Identification of Novel Auxin Responses during Arabidopsis Embryogenesis

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Thesis

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Think of the fierce energy concentrated in an acorn!
You bury it in the ground, and it explodes into an oak!
Bury a sheep, and nothing happens but decay.
GEORGE BERNARD SHAW

Contents

Introduction

Got Root?
Initiation of the Embryonic Root Meristem

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Abstract

Plant development relies on the activity of meristems, small groups of undifferentiated cells that produce all organs. The first meristems are formed in the embryo, and all subsequent development depends on their proper establishment, making embryonic meristem initiation a key step in plant life. The founder cells of the embryonic meristems are specified early in embryo development after the establishment of the body axis. Initiation of the root meristem in the early embryo is marked by the specification of a single cell, the hypophysis, and hence an attractive model to study meristem initiation. In this review, we will discuss the mechanisms that control embryo axis formation and root meristem initiation.

Plants are produced by their meristems, the plant equivalent of stem cell niches. These meristems are located at the growing tips of all higher plants, and generate leaves, shoots, flowers and roots. Even though the type of organ produced by each meristem is different, the underlying principle of meristem activity is a general one. On one hand, meristems must produce differentiated cells that are incorporated into the new organ, yet on the other hand, a population of undifferentiated stem cells must be maintained. To this end, meristems consist of two cell populations: a group of stem cells and a group of organizing cells that control the stem cells (Jürgens, 2003); (Stahl and Simon, 2005). A critical question in plant biology is how such a meristem is set up. The first meristems are found in the seedling, and are made during embryogenesis. We will first describe embryogenesis in the model plant *Arabidopsis thaliana* and then discuss the mechanisms controlling the events leading to the initiation of the root meristem.

Embryogenesis in *Arabidopsis*

Embryo development in *Arabidopsis* can be divided into three major phases of development. Immediately after fertilization, the basic body plan is established and consists of an apico-basal axis combined with a radial pattern of different tissues. Following this initial phase of pattern formation (Jürgens and Mayer, 1994), the embryo grows by cell division and elongation until it fills the seed at full maturity. Finally physiological processes of storage and desiccation prepare the seed for dormancy until it germinates.

The first microscopically observable event after fertilization is the rapid stretching of the zygote towards the central part of the endosperm (**Fig. 1A, 1B**). During this process, organelles of the zygote are relocated such that the nucleus is positioned in the apical part of the cell while the basal part holds the vacuole. A horizontal division in the upper half of the zygote yields two morphologically different daughter cells (**Fig. 1C**). The smaller cytoplasm-rich apical cell undergoes a relatively fast series of two vertical divisions and one horizontal division to form a spherical proembryo consisting of an upper and a lower cell tier of 4 cells each (**Fig. 1D, 1E**). This proembryo rests on top of an extraembryonic filamentous structure (suspensor) that is formed by descendants of the larger vacuolarized basal zygote daughter cell by a few rounds of horizontal divisions. Upon these initial cell divisions, the 8 cells of the proembryo undergo a round of periclinal divisions that divides the proembryo into an outer protodermal cell layer (epidermis precursors / protoderm) and an inner set of cells (ground tissue and vascular tissue precursors) (**Fig. 1F**). At about this stage, the uppermost suspensor cell bulges into the proembryo (**Fig. 1G**) and divides asymmetrically to yield a smaller apical and a larger basal cell (**Fig. 1H**).

At the now reached dermatogen stage, the embryo consists of three different domains anchored in the ovule via the suspensor. While cells of the uppermost domain give rise to the shoot apical meristem and most of the cotyledons, abaxial (lower) parts of the cotyledons, the hypocotyl and most of the embryonic root meristem are generated by the lower domain. The quiescent center and columella root cap of the root apical meristem are derived from the hypophysis (**Fig. 1H, 1I**). Cells of this lineage divide somewhat slower than the cells in the two other domains. A horizontal division at mid-globular stage (**Fig. 1H**), divides the hypophysis into a smaller apical lens-shaped cell and a larger basal cell. Two vertical divisions of the lens

shaped cell form the quiescent center, a small group of cells that control the surrounding stem cells of the root apical meristem (RAM). Descendants of the basal cell form stem cells and outer cell layers of the central root cap (columella) (**Fig. 1I**).
Since the formation of the RAM can be recognized somewhat earlier than the formation of the SAM and is not complicated by the formation of any attached organs we will focus in this review on the processes leading to the initiation of the RAM.

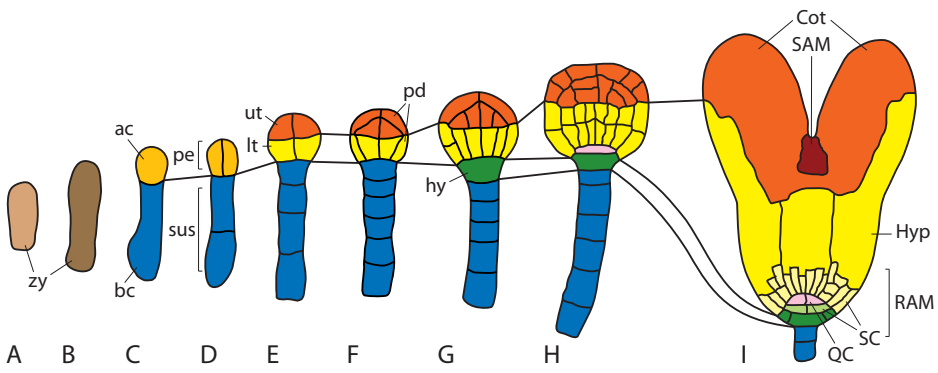

Figure 1: Stages of *Arabidopsis* **embryo development.**
From left to right, drawings represent successive stages of embryogenesis from the zygote (A) to the late heart stage (I). Clonally related regions are marked with distinct colors, and connected between embryos by lines.
Upon fertilization, the zygote (zy, A,B) elongates, and divides asymmetrically to yield a smaller apical cell (ac, C; 1-cell stage) and a larger basal cell (bc, C). These cells then undergo different developmental programs, with the basal cell giving rise to the filamentous suspensor (sus, D; 4-cell stage), and the apical cell generating the proembryo (pe, D). The 4 cells of the proembryo divide horizontally to generate an upper tier (ut, E; 8-cell stage) and a lower tier (lt, E) of 4 cells each. All 8 proembryo cells then divide periclinally to set apart the protoderm (pd, F; dermatogen-stage), the epidermis precursor. Subsequently, in the early globular stage (F), the uppermost suspensor cell bulges into the proembryo, which marks its specification as hypophysis (hy), the root meristem founder cell. Next, the hypophysis divides asymmetrically to generate a lens-shaped apical daughter and a larger basal daughter cell (H; transition stage). The lens-shaped daughter will generate the quiescent center, the root meristem organizing center. At the same time, the lateral flanks of the proembryo apex flatten, marking the future cotyledon primordial. At the heart stage (I), all seedling structures can be recognized, with two cotyledons (Cot) flanking the shoot apical meristem (SAM) at the embryo apex, the hypopcotyl (Hyp) in the center and the root apical meristem (RAM) at the embryo base. At this stage, the root meristem is already composed of a quiescent center (QC) that is surrounded by stem cells (SC) for all cell types in the root.

Formation of the embryonic root meristem

Obviously, the observed pattern of cell divisions and the establishment of different cell types are the outcome of an underlying genetically controlled program. While the embryo develops, certain sets of genes are switched on or off in the emerging cell types, thus marking differences in cell fate well before differentiation or division. In the following sections, we will discuss possible mechanisms of cell specification and pattern formation in the early *Arabidopsis* embryo that ultimately lead to the initiation of the root meristem.

Establishment of the embryo axis

Plant embryogenesis occurs in an extremely polar environment: the maternal tissues of the ovule are polar in that the egg apparatus is positioned at one end of the embryo sac, and the antipodes on the other. Nutrient supply from the mother plant to the developing seed occurs at this antipodal (chalazal) region, which again imposes polarity upon seed development. Furthermore, even before fertilization, the egg cell itself is highly polar, with the vacuole located at the basal and the nucleus at the apical end (Mansfield and Briarty, 1991). Hence, it is questionable whether zygote polarity is autonomously established at all, or if this is through maternal control. No zygotic mutants have so far been identified where polarity of the zygote is clearly disrupted, and it might be envisioned that such mutants would be maternal effect mutations.

Several mechanisms could account for differential specification of apical and basal daughter cells during zygote division. The two most extreme are 1) that two equivalent cells are generated by the anatomically asymmetric division, that become instructed for apical and basal fates by extrinsic cues, derived for example from maternal tissues, or from endosperm; 2) that intrinsic factors within the zygote (organelles, cell wall or membrane determinants, proteins, mRNAs, etc.) are unequally partitioned in the two daughters, thereby creating two cells that are immediately different.

Although not excluding other models, the only study (Haecker *et al.*, 2004), that addresses this problem, suggests the latter of the two mechanisms. While the expression of many genes is specifically induced in the apical or basal cell lineage after the division, the mRNAs of two *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes, namely *WOX2* and *WOX8,* are already present in the zygote and even in the egg cell (**Fig. 2A, 2B**). However, with zygote division, they become separated from each other. *WOX2* mRNA is restricted to the apical cell and its descendants, whereas *WOX8* mRNA is found only in the basal cell and its daughters (**Fig. 2C-E**). This pattern is also consistent with a scenario where the mRNAs are cleared before zygote division and asymmetrically reestablished after division, but the more probable explanation is that the mRNAs are differentially partitioned during zygote division. *wox8* mutants do not show defects in the early embryo, and it is therefore unclear what the relevance of its expression is for embryo polar axis formation. Redundancy of *WOX8* with its close homolog *WOX9* might explain the absence of a phenotype, particularly since *WOX9* is also expressed in the basal cell after zygote division (**Fig. 2C**). Interestingly, mutations in *WOX2* interfere with normal development of the proembryo, already at the 2-cell stage, suggesting that *WOX2* function is required in those cells where its mRNA accumulates. This is also the case

later in proembryo development. When the proembryo consists of 8 cells, it is composed of an upper and a lower tier of 4 cells each. These two tiers have distinct developmental fates as they will give rise to different parts of the seedling. This difference between upper and lower tier in the 8-cell proembryo is foreshadowed by domains of *WOX* gene expression.
When two tiers are formed, *WOX2* mRNA is expressed only in the upper tier (**Fig. 2E**). In *wox2* mutants, subsequent divisions in this upper tier are erroneous, suggesting that *WOX2* function is also required for normal patterning of this region.
At the same time, *WOX9* is activated in the lower tier in addition to its expression in the uppermost suspensor cell (**Fig. 2E**). Therefore, expression of *WOX2*, *8* and *9* marks a pattern along the apicobasal axis with four regions: An apical tier that expresses *WOX2*, a basal tier marked by *WOX9*, the uppermost suspensor cell that harbors both *WOX8* and *9* and the subtending suspensor cells where only *WOX8* is active.

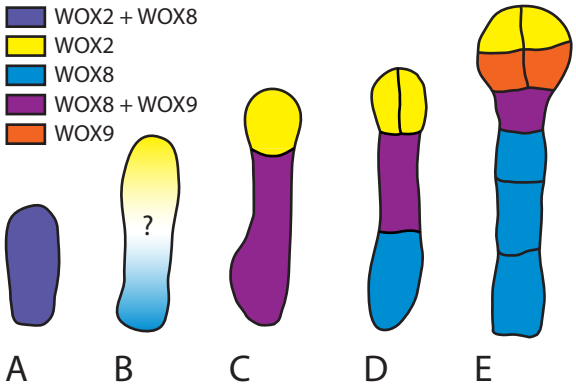

Figure 2: Cell specification in the early *Arabidopsis* embryo as revealed by WOX gene expression.
The mRNA accumulation of *WOX2, 8* and 9, and combinations thereof is depicted in different colors (see color legend), for early embryogenesis stages ranging from zygote (A) through 8-cell stage (E).
Both *WOX2* and *WOX8* are expressed in the zygote (A,B), but accumulate differentially upon division (C). In the basal cell, *WOX8* is co-expressed with *WOX9* (C), but after the division of the basal cell, WOX8 remains in the entire suspensor whereas WOX9 is restricted to the upper suspensor cell (D,E). After formation of an upper and a lower tier in the 8-cell proembryo (E), *WOX* expression marks 4 domains, with *WOX2* being expressed in the upper tier, *WOX9* in the lower tier, *WOX8* and *9* in the uppermost suspensor cell and *WOX8* in all other suspensor cells.

Development and maintenance of the suspensor

WOX8 and *9* mRNA accumulation reveals that expression patterns can be dynamically controlled within the suspensor, suggesting functional specialization of suspensor cells. Very little is known about how suspensor development is controlled, but it was recently shown that a MAP Kinase cascade might be involved.
The mitogen activated protein kinase kinase kinase (MAPKKK) YODA (YDA) is required for proper suspensor development (Lukowitz *et al.*, 2004). In *yda* mutants, the elongation of the zygote prior to division is impaired, allowing the generation of a normal sized apical daughter cell and only a shortened basal cell. While the apical cell develops normally until the 8-cell stage, the shortened basal cell fails to give rise to a proper suspensor. Cells of the basal lineage do not divide horizontally, but divide randomly, eventually leading to a loss

of the clear boundary between suspensor and proembryo at the 8-cell stage. Whereas these results can be explained by YDA being a regulator of cell elongation rather than suspensor cell fate, one finding suggests that YDA function is intimately connected with the latter. Plants where YDA is constitutively active show the formation of a suspensor-like cell file at the expense of proembryo development. Thus activity of YDA is necessary to promote extra-embryonic cell fate in the basal lineage while enhanced activity in the apical lineage is sufficient to activate a suspensor-like developmental program. Recently, good candidates were identified for the kinases that act downstream of YDA in a canonical MAP kinase cascade. Post-embryonically, YDA controls stomatal versus non-stomatal cell fate (Bergmann *et al.*, 2004). Mutations in both MAPK kinases MKK4 and MKK5 or in both MAP kinases MPK3 and MPK6 show phenotypes in stomatal development very similar to those in *yda* mutants (Wang *et al.*, 2007b). Furthermore, the *mpk3 mpk6* double mutant shows a *yda*-like embryo phenotype which suggest that both MAP kinases also act downstream of YDA during embryogenesis. Important remaining questions are what the input signals and output responses are. Two additional mutants with *yoda*-like early embryo defects, *grounded* (*grd*) and *short-suspensor* (*ssp*), have been isolated (Lukowitz *et al.*, 2004). These genes might encode such in- or output components and therewith make the YDA-MKK4/5-MPK3/6 cascade specific for early embryogenesis.

Mutations in *yda* prevent suspensor formation at the earliest stages, and consequently, *yda* mutants have at best a rudimentary suspensor. In another class of *Arabidopsis* mutants, the suspensor is initially properly specified, but not maintained. Here, suspensor cells start to proliferate, and in cases behave much like embryo cells. Roughly, these mutants can be divided into two categories. The first type of mutants includes loss-of-function mutations in the *SUSPENSOR1-3* (*SUS1-3*) (Schwartz *et al.*, 1994) and *RASPBERRY1-3* (*RSY1-3*) (Yadegari *et al.*, 1994; Apuya *et al.*, 2002) genes, as well as a host of mutations in housekeeping genes (www.seedgenes.org). In these mutants, suspensor proliferation is preceded by visible defects in the proembryo at the globular stage. This initiates a transformation of suspensor cells towards embryonic cell fate but when suspensor-derived embryos reach the equivalent of the globular stage, also these arrest, so that no viable second embryo is formed.

This is different in mutants of the *TWIN* (*TWN*) type, where the secondary embryo developing from the suspensor does develop into a mature embryo. Of the *TWN* class mutants, there is one, *twn2*, where the proembryo arrests at very early stages prior to the formation of suspensor-derived embryos (Zhang and Somerville, 1997). Two others, *twn1* and *amp1* do not affect the proembryo, but do show suspensor-derived embryos (Vernon and Meinke, 1994; Vernon *et al.*, 2001).

It has also been found that experimental interference with proembryo development, for example by acid treatment, X-ray irradiation (for review see (Yeung and Meinke, 1993)) or toxin expression (Weijers *et al.*, 2003), also leads to proliferation of suspensor cells. Therefore, it is likely that the proembryo is a source of signals that actively repress embryogenesis in suspensor cells.

Mutants of the first class (including *SUS* and *RSY*) affect the suspensor indirectly through impairment of general functions in the proembryo, very much like for example X-ray irradiation or toxin expression. The *TWN* class mutations however, might affect the suspensor more directly by interfering with embryo-dependent repression. This interpretation is supported by the isolation of only a handful of this type of mutants. *TWN2* encodes a valy-tRNA synthetase (Zhang and Somerville, 1997), *AMP1* encodes a glutamate carboxypeptidase (Hel-

liwell *et al.*, 2001), and the *TWN1* gene has not been identified yet. Although AMP1 might be involved in generating a presently unknown signaling molecule, the anonymous character of TWN1 and the supposed housekeeping function of TWN2 do not allow a mechanistic model to be drawn. In addition, it should be noted that for both *twn1* and *twn2* mutations, only a single allele has been reported, and it is therefore unclear whether or not these might be neomorphic.

Specification of hypophysis cell fate

During normal embryogenesis, the uppermost suspensor cell behaves rather differently from the other suspensor cells. This is observed at the anatomic level by its asymmetric division, but also by the activity of several genes. For example, the *WOX* genes are dynamically regulated during hypophysis establishment, with *WOX8* mRNA being lost specifically from the upper suspensor cell after hypophysis specification, and another member of the *WOX* family, *WOX5*, being switched on in this cell immediately upon its specification (Haecker *et al.*, 2004). Several other genes are specifically activated in the hypophysis, which suggests significant transcriptional reprogramming. Since at least one other suspensor-specific gene is not downregulated in the hypophysis (E. Rademacher and D. Weijers, unpublished), hypophysis fate does not seem to be specified at the full expense of suspensor fate, but rather in addition to it.

Specification of the upper suspensor cell as hypophysis not only marks the initiation of the root meristem, it is also of paramount importance for root formation. This is highlighted by the finding that the earliest defect in all rootless mutants is during hypophysis division. The two most well-studied rootless mutants are *monopteros* (*mp*) and *bodenlos* (*bdl*) (Berleth and Jurgens, 1993; Hamann *et al.*, 1999). Both mutations affect hypophysis-specific gene expression prior to division (D. Weijers, unpublished). Since *mp* and *bdl* mutants show almost identical embryo phenotypes, it has been suggested that MP and BDL act in a common pathway (Hamann *et al.*, 1999). This indeed appeared to be the case, since map-based cloning showed them to be an antagonistic pair of transcription factors that regulate gene expression in response to the plant hormone auxin (Hardtke and Berleth, 1998; Hamann *et al.*, 2002). *MP* encodes AUXIN RESPONSE FACTOR 5 (ARF5), which activates auxin-responsive genes. *BDL* encodes Aux/IAA12, a protein that binds MP and prevents activation of MP-dependent genes (Hamann *et al.*, 2002; Weijers *et al.*, 2005; Weijers *et al.*, 2006). Auxin activates MP by promoting the ubiquitin-proteasome-dependent degradation of BDL (Dharmasiri *et al.*, 2005). *mp* mutants are loss of function alleles whereas the *bdl* mutation prevents its auxin-dependent degradation, leading to constitutive inhibition of MP. Likewise, mutations in the ubiquitin pathway that prevent BDL degradation also impair hypophysis division and root formation (Dharmasiri *et al.*, 2003; Dharmasiri *et al.*, 2005).

Interestingly, MP does not act in the future hypophysis itself to specify its fate. Rather, MP and BDL act in the 8 inner cells of the lower tier of the 32-cell embryo (Fig 3) to control hypophysis specification in the adjacent suspensor cell (Weijers *et al.*, 2006). Hence, MP promotes signaling between a small group of proembryo cells and the neighboring suspensor cell. The non-autonomous control of hypophysis specification by factors in adjacent proembryo cells provides a very plausible explanation for why it is the uppermost suspensor cell that is specified and not any of the other cells.

This cell-cell communication could involve any possible signal, including hormones, secreted peptides or mobile mRNA or proteins. Several lines of indirect evidence suggest that auxin might be such a signal (Weijers *et al.*, 2006). An auxin-dependent reporter gene that is activated by ARF transcription factors (*DR5-GFP*) is switched on in the hypophysis at the time of its specification. Furthermore, the PIN1 auxin transporter is localized at the basal cell membranes of the inner 8 cells of the lower tier of the proembryo. Both the PIN1 protein in these 8 cells and the *DR5-GFP* activity in the hypophysis are lacking in *bdl* and *mp* mutant embryos, implying that this auxin transport is downstream of MP activity. There are however two observations that suggest auxin accumulation alone not to be sufficient for hypophysis specification. First, feeding developing *mp* or *bdl* embryos with auxin does not overcome the hypophysis division defect. Second, *DR5-GFP* is expressed in more than one suspensor cell. Yet, only the uppermost cell is specified as hypophysis. Therefore, we proposed the existence of a second embryo-derived signal involved in proembryo-hypophysis signaling (**Fig. 3**).
Particularly since the identity of a second signal is unknown, it is presently not clear how a double-input signaling system would outperform a system where only one signal is used to specify the adjacent cell. However, one could imagine a second signal that would promote the competence of the adjacent cell towards hypophysis fate and auxin to be a trigger that defines the precise timing of specification. Identification of the genes that are activated by MP prior to hypophysis specification, as well as dissection of the auxin response machinery within the hypophysis will be required to gain comprehensive understanding of this process, which is at the basis of root meristem formation. Naturally, root meristem formation is not complete with hypophysis specification, but requires further elaboration, definition of stem cells and differentiation of various cell types. For the sake of brevity, we have discussed here only the events leading to the establishment of the hypophysis, and therefore the initiation of the root meristem. Excellent reviews discuss those events that are required later to set up a

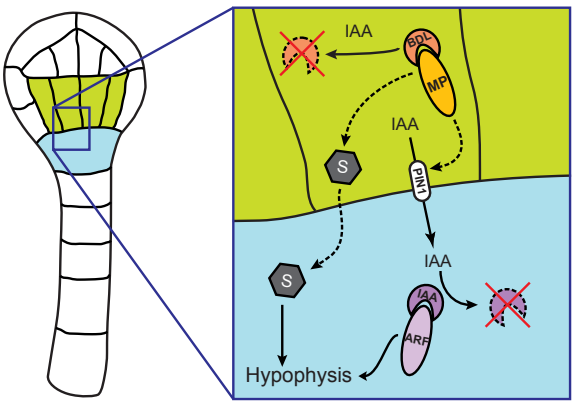

Figure 3: A model for auxin-dependent cell communication in hypophysis specification.
The future hypophysis (blue) is specified in response to signals from the adjacent central inner cells of the proembryo (green). In proembryo cells, auxin promotes degradation of BDL, thereby releasing the MP transcription factor. MP promotes transport of auxin to the adjacent suspensor cell through the membrane-localized PIN1 auxin transporter. Within this neighboring cell, auxin triggers the degradation of another Aux/IAA inhibitor (IAA), which sets free another ARF transcription factor. In parallel, MP promotes signaling to the future hypophysis through a second signal (S). Within this cell, ARF activity and the second signal (S) converge to specify its fate as hypophysis.

functional root meristem (Nakajima and Benfey, 2002; Willemsen and Scheres, 2004).

