genes in auxin mediated cell identity specification. It will be important to determine whether these genes are directly

controlled by ARF(s) that are expressed in similar expression domains. Given the large numbers of genes misregulated upon inhibition of auxin response in the suspensor, an interesting avenue for further research may be the epigenetic control of cell identity during embryogenesis. In summary, we have generated a useful resource and several important leads for further investigation into the mechanisms that control embryo and extra-embryonic cell identity.

MATERIALS AND METHODS

Plant Materials, Growth and Selection

Arabidopsis thaliana (Arabidopsis) plants (Columbia-0 ecotype for all wild type controls and transformations) were grown under standard conditions at 23°C in a 16-h-light/8-h-dark cycle. Selection for transgenes was performed on solid Murashige and Skoog medium supplemented with 25 mg/L kanamycin or 15 mg/L phosphinothricin where appropriate. The homozygous afb4-2 mutant was a kind gift from Mark Estelle (Greenham et al., 2011). Other insertion mutants used were obtained from the Nottingham Arabidopsis Stock Centre (Table 4). Selected insertion mutants were genotyped with an insertion primer (ATTTTGCCGATTTCGGAAC) and the gene specific primers listed in Table 5 (for details of design and use see http://signal.salk.edu/tdnaprimers.2.html). The M0171-GFP and UAS-bdl lines have been described previously (Chapter 3 and Weijers et al., 2006).

Table 4. Insertion mutant lines used in this study.
AGI = Arabidopsis Genome Initiative gene identification number.
NASC = Nottingham Arabidopsis Stock Centre stock ID number used for ordering lines.
Line = name of line, Line # = internal reference number.

AGI	Other Name	NASC #	Line	Line #	Insert location
AT1G68920	bHLH049	N661158	SALK_135188C	6.1	exon
AT1G68920	bHLH049	N663212	SALK_087424C	6.2	promoter
AT2G35310	-	N666125	SALK_057418C	13.1	promoter
AT3G54320	WRI1	N665205	SALK_008559C	16.1	intron
AT3G54320	WRI1	N585693	SALK_085693	16.2	intron
AT3G57800	bHLH060	N664650	SALK_063280C	18.1	exon
AT3G57800	bHLH060	N669084	SALK_134005C	18.2	intron
AT5G06250	-	N666657	SALK_088181C	24.1	intron
AT5G52600	-	N313460	GK-057A04	32.1	intron
AT1G15670	-	N652981	SALK_000312C	37.1	5' UTR
AT1G15670	-	N653586	SALK_014388C	37.2	exon
AT3G62100	IAA30	N668427	SALK_065384C	51.1	intron
AT4G04090	-	N831553	SAIL_712_E07	52.1	promoter
AT4G18740	-	N654149	SALK_038425C	54.1	exon
AT4G18740	-	N654343	SALK_106218C	54.2	promoter
AT4G24390	AFB4	-	Tillling afb4-2	55.3	exon
AT2G41240	BHLH100	N664325	SALK_150637C	75.1	promoter
AT2G41240	BHLH100	N656649	SALK_074568C	75.2	exon

Table 5. Primers used to genotype insertion mutant lines.
AGI = Arabidopsis Genome Initiative gene identification number.
NASC = Nottingham Arabidopsis Stock Centre stock ID number used for ordering lines.
Line = name of line, Line # = internal reference number.
LP (left primer) and RP (right primer) are the gene specific primers (5' to 3').

AGI name	NASC #	Line	Line #	LP	RP
AT1G68920	N661158	SALK_135188C	6.1	TTTCCGTCGTAACAACGAATC	CTAGTACCGGGTTGCAACAAG
AT1G68920	N663212	SALK_087424C	6.2	TAACACAGGGCAATGGAAAAG	TGCTGAAAACTCATCTTTCGC
AT3G57800	N664650	SALK_063280C	18.1	CGATACTACCCCTTCCTCACC	CTGAGACCGATTCATCTCAGC
AT3G57800	N669084	SALK_134005C	18.2	ATCATTGCCATGAGCTTGATC	ACAACGTCAAGTGGAGGTGAG
AT1G15670	N652981	SALK_000312C	37.1	ATCCCATTAACCGAACGGTAC	GAGTTCTTTAACCGGGTCGAC
AT1G15670	N653586	SALK_014388C	37.2	AAATGATTGCCAAAAAGAAAATG	GGAGGAACAAGGGCAATTTAG
AT3G62100	N668427	SALK_065384C	51.1	CGGAACAATTGTAATATCTCCG	AGGGAGAAGCTCATCGTCTTC
AT4G18740	N654149	SALK_038425C	54.1	TTGCGTAGCTGTAATTGGACC	TGCAACTTCTTTGAGCTCTGC
AT2G41240	N664325	SALK_150637C	75.1	TTGGTCGGTGTAAACGAGATC	TTGTGGTAGAAAAATGTGATTGC
AT2G41240	N656649	SALK_074568C	75.2	TCTCAATAGTCCACGTCCACC	AATGCTTGTGAAACTGTTGCC

Samples for Microarray

Embryo Dissection, Total RNA Isolation, and Microarray Experiments

72 hours following standard crosses of M0171-GFP flowers with UAS-bdl or wild type pollen, embryo isolations were performed using the dissecting microscope and fine forceps (Dumont 55 forceps, catalog no. 11295-55, Fine Science Tools) in a 5% Suc solution that contained 0.1% RNALater (Ambion, catalog no. AM7021) as described in previous study (Xiang et al, 2011), and the isolated embryos were pooled in a 1.5mL eppendorf tube sitting in dry ice. Each biological replicate contained 300-400 isolated embryos. Total RNA was extracted from each embryo sample following the protocol of RNAqueous-micro kit (Ambion, catalog no. 1927).

RNA Amplification and Labeling

The quantity of RNA isolated from the embryos was insufficient for preparation of probes for the microarray experiments. Therefore the mRNA was amplified prior to labeling. The mRNA amplification was conducted according to the protocol provided in the MessageAmp aRNA kit with minor modification (Ambion, catalog no. 1750). During the amplification, aminoallyl-UTP was incorporated into the newly synthesized aRNA; 3 µL of aminoallyl-UTP (50 mM) plus 2 µL of UTP (75 mM) instead of 4 µL of UTP were added. The purpose of incorporating aminoallyl-UTP is to provide a reactive chemical group to which the fluorescent dyes can be attached. After purification of the aRNA, the NHS-ester dyes were coupled to the modified bases of aRNA in a chemical reaction.

Microarray Experimental Design and Hybridization

The Arabidopsis 70-mer oligo array slides prepared by University of Arizona were used in all the microarray experiments (version ATV 3.7.2; http://ag.arizona.edu/microarray/). Antisense RNA labeling was performed following the protocol of Wellmer et al. (2004). The aRNA samples representing four biological replicates from experimental and control samples were labeled (two with cy3 and two with cy5) and hybridized to the slides following the protocol described in http://ag.arizona.edu/microarray. Hybridized slides were scanned sequentially

for Cy3- and Cy5-labeled mRNA targets with a ScanArray 4000 laser scanner at a resolution of 10 µm. The image analysis and signal quantification were performed using the QuantArray program (GSI Lumonics).

Microarray Analysis
Limma Software (Smyth, 2004) was used to normalize and to determine the modulated genes from microarray data.

Generation of Transcriptional Fusion Constructs
A Ligation Independent Cloning (LIC) strategy was used to generate the transcriptional fusion constructs in this chapter.

Construction of the Transcriptional Fusion and Suspensor Misexpression Vectors
Firstly, pPLVs (plant LIC vectors) were constructed based on a binary pGreenII (pGII) vector backbone with kanamycin (K) resistance or phosphinothricin (B) resistance (Hellens et al., 2000). A custom LIC site containing a unique HpaI restriction site used to linearize vector below (gaattctagttggaatgggttaacccaactccataaggatcc) was introduced via EcoRI and BamHI sites into the previously described pGIIK-SV40-3GFP-NOSt vector (Takada and Jürgens, 2007) to generate pGIIK-LIC-SV40-3GFP-NOSt used to make transcriptional fusion constructs. A custom LIC site also including a unique HpaI site (ctcgagctagttggaataggttaacccaactccatactgcag) was introduced, via XhoI and PstI, into pGIIB-pARF13-NOSt vector (generated in Chapter 3) to make pGIIB-pARF13-LIC-NOSt, used to make suspensor specific misexpression constructs.

Preparation of Vectors for Cloning
For a standard preparation, 2 to 4 µg of vector was cut with 1 µL HpaI fast cut restriction enzyme (Fermentas) in duplicate for 2 h at 37°C. Linearized vector was next purified from agarose gel using the QIAEXII gel extraction kit (Qiagen), and duplicates are pooled. Linearized vectors were then precipitated overnight (or minimum 2 h) using 0.5 volumes ammonium acetate (7.5 M) and 2.5 volumes of 100% ethanol at −20°C. The precipitated vector was pelleted by centrifugation for 30 min at maximum speed. The supernatant was removed, and the pellet was washed with 100 µL of 70% ethanol followed by a 100% ethanol wash. The pellet was next dried and resuspended in 50 µL of water (at 50°C for 5 to 10 min). For T4 treatment (New England Biolabs), 200 to 400 ng of linearized vector, 4 µL 10× T4 buffer, 4 µL 100 mM dCTP, 2 µL 100 mM dithiothreitol, 0.4 µL bovine serum albumin, 0.8 µL T4 DNA polymerase (New England Biolabs), and water to 40 µL total volume were mixed. The mixture was centrifuged at maximum speed for 1 min, incubated at 22°C for at least 30 min (up to 2 h), inactivated at 75°C for 20 min, and centrifuged again at maximum speed for 1 min. T4 treated vector could be stored at 4°C until further use.

Preparation of Promoter Fragments for Cloning
2.0 Kb promoters were amplified from genomic DNA by PCR using primers

that included LIC adapter sites (see Table 6). cDNAs were amplified from cDNA isolated from Arabidopsis leaves or ovules using the RNeasy Plant Mini Kit according to the manufacturers protocol (Qiagen). The primers used to amplify cDNAs (with LIC adapter sites) are listed in Table 7). PCR was performed in 50-µL volume in duplicate using Phusion Flash polymerase (Finnzymes; or another high-quality polymerase enzyme with proofreading) using the amplification protocol provided by the supplier. Fragments were next purified from agarose gel using a gel extraction kit, and duplicates pooled. For T4 treatment (New England Biolabs), 200 to 400 ng of purified fragment, 2 µL 10× T4 buffer, 2 µL 100 mM dGTP, 1 µL 100 mM dithiothreitol, 0.2 µL bovine serum albumin, 0.4 µL T4 DNA polymerase (New England Biolabs), and water to 20 µL total volume were mixed. The mixture was centrifuged at maximum speed for 1 min, incubated at 22°C for at least 30 min (up to 2 h), inactivated at 75°C for 20 min, and centrifuged again at maximum speed for 1 min. T4 treated fragments could be stored at 4°C until further use.

Annealing, Transformation in Escherichia coli, and Sequence Verification
To anneal the linearized, T4-treated vector and the T4-treated PCR fragment, 10 to 40 ng vector and insert were combined in a 1:3 M ratio for 30 min to 2 h at 22°C (usually about 1 to 3 µL each) or overnight at 4°C. The whole mixture was then transformed into electrocompetent DH5α E. coli cells (transformation efficiency >107 colony forming units/µg), plated on Luria-Bertani (LB)-agar plates with 25 mg/L kanamycin as antibiotic, and incubated at 37°C overnight. A T4-treated vector without added insert can be used to analyze the amount of background colonies. The next day, colonies were verified for inserts using colony PCR and positives grown overnight in 6 mL LB with 25 mg/L kanamycin. Plasmids are extracted (GeneJET plasmid mini prep kit from Fermentas) and checked by restriction digest and subsequent sequencing analysis.

Arabidopsis Transformation
A simplified plant transformation procedure allowing moderate throughput was used for all transformations. Plasmids were transformed into electrocompetent *Agrobacterium tumefaciens* GV3101 containing the pGreen helper plasmid pSOUP (Hellens et al., 2000) using standard protocols and plated on LB plates with the appropriate antibiotics. Following 2 d of growth at 28°C, a smear of multiple colonies was inoculated into 20 mL of liquid LB medium with the appropriate antibiotics and grown overnight at 28°C in a shaker. The next day, the volume of the culture was increased to 50 mL LB with antibiotics and grown, again at 28°C, to an OD600 of around 0.7 (0.5 to 0.9 is acceptable). If the optical density was too high, the cultures can be diluted to the correct OD600 using LB. Next, 2.5 g Sucrose and 10 to 20 µL Silwet was added to 50 mL of culture and shaken until the Sucrose dissolved. Five to ten plants were then floral dipped in this mixture, placed in a box, and covered with cling film for 1 d before further growth until T1 seeds could be harvested.

Table 6. Primers used to amplify 2.0 Kb promoters.

Internal #	AGI	SENSE PRIMER	ANTISENSE PRIMER
1	AT1G22590	TAGTTGGAATGGGTTCGAATTGGAAACCTTATCAGCTTT	TTATGGAGTTGGGTTCGAAAAAAAACTATATTTTCGTAA
2	AT1G48910	TAGTTGGAATGGGTTCGAATTGTGTAACCATAGAGATG	TTATGGAGTTGGGTTCGAATTCTTGTGTTTAGTTTGATA
3	AT1G61566	TAGTTGGAATGGGTTCGAAAAATTGAATAAAAGGGCTTC	TTATGGAGTTGGGTTCGAAGATTATGAACAAGCGCTAGT
4	AT1G68320	TAGTTGGAATGGGTTCGAAAATCATATATATCTTGTAGA	TTATGGAGTTGGGTTCGAAACTTAGTTTAAGTTTCCTAA
5	AT1G68510	TAGTTGGAATGGGTTCGAATTCTTATAAAAAATCATTTAAAA	TTATGGAGTTGGGTTCGAATGTGTTCTTGGAGAAAGTGG
6	AT1G68920	TAGTTGGAATGGGTTCGAAGAAGTTATTAGCATTAACTG	TTATGGAGTTGGGTTCGAACATTTTTTTTTTGTGAGCTA
7	AT1G76500	TAGTTGGAATGGGTTCGAATTATCCAATAAAAACCCATG	TTATGGAGTTGGGTTCGAACCGCATCTACTGACTCTTCT
9	AT2G12900	TAGTTGGAATGGGTTCGAAGTTGCATCATTTAGGCTTAG	TTATGGAGTTGGGTTCGAATGTTAAGGATTGGAGAAGAC
10	AT2G21420	TAGTTGGAATGGGTTCGAAATGTCGAAGTATTGGTTTGC	TTATGGAGTTGGGTTCGAAGGTTGTTTGGTGCATATATT
11	AT2G26850	TAGTTGGAATGGGTTCGAAGACAAACCTGTTGTGTTATT	TTATGGAGTTGGGTTCGAATCTGAAACAAAACAAAATTA
12	AT2G27250	TAGTTGGAATGGGTTCGAAAATTAACAAACTAGTTGATA	TTATGGAGTTGGGTTCGAAGAGAGATAAAGAGAGAAATA
13	AT2G35310	TAGTTGGAATGGGTTCGAATCCCTCGCATAATCACGCTC	TTATGGAGTTGGGTTCGAAGATACTGAAATTTCAAAATT
14	AT3G03770	TAGTTGGAATGGGTTCGAATAATGTAGTGTATATATATT	TTATGGAGTTGGGTTCGAATCCTTCACGAGTGATTATTA
15	AT3G25170	TAGTTGGAATGGGTTCGAAATGAGAAATATATATGACTT	TTATGGAGTTGGGTTCGAAGTGAATTAGGAGAAAAGTGA
16	AT3G54320	TAGTTGGAATGGGTTCGAACAAATCACTCTTCGCTAAGATTAC	TTATGGAGTTGGGTTCGAAAAGGAGGAAAAGGGCTAATTG
17	AT3G54800	TAGTTGGAATGGGTTCGAATTACACCAAACAACTTGCTC	TTATGGAGTTGGGTTCGAATGTTATTTTTTTCGATTGTTG
18	AT3G57800	TAGTTGGAATGGGTTCGAACACGTGATTAACACGCGGAT	TTATGGAGTTGGGTTCGAAAAAGAGAAAACCAATTTTGG
19	AT3G61150	TAGTTGGAATGGGTTCGAATCACAGACAACCAGATTCAG	TTATGGAGTTGGGTTCGAAGATAGTAACGACGAAAAAGG
20	AT3G61160	TAGTTGGAATGGGTTCGAATAAGTAGCTAAGCTAATCTC	TTATGGAGTTGGGTTCGAATTCTCGCCATTGGTCTATTA
21	AT4G28650	TAGTTGGAATGGGTTCGAATTTAGGTACTTATTTGTGGG	TTATGGAGTTGGGTTCGAAAAAGAAATAAACAAGATCTT
22	AT4G32540	TAGTTGGAATGGGTTCGAATCACTATCAAACCAAGATAAAGT	TTATGGAGTTGGGTTCGAATCTTGATGGATGATGGAAAA
23	AT4G34530	TAGTTGGAATGGGTTCGAATGGCTAAAACTCACCTTATA	TTATGGAGTTGGGTTCGAATAAAGGTGAGGGTAGAAGAG
24	AT5G06250	TAGTTGGAATGGGTTCGAAAAGGCAAAGCACCATGACTT	TTATGGAGTTGGGTTCGAAGAAAGAGAGAGGTGGGTACT
25	AT5G11320	TAGTTGGAATGGGTTCGAAACGTAAATAATAGGAATGAT	TTATGGAGTTGGGTTCGAAGTCGACTAATAAAAGCGAAA
26	AT5G11530	TAGTTGGAATGGGTTCGAAGTTACAAATACGTTTGAAGA	TTATGGAGTTGGGTTCGAATGAGGGTTTTTTTTTTGTTT
27	AT5G25830	TAGTTGGAATGGGTTCGAATAGCTACAGTTATTACATTAG	TTATGGAGTTGGGTTCGAAAAGTTTCGTTGATTAAAACT
28	AT5G26650	TAGTTGGAATGGGTTCGAATGATTTTACCCAAATTATGC	TTATGGAGTTGGGTTCGAATCAAAGGATGTTTATATAGC
29	AT5G26950	TAGTTGGAATGGGTTCGAACTCATGGCTCATGAAATCTT	TTATGGAGTTGGGTTCGAATGTTGTTGTTTACCCACAGA
30	AT5G39860	TAGTTGGAATGGGTTCGAAAGGGCAGCATGTAACACGAG	TTATGGAGTTGGGTTCGAATTCAAATATAAAGAAATTGA
31	AT5G47670	TAGTTGGAATGGGTTCGAAGGCGACTGTCGGAAACGAGT	TTATGGAGTTGGGTTCGAAAATATCCCTATAAATAATAA
32	AT5G52600	TAGTTGGAATGGGTTCGAATGGAATATTGAGAAATGAATG	TTATGGAGTTGGGTTCGAACTTTTATTTTTTCTTCCACA
33	AT5G56270	TAGTTGGAATGGGTTCGAACTCCGTAACGGGATATTTTG	TTATGGAGTTGGGTTCGAATATTTATAGTCTCTGTGTGT
34	AT5G57670	TAGTTGGAATGGGTTCGAATCTCTTAGGTGTCTCTAAAT	TTATGGAGTTGGGTTCGAATATACACTTGAAGAAGAAGA
91	AT3G05800	TAGTTGGAATGGGTTCGAATCATAAACCCCTTCCCAAGA	TTATGGAGTTGGGTTCGAAAAACATTTGGGAGAGATTGG
92	AT3G28857	TAGTTGGAATGGGTTCGAAGACAATTGTATATATGTCGA	TTATGGAGTTGGGTTCGAAAAGAAAATGAGAAAGTGATA
35	AT1G13960	TAGTTGGAATGGGTTCGAACACATTAAATGTTGCTAGTT	TTATGGAGTTGGGTTCGAAACTTTTTTGCCGTTTTGTTT
36	AT1G14600	TAGTTGGAATGGGTTCGAAATCGACGACATTTACGTCCC	TTATGGAGTTGGGTTCGAATCGAAAACCCTAATTCTTTT
37	AT1G15670	TAGTTGGAATGGGTTCGAATGTCTTAGAACAATCTCATG	TTATGGAGTTGGGTTCGAATAAAAATATCATTTAAATCA
38	AT1G31320	TAGTTGGAATGGGTTCGAAAAAACGATTTTATATATATCA	TTATGGAGTTGGGTTCGAAGGAGGGTCTCTAGGTGAAGT
39	AT1G62000	TAGTTGGAATGGGTTCGAATTGTCTTTTGTAATGTATTC	TTATGGAGTTGGGTTCGAATTCTTCCCAAGTCATAGACC
40	AT1G62220	TAGTTGGAATGGGTTCGAATGAATAAAAAACAGCCAATTG	TTATGGAGTTGGGTTCGAAGTGTGCTTCTTCCCAGATCA
41	AT1G80440	TAGTTGGAATGGGTTCGAATGAAGAAAATACTTGTTAAG	TTATGGAGTTGGGTTCGAAGGGAAAGAGGAGAGTGTGCC
42	AT1G80640	TAGTTGGAATGGGTTCGAAAACATCCCTAATTTCATAACCATCT	TTATGGAGTTGGGTTCGAAGTGGAGAGAGTGAGGAGGAG
43	AT2G18300	TAGTTGGAATGGGTTCGAATTAAATCCAAAAAAAAAAA	TTATGGAGTTGGGTTCGAAAAAGAGGATTTGCTATAAAT
44	AT2G18890	TAGTTGGAATGGGTTCGAATAGATCAACATACTTATATT	TTATGGAGTTGGGTTCGAAACTTTGTGAATAAAAACACA
45	AT2G23050	TAGTTGGAATGGGTTCGAAAAATTATCGTCTCGCAGGAT	TTATGGAGTTGGGTTCGAAGGTCACTTTAAAAAGAAACA
46	AT2G41170	TAGTTGGAATGGGTTCGAAAAATGATTATACACACTATT	TTATGGAGTTGGGTTCGAAGAACAATAATGAGGTTTAGG
47	AT2G45430	TAGTTGGAATGGGTTCGAAGTGAACTTTTGTGAAAAATC	TTATGGAGTTGGGTTCGAAAGGAGGAGGAGGTTAAGGTT
49	AT3G13960	TAGTTGGAATGGGTTCGAATATGTGTAGGTACCATCTTA	TTATGGAGTTGGGTTCGAATCTGTCACTCTTTTCCTCTG
50	AT3G54780	TAGTTGGAATGGGTTCGAAATGCAAAAATACGGAAATAC	TTATGGAGTTGGGTTCGAAGAGGATCAATGGAAGTGTTG
51	AT3G62100	TAGTTGGAATGGGTTCGAAGACATTGTTTGGATGTTTCG	TTATGGAGTTGGGTTCGAACTAAAACAGGTGATTAATGA
52	AT4G04090	TAGTTGGAATGGGTTCGAAGGCTGACCAGTTTGACACCT	TTATGGAGTTGGGTTCGAAAGCTATAGGCTCATATAACT
53	AT4G10270	TAGTTGGAATGGGTTCGAATTTCACCAAAGGAAACTATCAATGG	TTATGGAGTTGGGTTCGAATTAGAAAGTGAAGTGAATTG
54	AT4G18740	TAGTTGGAATGGGTTCGAAATCCAGCTATCATCACTGTC	TTATGGAGTTGGGTTCGAAAAATATTTATCCCCAAAAAA
55	AT4G24390	TAGTTGGAATGGGTTCGAAGCTGCAATTAACACAAGACA	TTATGGAGTTGGGTTCGAATCTCTCTCCATTTTCACCTC
56	AT4G31820	TAGTTGGAATGGGTTCGAAAATGTATTCAACCAACTCGA	TTATGGAGTTGGGTTCGAAAATTATGTATATACTTATAT
57	AT4G36240	TAGTTGGAATGGGTTCGAACTATATTGGTTTCAAGTGTA	TTATGGAGTTGGGTTCGAAAGCCGTATGAAAGCACTACA
58	AT5G18270	TAGTTGGAATGGGTTCGAATATAATACTAGCGAGTAAAG	TTATGGAGTTGGGTTCGAAATTGAAAGGGTGGAAAAAAT
59	AT5G48940	TAGTTGGAATGGGTTCGAATGAATGCCAGCGGAGTGATC	TTATGGAGTTGGGTTCGAAGGCTTAGGGTTTAGGGAAGT
60	AT5G49490	TAGTTGGAATGGGTTCGAAGTTTCGTGCACCGCCGAAGA	TTATGGAGTTGGGTTCGAAAAAAATAAGAAAATATTGAA
61	AT5G50915	TAGTTGGAATGGGTTCGAATCTTAGAGTGAAGTAATGTA	TTATGGAGTTGGGTTCGAAGAGAGAAGATTAAGGACTTG
62	AT5G66560	TAGTTGGAATGGGTTCGAAACGACCATCTCTACGAACAA	TTATGGAGTTGGGTTCGAAGGATATCGAAAAGGGGATC
67	AT1G29950	TAGTTGGAATGGGTTCGAAAGATTCACGCACGAAAAGCG	TTATGGAGTTGGGTTCGAACAGAAAACCCGTGCAAACG
68	AT1G68810	TAGTTGGAATGGGTTCGAACTAAGTTGCAAACTATCCATAC	TTATGGAGTTGGGTTCGAAATCACTTGTTTCTATGAAGAC
69	AT2G01420	TAGTTGGAATGGGTTCGAACCGAAATAAAGTTACAAAG	TTATGGAGTTGGGTTCGAATTTTTCCGGTGGGTTTTGG
74	AT1G05710	TAGTTGGAATGGGTTCGAAGCTTTGGAGTTGTTCTGTTG	TTATGGAGTTGGGTTCGAAAGAAACTGTGTCTGTTTTTG
75	AT2G41240	TAGTTGGAATGGGTTCGAAACCTCTCCTACGTGCATTGA	TTATGGAGTTGGGTTCGAATTTGAGTTTTAGATAGTTACTC
76	AT2G41130	TAGTTGGAATGGGTTCGAACATGGCTGGTTTTTGCTGGTTAAA	TTATGGAGTTGGGTTCGAAGGGTTCTGTCCTAAAAGATG
85	AT1G73590	TAGTTGGAATGGGTTCGAATGTCGACTTTTAGTATCAGC	TTATGGAGTTGGGTTCGAAGAGAAGAGACCACATTTTTA
96	AT2G42870	TAGTTGGAATGGGTTCGAATCTTCCATCTTCTTCTTCTT	TTATGGAGTTGGGTTCGAATGAAAGAAAGAGAGAGATGA

Table 7. Primers used to amplify cDNAs.

internal #	AGI	SENSE PRIMER	ANTISENSE PRIMER
6	AT1G68920	TAGTTGGAATAGGTTCATGGATTTAAGTGCGAAAGA	AGTATGGAGTTGGGTTCTCATGGCTCAACCTTCATAT
13	AT2G35310	TAGTTGGAATAGGTTCATGGCTAGAAACAGTGACAA	AGTATGGAGTTGGGTTCTTAGGGTTTTGAGACAAAGA
18	AT3G57800	TAGTTGGAATAGGTTCATGGATCTGACTGGAGGATT	AGTATGGAGTTGGGTTCTTACAGCTCCATTTTGACCT
22	AT4G32540	TAGTTGGAATAGGTTCATGGAGTCTCATCCTCACAA	AGTATGGAGTTGGGTTCTTAGGATTTAGAGGTAAAGA
23	AT4G34530	TAGTTGGAATAGGTTCATGAATGGAGCTATAGGAGG	AGTATGGAGTTGGGTTCTCAAACTCCTAAATTGCCAT
24	AT5G06250	TAGTTGGAATAGGTTCATGTCAGTCAACCATTACTC	AGTATGGAGTTGGGTTCTTATAAAAGAGTTAAAATTA
32	AT5G52600	TAGTTGGAATAGGTTCATGGAATGCAAAAGAGAAGA	AGTATGGAGTTGGGTTCCTAAAGCAGAGGAAAGAAAT

Microscopy

Embryo preparation and imaging of GFP (epi) fluorescence in embryos was conducted with either a Leica DMR microscope or a Zeiss LSM510 confocal microscope as previously described (Schlereth et al., 2010). Embryo phenotypes were analyzed using chloral hydrate cleared preparations (clearing solution of chloral hydrate, water and glycerol, 8:3:1) on a Leica DMR microscope equipped with differential interference contrast (DIC) optics.