Outlook

Molecular genetic studies in *Arabidopsis* have given a fairly detailed view of the processes leading to the initiation of the first meristem. We have discussed those events required for, and preceding specification of the uppermost suspensor cell as hypophysis, the founder cell for the root meristem. Intriguingly, this cell is specified in response to signals derived from adjacent cells in the proembryo. A very important question is to what extent this cell-communication based mechanism for root meristem initiation is universal in higher plants. *Arabidopsis* embryos have exceptionally regular cell divisions, and consist of much fewer cells than embryos of many other plant species (Johri *et al.*, 1992). Nonetheless, embryos of all higher plants do consist of proembryo and suspensor, and the root meristem is initiated at the junction of the two (Johri *et al.*, 1992). Cell communication-based hypophysis specification minimally requires that two regions with different fate (proembryo and suspensor) are present, and that these exchange signals. This does not require precise relative sizes of the regions, nor does it require regular cell divisions, and could hence be operational in most if not all higher plants. Interestingly, a rice homolog of the hypophysis-specific *Arabidopsis WOX5* gene has been isolated, and its mRNA accumulates at about the position that is expected to be the rice hypophysis equivalent (Kamiya *et al.*, 2003). Several other of the *Arabidopsis* "patterning genes" have homologs with matching expression patterns in other species (Lim *et al.*, 2000; Nardmann and Werr, 2006; Prigge and Clark, 2006), which does suggest conservation of patterning mechanisms across species.

Acknowledgments

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Scope of the Thesis

Apparently during embryogenesis various mechanisms are facilitated to coordinate morphogenesis in a spatio-temporal manner. A large number of factors contributing to diverse aspects of this phase in embryo development has been revealed by extensive screenings of embryos developing from mutagenised plants. However all these processes are fairly unconnected and research was focused on the specific output of each.
In plants physiological processes are controlled by the action of plant hormones that elicit specific responses at the sites they are perceived.
Over time the group of the five classical plant hormones auxin (IAA), gibberellins (GAs), cytokinins (CKs), ethylene and abscisic acid (ABA) has been enlarged by the discovery of additional hormonal compounds such as Polyamins, Brassinosteroids, Jasmonates, Salicylic acid and recently Strigolactones. Together these compounds offer a wide range of possible signals to control developmental processes.
However auxin has been shown to influence a far greater range of mechanisms than any other plant hormone. In addition it is the only one that is transported by a sophisticated intercellular network of carriers to ensure proper localization of its activity within the target tissues.
To this point auxin has been shown to be relevant for the control of root meristem and cotyledon formation, whereby both processes are controlled mainly by ARF5/MP and its inhibitor IAA12/BDL as explained before. Several lines of evidence such as the presence of auxin efflux carriers, the activity of an auxin responsive promoter and the larger number of *ARF*s suggest that more events in embryogenesis are auxin dependent.
Potentially the auxin transport network and a multitude of auxin responses facilitated by the ARFs would allow auxin to act as a spatio-temporal trigger that coordinates and directs embryo patterning.

In **Chapter II** we explore the involvement of auxin regulation in embryogenesis by cell type specific misexpression of stabilized *iaa12/bdl* and consequently inhibiting the respective auxin responses. This results in the identification of two novel auxin responses that have not been described previously. In particular, we provide evidence that auxin response is instrumental in maintaining cell fate in the suspensor.

Chapter III focuses on the *ARF* family and describes the expression patterns of all *ARF*s in the form of a comprehensive expression map. This not only demonstrates which ARFs are involved in the auxin responses found in Chapter II but also highlights a general mechanism to generate the well-known diversity of transcriptional responses to auxin stimuli.

In **Chapter IV**, we describe efforts to remove all ARF activity from the suspensor by creating multiple mutant combinations of *ARF*s that we found to be co-expressed in this cell type. The resulting embryonic phenotypes validate our expression-based selection of redundant ARFs and demonstrate the potency of our data in guiding the dissection of auxin response networks during embryogenesis.

ARF expression patterns diverge from early stages on, and the cohort of *ARF*s expressed in a cell determines the cell type-specific auxin response output (Chapter II). Therefore, the crucial outstanding question is how the different *ARF* expression patterns are set up. In **Chapter V** we investigate *ARF* expression in mutants that affect two early patterning pathways, and find that *ARF* expression domains are defined by at least three distinct mechanisms that provide both positive and negative transcriptional input.

In the **Summarizing discussion** we discuss implications of the work in this thesis and provide abstractions of how the simple auxin molecule can act as a versatile trigger in controlling different processes in the early embryo.

Chapter 2

Distinct Auxin Response Machineries control separate Cell Fates in the early Plant Embryo

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Abstract

Plant development in largely involves the activity of auxin to trigger specific processes. However in the morphogenetic phase of embryogenesis only specification of the root meristem and separation of cotyledons have been shown to depend on the action of an auxin response machinery. The core of such a machinery is formed by an inhibitory Aux/IAA and a transcription regulating auxin response factor (ARF). To unveil other morphogenetic auxin response machineries we employed the unspecific inhibition of ARFs by stabilized Aux/IAAs to inhibit auxin responses in various domains of the forming embryo. This revealed novel auxin responses involved in protoderm formation and suppression of secondary embryogenesis in the suspensor. We identified Aux/IAA10 and ARF13 as components of the suspensor auxin response machinery and with these we uncovered the first cell-autonomous process involved in suspensor cell fate maintenance.
Promoter swap experiments between the suspensor specific ARF13 and the embryo specific ARF5/MP demonstrated that ARFs posses intrinsic properties to modulate auxin responses. Hence cellular responses to auxin are prepatterned by the coordinated expression of distinct ARFs in suspensor and embryo.

Introduction

Embryogenesis in *Arabidopsis* distinguishes itself by a highly regular patterning of the two structures involved. These two structures - embryo and suspensor – develop from the zygote, but while the embryo emerges through a series of rapid cell divisions of the apical daughter cell, cells of the suspensor divide slowly and mostly do not differentiate into other cell types. In fact the role of most suspensor cells does not go beyond supporting the embryo and connecting it to maternal tissue. Only the uppermost suspensor cell becomes respecified and incorporated into the embryo as hypophysis, the founder cell of the root quiescent centre (QC) and columella.
This respecification crucially depends on the action of AUXIN RESPONSE FACTOR 5 / MONOPTEROS (ARF5/MP) which controls intercellular signaling events from the adjacent embryonic cells towards the later hypophysis. Like every canonical ARF, ARF5/MP is regulated in its activity by an interacting inhibitory Aux/IAA (here IAA12/BODENLOS (BDL)); high auxin levels enhance the degradation of the Aux/IAA and thus release the ARF from inhibition. Surprisingly, next to the ARF5/IAA12 controlled hypophysis specification, no other processes have been described to be auxin dependent during early embryogenesis. However activity of the DR5*rev* auxin response marker as well as the presence of auxin efflux carriers of the PINOID (PIN) family at early embryonic stages suggest auxin dependent signaling to be involved in further processes. In this chapter we have systematically probed auxin-dependent processes in the embryo by local misexpression of the ARF inhibitor protein iaa12/bdl. This revealed novel auxin responsive processes, including the maintenance of extra-embryonic cell fate in the suspensor. Our further analysis of the response identifies Aux/IAA and ARF components that regulate this endogenous response. Promoter swap experiments show that the suspensor-specific auxin response machinery is functionally distinct from the proembryonic auxin response machinery. Hence different early embryo lineages express distinct auxin response components, which endow these cells with unique developmental responses to the plant hormone auxin.

Results

Systematic cell-specific *iaa12/bdl* misexpression identifies novel auxin-responsive processes

To unravel the involvement of auxin in early morphogenetic processes in the *Arabidopsis thaliana* embryo, we took advantage of the limited specificity of ARF inhibition by Aux/IAA proteins (Weijers *et al.*, 2005). Inhibition of ARFs can be rendered auxin-independent by introducing a stabilizing mutation in Aux/IAA proteins that prevents auxin-dependent degradation. When strongly expressed, such a stabilized aux/iaa protein induces loss of most if not all ARF functions in the expression domain.
We achieved local expression of a stabilized aux/iaa (iaa12/bdl) by using the GAL4-*UAS* two-component gene expression system. In this approach, driver lines that express the *GAL4*

transcription factor in a specific domain are crossed with a line that expresses *iaa12/bdl* from a GAL4-dependent *UAS* promoter (Weijers *et al.*, 2006). The effects of local misexpression of *iaa12/bdl* can then be observed in F_1 embryos that originate from the cross. In total 17 different GAL4 driver lines were combined with a *UAS-iaa12* line, and as a control we crossed all driver lines with a *UAS-IAA12* line that expresses the non-stabilized IAA12/BDL protein. To cover most distinct areas of the early embryo, 14 *GAL4-GFP* enhancer trap lines were selected from the Haseloff collection based on their expression patterns during late embryogenesis (Haseloff). These patterns were first confirmed by GFP fluorescence at heart stage and extended to determine the patterns during earlier stages. In addition, we used an RPS5a driver line for ubiquitous expression and a SHOOT MERISTEMLESS (STM) driver line for hypophysis- or suspensor-specific expression (Weijers *et al.*, 2003). A complete overview of all lines used in this study is shown in table 1 and figures 1 and 2.
Expression of *iaa12/bdl* under the control of 6 of these driver lines led to several classes of phenotypic aberrations, while 11 other driver lines did not induce iaa12/bdl-dependent developmental abnormalities (Table 1). The validity of our approach is exemplified by the occurrence of *mp*-like monocotyledonous and rootless seedlings as when driving *iaa12/bdl* expression in cells normally expressing *ARF5/MP* (KS068, M0171, STM, RPS5a and Q0990; Table 1; Fig. 2, (Weijers *et al.*, 2006)). It is noteworthy that Q0990 drives *iaa12/bdl* expression only in provascular cells adjacent to the hypophysis (Fig. 2 w and x) and only affected root meristem formation while leaving ARF5/MP - dependent cotyledon initiation unaffected. This highlights the capability of our method to locally inhibit auxin responses in the embryo and dissect auxin responses in a cell type based manner.
Novel phenotypes were found when *iaa12/bdl* expression was driven by the RPS5a, J3281,

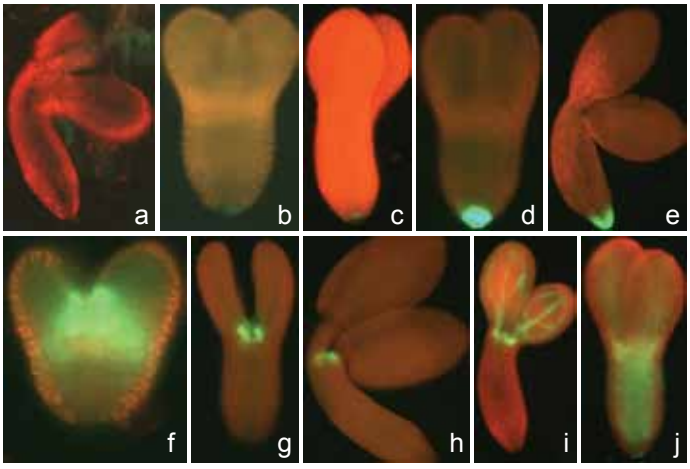

Figure 1: Expression domains of aphenotypic GAL4 driver lines with the following Haseloff Collection numbers:
a: M0136; b: Q0680; c: Q1630; d: M0223; e: J1092; f: M0148; g: M0028; h: M0167; i: M0164; j: J0571;

M0171, STM, and KS068 driver lines (Fig. 2). In these crosses, two distinct processes were affected. On one hand, ubiquitous expression (RPS5a and J3281) led to abnormal cell divisions during protoderm specification (Fig. 2 b and i). As a result, developing embryos often died at later stages but in some cases developed to seedlings with pleiotropic defects such as altered cotyledon numbers and the appearance of callus-like tissue (Fig. 2 e and j). Auxin response has not previously been associated with protoderm establishment, rendering this a novel auxin-dependent process.

Strikingly, expression of *iaa12/bdl* in the suspensor, using RPS5a, M0171, STM or KS068 driver lines, resulted in strong proliferation of suspensor cells and the formation of spherical structures within the former suspensor (Fig. 2 c, n and u). To test whether the observed phenotypes indeed result from ARF inhibition by iaa12/bdl and not from other properties intrinsic to IAA12/BDL, we investigated the effects of non-stabilized IAA12/BDL driven by the same driver lines. We did not observe defects in any of these control crosses, strongly suggesting that phenotypes arise from inhibition of ARFs by iaa12/bdl.

Suspensor proliferation has been reported as a consequence of death, arrest or ablation of the primary embryo, as for example in *suspensor (sus)*, *raspberry (rsp)* and *twin2 (twn2)* mutants. Although the potential of suspensor cells to divide and develop has been appreciated, the cell-autonomous pathways that control its proliferation are completely unknown. Interestingly, expression of *iaa12/bdl* in the proembryo alone never led to any suspensor phenotype, identifying auxin response as the first cell autonomous component in the maintenance of suspensor quiescence.

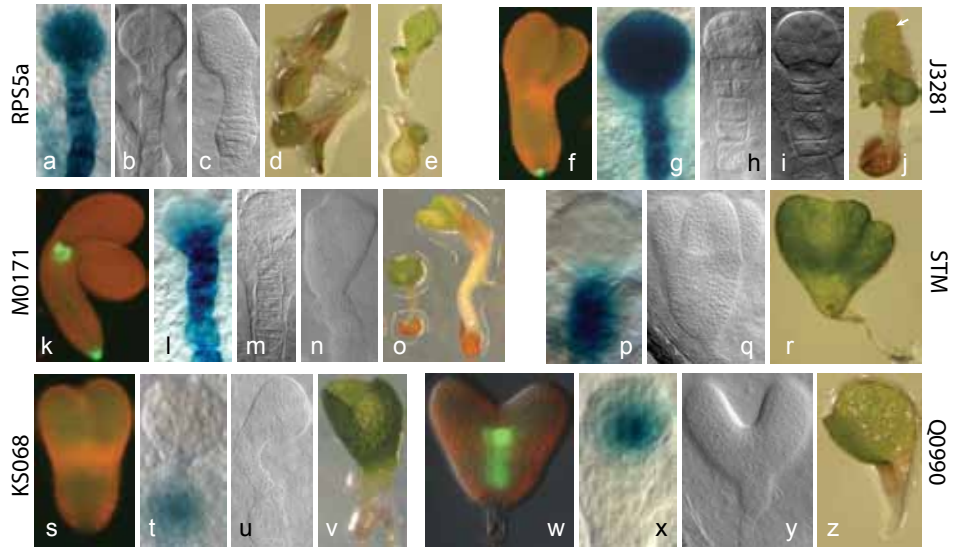

Figure 2: Phenotypes caused by *bdl* expression under control of certain GAL4 driver lines

Expression of *bdl* under control of RPS5a (a - e), J3281 (f - j), M0171 (k - o), STM (p - r), KS068 (s - v) and Q0990 (w - z) caused aberrant divisions in suspensor cells and the protoderm as well as fusion of cotyledons and disruptions in root meristem formation. Expression was monitored in all cases by GUS staining at dermatogen stage and GFP detection at late heart stage.

Ubiquitous expression of *bdl* in RPS5a and J3281 driver lines caused aberrations in the formation of the protoderm. Resulting seedlings often died, but if they developed further they were severly misshaped (d, e, j). In addition RPS5a driven *bdl* expression also resulted in aberrant proliferation of the suspensor thereby interfering with proper root meristem formation.

Auxin response controls suspensor cell fate maintenance

Considering the dramatic defect observed upon *iaa12/bdl* misexpression in the suspensor, we investigated the suspensor phenotype in greater detail. At first we asked whether suspensor cells are merely excessively dividing whilst maintaining their extra-embryonic fate or alternatively if suspensor cell fate is changed towards an embryonic fate. Few suspensor-specific genes have been reported, which is why we used the suspensor-specific GFP expression in the M0171 GAL4 driver line as a reporter for suspensor identity. While suspensor-specific *GFP* expression is maintained in M0171>>IAA12 embryos (Fig. 3 a), the GFP signal disappears from M0171>>iaa12 embryos (Fig. 3 b), suggesting at least partial loss of suspensor identity.
Cell proliferation in the suspensor upon *iaa12/bdl* expression was associated with elevated expression of the cytokinesis-specific syntaxin *KNOLLE* (*KN*) which is normally found predominantly in the embryo and rarely in the suspensor (Lukowitz *et al.*, 1996). The cell groups formed by these excessive divisions could either be de-differentiated cells or could actually follow an embryonic developmental program. To determine if the latter is the case, the expression of genes normally expressed in the proembryo was tested by *in-situ* hybridization. In *RPS5A>>iaa12* embryos, we found expression of the embryo-specific ARF5/MP (Hardtke and Berleth, 1998), *WUS* (Mayer *et al.*, 1998) and *STM* (Long *et al.*, 1996) genes in the proliferating cells at the position of the former suspensor (Fig. 3 d – f). This demonstrates the establishment of secondary embryonic tissue out of the former suspensor. While severe phenotypes induced by suspensor-specific or ubiquitous *iaa12/bdl* misexpression are lethal, under certain growth conditions we observed true twin embryos in these crosses (Fig. 2 d). Hence, inhibition of auxin response by *bdl* misexpression interferes with the maintenance of suspensor cell fate and leads to respecification of suspensor cells to embryo cells.

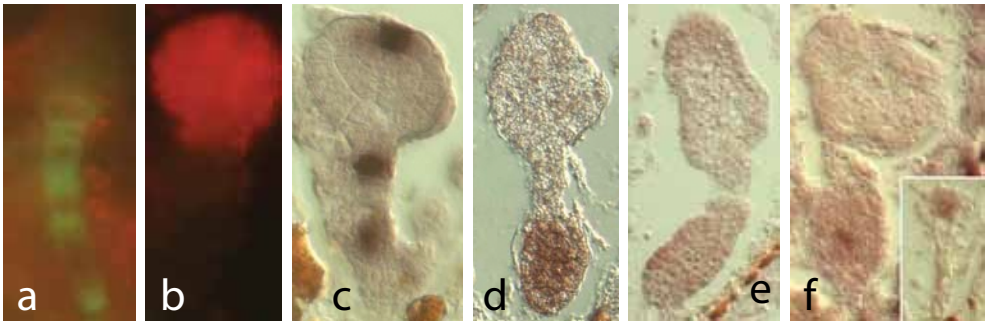

Figure 3: Marker expression in *M0171>>bdl*
GFP expression in the M0171 driver line is maintained when *BDL* is expressed (a) but absent upon expression of stabilized *bdl* (b). Cell proliferation in the suspensor is stimulated in *RPS5a>>bdl* embryos as demonstrated by enhanced expression of *KN* (c). The forming cell groups gain embryonic characteristics, which is highlighted by transcription of several embryo-specific genes (*ARF5/MP*: d, *STM*: e, *WUS*: f). The inset in f shows an wild type embryo.

IAA10 is an endogenous regulator of suspensor development

When misexpressing non-stabilized *IAA12/BDL* in suspensor cells, no defects are found, which shows that the TIR1/AFB-dependent Aux/IAA degradation machinery is active in suspensor cells. To identify the endogenous Aux/IAA that regulates suspensor development, we used public microarray expression data (Birnbaum *et al.*, 2003) to identify *Aux/IAA* genes that are preferentially expressed in the primary root tip columella cells. In the seedling root, QC and columella are the only descendants of the suspensor, and we argued that to some extent transcriptomes between these cells may overlap. The relative cell type specific, microarray-based expression level of each *Aux/IAA* was calculated as the particular expression level divided by the sum of expression levels in all 6 measured cell types in the root tip (Fig. 4). Six *Aux/IAA*s (*IAA7/AXR2, IAA10, IAA11, IAA17/AXR3, IAA20* and *IAA33*) were enriched in the columella over a 16,7% threshold defining the averaged expression level over all cell types. Out of this set, IAA20 and IAA33 were excluded since these proteins lack important conserved domains for auxin-dependent inhibition of ARF activity (Dreher *et al.*, 2006). Furthermore, reporter lines for *AXR2* and *AXR3* revealed that neither of the promoters is active during early embryogenesis ((Muto *et al.*, 2007); data not shown). For the remaining *IAA10* and *IAA11* expression patterns were determined by generating nuclear *3x GFP* (*n3xGFP*) fusions with either promoter (Takada and Jürgens, 2007).

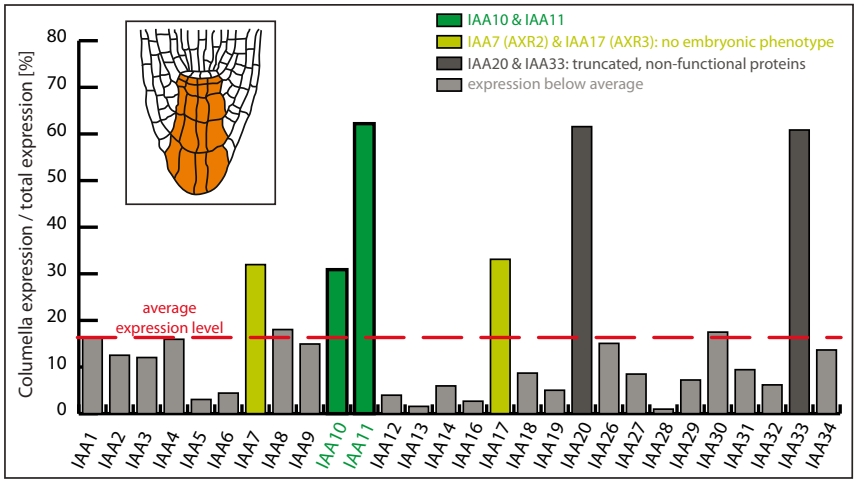

Figure 4: Relative Expression of *Aux/IAA*s in the Columella
Expression above the average expression level suggests a pronounced activity of the respective gene in columella cells

For both promoters, GFP was detected in the QC and central root cap of 6 day old roots (Fig. 5 b and i), validating the bioinformatic selection approach. Subsequently, *pIAA10* and *pIAA11* expression was analysed during embryogenesis. While *IAA11* expression is initiated after hypophysis specification and remains in the hypophysis derivatives (Fig. 5 j to l), the *IAA10* promoter marks suspensor cells as early as from the octant stage onwards, and remains active in suspensor derivatives until late embryogenesis (Fig. 5 c to e). The presence of the IAA10 protein in the suspensor was confirmed by fusing the promoter and stabilized

N-terminus (up to and excluding the C-terminal ARF interaction domain) to 3xGFP. The iaa10NT-3xGFP fusion protein was observed in the exact same cells as the transcriptional n3xGFP fusion (Fig 5 f, g).

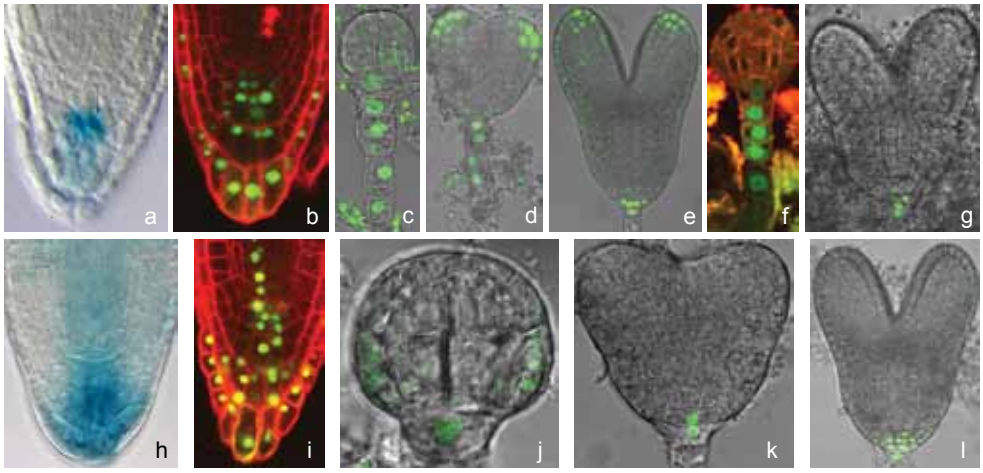

Figure 5: Expression patterns of *IAA10* and *IAA11* in roots and embryos
Signals steming from the expression of translational (a: *pIAA10::iaa10:GUS*) and transcriptional fusions (b: *pIAA10::n3GFP*) of *IAA10* were detected in the columella of roots of six day old seedlings. During embryogenesis *IAA10* expression was observed in tips of emerging cotyledons, the embryonic root pole and the suspensor when investigating *pIAA10::n3GFP* lines (c to e) and fusions of the N-terminus of iaa10 with 3GFP (f, g).
Activity of translational and transcriptional fusions with *IAA11* was present in the columella and vasculature of roots of 6 day old seedlings (h: *pIAA11::iaa11:GUS*; i: *pIAA11::n3GFP*). Expression controlled by the *IAA11* promoter was found solely in the columella of the forming embryonic root and the hypophysis (j - l).

To test the involvement of IAA10 and IAA11 in suspensor development, we engineered a stabilizing P to S mutation in domain II of each protein in the context of a genomic fragment containing the full promoter. Embryos of *pIAA11::iaa11* plants showed a low frequency of aberrant cell divisions in the hypophysis and embryonic root tip (Fig. 6 g to j) but never in the suspensor. Embryos of *pIAA10::iaa10* plants also showed abnormal hypophysis division (Fig. 6 a), and in addition displayed abnormal divisions in the suspensor, ranging from excessive divisions along the normal plane (Fig. 6 b) to misorientation of division planes in suspensor cells as early as from the one cell stage onwards (Fig. 6 c - f; Table 2). These abnormal vertical divisions resulted in the formation of extra cell files just beneath the primary embryo proper (Fig. 6 b to d). Combining *iaa10* and *iaa11* mutations did not enhance the *iaa10* phenotype confirming the discrete role of IAA10 in suspensor development and placing *iaa11* function at later processes of embryonic root development.
Despite the abundance of IAA10 in suspensor cells, and the suspensor defects induced by the *iaa10* mutation, we never observed proliferation of all suspensor cells or the formation of secondary embryos in *iaa10* plants, nor did we find persistent defects in seedlings. A likely explanation for this is the involvement of an auto-regulatory feedback loop. In this case constitutive iaa10 activity would negatively regulate suspensor cell fate maintenance, thereby attenuating its own suspensor-specific expression. Consistent with such a negative feedback loop, mutant iaa10-GUS protein can be detected during early stages, but GUS activity is lost at globular stage (not shown).

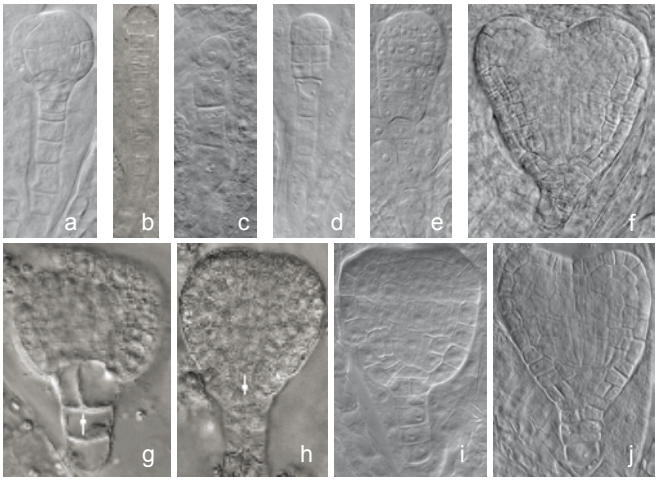

Figure 6: Embryo phenotypes of *iaa10, iaa11* and *iaa10 iaa11* mutants
When expressed from its genomic promoter stabilized *iaa10* induced phenotypes of differing strength. In weaker cases only the hypophyseal division was misoriented (a). In other cases suspensor cells divided faster than normal and longer suspensor cell files formed (b). Frequently aberrant divisions in the suspensor and enhanced proliferation led to the establishment of additional cell files adjacent to the embryo (c - f).
Expression of stabilized *iaa11* from its own promoter resulted in some cases in aberrant hypophysis divisions with subsequently disordered root poles (g, h).
iaa10 iaa11 double mutant embryos did not show a more severe phenotype than *iaa10* single mutants (i, j).

To determine the consequences of continuous presence of stabilized iaa10 protein in suspensor cells, *iaa10* was expressed from a suspensor-specific promoter. In such pSUSP::*iaa10* lines, two classes of phenotypes were observed. One class included stronger suspensor proliferation defects (Fig. 7 a - c), resembling those in the M0171>>*iaa12* embryos. Here, secondary embryo-like structures could be observed (Fig. 7 c), showing that auxin-dependent degradation of IAA10 is required for suspensor fate maintenance. The other class of phenotypes included hypophysis division defects (Fig. 7 d) and the occurrence of rootless seedlings (Fig. 7 e). In contrast to the rootless *arf5/mp* mutant, *pSUSP::iaa10* embryos were normal in all areas except the hypophysis derivatives (Fig. 7 d, e). This result highlights the importance of auxin response in both proembryo (ARF5/MP-dependant) and hypophysis (IAA10 - dependent) for root initiation. In summary, IAA10 forms the Aux/IAA part of a novel suspensor specific auxin response machinery that is required for both suspensor maintenance and root initiation.

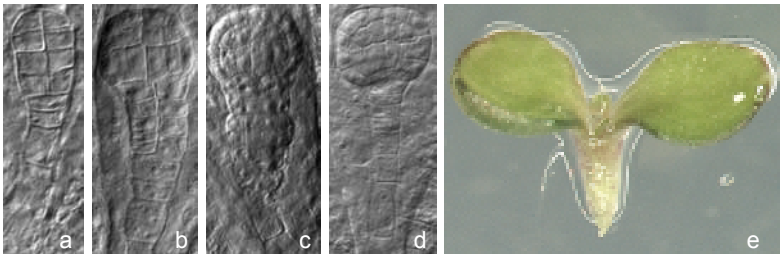

Figure 7: *pSUSP::iaa10* embryos phenotypically resemble M0171>>bdl embryos
Expression of iaa10 from a suspensor specific promoter resulted in two classes of embryonic phenotypes. On one hand aberrant divisions in the suspensor caused the formation of extra cell groups (a - c), while in other embryos solely the hypophysis divided in an aberrant plane (d). However these plants failed to establish a proper root meristem and hence remained rootless (e).

ARF activity is required for normal suspensor development

Typically Aux/IAAs exert influence on transcription by inhibiting the activity of ARFs. To this point none of the 23 ARFs found in *Arabidopsis* has been reported to be expressed in the suspensor. Furthermore despite the availability of T-DNA insertion lines for all ARFs not all knock-out mutants have been analyzed in terms of aberrant suspensor development. As a first step in identifying the ARF(s) that mediate auxin response in the suspensor, all available *ARF* knock-out lines were inspected for suspensor and embryo defects (Table 3). Even though we observed all embryonic and post-embryonic aberrations that had been reported for several ARFs, we did not find any changes in suspensor development in any of the *ARF* mutants (no significant defects in over 100 embryos observed for each line). Hence suspensor auxin response must be mediated by redundant ARFs.

Redundancy of functionality has already been demonstrated for almost all sister pairs in the family. But a cluster of eight highly similar genes has not been tackled so far. Genes in this cluster (*ARF12* to *ARF15*, *ARF20* to *ARF23*) share more than 90% identity in their genomic sequences including 1 kb upstream and 0.3 kb downstream of the particular open reading frames. RT-PCR analysis has shown that at least one of these 8 *ARF*s is expressed in siliques during early embryo development (Okushima *et al.*, 2005), but the high sequence homology does not allow the discrimination of individual *ARF*s by PCR. This high similarity appears to be the outcome of several recent tandem duplications (Remington *et al.*, 2004), which also positioned all genes in short genetic distance on chromosome I. The generation of higher order combinations of the respective knock-out lines therefore is rather difficult if not impossible.

Given the reported expression of these *ARF*(s) in young siliques, this cluster is a good candidate to redundantly control suspensor development. To test this hypothesis, an RNAi construct targeted specifically against the middle region of the clustered *ARF*s was expressed under the control of *35S* or *RPS5a* promoter (Table 4). Although phenotypes were variable, several lines were recovered that displayed abnormal suspensor development (Table 5; Fig. 8), in extreme cases showing suspensor overproliferation as in the M0171>>*iaa12* or pSUSP-*iaa10* lines (Fig. 8 c). For reasons described above, down-regulation of individual ARFs could not be tested in these RNAi lines. Nonetheless, since single mutants did not show this phenotype, at least two of the clustered ARFs are involved in suspensor development.

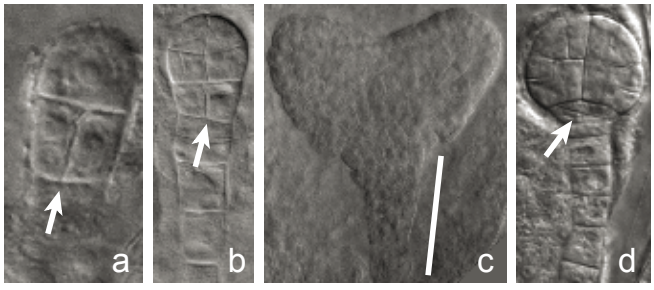

Figure 8: Embryo phenotypes caused by overexpression of RNAi against cluster I' *ARF*s
Expression of an RNAi fragment targeted against cluster I' ARFs under the control of the *35S* promoter induced aberrant cell divisions (arrows) in suspensor cells adjacent to the embryo (a, b) or in the hypophysis (d). In some cases this led to overproliferation of the suspensor cell file (c).

With this indication we set out to analyze the expression patterns of all eight genes during embryogenesis. To this end we fused approximately 2 kb long promoter fragments to nuclear localized 3xGFP and analyzed GFP signals during embryogenesis in at least ten independent lines for each ARF. For all constructs we found GFP signals in the embryo surrounding endosperm (ESE) at globular stage (Fig. 9). Strikingly, in addition to this ESE-specific expression pattern, *ARF13* was also expressed in the suspensor. *pARF13* activity could be observed from the octant stage onward, and persisted in suspensor cells until early heart stage (Fig. 9 g – i). To exclude that ARF activity in the ESE contributes to auxin-dependent suspensor development, auxin responses in this tissue were inhibited by driving the expression of three phylogenetically distant stabilized Aux/IAA mutant proteins (iaa3/shy2, iaa12/bdl and iaa17/axr3) under control of three driver lines (KS117, N9185, N9319) that are active in the endosperm (Ingouff *et al.*, 2005). None of the combinations induced suspensor defects (no significant defects in over 50 F_1 embryos observed for each combination), suggesting that only suspensor-expressed *ARF*s contribute to cell fate maintenance. As single knock-out lines for *ARF13* were aphenotypic, additional non-cluster *ARF*s are presumably expressed in the suspensor.

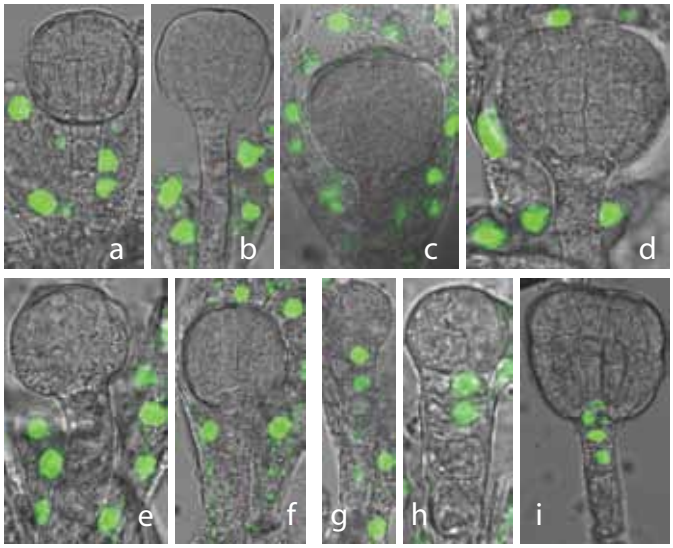

Figure 9: Expression patterns of cluster I' *ARF*s
2kb long promoter fragments of *ARF12* (a), *ARF14* (b), *ARF15* (c), *ARF20* (d), *ARF21* (e), *ARF22* (f) and *ARF23* (not shown) control expression in the Embryo Surrounding Endosperm. Only the *ARF13* promoter is also active in the suspensor cell file (g - i).

Apical and basal cell lineages express functionally distinct auxin response machineries

Suspensor and embryo cells follow completely different developmental programs, yet both embryo and suspensor development involve an Aux/IAA-ARF module. To determine whether the distinct expression of *ARF5/MP* in the proembryo and *ARF13* in the suspensor contributes to the different developmental progression of embryo and suspensor cells, we either misexpressed *ARF5/MP* in the suspensor from the *ARF13* promoter or we expressed *ARF13* ubiquitously from the *RPS5A* promoter. *pARF13::ARF5* expression strongly interfered with normal suspensor development and led to embryos with shorter suspensors, roughly half the

size of wild type suspensors. In contrast, *pRPS5A::ARF13* embryos showed various defects in the proembryo, in extreme cases leading to embryo arrest (Fig. 10).
In conclusion, developmental information is contained in each particular ARF and the outcome of the auxin response in the two early embryonic lineages is prepatterned by the expression patterns of *ARF5/MP* and *ARF13*.

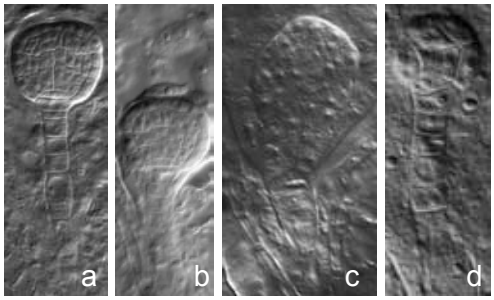

Figure 10: Missexpression of *ARF5/MP* and *ARF13* leads to embryo phenotypes
Expression of *ARF5/MP* in the suspensor (*pARF13::ARF5*) caused reduced suspensor cell elongation (b), while ubiquitous *ARF13* expression from the *RPS5a* promoter (c, d) resulted in various embryonic defects. (a: wild type)

Discussion

Our systematic approach of repressing ARF activity and thus auxin responses in specific domains during embryogenesis by expressing *iaa12/bdl* in various subdomains revealed novel auxin responses during embryogenesis of *Arabidopsis.*
In the suspensor, persistent inhibition of the auxin response machinery caused at least a partial loss of suspensor cell fate and the induction of embryo specific markers. These alterations in the transcriptomes of suspensor cells resulted in the onset of aberrant cell divisions and under certain circumstances led to the development of secondary embryos. Since the primary embryo did not show abnormal development before the aberrant divisions in the suspensor and even survived the formation of viable secondary embryos, we have uncovered the first cell-autonomous process involved in suspensor cell fate maintenance.
Auxin stimuli are typically perceived and translated into transcriptional changes by a modular switch with an interacting Aux/IAA – ARF pair at its core. By following a lead derived from publicly available expression data we were able to identify *IAA10* as the Aux/IAA component of the suspensor auxin response machinery.
With *ARF13*, which was found to be expressed specifically in the suspensor, we identified the first ARF counterpart of the suspensor auxin response machinery. However T-DNA insertion lines did not show phenotypic aberrations as observed by repressing suspensor specific ARF activity either by expression of stabilized *aux/iaa*s or RNA interference constraining translation of *ARF* transcripts.
Hence we propose that more ARFs participate in the suspensor auxin response or alternatively that redundant factors are upregulated to compensate for loss of ARF13 activity (Fig. 11). The phylogenetic tree does not highlight further candidates for this functionality and conclusive expression data is lacking for the suspensor. As demonstrated by our promoter swap experiments with *ARF5/MP* and *ARF13*, the redundant ARFs must have intrinsic properties that enable proper control of suspensor cell fate maintenance, since otherwise – as by misexpression of *ARF5/MP* – suspensor cell development is disturbed.
How the specific expression patterns of *ARF*s expressed in the suspensor or the embryo are

established, refined and coordinated during development is currently unclear and regulatory factors upstream of the *ARF*s have not been identified yet. With our promoter *n3xGFP* lines we now have sensitive markers at hand to investigate changes in expression patterns in various mutant backgrounds. This certainly will further elucidate regulatory networks controlling embryogenesis.

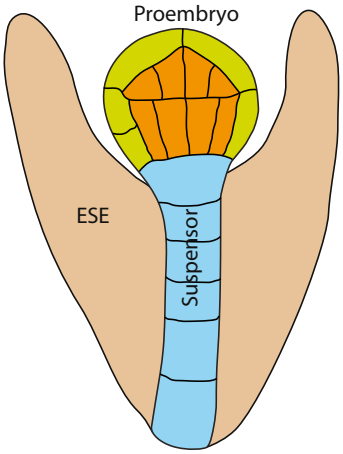

Figure 11: Components of distinct auxin reponse machineries present during embryogenesis
All *ARF*s of the cluster I' are expressed in the embryo surrounding endosperm (ESE), of these *ARF13* is also expressed in the suspensor and hypophysis where it is regulated by its inhibitor IAA10. Two more auxin response machineries regulated patterning of the embryo. Auxin responses in the inner cells are regulated by ARF5/MP and IAA12/BDL while a yet unknown auxin response machinery controls protoderm development.

Materials & Methods

Microscopy

For fluorescence microscopy ovules were transferred from siliques into a drop of PBS buffer containing 4% (w/v) PFA, 5% glycerol (v/v) and FM4-64 at a concentration of 1 µM on a microscope slide. After applying the cover slip, embryos were squeezed out of the ovules by short strokes with a pencil onto the cover slip. Free embryos were then investigated for GFP signals by using a Carl Zeiss LSM510 confocal laser scanning microscope (software version 3.2 SP2) 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 530 nm while FM4-64 signals were taken after passing a longpass filter of 650 nm.

For DIC microscopy ovules were mounted in a clearing solution of chloral hydrate, water and glycerol (8:3:1), left overnight at room temperature and subsequently investigated at a Leica DRM microscope equipped with differential interference contrast (DIC) optics.

Plant growth and material

Seeds were normally sterilized by rinsing with 70% ethanol, followed by three steps of washing with autoclaved water. In addition seeds of T1 plants were incubated for 10 minutes in 1% hypochlorite / 0.01% Tween-20 before washing. After plating on ½ MS medium seeds were transferred to a growth chamber with long day light regime and a constant temperature of 22 ºC. After two weeks seedlings typically were transferred to soil and further grown at the same light and temperature conditions as before.
Wild type plants were of the Columbia ecotype (Col-0) and transformed by floral dip method as described by (Clough and Bent, 1998). Selection of transgenic plants was carried out on ½ MS plates containing either 50 mg/l kanamycin or 15 mg/l phosphinotricin.
References and order numbers of all T-DNA and GAL4 driver lines used can be found in tables 1 and 2.

Analysis of GUS expression and *in-situ* hybridization

Both assays were performed as described in (Hamann *et al.*, 2002).
Primers used to generate the *in-situ* probe can be found in table 6.

Molecular Cloning

The general procedure for cloning was to first amplify the respective fragment from BAC clones or genomic DNA using Pwo DNA Polymerase (Fermentas) and subclone it into pBluescript SK (-). The resulting constructs were then sequenced by ServiceXS (Leiden, the Netherlands) and checked for potential mutations. Correct fragments were cloned into binary pGreenII vectors (Hellens *et al.*, 2000) carrying the desired marker gene for later plant selection and then used for plant transformation.
During this process DNA purification was achieved by using the High Pure PCR Product Purification Kit (Roche) for recovery of DNA from agarose gels or the GeneJet Plasmid Miniprep Kit (Fermentas) for extraction of DNA from cell cultures. Ligation of DNA fragments was carried out by incubating the respective fragments for 1 hour at room temperature with T4-DNA ligase (Fermentas).
Plasmids were amplified by culturing transformed *Eschericha coli* XL1-Blue strains (Stratagene) in LB medium at 37 ºC.
Primers used for amplification of fragments as well as the original DNA sample and the fragment length can be found in table 6.

Site directed mutagenesis

Point mutations in the degron of IAA10 and IAA11 were inserted by site-directed mutagenesis as described in (Sawano and Miyawaki, 2000). The primers used hereby were: for IAA10 CTGTAGGTTGG**A**CGCCTCTACGG
and for IAA11 GTCCTTATTGGTG**A**CCATCCCAC;
both 5' phosphorylated. The exchanged nucleotide is depicted in bold letters.

Acknowledgments

The authors acknowledge SALK, SAIL, FLAG, GABI for making the T-DNA insertion lines available, the NASC stock centre for distributing the GAL4 driver lines, and Frederic Berger and Ottoline Leyser for materials. This work was supported by grants from the Netherlands Organization for Scientific Research (NWO; VIDI, ALW- Open Competition) to DW.

Tables

Table 1: GAL4 driver lines

Driver line	ABRC Seed Stock #	Expression pattern		Embryo Phenotype	Reference
		at globular stage	at torpedo stage		
J0571	N9094	n. d.	cortex + endodermis with initials	-	(Haseloff)
J1092	N9147	n. d.	QC + “inner” root cap	-	(Haseloff)
J3281	N9128	Ubiquitous	Vasculature + central root cap	+	(Haseloff)
KS068	N9256	Suspensor	Suspensor, shoot apex	+	(Haseloff)
M0028	N9274	n. d.	SAM	-	(Haseloff)
M0136	N9302	n. d.	Basal half of embryo	-	(Haseloff)
M0148	N9303	n. d.	Cotyledon junction	-	(Haseloff)
M0164	N9307	n. d.	Cotyledon junction, early in cotyledons	-	(Haseloff)
M0167	N9308	n. d.	Cotyledon junction	-	(Haseloff)
M0171	N9312	Suspensor	SAM, cotyledon junction, RAM, suspensor	+	(Haseloff)
M0223	N9336	n. d.	Root cap in embryo and seedling	-	(Haseloff)
Q0680	N9209	n. d.	Central root cap + vasculature	-	(Haseloff)
Q0990	N9217	Inner cells of the proembryo	Central cell and stele initials, vasculature	+	(Haseloff)
Q1630	N9227	n. d.	C1, c2 of columella, vasculature	-	(Haseloff)
RPS5a	n. a.	Ubiquitous	Ubiquitous	+	(Weijers *et al.*, 2003)
STM	n. a.	Suspensor	Suspensor	+	
KS117	n. a.	Endosperm	n. d.	-	(Ingouff *et al.*, 2005)
J2641	N9185	Endosperm	n. d.	-	(Ingouff *et al.*, 2005)
M0186	N9319	Endosperm	n. d.	-	(Ingouff *et al.*, 2005)

n. a.: not available
n. d.: not determined

Table 2: Phenotypes in *iaa10* embryos

Stage	aberrant division of hypophysis	extra divisions in suspensor	shortened suspensor	aborted ovules	wild type
Up to dermatogen	2	6	8	21	84
Globular	5	2	1	0	39
Heart stage and older	2	0	0	0	6
Sum	9	8	9	21	129

Table 3: T-DNA lines

Gene name	Locus	Allele	Stock #	References
ARF1	At1g59750	*arf1-5*	SALK_079046	(Ellis *et al.*, 2005)
ARF2	At5g62000	*arf2-8*	SALK_108995	(Ellis *et al.*, 2005)
ARF3	At2g33860	*arf3-3*	SALK_031544	
ARF4	At5g60450	*arf4-2*	SALK_070506 C	(Pekker *et al.*, 2005)
ARF5/MP	At1g19850	*MP-B4149*		(Weijers *et al.*, 2005)
ARF6	At1g30330	*arf6-1*	CS24606	(Okushima *et al.*, 2005)
ARF7	At5g20730	*arf7-1*	SALK_040394	(Okushima *et al.*, 2005)
ARF8	At5g37020	*arf8-4*	*fwf*	(Vivian-Smith *et al.*, 2001)
ARF9	At4g23980	*arf9-1*	CS24609 / SAIL_881_H05	(Okushima *et al.*, 2005)
		arf9-2	CS24610 / SAIL_1207_H04	(Okushima *et al.*, 2005)
ARF10	At2g28350	*arf10-1*	SALK_143232	(Okushima *et al.*, 2005)
ARF11	At2g46530	*arf11-2*	SALK_063778	
ARF12	At1g34310	*arf12-2*	SAIL_1161_E12	(Okushima *et al.*, 2005)
ARF13	At1g34170	*arf13-1*	SALK_005960	(Okushima *et al.*, 2005)
		arf13-2	SALK_138188	
ARF14	At1g35540	*arf14-1*	FLAG_497G08	
ARF15	At1g35520	*arf15-101*	SALK_121828	
ARF16	At4g30080	*arf16-1*	SALK_021448	(Weijers *et al.*, 2005)
		arf16-2	SALK_021432	(Weijers *et al.*, 2005)
ARF17	At1g77850	*arf17-1*	SALK_062511	
ARF18	At3g61830	*arf18-3*	GABI_699B09	C. Gasser p.c.
ARF19	At1g19220	*arf19-1*	CS24617	(Okushima *et al.*, 2005)
		arf19-2	CS24618	(Okushima *et al.*, 2005)
ARF20	At1g35240	*arf20-1*	SALK_019051	(Okushima *et al.*, 2005)
ARF21	At1g34410	*arf21-101*	SALK_020702	
ARF22	At1g34390	*arf22-101*	SALK_123566	
ARF23	At1g43950	n.a.		