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SUPPLEMENTARY INFORMATION

Global analysis of M0171-bdl datasets

BINGO Cytoscape analysis
The datasets were initially subjected to BiNGO (Biological Networks Gene Ontology Tool) to assess overrepresentation of GO (Gene Ontology) terms. Default settings were used (hypergeometric test, Benjamini & Hochberg False Discovery Rate for correction of multiple testing, significance level of 0.05) and the whole genome annotation as reference set together with GO-SLIM ontology terms for *Arabidopsis thaliana*. No strong over- or underrepresentation of specific functions was found, except "transcription factor activity" (GO:0003700), which was enriched in the 2.0 fold downregulated dataset (not shown). Therefore, the transcriptomic response appears to represent a global effect, rather than the regulation of a specific subset of gene (functions).

Comparison to previously generated datasets

Next, to validate the array data as being representative for suspensor to embryo transformation, to aid the identification of relevant signatures in the transcriptomics data, and help selecting genes for further study, we compared the gene lists with several previously published microarray experiments.

Auxin
The stabilization of bdl in the suspensor is presumed to override any Aux/IAA specificity and therefore inhibit all ARF activity and auxin responsiveness in these cells (Weijers et al., 2005; Chapter 3). Thus the resulting sets of misregulated genes should have a degree of overlap with auxin-responsive genes identified in previous microarray experiments. The Arabidopsis Hormone Database (AHD2.0) is an online resource for the identification of genes involved in auxin response as well as auxin auxin-responsive genes (Jiang et al., 2011). Consolidation of eight independent microarray based experiments involving auxin treatments and/or auxin response mutants led to the identification of 1245 auxin responsive Arabidopsis genes in the AHD2.0, equating to 3.7% of all 33,602 Arabidopsis genes in the current (TAIR10) genome annotation release. The microarray sample tissues and therefore subsets of auxin response components (ARFs and Aux/IAAs) vary; potentially influencing auxin mediated transcriptional responses. Therefore only a very global analysis for enriched genes was made, by combining the M0171-bdl up- and downregulated gene lists before comparison.
As expected, the M0171-bdl combined dataset was enriched for auxin responsive genes, in total 6.7% (162) of the 2416 misregulated genes were auxin responsive in AHD2.0. Although most of the misregulated genes have not been flagged as

auxin-responsive, the enrichment is relevant, as in contrast to the previous array experiments we here inhibit endogenous auxin response rather than providing excess external auxin. Well-known primary auxin response genes include members of the *Aux/IAA*, *SAUR* and *GH3* gene families (reviewed in Hagen and Guilfoyle 2002). Genes in these families are also present in the M0171-datasets (Suppl. Table S2), including *IAA12* (representing bdl; 10.6 fold up), *IAA17* (3.2 fold up), and 4 downregulated Aux/IAAs. The latter include two non-canonical Aux/IAAs (*IAA20* and *IAA30*), which will be further discussed in Chapter 5. In total 5 SAURs are misregulated, 2 positively and 3 negatively (Suppl. Table S2). All four misregulated GH3 family members (GH3.3-GH3.5, GH3.17) are downregulated.

In summary the enrichment of the M0171-bdl datasets for auxin responsive genes, including well known primary auxin responsive genes from several different families strongly infers that the microarray-based approach was successful in identifying genes that are misregulated when auxin response is inhibited in the suspensor.

Auxin and Transcription Factors

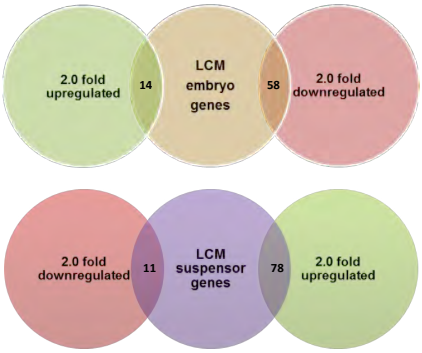
Previous analysis of microarray experiments that investigate the transcriptional response to auxin revealed that in addition to the well-known early auxin responsive genes, several families of transcriptional regulators are also overrepresented (Paponov et al., 2008). AP2 type and bHLH family transcription factors have subsequently been identified as direct targets of ARFs (Cole et al., 2009; Schlereth et al., 2010). The Plant Transcription Factor Database v2.0 (Zhang et al., 2011) was used to assess overrepresentation of transcription factors in the AHD2.0 database list of auxin responsive genes. This database contains 1695 annotated Arabidopsis genes as encoding for transcription factors, which equates to 5.0% of all Arabidopsis genes. Of these, 1578 are represented on the Operon array chip used. In the auxin-responsive gene list from AHD2.0, 9.8% of the genes were transcription factors, equating to an approximately twofold enrichment, and confirming earlier findings (Paponov et al., 2008; Schlereth et al., 2010). On a global scale, transcription factors were mildly enriched in the M0171>>bdl datasets (179/2416 genes, 7.4%). However, when considering only the top 10% misregulated genes (137 genes ≥4.2 fold up and 105 genes ≥2.9 fold down), we did find a strong enrichment of transcriptional regulators also enriched in both M0171-bdl datasets. Among the top 10% down-regulated genes, 17.1% encode transcriptional regulators while this number is 21.2% of the top 10% of upregulated genes. This significant overrepresentation of transcription factors in the most strongly misregulated genes of the M0171-bdl datasets is highly significant, as transcription factors are key regulators of changes in cell identity.

Given the enrichment of both auxin-regulated genes and transcriptional regulators, we next mined the M0171-bdl datasets for members of auxin-responsive transcription factor families (Suppl. Table S3). In total 67 transcription

factors belonging to auxin-responsive families were found to be at least 1.5 fold misregulated. Of these 14 were at least 2.0 fold upregulated and 22 were at least 2.0 fold downregulated. As these are good candidates for mediating auxin-dependent cell identity change, nine upregulated and ten downregulated genes were selected for further analysis. These included members of the bHLH, LBD, Homeobox and WRKY transcription factor families (Suppl. Table S3).

Embryonic datasets
Inhibition of auxin response in the suspensor is expected to downregulate those genes whose expression is activated by ARFs in the suspensor, thus leading to changes in cell identity. However, despite selecting an early time point for harvesting embryos, we noticed that the expression of several genes not expressed in the suspensor was downregulated (e.g. *PIN1*, *MP*). As pro-embryo and suspensor are connected and cell-cell signaling occurs between the two lineages the downregulated dataset will include false positives, i.e. embryo specific genes that are downregulated. Genes that are upregulated upon auxin response inhibition should include those that are instructive for a cell identity change and could normally be expressed in (subdomains of) the early pro-embryo and/or actively repressed in the suspensor. To help in identifying these two types of genes, we made use of a dataset generated by laser capture microdissection (LCM) of globular stage embryos and suspensor cells (Le et al., 2010). This dataset has been deposited in the Gene Expression Omnibus as GSE11262. Within this dataset, 372 genes could be assigned as present in globular stage embryo cells and absent in globular stage suspensor cells (i.e. pro-embryo specific) and 1185 genes as present in globular stage suspensor cells and absent in globular stage embryo cells (i.e. suspensor-specific).

Through comparing the M0171>>bdl dataset with the pro-embryo/suspensor dataset, it appears that the majority of upregulated genes are assigned to globular stage suspensor cells and the majority of downregulated genes are assigned to globular stage embryo cells (Suppl. Fig. S1). These patterns do not conform to the criteria for genes involved in suspensor-embryo transformation. Nevertheless the list of 14 upregulated genes that are normally predicted to be expressed exclusively in embryo cells include four transcription factors, *SHOOTMERISTEMLESS* (*STM*), which was shown to be transcribed in proliferating M0171-bdl suspensor cells in Chapter 3 (discussed below), and three others which were included in the subset were selected as strong candidates for further analysis (Table 1).



Supplemental Figure S1. Venn diagrams for overlap between M0171-bdl and LCM datasets.
Venn diagrams showing overlap between the genes misregulated with a cutoff of 2.0 fold in the M0171-bdl dataset (circles labeled 2.0 fold upregulated and 2.0 downregulated) and LCM datasets. The LCM datasets included 372 genes assigned as present in globular stage embryo cells and absent in globular stage suspensor cells (LCM embryo genes) and 1185 genes as present in globular stage suspensor cells and absent in globular stage embryo cells (LCM suspensor genes).

Unpublished datasets of Zygote vs Dermatogen stage embryos
When auxin response is inhibited in suspensor cells these undergo a cell identity transformation that ultimately leads to acquisition of embryonic identity. The route by which cell identity is changed from extra-embryonic to embryonic is unknown but several pathways can be envisioned. Firstly, upon induction, the cells could directly acquire embryonic cell identity. Secondly, the cells could undergo dedifferentiation to zygote cell identity and subsequently develop embryonic cell identity. We therefore compared the M0171-bdl dataset to datasets containing genes enriched in the zygote and dermatogen stage embryos.

Previously, zygotes and dermatogen stage embryos were dissected, RNA isolated and hybridized to Operon arrays (R. Datla personal communication). Two datasets were generated, with 260 genes enriched at dermatogen stage relative to zygote and 251 genes depleted at dermatogen stage relative to zygote (i.e. zygote enriched). Similarities between the dermatogen enriched dataset and the M0171-bdl datasets were evident and expected due to continuity between dermatogen and early globular stage transcriptomes. However when comparing the M0171-bdl datasets to the zygote enriched dataset there was a clear difference in the amount of overlap, with upregulated dataset containing 50 genes found in the zygote dataset and the downregulated dataset containing just seven. This suggests that the transcriptomic effect induced by bdl misexpression in suspensor cells shares significant similarity with the zygote transcriptome. Several of the zygote enriched, upregulated genes were selected for further analysis (Table 1).

Comparison to hypophysis derivative transcriptomes
The hypophysis derivatives, namely the quiescent centre (QC) and columella root cap are the only descendants of suspensor cells that are maintained after embryogenesis. Therefore some continuity in the transcriptomes of these root meristem cells and the suspensor could be expected and informative when further investigating the M0171-bdl downregulated dataset, which is expected to include suspensor-specific genes. Two previously published datasets identified 290 QC enriched genes and 300 QC- and 90 columella-enriched genes respectively (Nawy et al., 2005; Brady et al., 2007). These datasets were combined to give 626 unique entries that are enriched in basal descendants. In total 33 genes were shared by the M0171-bdl downregulated dataset and the basal descendent dataset. Of these, four were included in the subset for further investigation (Table 1).

Comparison to known regulators of (somatic) embryogenesis

Previously, somatic embryogenesis has been used to investigate the mechanisms by which somatic cells can acquire embryonic cell identity. Application of exogenous auxin is the most well characterized inducer of somatic embryogenesis (reviewed in Karami and Saidi, 2010). Despite considerable effort, only a handful of key somatic embryogenesis regulators, i.e. capable of inducing somatic embryogenesis when ectopically expressed, have been identified to date (reviewed by Karami et al., 2009 and Zhang and Ogas, 2009). Of these, two are misregulated in the M0171-bdl datasets.

LEC1-LIKE (L1L), the closest relative of LEAFY COTYLEDON (LEC1), and LEC1 itself are capable of inducing somatic embryogenesis when ectopically expressed (Lotan et al., 1998; Kwong et al., 2003). L1L is 2.5 fold upregulated in the M0171-bdl dataset. Also known as NF-YB6, L1L is a subunit of NF-Y transcription factor complex made up of NF-YA, NF-YB and NF-YC subunits, of which there are 10, 13 and 10 homologs in Arabidopsis respectively (Siefers et al., 2009). Interestingly, five other subunits are also misregulated in the microarray (not shown).

In differentiating tissues, ectopic STM function (together with WUS) has been shown to activate meristem activity (Gallois et al., 2002). Several identified factors associated with somatic embryogenesis have also been identified. FUSCA3 (FUS3), a B3 domain transcription factor, is implicated in somatic embryogenesis as direct somatic embryo development induction from immature zygotic embryos is practically abolished in fus3 loss of function mutants (Gaj et al., 2005). FUS3 is 1.9 fold upregulated in the M0171-bdl dataset, however was previously shown to be expressed in the suspensor and therefore is not expected to trigger suspensor to embryo transformation (Kroj et al., 2003).

Supplemental Table S1. M0171-bdl datasets.

Downregulated Genes

AGI	FC	q-value(%)
At3g13960	25.4	0.39
At1g80100	19.6	0.39
At2g01420	15.5	0.39
At1g15760	8.6	0.39
At2g21050	8.3	0.39
At3g15540	8.1	0.39
At2g45190	8.0	0.39
At1g53860	7.9	0.39
At1g24590	7.4	0.39
At5g05250	7.4	0.39
At4g29030	7.0	1.28
At3g19380	7.0	1.28
At3g02000	6.8	1.28
At1g68870	6.7	1.28
At2g14247	6.5	1.28
At1g10060	6.1	1.28
At1g71691	6.0	1.28
At5g45720	5.9	1.28
At1g52070	5.7	1.28
At1g70830	5.6	1.28
At2g33530	5.6	1.28
At4g31760	5.5	1.28
At4g31820	5.5	1.28
At1g29270	5.5	1.28
At3g51060	5.4	1.28
At2g26180	5.2	1.28
At3g54780	5.1	1.28
At1g73590	5.1	1.28
At5g50915	5.0	1.28
At5g01240	4.9	1.28
At5g14750	4.9	1.28
At2g39060	4.8	1.28
At3g16500	4.8	1.28
At4g36630	4.8	1.28
At5g66940	4.8	1.28
At1g23020	4.7	1.28
At3g53450	4.7	1.28
At1g68480	4.7	1.28
At4g23750	4.7	1.28
At5g55340	4.6	1.28
At3g24225	4.5	1.28
At1g28110	4.4	1.28
At1g75500	4.2	1.28
At3g57010	4.2	1.28
At1g21090	4.1	1.28
At5g12330	4.1	1.28
At2g06850	4.0	1.28
At1g37140	3.9	1.28
At5g66350	3.9	1.28
At1g12980	3.9	1.28
At2g18890	3.8	1.28
At5g05220	3.8	1.28
At1g13400	3.8	1.28
At2g25790	3.8	1.28
At3g12970	3.7	1.28
At3g55560	3.7	1.28
At4g24780	3.6	1.28
At2g42870	3.6	1.28
At1g66090	3.6	1.28
At1g20230	3.6	1.45
At1g53815	3.6	1.28
At1g70510	3.6	1.45
At1g62420	3.6	1.45
At5g18270	3.5	1.28
At2g04920	3.5	1.45
At3g62100	3.5	1.28
At4g29020	3.5	1.28
At5g43870	3.5	1.28
At1g70720	3.5	1.45
At1g48870	3.4	1.28
At1g29950	3.4	1.28
At3g15680	3.4	1.28
At1g55200	3.4	1.28
At1g47400	3.3	1.28
At1g78430	3.3	1.28
At2g32280	3.3	1.28
At5g51560	3.3	1.28
At2g03090	3.3	1.28
At1g80440	3.2	1.28
At5g50570	3.2	1.28
At1g19200	3.2	1.90
At5g07780	3.2	1.45
At1g12030	3.2	1.45
At1g28070	3.2	1.28
At3g24450	3.2	1.28
At4g11140	3.2	1.28
At5g56460	3.2	1.45
At1g72940	3.2	1.45
At2g19810	3.1	1.90
At2g23050	3.1	1.45
At3g14370	3.1	1.47
At1g51190	3.1	1.28
At1g15670	3.1	1.45
At5g01075	3.1	1.28
At1g17140	3.1	1.45
At5g48940	3.0	1.45
At2g07751	3.0	1.45
At3g21250	3.0	1.90
At5g53450	3.0	1.45
At1g56680	3.0	1.45

Continued

AGI	FC	q-value(%)
At1g28130	2.9	2.32
At3g18850	2.9	1.45
At5g15230	2.9	1.45
At5g22810	2.9	1.45
At3g56360	2.9	1.45
At2g33510	2.9	1.28
At2g39660	2.9	1.90
At4g05410	2.9	1.45
At3g51750	2.9	1.45
At1g52290	2.9	1.45
At1g49740	2.8	1.45
At5g13700	2.8	1.90
At1g13620	2.8	1.47
At5g19650	2.8	1.90
At1g48300	2.8	1.47
At2g37590	2.8	1.47
At1g14600	2.8	1.90
At5g26670	2.8	2.13
At4g09160	2.7	1.45
At5g54840	2.7	1.45
At1g68810	2.7	1.90
At5g50220	2.7	2.32
At1g55450	2.7	2.13
At3g06770	2.7	1.45
At1g31320	2.7	1.47
At4g09290	2.6	1.47
At3g24660	2.6	1.45
At1g74580	2.6	2.73
At3g56160	2.6	2.32
At3g42340	2.6	1.90
At2g07681	2.6	1.47
At1g78190	2.6	1.90
At4g15910	2.6	1.90
At3g07270	2.6	2.13
At1g60790	2.6	1.90
At4g04090	2.6	1.47
At3g26932	2.6	1.90
At5g56220	2.6	1.47
At3g20840	2.6	1.90
At1g78860	2.6	1.90
At1g02810	2.6	1.47
At2g07675	2.6	1.47
At5g28640	2.5	1.90
At1g77855	2.5	2.52
At2g18300	2.5	2.52
At5g18180	2.5	2.32
At1g62770	2.5	2.32
At2g05000	2.5	1.90
At2g26550	2.5	2.73
At3g48675	2.5	1.90
At4g00950	2.5	2.32
At2g06390	2.5	1.47
At3g57500	2.5	1.90
At4g16447	2.5	2.13
At5g13290	2.5	1.90
At5g05940	2.5	1.47
At5g41070	2.5	1.90
At2g15820	2.5	2.73
At1g80370	2.4	1.90
At2g26520	2.4	1.90
At2g45430	2.4	2.32
At1g70850	2.4	2.13
At5g43810	2.4	1.90
At5g52890	2.4	3.43
At1g29980	2.4	1.90
At4g38660	2.4	1.90
At5g67260	2.4	1.90
At1g30040	2.4	2.13
At2g26150	2.4	2.13
At1g71870	2.4	1.90
At4g26530	2.4	2.32
At1g10990	2.4	2.32
At1g67040	2.4	1.90
At5g22860	2.4	2.13
At3g17360	2.4	2.52
At1g70970	2.4	2.13
At3g47320	2.4	2.13
At3g04670	2.4	1.90
At5g61130	2.4	1.90
At1g62000	2.4	2.13
At1g67340	2.4	2.73
At2g26710	2.4	2.73
At3g56760	2.3	3.43
At1g16220	2.3	2.13
At5g59790	2.3	2.13
At1g31040	2.3	1.90
At2g42110	2.3	2.13
At5g26692	2.3	2.73
At5g06940	2.3	2.13
At1g44860	2.3	3.43
At1g79580	2.3	2.73
At4g27260	2.3	2.32
At3g25900	2.3	2.13
At3g43800	2.3	2.13
At3g08570	2.3	2.32
At1g69700	2.3	2.13
At5g17970	2.3	2.13
At2g16850	2.3	1.90
At1g15080	2.3	2.13
At3g15720	2.3	2.32

Continued

AGI	FC	q-value(%)
At1g65710	2.3	2.32
At2g37880	2.2	2.52
At3g51920	2.2	3.13
At4g16370	2.2	2.13
At4g17470	2.2	2.52
At2g11910	2.2	2.32
At1g30135	2.2	2.32
At4g29360	2.2	2.13
At2g22680	2.2	2.13
At4g29190	2.2	2.52
At3g59190	2.2	3.13
At4g18740	2.2	2.52
At5g60200	2.2	2.32
At2g19090	2.2	2.13
At1g11400	2.2	2.73
At2g14590	2.2	3.13
At5g18310	2.2	2.32
At5g43730	2.2	3.31
At1g80640	2.2	2.32
At5g37260	2.2	2.13
At2g41130	2.2	3.31
At4g34160	2.2	2.32
At5g20330	2.2	2.32
At5g13830	2.2	2.32
At1g69295	2.2	2.32
At3g17680	2.2	2.13
At1g52930	2.2	2.32
At1g59030	2.2	3.13
At4g00430	2.2	2.32
At1g56020	2.2	2.32
At2g42840	2.2	2.52
At1g13960	2.2	2.32
At3g49670	2.2	2.32
At3g29030	2.2	2.13
At3g55750	2.2	2.32
At5g41663	2.2	3.62
At4g27010	2.2	2.32
At2g37940	2.2	2.32
At2g44860	2.2	2.13
At1g68740	2.2	2.52
At1g14640	2.2	3.54
At1g63100	2.2	2.32
At4g13235	2.2	2.32
At3g49410	2.2	2.52
At5g46790	2.2	2.73
At2g34910	2.2	2.52
At4g16990	2.2	3.31
At1g06390	2.1	2.13
At2g42050	2.1	2.32
At4g08910	2.1	2.13
At2g01630	2.1	2.32
At1g70895	2.1	2.52
At1g70890	2.1	2.32
At4g11350	2.1	3.13
At5g49490	2.1	2.52
At2g02240	2.1	2.73
At5g62710	2.1	3.62
At2g38370	2.1	2.32
At1g62220	2.1	2.32
At5g38790	2.1	2.32
At1g44740	2.1	2.73
At4g38140	2.1	3.54
At5g20160	2.1	2.52
At1g53360	2.1	2.52
At2g29350	2.1	2.73
At2g18500	2.1	2.52
At2g41170	2.1	2.32
At3g04770	2.1	2.32
At4g24390	2.1	2.52
At2g29310	2.1	2.73
At4g10270	2.1	2.32
At2g30700	2.1	3.13
At2g45160	2.1	2.73
At5g66560	2.1	2.52
At1g10040	2.1	3.62
At1g05710	2.1	2.32
At3g07540	2.1	3.31
At1g62720	2.1	4.42
At1g68760	2.1	2.32
At5g43630	2.1	2.32
At2g27840	2.1	2.52
At1g03170	2.1	2.32
At2g45450	2.1	2.52
At2g33370	2.1	2.32
At1g66250	2.1	2.32
At2g16570	2.1	2.52
At3g02010	2.1	2.52
At1g22050	2.1	2.52
At4g20940	2.0	2.52
At4g26990	2.0	4.42
At5g48500	2.0	2.32
At1g57610	2.0	2.52
At1g25290	2.0	3.83
At4g03100	2.0	2.32
At4g02290	2.0	2.52
At1g61095	2.0	2.52
At5g66600	2.0	4.42
At3g13500	2.0	2.52
At1g28310	2.0	3.54
At5g13740	2.0	3.62

Continued

AGI	FC	q-value(%)
At3g60390	2.0	3.43
At2g34320	2.0	4.68
At2g18340	2.0	2.32
At2g41650	2.0	2.52
At2g47930	2.0	2.52
At1g71692	2.0	3.54
At1g70710	2.0	2.52
At4g32880	2.0	3.62
At2g20490	2.0	2.52
At3g21850	2.0	2.73
At1g12560	2.0	2.73
At3g27580	2.0	3.43
At2g17580	2.0	2.52
At3g03920	2.0	2.73
At1g28580	2.0	3.83
At3g25670	2.0	3.54
At4g25240	2.0	2.52
At3g42723	2.0	3.43
At2g41240	2.0	3.13
At2g34710	2.0	2.73
At2g23170	2.0	4.42
At4g36020	2.0	3.13
At4g25980	2.0	4.68
At2g38810	2.0	2.52
At4g25390	2.0	2.52
At5g61200	2.0	3.13
At5g48350	2.0	2.73
At1g69420	2.0	2.52
At1g72890	2.0	3.54
At4g22560	2.0	3.13
At1g15000	2.0	3.31
At1g07220	2.0	2.73
At4g16280	2.0	4.68
At1g16070	2.0	3.43
At4g38480	2.0	3.94
At4g04920	2.0	3.13
At5g19300	2.0	2.52
At5g05510	2.0	2.73
At3g21170	2.0	3.62
At5g37478	2.0	3.54
At1g28080	2.0	3.31
At1g25530	2.0	2.52
At3g15300	2.0	3.54
At1g18630	2.0	2.73
At1g62900	2.0	3.43
At2g31920	2.0	2.73
At2g34390	2.0	3.62
At5g13680	2.0	2.73
At1g02870	2.0	3.13