Table 4: Specificity of the RNAi fragment

A BLAST search was performed using BLASTN 2.2.17 on the TAIR9 DNA Genes database including introns and UTRs.

Gene number	Gene name	Score (bits)	e value
AT1G35520.1	**ARF15**	234	4,00E-61
AT1G35240.1	**ARF20**	220	6,00E-57
AT1G35540.1	**ARF14**	212	2,00E-54
AT1G34390.1	**ARF22**	204	4,00E-52
AT1G34410.1	**ARF21**	204	4,00E-52
AT1G43950.1	**ARF23**	202	1,00E-51
AT1G34310.1	**ARF12**	188	2,00E-47
AT1G34170.3	**ARF13**	82	4,00E-15
AT1G34170.2	**ARF13**	82	4,00E-15
AT1G34170.1	**ARF13**	82	4,00E-15
AT1G24310.1		38	0.049
AT5G36890.2	BGLU42	34	0.77
AT5G36890.1	BGLU42	34	0.77
AT4G21590.2	ENDO3	34	0.77
AT4G21590.1	ENDO3	34	0.77
AT3G10040.1		34	0.77
AT3G19630.1		34	0.77
AT3G42500.1		34	0.77
AT2G20250.1		34	0.77
AT5G17790.1	VAR3	32	3.0
AT5G20990.1	B73, SIR4, CNX, CHL6, CNX1	32	3.0
AT4G23980.2	**ARF9**	32	3.0
AT4G23980.1	**ARF9**	32	3.0
AT4G20790.1		32	3.0
AT4G07338.1		32	3.0
AT4G14750.1	IQD19	32	3.0
AT3G43920.3	DCL3, ATDCL3	32	3.0
AT3G43920.2	DCL3, ATDCL3	32	3.0
AT3G43920.1	DCL3, ATDCL3	32	3.0
AT3G11490.1		32	3.0
AT3G29320.1		32	3.0
AT2G23230.1		32	3.0
AT1G19220.1	**ARF19**	32	3.0
AT1G19780.1	ATCNGC8, CNGC8	32	3.0
AT1G53370.1		32	3.0
AT1G53360.1		32	3.0
AT1G67500.1	ATREV3	32	3.0

Table 5: Phenotypes in ARF-RNAi lines

Line	N	% Phenotypic aberrations
p35S::ARF-RNAi # 31	205	7.3
p35S::ARF-RNAi # 46	100	14
pRPS5a::ARF-RNAi # 7	139	7.2
pRPS5a::ARF-RNAi # 8	52	3

Table 6: Primers used for cloning

Fragment	Primer orientation	Primer	Fragment Length
IAA10 promoter	Sense	GAATTCAGTGGATC	1013 bp
	Antisense	AGGAACGAAAAATCGGAAAC	
IAA10 locus	Sense	AGTGGATCGAAGCATACGACCTTG	2222 bp
	Antisense	CTTACCTACTCCAGCTCCAATTGATG	
IAA11 promoter	Sense	AGTTGAAGTTGAGAAGAAGA	1442 bp
	Antisense	GACGGGTGGGATTTGTCTCT	
IAA11 locus	Sense	AGTTGAAGTTGAGAAGAAG	3687 bp
	Antisense	TAATATCATCTGAGCTGTAA	
ARF5 promoter	Sense	CAGGTACCATATATCTTAGTGACAAACGCG	2071 bp
	Antisense	CAGGGCCCACAGAGAGATTTTTCAATG	
ARF5 locus	Sense	GACTCGAGGCTTCATTGTCTTGTGTTG	2709 bp
	Antisense	CCACTAGTTGAAACAGAAGTCTTAAGATCG	
ARF13 promoter	Sense	AGAGTCAAAAAAATGATTTTGTTTGTGATTA	1808 bp
	Antisense	GAGCTCTTATTGCAA	
ARF13 locus	Sense	ATGGAAAATAATGGAGAAATGAATGC	3279 bp
	Antisense	AATGTATTTCAAAGA	
RNAi fragment	Sense	ATAGATCCAAGAGCTATATG	192 bp
	Antisense	CACGACATTGAAGCTTAGAAGGAAG	
ARF15 Probe	Sense	TCATCACATCTCTTGCCTCCT	402 bp
	Antisense	GTACAAGTTCTAGTAGAAC	

Chapter 3

An Atlas of *ARF* expression during Embryogenesis

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Abstract

A multitude of developmental processes is regulated by auxin-dependent signalling events, which are mostly mediated by changes in gene expression. Auxin controls gene expression by promoting degradation of Aux/IAA proteins which normally inhibit DNA-binding AUXIN RESPONSE FACTORs (ARF). Hence, auxin releases the inhibition of this family of transcription factors. ARFs are the executors of auxin-dependent transcription and therefore form the pivotal point in translating auxin signals into specific transcriptional responses.
Analysis of single *ARF* knock-out mutants and generation of double mutants for ARF sister pairs has demonstrated redundant functions. However the phenotypes observed so far can only account for part of the auxin responses that occur during plant development. Further, less intuitive redundancies between ARFs are plausible, but the comprehensive unguided generation of multiple knock-out lines among the 23 *ARF* genes is unrealistic.
We reasoned that only those *ARF*s that are co-expressed in the relevant cell type would be able to mediate local auxin responses. Therefore we generated transcriptional fusions of all *ARF* promoters to a sensitive nuclear *GFP* reporter and determined the respective expression patterns at cellular resolution in the developing embryo. We found 7 *ARF*s (*ARF1, ARF2, ARF5/MP, ARF6, ARF9, ARF13* and *ARF18*) to be expressed in various cell types of the embryo up to the globular stage. Surprisingly, every cell type in the globular embryo expresses a unique set of *ARF*s. The number of *ARF* genes expressed, and the complexity of the combinatorial patterns further increased at later stages of embryogenesis, and ensured a unique set of ARFs for each cell type even at later stages. The *ARF* expression map predicts redundant ARF functions and will hence serve as the basis for reverse-genetic analysis of ARF functions in embryogenesis. The combinatorial patterns of *ARF* expression suggest that cellular auxin responses are conditioned by the prepattern of *ARF* expression.

Introduction

Growth and development of a plant calls for the coordinated establishment of specialized cell types forming the respective tissues and organs. Instructive information for cell fate decisions can be passed on to a cell either from its ancestors (a lineage-based mechanism) or be derived from its position in the respective organ (a position-based mechanism) (Scheres, 2001). Position-based mechanisms often facilitate plant hormones to control development in a spatio-temporal manner.

The most prominent plant hormone is auxin (indole-3 acetic acid), which is involved in most, if not all developmental and growth processes (Vanneste and Friml, 2009). Its controlled distribution in the plant is achieved by an elaborate network of auxin efflux carriers of the PIN-FORMED (PIN) family. Asymmetric localization of these proteins, and thus the direction of auxin transport, is actively maintained in plant cells. Redistribution of the PINs allows for selective generation of auxin concentration maxima whereby positional information is conferred onto the targeted cells (Petrasek and Friml, 2009). Currently two classes of proteins have been reported to bind auxin and act as receptors for this plant hormone. The AUXIN BINDING PROTEIN1 (ABP1) is involved in cell elongation by mediating the activation of ion fluxes across the plasma membrane in response to auxin. However the physiological mechanisms underlying this process remain unclear (Braun *et al.*, 2008).
On the other hand auxin is bound by several related F-Box proteins (TRANSPORT INHIBITOR PROTEIN1 (TIR1) and AUXIN SIGNALING F-BOX proteins (AFB1, 2 and 3)) which form the selective component of an SCF ubiquitin-ligase (E3) complex. This complex facilitates the ubiquitination and subsequent degradation of AUXIN/INDOLE-3 ACETIC ACID (Aux/IAA) proteins upon high auxin levels in the cell. At low auxin levels Aux/IAAs inhibit the activity of AUXIN REPONSE FACTORs (ARF) by binding via two shared protein interaction domains at the C-terminus of both protein families. Eventually ARFs form the pivotal point in auxin dependent transcriptional regulation by binding to target gene promoters and stimulating their expression via differing regulatory domains found between N-terminal DNA binding and C-terminal protein interaction domains. Hence, through binding to $SCF^{(TIR1/AFB)}$, auxin stimulates the degradation of ARF inhibitors, thereby releasing these transcription factors from inhibition. This auxin-dependent transcription mediates the majority of auxin responses, since mutations in components of this machinery cause dramatic auxin-related defects at all stages that have been analysed (Dharmasiri *et al.*, 2005).

However, despite this thorough understanding of how auxin triggers transcriptional changes, for many auxin regulated processes the involved ARFs have not been identified. There are 23 *ARF* genes in the *Arabidopsis thaliana* genome, and mutant phenotypes have been observed for only a handful (Guilfoyle and Hagen, 2007). Presumably, ARFs act redundantly to control growth and development. Indeed, the phenotypes of double mutants between closely related ARFs suggest that this is the case. Nonetheless, a limited number of mutant combinations has been generated, and most auxin responses await identification of the ARF component. Analysing auxin responses by generating all possible ARF mutant combinations to overcome redundancies is impossible and clues to narrow down the number of ARFs involved in a particular process are needed.

To begin dissecting ARF function in development systematically, we have mapped the expression of all *ARF*s in critical stages of embryogenesis at cellular resolution and generated an atlas of *ARF* expression. This provides a detailed first view of the combinatorial complexity in auxin responses and allows the systematic generation of plants lacking combined ARF activity in a specific cell type.

Results and Discussion

The ARF transcription factor family

The genome of *Arabidopsis* contains 23 *ARF*s including one potential pseudogene (*ARF23*) that results from a premature stop codon in the coding sequence. Phylogenetic analysis reveals that this family divides into three subgroups (Fig. 1, (Remington *et al.*, 2004; Okushima *et al.*, 2005)) with the main difference lying within the divergent middle regions of the ARF proteins.

Hence all ARFs containing a glutamine-rich regulatory domain (ARF5/MP, ARF6, ARF7, ARF8 and ARF19) cluster together. In protoplast transfection assays these ARFs have been shown to activate reporter gene expression upon auxin stimuli and therefore are regarded as transcriptional activators (Tiwari *et al.*, 2003).

Middle regions of all other ARFs lack glutamine enrichment and some act as repressors in protoplast transfection assays. However, whether the activity observed on a synthetic promoter in protoplasts fully captures the physiological activity of the ARFs remains an open question.

The majority of ARFs clusters together in one phylogenetic subgroup consisting of ARF1 to ARF4, ARF9, ARF11, ARF12 to ARF15, ARF18 and ARF20 to ARF23. Half of this group (ARF12 to ARF15 and ARF20 to ARF23; together named class I') presumably resulted from a recent series of tandem duplications (Hagen and Guilfoyle, 2002) and therefore share more than 80% protein identity (Okushima *et al.*, 2005). Notably the genomes of *Populus trichocarpa* and *Oryza sativa* do not hold an orthologue even to ARF13 which is basal to the described class of ARFs (Kalluri *et al.*, 2007; Wang *et al.*, 2007a). Finally the third and basal most group is formed by ARF10, ARF16 and ARF17. The latter of these three lacks the C-terminal interaction domains III/IV, as does ARF3. The role and regulation of these ARFs and their domains is not well understood.

As mentioned earlier, most of the *ARF* single mutants are aphenotypic. This implies that these genes are either non-functional or that their function is masked by redundancies. Often closely related genes account for redundant functionality and indeed most of the ARFs in *Arabidopsis* group together with a sister *ARF*. The respective double knock-out mutants (*arf1/arf2, arf5/arf7, arf7/arf19, arf6/arf8, arf10/arf16*) show either an enhancement or novel phenotypes, demonstrating that redundancies are common in the ARF family (Hardtke *et al.*, 2004; Ellis *et al.*, 2005; Nagpal *et al.*, 2005; Wang *et al.*, 2005; Wilmoth *et al.*, 2005). Furthermore recent work on post-transcriptional gene regulation by small RNAs suggests simultaneous regulation of ARF6/ARF8, ARF10/ARF16/ARF17 and ARF3/ARF4 by *micro*-RNA167, *micro*RNA160 or TAS3 *ta-si* RNAs (Fahlgren *et al.*, 2006; Wu *et al.*, 2006; Liu *et*

al., 2007) highlighting the interchangeable properties of theses factors
Nonetheless many auxin responses are not explained by the known single and double mutant phenotypes, leaving a multitude of combinatorial options open to investigation. Since alone the generation of all double mutant combinations counts up to 506 (= 23*22) different possibilities, unravelling auxin responses by random combination of ARF mutants would be an enormous task. Clearly this effort can be reduced, if it is determined which ARFs co-exist *in vivo*. We therefore chose to first determine the expression patterns of all ARFs to guide combinations of particular interest.

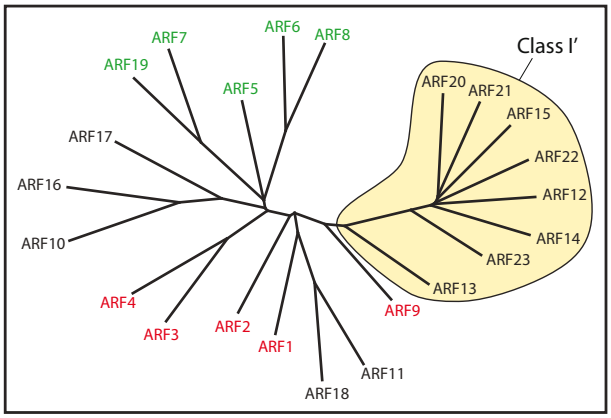

Figure 1: Phylogenetic tree of the ARF family in *Arabidopsis*
The ARF family subdivides in three groups. Based on protoplast assays ARFs with a glutamine rich middle domain have been implicated to act as transcriptional activators (in green). Of the remaining ARFs some have been proposed to act as repressors (in red). Due to differing DNA binding domains ARF10, 16 and 17 form a separate subgroup while the third and biggest group is dominated by a cluster of eight highly similar genes (Class I'; yellow background).

Experimental procedure

The basic prerequisite to protein activity is its transcription and translation in the particular cell. Under the assumption that the protein is not transported from cell to cell, the transcriptional domain depicts its broadest range of activity. This domain might be further defined in both temporal and spatial resolution by posttranscriptional mechanisms such as post transcriptional gene silencing (PTGS), protein modification and degradation or the presence of inhibitors (examples: *micro*RNAs mentioned above, phosphorylation of ARF2 by BIN2, interaction with Aux/IAAs; (Weijers *et al.*, 2005; Vert *et al.*, 2008)).
To define the sites of ARF expression during embryogenesis we cloned two kb long promoter fragments, starting immediately upstream of the translational start site, in front of a triplet of green fluorescent protein (GFP), carrying the SV40 nuclear localization signal.
Next to higher signal intensities, as a result of concentrating the GFP in the nuclei, this approach should allow for a cellular resolution that can not be achieved with other techniques, in particular GUS staining or *in-situ* hybridization (Takada and Jürgens, 2007). The two kb long promoter fragments should include most of the *cis*-regulatory elements since most of them (~ 90%) have been reported to reside in the first kb upstream of the transcriptional start site (Megraw *et al.*, 2006).
For each promoter fragment we obtained at least ten independent transgenic lines. All these lines were checked for divergence in the respective expression patterns and eventually two lines showing strong and representative signals were selected. Homozygous offspring of these lines was used to record expression patterns by confocal microscopy.

Patterns of ARF expression

General overview

Using confocal microscopy we recorded *GFP* expression driven from each of the *ARF* promoters during embryogenesis from the octant stage onwards.
We found the promoters of *ARF1*, *ARF2*, *ARF5/MP*, *ARF6*, *ARF9*, *ARF13* and *ARF18* to be active during early embryogenesis. While suspensor specific expression from the *ARF13* promoter ceased with the degradation of this structure, all other *ARF* promoters remained active at late heart stage even though their activity was in some cases restricted to a few cells. With the full definition of the basic body plan at late heart stage the initial set of ARFs was extended by the activation of five additional promoters (*ARF3, ARF4, ARF7, ARF10* and *ARF11*) in various cell types. Notably, in addition to the set of embryo-expressed ARFs, all class I' *ARF*s (*ARF12* to *ARF15* and *ARF20* to *ARF23*) are specifically expressed in the micropylar endosperm at globular stage of embryonic development. Interestingly, out of these we found only the *ARF13* promoter to be active in the suspensor as mentioned above (Fig. 2, 3).
Next to the involvement of *ARF* promoters in embryogenesis we also noted expression of several *ARF*s in tissues of the ovule. Integuments displayed ubiquitous *ARF1, ARF2, ARF5* and *ARF8* promoter activity, whereas the endosperm was ubiquitously marked by expression of the *ARF17* promoter (Fig. 5).
In summary, expression from all but the *ARF16* and *ARF19* promoter fragments was observed either in the embryo or tissues of the ovule. Specific patterns will be described in detail in the following sections.

Apico-basal differentiation of ARF expression at the octant stage

At the octant stage the basic domains of the embryo have been established and one can distinguish an apical and a basal cell tier as well as the extra-embryonic suspensor. Despite the fact that suspensor and proembryonic cells stem from different ancestors, the expressed sets of *ARF*s overlapped in these experiments. In particular we found expression of *ARF1*, *ARF6* and *ARF18* in all cells at this stage. The onset of cellular differentiation was reflected by the specificity of *ARF2*, *ARF9* and *ARF13* promoters. These promoters were activated only in the suspensor (Fig. 2).

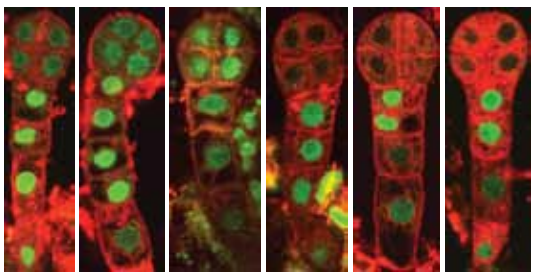

Figure 2: *ARF* expression at the octant stage
Expression of *n3GFP* driven by the promoters of *ARF1*, *ARF6* and *ARF18* was detectable in all cells. *ARF2*, *ARF9* and *ARF13* promoters controlled expression exclusivley in the suspensor cell file.

Cell fates correlate with distinct sets of active ARF promoters at the globular stage

Radial patterning of the embryo takes place before the globular stage when protoderm as well as ground tissue are separated. At the level of *ARF* expression this is depicted by a refinement of promoter activity. *ARF1* and *ARF18* promoter remain to be active ubiquitously but *ARF6* expression is no longer found in the upper half of the proembryo even though expression remains in the basal half and suspensor cells. A somewhat similar situation was observed for the *ARF2* and *ARF9* promoters, expression of which expanded to the protodermal cells of the lower proembryo tier yet remained unchanged in the suspensor. Finally specification of inner and outer cell layers is highlighted by the restriction of *ARF5/MP* promoter driven expression to the inner cells of the proembryo. Essentially this leaves every cell type present in the globular embryo with a distinct set of expressed *ARF*s (Fig. 3).
ARF1 and *ARF18* are ubiquitously expressed and mark the apical protoderm where no other *ARF*s are expressed. In the inner apical cells they are joined by *ARF5/MP* activity, which also is present in the inner cells of the basal tier but here in combination with *ARF6*. *ARF6* expression distinguishes the basal cells from the apical ones but in the protodermal cells of the basal tier *ARF2* and *ARF9* raise the number of expressed *ARF*s in a certain cell type to 5. Finally expression of the *ARF13* promoter in addition to the set present in basal protodermal cells marks suspensor cell fate and the hypophysis.

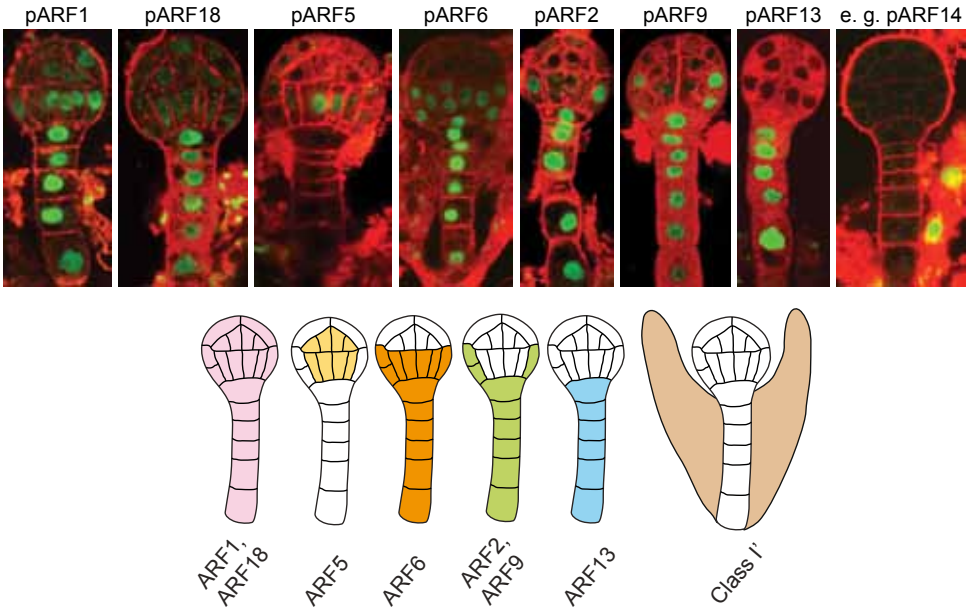

Figure 3: *ARF* expression at the globular stage
At globular stage *ARF1* and *ARF18* promoters remained active in all cells of the embryo. *ARF5* expression was found in the inner cells of the basal domain of the embryo. Expression of *ARF6* was restricted to the basal domain of the embryo and the suspensor. *ARF2* and *ARF9* were expressed like *ARF6* with the exception that both were not activated in the inner cells of the basal domain. The *ARF13* promoter was active in the suspensor and the ESE, while activity of all other promoters of Class I' *ARF*s (*ARF12, 14, 15, 20, 21, 22* and *23*) was found in the ESE only.