Upregulated Genes

AGI	FC	q-value(%)
At2g27250	44.8	0.00
At2g31980	30.8	0.00
At4g21020	26.6	0.00
At4g27160	24.9	0.00
At3g54800	24.4	3.62
At3g62230	24.1	0.00
At4g27150	23.4	0.00
At3g22640	22.6	0.00
At3g27660	22.1	0.00
At1g52690	20.9	0.00
At4g34135	19.3	0.00
At2g26400	17.7	0.00
At4g15390	17.2	0.00
At3g63040	15.6	0.00
At2g23190	15.1	0.00
At4g36700	14.6	0.00
At3g12430	13.7	0.00
At4g32510	13.2	3.43
At2g40600	13.0	3.83
At1g75830	12.4	0.00
At3g17520	12.3	0.00
At3g01570	12.1	0.00
At5g44630	11.9	0.00
At4g27140	11.9	0.00
At4g27170	11.6	0.00
At4g23680	11.4	0.00
At5g46070	11.2	4.09
At1g47540	10.8	0.00
At5g12910	10.8	3.31
At1g04550	10.6	0.00
At3g58410	10.5	0.00
At1g02450	10.2	0.00
At3g07850	9.9	0.00
At3g21180	9.9	4.68
At4g34530	9.9	0.00
At2g27990	9.8	0.60
At5g43430	9.6	2.13
At3g49260	9.6	0.00
At3g54940	9.5	0.00
At5g59230	9.4	2.32
At2g40990	9.3	3.94
At2g38870	9.0	0.00
At1g43780	8.8	0.00
At4g36490	8.7	0.00
At1g55920	8.7	0.00
At2g15490	8.5	0.00
At1g32560	8.4	0.00
At4g29270	8.0	0.00
At5g11930	8.0	0.00
At3g22490	8.0	0.60
At5g11320	7.9	0.00
At1g65490	7.9	0.00
At2g36710	7.2	2.13
At4g11940	7.2	0.21
At5g19580	7.2	0.00
At2g36020	7.0	0.00
At4g28405	6.9	0.00
At3g09260	6.8	0.60
At1g68170	6.7	0.00
At3g51590	6.7	0.00
At1g77100	6.5	0.00
At1g15520	6.4	2.13
At5g17700	6.3	0.00
At4g36880	6.3	0.00
At5g25830	6.3	0.60
At1g34095	6.2	0.00
At5g08030	6.1	0.60
At3g62170	6.0	0.00
At4g32540	5.9	0.00
At2g38920	5.9	0.60
At3g44790	5.9	4.42
At2g47040	5.9	0.00
At5g07500	5.7	0.00
At4g16690	5.7	0.60
At5g40420	5.7	0.00
At5g27420	5.7	1.47
At5g12380	5.6	3.83
At2g38940	5.6	0.00
At1g53690	5.5	0.00
At5g51210	5.5	0.00
At5g15110	5.4	0.00
At1g55740	5.4	0.00
At3g05610	5.3	0.00
At5g39860	5.2	0.00
At4g30340	5.2	4.09
At1g65500	5.2	0.00
At1g76470	5.2	0.00
At3g05930	5.2	0.00
At5g26717	5.2	0.00
At1g65610	5.1	0.60
At2g42340	5.1	5.49
At1g61320	5.1	2.52
At5g04460	5.1	2.52
At5g24790	5.1	1.18
At2g34340	5.0	0.00
At2g43680	5.0	3.83
At1g69600	5.0	0.00
At5g50030	5.0	0.00
At1g12080	5.0	0.00
At5g13310	5.0	4.09

Continued

AGI	FC	q-value(%)
At1g28670	4.9	0.00
At2g32990	4.9	0.00
At1g48130	4.9	0.00
At3g23090	4.8	0.00
At3g42725	4.8	0.39
At1g09155	4.8	3.31
At5g09210	4.8	0.00
At4g25140	4.8	0.00
At1g75450	4.8	2.32
At5g16570	4.8	0.00
At2g47030	4.8	0.00
At3g29970	4.7	5.13
At2g21840	4.7	3.13
At1g02230	4.7	0.60
At5g44120	4.7	0.60
At3g01270	4.7	0.00
At1g52090	4.7	4.09
At3g26110	4.6	0.00
At1g33750	4.6	0.00
At2g27380	4.6	0.00
At5g28680	4.5	0.00
At2g47050	4.5	0.00
At3g03520	4.5	3.62
At5g01600	4.5	0.00
At3g20580	4.5	0.00
At1g32780	4.5	0.21
At5g62340	4.4	0.00
At4g25810	4.4	2.13
At5g48330	4.4	0.75
At5g04180	4.3	0.00
At1g48470	4.3	0.00
At4g35010	4.3	0.39
At2g31500	4.3	0.00
At4g02250	4.3	0.00
At4g15750	4.3	1.47
At3g07820	4.3	0.92
At2g16730	4.2	0.60
At3g57690	4.2	0.75
At1g04920	4.2	0.00
At1g22100	4.2	0.00
At3g45310	4.2	0.00
At3g54320	4.2	0.60
At1g61563	4.2	0.00
At4g06724	4.1	4.42
At1g29640	4.1	2.32
At3g61160	4.1	0.00
At5g01300	4.1	0.00
At5g24530	4.1	0.00
At1g65090	4.1	0.00
At4g30670	4.0	0.39
At3g25170	4.0	0.00
At1g65550	4.0	1.18
At1g09750	4.0	0.00
At2g29090	4.0	1.90
At5g05190	4.0	3.83
At1g70410	4.0	0.39
At4g33600	4.0	0.00
At5g56140	3.9	2.73
At4g25433	3.9	0.00
At5g23320	3.9	0.75
At5g12420	3.9	0.00
At1g14420	3.9	0.00
At1g33280	3.9	1.28
At3g49540	3.9	0.39
At2g16660	3.9	0.21
At4g18596	3.9	0.00
At1g09790	3.9	0.60
At3g23870	3.8	0.00
At5g06250	3.8	0.39
At5g28000	3.8	0.92
At3g14040	3.8	1.18
At3g52970	3.7	1.45
At1g35400	3.7	5.13
At1g78500	3.7	0.21
At5g27200	3.7	0.21
At3g49040	3.6	2.13
At1g17620	3.6	5.13
At5g53870	3.6	0.21
At1g35560	3.6	3.13
At4g40020	3.6	3.62
At3g46260	3.6	3.62
At5g27910	3.6	0.75
At3g28150	3.6	0.39
At5g38850	3.6	3.54
At3g28750	3.5	0.39
At3g61890	3.5	4.42
At1g48910	3.5	0.60
At4g27520	3.5	1.28
At1g78520	3.5	0.39
At5g51690	3.5	3.54
At5g44580	3.4	0.60
At4g37360	3.4	0.39
At5g52340	3.4	3.62
At2g26850	3.4	0.39
At3g06090	3.4	0.39
At3g28430	3.4	4.09
At5g01610	3.4	0.60
At1g08140	3.4	0.92
At1g78260	3.4	0.60
At3g13784	3.4	5.13

Supplemental Table S1 Continued.

Continued

AGI	FC	q-value(%)
At4g34830	3.4	2.32
At4g28650	3.3	0.75
At2g28680	3.3	0.21
At1g02790	3.3	0.75
At2g25770	3.3	0.39
At3g28345	3.3	0.39
At1g56600	3.3	3.13
At2g30230	3.3	1.28
At1g28660	3.3	0.39
At1g62290	3.3	0.39
At1g66570	3.3	2.73
At2g23510	3.3	0.60
At2g24450	3.3	0.60
At3g22410	3.3	1.18
At3g05150	3.3	0.60
At5g42300	3.3	2.73
At3g58330	3.2	0.60
At5g39050	3.2	0.60
At1g25480	3.2	4.42
At1g06850	3.2	2.32
At1g04250	3.2	2.73
At2g41340	3.2	1.90
At2g14560	3.2	0.60
At1g58310	3.2	0.60
At1g44760	3.2	0.39
At5g01520	3.2	3.31
At1g79160	3.2	0.39
At2g14290	3.2	0.60
At2g38530	3.2	0.39
At1g78320	3.2	0.60
At4g17500	3.1	2.13
At3g51420	3.1	0.60
At2g40610	3.1	1.18
At3g12580	3.1	0.60
At5g38160	3.1	0.39
At2g03980	3.1	0.60
At5g43935	3.1	4.68
At4g01890	3.1	5.13
At1g22590	3.1	0.60
At2g47115	3.1	5.49
At2g32270	3.1	0.60
At1g17060	3.0	0.60
At2g36550	3.0	0.60
At3g44910	3.0	5.13
At4g18020	3.0	2.73
At3g05260	3.0	4.09
At1g10770	3.0	3.62
At1g67810	3.0	0.60
At3g14205	3.0	3.62
At1g68320	3.0	0.60
At4g03965	3.0	0.60
At1g35750	2.9	2.13
At1g04560	2.9	1.18
At1g28640	2.9	0.60
At1g73190	2.9	1.45
At5g18900	2.9	2.73
At2g38060	2.9	5.13
At3g05800	2.9	3.13
At5g66020	2.9	0.75
At4g09760	2.9	3.83
At2g24762	2.9	4.68
At3g20220	2.9	0.60
At1g27170	2.9	0.60
At3g57020	2.9	2.73
At3g16180	2.9	0.60
At5g59340	2.9	0.75
At4g21900	2.9	0.60
At2g23790	2.9	3.62
At1g70560	2.9	0.60
At1g54860	2.9	1.28
At3g13790	2.9	0.60
At1g11740	2.9	0.60
At4g23690	2.9	0.92
At1g54550	2.8	4.68
At3g49160	2.8	0.92
At1g44830	2.8	1.28
At1g01490	2.8	0.60
At5g66670	2.8	1.18
At1g18010	2.8	1.18
At4g13000	2.8	0.75
At1g47840	2.8	0.60
At1g68510	2.8	0.60
At2g21420	2.8	2.13
At4g19380	2.8	0.60
At5g16410	2.8	1.45
At4g04460	2.8	0.60
At4g09930	2.8	0.60
At1g28650	2.8	0.92
At2g46950	2.8	0.60
At3g55890	2.8	3.83
At1g10370	2.8	1.47
At5g22920	2.8	2.32
At5g12460	2.8	2.13
At3g29240	2.8	2.73
At2g25940	2.8	0.75
At3g12880	2.8	0.60
At4g34860	2.7	3.54
At2g38380	2.7	3.54
At5g01360	2.7	0.60
At5g49680	2.7	2.73

Continued

AGI	FC	q-value(%)
At1g27040	2.7	1.18
At5g66520	2.7	1.28
At1g05170	2.7	0.60
At1g06030	2.7	0.60
At1g63060	2.7	1.28
At3g25165	2.7	2.32
At5g09640	2.7	0.60
At2g17190	2.7	4.68
At5g26650	2.7	1.18
At1g78380	2.7	0.60
At5g23840	2.7	4.68
At2g04100	2.7	0.75
At1g58643	2.7	0.60
At2g02120	2.7	0.60
At5g51950	2.7	1.28
At1g53930	2.7	0.75
At1g53950	2.7	0.60
At2g48110	2.7	5.13
At5g25820	2.7	0.60
At5g14780	2.7	0.60
At3g13400	2.7	0.92
At5g06270	2.6	0.60
At1g61566	2.6	1.47
At3g21620	2.6	0.60
At3g20270	2.6	0.60
At2g35500	2.6	5.13
At5g59110	2.6	0.60
At2g19360	2.6	5.49
At1g63300	2.6	5.13
At1g51250	2.6	3.83
At1g11765	2.6	1.47
At5g49190	2.6	0.60
At3g17265	2.6	4.42
At1g80580	2.6	3.94
At5g07950	2.6	5.49
At2g18660	2.6	3.54
At5g44020	2.6	1.28
At4g02190	2.6	5.49
At3g09330	2.6	1.28
At5g23405	2.6	4.68
At3g11160	2.6	0.75
At4g23600	2.6	3.43
At3g42640	2.6	1.28
At5g03590	2.6	1.28
At5g56270	2.5	1.28
At2g43510	2.5	0.75
At5g63240	2.5	0.60
At1g74550	2.5	0.75
At4g32380	2.5	0.60
At5g41800	2.5	3.13
At1g65810	2.5	0.60
At1g68920	2.5	5.49
At5g45200	2.5	2.52
At3g02970	2.5	2.73
At2g01610	2.5	4.09
At2g43180	2.5	4.42
At3g21700	2.5	1.28
At2g37100	2.5	1.47
At1g76290	2.5	1.28
At5g64530	2.5	1.18
At1g33055	2.5	1.18
At5g07430	2.5	1.18
At5g26950	2.5	1.47
At3g44300	2.5	0.92
At1g66500	2.5	3.13
At5g47670	2.5	1.28
At2g24600	2.5	3.43
At1g17745	2.5	2.73
At5g50490	2.5	0.92
At5g19710	2.5	5.49
At4g37800	2.5	0.92
At2g40730	2.5	3.62
At5g26700	2.5	1.18
At5g63750	2.5	1.18
At3g28857	2.4	0.92
At2g45800	2.4	0.92
At5g19140	2.4	0.75
At5g52560	2.4	3.83
At1g70540	2.4	1.47
At5g20230	2.4	3.54
At2g39705	2.4	0.92
At3g28030	2.4	3.31
At4g20530	2.4	0.92
At5g48510	2.4	1.45
At4g21960	2.4	1.18
At4g23070	2.4	1.18
At2g35310	2.4	0.92
At2g25310	2.4	3.83
At4g39270	2.4	4.42
At1g54010	2.4	0.92
At4g22235	2.4	0.92
At3g61150	2.4	1.18
At1g10550	2.4	1.18
At4g24510	2.4	1.18
At3g57800	2.4	3.31
At1g68440	2.4	0.92
At1g24470	2.4	0.92
At3g13080	2.4	2.32
At4g26710	2.4	3.83
At4g00330	2.4	3.83

Continued

AGI	FC	q-value(%)
At2g14160	2.4	1.18
At2g37760	2.4	1.18
At1g23350	2.4	0.92
At1g35140	2.4	2.32
At1g76500	2.3	1.18
At1g60540	2.3	1.47
At1g22430	2.3	1.18
At3g09340	2.3	1.18
At2g46450	2.3	1.90
At2g02990	2.3	0.92
At2g05420	2.3	2.32
At5g42220	2.3	4.42
At3g14990	2.3	1.28
At2g43520	2.3	1.47
At2g02780	2.3	5.13
At1g24520	2.3	2.13
At5g42030	2.3	4.09
At4g32870	2.3	0.92
At2g45830	2.3	3.43
At2g31570	2.3	1.18
At5g39180	2.3	1.18
At3g02410	2.3	1.28
At2g38750	2.3	1.18
At4g21380	2.3	1.18
At1g63360	2.3	1.45
At5g38950	2.3	1.28
At5g14860	2.3	1.18
At1g35290	2.3	1.18
At5g54140	2.3	4.68
At3g30775	2.3	1.18
At1g29600	2.3	1.45
At1g22620	2.3	2.52
At3g26860	2.3	1.28
At4g13050	2.3	1.28
At5g52230	2.3	1.45
At5g17330	2.3	1.18
At1g60970	2.3	1.18
At4g23090	2.3	1.18
At2g23970	2.3	1.18
At1g28420	2.3	3.43
At4g14740	2.3	1.28
At1g08630	2.3	1.18
At1g50650	2.3	1.47
At1g62360	2.3	1.18
At1g02820	2.3	2.13
At3g10200	2.2	1.28
At5g39530	2.2	4.09
At2g04230	2.2	2.73
At3g17810	2.2	1.28
At4g20830	2.2	1.45
At2g19000	2.2	3.83
At1g05320	2.2	1.28
At2g12940	2.2	1.28
At5g06530	2.2	2.73
At5g07550	2.2	2.32
At5g11530	2.2	3.43
At3g15760	2.2	1.47
At5g53030	2.2	3.43
At5g57670	2.2	4.42
At3g08940	2.2	1.28
At2g44560	2.2	1.18
At3g05625	2.2	2.52
At4g22590	2.2	1.45
At4g15500	2.2	2.32
At1g06980	2.2	1.28
At3g02110	2.2	1.18
At2g25890	2.2	1.45
At2g27180	2.2	1.28
At1g25450	2.2	1.28
At5g41900	2.2	3.54
At3g19350	2.2	1.28
At3g54890	2.2	3.31
At4g24040	2.2	3.54
At1g68610	2.2	2.13
At3g01900	2.2	1.28
At3g61010	2.2	1.90
At1g76090	2.2	1.28
At1g26795	2.2	1.28
At1g12180	2.2	1.18
At5g59220	2.2	1.45
At5g46230	2.2	1.28
At4g34050	2.2	1.18
At5g02190	2.2	2.13
At1g64830	2.2	1.28
At5g39400	2.2	2.73
At1g17780	2.2	2.32
At1g66460	2.2	1.28
At1g22210	2.2	2.73
At5g46110	2.2	1.28
At1g68110	2.2	1.90
At5g47550	2.2	1.28
At1g67330	2.2	1.47
At4g15053	2.2	2.13
At1g69860	2.2	1.47
At1g03620	2.2	3.31
At3g01700	2.2	1.90
At1g62660	2.2	3.31
At1g13830	2.2	1.28
At3g19020	2.2	4.09
At1g71450	2.2	4.68

Continued

AGI	FC	q-value(%)
At2g01031	2.2	1.28
At5g24480	2.1	1.45
At2g44240	2.1	3.43
At5g02020	2.1	3.62
At4g37250	2.1	1.47
At2g34970	2.1	2.13
At4g00990	2.1	1.28
At1g04700	2.1	5.49
At5g38930	2.1	1.28
At4g13840	2.1	1.28
At3g23730	2.1	1.28
At3g15820	2.1	1.28
At1g36970	2.1	4.42
At1g78370	2.1	1.28
At5g25470	2.1	3.43
At2g35300	2.1	2.73
At1g48930	2.1	1.45
At5g61190	2.1	1.90
At5g05460	2.1	1.90
At3g19000	2.1	1.28
At3g03770	2.1	5.13
At5g51550	2.1	1.90
At3g03270	2.1	1.28
At2g44550	2.1	1.28
At5g19980	2.1	2.32
At3g63200	2.1	1.45
At5g50480	2.1	3.83
At2g42000	2.1	5.49
At2g27080	2.1	2.32
At4g24220	2.1	2.52
At1g01130	2.1	1.90
At2g41190	2.1	1.90
At3g60130	2.1	1.45
At1g19320	2.1	2.52
At5g25450	2.1	2.32
At1g03630	2.1	1.45
At5g02160	2.1	1.45
At5g39030	2.1	2.13
At1g20620	2.1	1.47
At5g62620	2.1	1.90
At3g53990	2.1	1.45
At1g03210	2.1	2.73
At5g59700	2.1	3.94
At5g48000	2.1	4.42
At3g05320	2.0	5.49
At1g51010	2.0	3.43
At3g22235	2.0	2.52
At4g35650	2.0	1.47
At1g55090	2.0	4.09
At4g14780	2.0	2.13
At1g78460	2.0	1.47
At2g47710	2.0	1.47
At1g53790	2.0	3.13
At3g20865	2.0	1.47
At2g12900	2.0	1.45
At4g39940	2.0	4.68
At5g53080	2.0	4.68
At1g22110	2.0	2.32
At2g24610	2.0	5.13
At4g30960	2.0	1.47
At3g61470	2.0	1.47
At5g05030	2.0	1.47
At2g22660	2.0	1.47
At3g55740	2.0	2.32
At1g71015	2.0	4.09
At5g11100	2.0	2.73
At3g61230	2.0	2.13
At1g41830	2.0	5.49
At5g52600	2.0	1.90
At2g17780	2.0	4.68
At5g57910	2.0	5.13
At1g74100	2.0	3.62
At4g05250	2.0	1.90
At1g15150	2.0	1.47
At4g34580	2.0	1.90
At1g03230	2.0	3.54
At5g39440	2.0	2.52
At1g18000	2.0	1.90
At4g17940	2.0	2.32
At5g37770	2.0	1.47
At1g07720	2.0	2.32
At4g23500	2.0	1.90
At1g55020	2.0	2.13
At2g26070	2.0	1.47
At5g25770	2.0	3.13
At3g50820	2.0	2.13
At5g51010	2.0	2.13
At1g06020	2.0	5.49
At3g13390	2.0	2.73
At1g68795	2.0	3.83
At2g05100	2.0	3.13
At3g58980	2.0	2.13
At4g35560	2.0	1.90
At1g02930	2.0	2.13
At1g34790	2.0	1.90
At1g35513	2.0	2.13
At5g07020	2.0	3.62
At1g78995	2.0	2.13
At2g21140	2.0	2.73
At2g44670	2.0	4.09

Continued

AGI	FC	q-value(%)
At4g25100	2.0	1.47
At4g19200	2.0	1.47
At3g22550	2.0	4.09
At1g27560	2.0	5.13
At2g35290	2.0	2.32
At2g29300	2.0	4.42
At1g67750	2.0	2.32
At2g44580	2.0	1.47
At3g16490	2.0	3.31
At1g01430	2.0	1.47
At5g54350	2.0	3.83
At4g03510	2.0	3.31
At1g25400	2.0	1.90
At1g33700	2.0	2.13
At5g35460	2.0	1.90
At1g72130	2.0	2.52
At3g45600	2.0	1.47
At1g24540	2.0	1.90
At2g34420	2.0	1.90
At4g09650	2.0	1.90
At5g53240	2.0	2.52

AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. FC = fold change in M0171-bdl dataset. q-value (%) = False Discovery Rate.

Supplemental Table S2. Primary Auxin Responsive Genes Misreguated in the M0171-bdl datasets.

AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. FC = fold change in M0171-bdl dataset.

Gene Family	AGI	Other name(s)	FC
AUX/IAA	AT3G15540	IAA19	8.1 down
AUX/IAA	AT3G16500	IAA26	4.8 down
AUX/IAA	AT3G62100	IAA30	3.5 down
AUX/IAA	AT2G46990	IAA20	1.7 down
AUX/IAA	AT1G04550	IAA12	10.6 up
AUX/IAA	AT1G04250	IAA17	3.2 up
GH3	AT1G28130	GH3,17	2.9 down
GH3	AT4G27260	GH3.5, WES1	2.3 down
GH3	AT2G23170	GH3,3	2.0 down
GH3	AT1G59500	GH3,4	1.7 down
SAUR	AT5G27780	-	1.9 down
SAUR	AT1G19840	-	1.7 down
SAUR	AT5G10990	-	1.7 down
SAUR	AT3G20220	-	2.9 up
SAUR	AT1G79130	-	1.7 up

Supplemental Table S3. Members of Auxin Responsive Transcription Factor Families Misregulated in the M0171-bdl datasets.

AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. FC = fold change in M0171-bdl dataset.

Gene Family	AGI	Other name(s)	FC
ARF	AT1G19850	ARF5, MP	1.8 down
ARF	AT2G33860	ARF3, ETT	1.6 down
ARF	AT2G28350	ARF10	1.6 down
ARF	AT1G34170	ARF13	1.9 up
ARF	AT1G35520	ARF15	1.8 up
ARF	AT1G34410	ARF21	1.8 up
ARF	AT1G35240	ARF20	1.8 up
ARR	AT4G31920	ARR10	1.8 down
ARR	AT1G19050	ARR7	1.7 down
ARR	AT3G16857	ARR1	1.6 down
ARR	AT3G48100	ARR5	1.8 up
bHLH	AT5G50915	bHLH137	4.6 down
bHLH	AT2G42870	bHLH165	4.2 down
bHLH	AT1G29950	bHLH144	3.5 down
bHLH	AT1G68810	bHLH030	2.7 down
bHLH	AT2G18300	bHLH064	2.5 down
bHLH	AT1G05710	bHLH153	2.2 down
bHLH	AT2G41130	bHLH106	2.2 down
bHLH	AT2G41240	bHLH100	2.0 down
bHLH	AT3G47640	bHLH047	1.8 down
bHLH	AT2G42280	bHLH130	1.5 down
bHLH	AT4G34530	bHLH63	9.9 up
bHLH	AT5G39860	bHLH136	5.2 up
bHLH	AT3G05800	bHLH150	2.9 up
bHLH	AT1G68920	bHLH49	2.5 up
bHLH	AT3G57800	bHLH60	2.4 up
bHLH	AT3G28857	bHLH164	2.4 up
bHLH	AT3G07340	bHLH62	1.6 up
bHLH	AT5G08130	bHLH46	1.6 up
C2C2(Zn)DOF	AT5G66940	-	4.8 down
C2C2(Zn)DOF	AT2G37590	DOF2.4	2.8 down
C2C2(Zn)DOF	AT5G60200	TMO6	2.2 down
C2C2(Zn)DOF	AT1G28310	-	2.0 down
C2C2(Zn)DOF	AT1G07640	OBP2	1.6 down
HOMEOBOX	AT1G70510	KNAT2	3.6 down
HOMEOBOX	AT4G32880	ATHB8	2.0 down
HOMEOBOX	AT3G60390	HAT3	2.0 down
HOMEOBOX	AT2G34710	ATHB14, PHB	2.0 down
HOMEOBOX	AT5G06710	HAT14	1.8 down
HOMEOBOX	AT1G52150	ATHB15, CNA, ICU4	1.8 down
HOMEOBOX	AT2G27990	BLH8, PNF	9.8 up
HOMEOBOX	AT3G61890	ATHB12	3.5 up
HOMEOBOX	AT5G59340	WOX2	2.9 up
HOMEOBOX	AT3G61150	HDG1, HD-GL2-1	2.4 up
HOMEOBOX	AT1G28420	HB-1	2.3 up
HOMEOBOX	AT1G62360	STM, BUM1, SHL, WAM1, BUM, WAM	2.3 up
HOMEOBOX	AT5G45980	WOX8, STPL	1.9 up
HOMEOBOX	AT1G26960	AtHB23	1.8 up
HOMEOBOX	AT5G02030	LSN, PNY, HB-6, BLR, RPL, BLH9, VAN	1.8 up
HOMEOBOX	AT4G00730	ANL2	1.8 up
HOMEOBOX	AT5G65310	ATHB5	1.8 up
HOMEOBOX	AT2G32370	HDG3	1.6 up
HOMEOBOX	AT1G05230	HDG2	1.6 up
LBD	AT1G31320	LBD4	2.7 down
LBD	AT4G00210	LBD31	1.8 down
LBD	AT1G68510	LBD42	2.8 up
LBD	AT5G35900	LBD35	1.7 up
MYB-rel	AT5G37260	RVE2, CIR1	2.2 down
MYB-rel	AT1G18330	EPR1	1.6 down
MYB-rel	AT1G75250	ATRL6	1.5 down
MYB-rel	AT5G06110	-	1.5 down
MYB-rel	AT5G56840	-	1.7 up
MYB-rel	AT1G08810	MYB60	1.7 up
WRKY	AT3G04670	WRKY39	2.4 down
WRKY	AT1G13960	WRKY4	2.2 down
WRKY	AT5G56270	WRKY2	2.5 up
WRKY	AT1G55600	WRKY10	1.7 up

Chapter

Convergent regulation of auxin homeostasis in Arabidopsis embryogenesis

Annemarie S. Lokerse, Anna M. Keller and Dolf Weijers

ABSTRACT

During early Arabidopsis embryogenesis a simple structure is formed in which two distinct cell lineages are initially specified, embryonic and extra-embryonic. The finding that specific inhibition of auxin response induces an identity change in extra-embryonic cells towards an embryonic identity provided an excellent model in which to study cell identity specification and maintenance. The transcriptional responses that accompany early changes in cell identity were captured with a microarray-based approach, using dissected early embryos in which auxin response was inhibited in the extra-embryonic lineage. In this chapter we investigate the prevalence of auxin homeostasis and signaling components in the resulting dataset. A comprehensive analysis of the dataset revealed a strong enrichment for genes encoding components of auxin biosynthesis, transport, (de)conjugation pathways as well as core response components. Strikingly, in most cases the predicted effect of changes in expression of individual genes was an increase in intracellular auxin concentration. To our knowledge this is the first time that such a global and convergent regulation of gene families involved in auxin homeostasis and signaling has been shown. Furthermore we document the dynamic patterns of expression of selected auxin-related genes during early embryogenesis. An insertion mutant line of one of these downregulated genes, *IAA20* (which encodes an unusual member of the Aux/IAA family), has a low but significant cell patterning defect at the junction between the embryonic and extra-embryonic cells, indicative of a disruption in correct cell identity specification.