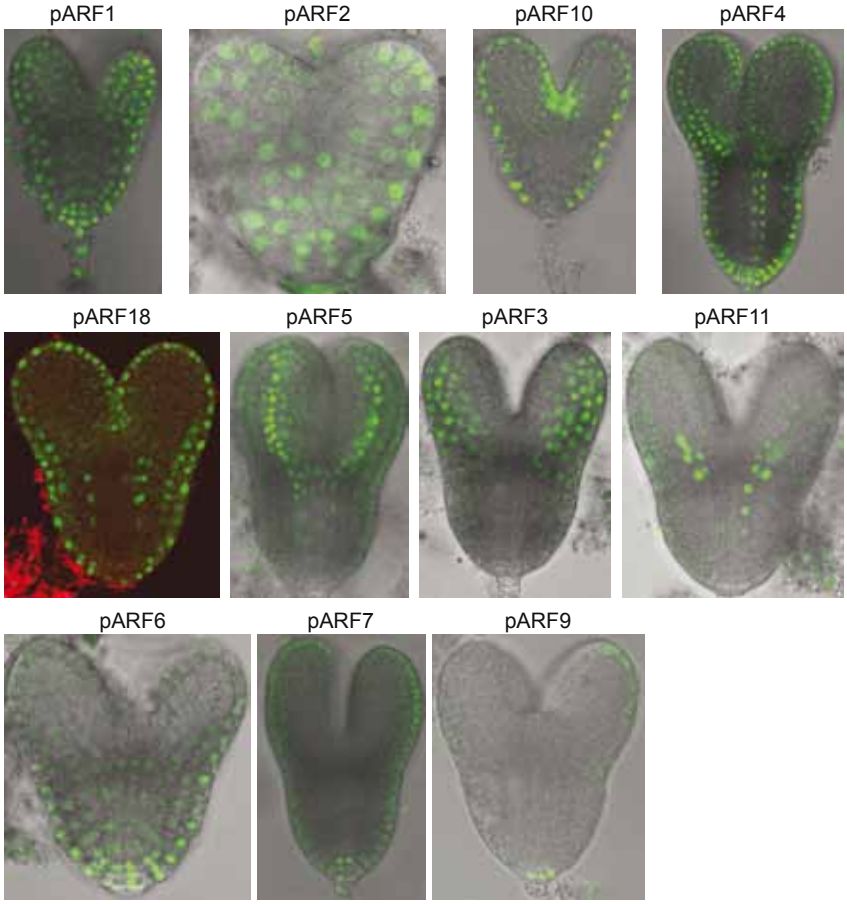

Figure 4: *ARF* expression at late heart stage
The pictures represent expression patterns of the 11 *ARF* promoters we found to be active at heart stage. Transcriptional fusions of *n3GFP* with promoters of the remaining 12 *ARF*s did not lead to a detectable signal at this stage.

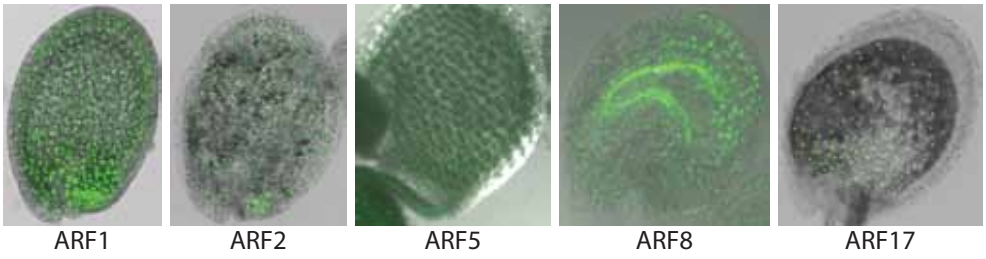

Figure 5: *ARF* promoter activity in tissues of the ovule
Expression under control of the *ARF1, 2, 5* and *8* promoters was found in the integuments, while *ARF17* expression was specific to the endosperm.

Diversification of *ARF* expression patterns in late stages of embryogenesis

The domain / cell type specific expression patterns found at early stages of embryogenesis are refined and complicated by the additional activity of further *ARF* promoters that are activated at late heart stage (Fig. 4).
At this developmental time point all cell types of the later seedling have been formed and subsequently only the *ARF1* promoter resides in all cells. Tissues with most *ARF* promoter activity are the epidermis and the stele. In the latter we observed expression of *GFP* under the control of the *ARF4, ARF5/MP, ARF11* and *ARF18* promoters. Two of these promoters (*ARF4* and *ARF18*) were also active in the epidermis as was the *ARF2* promoter and in only some sections the *ARF6* and *ARF10* promoters.
Abaxial sides of the cotyledons were marked by *ARF3* promoter activity and expression from the *ARF9* promoter could be found only in the outermost layer of the columella.
Remarkably we only found the *ARF1* promoter to be active in the SAM while the QC displayed activity of *ARF6*, *ARF7* and *ARF10* promoters.

ARF Promoter activity reflects endogenous expression domains

Our approach of analysing *ARF* expression by utilizing promoter fragments for transcriptional fusions might miss regulatory elements that lie further upstream or are embedded in the coding sequence of the gene.

Sequences potentially holding such elements were included in earlier studies of ARF5/MP protein distribution in the embryo. In these experiments a 6xHA tag was inserted into a genomic fragment spanning the entire *ARF5/MP* gene including 4.1 kb upstream and 0.9 kb downstream sequences (Weijers *et al.*, 2006). Immunolocalization of the generated fusion protein unveiled the presence of ARF5/MP in exactly the same cells that are marked by expression from the promoter construct used in this study. Similarly, insertion of GFP in the middle region of ARF5/MP within the same genomic fragment shows the same protein accumulation as observed here with a 2 kb promoter fragment (Schlereth *et al.*, 2009). In both cases, the expression is sufficient to complement the *arf5/mp* mutant phenotype.
In a second *ARF* fusion construct created to test the quality of our promoter assay we combined *GUS* with the coding sequence for *ARF15* under the control of the promoter fragment used previously. The respective *pARF15::GUS:ARF15* embryos and ovules displayed staining solely in the micropylar endosperm as predicted by the transcriptional fusion (Fig. 6).

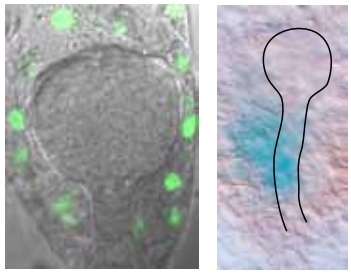

Figure 6: *ARF15* expression in the ESE
Expression of both a transcriptional and a translational fusion of *ARF15* is found exclusivley in the ESE.

This consistency of data implies that protein abundance of at least two subgroups of the ARF family is exclusively regulated by transcriptional control. Moreover all regulatory elements facilitating this transcriptional regime seem to be present in the 2 kb promoter fragments we used.
Additional expression data for other *ARF*s during embryogenesis is only available for *ARF7* and *ARF16*. In the case of *ARF7*, this data is derived from *in-situ* hybridization (Hardtke *et al.*, 2004). The signals as observed by these authors are very weak and diffuse, which makes it difficult to compare the patterns with those observed here.

GFP:ARF16 fusion proteins were found in the embryonic root cap from the heart stage onwards by Wang *et al*. (Wang *et al.*, 2005). To create the fusion protein the authors used a 2 kb long promoter fragment and ARF16 cDNA. Therefore essentially the same regulatory sequences as in our promoter study controlled expression in their investigation. However degradation of transcripts through interference of microRNA160 is claimed by the authors. Hence, the observed presence of GFP:ARF16 does not reflect the full extent of transcription from the *ARF16* promoter.
Careful inspection of embryos carrying our transcriptional fusion constructs did not indicate any *ARF16* expression. Nevertheless we observed expression patterns in roots that matched the ones reported by Wang *et al.*. A possible explanation to this might be that we did not find a strong line for embryonic *ARF16* expression and thus did not detect low levels of *ARF16* promoter activity.

Next to the detailed study of single gene expression patterns, a broad overview of *ARF* expression in a certain tissue can be achieved by performing gene expression profiling with DNA microarrays. Recently two groups combined this method with laser-capture microdissection to extract and analyze the transcriptome of various embryonic domains and structures (Lindsey lab: (Casson *et al.*, 2005; Spencer *et al.*, 2007), Goldberg lab: (Goldberg)). Both groups used the Affymetrix GeneChip Arabidopsis ATH1 Genome array which covers approximately 22.800 genes but lacks probes for *ARF14*, *15*, *20* and *22*.
Keith Lindseys group extracted mRNA from sections of globular (apical and basal), heart (cotyledon and root tip) and torpedo (cotyledon tip, root tip and SAM) stage embryos. Three replicates were preformed for each tissue and data analysis was carried out by the team. The authors suggest the use of a level of 75 as minimal transcript presence signal to cancel out any noise in the data (as previously reported in (Birnbaum *et al.*, 2003)). When we extracted the expression levels of all ARFs from the data provided by the Lindsey lab, only *ARF3*, *ARF8* and *ARF12* expression levels exceeded the noise threshold advised by the authors. Of these only *ARF3* expression (102 ± 22 vs. 706 ± 455) showed an upregulation in the apical half of the embryo whereas *ARF8* (94 ± 7 vs. 105 ± 7) and *ARF12* (347 ± 4 vs. 395 ± 44) levels were equal in both domains.
Hence the *ARF* expression data contained in this data set did not qualify for any comparison with our observations and we did not take it into further account.

A focus on transcriptional changes during seed development was taken by the Goldberg lab by extracting mRNA from various tissues within the developing ovule. Hereby a distinction between different domains of the embryo was not made but therefore complete embryos of different stages (pre-globular, globular, heart and torpedo stage) were isolated as well as the

suspensor at globular stage of embryo development and various sections of ovule tissues. To this point the group performed only duplicates of their experiments causing relatively high deviations in some of the recorded expression levels.
Next to these variations we first checked the available data for expression above noise with a threshold of 75 as mentioned before. This revealed that expression levels of most ARFs were above noise and changing between the distinct tissues.
To further test the quality of this data set we continued by analyzing the expression levels of several marker genes with differential regulation during embryo development for reproduction of described expression patterns.
WOX2, WOX8 and WOX9 are known to be arranged along the apico-basal axis of the embryo, with WOX2 being found only in apical embryonic cells and WOX8 only in the suspensor while WOX9 resides in the suspensor and the basal half of the proembryo (Haecker *et al.*, 2004). *PIN7* on the other hand is exclusively expressed in the suspensor whereas PIN1 and IAA12/BDL are found only in the embryo (Hamann *et al.*, 2002; Friml *et al.*, 2003).
We found that differential expression of these genes between several stages of embryonic development (octant, globular, torpedo) and different domains (micropylar endosperm, suspensor and embryo proper at globular stage) was correctly reflected in the data. However there are some deviations. Namely *IAA12/BDL* is not expressed in the suspensor nor the endosperm just as expression of *WOX8* has not been reported for the endosperm (Fig. 7). Thus the Goldberg data confirms an overall trend of expression patterns but might also contain some false positives.
Extraction of *ARF* expression data for pre-globular, globular and torpedo stage embryos as

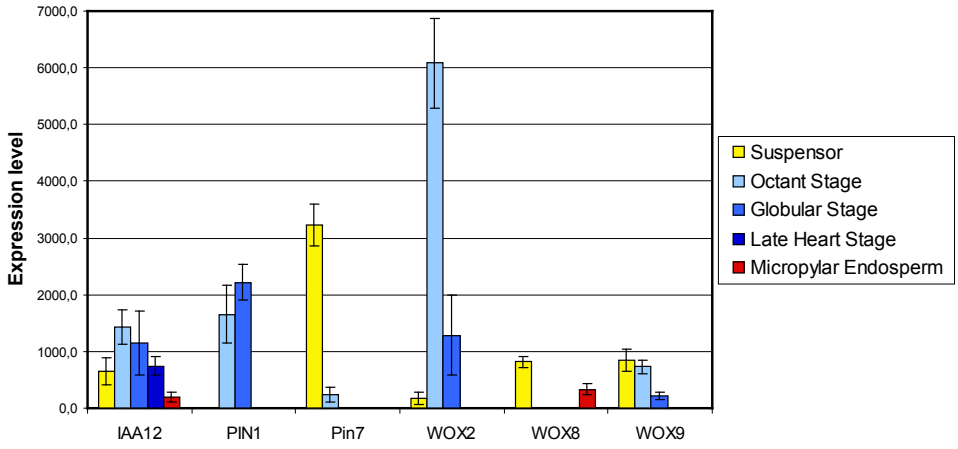

Figure 7: Expression levels of several marker genes in the Goldberg data set
Expression peaks of all genes correlate with reported expression patterns. Low levels of expression often account for false positives as is the case for *IAA12/BDL* expression in suspensor and endosperm and *WOX8* expression in the endosperm.
Bars represent the average expression level of each gene measured in two independent experiments. Measurements were only taken into account if they were judged as "present" in at least one experiment. Error bars denote a 95% confidence interval around the average level.

well as for the suspensor and the micropylar endosperm showed that some *ARF*s are highly expressed (above 2000 and up to 22000) in various preparations while in many cases levels below 1000 are reached (Fig. 8).
Of the highly expressed *ARF*s only *ARF2* seems to be expressed in all relevant samples, closely representing the expression patterns we found.
The highest expression of *ARF*s spotted on the chip is recorded for members of class I' cluster, namely *ARF12*, *ARF13* and *ARF21*. According to the chip data all three are expressed at high levels in the suspensor and in the micropylar endosperm. This fits with our observations, except that we only found *ARF13* in the suspensor whereas the others were present only in the micropylar endosperm.
Expression patterns of all other *ARF*s match in some aspects our findings but at the same time show deviations that make any interpretation speculative.
Although the Goldberg data is available, a concluding publication has not been published yet. It is therefore possible that the data is still in the process of being revised and final adjustments might change the overall picture.

Comprehensive *ARF* expression map implies diversification of auxin responses by variation in ARF combinations

Only a handful *ARF*s had been shown to be expressed during embryogenesis. By studying *GFP* expression driven from all *ARF* promoters at various embryonic stages we greatly expanded the current picture and can now provide a comprehensive overview of potential ARF activity in this developmental window.
The 2 kb fragments we used to drive *GFP* expression proved to be sufficient to render complete *ARF* expression patterns when compared with other independent methods of expression analysis. It is very likely that the maximal domain of ARF activity shown by our studies is modulated and narrowed down post-transcriptionally. This might occur via post transcriptional gene silencing (PTGS) as demonstrated for *ARF3/4*, *ARF6/8* and *ARF10/16/17* or protein modification as shown for ARF2. Finally inhibition of most of the ARFs by Aux/IAAs is regulated in a time and space dependent manner through auxin.

Nonetheless our results give a first indication on how the broad range of distinct auxin responses is achieved by plant cells.
Based on the fact that every cell type in the developing embryo possesses a specific set of ARFs the most simplistic conclusion is that combining different ARFs results in different outputs onto auxin stimuli (Fig. 9). This conclusion implicates that ARFs show some degree of specificity and distinctive activity to stimulate different transcriptional responses.
Several intrinsic features of the ARF proteins can be envisioned to generate such a diversification. On one hand the varied amino acid composition in the middle region and the resulting different transcriptional activities of the ARFs in protoplast assays highlight their differential influence on transcription. In addition, variation in the DNA binding domain might allow each ARF to bind a specific set of target genes. Finally the distinct structure of an ARF might enable specific interactions with other proteins that in turn influence transcriptional activity (for example specific interaction of the kinase BIN2 with ARF2 and ARF9).

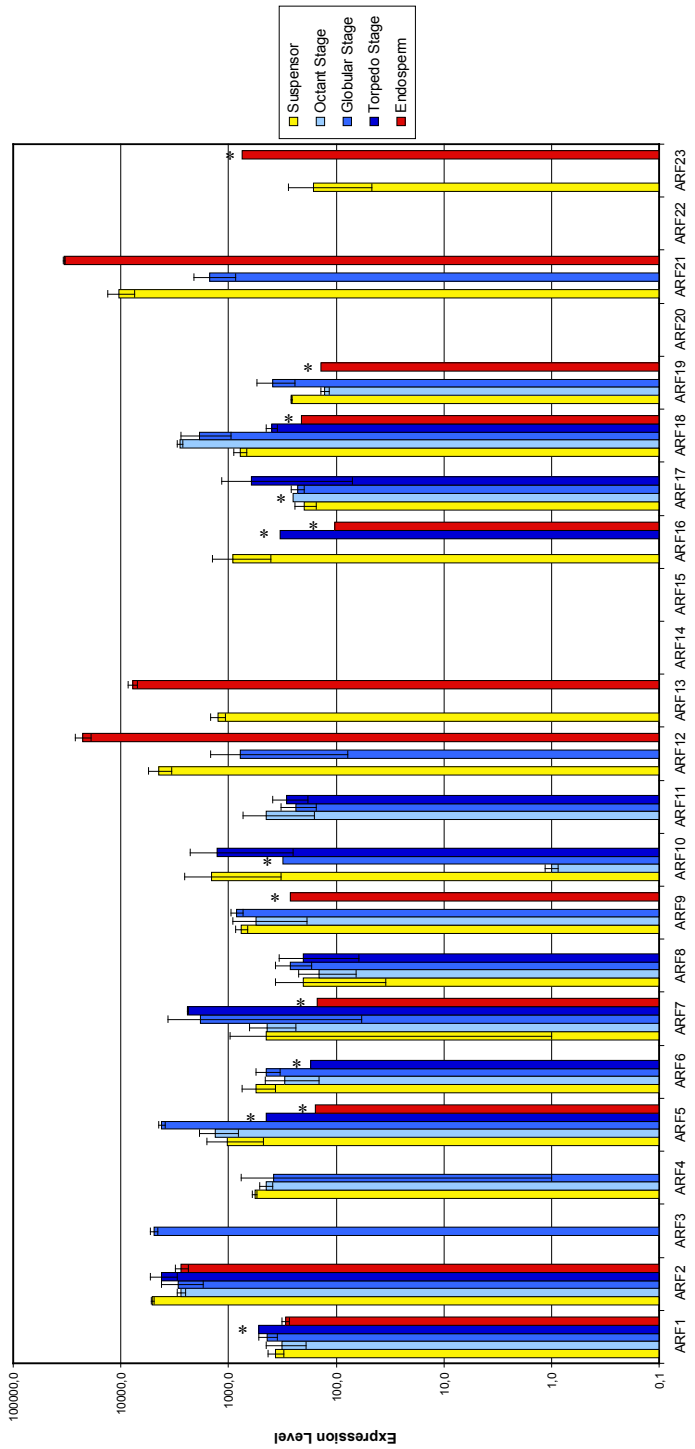

Figure 8: *ARF* expression levels as represented in the Goldberg data set
ARF expression levels in the analysed tissues and stages highlight expression of class I *ARF*s in suspensor and endosperm. They also correlate in other aspects with the expression patterns revealed by our transcriptional fusions (Fig. 2 – 3), however numerous deviations hinder a conclusive interpretation.
Measurements were only taken into account if they were judged as "present" in at least one experiment. Bars represent the average expression level of each gene measured in two independent experiments. A star on top of the bar indicates samples for which only one measurment was recorded.
Error bars denote a 95% confidence interval around the average level.
Maximum peaks are: *ARF12* – endosperm: 22540, *ARF13* – endosperm: 7750, *ARF21* – suspensor: 10350 and endosperm: 33830

A road map to engineering cell fate decisions

The observation that subsets of ARFs correlate with distinct cell fates holds a second noteworthy result. Namely that the amount of ARFs employed by a cell raises the further it is away from the top of the embryo. In globular embryos apical cells show expression of only two ubiquitous *ARF* promoters (*ARF1* and *ARF18*) while suspensor cells have four additional *ARF* promoters (*ARF2*, *ARF6*, *ARF9* and *ARF13*) turned on. Moreover, in heart stage embryos the SAM is the only site with only one *ARF* promoter (*pARF1*) being active while the RAM displays expression from eight *ARF* promoters. Even though at this stage one can only speculate about the relevance of this correlation, the expression data of all *ARF*s allows clear predictions to be made for future functional investigations. This includes selection of co-expressed *ARF*s for higher-order mutant analysis, but also cell-specific misexpression of *ARF*s. When misexpressed, *ARF5/MP* induces strong defects (Hardtke *et al.*, 2004; Weijers *et al.*, 2006, Chapter 2), which suggests that changing ARF composition in cells upsets their normal developmental program. From the expression atlas, one can predict sites where misexpression of one or two *ARF*s should cause specific defects, since the ARF composition of another cell type would be mimicked.
From classical tissue culture studies, it is well established that development of a shoot is favoured by cytokinin treatment, while root formation is an output of auxin treatment. In this light, it is interesting to see that the number of *ARF*s expressed in the future root pole of the embryo exceeds by far the number of *ARF*s expressed in the presumptive shoot meristem. By definition the root auxin response capacity is more elaborate or more refined. Strikingly, we find that the shoot apical meristem in the heart-stage embryo is almost devoid of *ARF* expression. This could mean that this group of cells has a limited capacity to responde to auxin. Whilst speculative, this finding was only revealed because all *ARF*s were investigated, predicts that misexpression of *ARF*s in the embryonic shoot apical meristem might disturb the formation of the meristem.

Finally, even though our analysis did not cover all tissues and organs in which auxin responses play a vital role, the availability of this collection of lines will prove to be of great value in further dissecting auxin response networks throughout the plant.

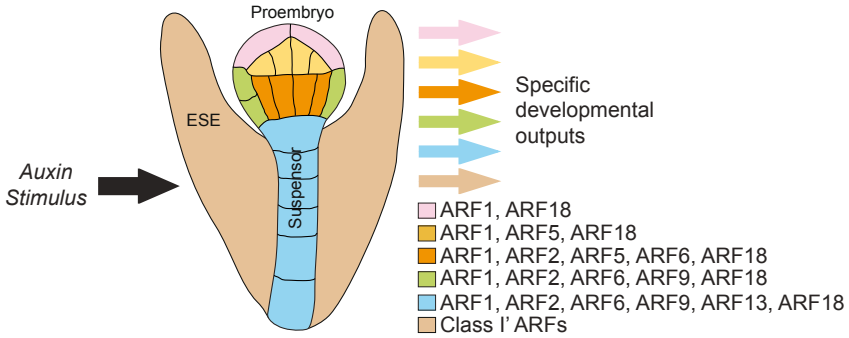

Figure 9: Distinct auxin response machineries permit cell type specific outputs
Auxin stimuli are translated into transcriptional responses by cell type specific auxin response machineries that are composed of varying ARF factors. The lowest amount of ARFs is found in the apical protoderm, while a maximum of 8 ARFs (Class I' = ARF12 to ARF15 and ARF20 to ARF23) is found in the embryo surrounding endosperm (ESE). The number of co-expressed *ARF*s coincides with the cellular postion along the apico-basal axis.

Materials & Methods

Cloning

All promoter fragments were amplified by PCR from genomic DNA or the respective BAC clone and initially ligated into pGEM-T easy (Promega). The cloned fragments were checked by sequencing (ServiceXS, Leiden) and in a second step subcloned into pGreenII / Kan SV40:3xGFP (Takada and Jürgens, 2007) thereby forming GIIK pARFXX::SV40x3GFP. Primers and restrictions sites used in this process are listed in table 1 (restriction sites are in bold).

The so formed binary vectors were used to transform plants of the Columbia ecotype (Col-0) via floral dip (Clough and Bent, 1998). In the resulting transgenic T_1 generation expression patterns were analyzed during embryogenesis (T_2 embryos) in at least ten independent plants for each ARF. Offspring of two typical lines for each construct was further used to study GFP expression in detail using confocal microscopy.

Plant growth conditions

Plants were grown under a 16 hour light / 8 hour night light regime at 22 ºC. Typically seeds were germinated and grown for the first two weeks on 1/2 MS medium containing 1% sucrose, 0.8% agar and kanamycin (c = 50 mg/l) if selection was required. Afterwards seedlings were transferred to soil and grown under the same conditions in a walk-in growth chamber.

Microscopy

Ovules were removed from siliques with a fine needle and transferred to PBS buffer containing 4% paraformaldehyde and 5% glycerol. Staining of membranes was achieved by adding the lipophillic dye FM4-64 to a final concentration of 1 μM. Images were taken with a confocal microscope (LSM510, Zeiss) using an argon laser for excitation at 488 nm and by collecting signals at 505 – 530 nm (GFP) and above 650 nm (FM4-64).

Acknowledgements

This work was financially supported by grants from the Netherlands Organization for Scientific Research (NWO; VIDI, ALW- Open Competition) to D.W.. We acknowledge Sacco de Vries for support and the Arabidopsis Biological Resource Center for BAC clones.

Table

Table 1: Primers and DNA templates used for amplification of the ARF promoters
Bold letters indicate additional bases that were used to establish restriction sites (bold and italic) necessary for cloning.

Gene	Primer orientation	Sequence (5' extension in bold)	Position (relative to START codon)	BAC number
ARF1	Sense	**CA*GGTACC***TTAAAGCTAACTCGC	-2088	genomic
	Antisense	**CA*GGGCCC***TAGGAATCTACTTAC	-1	
ARF2	Sense	**CA*GGGCCC***TGAATGAAAGAGTCG	-2084	genomic
	Antisense	**CA*CTGCAG***ACCTTCCGAAGCTCAGATC	-1	
ARF3	Sense	**CA*GGTACC***ATCTCTCGTTTACTTTGATGC	-2056	genomic
	Antisense	**CA*GGGCCC***TAAAGAGAGAGAAACAG	-1	
ARF4	Sense	**CA*GGTACC***TTTTAGTCGTTGCGGG	-2099	MUF9
	Antisense	**CA*GGGCCC***TGAAAAAGCTTCTCTTTTAAG	-1	
ARF5	Sense	**CA*GGTACC***ATATATCTTAGTGACAAACGCG	-2071	genomic
	Antisense	**CA*GGGCCC***ACAGAGAGATTTTTCAATG	-1	
ARF6	Sense	**CA*GGGCCCC***TCTGTATATAATAAACAAGC	-2132	T4K22
	Antisense	**CA*CTGCAG***TTTTTATTCTAACTTAAAAAGC	-1	
ARF7	Sense	**CA*GGTACC***TTTAAACGTTAGCTAGGCC	-2044	genomic
	Antisense	**CA*GGGCCC***GATCACTCAACTTTACTTTC	-1	
ARF8	Sense	**CA*GGTACC***TCCATATGGAGTACAGTGAGG	-2084	genomic
	Antisense	**CA*GGGCCC***GTCTAATTCAACTTCAAGAAACC	-1	
ARF9	Sense	**C*GGTACC***TGGTGGTGGGTTTTAAGG	-2295	T32A16
	Antisense	**T*GGGCCC***CAGCTGATTAAATCTTCTATCAGTCACACC	-1	
ARF10	Sense	**CA*GGTACC***AAAACTTAGGCCTTAGATGG	2078	T1B3
	Antisense	**CA*GGGCCC***CTAGACGAAGTTGTGTAAC	-1	
ARF11	Sense	**C*GGTACC***GATAATCATATTATAACT	-2075	F11C10
	Antisense	**T*GGGCCC***CGAAGAAACCAAAAAAAATCC	-1	
ARF12	Sense	**A*GGTACC***AATCTTATAAGAAATTAAGAAGAGAATAGA	-2012	F23M19
	Antisense	**A*GGGCCC***AAGCTCGTATCTAAA	-1	
ARF13	Sense	**A*GGTACC***AGAGTCAAAAAAATGATTTTGTTTGTGATTA	-1802	F12G12
	Antisense	**C*GGGCCC***GAGCTCTTATTGCAA	-1	
ARF14	Sense	**C*GGTACC***AACAAAAACAAAATC	-2017	F15O4
	Antisense	**A*GGGCCC***AAGCTCGTACCTAAA	-1	
ARF15	Sense	***GGTACC***GATGGGTTAATTGGGTAAACCATTGAAACC	-1744	F15O4
	Antisense	***CTGCAG***CATAACCTCTTATCCAAAGATCGGTATACC	-1	
ARF16	Sense	**CA*GGTACC***TTGGATTTTTGTTTACGTATGG	-2045	genomic
	Antisense	**CA*GGGCCC***ATTTTTTGTGACCGTT	-1	
ARF17	Sense	**CA*GGTACC***TAACCATGATGTGATAGTGGG	-1795	genomic
	Antisense	**CA*GGGCCC***AGGTATTTGTTTTCAGTG	-1	
ARF18	Sense	**C*GGTACC***CGTCTCCGCTTTGCAAGG	-2265	F21F14
	Antisense	**T*GGGCCC***TGAAGAACCCAGATGAGAACTG	-1	
ARF19	Sense	**CA*GGTACC***GTTAATTCGTTTTGATGAGG	-2063	genomic
	Antisense	**CA*GGGCCC***GGTTTATAGAAAGAACG	-1	
ARF20	Sense	**C*GGTACC***GATATCCGCTCTATG	-2004	T9I1
	Antisense	**A*GGGCCC***AACCTCGTATCCAAA	-1	
ARF21	Sense	**C*GGTACC***GCTTAATCTAACAAC	-2006	F7P12
	Antisense	**A*GGGCCC***AAGCTCGTATCTAAA	-1	
ARF22	Sense	**A*GGTACC***CGTCGTCAACCTCTG	-1711	F7P12
	Antisense	**A*GGGCCC***AAGCTCGTATCTAAA	-1	
ARF23	Sense	**C*GGTACC***GGAAATTCACATCAT	-805	F9C16
	Antisense	**A*GGGCCC***AAGCTCGTATCCAAA	-1	

Chapter 4

Phylogenetically divergent co-expressed *ARF*s redundantly control embryonic and post-embryonic Development

Eike H. Rademacher, Willy van den Berg,
Annemarie S. Lokerse and Dolf Weijers

Abstract

Comprehensive mapping of ARF expression during embryogenesis of *Arabidopsis* demonstrated that six ARFs (ARF1, 2, 6, 9, 13 and 18) are active in suspensor cells. Unspecific inhibition of ARF activity in the suspensor by expression of stabilized Aux/IAAs caused loss of suspensor cell fate and proliferation of the cell file. To determine which of the six suspensor-expressed ARFs contribute to suspensor cell fate maintenance we set out to ultimately generate the respective hexuple mutant as well as intermediate mutant combinations. To this point two triple mutant lines - *arf1-5 arf2-8 arf6-1* and *arf9-1 arf13-2 arf18-2* - have been established. Of these the *arf9-1 arf13-2 arf18-2* triple mutant did not show any phenotypic aberrations while *arf1-5 arf2-8 arf6-1* plants show enhancement of postembryonic phenotypes reported for the *arf1-5 arf2-8* double mutant. In addition, embryos of this genotype are impaired in proper cell organization at the basal pole of the embryo but still develop to viable seedlings. Our findings highlight a novel auxin response machinery in which a predicted activator (ARF6) and predicted repressors (ARF1 and 2) act redundantly to control developmental processes. Furthermore, these findings highlight the value of our ARF expression map for revealing unsuspected redundancies among phylogenetically distant ARFs.

Introduction

Auxin response factors (ARFs) form the pivotal point in translating auxin stimuli into various transcriptional responses of the cell. A wealth of experiments demonstrated that at steady state, ARFs are inhibited by Aux/IAA proteins which in turn are degraded upon elevated auxin concentrations. Thus, auxin releases the ARFs from inhibition. While this mode of action explains how auxin stimuli are perceived generically, it fails to explain how the multitude of auxin responses is generated in plant development. One major factor in generating cellular diversity of auxin responses may be the regulation of the 23 *ARF*s present in the *Arabidopsis thaliana* genome. If cells are endowed with unique sets of ARFs, whose activities are individually controlled, these may condition auxin responses such that the cell alters the expression of a particular subset of auxin-responsive genes.

One way to alter ARF activity lies in the modification of their binding affinity to, or transcriptional activation / repression potential of the respective target genes. For example, the BIN2 protein kinase phosphorylates ARF2 and thereby changes the binding of the latter to DNA, which in turn affects the transcriptional responses mediated by ARF2 (Vert *et al.*, 2008). Changes in the binding to target gene promoters might also be achieved by remodeling the chromatin structure of the respective genes. Such a mechanism has been implicated by Fukaki *et al.* (Fukaki et al., 2006) who showed the involvement of the chromatin remodeling factor PICKLE (PKL) in the modulation of ARF7/ARF19 dependent lateral root formation.

Another regulatory control layer in generating cell type-specific auxin responses arises from the combinatorial utilization of ARFs with partially overlapping functionality. This would allow the creation of an enormous amount of distinct combinations and corresponding transcriptional responses (already 2048 if not more than 3 out of 23 ARFs act together). A first glimpse into the joint activity of ARFs stems from the generation of double mutants lacking the activity of two (closely related) sister ARFs. Such double mutant plants (*arf1 arf2; arf3 arf4; arf5 arf7; arf6 arf8; arf7 arf19*) show partially enhanced phenotypes demonstrating overlapping functionality of the knocked-out *ARF*s (Hardtke *et al.*, 2004; Ellis *et al.*, 2005; Nagpal *et al.*, 2005; Pekker *et al.*, 2005; Wilmoth *et al.*, 2005).

Our cellular expression atlas for the *ARF* family (Chapter 3) greatly enhanced the available knowledge of co-expressed *ARF*s. Based on this data set we were able to define cell type-specific combinations of distantly related *ARF*s. Importantly, many cell types express more than two *ARF*s, such that redundant functions would probably not even be found in systematic double mutant analysis.

In particular we focused on early embryogenesis and found a subset of seven *ARF*s (*ARF1, ARF2, ARF5/MP, ARF6, ARF9, ARF13* and *ARF18*) to be expressed in various expression patterns throughout the embryo. Thereby a total of five different cell type specific ARF combinations can be distinguished coinciding with the cell fates found at dermatogen stage of embryogenesis. The maximum of overlapping *ARF* expression is present in the suspensor where ARF1, 2, 6, 9, 13 and 18 seem to control auxin dependent transcription.

During early embryogenesis the uppermost suspensor cell adopts hypophysis cell fate and becomes the founder cell of the root meristem. For both the respecification of the uppermost suspensor cell as well as the initial maintenance of its suspensor cell fate distinct auxin responses are employed. Extensive work on ARF5/MP demonstrated the involvement of a proembryo-localized auxin response machinery in the non cell autonomous specification of the hypophysis (Weijers *et al.*, 2006). However the suspensor-specific auxin response ma-

chinery has yet not been completely identified. In previous chapters we have described our efforts to first characterize the impact of auxin responses in the suspensor and subsequently identify the transcription factors involved. Here we report our progress towards generating a hexuple mutant plant line that lacks the activity of all six *ARF*s (*ARF1, 2, 6, 9, 13, 18*) that are expressed in the suspensor. Various combinations of *arf* mutants have been generated and revealed unexpected redundancies that could not have been deduced from the phylogenetic relationships of the ARFs. In addition to validating the predictive value of our *pARF-GFP* expression data, this allows further insights into auxin dependent transcriptional networks.

Results

ARF1, 2 and 6 redundantly control post-embryonic plant development

In generating the *arf1, 2, 6, 9, 13, 18* hexuple mutant, we chose to first generate two triple mutant combinations. One of these was an *arf1 arf2 arf6* triple mutant. Even though ARF1 and 2 have been shown to repress an auxin-dependent reporter gene in protoplasts, whereas ARF6 acts as an activator in the same assay, we found striking overlap in expression of these three *ARF* genes in the early embryo (Chapter 3). Likewise, extensive co-expression of these *ARF*s was found in the primary root tip (Barbara Möller, unpublished).
The co-expression found in our *pARF-GFP* survey is corroborated by expression data derived from the Developmental Map data set of the Arabidopsis eFP Browser (Winter *et al.*, 2007), which shows that *ARF1*, *ARF2* and *ARF6* are ubiquitously expressed throughout the plant.
According to the eFP browser, *ARF1* is expressed to highest levels during late stages of seed development as well as during the transition of the shoot apex from vegetative growth to an inflorescence and the following early stages of flower development.
In almost all tissues *ARF2* is expressed at higher levels than *ARF1*. The most prominent peaks are found in cauline and senescent leaves. Finally, *ARF6* expression reaches its highest peaks in the shoot apex and during early flower development. But differing from *ARF1, ARF6* continues to be expressed at high levels in the petals and carpels of the mature flower. What these microarray data collectively show is that the expression of *ARF1, 2* and *6* overlaps throughout plant development. For this reason, while generating the triple mutant for analyzing embryogenesis, we also scored post-embryonic development to determine the extent of functional redundancy between these genes.

Initially, we confirmed the developmental phenotypes that have been reported for the *arf2-8* and *arf6-1* single mutants. Plants carrying a null allele of ARF6 (*arf6-1*) show delayed stamen filament elongation and anther dehiscence (Nagpal *et al.*, 2005), whereas the loss of ARF2 in *arf2-8* plants causes a delay in rosette leaf senescence and floral organ abscission (Ellis *et al.*, 2005). In contrast, knock-out lines for *ARF1* do not show any phenotypic irregularities, but enhance the *arf2* mutant phenotype (Ellis *et al.*, 2005). Under our growth conditions, we confirmed these characteristics for the *arf1-5*, *arf2-8* and *arf6-1* single mutants, and in the *arf1-5 arf2-8* double mutant. We crossed *arf6-1* with *arf1-5 arf2-8* and recovered all

double and the triple mutant combinations in the F_2 generation. *arf1-5* and *arf2-8* mutants have been reported to be null or show severe reduction in mRNA level (Ellis *et al.*, 2005; Okushima *et al.*, 2005).

Interestingly, when comparing these genotypes with wild type and the single mutants, clear enhancement of post-embryonic phenotypes can be observed. The genotypes span a range of increasing defects with the wild type Columbia phenotype on one end of the spectrum, and the severely affected *arf1-5 arf2-8 arf6-1* triple mutant plants on the other end (Fig. 1 and 2). In between these extremes one finds (in order of increasing impairment) first the single mutants for *arf1-5*, *arf6-1* and *arf2-8* with the above described phenotypes followed by the double mutants lacking functionality of *arf1-5* and *arf6-1*, *arf2-8* and *arf6-1* or *arf1-5* and *arf2-8*. Plants of all three double mutant genotypes as well as of the triple mutant genotype are considerably delayed in rosette leaf senescence (not shown). Consequently this delayed decay allows the plants to grow to much greater size even though maximal length is reached noticeably later than in wild type plants due to a concomitant retardation in the initiation of flowering by approximately 3 weeks in the case of the triple mutant (Fig. 1). The delay in flowering was also reflected in the number of rosette leaves formed until the first shoot had emerged. On average wild type plants developed 8.6 rosette leaves before flowering whereas triple mutant plants showed 20.8 rosette leaves at that moment.

Along the growing stems in all mutant combinations, less side branches are formed (Fig. 1). Furthermore the number of flowers emerging from the shoot apical meristem is reduced in combinations involving the *arf2-8* null allele.
Another clear case of redundancy is found during flower development. While *arf1-5 arf6-1* flowers open and display normal-sized petals, flowers of *arf2-8 arf6-1, arf1-5 arf2-8* and *arf1-1 arf2-8 arf6-1* plants remain closed throughout development and show a reduction in petal length (Fig. 2). In all these combinations the outgrowth of stamen filaments is delayed or reduced so that the plants are impaired in self fertilization. In the cases of all double mutants, the resulting lower fertility can easily be overcome by manual pollination. However

Figure 1: Overall plant structure after 6 weeks of growth
After 6 weeks of growth double and triple mutant plants are delayed in development when compared to wild type plants. All mutant plants also show a reduction in the onset of side branches as well as in the growth rate of their primary shoots.

in the triple mutant anthers do not dehisce (Fig. 2) so that this mutant combination is fully male sterile.
In addition to the stamen phenotype, *arf1-5 arf2-8 arf6-1* flowers also fail to develop stigmatic papillae and have a stunted gynoecium. These flowers therefore resemble to some extent the floral phenotype found in *arf6 arf8* double mutant plants (Nagpal *et al.*, 2005). In summary, ARF1, 2 and 6 show redundant activities in flowering control, senescence, shoot branching, anther and gynoecium development.

Figure 2: Aberrations in inflorescence and flower development
The overall organization of inflorescences of all higher order mutant plants is normal. But the distances between mature flowers are enlarged in all combinations including *arf2-8*. While *arf1-5 arf6-1* flowers open but fail to self-polinate due to shorter stamen filaments, flowers of *arf2-8 arf6-1*, *arf1-5 arf2-8* and *arf1-5 arf2-8 arf6-1* plants do not open. Furthermore outgrowth of the carpels is reduced in *arf1-5 arf2-8* and *arf1-5 arf2-8 arf6-1* plants so that the gynoeceum never extends over the closed sepals and petals.

Redundant control of embryo development by ARF1, ARF2 and ARF6

Our expression data for ARF1, ARF2 and ARF6 predicts the three genes to be redundantly active in the basal protoderm of the proembryo as well as in the suspensor at the globular stage (Chapter 3; Fig. 3).
While in post-embryonic development, double mutant combinations revealed redundant ARF functions, this is not the case in the embryo as none of the single (*arf1-5, arf2-8, arf6-1*) or double mutant (*arf1-5 arf2-8, arf1-5 arf6-1, arf2-8 arf6-1*) combinations displayed significant phenotypic aberrations during embryogenesis.
As the triple homozygote is sterile, we manually self-fertilized plants that are homozygous for *arf1-5* and *arf6-1* and heterozygous for *arf2-8* to obtain a population that segregates 25% triple mutant embryos. In such pollinations we observed a novel phenotype, not observed in

any of the controls. Approximately 18% of the embryos (20 of 112) displayed abnormal divisions in the basal domain of the proembryo and in the suspensor (Fig. 3). When corrected for the heterozygosity of *arf2-8*, this fraction represents more than half of the triple homozygous embryos.
Aberrant cell divisions were already observed at the four cell stage (Fig. 3 a). It can not be unequivocally determined whether the early defects result from a wrong horizontal division of the apical cell or if they are rather derived from the basal cell lineage due to a misoriented vertical division. Despite being distorted at the basal end, triple mutant embryos did not abort as evidenced by the full seed set and absence of ovules aborted at later stages. In fact embryos of all genotypes, including the triple mutant developed into viable seedlings without apparent strong patterning defects (Fig. 1, 2).
In conclusion, novel embryo phenotypes that are observed in those cells of the *arf1-5 arf2-8 arf6-1* triple mutant that co-express all three *ARF*s, revealed functional redundancy between these three phylogenetically distant *ARF*s.

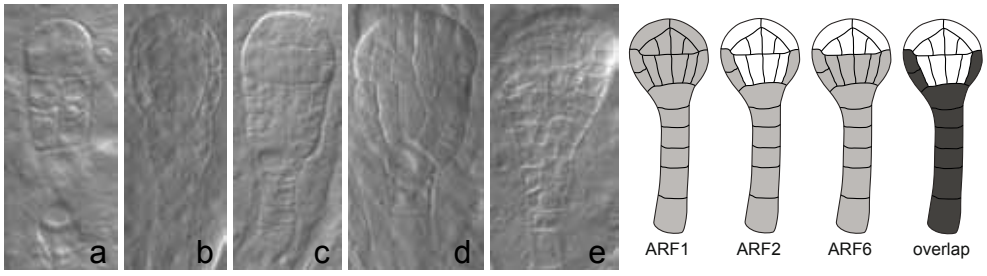

Figure 3: Embryo phenotype of *arf1-5 arf2-8 arf6-1* plants.
Normaski images of cleared ovules derived from *arf1-5* -/- *arf2-8* +/- *arf6-1* -/- plants. Embryos show extra cells beetween an apical embryonic domain and a suspensor like cell file. Morphological features of theses cells such as being small and fast dividing and not having a big vacuole suggest that these cells partially lost suspensor cell fate. As highlighted by the depicted expression schemes of *ARF1, 2* and *6* the phenotypic aberrations match with the expression overlap of all three genes.

Loss of ARF9, 13 and 18 does not cause developmental defects

In our effort to generate an *arf1, 2, 6, 9, 13, 18* hexuple mutant, we also generated plants homozygous for T-DNA insertions in *ARF9*, *ARF13* and *ARF18*, as well as all double mutants. *ARF9*, *13* and *18* are closely related in sequence, and cluster together in the phylogenetic tree, but are distinct from *ARF1, 2* and *6* (see Fig. 1 in Chapter 3). Therefore, it is conceivable that these *ARF*s form a sub-function of the suspensor-expressed *ARF*s.
We isolated several T-DNA insertion lines from the SALK collection. In particular these were *arf9-1*, *arf9-2*, *arf9-101* and *arf9-102*, *arf13-1*, *arf13-2*, *arf18-1* and *arf18-2*.The insertion sites of all lines were verified by sequencing insertion-specific PCR fragments. All insertion lines contained an insertion at the expected position (Fig. 4). In order to select mutants for constructing the higher-order mutant, we first set out to test whether ARF mRNA is lost or reduced in the mutant lines. The *arf18* T-DNA insertion lines have been characterized by C. Gassers lab. RT-PCR demonstrated that *arf18-2* is a full knock-out while in *arf18-1* mRNA levels are only reduced (C. Gasser, personal communication). Consequently we used *arf18-2* for the generation of higher order mutants. We attempted to design primers that would specifically recognize either *ARF9* or *ARF13* mRNA. However, due to the extremely

high similarity of mRNAs encoded by the *ARF9, 12, 13, 14, 15, 20, 21, 22* and *23* genes, it was impossible to discriminate between the transcripts by means of RT-PCR. We therefore considered *arf9-1* and *arf13-2* to be most likely the strongest knock-out candidates based on the location of the insertion in an N-terminal exon (Fig. 4) and used these two lines for the generation of double and triple mutant plants.

We investigated all single, double and the triple mutants for visible seedling and vegetative phenotypes, as well as for aberrations during embryogenesis. None of the lines showed abnormal development. Therefore, either the *arf9-1* and/or the *arf13-2* lines still express a sufficiently high level of transcript, or these three ARFs are not limiting for normal embryo development. As these three *ARF*s have overlapping expression in the suspensor, where also *ARF1, 2* and *6* are expressed, it is possible that ARF9, 13 and 18 function will only become apparent in a hexuple mutant. The *arf1 arf2 arf6* and *arf9 arf13 arf18* triple mutants were crossed and F_1 plants have been selected that are heterozygous for all six mutations. These plants are aphenotypic, and further generations will be required to determine the hexuple mutant embryo phenotype.

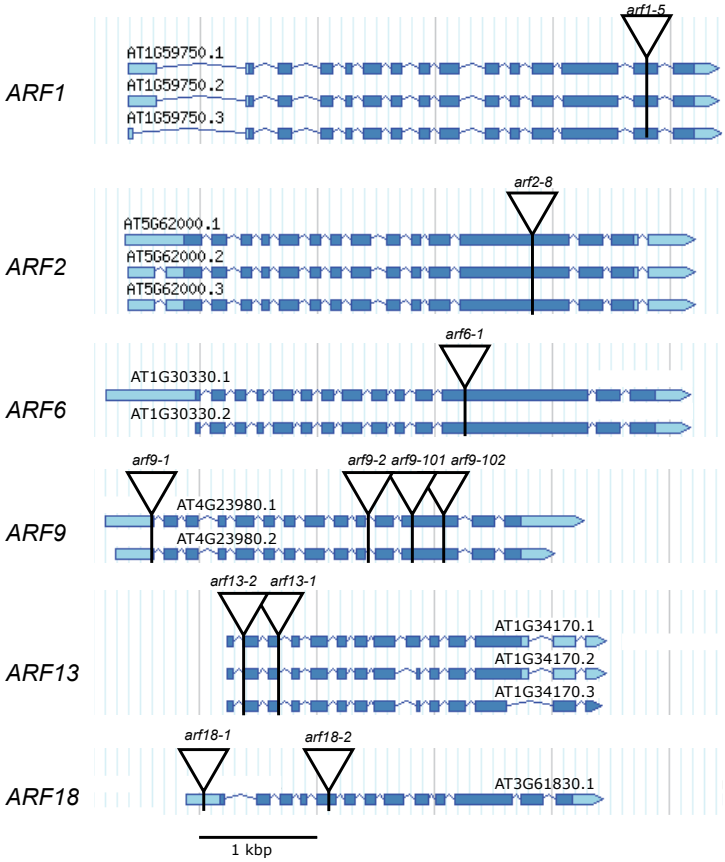

Figure 4: Location of the T-DNA insertions in the 6 *ARF* genes
Pictures have been adapted from TAIRs GBrowse.
Exons are depicted as dark blue boxes; 5' and 3' UTRs are represented by light blue boxes.