INTRODUCTION

Auxin plays a role in many aspects of plant growth and development. Although the relatively simple mechanism of the transcriptional response to auxin is rather generic, the outputs of auxin response are highly context-dependent. In Chapter 3 we identified a novel auxin response, which is required in the context of the extra-embryonic suspensor cells for cell identity maintenance, including repression of embryonic capacity.

The context dependent nature of auxin response can be envisioned to result from the generation of specificity at several different levels (reviewed in Chapter 1 and Del Bianco and Kepinski, 2011). For example, in Chapter 2 we show that in the embryo, different cell types express distinct subsets of *AUXIN RESPONSE FACTOR*s (*ARFs*). In addition to *ARFs*, the differential expression of other core components of the response machinery during embryogenesis could also be expected, and thereby generate specificity. These components are represented by members of multi gene families, and in addition to differential regulation, functional specificity, including interaction specificity, has been demonstrated (Weijers et al., 2005b; Muto et al., 2007; Vernoux et al., 2011). Moreover, the executors of auxin response, namely the ARFs, are expected to have binding

preferences to *cis*-regulatory elements found in the promoters of auxin responsive genes, an area that needs further investigation.

In Chapter 4 we showed that following the specific inhibition of auxin response in the suspensor, auxin responsive genes were enriched among the misregulated gene datasets, as expected. An additional, immediately evident observation following the global analysis of misregulated gene datasets was the overrepresentation of genes involved in auxin homeostasis and signaling. These findings are not mutually exclusive, indeed several gene families involved in auxin homeostasis and signaling were themselves first characterized as being auxin responsive (e.g. *Aux/IAAs* and *GH3* genes discussed below).

Auxin responses are ultimately dependent on the concentration of auxin in a cell, although it has yet to be shown how linear the relationship between auxin concentration and response is. Cellular auxin homeostasis depends on *de novo* auxin biosynthesis, degradation, (de)conjugation and intercellular polar transport.

There are several pathways leading to the synthesis of the most abundant auxin, indole 3-acetic acid (IAA), none of which has been fully elucidated to date. IAA is synthesized from indole via tryptophan-dependent or tryptophan independent pathways, the latter of which remains largely uncharacterized. Currently Arabidopsis genes encoding key enzymes have been identified in four routes downstream of tryptophan, the indole-3-acetaldoxime (IAOx) pathway, the tryptamine (TAM) pathway, the indole-3-pyruvic acid (IPA) pathway, and the indole-3-acetamide (IAM) pathway (Suppl. Table S1). Of these, four members of the *YUCCA* (*YUC*) gene family, which encode flavin monooxygenases in the TAM pathway, have been shown to be embryo expressed (Cheng et al., 2007a). *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA1*) and *TRYPTOPHAN AMINOTRANSFERASE RELATED* (*TAR*) 1 and 2, encode rate limiting enzymes that function in the IPA branch. The specific expression of *TAA1* in the future apical and root meristems during embryogenesis, clearly infers a role for local auxin biosynthesis in specification or maintenance of these cells (Stepanova et al., 2008). Indeed, *taa1 tar1 tar2* triple mutants display embryo defects in both apical and basal regions. Aberrant embryos also result from combining *yuc1 yuc4 yuc10* and *yuc11* mutations, thus the TAM and IPA pathways appear to have non-redundant roles in embryogenesis (Cheng et al., 2007a).

In general, auxin conjugates are thought to be inactive storage forms of auxin (reviewed by Ludwig-Müller, 2011). Several gene families encode enzymes which function in the synthesis or hydrolysis of auxin conjugates (reviewed by Woodward and Bartel, 2005). Enzymes that conjugate IAA to amino acids include those encoded by the *GH3* family. As expected, overexpression or disruption of *GH3* members leads to phenotypes consistent with decreased free auxin levels and hypersensitivity to auxin respectively (Staswick et al., 2005). Conversely,

a family of amidohydrolases with differing specificity, reside in the ER lumen and release IAA from IAA conjugates (reviewed by Woodward and Bartel, 2005). Finally, selected auxin conjugates are probably also intermediates in IAA degradation.

Polar auxin transport is the most well elucidated auxin homeostasis regulator (reviewed by Grunewald and Friml, 2010). Not only their specific expression, but also the dynamic, polar subcellular localization of auxin transporters is crucial for their function in imparting direction to auxin fluxes and the generation of auxin maxima and minima. Polar auxin efflux is mainly mediated by the PIN proteins, of which four are dynamically expressed during embryogenesis (Friml et al., 2003). Functional redundancy exists between the four PINs and higher order mutants display embryo defects (Friml et al., 2003). An elegant control mechanism of the apical-basal targeting of PINs is mediated by antagonistically acting AGC kinases and PP2A phosphatase, which influence the phosphorylation state and thereby membrane localization of the PINs (Benjamins et al., 2001; Michniewicz et al., 2007; Dhonukshe et al., 2010). AGC kinases are also expressed during embryogenesis and are essential for the formation of cotyledons (Cheng et al., 2008). *PP2A* is expressed throughout the embryo from 8-cell stage onwards and loss of function analysis revealed apical and basal aberrations from this stage onwards, similar to embryos with defects in auxin transport (Michniewicz et al., 2007). Recently, a family of NPH3-like genes, the *NPYs* (*NAKED PINS IN YUC MUTANTS*) which were previously shown to be important for auxin mediated organ formation, have also been characterized as regulators of PIN membrane localization and polarity (Cheng et al., 2008; Furutani et al., 2011). Finally, auxin cellular influxes are mediated by AUXIN RESISTANT 1 (AUX1) and three homologues LAX1, LAX2 and LAX3 (LIKE AUXIN RESISTANT) which have recently been shown to be redundantly required for correct patterning of the quiescent centre and columella (Ugartechea-Chirino et al., 2010).

Feedback from auxin signaling to auxin homeostasis is a well-established phenomenon. *GH3* family genes acting as auxin conjugators are induced by auxin, presumably to recover homeostasis following an auxin maxima-induced response. Similarly, it has long been known that transcription of the majority of Aux/IAA repressors is highly auxin inducible, enabling the reestablishment of transcriptional repression following an auxin response. Moreover, auxin also upregulates the expression of auxin transporters in both the *PIN* and *LAX* families (Vieten et al., 2005; Swarup et al., 2008). Thus, already at the level of transcription, there appears to be multiple feedbacks from auxin signaling to auxin homeostasis, providing a mechanism to self-regulate auxin response. Nevertheless, although these self-regulatory feedbacks have been observed, it is unclear to what extent these occur within the same context, and whether these functionally converge, or rather represent independent regulatory events. Importantly, auxin-regulation of auxin homeostatic components has mostly been observed upon auxin treatment. Such conditions of excessive auxin activity

may not necessarily represent physiologically relevant regulation.

In this chapter we investigate feedback as represented by misregulation of genes involved in auxin homeostasis and signaling resulting from the targeted inhibition of auxin response in the suspensor. The early embryo is an excellent model system in which to investigate the regulation of auxin homeostasis. Importantly cell types in the early embryo are auxin-responsive and expression of genes encoding both auxin biosynthesis and auxin transport components has already been documented. Moreover, the early embryo represents a simple tissue with few cell types and the transcriptional datasets generated from early embryos will presumably not be as convoluted as those generated from more complex tissues. Furthermore, the nature of the dataset generated here (inhibition of auxin response) allows probing the extent of auxin regulation of gene transcription under physiological conditions. We show that many auxin homeostatic genes are misregulated upon ARF inhibition and document expression patterns of selected genes during embryogenesis, including many members of the Aux/IAA family. In addition to *IAA30*, the suspensor expression of which is lost in proliferating suspensor cells (Chapter 3), an auxin biosynthesis gene is ectopically expressed in the ARF13-iaa10 background. Finally, loss of function and misexpression approaches are taken to investigate the role of auxin homeostasis and signaling components in conferring the suspensor proliferation phenotype that occurs upon the inhibition of auxin response. Our results show a striking degree of convergence of transcriptional responses involving members of 15 gene families that collectively re-establish auxin activity upon auxin response inhibition.

RESULTS

Identification of genes involved in auxin homeostasis that are misregulated in the M0171-bdl dataset

A comprehensive list of genes involved in auxin homeostasis and core signaling was generated, including genes annotated to GO terms related to auxin as well as those identified through an extensive literature search (Suppl. Table S1). Of these 158 genes, 13 were found to be upregulated and 26 were downregulated (cut off 1.5 fold misregulated and False Discovery Rate (FDR) <5.5%) upon suspensor-specific auxin response inhibition in the M0171-bdl microarray based experiment detailed in Chapter 4 (Table 1).

Interestingly, none of the upregulated auxin homeostasis and response genes were annotated as auxin-responsive in the Arabidopsis Hormone Database 2.0 (AHD2.0). As the main criteria for auxin-regulation in AHD2.0 is response to exogenously added auxin, this difference may reflect the regulation of these genes by physiological auxin concentrations. Conversely, 13 of the 26 downregulated genes were present in the auxin-responsive AHD2.0 dataset,

which is a clear enrichment as in total 36 of the 158 genes in the list are annotated as auxin-responsive in the AHD2.0. Regardless, the convergent misregulation of 39 auxin homeostasis and signaling genes upon inhibition of auxin response in the suspensor clearly points towards the existence of an intricate feedback network between auxin response and homeostasis during Arabidopsis embryogenesis.

Table 1. Auxin homeostasis and core signaling genes misregulated in the M0171-bdl datasets

AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. FC = fold change in M0171-bdl dataset. AHD2.0 auxin = auxin responsiveness in AHD2.0 database.

AGI	other name(s)	Function	FC	AHD2.0 auxin
AT4G32540	YUC1	Auxin biosynthesis enzyme	5.9 up	-
AT5G11320	YUC4	Auxin biosynthesis enzyme	7.9 up	-
AT1G48910	YUC10	Auxin biosynthesis enzyme	3.5 up	-
AT1G70560	TAA1	Auxin biosynthesis enzyme	2.9 up	-
AT3G44300	NIT2	Auxin biosynthesis enzyme	2.5 up	-
AT5G66350	SHI	Regulation of auxin biosynthesis	3.9 down	-
AT3G51060	STY1	Regulation of auxin biosynthesis	5.4 down	-
AT5G12330	LRP1	Regulation of auxin biosynthesis	4.1 down	+
AT2G18120	SRS4	Regulation of auxin biosynthesis	1.9 down	-
AT2G23170	GH3.3	Auxin conjugation	2.0 down	+
AT1G59500	GH3.4	Auxin conjugation	1.7 down	+
AT4G27260	GH3.5/WES1	Auxin conjugation	2.3 down	+
AT5G54140	ILL3	Auxin deconjugation	2.3 up	-
AT4G16690	AtMES16	Auxin deconjugation	5.7 up	-
AT1G73590	PIN1	Polar auxin efflux carrier	5.1 down	+
AT2G01420	PIN4	Polar auxin efflux carrier	15.5 down	+
AT1G23080	PIN7	Polar auxin efflux carrier	1.9 down	+
AT3G14370	WAG2	Regulation of PIN polarity	3.1 down	-
AT4G31820	NPY1/MAB4	Regulation of PIN polarity and internaliation	5.5 down	-
AT2G23050	NPY4/MEL4	Regulation of PIN polarity	3.1 down	-
AT1G17140	ICR1	Regulation of PIN polarity	3.1 down	
AT3G27580	D6PKL3	Regulation of polar auxin transport	2.0 down	+
AT5G01240	LAX1	Auxin influx carrier	4.9 down	-
AT2G21050	LAX2	Auxin influx carrier	8.3 down	+
AT4G24390	AFB4	Auxin receptor	2.1 down	-
AT5G49980	AFB5	Auxin receptor	1.7 down	-
AT2G33860	ARF3	Auxin Response Factor	1.6 down	-
AT1G19850	ARF5	Auxin Response Factor	1.8 down	-
AT2G28350	ARF10	Auxin Response Factor	1.6 down	+
AT1G34170	ARF13	Auxin Response Factor	1.9 up	-
AT1G35520	ARF15	Auxin Response Factor	1.8 up	-
AT1G35240	ARF20	Auxin Response Factor	1.8 up	-
AT1G34410	ARF21	Auxin Response Factor	1.8 up	-
AT1G04550	IAA12/BDL	Aux/IAA transcriptional regulator	10.6 up	-
AT1G04250	IAA17/AXR3	Aux/IAA transcriptional regulator	3.2 up	-
AT3G15540	IAA19/MSG2	Aux/IAA transcriptional regulator	8.1 down	+
AT2G46990	IAA20	Aux/IAA transcriptional regulator	1.7 down	-
AT3G16500	IAA26/PAP1	Aux/IAA transcriptional regulator	4.8 down	+
AT3G62100	IAA30	Aux/IAA transcriptional regulator	3.5 down	+

Expression patterns of selected auxin homeostasis genes in wild-type embryos

Of the 39 misregulated auxin homeostasis genes, nine were selected for embryo expression analysis in Chapter 4. The expression of YUC1/4/10, PIN1/4, IAA30 and AFB4 are discussed in Chapters 3 and 4. The expression patterns of the remaining two genes, *NPY1* and *NPY4* are shown in Fig. 1. In addition, transcriptional reporter lines were made and analyzed for another *NPY* family member, *NPY2*, which is not misregulated in the M0171-bdl datasets (Fig. 1).

NPY1 and *NPY4* are expressed in both apical and basal cell derivatives and therefore are unlikely to directly regulate suspensor cell identity. *NPY1* is initially expressed at globular stage, in the basal protodermal cells as well as the basal cell following hypophysis division, later expanding into the ground tissue of the basal tier of the embryo (Fig.1). *NPY4* is expressed in a somewhat opposite manner, in the inner (prevasculature) cells of the basal tier of the embryo and in the apical cell following hypophysis division (Fig.1). *NPY2* is specifically expressed in the suspensor and later becomes limited to the apical cells following hypophysis division, i.e. the quiescent centre (Fig.1). Thus *NPY1* and *NPY2* expression overlaps in the quiescent centre. Importantly, the expression domains perfectly match the protein localization patterns that were recently reported (Furutani et al., 2011), further validating the transcriptional fusion approach.

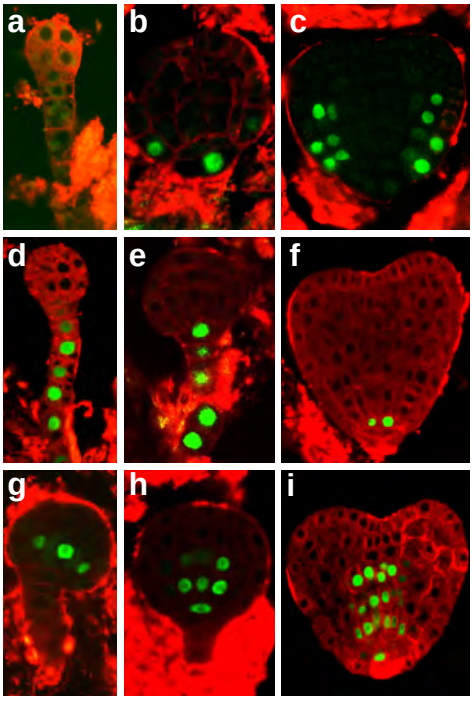


Figure 1. Expression of *NPY* genes during early embryogenesis.
Transcriptional fusions of 2.0 Kb promoters of *NPY1* (a-c), *NPY2* (d-f) and *NPY4* (g-i) to nuclear-localised triple GFP (green signal). Red signal from membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

The expression of *PIN1* and *PIN4* was analyzed and discussed in Chapter 4. The analysis of *PIN* gene expression was extended to *PIN7*, which was 1.9 fold downregulated in the M0171-bdl dataset and therefore just missed the 2.0 cutoff criteria set for the selection of genes for expression analysis in Chapter 4. PIN7 protein has been previously localized to the basal cell following zygote division and subsequently is specifically expressed in suspensor cells and in the hypophysis when specified (Friml et al., 2003). Analysis of the transcriptional fusion lines generated in this study also found *PIN7* expression in suspensor cells and in the hypophysis and its derivatives. Interestingly, at later stages, *PIN7* becomes expressed in the prevasculature, which to our knowledge has not yet been reported (Fig. 2).

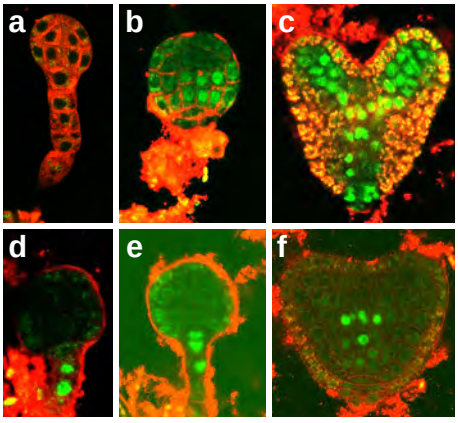


Figure 2. Expression of *AFB5* and *PIN7* during early embryogenesis.
Transcriptional fusions of 2.0 Kb promoters of *AFB5* (a-c) and *PIN7* (d-f) to nuclear-localised triple GFP (green signal). Red signal from membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

In Chapter 3 we show that the expression of *IAA30* is initially suspensor specific and lost in proliferating suspensor cells in the pARF13-iaa10 mutant background. However, subsequent investigation of an *IAA30* loss of function line did not uncover any aberrant embryo development (Chapter 4). One possible reason for this is functional redundancy. Indeed IAA30 has a homolog, IAA20, which shares 83% similarity at the amino acid level, making these Aux/IAAs the most closely related pair of all 29 in the Aux/IAA family. Like IAA30, IAA20 also lacks the domain required for auxin mediated degradation. *IAA20* is 1.7 fold downregulated in the M0171-bdl dataset. Transcriptional reporter lines were made for *IAA20* and expression was detected in the hypophysis derivatives and later in the prevasculature of the embryo (Fig. 3). Thus the expression of *IAA20* and *IAA30* overlap in the hypophysis derivatives and prevasculature.

Finally, the auxin receptor *AFB4* was found to be expressed in the hypophysis, hypophysis derivatives and later in the prevasculature (Chapter 4) and also has a close homolog, *AFB5*, which like *IAA20* is 1.7 fold downregulated in M0171-bdl dataset. AFB4 and AFB5 share 78% identity at amino acid level. Transcriptional reporter lines revealed *AFB5* expression in the prevasculature and hypophysis derivatives, similar to *AFB4* transcriptional fusions (Fig. 2).

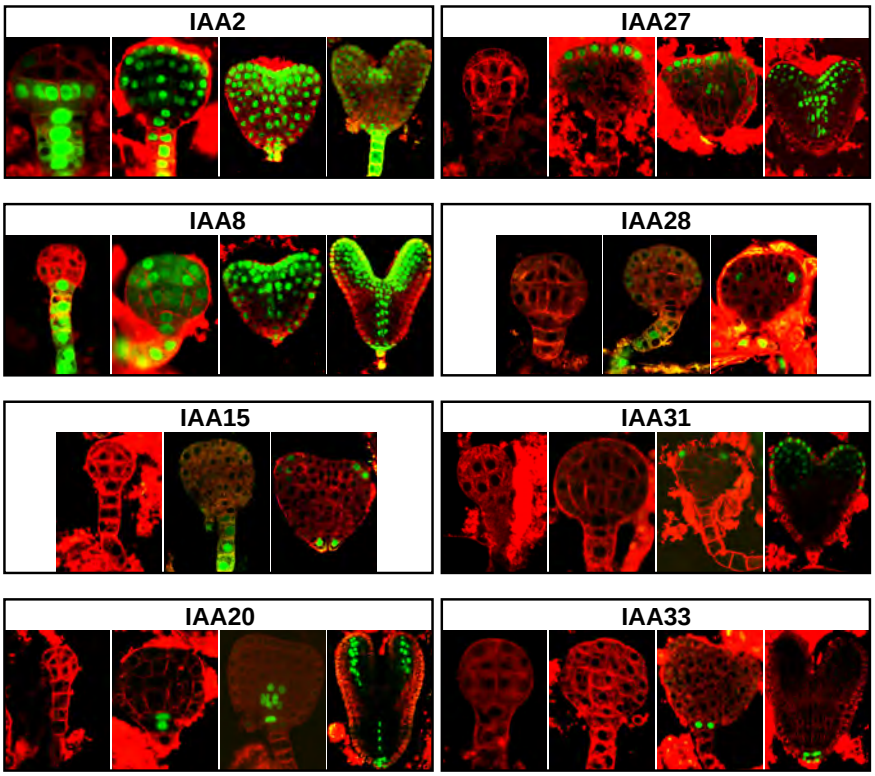


Figure 3. *Aux/IAA* expression patterns during Arabidopsis embryogenesis.
Transcriptional fusions of 2.0 Kb IAA promoters to nuclear-localised triple GFP as detailed in the text and reported with green signal. Red signal from membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

The specific expression patterns of *IAA20* and *IAA30*, as well as previously published embryonic expression patterns of other Aux/IAAs prompted us to initiate the systematic expression analysis of all remaining Aux/IAAs during Arabidopsis embryogenesis. In total transcriptional reporters for 8 Aux/IAAs were analyzed in this chapter (Fig. 3). Together with data in other chapters and previously published expression analysis, the embryonic expression patterns of 18 of the 29 Aux/IAAs are now known (Table 2). Interestingly, all but two Aux/IAAs (*IAA3* and *IAA17*) were expressed during early embryogenesis and to date 11 Aux/IAAs are expressed in the descendants of the basal cell lineage (i.e. suspensor and/or hypophysis and/or hypophysis derivatives). *IAA33* has the most limited pattern of expression during embryogenesis, it only becomes expressed in the future columella, i.e. once the basal cell arising from the hypophysis division has itself divided (Fig. 3). Aux/IAA expression patterns during embryogenesis will guide the selection of novel mutant combinations and when complete serve as a basis for testing functionally relevant interactions with their targets, the ARFs.

In summary, guided by the misregulation in the M0171>>bdl dataset, we have analyzed expression patterns of 13 genes encoding components of auxin homeostasis in wild-type embryos. Interestingly, although patterns diverge, there appears to be a prevalence of genes that are expressed in the prevasculature, suspensor and hypophysis.

Auxin biosynthesis in proliferating suspensor cells

For those genes whose expression is lost or downregulated in M0171>>bdl embryos, the expression can only be affected within the normal expression domain. As shown in Chapter 3, *IAA30* represents such an example where expression is lost from a part of the expression domain. For genes whose expression is upregulated in M0171>>bdl embryos however, it is unpredictable whether this represents enhanced or ectopic expression. To determine if upregulation can reflect ectopic expression, we investigated the expression pattern of auxin biosynthesis enzyme *YUC1* in the pARF13-iaa10 mutant background. *YUC1* is 5.9 fold upregulated in the microarray dataset. Strikingly, in pARF13-iaa10 embryos *YUC1* becomes expressed in proliferating suspensor cells (Fig. 4). Interestingly, *YUC1* expression is detected in all suspensor cells as early as the first aberrant cell divisions (Fig. 4c). This suggests that upregulation of *YUC1* occurs relatively early during the suspensor identity change, in some cases preceding aberrant cell divisions, the most obvious consequence of this transition.

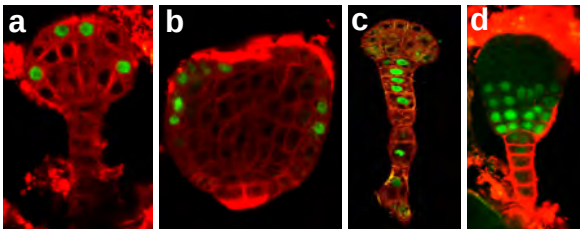


Figure 4. *YUC1* is misexpressed in the ARF13:iaa10 mutant background.
In phenotypic pARF13:iaa10 embryos (c,d), *YUC1* becomes expressed in suspensor cells as opposed to apical expression pattern seen in wild-type embryos (a,b). *YUC1* expression reported by GFP (green signal), FM4-64 stained membranes (red signal).