Discussion

Based on our *ARF* expression atlas we set out to generate the hexuple knock-out mutant for all *ARF*s co-expressed in the suspensor. While the final mutant line homozygous for all alleles is not yet available, intermediate double and triple mutants already allow for further insights into ARF functionality. The six ARFs found in the suspensor fall into three phylogenetic subgroups. ARF1 and ARF2 are closely related to each other and form a sister pair within the ARF tree. On the other hand, ARF9, ARF13 and ARF18 are directly related to the previous pair but have been further modified during evolution of *Arabidopsis*.
Finally, ARF6 is distantly related to the five above mentioned ARFs. Due to the amino acid composition of its middle region and its relationship with other transcriptional activators it might be the only transcriptional activator found in the suspensor (Tiwari *et al.*, 2003; Remington *et al.*, 2004).

To this point we have generated the triple mutant knock-out combinations *arf1-5 arf2-8 arf6-1* and *arf9-1 arf13-2 arf18-2*. The later of these did not show any visible phenotype during embryogenesis or post-embryonic development of the seedling. Several reasons for this can be envisioned. Firstly, the alleles we used to create the triple mutant may not be full knock-outs and still provide a sufficient activity of the respective ARF. Indeed, we were not able to verify the reduction of transcription of the loci by RT-PCR due to the close homology of *ARF9* and *ARF13* to each other and seven other *ARF*s expressed in the embryo-surrounding endosperm (Chapter 3). Nevertheless the location of the T-DNA insertions in a 5'-located exon should result in at least strong impairment of transcription from the loci. Additionally, we also generated combinations of other *ARF9* and *ARF13* alleles and also there we did not find phenotypic aberrations (not shown).
Clearly, the lack of ARF9, ARF13 or ARF18 function does not affect plant development in a severe manner. Even though all three ARFs are closely related in phylogenetic terms, inspection of our *ARF* expression atlas shows that expression patterns of *ARF9* and *ARF18* resemble the ones found for *ARF2* or *ARF1* respectively. Hence these factors have the potential to fully cover the loss of *arf9* and *arf18* and thereby suppress any phenotype involving the *arf9 arf18* double mutant. The ongoing analysis of further *arf* knock-out allele combinations will certainly uncover the particular contributions of such redundancies. Furthermore, microarray analysis on embryos that misexpress the ARF-inhibitor *iaa12/bdl* in suspensor cells revealed that several of the ARFs that are closely related to ARF9 and 13 are upregulated upon ARF inhibition (Annemarie S. Lokerse and Dolf Weijers, unpublished observations). Such conditional redundancy might also explain the failure to find suspensor defects in the *arf9 arf13 arf18* triple mutant.

Contrary to *ARF9*, *ARF13* and *ARF18*, the knock-out of *ARF1*, *ARF2* or *ARF6* does lead to phenotypic aberrations during plant development. Our analysis of *arf1-5 arf6-1*, *arf2-8 arf6-1* and *arf1-5 arf2-8 arf6-1* double and triple mutants revealed that ARF6 is partially redundant to both ARF1 and ARF2 in a range of developmental processes. This is perhaps not surprising considering the extensive co-expression of the three *ARF* genes (Chapter 3 and Barbara Möller, unpublished), but is less trivial in the light of ARF6 being predicted to be a transcriptional activator while ARF1 and ARF2 appear to function as repressors (Tiwari *et al.*, 2003). Though unexpected, redundancy between activator ARFs and repressor ARFs

is not entirely without precedent: ARF7 and ARF19 have been reported to act redundantly with ARF2 during leaf senescence and anther development (Ellis *et al.*, 2005). However, in that particular case, no double mutant controls were included. The enhancement of the *arf2* mutant phenotype may therefore be caused by the *arf19* mutation alone. ARF19 is peculiar among the ARFs since it has both the middle region signature of a transcriptional activator and an EAR domain that in their proteins mediates transcriptional repression (reviewed in (Lokerse and Weijers, 2009)).
Two scenarios could explain the redundant action of ARF1, 2 and 6. On one hand the middle region of an ARF might not strictly define its transcriptional activity as its effect on the transcriptome could be modulated by interacting factors or protein modifications. In this scenario, the *in vivo* activity of an ARF will depend on context-dependent cofactors.
On the other hand, repressor and activator ARFs might influence the same mechanism by working on both sides of a balanced steady state. In this case a new developmental program is induced by up- as well as downregulation of differing target genes, the composition of which will define the novel state of the cell. This would give a robust response that could be maintained at a less intense level even if some of the ARFs are missing. However such a mechanism requires differential binding of ARFs to target gene promoters, a possibility that has not been explored to date. Furthermore, one prediction of this scenario is that single or double mutants would show perhaps opposite transcriptional defects. The resolution of our analysis has not allowed detection of such molecular phenotypes, but this is a hypothesis worth testing experimentally.

The phenotypic analysis of higher order knock-out mutant combinations conducted in this study proved the importance and capability of our *ARF* expression atlas for the guided dissection of uncharacterized auxin responses. It furthermore confirmed the redundant action of unrelated ARFs as predicted by the co-expression patterns.
About the relevance of higher order redundancies among the ARFs one can only speculate at this point. Since no two *ARF* genes are completely similar a simple idea might be that even though greatly overlapping in function every single ARF delivers an additional specific, albeit small contribution to the respective developmental process. A complementary approach to test this hypothesis would be to complement a phenotypic *ARF* mutant (such as *arf5/mp*) with each *ARF* cDNA driven from the correct cis-elements, and determine to what extent co-expressed *ARF*s can rescue the mutant phenotype.
On top of this co-expression-based redundancy, additional regulatory feedback loops between the *ARF*s could further contribute to redundancy within the family. One example is the upregulation of *ARF* genes in a microarray on suspensor-specific *iaa12/bdl* misexpression (Annemarie S. Lokerse and Dolf Weijers, unpublished observations). A similar case has been reported for the PIN auxin transport proteins. Loss of a *PIN* gene leads to upregulation of other *PIN* genes, likely through auxin accumulation and auxin-dependent transcription (Vieten *et al.*, 2007). While some *ARF*s respond to auxin treatment (*ARF5/MP*: (Wenzel *et al.*, 2007), *ARF16*: (Wang *et al.*, 2005), *ARF19*: (Wilmoth *et al.*, 2005; Li *et al.*, 2006)), others may be feedback-controlled through changes in cell identity; there is ample opportunity for further conditional redundancy in this gene family. With the availability of a line that segregates mutations in all *ARF*s expressed in a cell type, the suspensor, we will soon see the full extent of transcriptional complexity in *ARF* regulation. In a more optimistic case, we will be able to determine the consequence of removing all ARF activity from this cell type.

Materials & Methods

Plant growth and material

Seeds were sterilized by rinsing with 70% ethanol, followed by three steps of washing with autoclaved water. After plating on ½ MS medium, seeds were transferred to a growth chamber with long day light regime (16 h light / 8 hours dark) and a constant temperature of 22 ºC. After approximately two weeks seedlings were transferred to soil and further grown at the same light and temperature conditions as before.
Wild type plants were of the Columbia ecotype (Col-0). The stock names of all T-DNA lines can be found in Table 1. All T-DNA lines were genotyped by triplex PCR using a matching left-border primer and the respective genomic primers as listed in Table 2. The PCR program consisted of an initial denaturation step for 3 min at 95 ºC, then amplification of the fragments in 35 cycles of 95 ºC for 30 sec, 60 ºC for 30 sec, 72 ºC for 1 min 30 sec and a final elongation step at 72 ºC for 3 min. PCR products were analyzed by standard agarose gel electrophoresis.

Microscopy

For DIC microscopy ovules were mounted in a clearing solution of chloral hydrate, water and glycerol (8:3:1), left overnight at room temperature and subsequently investigated at a Leica DRM microscope equipped with differential interference contrast (DIC) optics.

Delay in flowering time

Flowering time was defined as the first shoot being 1 cm long. Measurements were averaged over 13 wild type plants and 8 *arf1 arf2 arf6* triple mutant plants.

Tables

Table 1: T-DNA insertion lines

Allele	Stock name
arf1-5	SALK 079046
arf2-8	SALK 108995
arf6-1	CS24606
arf9-1	CS24609 / SAIL 881 H05
arf9-2	CS24610 / SAIL 1207 H04
arf9-101	SALK 019903
arf9-102	SALK 032472
arf13-1	SALK 005960
arf13-2	SALK 138188
arf18-1	SAIL 81 A06
arf18-2	GABI 513E07

Table 2: Genotyping primers for T-DNA insertions

Primer name	Sequence
LBb1.3 (left border primer for SALK lines)	TGGTTCACGTAGTGGGCCATCG
SAIL LB1 (left border primer for SAIL lines)	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
o8409 (left border primer for GABI-KAT lines)	ATATTGACCATCATACTCATTGC
arf1-5 LP	CAGACACTCCTTCCTCAGTGC
arf1-5 RP	GCCCTCTGTATTCCCATTTTC
arf2-8 LP	ATGAAGATTTTGCGAACCATG
arf2-8 RP	TTACACAGATTTGCTCTCCGG
arf6-1 LP	ATCATTCATCATCTGATCAAAATGCAGG
arf6-1 RP	ACGCTTTGATGGCCAACC
arf9-1 LP	TGAGGAAAACAATTATTGTTGTGC
arf9-1 RP	AAGTCGACTTGTTGCGTTGAC
arf13-2 LP	ACGAGGTTTCCACTTTCCATC
arf13-2 RP	GCAAGCAACAAAATTAGATCTTTC
arf18-2 LP	TCTTCCTCCAAAGATACTTTGTCG
arf18-2 RP	ATCCCCATTCTCACCCCTG

Chapter 5

Distinct Control of *ARF* Expression in the early Embryo by the SSP-YODA and WOX Pathways

Eike H. Rademacher, Cristina Llavata Peris and Dolf Weijers

Abstract

Differential expression of *ARF*s in distinct cell types of the *Arabidopsis* embryo implicates that cellular auxin responses are prepatterned by the respective *ARF* expression domains. How these expression domains are established is currently unknown and factors regulating *ARF* expression have not yet been described. Here we analyze changes in the expression of all *ARF*s involved at early steps of embryogenesis in two mutant backgrounds. Both, the SSP-YDA pathway and the *WOX* genes (*WOX8*, *WOX9* and *WOX2*), independently control the formation of the apico-basal axis during embryogenesis. We found that misspecification of the basal lineage in mutants with disruptions in the SSP-YDA pathway coincides with expanded expression of the embryo-specific *ARF5/MP* gene, while the expression of suspensor *ARF*s is unaffected in this mutant. Conversely, mutations in *WOX8* and *WOX9* disrupt expression of *ARF9* and *ARF13* in the suspensor. *ARF1, 2* and *6* expression is not changed in any of the two mutant backgrounds, implying the existence of a third yet unknown control mechanism of *ARF* expression in the suspensor. Our results suggest that three independent pathways are employed to establish and maintain proper suspensor cell fate by regulating specific sets of *ARF*s.

Introduction

Plant embryos do not freely float in a nutritious liquid inside the ovule. Rather, embryos are anchored within the seed and connected to maternal tissue via an extra-embryonic structure, called the suspensor. This suspensor develops simultaneously with the embryo from a larger basal cell that is formed by the asymmetric division of the zygote. Next to positioning the developing embryo in the ovule, the suspensor also serves as vital transport route for nutrients and potentially for developmental signals to the proembryo. Its rather passive involvement in embryogenesis is reflected in the slow rate and uniform orientation of cell divisions as well as in the advanced differentiation exhibited by the respective cells. Importantly, the suspensor is a transient structure: cell death has been reported in later embryonic stages and the suspensor is generally not part of the seedling.

It has been established decades ago that suspensor cells have developmental potential beyond the rather restricted activity that is normally observed (Haccius, 1978). Genetic or experimental ablation or impairment of embryo cells induces embryo-like divisions in the suspensor, eventually giving rise to secondary embryos. Hence, the suspensor can be regarded as a transient pool of stem cells that can switch to embryo identity if the need arises. Many mutants in *Arabidopsis thaliana* show suspensor proliferation. However, all but a few of these first display defects in proembryo development. Among the exceptions is *twin1*, which shows secondary embryogenesis without prior arrest of the proembryo. As the name implies, proliferation of the suspensor generates secondary embryos and hence twins are formed within one ovule. Despite this impressive developmental fate change, the *TWIN1* gene has not yet been identified (Vernon *et al.*, 2001). The *AMP1* gene is also required for suspensor development, since the *amp1* mutant shows low penetrance of twinning derived from suspensor division. The *AMP1* gene encodes a glutamate carboxypeptidase, whose expression and function in the embryo are unclear (Chaudhury, 1993; Helliwell *et al.*, 2001). Hence, mechanisms that autonomously control suspensor fate maintenance are elusive. The only other case of aberrant proliferation and eventual embryo formation in the suspensor of a viable primary embryo is the inhibition of suspensor specific auxin responses by ectopic expression of stabilized *Aux/IAA*s as described in this thesis (Chapter 2). Interestingly, in this case, the loss of suspensor fate is induced by local inhibition of ARFs in suspensor cells, and hence identifies the first cell-autonomous component in suspensor fate maintenance.

Proper maintenance of suspensor cells is of great importance for correct development of the embryo. However, fundamental to the maintenance of suspensor cell fate is the correct initial specification of apical (embryo) and basal (suspensor) cell lineages. The knowledge on factors that control early embryogenesis is sketchy, but two groups of factors have been rigorously shown to be involved in the establishment and separation of the corresponding initial cell lineages. On one hand the YODA signalling cascade that includes the SHORT SUSPENSOR (SSP) receptor-like kinase, the YODA (YDA) MAPKKK, and the MAPKs 3 and 6 (MPK3 / 6) controls the elongation and asymmetric division of the zygote. The activity of this pathway is initiated by the temporally restricted translation of pollen derived *SSP* mRNA in the zygote (Bayer *et al.*, 2009). Disruptions at any step of the signalling cascade lead to reduced outgrowth of the zygote. As a consequence of this, cells of the basal lineage do not fully differentiate into suspensor cells as shown by loss of a suspensor-specific marker gene, remain small and in some cases divide in aberrant division planes. Which genes and cellular processes act downstream of the SSP-YDA pathway is currently unknown.

Other recent work has established a role for homeodomain transcription factors in setting up early fates in the *Arabidopsis* embryo. Expression of several genes of the *WUSCHEL RELATED HOMEOBOX* clade of transcription factors is separated during the division of the zygote (Haecker *et al.*, 2004). Embryos lacking the apically expressed *WOX2* develop normally up to the 8-cell stage but are then impaired in proper protoderm and subsequent cotyledon development. In contrast the combined loss of WOX8 and WOX9 function in the basal cell lineage disturbs embryo development at much earlier stages (Breuninger *et al.*, 2008). Already at the 2-cell stage aberrant divisions of the proembryo can be observed and later on enlargement of suspensor cells greatly affects the growth of the embryo, leading eventually to abortion.
Even though the YODA cascade is involved in specifying the asymmetric division of the zygote, a result of which potentially includes the unequal distribution of WOX2, 8 and 9, both pathways do not appear to function in a linear relationship. This has been shown by the generation of *yda wox8 wox9* triple mutant plants. In these mutants zygote elongation is impaired as in *yda* lines but the following development of the two daughter cells is fully arrested. This indicates that both pathways function at least partially independently of each other and therefore might influence the establishment of apical and basal cell fates in distinct ways (Breuninger *et al.*, 2008).
In this chapter we have investigated the regulation of *ARF* expression patterns in the early suspensor by the SSP-YDA and the WOX pathways. As these pathways probably control distinct aspects of early fate specification, the regulation of six *ARF* genes in the suspensor may shed light on mechanisms of transcriptional regulation. Analysis of the expression of all embryonic *ARF*s in the *ssp* mutant, and of the suspensor-expressed *ARF*s in the *wox8 wox9* double mutant allowed us to confirm the distinct activities of SSP-YDA and WOX pathways in controlling *ARF* expression. Based on our findings we propose a model of regulatory networks controlling the differentiation and maintenance of the two basic embryogenic cell lineages.

Results

ARF expression in the suspensor is established independently of the SSP-YDA signaling pathway

Activity of the YODA signaling pathway is crucial to establish the basal cell lineage. Disruptions in the signaling cascade result in reduced elongation of the zygote. As a result developing suspensor cells also do not elongate but start to divide in aberrant division planes and form surplus cell files adjacent to the proembryo.
Direct influence of the YODA pathway onto embryogenesis appears to be restricted to the initial establishment of the apical and basal lineages. Yet it is possible that the transient activity of the pathway controls later developmental processes by initiating self-perpetuating feedback loops within each cell lineage. Positive feedback loops promote the establishment of multiple states after induction by an external stimulus. They essentially are the memory of a cell, since their maintained state reflects events in the history of a cell. Negative feedback loops on the other hand tend to maintain homeostasis by dampening disturbances through

external stimuli and returning to a steady state (Thomas *et al.*, 1995; Ferrell, 2002). Auxin response machineries could be subjected to various such regulatory loops as suggested for example by the reduced expression of *IAA10* in the *iaa10* gain of function mutant (Chapter 3).
To study how the SSP-YDA pathway affects establishment of *ARF* expression patterns during early embryogenesis we employed the fact that triggering of the pathway is strictly dependent on *SSP* mRNA delivered by the fertilizing pollen. Absence of *SSP* transcripts in *ssp-2* pollen therefore results in *yda*-like phenotypes ranging from the complete absence of a recognizable suspensor to the formation of rudimentary or shortened suspensors with in some cases aberrant cell division patterns.
Inhibition of ARF activity in the suspensor also causes aberrant divisions within the suspensor at early stages of embryogenesis, which is why we investigated the expression patterns of all suspensor-expressed *ARF*s (*ARF1, 2, 6, 9, 13* and *18*) after pollination with pollen derived from homozygous *ssp-2* plants. In addition, since loss of suspensor cell fate in the *ssp* mutant has only been demonstrated based on one molecular marker, *SUC3*, (Bayer *et al.*, 2009), we also included the suspensor-specific *pIAA10-GFP* reporter as an independent marker for suspensor cell fate.
We pollinated two independent homozygous lines each for all *pARF-GFP* and the *pIAA10-GFP* construct with wild type pollen or pollen from *ssp-2* plants and compared the expression patterns in F_1 embryos derived from these crosses. Since the embryo preparation procedure did not allow us to distinguish between embryos with moderately shortened suspensors without aberrant division patterns or wild type embryos that theoretically could have resulted from a low level of self-pollination or cross-pollination by adjacent plants, we focused on embryos with clear mutant aberrations in suspensor development.
While *pIAA10-GFP* expression was observed in control crosses (Fig. 1 a), no signal was detected in the *ssp* mutant (Fig. 1 b, c), further supporting the conclusion that suspensor cell fate is compromised in this mutant.
All *ssp-2* mutant embryos (including aphenotypic ones) showed wild type-like expression of *ARF1, 6, 9, 13* and *18* at octant, dermatogen and globular stage (Fig. 1 h - m). Expression of *ARF2* was expanded from the wild type pattern in suspensor and protoderm of the basal embryonic half (Fig. 1 i) to include the inner cells of the basal embryo domain at globular stage. This however might be an indirect result from misspecification of these cells in the *ssp* mutant.
Based on this expression analysis, it appears that the SSP-YDA signaling pathway is not required for the activation of *ARF* expression in the suspensor.

Signaling via the SSP-YDA pathway restricts *ARF5/MP* expression to the embryo

Post-embryonicaly, the YODA pathway acts negatively on the specification and establishment of meristemoids during stomatal development (Bergmann *et al.*, 2004). Therefore, alternatively to controlling apical-basal differentiation of *ARF* expression by activating the expression of *ARF*s in the suspensor, the SSP-YDA cascade could also act by inhibiting the expression of embryo-specific *ARF*s in the suspensor. The only *ARF* that qualifies as being embryo-specific is *ARF5/MP*, whose initial expression according to *in-situ* hybridization can be detected in all cells of the 8-cell embryo (Hamann *et al.*, 2002), and whose promoter-*GFP*

expression remains restricted to the proembryo after being first detectable at the 16-cell stage (Chapter 3). We crossed two independent homozygous *pARF5-n3GFP* lines with pollen of wild type plants or *ssp-2* homozygotes and determined *ARF5/MP* expression in developing F_1 embryos. Strikingly, in contrast to the proembryo-specific expression observed in controls pollinated with wild type pollen (Fig. 1 d, f), *ssp-2* embryos showed expansion of *ARF5/MP* expression into cells that take the position of suspensor cells in wild type development. This aberrant expression could be detected at globular stages, and persisted in cells of the forming root pole up to torpedo stage (Fig. 1 e, g).
This result suggests that indeed the SSP-YDA pathway negatively regulates the expression of *ARF5/MP* in the suspensor / basal lineage. This finding is in agreement with the observation that misexpression of *ARF5/MP* from the suspensor specific *ARF13* promoter causes *ssp*-like short suspensor phenotypes (Chapter 2). In fact, these two findings taken together imply that perhaps *ssp* suspensor defects result in part from a failure to suppress *ARF5/MP* expression.

Proper establishment of the suspensor auxin response machinery is lost in *wox8 wox9* mutants

Different to mutants of the SSP-YDA pathway, elongation and division of the zygote is not affected in *wox2* or *wox8 wox9* mutant plants. In contrast, while *WOX* genes may not control the first asymmetric division, *wox8 wox9* mutants are severely disturbed in subsequent developmental steps of the apical and basal cell lineages so that the embryo is eventually aborted.

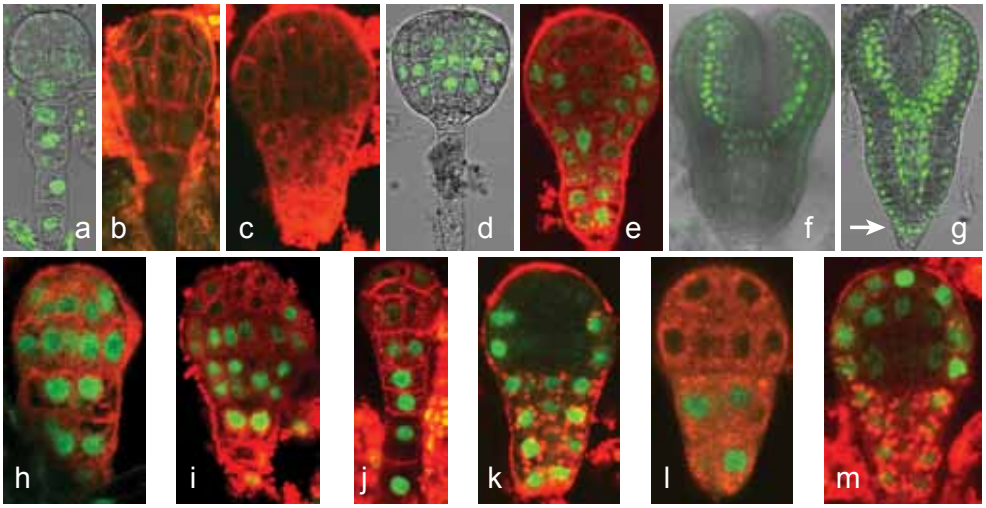

Figure 1: Alterations of *IAA10* and *ARF* expression patterns in *ssp* embryos
In *ssp* mutant embryos the suspensor marker *pIAA10::n3GFP* (wild type expression shown in a) is not expressed, marking at least partial loss of suspensor cell fate in the respective cells (b, c).
A change in the cell fate of suspensor cells is further highlighted by the expansion of the embryo specific *pARF5::n3GFP* marker into these cells (d: wild type embryo, e: *ssp* embryo). *ARF5* expression persists in additional cells at the root pole even at later stages (g), where it is not expressed in control embryos (f).
Expression of all other *ARF*s normally expressed during early embryogenesis remained unchanged in *ssp* mutant embryos with the exception of *ARF2* expression expanding into the inner domain of the basal half (h: *pARF1*; i: *pARF2*; j: *pARF6*; k: *pARF9*; l: *pARF13*; m: *pARF18*).