To further investigate the potential role of auxin biosynthesis in conferring the suspensor proliferation phenotype seen in M0171-bdl and pARF13-iaa10, *YUC1* and *YUC10* were misexpressed using the suspensor specific pARF13 promoter. As discussed in chapter 4, misexpression leading to suspensor proliferation phenotypes can manifest seedling phenotypes such as twinning, rootlessness and cotyledon number defects. However, a screen of all T1 seed following the transformation of five Arabidopsis plants per construct did not reveal seedling phenotypes. Subsequently, embryos from T2 ovules dissected from T1 plants

were also screened (>100 ovules per line), however suspensor proliferation (or other defects) were not found. In summary, although *YUC1* is misexpressed in suspensor cells in which auxin response has been inhibited, a causal relationship between *YUC1* expression and proliferation phenotype has not yet been established. Nonetheless, our data show that transcriptional rewiring of the auxin homeostasis network upon auxin response inhibition involves both gene inhibition (*IAA30*) and ectopic gene activation (*YUC1*).

Effect of loss of function mutants in genes involved in auxin response on suspensor development/embryogenesis

Previously, generation and analysis of higher order mutants within the *PIN*, *YUC* and *NPY* gene families have revealed important and redundant functions for these genes in embryogenesis and organogenesis (Friml et al., 2003; Cheng et al., 2007a; Cheng et al., 2007b; Cheng et al., 2008; Furutani et al., 2011). Furthermore, the loss of function *afb4-2* mutant was investigated and discussed in Chapter 4, highlighting the importance of correct auxin homeostasis and signaling in development. Some phenotypic similarities are observed between previously reported mutants in these families and the M0171>>bdl phenotypes. Nonetheless, the misregulation of only these genes can not account for the suspensor proliferation phenotypes.

To determine if other auxin homeostasis components contribute to the observed phenotypes, we investigated an insertion line available for IAA20 (iaa20-1, CSHL GT13391), in which the insertion point was annotated to the first exon and was therefore likely to be a true loss of function allele. Out of a segregating population, embryos derived from four different wild type plants and three plants homozygous for the insertion were screened. Striking phenotypes were seen in the *iaa20-1* line (Fig. 5) which resembled those seen in M0171>>bdl and pARF13-iaa10 mutant backgrounds. The abberant phenotype was seen at a very low frequency (5%, n=885), however was never observed in wild type embryos (n=289) collected from plants grown under identical conditions. As this low penetrance could be due to redundancy with the highly homologous and co-expressed *IAA30* gene, we attempted to generate an *iaa20 iaa30* double mutant. Unfortunately, difficulties in characterising the genetic background of *iaa20 iaa30* double mutants precluded analysis of any genetic interation between these Aux/IAAs to date.

In summary, loss of function mutations in several of the downregulated genes cause phenotypes that resemble M0171>>bdl embryos. Each of these is incompletely penetrant and none displays the dramatic suspensor proliferation phenotype. Therefore we conclude that this phenotype is likely the consequence of a global disruption of the auxin homeostatic network.

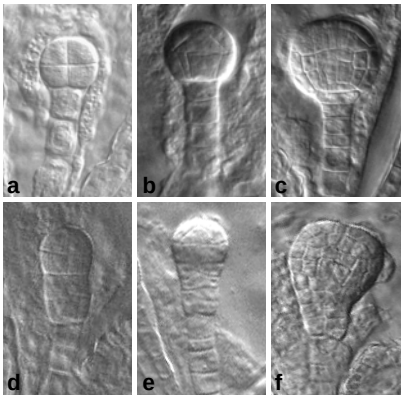


Figure 5. Embryo phenotype of the iaa20-1 mutant.
Extra cell divisions occur in the basal cell descendants, leading to suspensor cell proliferation in iaa20-1. Wild-type (a-c), iaa20-1 mutant. (d-f).

DISCUSSION

In this chapter we have investigated the observation made in Chapter 4 that upon inhibition of auxin response in the suspensor, many genes involved in the maintenance of auxin homeostasis are misregulated. A comprehensive analysis of all auxin homeostasis and signaling genes revealed that gene misregulation upon auxin response inhibition is found in all of the main facets that contribute to auxin homeostasis, namely biosynthesis, conjugation and transport.

Specifically, genes involved in three of the four Trp-dependent auxin biosynthesis pathways were upregulated in the M0171-bdl dataset, strongly inferring that inhibition of auxin response leads to an increase in auxin levels. Of these, YUC1 misexpression in proliferating suspensor cells was verified. Moreover, three genes that encode enzymes that conjugate auxin were downregulated and conversely two genes that encode auxin conjugate hydrolases were upregulated, the net effect of which would also presumably be an increase in free auxin levels. This correlation is further elaborated upon with the finding that all ten genes that are misregulated and associated with auxin transport are in fact downregulated.

An exception to the correlation between positive and negative misregulation and the effect on auxin levels are 4 genes belonging to the *SHORT INTERNODES* gene family that encode transcriptional regulators of auxin biosynthesis (Sohlberg et al., 2006). Their downregulation is difficult to reconcile with the upregulation of *YUC4*, which is a direct target of one of these genes, *STYLISH1* (Eklund et al., 2010). A possible explanation for this is that suspensor and apical embryo expression domains of YUC4 result from different mechanisms of transcriptional control.

The profound misregulation of multiple gene families involved in auxin homeostasis occurs in such a way that infers cells are responding to a

perceived depletion in auxin (the result of which would be a decrease in auxin response). Specifically genes that will increase the intracellular level of auxin are upregulated and genes that are involved in auxin transport are downregulated. This is graphically represented in the model presented in Fig. 6. Although feedback from auxin to the expression of genes controlling auxin homeostasis has long been appreciated, this is the first time such a convergent misregulation has been demonstrated and has been coupled to a biologically relevant output, i.e. that cells subsequently undergo a dramatic identity change. This is likely a reflection of the experimental setup, that is a temporally defined and cell specific response was elicited and samples for genomic analysis were collected from relatively simple tissue (the early embryo). The large degree of convergent homeostatic control that is revealed by these experiments also provides an explanation for why embryos are relatively inert to perturbations in biosynthesis and transport components. In part, genetic redundancy explains the absence of phenotypes in single or double mutant combinations. However, often the redundant genes are not co-expressed, as is the case for the *NPY*, *YUC* and *PIN* genes (Furutani et al., 2011; Cheng et al., 2007a; Friml et al., 2003). Based on our work, another plausible explanation for genetic robustness is the existence of an elaborate homeostatic gene network in which members of more than 10 gene families are regulated in such a way that reduced auxin activity efficiently triggers enhanced auxin levels, reduced auxin transport and increased auxin responsiveness.

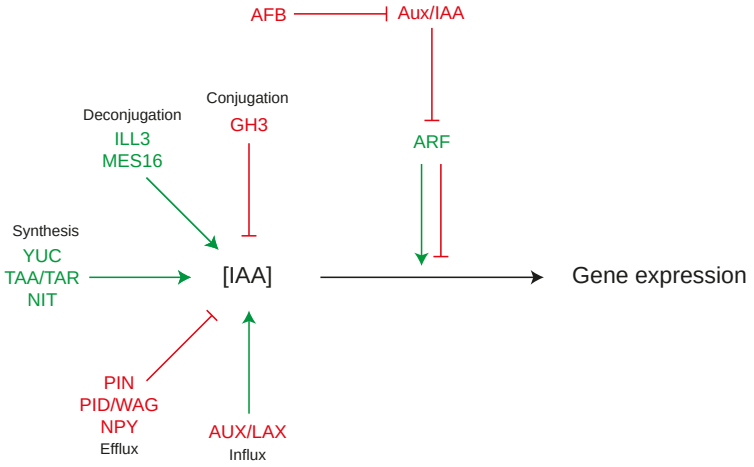


Figure 6. Overview of Convergent Regulation of Auxin Homeostasis.
Green arrows represent positive effect on IAA concentration or in auxin response and red bars represent negative effect on IAA concentration or in auxin response. Gene families in green were upregulated in the the M0171-bdl dataset and gene families coloured in red were downregulated. In most cases upregulated gene families have a positive effect on IAA concentration or in response and downregulated genes have a negative effect, suggesting convergent regulation functions to re-establish auxin homeostasis.

In general, the expression analysis of auxin homeostasis genes described in this thesis and previously published research reveals highly dynamic embryonic expression patterns, perhaps reflecting the dynamic auxin fluxes that occur during embryo development. The PIN and NPY transcriptional fusion lines revealed patterns that were in keeping with previously described protein localization patterns, validating our transcriptional fusion approach. Interestingly, feedback within the PIN family has been demonstrated. Not only does auxin positively control the expression of PINs in a tissue specific manner, but also negatively regulates the abundance of PIN proteins post-transcriptionally (Vieten et al., 2005). Additionally, ectopic expression of PINs occurs in pin mutant backgrounds, adding robustness to the auxin transport network (Vieten et al., 2005). Our microarray results also suggest that auxin response may regulate the expression of the NPY family of PIN polarity regulators, providing another opportunity of feedback, as has already been demonstrated for the AGC kinase PINOID and its homologs (Benjamins et al., 2001; Vieten et al., 2005).

The dynamic expression of *PIN4*, *PIN7* and others in both apical and basal lineages during embryogenesis makes it hard to interpret any potential changes in the expression pattern in the ARF13:iaa10 mutant background, and therefore this analysis was not performed. However, normally apical *YUC1* was misexpressed in the ARF13-iaa10 mutant background, inferring that our approach and misexpression data generated include actual autonomous gene expression changes that occur during suspensor identity transformation. Clearly non-autonomous changes in gene expression also occur, reflecting the continuity between suspensor and embryo, especially with regards to auxin. Interestingly, the non-autonomous changes (such as downregulation of PIN1) are also consistent with the correlation between expression and the positive effect on individual intracellular auxin levels (and thereby auxin response).

Previously published loss of function analysis (as discussed in the introduction) as well as loss of function phenotypes described for the multiple IPA auxin biosynthesis enzyme mutant (Chapter 3), *afb4-2* mutant (Chapter 4) and in *iaa20-1* (this chapter) demonstrate that perturbations in individual facets of auxin homeostasis are capable of effecting auxin-mediated patterning and suspensor cell identity. However, the lack of phenotype upon misexpression of *YUC1* in the suspensor suggests that an increase in auxin (biosynthesis) in the suspensor alone is not sufficient to induce suspensor cell identity changes. This is presumably because the embryo has a robust, highly redundant transport system to efficiently re-distribute any extra auxin (Weijers et al., 2005a). Further experiments, such as expression of ARF13:iaa10 in auxin biosynthesis mutants, could provide a causal link between auxin biosynthesis and the suspensor phenotypes that was not revealed by suspensor specific misexpression of *YUC1*.

Underlying the auxin responses and effect of inhibition thereof is a matrix of differentially expressed auxin response components. Not only ARFs

(as documented in Chapter 2), but also auxin receptors and Aux/IAAs are dynamically expressed during embryogenesis. Moreover, not all auxin receptors and Aux/IAAs are biochemically equivalent. Indeed dramatic examples of this are IAA20 and IAA30 which represent six of the 29 Arabidopsis Aux/IAAs which are labeled as "noncanonical" due to their lack of one or more conserved Aux/IAA domains. These noncanonical Aux/IAAs (nc-IAAs) also occur in other plant genomes (Jain et al. 2006; Wang et al., 2010) and are difficult to place within with the generic auxin response pathway. For example, IAA20 and IAA30 do not possess domain II and are therefore cannot be degraded in response to auxin via the conventional pathway. *IAA20* and *IAA30* expression is auxin-inducible (Sato and Yamamoto, 2008; Chapter 2) and IAA20 protein has been shown to be long-lived and its stability unresponsive to auxin (Dreher et al., 2006). These proteins do contain the domains required for ARF interaction and repression. Recently, a systematic yeast-2-hybrid approach has confirmed the ability of nc-Aux/IAAs to interact with ARFs (Vernoux et al., 2011). Depending somewhat on the degree of selectivity between nc-IAAs and ARFs, it is likely that if nc-IAAs are present in a cell, it would in effect be blind to auxin. One possible justification for the presence of nc-IAAs is that they represent an off switch (or dampener) to auxin response, i.e. following a cellular auxin response nc-IAAs expression is induced by auxin and sequester ARFs, even in cases where intracellular levels of auxin remain high (and canonical Aux/IAAs would be continuously degraded). The co-incidence of *IAA30* with auxin response maxima (as discussed in Chapter 3) corroborates this hypothesis. At least in the context of embryogenesis, nc-Aux/IAAs have very specific expression patterns. The low penetrant *iaa20* mutant phenotype occurs at the junction between embryo and suspensor and it will interesting to determine if contribution in either or both domains are needed to rescue the mutant phenotype.

In summary, a major consequence of the inhibition of auxin response in the suspensor is the convergent misregulation of many auxin homoeostasis components. The initial validation of microarray data demonstrating this finding comes from the analysis of loss of function mutants *afb4-2* (Chapter 4) and *iaa20-1* as well as the misexpression of *IAA30* (Chapter 2) and *YUC1* in the ARF13:iaa10 mutant background. Further work is needed to dissect the relative importance of additional individual components and auxin homoeostasis nodes in the suspensor to embryo transformation.

MATERIALS AND METHODS

Plant Materials, Growth and Selection

Arabidopsis thaliana (Arabidopsis) plants (Columbia-0 ecotype for all wild-type controls and transformations unless otherwise stated) were grown under standard conditions at 23°C in a 16-h-light/8-h-dark cycle. Selection for transgenes was performed on solid Murashige and Skoog medium supplemented with 25 mg/L kanamycin or 15 mg/L phosphinothricin where

appropriate. The iaa20-1 insertion line GT13391 was obtained from the Cold Spring Harbor Laboratory (http://genetrap.cshl.edu/) and genotyped using an insertion primer (TCCGTTCCGTTTTCGTTTTTTAC) and two flanking gene specific primers (CGTCACGATAACCTTTCATGC and CCTATGAAATAGTGATGGGCG). Representative lines carrying transcriptional reporters (~3 Kb upstream sequences fused to a double GFP) for IAAs 2, 8, 15, 27, 28 and 31 were obtained from Dr Martin Kieffer (University of Leeds). Transcriptional fusion lines for *YUC1* were generated in Chapter 4.

Generation of Transcriptional Fusion Constructs
A Ligation Independent Cloning strategy using the pGIIK-LIC-SV40-3GFP-NOSt generated in Chapter 4 was used to generate all transcriptional fusions (aside from the IAA reporters above). 2.0 Kb promoter fragments were amplified with the primers listed in Table 3, cloned and verified according to the methods detailed in Chapter 4.

Arabidopsis transformation
A simplified plant transformation procedure allowing moderate throughput, as detailed in Chapter 4 was used for all transformations.

Microscopy
Embryo preparation and imaging of expression as reported by n3GFP in embryos was conducted with a Zeiss LSM510 confocal laser scanning microscope as previously described (Schlereth et al., 2010). Embryo phenotypes were analyzed using chloral hydrate cleared preparations (clearing solution of chloral hydrate, water and glycerol, 8:3:1) on a Leica DMR microscope equipped with differential interference contrast (DIC) optics. In roots, gene expression or protein accumulation, as reported by n3XGFP, 2GFP or sYFP, was analyzed in homozygous T3 lines carrying a single T-DNA insert as determined by segregation of kanamycin or phosphinothricin resistance. Four- to five-day-old vertically grown seedlings were incubated in water containing 1 µM FM4-64 (Invitrogen) for 1 min and subsequently imaged on a Zeiss LSM510 confocal laser scanning microscope.

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SUPPLEMENTARY INFORMATION

Supplemental Table S1. Genes involved in auxin homeostasis and core signaling.
AGI = Arabidopsis Genome Initiative gene identification number. Other name(s) = abbreviated common names for genes. FC = fold change in M0171-bdl dataset. AHD2.0 auxin = auxin responsiveness in AHD2.0 database. References in main reference list.

AGI	Other name(s)	Function	FC	AHD2.0 auxin	References
AT4G32540	YUC1	Auxin biosynthesis enzyme	5.9 up	-	Zhao et al., 2001
AT4G13260	YUC2	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT1G04610	YUC3	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT5G11320	YUC4	Auxin biosynthesis enzyme	7.9 up	-	Zhao et al., 2001
AT5G43890	YUC5	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT5G25620	YUC6	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT2G33230	YUC7	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT4G28720	YUC8	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT1G04180	YUC9	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT1G48910	YUC10	Auxin biosynthesis enzyme	3.5 up	-	Zhao et al., 2001
AT1G21430	YUC11	Auxin biosynthesis enzyme	-	-	Zhao et al., 2001
AT1G70560	TAA1	Auxin biosynthesis enzyme	2.9 up	-	Stepanova et al., 2008; Tao et al., 2008
AT1G23320	TAR1	Auxin biosynthesis enzyme	-	-	Stepanova et al., 2008; Tao et al., 2008
AT4G24670	TAR2	Auxin biosynthesis enzyme	-	-	Stepanova et al., 2008; Tao et al., 2008
AT4G39950	CYP79B2	Auxin biosynthesis enzyme	-	+	Zhao et al., 2002
AT2G22330	CYP79B3	Auxin biosynthesis enzyme	-	+	Zhao et al., 2002
AT3G44310	NIT1	Auxin biosynthesis enzyme	-	-	Vorwerk et al., 2001
AT3G44300	NIT2	Auxin biosynthesis enzyme	2.5 up	-	Vorwerk et al., 2001
AT1G08980	AMI	Auxin biosynthesis enzyme	-	-	Pollmann et al., 2003
AT5G17690	LHP1/TFL2	Regulation of auxin biosynthesis	-	-	Rizzardi et al., 2011
AT5G66350	SHI	Regulation of auxin biosynthesis	3.9 down	-	Eklund et al., 2010
AT3G51060	STY1	Regulation of auxin biosynthesis	5.4 down	-	Eklund et al., 2010
AT4G36260	STY2	Regulation of auxin biosynthesis	-	-	Eklund et al., 2010
AT5G12330	LRP1	Regulation of auxin biosynthesis	4.1 down	+	Eklund et al., 2010
AT2G21400	SRS3	Regulation of auxin biosynthesis	-	-	Eklund et al., 2010
AT2G18120	SRS4	Regulation of auxin biosynthesis	1.9 down	-	Eklund et al., 2010
AT1G75520	SRS5	Regulation of auxin biosynthesis	-	-	Eklund et al., 2010
AT3G54430	SRS6	Regulation of auxin biosynthesis	-	-	Eklund et al., 2010
AT1G19790	SRS7	Regulation of auxin biosynthesis	-	-	Eklund et al., 2010
AT2G46870	NGA1	Regulation of auxin biosynthesis	-	-	Trigueros et al., 2009; Alvarez et al., 2009
AT3G61970	NGA2	Regulation of auxin biosynthesis	-	-	Trigueros et al., 2009; Alvarez et al., 2009
AT1G01030	NGA3	Regulation of auxin biosynthesis	-	-	Trigueros et al., 2009; Alvarez et al., 2009
AT4G01500	NGA4	Regulation of auxin biosynthesis	-	-	Trigueros et al., 2009; Alvarez et al., 2009
AT1G28300	LEC2	Regulation of auxin biosynthesis	-	-	Stone et al., 2008
AT4G27330	NZZ/SPL	Negative regulation of auxin biosynthesis	-	-	Li et al., 2008
AT4G37390	GH3.2	Auxin conjugation	-	+	Staswick et al. 2005
AT2G23170	GH3.3	Auxin conjugation	2.0 down	+	Staswick et al. 2005
AT1G59500	GH3.4	Auxin conjugation	1.7 down	+	Staswick et al. 2005
AT4G27260	GH3.5/WES1	Auxin conjugation	2.3 down	+	Staswick et al. 2005
AT5G54510	GH3.6/DFL1	Auxin conjugation	-	+	Staswick et al. 2005
AT1G28130	GH3.17	Auxin conjugation	-	-	Staswick et al. 2005
AT2G23260	UGT84B1	Auxin conjugation	-	-	Jackson et al., 2001
AT5G55250	IAMT1	Auxin conjugation	-	-	Qin et al., 2005
AT3G02875	ILR1	Auxin deconjugation	-	-	Bartel and Fink, 1995
AT5G56650	ILL1	Auxin deconjugation	-	-	LeClere et al., 2002
AT5G56660	ILL2	Auxin deconjugation	-	-	LeClere et al., 2002
AT5G54140	ILL3	Auxin deconjugation	2.3 up	-	LeClere et al., 2002
AT1G51780	ILL5	Auxin deconjugation	-	-	LeClere et al., 2002
AT1G44350	ILL6	Auxin deconjugation	-	-	LeClere et al., 2002
AT1G51760	IAR3	Auxin deconjugation	-	-	Davies et al., 1999
AT2G23620	AtMES1	Auxin deconjugation	-	-	Yang et al., 2008
AT2G23600	AtMES2	Auxin deconjugation	-	-	Yang et al., 2008
AT2G23610	AtMES3	Auxin deconjugation	-	-	Yang et al., 2008
AT2G23560	AtMES7	Auxin deconjugation	-	-	Yang et al., 2008
AT4G37150	AtMES9	Auxin deconjugation	-	-	Yang et al., 2008
AT4G16690	AtMES16	Auxin deconjugation	5.7 up	-	Yang et al., 2008
AT3G10870	AtMES17	Auxin deconjugation	-	+	Yang et al., 2008
AT5G58310	AtMES18	Auxin deconjugation	-	-	Yang et al., 2008
AT3G23140	URO	Regulation of auxin deconjugation	-	-	Sun et al., 2010
AT1G73590	PIN1	Polar auxin efflux carrier	5.1 down	+	Gälweiler et al., 1998
AT5G57090	PIN2	Polar auxin efflux carrier	-	-	Müller et al., 1998
AT1G70940	PIN3	Polar auxin efflux carrier	-	+	Friml et al., 2002a
AT2G01420	PIN4	Polar auxin efflux carrier	15.5 down	+	Friml et al., 2002b
AT5G16530	PIN5	intracellular auxin distribution	-	-	Mravec et al., 2009
AT1G77110	PIN6	intracellular auxin distribution	-	-	Mravec et al., 2009
AT1G23080	PIN7	Polar auxin efflux carrier	1.9 down	+	Friml et al., 2003
AT5G15100	PIN8	intracellular auxin distribution	-	-	Mravec et al., 2009
AT2G34650	PID	Regulation of PIN polarity	-	+	Friml et al., 2004
AT2G26700	PID2	Regulation of PIN polarity	-	-	Cheng et al., 2008
AT1G53700	WAG1	Regulation of PIN polarity	-	-	Cheng et al., 2008; Dhonukshe et al., 2010
AT3G14370	WAG2	Regulation of PIN polarity	3.1 down	-	Cheng et al., 2008; Dhonukshe et al., 2010
AT4G31820	NPY1/MAB4	Regulation of PIN polarity	5.5 down	-	Cheng et al., 2007; Furutani et al., 2011
AT2G14820	NPY2/MEL3	Regulation of PIN polarity	-	-	Furutani et al., 2011
AT5G67440	NPY3/MEL2	Regulation of PIN polarity	-	-	Furutani et al., 2011
AT2G23050	NPY4/MEL4	Regulation of PIN polarity	3.1 down	-	Furutani et al., 2011
AT4G37590	NPY5/MEL1	Regulation of PIN polarity	-	+	Furutani et al., 2011
AT1G69960	PP2A	Regulation of PIN polarity	-	-	Michniewicz et al., 2007
AT5G55910	D6PK	Regulation of PIN polarity	-	-	Zourelidou et al., 2009
AT4G26610	D6PKL1	Regulation of PIN polarity	-	-	Zourelidou et al., 2009

Supplemental Table S1. Continued

AGI	Other name(s)	Function	FC	AHD2.0 auxin	References
AT5G47750	D6PKL2	Regulation of PIN polarity	-	-	Zourelidou et al., 2009
AT3G27580	D6PKL3	Regulation of PIN polarity	2.0 down	+	Zourelidou et al., 2009
AT1G17140	ICR1	Regulation of PIN polarity	3.1 down	-	Hazak et al., 2010
AT2G38120	AUX1	Auxin influx carrier	-	-	Bennet et al., 1996
AT5G01240	LAX1	Auxin influx carrier	4.9 down	-	Parry et al., 2001
AT2G21050	LAX2	Auxin influx carrier	8.3 down	+	Parry et al., 2001
AT1G77690	LAX3	Auxin influx carrier	-	-	Swarup et al., 2008
AT2G36910	ABCB1	Nonpolar auxin efflux carrier	-	-	Noh et al., 2001
AT2G47000	ABCB4	Nonpolar auxin efflux carrier	-	-	Santelia et al., 2005
AT3G28860	ABCB19	Nonpolar auxin efflux carrier	-	-	Noh et al., 2001
AT1G59870	ABCG36	Auxin precursor efflux carrier	-	-	Strader and Bartel., 2009
AT3G53480	ABCG37	Auxin precursor efflux carrier	-	-	Ruzicka et al., 2010
AT2G31190	WXR/RUS2	Required for auxin polar transport	-	-	Ge et al., 2010
AT4G00220	JLO	Regulation of PIN expression	-	-	Borghi et al., 2007
AT3G62980	TIR1	Auxin receptor	-	-	Dharmasiri et al., 2005a; Kepinski and Leyser, 2005
AT4G03190	AFB1	Auxin receptor	-	-	Dharmasiri et al 2005b
AT3G26810	AFB2	Auxin receptor	-	-	Dharmasiri et al 2005b
AT1G12820	AFB3	Auxin receptor	-	-	Dharmasiri et al 2005b
AT4G24390	AFB4	Auxin receptor	2.1 down	-	Greenham et al., 2011
AT5G49980	AFB5	Auxin receptor	1.7 down	-	Greenham et al., 2011
AT1G15750	TPL	Aux/IAA interactor	-	-	Szemenyei et al., 2008
AT1G80490	TPR1	Aux/IAA interactor	-	-	Szemenyei et al., 2008
AT3G16830	TPR2	Aux/IAA interactor	-	-	Szemenyei et al., 2008
AT5G27030	TPR3	Aux/IAA interactor	-	-	Szemenyei et al., 2008
AT3G15880	TPR4	Aux/IAA interactor	-	-	Szemenyei et al., 2008
AT3G50060	MYB77	ARF interactor	-	-	Shin et al., 2007
AT1G59640	BPEp	ARF interactor	-	-	Varaud et al., 2011
AT1G59750	ARF1	Auxin Response Factor	-	-	Ulmasov et al., 1997; Guilfoyle and Hagen, 2007
AT5G62000	ARF2	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT2G33860	ARF3	Auxin Response Factor	1.6 down	-	Guilfoyle and Hagen, 2007 (references therin)
AT5G60450	ARF4	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G19850	ARF5	Auxin Response Factor	1.8 down	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G30330	ARF6	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT5G20730	ARF7	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT5G37020	ARF8	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT4G23980	ARF9	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT2G28350	ARF10	Auxin Response Factor	1.6 down	+	Guilfoyle and Hagen, 2007 (references therin)
AT2G46530	ARF11	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G34310	ARF12	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G34170	ARF13	Auxin Response Factor	1.9 up	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G35540	ARF14	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G35520	ARF15	Auxin Response Factor	1.8 up	-	Guilfoyle and Hagen, 2007 (references therin)
AT4G30080	ARF16	Auxin Response Factor	-	+	Guilfoyle and Hagen, 2007 (references therin)
AT1G77850	ARF17	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT3G61830	ARF18	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G19220	ARF19	Auxin Response Factor	-	+	Guilfoyle and Hagen, 2007 (references therin)
AT1G35240	ARF20	Auxin Response Factor	1.8 up	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G34410	ARF21	Auxin Response Factor	1.8 up	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G34390	ARF22	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT1G43950	ARF23	Auxin Response Factor	-	-	Guilfoyle and Hagen, 2007 (references therin)
AT4G14560	IAA1	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT3G23030	IAA2	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT1G04240	IAA3/SHY2	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT5G43700	IAA4	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT1G15580	IAA5	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT1G52830	IAA6/SHY1	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT3G23050	IAA7/AXR2	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT2G22670	IAA8	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT5G65670	IAA9	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT1G04100	IAA10	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT4G28640	IAA11	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT1G04550	IAA12/BDL	Aux/IAA transcriptional regulator	10.6 up	-	Liscum and Reed, 2002 (references therein)
AT2G33310	IAA13	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT4G14550	IAA14/SLR	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT1G80390	IAA15	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT3G04730	IAA16	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT1G04250	IAA17/AXR3	Aux/IAA transcriptional regulator	3.2 up	-	Liscum and Reed, 2002 (references therein)
AT1G51950	IAA18	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT3G15540	IAA19/MSG2	Aux/IAA transcriptional regulator	8.1 down	+	Liscum and Reed, 2002 (references therein)
AT2G46990	IAA20	Aux/IAA transcriptional regulator	1.7 down	-	Liscum and Reed, 2002 (references therein)
AT3G16500	IAA26/PAP1	Aux/IAA transcriptional regulator	4.8 down	+	Liscum and Reed, 2002 (references therein)
AT4G29080	IAA27/PAP2	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT5G25890	IAA28	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT4G32280	IAA29	Aux/IAA transcriptional regulator	-	+	Liscum and Reed, 2002 (references therein)
AT3G62100	IAA30	Aux/IAA transcriptional regulator	3.5 down	+	Liscum and Reed, 2002 (references therein)
AT3G17600	IAA31	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT2G01200	IAA32	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT5G57420	IAA33	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)
AT1G15050	IAA34	Aux/IAA transcriptional regulator	-	-	Liscum and Reed, 2002 (references therein)

Chapter

Expression of bHLH genes in the Arabidopsis embryo and regulation during suspensor to embryo transformation

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ABSTRACT

Arabidopsis embryogenesis is the developmental process that begins with the division of the zygote and ends when the fundamental organs for subsequent seedling growth are established. In the embryo successive rounds of cell division generate apical –basal and radial polarity and specify the cell types required for the basic body plan. Interestingly, the very first division distinguishes two very different cell lineages, namely the embryo and the extra-embryonic suspensor. Little is known about the specification of these initial cells, however recently we have shown that auxin response plays a role in the maintenance of the suspensor cell lineage. Surprisingly, the cell autonomous mechanism through which auxin functions includes active repression of embryonic identity. Previously, we generated a unique microarray dataset of genes that are misregulated upon specific inhibition of auxin response in the extra-embryonic cell lineage. Within this dataset we found a significant overrepresentation of transcription factors, which are key regulators of changes in cell identity. In this chapter we focus on one family of auxin responsive transcription factors, the bHLH superfamily. We expand our expression analysis to include all bHLH superfamily members misregulated in the dataset as well as several closely related members. We document several specific expression patterns, consistent with a role for bHLHs as embryo/suspensor cell identity determinants. Furthermore we show that the lineage-specific expression of two bHLHs is changed when auxin response is inhibited in the suspensor.

INTRODUCTION

The mechanisms by which plant cells acquire and maintain distinct identities is an intriguing area of plant research relevant not only to embryonic development but also post embryonically, where specification events and switches in cell fates occur during organogenesis and major phase transitions (Van Lijsebettens and Van Montagu, 2005). Molecular-genetic approaches have revealed the importance of transcription factors in cell identity transitions and the importance of hormones, including auxin, in cell-cell signaling essential for conferring positional information. Indeed, the most well elucidated homeotic factors are transcription factors which are increasingly being placed into complexes and networks (as reviewed by Kaufmann et al., 2010). At the top of a network are so called "master regulators", transcription factors which coordinate changes in the expression of many genes to initiate a new developmental program (and antagonistically suppress earlier ones). These regulators are perhaps most easily defined as such when their ectopic expression is necessary and sufficient to cause developmental reprogramming/cell identity change, as is the case for somatic embryo inducer LEC1 (discussed in Chapter 4).

Interestingly, in Chapter 2 we show that each morphologically distinct cell type in the early Arabidopsis embryo expresses a unique subset of *AUXIN RESPONSE*

FACTORS (*ARFs*), however it remains to be determined to which extent ARF sets instruct the specification of a cell type, or result from it. Nonetheless, in Chapter 3 we identified auxin as the first autonomous regulator of suspensor cell identity maintenance. The suspensor, derived from the basal cell following asymmetric division of the zygote, is initially extra-embryonic in identity, in sharp contrast to the early embryonic cells which develop from the apical daughter of the zygote. Specific inhibition of auxin response in the suspensor induces a cell identity change and suspensor cells acquire an identity that strongly resembles that of embryonic cells (Chapter 3).

In Chapter 4 we identified members of auxin responsive transcription factor families that are misregulated upon targeted inhibition of auxin response in the suspensor. Subsequent analysis of the embryonic expression patterns of 35 selected transcriptional regulators identified 19 which are specifically expressed in apical or basal cell lineages during early embryo development. Amongst these were seven basic Helix-Loop-Helix (bHLH) superfamily proteins.

bHLH proteins are defined by an N-terminal domain rich in basic amino acids, followed by a Helix-Loop-Helix (HLH) region containing two amphipathic α-helices linked by a variable loop region (as reviewed by Carretero-Paulet et al., 2010). Most bHLHs function as transcription factors, the basic region and HLH regions functioning in DNA binding and protein-protein interactions respectively. Protein interaction via the HLH most often results in the formation of homo- or heterodimers with other bHLH proteins, and this appears to be important for DNA recognition and contributes to DNA binding specificity. bHLH transcription factors are widely found in eukaryotic genomes and in the Arabidopsis genome 167 bHLHs, including some recently annotated atypical bHLHs, have been identified to date (Carretero-Paulet et al., 2010). Atypical bHLHs are in general non DNA binding and dimerization to these proteins inhibits DNA binding activity (Massari and Murre, 2000).

In Arabidopsis, all but 3 orphans are classified into 28 subfamilies based on the bHLH domains, and additional conserved motifs outside of these domains strengthen this classification. These motifs may mediate the interactions to the increasing multitude of bHLH interactors identified recently, which now include WD40, MYB, AP2/ERF-type, BES1 and JAZ proteins (reviewed by Balkunde et al., 2010; Chandler et al., 2009; Yin et al., 2005; Qi et al., 2011). Intriguingly, a bHLH named BIGPETALp (bHLH031) has recently been shown to interact with ARF8, and together regulate petal growth (Varaud et al., 2011). The interaction occurs through a motif unique to this bHLH, thus it is unclear whether other bHLHs will also be characterized as ARF interactors.

The ever increasing functional characterization of Arabidopsis bHLHs has revealed roles for these transcription factors in many biological processes, including hormone signaling and cell identity specification during development.

The stomata development and patterning pathway is a well elucidated system for studying cell fate determination. Three closely related bHLHs, SPEECHLESS, MUTE and FAMA each have distinct roles in key successive transitional states of the stomatal lineage (reviewed by Pillitteri and Torii, 2007). These three bHLHs are thought to function through dimerization with two other bHLHs (SCREAM and SCREAM2) which themselves redundantly initiate stomatal development (Kanaoka et al., 2008). Auxin signaling has been linked to bHLH transcription factors in several different ways. The auxin inducible bHLH054 has been characterized as ROOT HAIR DEFECTIVE 6-LIKE 4 and its function is required for auxin stimulated root hair growth (Yi et al., 2010). bHLHs also play a role in the specification of the female reproductive tract. Expression and loss of function analysis has implicated three HECATE (HEC1, HEC2, HEC3) and the SPATULA bHLHs in this process (Gremski et al., 2007; Heisler et al., 2001). The HEC proteins can heterodimerize with SPT and their ectopic overexpression phenotype of pin-shaped inflorescences implicates auxin in female tissue patterning (Gremski et al., 2007). Recently, bHLHs HALF-FILLED (HAF) and closely related BRASSINOSTERIOD ENHANCED EXPRESSION1 (BEE1) and BEE3 have also been implicated in reproductive tract development (Crawford and Yanofsky 2011). They are expressed in overlapping patterns within the reproductive tract but each have a slightly different expression pattern suggesting subtle differences in function. *HAF* expression has been tentatively placed downstream of the HEC genes and of auxin signaling through ARF6 and ARF8 (Crawford and Yanofsky, 2011). Post fertilization, *INDEHISCENT* (*IND/bHLH040*) is expressed in narrow stripes that will become the Arabidopsis fruit valve margins important for fruit opening. IND plays a role in specifying these cells through generating an auxin minimum by regulating auxin transport. Specifically, IND mediates changes in PIN polarity by regulating the expression of PIN polarity regulator kinases (Sorefan et al., 2009).

Several bHLHs have been implicated in Arabidopsis embryo development. BIM1 (BES interacting Myc-like protein 1) which interacts with BES1 (a transcription factor that executes transcriptional changes in response to brassinosteroids) was also shown to complex with embryonic patterning AP2/ERF-type transcription factors DORNROESCHEN (DRN) and DORNROESCHEN-LIKE (DRNL) and HD-ZIP transcription factor PHAVOLUTA (Chandler et al., 2009). *TARGET OF MONOPTEROS* (*TMO7*), characterized as a direct target of MP/ARF5, encodes a small, atypical bHLH (bHLH135) that was found to move from the pro-embryo into the uppermost suspensor cells (Schlereth et al., 2010). This movement was shown to be important for changing the identity of the uppermost suspensor cell to hypophysis, the precursor of the quiescent centre and distal root meristem cells (Schlereth et al., 2010).

As several previously characterized bHLHs have been shown to play a role in cell specification downstream of hormones including auxin, we decided to further explore this family of transcription factors. We expanded our transcriptional

expression analysis to include all bHLHs misregulated in the M0171-bdl datasets as well as additional bHLHs, closely related to those misregulated. Subsequently, loss of function and reporter approaches were adopted to further investigate those bHLHs expressed during embryo development. We show here that suspensor to embryo transformation is accompanied by changes in expression of several bHLH genes, suggesting their involvement in cell identity changes.

RESULTS

Expression of selected bHLH superfamily proteins

The M0171-bdl datasets generated in Chapter 4 were mined for all bHLH proteins, using the 167 genes currently annotated in the superfamily (Carretero-Paulet et al., 2010). Of these, 144 were represented on the microarray chip and of these, 18 were found to be misregulated (>1.5 fold misregulated and False Discovery Rate [FDR] <5.5%). The expression of the 14 bHLHs misregulated over 2-fold was already investigated in Chapter 4. Of these 14, three were exclusively expressed in apical cell derivatives and upregulated upon inhibition of auxin response, and therefore possible factors acting in the suspensor to embryo transformation pathway. Furthermore, two bHLHs were expressed in basal cell derivatives and downregulated upon inhibition of auxin response in the suspensor, and were therefore potential candidates involved in suspensor cell identity maintenance. The remaining nine did not satisfy these criteria. No expression was detected in transcriptional fusion lines for seven bHLHs. The final two bHLHs had expression patterns that precluded a cell-autonomous function in suspensor or embryo identity, but had interesting expression patterns nonetheless (discussed below).

Here we elaborate the analysis of bHLH gene expression in the Arabidopsis embryo. Despite their fundamental importance in cell fate decisions, surprisingly few bHLH genes have been studied during embryogenesis. The representation in the M0171>>bdl dataset suggests that bHLH expression patterns are correlated with (changes in) cell identities. We have therefore generated a further set of transcriptional reporters to determine if indeed these genes mark cell identities in the embryo, and to address if and how suspensor to embryo transformation changes these identities.

Two bHLHs belonging to subfamilies 15 and 25 respectively, *bHLH153* and *bHLH137*, were downregulated in the M0171-bdl dataset however were expressed in the embryo, not suspensor as expected (Fig. 1). Specifically, *bHLH153* is initially expressed in the inner cells of the embryo, however as these cells divide expression is limited to the future ground tissue. This is pattern is continued post-embryonically as endodermis specific expression in the root meristem (Fig. 1). *bHLH137* is expressed in the outer, protoderm cells of the basal tier of the embryo (Fig. 1).

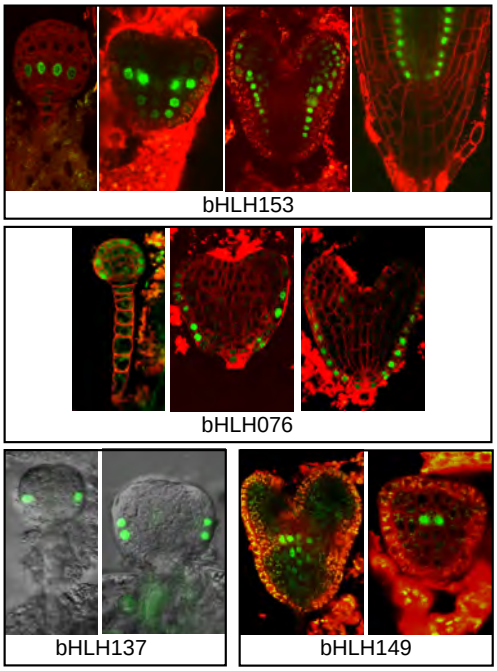

Figure 1. Embryo expression of selected bHLHs misregulated in the M0171-bdl dataset.
Expression as reported by transcriptional fusions of 2.0 Kb promoter of 9 genes to nuclear-localised triple GFP (green signal). Red signal reports membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

The bHLH subfamily 13 includes MP target *TMO5*, which functions in the embryonic root initiation pathway (Schlereth et al., 2010). Two other members in this subfamily (*TMO5-LIKE1* and *TMO5-LIKE3*) were downregulated in M0171-bdl dataset. However, neither genes have a documented embryonic expression pattern to date. Post-embryonically *TMO5* and *TMO5-LIKE1* expression does overlap in the root vasculature (Möller, De Rybel et al., unpublished).

In addition to the aforementioned subfamilies, the 18 bHLHs misregulated in the bHLH belonged to 8 other subfamilies (as annotated by Carretero-Paulet et al., 2010). Thus, in addition to the 4 genes misregulated between 1.5 and 2.0 fold, transcriptional fusion lines were made for eight additional selected members of these subfamilies (Table 1). Specifically these included members of subfamilies 16,19 and 25. An overview of all of the bHLHs investigated in the Chapter 4 and this chapter is given in Table 1.

Subfamily 16 consists of 6 bHLHs which at less than 100 amino acids each are the smallest bHLHs in the Arabidopsis genome. Perhaps owing to its small size, subfamily 16 member TMO7 moves from its site of transcription, as discussed in the introduction. Two members were upregulated in the M0171-bdl dataset, *bHLH136* and *bHLH164*. These genes are also known as *PACLOBUTRAZOL RESISTANCE1* and *PACLOBUTRAZOL RESISTANCE5* (*PRE1* and *PRE5)* respectively and have previously been implicated in developmental responses to gibberellin

Table 1. Overview of bHLH Expression.
AGI = Arabidopsis Genome Initiative gene identification number.
bHLH numbering and subfamily annotation according to Carretero-Paulet et al., 2010.
Other name(s) = abbreviated common names for genes also used in main text.
FC = fold change in M0171-bdl dataset, nc = no change
References in main reference list at end of chapter.

AGI	bHLH	bHLH subfamily	Other name(s)	FC	Expression during Embryogenesis	Reference
AT3G47640	bHLH047	4	PYE	1.8 down	No expression detected	This chapter
AT2G41240	bHLH100	12	-	2.0 down	Suspensor, later also basal tier protoderm	Chapter 4
AT1G68810	bHLH030	13	TMO5-LIKE1	2.7 down	Not analysed	Chapter 4
AT2G41130	bHLH106	13	TMO5-LIKE3	2.2 down	Not analysed	Chapter 4
AT5G08130	bHLH046	14	BIM1	1.6 up	No expression detected	This chapter
AT1G05710	bHLH153	15	-	2.1 down	Inner cells of embryo	Chapter 4
AT5G15160	bHLH134	16	TMO7-LIKE2/PRE2/BNQ2	nc	No expression detected	This chapter
AT1G74500	bHLH135	16	ATBS1/TMO7/PRE3	nc	Inner cells of embryo proper	Schlereth et al., 2010
AT5G39860	bHLH136	16	PRE1 BNQ1	5.2 up	No expression detected	Chapter 4
AT3G47710	bHLH161	16	PRE4	nc	No expression detected	This chapter
AT1G26945	bHLH163	16	KDR	nc	No expression detected	This chapter
AT3G28857	bHLH164	16	PRE5	2.4 up	No expression detected	Chapter 4
AT1G29950	bHLH144	17	-	3.4 down	No expression detected	Chapter 4
AT3G17100	bHLH147	19	AIF3	nc	No expression detected	This chapter
AT3G06590	bHLH148	19	AIF2	nc	No expression detected	This chapter
AT1G09250	bHLH149	19	AIF4	nc	Inner cells of embryo	This chapter
AT3G05800	bHLH150	19	AIF1	2.9 up	No expression detected	Chapter 4
AT2G42870	bHLH165	21	PAR1	3.6 down	Suspensor derivatives	Chapter 4
AT1G68920	bHLH049	25	-	2.5 up	Inner cells of embryo	Chapter 4
AT3G57800	bHLH060	25	-	2.4 up	Embryo, protoderm	Chapter 4
AT3G07340	bHLH062	25	-	1.6 up	No expression detected	This chapter
AT4G34530	bHLH063	25	CIB1	9.9 up	Embryo, apical and basal tier junction	Chapter 4
AT2G18300	bHLH064	25	-	2.5 down	No expression detected	Chapter 4
AT1G26260	bHLH076	25	CIB5	nc	Embryo, basal protoderm	This chapter
AT5G50915	bHLH137	25	-	5.0 down	Embryo, basal protoderm	Chapter 4
AT2G42280	bHLH130	27	-	1.5 down	No expression detected	This chapter

(Lee et al., 2006). However no embryo expression was detected in lines carrying transcriptional fusions of these bHLHs (data not shown). Additional reporters were made to complete the expression analysis of this subfamily however again, none of the *TMO7* homologs showed any expression during embryo development (data not shown) although expression was detected in the root meristem (Fig. 2). The upregulation of *PRE1* and *PRE5* in M0171>>bdl embryos therefore does not seem to reflect a cell identity change towards an embryonic cell type.

In a yeast two-hybrid screen, four closely related basic helix-loop-helix (bHLH) transcription factors that comprise subfamily 19 were shown to be able to interact with TMO7 and were named ATBS1 Interacting Factors (AIF1-4; Wang et al., 2009). *AIF1* was 2.9 fold upregulated in the M0171-bdl datasets. We analyzed the expression of the four AIF1-4 genes in the embryo and found that *AIF1*, but not any of its relatives was expressed in the embryo (Fig. 1). *bHLH148/AIF2*, *bHLH147/AIF3*, and *bHLH149/AIF4* are all expressed in the root cap and lower columella cells (Fig. 3), which are descendants of the suspensor, but no

expression in the embryo could be detected. Hence, *AIF1* represents another bHLH gene that is normally expressed in the pro-embryo and is upregulated upon auxin response inhibition in the suspensor.

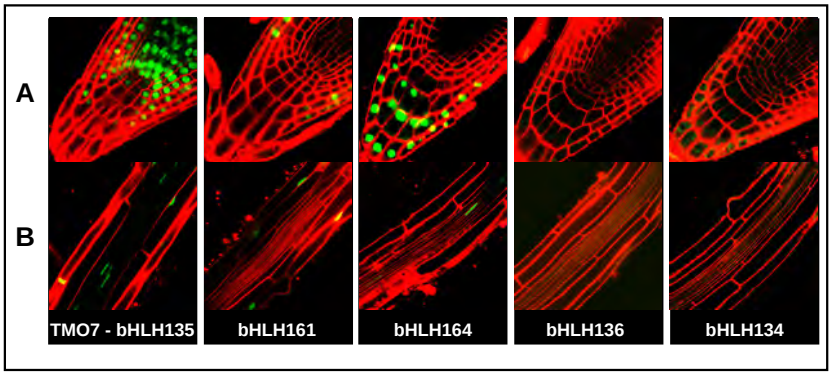

Figure 2. Root expression of selected subfamily 16 bHLHs.
Expression as reported by transcriptional fusions of 2.0 Kb promoters to nuclear localized triple GFP (green signal). Red signal reports membranes stained with FM4-64 dye. Root tip (A) and mature root (B). Expression patterns are detailed in the text.

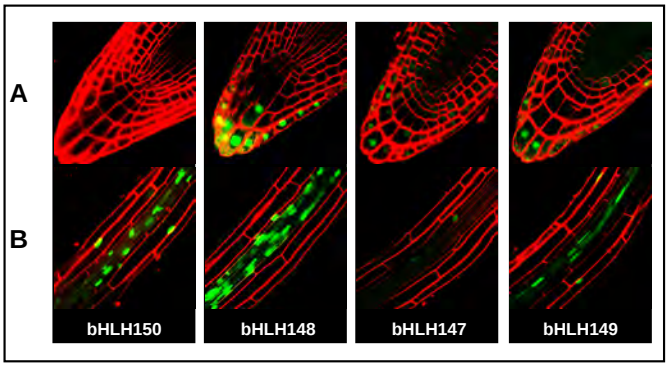

Figure 3. Root expression of selected subfamily 19 bHLHs.
Expression as reported by transcriptional fusions of 2.0 Kb promoters to nuclear localized triple GFP (green signal). Red signal reports membranes stained with FM4-64 dye. Root tip (A) and mature root (B). Expression patterns are detailed in the text.

In the M0171-bdl dataset six bHLHs belonging to subfamily 25 were misregulated. The expression pattern of five of these bHLHs was investigated in Chapter 4, and three upregulated members were found to be expressed in the embryo. In this chapter we generated transcriptional reporter lines for *bHLH062* which was 1.6 fold upregulated in the M0171-bdl dataset, however no expression was detected (data not shown). An additional reporter was made for subfamily

25 member *bHLH076* and expression was mainly detected in the protoderm, and thus has overlapping expression pattern with *bHLH137* (Fig. 1) and *bHLH060* as reported in Chapter 4.

Additionally, the expression of the remaining three bHLHs that were misregulated in the M0171-bdl dataset under the initial 2.0 cutoff for selection were analyzed, however no embryo expression was detected (data not shown). One of these, bHLH046, is also known as BIM1 and interacts with BES1 and plays a role in brassinosteriod-regulated gene expression (Yin et al., 2005). As mentioned in the introduction, BIM1 also interacts with DRN and DRNL and *in situ* hybridization detected expression in the embryo from early globular stage (Chandler et al., 2009). As the transcriptional lines generated do show expression in the root meristem (below), several other scenarios could explain this discrepancy, including that not all cis-regulatory elements required for *BIM1* embryo expression are contained with the 2.0 Kb upstream region used in the transcriptional fusion. Further work is required to address this difference. We subsequently checked the expression of all 4 bHLHs misregulated under 2.0 fold in the root meristem and found expression for three (Fig. 4). Both *bHLH046* and *bhLH130* were expressed in the apical meristem and excluded from the suspensor derived QC and columella. *bHLH062* was specifically expressed in the lateral root cap (Fig. 4). No expression was detected for *bHLH047* which has previously been characterized as *POPEYE* (*PYE*), a regulator of response to iron deficiency in roots and trace levels of pPYE:GFP expression have been reported in the root vasculature, columella root cap, and lateral root cap under iron sufficiency (Long et al., 2010).

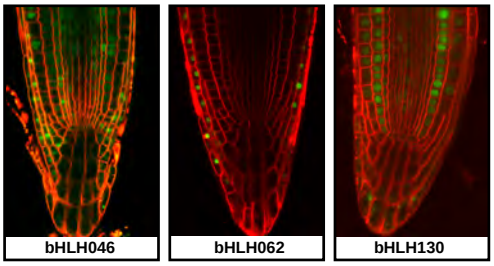

Figure 4. Root meristem expression of selected bHLHs misregulated in the M0171-bdl dataset.
Expression as reported by transcriptional fusions of 2.0Kb promoter to nuclear-localised triple GFP (green signal). Red signal reports membranes stained with FM4-64 dye. Expression patterns are detailed in the text.

Cell autonomy of bHLH transcription factors

While some bHLH proteins act cell-autonomously in the cells where the gene is transcribed (e.g. TMO5; Schlereth et al., 2010) others move to adjacent cells (e.g. TMO7, UPBEAT1; Schlereth et al., 2010; Tsukagoshi et al., 2010). To determine whether the bHLH genes identified here as regulated during suspensor to embryo transformation encode cell-autonomous or cell non-autonomous proteins, we generated translational fusions of several proteins to the sensitive sYFP2 protein. In both the embryo and root meristem, the pbHLH049:bHLH049-

sYFP2 protein localization domain was identical to the transcriptional fusion expression patterns (Fig. 5), demonstrating that this protein likely does not move. Furthermore, transcriptional and translational fusion lines were generated for bHLH134 (an example of subfamily 16) and bHLH150 (an example of subfamily 19). In both cases the analyzed protein localization domains fully overlapped with the promoter expression domains (Fig. 5). In conclusion, no evidence for protein movement was found for those bHLH genes that are misregulated in M0171>>bdl embryos. Rather, these proteins intrinsically mark the cells in which they are expressed, and could therefore be identity determinants.

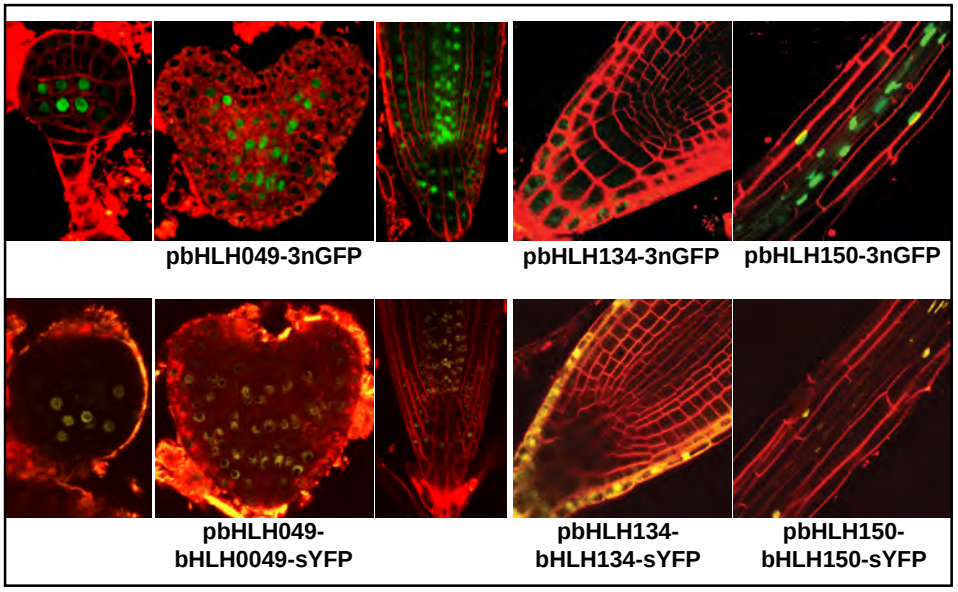

Figure 5. Comparison of expression patterns generated from transcriptional and translational fusions of selected bHLH proteins.
Signals from transcriptional and translational fusions (in green and yellow respectively), showing that the transcriptional and translation fusions domains of bHLH049, bHLH134 and bHLH150 are identical. Red signal reports membranes stained with FM4-64 dye.

Placing bHLHs into the context of suspensor/embryo identity

Being generally cell-autonomous proteins, whose expression is altered during suspensor to embryo transformation, these bHLH proteins are good candidates for being involved in the actual cell fate transformation. To determine whether expression patterns are consistently changed during this fate transformation, we analyzed expression of two genes in pARF13-iaa10 embryos. For this experiment we selected a bHLH gene (*bHLH100*) that is normally expressed in suspensor cells and downregulated in M0171>>bdl embryos, as well as one (*bHLH060*) that is

normally expressed in the pro-embryo and upregulated in M0171>>bdl embryos. As predicted by the microarray, both transcriptional fusions were misexpressed in this background. Ectopic *bHLH060* expression is detected in aberrantly dividing cells of the suspensor in ARF13:iaa10 mutant embryos (Fig. 6 a vs b,c). Conversely, suspensor expression of *bhLH100* is lost in proliferating suspensor cells in the ARF13:iaa10 mutant background (Fig. 6 d vs e,f). Therefore, even though these two genes represent only 2 examples, it appears that suspensor to embryo transformation is accompanied by loss and gain of 2 lineage-specific bHLH transcription factors.

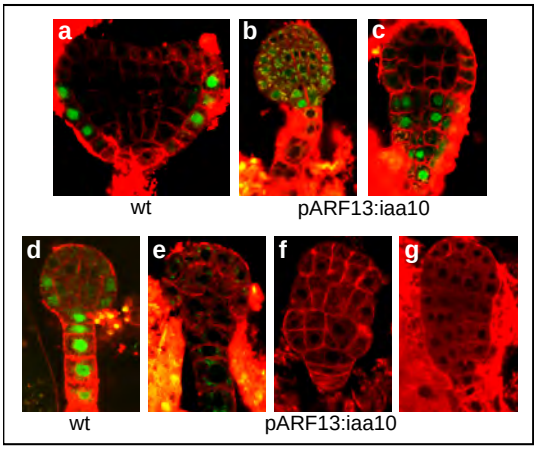

Figure 6. Change in expression of bHLHs in the pARF13:iaa10 mutant background. Expression as reported by transcriptional fusions of 2.0 Kb promoter to nuclear localised triple GFP (green signal). The expression domain of bHLH060 is expanded from the embryo in wild type (wt) (a) to include suspensor cells in phenotypic pARF13:iaa10 embryos (b,c). Suspensor expression of expression of bHLH100 in wildtype (wt) (d) is lost in phenotypic pARF13:iaa10 embryos (e,f). Red signal reports membranes stained with FM4-64 dye.

bHLH functional analysis

Next we investigated the function of the bHLHs shown to be expressed in the embryo in this chapter using a loss of function approach. Insertion lines for two additional bHLHs with expression patterns documented in this chapter were investigated (Table 2, Materials and Methods). The available lines were analyzed for seedling and embryo phenotypes as described in Chapter 4; however no aberrant phenotypes were detected. Recently, higher order mutants made with subfamily 13 members including *TMO5-LIKE1* have shown cell division defects in the embryo (Möller, De Rybel et al., unpublished) highlighting the redundancy within the bHLH superfamily. As most bHLH proteins discussed in this chapter have at least one close homolog, it is likely that genetic redundancy masks functions during embryogenesis. Finally, to determine if ectopic expression of *bHLH060* in suspensor cells is sufficient to (partially) convert these cells to embryo-like identity, we expressed it from the suspensor specific *ARF13* promoter. As with all other genes individually misexpressed in the suspensor (Chapter 4), *bHLH060* misexpression did not induce suspensor defects or any other morphological aberrations in embryos or seedlings. Hence, bHLH misregulation is part of a complex transcriptional response that can not be causally linked to the misregulation of individual genes.

DISCUSSION

In this chapter we expanded the expression analysis of bHLH transcription factors during embryogenesis. Combined with the results in Chapter 4, we have identified 9 bHLHs as being expressed during embryogenesis. Possible explanations as to why several bHLHs that are present in the M0171-bdl dataset were not found to be expressed during embryogenesis are given in Chapter 4 and will not be discussed here. *bHLH100* is initially expressed exclusively in the suspensor, and later the expression domain is expanded to include apical, embryo cells. Another basally expressed bHLH, *bHLH165*, is restricted to the basal cell following hypophysis division and has also been detected in ground tissue initials at later stages of embryogenesis. The remaining seven bHLHs are expressed in the embryo, and are never expressed in the suspensor. In general three embryo specific expression patterns are detected. Firstly, protoderm specific expression was determined for *bHLH137*, *bHLH076* and *bHLH060*. Next, ground tissue expression was detected in transcriptional fusion lines for *bHLH153* and *bHLH063*. Finally prevasculature expression was detected in transcriptional fusion lines of *bHLH149* and *bHLH049*. At around globular stage *bHLH153* and *bHLH049* are coexpressed in the inner cells of the basal tier of the embryo, and subsequently restricted to ground tissue or prevasculature respectively. These expression results will be informative for further experiments such as the generation of higher order mutants. Mostly unexplored to date is the post-embryonic expression patterns in these lines, which could infer roles for several bHLHs at later stages. For example *bHLH153* is specifically expressed in the root endodermis and may be important for the specification or maintenance of this cell type.

Importantly expression patterns as detected by the transcriptional fusions match the translations fusions analyzed to date, supporting our transcriptional fusion approach, and qualifying the factors studied here as intrinsic cell identity markers.

So far, the number of bHLH genes for which a gene expression pattern or function had been described in the embryo is very limited. With this study we greatly expand this number, and identify several bHLH genes whose expression is restricted to singly cell types or small embryo domains. Importantly, most of these are regulated by the pathways that control the suspensor to embryo transformation. This shows that cell identities and their transformation in embryos are associated with bHLH gene expression as is the case for example in the root or shoot epidermis (reviewed by Schellmann et al., 2007). Although we have shown that apical and basal bHLHs (*bHLH60* and *bHLH100*) are misexpressed or lost in the ARF13:iaa10 background respectively, conclusive evidence that these and other bHLHs expressed in the embryo play a role in cell identity specification remains to be demonstrated. It will also be important to place these bHLHs into an auxin response pathway, either as direct or indirect

targets of ARFs. The bHLH expression patterns documented in this chapter provide a starting point to elucidate these important remaining questions.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana plants (Columbia-0 ecotype) were grown under standard conditions at 23°C in a 16-h-light/8-h-dark cycle. Selection for transgenes was performed on solid Murashige and Skoog medium supplemented with 25 mg/L kanamycin or 15 mg/L phosphinothricin where appropriate. The afb4-2 homozygous mutant line used was previously published (Greenham et al., 2011). Other details of insertion lines used are in Table 2 and primers used for genotyping are listed in Table 3.

Table 2. Insertion lines used.
AGI = Arabidopsis Genome Initiative gene identification number.
bHLH numbering according to Carretero-Paulet et al., 2010.
Other name = abbreviated common names for genes also used in main text.
NASC = Nottingham Arabidopsis Stock Centre stock ID number used for ordering lines.
Line = name of line, Line # = internal reference number.

AGI	bHLH	Other Name	NASC	Line	Line #
AT1G26260	bHLH076	CIB5	N590179	SALK_090179	100.1
AT1G26260	bHLH076	CIB5	N815870	SAIL_340_H02	100.2
AT5G50915	bHLH137	-	N656019	SALK_141414C	61.1
AT5G50915	bHLH137	-	N680151	SALK_113830C	61.2
AT1G09250	bHLH149	-	not available	-	-
AT1G05710	bHLH153	-	not available	-	-

Table 3. Genotyping primers for insertions lines.
AGI = Arabidopsis Genome Initiative gene identification number.
NASC = Nottingham Arabidopsis Stock Centre stock ID number used for ordering lines.
Line = name of line, Line # = internal reference number.
LP (left primer) and RP (right primer) are the gene specific primers (5′ to 3′) used in combination with an insertion specific primer for genotyping and were designed using the T-DNA Primer Design program at the Salk Institue Genomic Analysis Laboratory website (http://signal.salk.edu/tdnaprimers.2.html).

AGI	NASC	Line	Line #	LP	RP
AT1G26260	N590179	SALK_090179	100.1	CAAAATCTGCTTCCTCCTCTG	GTTCTTGCAAGATCTTGTGCC
AT1G26260	N815870	SAIL_340_H02	100.2	TGGAGTTCCAGATTCAACTGG	GACATGTTGTGGGGAAATGTC
AT5G50915	N656019	SALK_141414C	61.1	GCCTTCCCTGTTACCTATTCG	TCTAACATAAATTACCCGCCG
AT5G50915	N680151	SALK_113830C	61.2	AGGGAAAAGATAAGTGAGCGG	CGTAGATTTTATTCCCTCCGTG

Generation of transcriptional and translational fusion constructs

A Ligation Independent Cloning (LIC) strategy was used to make the transcriptional and translational fusions used in this chapter. Construction of the vector for transcriptional fusions, subsequent cloning of promoter fragments (of which the primers can be found in Table 4) and verification of constructs was performed as detailed in Chapter 4. The hygromycin resistant pGII-H-NOSt (Hellens et al., 2000) served as a base vector into which a LIC site (gaattctagttggaatgggttaacccaactccataaggatcc) was introduced using EcoRI and BamHI restriction enzyme sites. Subsequently, a fragment encoding super YELLOW FLUORESCENT PROTEIN (sYFP; Kremers et al., 2006) was introduced into the BamHI site at the 3′ flank of the LIC site to generate pGII-H-LIC-sYFP-NOSt. The primers listed in Table 4 were used to amplify the gene loci (2.0 Kb promoter and CDS without stop codon) from Arabidopsis genomic DNA, which were then introduced into the vector using LIC according to the method given in Chapter 4. Restriction analysis and sequencing confirmed the integrity of the fusion constructs prior to Arabidopsis transformation.

Table 4. Promoter and translational fusion primers.

AGI = Arabidopsis Genome Initiative gene identification number.
bHLH numbering according to Carretero-Paulet et al., 2010.
FWD and REV = forward and reverse primers 5′ to 3′.

AGI	bHLH		Sequence
Transcriptional fusions			
AT3G47640	bHLH047	FWD	TAGTTGGAATGGGTTCGAAGGTGCAAACGAACCGGCAAA
		REV	TTATGGAGTTGGGTTCGAAGAAATTGTTTTTTTTGGAGGAAG
AT5G08130	bHLH046	FWD	TAGTTGGAATGGGTTCGAATGTGACTCCGCGAATGATTTTCTC
		REV	TTATGGAGTTGGGTTCGAATTGGCGTGTCAAGGTACTGT
AT5G15160	bHLH134	FWD	TAGTTGGAATGGGTTCGAATAAGAAAAGCGAGAGGTCATGTG
		REV	TTATGGAGTTGGGTTCGAAGTTTGATTTGAAAAAATATTC
AT3G47710	bHLH161	FWD	TAGTTGGAATGGGTTCGAACCAAAATTATGAACACTCATATACGG
		REV	TTATGGAGTTGGGTTCGAATATGTAATATATATGATATGG
AT1G26945	bHLH163	FWD	TAGTTGGAATGGGTTCGAAGGCAAATAAATAAAGTATTCAGAAG
		REV	TTATGGAGTTGGGTTCGAACTTCTTTCTTGATATATTATAAGTGTGTTTGTTTGGG
AT3G17100	bHLH147	FWD	TAGTTGGAATGGGTTCGAAGCCGTTCGTCAAAAGTATGTAATG
		REV	TTATGGAGTTGGGTTCGAAAATCGCCGATCAAATCTCAGTGAG
AT3G06590	bHLH148	FWD	TAGTTGGAATGGGTTCGAAGCTGGAATTATGATTTACAG
		REV	TTATGGAGTTGGGTTCGAAAACCAAATAATTGGCTCTGATCTCC
AT1G09250	bHLH149	FWD	TAGTTGGAATGGGTTCGAAGTATTGCCGAAAAAAGTATGG
		REV	TTATGGAGTTGGGTTCGAATAAATCCGATTTAAAACCAGAG
AT3G07340	bHLH062	FWD	TAGTTGGAATGGGTTCGAAAACATCAAAAGTGAAGAGGG
		REV	TTATGGAGTTGGGTTCGAAGTTAATGGGCAGTAATGAGG
AT1G26260	bHLH076	FWD	TAGTTGGAATGGGTTCGAATATTTTTTAGACGATCGGGT
		REV	TTATGGAGTTGGGTTCGAATCTCTTAAAAGTAGTAAAGC
AT2G42280	bHLH130	FWD	TAGTTGGAATGGGTTCGAATGTTATAGATAACTAATCAT
		REV	TTATGGAGTTGGGTTCGAATATTTATCGTCTTTTTGCTTC
Translational fusions			
AT1G68920	bHLH049	FWD	TAGTTGGAATGGGTTCGAAGAAGTTATTAGCATTAACTG
		REV	TTATGGAGTTGGGTTCGAACTGGCTCAACCTTCATATTTG
AT5G15160	bHLH134	FWD	TAGTTGGAATGGGTTCGAACCGTAGTAAAACGATGGAGATAAG
		REV	TTATGGAGTTGGGTTCGAACTCCATTAATCAAGCTCCTAATAAC
AT3G05800	bHLH150	FWD	TAGTTGGAATGGGTTCGAACGGTCGTTAGCTAAATTTTGGGCC
		REV	TTATGGAGTTGGGTTCGAACTAAAGCCGAGCCGAGATTAGTAGACG

Arabidopsis Transformation
A simplified plant transformation procedure allowing moderate throughput, as detailed in Chapter 4 was used for all transformations.

Microscopy
Embryo preparation and imaging of fluorescent reporters in embryos was conducted with either a Zeiss LSM510 confocal laser scanning microscope as previously described (Schlereth et al., 2010). Embryo phenotypes were analyzed using chloral hydrate cleared preparations (clearing solution of chloral hydrate, water and glycerol, 8:3:1) on a Leica DMR microscope equipped with differential interference contrast (DIC) optics. Gene expression or protein accumulation was analyzed in roots of homozygous T3 lines. Four- to five-day-old vertically grown seedlings were incubated in water containing 1 µM FM4-64 (Invitrogen) for 1 min and subsequently imaged on a Zeiss LSM510 confocal laser scanning microscope.

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Chapter

Summarizing Discussion

Annemarie S. Lokerse

Investigation into the mechanisms underlying the *de novo* formation and specification of cell identities is essential to understand plant growth and development. Physically and intrinsically asymmetric cell divisions, as well as external cues/positional information, play essential roles in the generation of daughter cells with different fates (reviewed by De Smet and Beeckman, 2011). During *Arabidopsis thaliana* (Arabidopsis) embryogenesis, practically every round of cell division gives rise to new cell identities, including the *de novo* formation of meristems, making this an excellent model to study cell specification events. Indeed, the very first asymmetric cell division of the zygote gives rise to two distinct, even opposite, cell identities. The smaller apical cell and its progeny adopt an embryonic cell identity, whereas the larger basal cell and its descendants (collectively, the suspensor) are non-embryonic during early embryogenesis. Plasticity in this bipolar classification is demonstrated at around early globular stage, when the non-embryonic cell subjacent to the proembryo undergoes an identity change and is incorporated into the proembryo (reviewed by Peris et al., 2010). However, prior to this event, the embryonic and non-embryonic lineages represent distinct sets of cells and little is known about what distinguishes these at a molecular level.

One molecule that is dynamically and differentially distributed in the apical and basal cell lineages during Arabidopsis embryogenesis is the plant hormone auxin. The earliest reported effect of perturbations in auxin transport or response occurs at one cell stage, when the apical cell undergoes a transverse instead of longitudinal division (reviewed by Möller and Weijers, 2009). This aberrant division occurs (albeit infrequently) in *mp/arf5* and *bdl* mutants. *MP* and *BDL* encode interacting representatives of AUXIN RESPONSE FACTORS (ARFs) and their inhibitors the Aux/IAAs. These components are the core effectors of auxin responses, Aux/IAAs being degraded in an auxin dependent manner, freeing ARFs which function at the level of transcription, both positively and negatively regulating the expression of auxin responsive genes.

Indeed the elegantly simple mechanism that enables transcriptional responses to the hormone auxin has been well elucidated. However as auxin response becomes implicated in increasingly diverse developmental and other programs, it is clear that specificity must be generated within this mechanism, as discussed in **Chapter 1**. Large gene families encode the core auxin response components, the ARFs and Aux/IAAs, and to a lesser extent the auxin receptors. Within these families functional specificity has been demonstrated (Weijers et al., 2005; Muto et al., 2007), as has a large degree of functional redundancy (Okushima et al., 2005; Overvoorde et al., 2005). Conceptually, specificity in auxin response (and spatially and temporally distinct developmental outputs) could be generated through the combinatorial interactions within and between these core components. Therefore initial characterization of an auxin response within a cell or tissue should include establishment of the subset of auxin response components present and preferably, a network of their interactions.

As the existing knowledge regarding spatial and temporal auxin component expression was extremely limited, we generated a set of sensitive transcriptional reporter lines for all ARFs to facilitate a simple and comprehensive identification of the ARF complement within a cell/tissue of interest (**Chapter 2**). This family was chosen as they are the DNA-binding transcription factors which are ultimately the executors of of auxin response, determining which genes are responsive to auxin, at the level of transcription.

Our analysis of ARF expression in the root meristem revealed both ubiquitous and specific ARF expression patterns and ARF subsets that distinguished the actively dividing cells from those undergoing elongation. Moreover, a striking correlation between cell type and ARF expression patterns was revealed in the early embryo, where each cell type expressed a unique ARF complement. The atlas of ARF expression in the root meristem and early embryo (and future expression analysis in other tissues) will guide functional characterization at cellular resolution. Furthermore, cell identity predictions can be tested through precise manipulations of the ARF composition in a cell type. Further work is required to determine how these ARF expression patterns are established and how instructive ARFs are for early embryo cell specification events.

Regardless, it is clear that cell specific ARF combinations could translate auxin into the various auxin-triggered developmental responses in the embryo, such as cotyledon initiation, vascular development and hypophysis specification. In the latter, MP/ARF5 and BDL/ IAA12, expressed in the inner cells of the proembryo, mediate a non-cell autonomous auxin response. This response includes upregulation of an auxin efflux transporter, *PIN1*, and the transport of auxin and a mobile transcriptional regulator to the subjacent non-embryonic file of cells, the uppermost of which is specified as the hypophyisis and incorporated into the embryo where it will further divide to give rise to the QC and distal meristem initials (Weijers et al., 2006). This file of non-embryonic cells are the descendants of the basal cell following zygote division and are also known as the suspensor. In addition to the donation of its uppermost cell to the embryo, the suspensor plays a role in connecting the embryo to its surrounding tissues (reviewed by Kawashima and Goldberg, 2010).

The transport of auxin into the suspensor infers the existence of an autonomous auxin response in these cells, and a goal of the work presented in this thesis was to identify and characterize this response. Transcriptional fusion analysis revealed that six ARFs are expressed in the suspensor, of which only ARF13 expression is exclusively in the suspensor throughout embryogenesis (**Chapter 2**). The striking overlap in the expression pattern of ARFs 1,2 and 6 was informative in that triple mutants in arf1/2 and distantly related arf6 resulted in aberrant divisions at the embryo-suspensor junction, not detected in single or double mutant combinations (**Chapter 3**). This redundancy was unexpected since ARF1 and 2 have been characterized as transcriptional repressors and ARF6

as a transcriptional activator in protoplast-based assay (Tiwari et al., 2003). The *in vivo* identification of downstream targets of these ARFs will help to clarify their functional redundancy in embryo development.

In lieu of a hextuple ARF suspensor mutant, and to overcome any conditional redundancy that may occur in this mutant, a GAL4-UAS two component system was used to specifically inhibit all ARFs and thereby auxin response in the suspensor. This was achieved by expressing a stabilized Aux/IAA (*iaa12*) under the control of the UAS promoter and crossing this line to the suspensor specific driver line M0171, capable of activating the UAS promoter. This approach was previously used to inhibit auxin response subdomains in the proembryo and given the limited specificity of stabilized Aux/IAAs is expected to completely abolish auxin response in a given subdomain (Weijers et al., 2006). Targeted inhibition of auxin response in the suspensor triggered aberrant cell divisions throughout the cell file and proliferation into spherical tissues, which occasionally yielded twin embryos and seedlings (**Chapter 3**). Subsequently, the expression of embryo-specific markers was detected in the proliferating suspensor cells. Finally, IAA10 was identified as a suspensor expressed Aux/IAA and suspensor specific expression of stabilized iaa10 also lead to suspensor proliferation phenotype. Thus, inhibition of the autonomous auxin response in the suspensor was found to interfere with the maintenance of suspensor cell identity and leads to acquisition of embryonic identity.

Developmental plasticity is a well-known characteristic of the suspensor. Most often, suspensor cell proliferation and the formation of secondary embryos are a consequence of defects in the embryo, inferring the presence of embryo-derived signals that normally inhibit the embryonic program in suspensor cells (Schwartz et al., 1994; Yadegari et al., 1994; Vernon and Meinke, 1994; Zhang and Somerville, 1997). Importantly, the research presented in this thesis has identified auxin response as the first cell autonomous mechanism required for suspensor cell identity maintenance, in part through inhibition of an apical, embryo identity.

As very little is known about what distinguishes the apical and basal cell derivatives, the finding that auxin response autonomously regulates suspensor cell identity provided an excellent model system for further investigation into determinants of embryo and suspensor identity. We adopted a microarray-based approach, coupled to a novel embryo dissection technique to detect early changes in the transcriptome (potentially including master regulators) when auxin response was specifically inhibited in only one cell type, the suspensor. A narrow window of opportunity was available for selecting a time point for harvesting embryos (resulting from crosses between M0171 driver and UAS-bdl) for the microarrays. Specifically, this window occurs from when the M0171 driver expression is active (as evidenced by GFP expression) until morphological changes are evident in the suspensor/embryo cells. The time point of 72 h was

chosen, at which stage the M0171 driver was first active (ie 16 cell embryo) and the first visible phenotypic difference, (ie. an aberrant cell division) occurred at low frequency. Despite this, an unexpectedly large number of genes (2416 or ~7% of all Arabidopsis genes) were significantly misregulated and included in the M0171-bdl dataset (**Chapter 4**).

The fact that such a large number of genes were misregulated is reflected in the subsequent transcriptional fusion approach used. This approach was used both to validate the dataset generated and in an attempt to identify key regulators of cell identity. An important prerequisite for a function in cell identity would be embryo or suspensor specific expression, together with appropriate positive or negative misregulation. Specifically, genes involved in suspensor cell identity should be expressed in the suspensor and downregulated and genes involved in suspensor to embryo cell identity could be expressed in the embryo and should be upregulated in the M0171-bdl dataset. The transcriptional fusion approach was selected for several reasons. Firstly, the generation of transcriptional fusion lines was comparatively simple and fast and thus enabled a relatively large number of genes (70) to be screened. This was important, not only because we needed to cast as wide a possible net given the large number of misregualted genes, but also as an initial global analysis we identified false positives, i.e. genes downregulated in the embryo as a consequence of auxin response inhibition in the suspensor. In our hands, transcriptional fusions to a triple GFP reporter are the most sensitive method to detect and image expression which was important as we anticipated that many genes could have low expression levels in the embryo. The nuclear localization signal in the reporter aided the analysis of expression patterns at cellular resolution. In total, 40 genes were found to be expressed during embryogenesis, and in general their patterns did not contradict previously published expression patterns when available. This represents a massive increase in the number of genes that are known to be expressed in the Arabidopsis embryo at cellular resolution.

The initial global analysis of the resulting unique datasets, which included both up and downregulated genes, found enrichment for genes involved in auxin homeostasis. In general, the genes in more than 10 families involved in auxin homeostasis were misregulated in such a way that the predicted effect of each individual misregulation would be an increase in free auxin concentration in the cell. Although feedback from auxin response to auxin homeostasis has been previously demonstrated, this is the first time such a convergent feedback has been documented and coupled to a biologically relevant output, i.e. cell identity change.

Amongst the auxin homeostasis genes that were misregulated were two closely related, non-canonical Aux/IAAs (nc-IAAs), IAA20 and IAA30. Expression of *IAA30* was detected in suspensor cells, and both *IAA20* and *IAA30* were expressed in hypophysis derivatives and prevasculature. Later, expression of *IAA30* was also

evident in cotyledon tips. Another nc-IAA, *IAA33*, was exclusively expressed once the basal cell arising from the hypophysis division had itself divided, the most specific expression pattern for an auxin response component to date. Interestingly, an aberrant embryo phenotype, albeit at low frequency, was evident in an *iaa20* insertion line, making this one of the few loss of function aux/iaa mutants with a developmental phenotype (**Chapter 5**; Tian and Reed, 1999; Nagpal et al., 2000). nc-IAAs are a true anomaly in the auxin response pathway in that they are able to interact with ARFs and canonical Aux/IAAs (Vernoux et al., 2011), but cannot be degraded in response to auxin (e.g. IAA20, Dreher et al., 2006). Moreover, *IAA20/30* expression is induced by auxin (Sato and Yamamoto, 2008) and, at least in the embryo, IAA20/30 expression is similar to auxin response maxima as reported by the artificial auxin responsive reporter, DR5-GFP (**Chapter 3**). This may provide a clue to their function, which could be to fine-tune or modulate auxin maxima responses. Their effect would depend on their interaction partner(s); interactions with ARFs could, irrespective of auxin concentration, directly inhibit ARF activity or potential for ARF hetero- or homo-dimerization, in effect switching an auxin response off. Conversely, interaction with canonical Aux/IAAs could sequester them, preventing their interaction with ARFs in which case ARFs would be free to continue effecting transcriptional changes. Regardless, the 6 nc-IAAs in the Arabidopsis genome, the expression of IAA20, IAA30 and IAA33 in subdomains of the embryo and the iaa20 mutant phenotype mean that these nc-IAAs cannot easily be disregarded. Furthermore, it will be interesting to investigate whether these proteins have any degrons mediating a conditionally induced degradation. Further expansion of the Aux/IAA expression analysis revealed that similar to ARFs, many Aux/IAAs are also dynamically and differentially expressed in the Arabidopsis embryo (**Chapter 5**).

Two closely related, monophyletic auxin receptor family members, *AFB4* and *AFB5*, were also downregulated in response to the suspensor specific inhibition of auxin response. Transcriptional fusions reported their expression in hypophysis descendents and prevasculature and the afb4-2 mutant displayed erroneous divisions at the apical-basal embryo boundary (**Chapter 4** and **5**). AFB4 was recently characterized as a negative regulator or auxin signaling, revealing yet more diversity and complexity in the auxin response pathway (Greenham et al., 2011).

The finding that members of auxin responsive transcription factor families were clearly overrepresented in the microarray datasets, led us to survey the members of one such family, the bHLHs in **Chapters 4** and **6**. We showed that 9 bHLHs were expressed in either subdomains of the embryo or in the suspensor, in 6 different patterns. These specific expression patterns highlight the need for expression analysis at cellular resolution. The expression patterns documented in Chapters 4 and 6 could be used in combination with newly developed methods (Deal and Henikoff, 2011) to isolate subdomains of the embryo, prior to transcriptional (or other) profiling.

However, the majority of the selected genes were not expressed in a pattern in keeping with a role in auxin mediated suspensor cell identity maintenance or embryo transformation. This is likely a direct consequence of the fact that an unexpectedly large number of genes were misregulated upon inhibition of auxin response. There are several facets to an interpretation of this result. Firstly, that cell identity transformation is a complex response and a massive reprogramming of the transcriptional profile of suspensor (and embryo) cells precedes any gross morphological changes, inferring that these cells have a high level of transcriptional plasticity. It must also be acknowledged that the identification of informative transcriptional changes was impeded by the continuity between embryo and suspensor cells, enabling secondary transcriptional changes in embryo expressed genes, which were subsequently incorporated into the M0171-bdl datasets.

Several examples of the embryo-suspensor continuity have already been touched upon in this discussion, including that mutations in embryo genes can result in suspensor proliferation and that auxin is dynamically transported during embryogenesis. Recently, an embryonic auxin response was found to mediate the transport of a mobile transcriptional regulator from the embryo cells adjacent to the suspensor to the uppermost suspensor cell where it functions in hypophysis identity specifcation (Schlereth et al., 2010). Another example that illustrates the interconnectedness of embryo and suspensor comes from the analysis of *WOX* (*WUSCHEL RELATED HOMEOBOX*) clade mutants. Following the asymmetric division of the zygote *WOX2* becomes restricted to the apical cells and *WOX8* and *WOX9* become restricted to the basal cells (Haecker et al., 2004). In addition to aberrant cell divisions in the basal lineage, *wox8 wox9* double mutants exert a non-cell-autonomous effect on the apical lineage, which includes loss of *WOX2* expression and incorrect orientation of the apical cell division (Breuninger et al., 2008). That continuity was responsible for secondary changes in embryo expressed auxin responsive genes was recently verified by the considerable overlap between the datasets generated here and those generated by inhibiton of auxin response in the inner cells of the proembryo (Möller and Weijers, unpublished data).

Despite these complications we did identify several embryo and suspensor specific genes which were misregulated in keeping with a function in the acquisition or maintenance of cell identity respectively. However, aside from the *iaa20-1* and *afb4-2* mutants described above, subsequent functional characterization by loss of function and misexpression were unsuccessful in consolidating a role for these genes as cell identity regulators. The phenomenon of genetic redundancy in Arabidopsis (reviewed by Briggs et al., 2006) is the most likely explanation as to the absence of mutant phenotype in single loss of function mutants investigated in **Chapters 4, 5** and **6**. The current ongoing generation of higher order mutants may reveal regulatory roles for the remaining candidate genes. Upregulated genes expressed in wild type embryos were

specifically misexpressed in the suspensor using the ARF13 promoter, yet did not induce developmental abnormalities. This is perhaps not surprising, given the large number of genes misregulated in M0171>>bdl embryos prior to the first morphologically evident phenotypes.

The relatively large number of genes that were misregulated upon inhibition of auxin response in the suspensor could also infer that the transcriptional programs of embryonic and suspensor cells are under differing epigenetic control and/or that epigenetic reprogramming may be involved in identity transformations. Epigenetic regulatory mechanisms are used to coordinate changes in gene expression throughout development (reviewed by Feng et al., 2010). Epigenetic control of gene expression is mediated through changes in chromatin structure, including DNA methylation and a plethora of histone modifications. Interestingly, previous research has shown that DNA methyltransferase mutants display defects in early apical and basal embryo patterning, including suspensor proliferation (Xiao et al., 2006). Polycomb repressive complexes (PRC2) are conserved in higher eukaryotes and generally repress transcription via methylation of histone H3 lysine 27 (H3K27). Recently a global comparison between meristematic and differentiated tissues revealed differential H3K27 trimethylation status of hundreds of H3K27 target genes, inferring that this could be a major determinant of tissue specific expression patterns in plants (Lafos et al., 2011). A similar approach could be used to compare embryo and suspensor expressed genes. Interestingly, in addition to direct negative regulation of genes by their H3K27 methylation status, positive control of gene expression was affected by restricting the expression of many miRNA genes. Identification of both direct negative and indirect positive regulation of auxin homeostasis and response components now provides an interesting link between auxin and epigenetic control mechanisms (Lafos et al., 2011). Changing histone modifications can also be achieved through replacement of histones with histone variants (H3.3s) that can be incorporated in the absence of DNA replication and are associated with transcriptional activation (reviewed by Feng et al., 2010). Histone exchange appears to be important for epigenetic reprogramming during fertilization and intriguingly occurs differently in the zygote and endosperm (Ingouff et al., 2007). Tissue specific expression of H3.3 variants is likely, and a H3.3-like variant was 10.8 fold upregulated in the M0171-bdl dataset (data not shown), perhaps warranting further investigation. Recently epigenetic factors involved in repressing the embryonic program post germination have been shown to also play a role in the repression of the maturation phase during early embyogenesis (Willmann et al., 2011). Through analysis of a miRNA biogenesis mutant, miRNAs were placed upstream of transcription factors and epigenetic regulators that control the maturation program and were shown to function as early as eight cell stage (Willmann et al., 2011; Nodine and Bartel. 2010).

In summary, we have shown that specificity in auxin responses during embryogenesis are at least in part generated through the spatial and temporal expression of core auxin response components. We have identified a subset of auxin response components that function in a novel auxin response, suspensor cell identity maintenance. Surprisingly, we found that the auxin controlled maintenance of suspensor cell identity includes repression of the embryonic program. This finding gave us an experimental system in which to investigate suspensor cell identity and embryonic transformation. Targeted and specific inhibition of auxin response in the suspensor was coupled to new embryo dissection techniques and a microarray based approach was used to generate a unique dataset which was subsequently mined for cell identity regulators. Unexpectedly, inhibition of auxin response induced the misregulation of thousands of genes, prior to gross morphological changes, revealing a high degree of transcriptional plasticity in these cells. This complicated the identification of regulators. Moreover, the dataset also included secondary/ indirect changes in embryo expressed genes, which were inevitable given the connectivity and developmental connectedness between the embryo and suspensor. In the future, transcriptional profiling of isolated embryo and suspensor cell types (using transcriptional fusion markers generated in this research) could be performed to circumvent this issue. Regardless, one of the most striking findings was the convergent regulation of members of many gene families involved in all facets of auxin homeostasis. It appears that transient auxin response inhibition is sensed as an auxin minimum and in general auxin homeostasis genes were activated or repressed in such a way that would increase cellular auxin levels (and response). Finally, many bHLH superfamily members were misregulated upon the inhibition of suspensor auxin response and subsequently found to have specific expression patterns in the embryo, validating further research to place these factors into the auxin response pathways controlling cell identity in the embryo.

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SUMMARY

Auxin is a structurally simple molecule, yet it elicits many different responses in plants. In **Chapter 1** we have reviewed how specificity in the output of auxin signaling could be generated by distinct regulation and the unique properties of the members of the Aux/IAA and ARF transcription factor families.

In **Chapter 2** we further investigated the generation of specificity in auxin responses by generating a set of sensitive transcriptional reporter lines for all Arabidopsis ARFs. This facilitated a comprehensive identification of the ARF complement within a cell/tissue of interest. Our analysis of ARF expression in the root meristem revealed both ubiquitous and specific ARF expression patterns and ARF subsets that distinguished the actively dividing cells from those undergoing elongation. Moreover, a striking correlation between cell type and ARF expression patterns was revealed in the early Arabidopsis embryo, where each cell type expressed a unique ARF complement.

In **Chapter 3** we characterized a novel cell-autonomous auxin response is required for hypophysis specification and root meristem initiation, and identify Aux/IAA and ARF transcription factors that mediate this response. We show that auxin response components in the proembryo and the suspensor are intrinsically different, and their regulated, lineage-specific expression creates a prepattern enabling different developmental auxin responses. Surprisingly, we find that, in addition to mediating hypophysis specification, auxin response also acts to maintain suspensor cell identity. We show that auxin controlled maintenance of suspensor cell identity includes repression of the embryonic program. This finding gave us an experimental system in which to investigate suspensor cell identity and embryonic transformation.

In **Chapter 4** the targeted and specific inhibition of auxin response in the suspensor was coupled to new embryo dissection techniques and a microarray based approach was used to generate a unique dataset which was subsequently mined for cell identity regulators. Unexpectedly, inhibition of auxin response induced the misregulation of thousands of genes, prior to gross morphological changes, revealing a high degree of transcriptional plasticity in these cells. This complicated the identification of regulators. Moreover, the dataset also included secondary/indirect changes in embryo expressed genes, which were inevitable given the connectivity and developmental connectedness between the embryo and suspensor.

One of the most striking findings from analysis of the dataset generated in Chapter 4 was the convergent regulation of members of many gene families involved in all facets of auxin homeostasis, as investigated in **Chapter 5**. It appears that transient auxin response inhibition is sensed as an auxin minimum and in general auxin homeostasis genes were activated or repressed in such a

way that would increase cellular auxin levels (and response).

Finally, many bHLH superfamily members were misregulated upon the inhibition of suspensor auxin response and subsequently found to have specific expression patterns in the embryo, the focus of in **Chapter 6**. Several bHLHs were shown to lose their lineage specific expression patterns upon inhibition of auxin response in the suspensor, validating further research to place these factors into the auxin response pathways controlling cell identity in the embryo.

SAMENVATTING

Het plantenhormoon auxine is een klein en structureel eenvoudig molecuul, maar kan desondanks een breed scala aan processen aansturen. In **Hoofdstuk 1** wordt beschouwd hoe de unieke eigenschappen en regulatie van ARF en Aux/IAA transcriptiefactoren kunnen leiden tot specifieke respons op auxine.

In **Hoofdstuk 2** wordt een eerste stap gezet richting het systematisch analyseren van de rol van ARF transcriptiefactoren in het genereren van een specifieke auxine-respons. Hiertoe is een collectie gen-expressie reporters gemaakt die het mogelijk maakt de expressiepatronen van alle 23 ARF genen in Arabidopsis gevoelig te detecteren. Deze analyse heeft geleid tot de identificatie van de ARFs die in verschillende cellen en weefsels actief zijn. Zo blijkt in het wortelmeristeeem een combinatie van brede en meer specifiek tot expressie komende ARFs actief te zijn, zodanig dat verschillende combinaties van ARFs actief zijn in delende cellen en de cellen in de strekkingszone. Daarnaast is gevonden dat alle celtypen in het vroege embryo een unieke set ARF transcriptiefactoren bezitten.

In **Hoofdstuk 3** wordt laten zien dat een tot nog toe onbekende auxine-respons nodig is in de suspensor voor specificatie van de hypofyse en derhalve voor de aanleg van het wortelmeristeem. Daarnaast zijn de Aux/IAA en ARF transcriptiefactoren ontdekt die deze respons mogelijk maken. Interessant genoeg blijken deze ARF transcriptiefactoren functioneel verschillend te zijn van de ARF transcriptiefactor die in het naastliggende embryo actief is. Derhalve blijkt dat de gereguleerde expressie van functioneel verschillende ARF transcriptiefactoren een “pre-patroon” genereert waardoor verschillende auxine-responsen in deze twee celtypes optreden. Naast het reguleren van hypofyse-aanleg, blijkt auxine-respons in de suspensor ook belangrijk te zijn voor het onderdrukken van embryo-identiteit. Het reduceren van auxine-respons in suspensorcellen leidt tot een transformatie naar embryo-identiteit. Deze bevinding heeft ons in staat gesteld om de genetische basis van suspensor-identiteit en embryo-transformatie te onderzoeken.

Het onderzoek beschreven in **Hoofdstuk 4** was erop gericht de genen te identificeren die gereguleerd worden tijdens de transformatie van suspensor naar embryo identiteit zoals die optreedt na remming van ARF transcriptiefactoren in de suspensor. Zulke genen zouden betrokken kunnen zijn bij de celtransformatie en zouden belangrijke regulatoren van suspensor- of embryo-identiteit kunnen zijn. Hiertoe is de gereguleerde expressie van een ARF inhibitor gekoppeld aan een nieuwe embryo dissectie methode, en is met behulp van microarrays een transcriptieprofiel gegenereerd. Hoewel voor dit experiment embryo’s waren geoogst waarin nog geen fenotypische abnormaliteiten waarneembaar waren, bleken duizenden genen anders gereguleerd te zijn. Dit resultaat toont aan dat er een enorme plasticiteit bestaat

van transcriptie tijdens de embryo ontwikkeling. Deze complicatie heeft de selectie van genen voor verder onderzoek bemoeilijkt, mede omdat weinig andere bronnen van data beschikbaar zijn. Markant genoeg bleken vele van de gedereguleerde genen normaal in het embryo tot expressie te komen. Dit duidt op indirecte effecten van het interfereren met suspensorfunctie op het naastliggende embryo, en onderstreept het belang van communicatie tussen embryo en suspensor.

Een van de meest in het oog springende consequenties van het inhiberen van ARF activiteit in de suspensor op transcriptie was de hoge mate waarin genen betrokken bij auxine activiteit gedereguleerd waren. In **Hoofdstuk 5** wordt beschreven dat meerdere leden van verschillende genfamilies werden gedereguleerd, zowel positief als negatief. Interessant genoeg bleek er een hoge mate van convergentie te bestaan, zodanig dat genen die een positieve werking op auxine activiteit of concentraties uitoefenen doorgaans omhoog gereguleerd werden, terwijl negatieve regulatoren neer-gereguleerd werden. Het onderbreken van auxine activiteit heeft een netwerk van regulatie blootgelegd waardoor reductie in auxine activiteit wordt gecompenseerd door genregulatie van vele componenten in biosynthese, inactivatie, transport en respons.

Tenslotte was een ander opvallend resultaat van het inhiberen van ARF activiteit dat genen behorend tot de bHLH superfamilie van transcriptiefactoren sterk gereguleerd bleken. Inspectie van de expressiepatronen van een aantal van deze genen in **Hoofdstuk 6** laat zien dat de meeste van deze bHLH genen specifiek in celtypes van het embryo of in de suspensor actief zijn. Voor die bHLH transcriptiefactoren waarvan het effect van suspensor-specifieke ARF inhibitie is onderzocht, bleek de celtype-specifieke expressie verstoord. Gezien de belangrijke rol van bHLH factoren in het specificeren van celidentiteit in plant en dier, biedt de identificatie van deze factoren een aanknopingspunt voor vervolgonderzoek naar de mechanismen van suspensorspecificatie en embryo-transformatie.

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Lotte, Ruud and Dr Ana, your friendship gave me the support, empathy, much needed perspective, and most importantly daily opportunities for recuperation, without which I couldn't have completed this thesis. I look forward to your PhD defences, Lotte and Ruud. Tineke you're a great friend despite "Tineke time" – but you better be on time for my defence! Dr Kamila and Dr Tomek thanks for your friendship and for teaching me about mushrooms! I hope the tide will turn

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Mark, falling in love with you was the nicest distraction from a PhD that I could ever have wished for, life is wonderful with you in the world! Thanks also to your family for making me feel welcome. I would also like to thank all of my extended family in the Netherlands for welcoming me and helping me to get settled in the Netherlands.

Finally to my Mum and Dad and my brothers Joost and Daan, thank you for always believing in me and for teaching me the values that make me the person that I am today.

CIRRICULUM VITAE

Annemarie Suzanne Lokerse was born on the 12th of December 1978, in Zeist, the Netherlands. At the age of three she immigrated to Auckland, New Zealand. She completed her secondary education at Howick College in Auckland in 1996 and started studying at the University of Auckland in 1997. In 2000 she graduated with a Bachelor Degree of Science. In 2004 she graduated again, with a Master Degree of Science, majoring in Biological Sciences. Shortly thereafter she moved to Christchurch, New Zealand and worked as a Research Fellow for six months in the Bio-Protection Research Centre based at Lincoln University. For the following three years she worked as a Research Technican for Professor Tony Conner in the Plant Genetic Technologies team at the New Zealand Institute for Crop and Food Research in Lincoln. In April 2007 she relocated to the Netherlands and began her PhD studies in the Biochemistry Department at the University of Wageningen. The results obtained over the next four years are presented in this thesis. Following her PhD she intends to pursue a career in plant science.

PUBLICATIONS

Different auxin response machineries control distinct cell fates in the early plant embryo. Rademacher EH*, Lokerse AS*, Schlereth A, Llavata-Peris CI, Bayer M, Kientz M, Freire Rios A, Borst JW, Lukowitz W, Jürgens G and Weijers D. Developmental Cell, in press. * equal author contribution.

A cellular expression map of the Arabidopsis AUXIN RESPONSE FACTOR gene family. Rademacher EH*, Möller B*, Lokerse AS*, Llavata-Peris CI, van den Berg W and Weijers D. The Plant Journal. 2011 68(4) 597-606. * equal author contribution.

A versatile set of Ligation-Independent Cloning vectors for functional studies in plants. De Rybel B, van den Berg W, Lokerse AS, Liao CY, van Mourik H, Möller B, Llavata-Peris CI and Weijers D. Plant Physiology. 2011 156(3) 1292-1299.

miR390, Arabidopsis TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD and Maizel A. Plant Cell. 2010 22(4) 1104-1117.

Auxin enters the matrix - assembly of response machineries for specific outputs. Lokerse AS and Weijers D. Current Opinion in Plant Biology. 2009 12(5) 520-526.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Annemarie Lokerse
Date: 14 December 2011
Group: Laboratory of Biochemistry, Wageningen University and Research centre

1) Start-up phase	date
► **First presentation of your project**	
Introduction given to Lab of Biochemistry members	2007
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Review published Lokerse, A.S and Weijers, D. (2009) Auxin enters the matrix - assembly of response machineries for specific outputs	2009
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7,5 credits**

2) Scientific Exposure	date
► **EPS PhD Student Days**	
EPS PhD student day, Wageningen University	Sep 13, 2007
First Joint Retreat of PhD Students in Experimental Plant Sciences	Oct 02-03, 2008
EPS PhD student day, Naturalis Leiden	Feb 26, 2009
► **EPS Theme Symposia**	
EPS theme 1 Symposium 'Developmental Biology of Plants', Wageningen University	Oct 11, 2007
EPS Theme I Symposium 'Developmental Biology of Plants' Leiden University	Jan 20, 2011
► **NWO Lunteren days and other National Platforms**	
ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 02-03, 2007
ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 07-08, 2008
ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 06-07, 2009
ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 19-20, 2010
ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
NVMB Spring Symposium - Mechanisms in Cell Specification and Pattern Formation	May 18, 2007
EPS Flying Seminar: Hiroo Fukuda (Department of Biological Sciences, The University of Tokyo, Japan)	Nov 26, 2007
Minisymposium Biochemistry Department	Feb 21, 2008
Thursday Seminars Biochemistry: Prof Maarten Jongsma (Bioscience, PRI)	Feb 17, 2008
EPS Flying Seminar: Prof Richard Vierstra (Laboratory of Genetics, University of Wisconsin-Madison, USA)	Apr 14, 2008
Thursday Seminars Biochemistry: Dr Delphine Chinchilla, (University of Basel, Switzerland).	Apr 24, 2008
EPS Flying Seminar: Prof Simon Gilroy (Department of Botany, University of Wisconsin-Madison, USA)	May 19, 2008
Joint Meeting groups Prof Sacco de Vries and Prof Gerco Angenent	May 28, 2008
Thursday Seminars Biochemistry: Dr Niko Geldner (Laboratory of Plant Cell Biology, University of Lausanne, Switzerland).	May 29, 2008
EPS Flying Seminar: Prof Zhenbiao Yang (University of California, Riverside, USA).	Jun 23, 2008
Thursday Seminars Biochemistry: Dr Jan Lohmann (Max Planck Institute, Tübingen, Germany)	Jun 26 2008
Thursday Seminars Biochemistry: Dr Enrico Scarpella (University of Edmonton, Canada)	Oct 16, 2008
Thursday Seminars Biochemistry: Dr Ana Cano-Delgado (CSIC, Barcelona, Spain)	Feb 26, 2009
Thursday Seminars Biochemistry: Dr Sabrina Sabatini (La Sapienza, Rome, Italy)	Mar 26, 2009
Joint Meeting groups dr. Dolf Weijers and prof. Ben Scheres (Utrecht University)	Jun 22, 2009
Thursday Seminars Biochemistry: Prof Dorus Gadella (UVA, Sils, Amsterdam)	Oct 01, 2009
Thursday Seminars Biochemistry: Dr Bruno Mueller (Institute of Plant Biology, University of Zurich)	Oct 29, 2009
Thursday Seminars Biochemistry: Prof Klaus Harter (ZMPB, Tübingen University, Germany)	Dec 10, 2009
Thursday Seminars Biochemistry: Roeland Boer (Institute for Research in Biomedicine, Barcelona)	Apr 22, 2010
Thursday Seminars Biochemistry: Dr Teva Vernoux (ENS Lyon, France)	Sep 16, 2010
Thursday Seminars Biochemistry: Dr Richar Smith (Institute of Plant Sciences, Bern, Switzerland)	Nov 25, 2010
Joint Meeting groups dr. Dolf Weijers and prof. Ben Scheres (Utrecht University)	Feb 11, 2011
► **Seminar plus**	
Richard D. Vierstra (University of Wisconsin-Madison) "The Expanding Universe of Ubiquitin Fold Proteins"	Apr 14, 2008
► **International symposia and congresses**	
5th Symposium of SFB446: Mechanisms of Cell Behaviour, ZMBP, Tübingen, Germany	May 02-03, 2008
Auxin 2008, Marrakech, Morocco	Oct 04-09, 2008
Molecular Aspects of Plant Development 2010, Vienna, Austria	Feb 23-26, 2010
► **Presentations**	
Poster at Auxin 2008	Oct 04-09, 2008
Oral Presentation at Molecular Aspects of Plant Development Conference, 2010	Feb 25, 2010
Oral Presentation at ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands 2011	Apr 05, 2011
► **IAB interview** (Prof.dr. Angenent)	Dec 02, 2009
► **Excursions**	
Scientific PhD Excursion group prof. Sacco de Vries, Barcelona	Apr 21-25, 2009
Subtotal Scientific Exposure	*16,6 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
International PhD Summer School 'Environmental Signaling; Arabidopsis as a model'	Aug 27-29, 2007
PhD Course Bioinformatics - A User's Approach	Aug 30-Sep 03, 2010
► **Journal club**	
Weekly journal club (Dolf Weijers group)	2007-2011
► **Individual research training**	
Subtotal In-Depth Studies	*5,4 credits**

4) Personal development	date
► **Skill training courses**	
Information Literacy including Endnote	Dec 07-08, 2009
Techniques for Writing and Presenting a Scientifc Paper	Jun 29-Jul 02, 2011
Advanced Course Guide to Scientific Artwork	Nov 04-05, 2010
Adobe InDesign Course	Nov 15, 2010
Educational Staff Development Course: Teaching and Supervising Thesis Students	Jun 17-18, 2010
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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