Several embryonic markers including *WOX2* have been shown to be lost in *wox8 wox9* embryos (Breuninger *et al.*, 2008). Since *WOX8* and *9* are expressed exclusively in the basal cell lineage during early stages, these genes might regulate the generation of an instructive signal from the basal cell lineage that triggers embryonic development in the adjacent apical cell. In analogy to the role of ARF5/MP in promoting cell-cell communication during hypophysis specification, suspensor specific ARFs might play an important role in such a cellular communication. To define the role of WOX8 and 9 in establishment of *ARF* expression in the suspensor, we crossed two independent promoter-*GFP* lines for each suspensor-expressed *ARF* with *wox8-1* (-/-) *wox*9-*1* (+/-) double mutant plants. In the F_1 generation, plants that carry the promoter-GFP transgene and both *wox8* and *wox9* mutations were selected by PCR. F_2 plants were selected that are homozygous for the promoter-*GFP* transgene (by fluorescence microscopy on F_3 embryos), and that segregate approximately 25% defective embryos. As reported (Breuninger *et al.*, 2008) in these plants, embryos developed into fingerlike structures due to repeated aberrant divisions in the embryo and suspensor, confirming the presence of both *wox8* and *wox9* mutations.

Investigation of *ARF* expression patterns in phenotypic embryos from two-cell stage to globular stage showed that expression of *ARF1, 2* and *6* is not altered and resembles the patterns found in all other wildtypic embryos that segregate in the same plant and are homozygote for *wox8* or *wox9* (Fig. 2 i/j, k/l, m/n).

In contrast, expression of *ARF9* and *ARF13* was absent from octant to globular stage embryos with unambiguous abnormal phenotypes (Fig. 2 a - h). At the same time all other embryos prepared from the same silique but being phenotypically normal did show normal expression

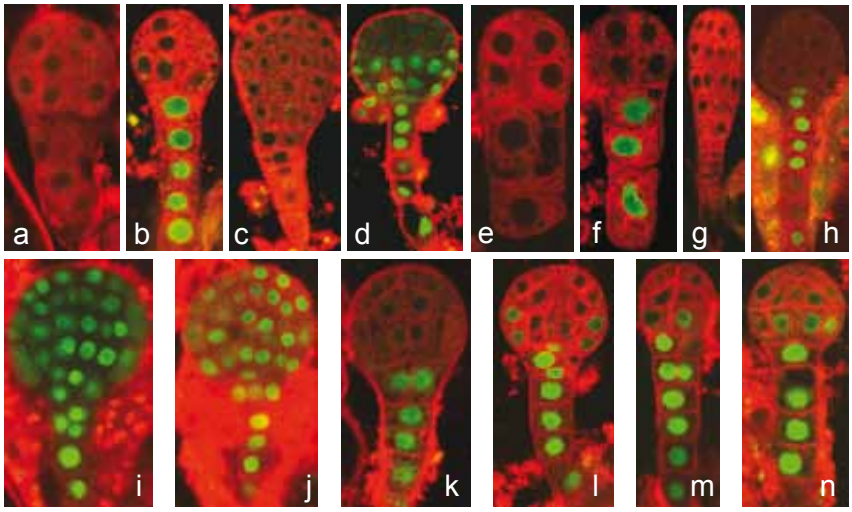

Figure 2: *ARF* expression in *wox8 wox9* mutant embryos

Expression from the *ARF9* (a - d; b and d: wild type embryos) or the *ARF13* promoter (e - h; f and h: wild type embryos) is lost in *wox8 wox9* double mutant embryos, while expression of *n3GFP* under the control of *ARF1* (i), *ARF2* (k) or *ARF6* (m) promoters is unchanged. In single mutant embryos *ARF1, 2* and *6* are expressed normally (j, l and n).

of both *ARF*s.
In summary, WOX8 and WOX9 are required for proper expression of *ARF9* and *ARF13*, which participate in auxin response in the suspensor. Interestingly, neither WOX8 and WOX9 nor the SSP-YDA signaling pathway controls the expression of *ARF1, 2* and *6*. This implies that a third, yet unknown regulatory pathway exists to specify the expression patterns of these three genes during early embryogenesis.

Early WOX activity is established independent of ARF functionality

Proper *ARF* expression in the suspensor (ARF9 and 13) requires WOX8 and 9 activity. However, these *WOX* genes are themselves suspensor-specific at early stages and ARF activity in the suspensor is required for cell fate maintenance. Therefore we tested if in turn, sustained *WOX* expression in the suspensor also requires proper ARF activity.
We crossed reporters for *WOX2, 8* and *9* into the *iaa10* mutant. As all 6 suspensor-expressed *ARF*s may act redundantly, we chose to downregulate ARF activity by the stabilized iaa10 mutant protein. This mutation causes a mild phenotype in suspensor maintenance, which can be observed both phenotypically by aberrant and excessive cell divisions, and by loss of the suspensor-specific expression of the *IAA10* promoter (Chapter 3).
In *iaa10* mutant embryos, we could not find changes in the expression patterns of *WOX2, 8* or *9*, despite the obvious iaa10-induced proliferating suspensor defect (Fig. 3 b, d, f). To test if perhaps the weak *iaa10* mutant phenotype, and hence remaining ARF activity, precludes detecting ARF-dependent *WOX* expression, we tested *WOX8* transcripts by *in-situ* hybridiza-

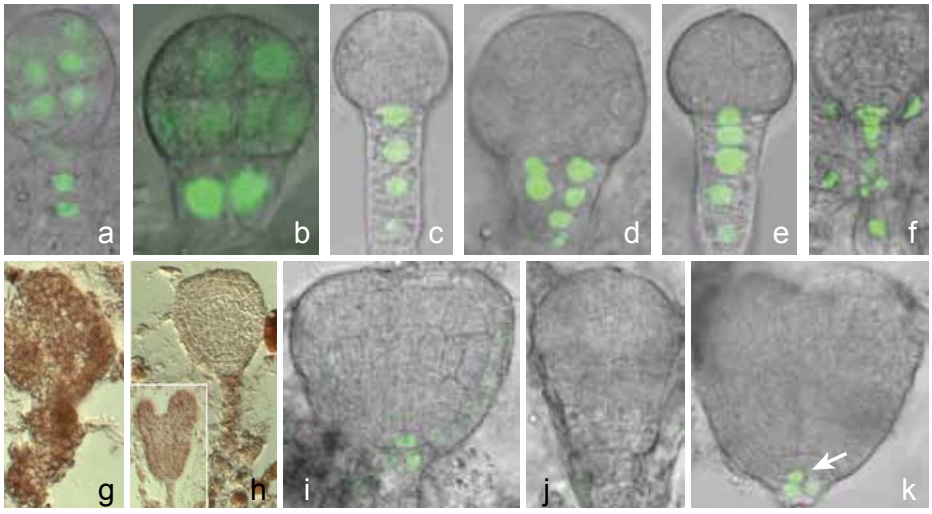

Figure 3: Regulation of *WOX* expression by the suspensor auxin response machinery
Activity of *pWOX2::n3YFP* (b), *pWOX8::n3YFP* (d) and *pWOX9::n3YFP* (f) is unchanged in *iaa10* mutant embryos showing aberrant cell divisions at the suspensor-embryo junction when compared to expression in wild type (a, c or e).
In line with this, enhanced repression of the suspensor specific auxin response in *RPS5a>>iaa12* plants did also not affect *WOX8* expression as shown in g (h displays *WOX8* expression in wild type embryos and the inset in h shows staining after incubation with the sense probe).
Expression of *WOX5* is not detactable in phenotypic *iaa10* embryos before heart stage (j). Different to wild type expression (i) at this stage, *WOX5* expression is shifted out of the QC (arrow) and established in cells below it (k).

tion of embryos overexpressing stabilized *iaa12/bdl* in both proembryo and suspensor from the *RPS5a* promoter (Chapter 2). These embryos generally have stronger phenotypes than the *iaa10* embryos and hence might show a stronger inhibition of suspensor ARF activity. Nonetheless, despite strong phenotypes, *WOX8* expression was not downregulated in suspensor cells (Fig. 3 g).
Therefore we conclude that *WOX2, 8* and *9* are regulated independently of the suspensor auxin response machinery. This finding also puts forward the interesting notion that *WOX8* / *WOX9* expression is maintained even when cells lose suspensor identity. This suggests that *WOX8* and *9* expression is controlled by the position rather than the fate of the suspensor cells.

WOX5 expression depends on suspensor ARF activity

While WOX2, 8 and 9 control the formation of the apico-basal axis, other *WOX* genes act later in embryogenesis. Among these is *WOX5*, whose expression is first detected in the hypophysis and later restricted to the quiescent center (QC) of the embryonic root. Since a stabilizing mutation in the suspensor-specific ARF inhibitor IAA10 causes a failure in establishing a proper embryonic root meristem (Fig. 3 j), we tested if *WOX5* expression is controlled by the suspensor ARF machinery. A *pWOX5-GFP* reporter was introduced into the *iaa10* mutant, and GFP fluorescence was analyzed in phenotypically abnormal mutant embryos. We found that at late globular stage *WOX5* expression was established in the root pole of the majority of *iaa10* embryos. During establishment of the root meristem however, *WOX5* expression was shifted to one cell layer below the prospective quiescent center (Fig. 3 k). This indicates that proper establishment of QC-localized *WOX5* expression depends on the activity of ARFs in these cells. The activation of *WOX5* expression one cell layer lower may indicate the restoration of an embryonic root meristem at a morphological different point.
Thus, while *WOX5* expression per se seems not to be influenced by ARF activity, the positional information that guides spatially correct *WOX5* activation in the QC does require the suspensor ARF machinery.

Discussion

Lineage- and position-dependent inputs define *ARF* expression patterns

Embryogenesis is disturbed in the *wox8 wox9* double mutant as well as in mutants with insufficient signaling via the SSP-YDA pathway. In both cases malfunction of suspensor-specific processes also indirectly causes deviations in the proper development of the embryo. However as judged from the differing phenotypes, SSP-YDA signaling and WOX activity probably regulate distinct aspects in the establishment and maintenance of the basal cell lineage. SSP-YDA signaling drives the elongation of the zygote and subsequently is involved in the separation of apical and basal cell fates. WOX2 and WOX8 / 9 are factors which are distributed specifically to the apical or basal cell lineages. This suggests that WOX genes may function downstream of the SSP-YDA signaling cascade. However, embryos of *yda wox8 wox9*

triple mutant plants die after an initial *yda*-like development before the first division of the apical cell. Therefore it is conceivable that these two inputs do not work in a linear pathway but might belong to independent signaling events that separate (SSP-YDA signaling) and specify (WOX activity) the apico-basal dualism during early embryogenesis.
In line with this are the distinct effects on *ARF* expression that we found in *ssp* or *wox8 wox9* mutants. In *ssp* embryos, expression of all *ARF*s that are expressed in the suspensor is normally activated and maintained. The only exception to this is the aberrant expression of the normally embryo-specific *ARF5/MP* in mutant suspensor cells. Unfortunately, the low activity of the *pARF5* reporter prior to the 16-cell stage hampered the analysis of the effect of *ssp* on early *ARF5/MP* misexpression. Therefore we can at present not distinguish whether *ARF5/MP* misexpression induces the *ssp* suspensor defect or results from it. Ectopic expression of *ARF5/MP* in the suspensor was not sufficient to induce the strong *ssp / yda* phenotypes (proliferation) but did result in a *ssp*-like shortening of suspensor cells (Chapter 2). In conclusion, suppression of *ARF5/MP* in the suspensor is controlled by the SSP-YDA signaling pathway and necessary to allow proper development. The generation of an *ssp mp* double mutant should allow the determination of whether *ARF5/MP* misregulation is causal to the short suspensor defect in *ssp*.
Contrary to the situation in *ssp* embryos, loss of both *WOX8* and *WOX9* abolishes expression of *ARF9* and *ARF13*. Since a loss of several embryonic markers in the apical cell lineage has been demonstrated in this double mutant (Breuninger *et al.*, 2008), a model emerges in which WOX8 and WOX9 are employed to generate a signal that induces embryonic development in the apical lineage. Such a signal could be the directed transport of auxin via the suspensor to the proembryo as implicated by the localization of PIN7 on the apical side of suspensor cells (Friml *et al.*, 2003). In such a model, ARF9 and ARF13 might have a similar role in controlling *PIN7* expression as ARF5/MP has for *PIN1* expression in the proembryo (Weijers *et al.*, 2006).

Summarizing the above conclusions we propose a model for *ARF* expression regulation during early embryogenesis in which lineage- and position-dependent clues converge to ensure proper development. Importantly, the specific expression of different ARFs in the apical and basal lineage requires both positive and negative inputs at the transcriptional level (Fig. 4).
First, activity of the SSP-YDA signaling pathway establishes the apical and basal cell lineages, in part through the suppression of *ARF5/MP* in the basal cell lineage, which is maintained during further development in a lineage-dependent manner. On top of this, positional information is perceived from the unequal activation of *WOX* expression in the two lineages, one output of which is the suspensor-specific activation of *ARF9* and *ARF13*. Analogous to ARF5/MP-dependent *PIN1* expression in the proembryo (Weijers *et al.*, 2006), ARF9 and 13 in turn might regulate *PIN7* expression and the subsequent generation of an auxin maximum in the proembryo. A lack of auxin transport to the proembryo would lead to elevated auxin levels in the suspensor and an induction of embryo-like development. Notably the involvement of flexible position dependent auxin responses would also allow the generation of secondary embryos in the case of reduced auxin transport at later stages of development of the primary embryo. Importantly, this working model puts forward several testable hypotheses that will allow addressing the mechanistic connections of the SSP-YDA, WOX and ARF pathways in early embryo development.

Finally expression of *ARF1, 2* and *6* is unchanged in either *ssp* or *wox8 wox9* mutants. Two possibilities arise. First, the SSP-YDA and WOX pathways redundantly control *ARF1, 2* and *6* expression, in which case an *ssp wox8 wox9* triple mutant would show loss of expression. Alternatively, a yet unknown pathway promotes expression of these three *ARF* genes independent of the SSP-YDA and WOX pathways. It is interesting to note that of all *ARF*s expressed in the early embryo *ARF1, 2* and *6* are the most ubiquitous (Chapter 3). It is possible that a cell fate-independent mechanism drives initial ubiquitous expression of these *ARF*s. In this case, these genes do probably not contribute to the establishment or maintenance of specific cell fates during early embryogenesis and might thus serve either as a buffer system for the lineage specific auxin responses (ARF5/MP *versus* ARF9/13), or by preventing the expression of auxin responsive genes necessary for later developmental processes, or by setting a baseline auxin response level that is required for general cellular auxin-dependent processes.

Our results provide important insights into regulatory networks governing early embryogenesis. Further research revealing the downstream targets of cell fate specific ARFs will demonstrate how all three mechanisms of regulation of *ARF* expression are interlinked to ensure proper development of the embryo.

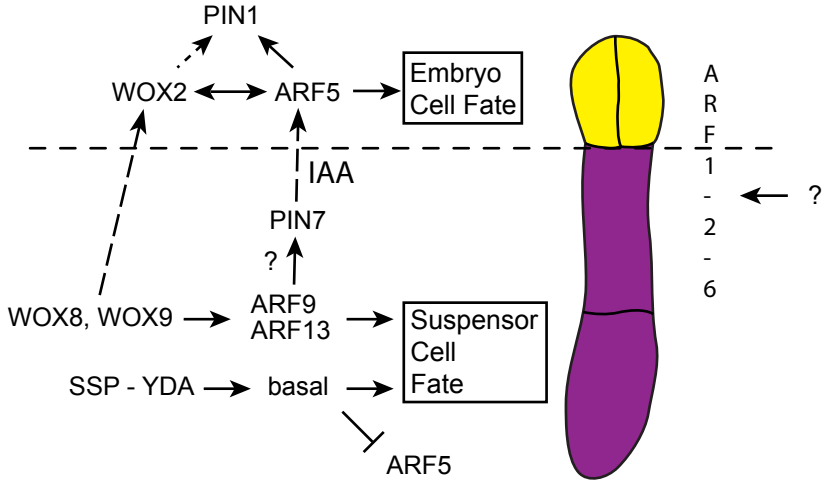

Figure 4: Three distinct mechanisms control early embryonic *ARF* expression
While the SSP-YDA signaling pathway represses *ARF5* expression in the basal cell lineage, WOX8 and WOX9 positively control expression of *ARF9* and *13* in these cells.
wox8 wox9 mutants lack expression of *WOX2* and *PIN1* in the apical cell lineage, which indicates the generation of an instructive signal from the basal cells to the apical cells. In analogy to the *ARF5* controlled *PIN1* expression such a signal might be the *ARF9 / ARF13* dependent *PIN7* expression to direct auxin flux to the apical cell lineage. *ARF1, 2* and *6* are ubiquitously expressed through out the embryo until octant stage. Mechanism regulating this broad expression have not been identified yet.

Material & Methods

Plant growth and material

Seeds were sterilized by rinsing with 70% ethanol followed by three steps of washing with water. After plating on ½ MS medium seeds were transferred to a growth chamber with long day light regime (16 h light / 8 h dark) and constant temperature of 22 ºC. The seedlings were transferred to soil after approximately two weeks on plate and then grown under the same conditions in a walk-in growth chamber. Wild type plants were of the Columbia ecotype (Col-0).
Marker lines for *WOX2*, *WOX5*, *WOX8* and *WOX9* have been kindly provided by T. Laux and are described in Breuninger *et al.* (2008). The *ARF* marker lines have been described in Chapter III of this thesis. The *iaa10* mutant has been described in Chapter II of this thesis.
Seedlings of the *wox8 wox9* double mutant were genotyped as described in Breuninger *et al.* (2008). Genotyping of the *ssp-2* (SALK 051462) allele was performed by PCR with the following primers:
LB1.3 (left border primer for SALK lines): TGGTTCACGTAGTGGGCCATCG;
ssp-2 LP : TTAGAGACCACACGAGAAGGC;
ssp-2 RP: TAACATGGCTTGGTCTGATCC).
For pollination young flower buds were opened and emasculated before dehiscence of the anthers. After an overnight rest and the outgrowth of the papillae the remaining gynoecia were manually pollinated by applying pollen of the respective genotype.

Microscopy

For fluorescence microscopy ovules were transferred from siliques into a drop of PBS buffer containing 4% (w/v) PFA, 5% (v/v) glycerol and FM4-64 at a concentration of 1 μM on a microscope slide. After applying the cover slip, embryos were squeezed out of the ovules by short strokes with a pencil onto the cover slip. Free embryos were then investigated for GFP signals by using a Carl Zeiss LSM510 confocal laser scanning microscope (software version 3.2 SP2) 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 530 nm while FM4-64 signals were taken after passing a longpass filter of 650 nm.
For DIC microscopy ovules were mounted in a clearing solution of chloral hydrate, water and glycerol (8:3:1), left overnight at room temperature and subsequently investigated at a Leica DRM microscope equipped with differential interference contrast (DIC) optics.

Acknowledgments

The authors would like to thank Thomas Laux for providing seeds of the *wox8 wox9* mutant and the WOX marker lines as well as Wolfgang Lukowitz for providing seeds of the *ssp-2* mutant. This work was supported by grants from the Netherlands Organisation for Scientific Research (NWO; ALW-VIDI 864.06.012) and the European Union 7th Framework program (ITN "SIREN" Contract number 214788-2).

Chapter 6

Summarizing Discussion

Summarizing Discussion

The first division of the zygote of *Arabidopsis thaliana* separates two cellular lineages with distinct developmental characteristics. While most of the embryo emerges from the apical daughter cell of the zygote, the basal daughter forms the suspensor, and its descendants only contribute to the embryonic root meristem. In fact solely the uppermost suspensor cell is respecified to become the hypophysis and be incorporated into the embryo as founder cell of the root meristem.
Crucial factors for the correct specification of the hypophysis are IAA12/BDL and ARF5/MP which both are involved in auxin-dependent signaling from the embryo to the uppermost suspensor cell. Even though an elaborate PIN auxin transport network (Friml *et al.*, 2003) has been reported, and gene expression responses to auxin have been visualized through the auxin-dependent DR5 promoter, it was unknown which developmental steps in embryogenesis are controlled by auxin. A major aim of the work described in this thesis was to define the extent of auxin regulation in embryo pattern formation. Emphasis was placed on the transcription factors involved: the ARF transcriptional regulators and their Aux/IAA inhibitors.

The approach we chose to chart auxin-dependent processes was to misexpress stabilized *aux/iaa* proteins. Since the interaction specificity of these inhibitors with the ARF transcription factors is limited (Weijers *et al.*, 2005), this allowed us to devise a method of inhibiting any auxin response by driving *iaa12/bdl* expression in various embryonic subdomains under the control of several GAL4 driver lines. Thereby the unspecific inhibition of ARF activity by iaa12/bdl was a crucial prerequisite to overcome potential redundant action of ARFs and hence allowed us to reveal auxin responses that previously were inaccessible.
In **chapter 2** we describe the application of this approach and our finding, that in addition to the ARF5/MP-dependent processes of root meristem and cotyledon formation (Berleth and Jurgens, 1993), at least two more processes are auxin-controlled. In particular we find proper auxin response to be required for protoderm formation and the maintenance of suspensor cell fate.
Strikingly, expression of *iaa12/bdl* in the suspensor cell file induces aberrant divisions of these cells, redirecting cell fate to a more embryonic one and in some cases stimulating the formation of intact secondary embryos. We further identified the Aux/IAA10 and ARF13 proteins as components of a suspensor-specific auxin response machinery, likely the one whose inhibition causes loss of suspensor cell fate maintenance.
To this point suspensor proliferation and suspensor-derived embryogenesis have been attributed to a lack of suppression maintained by signals originating from the primary embryo (Schwartz *et al.*, 1994). Importantly, nothing is known about the mechanisms that operate in suspensor cells to maintain the fate. With the suspensor-specific auxin response machinery consisting in part of IAA10 and ARF13 we described the first cell-autonomous process necessary to maintain suspensor cell fate and highlight auxin as one of the involved signals.

Yet from investigating T-DNA insertion lines for *ARF13* as well as RNAi lines that target *ARF13* and other *ARF*s, it became apparent that other ARFs must act redundantly with ARF13 in suspensor maintenance. An initial analysis of available T-DNA insertion lines for all *ARF*s did not identify additional factors.

Among the ARFs, redundancies could be predicted between close homologs. However, none of the double mutants in "sister" ARFs showed suspensor defects. There may however be profound redundancy between any and all ARFs that are co-expressed in a cell, but knowledge on *ARF* expression was very limited, particularly on a cellular scale. Therefore, to identify those *ARF*s expressed in the suspensor, in **Chapter 3** we chose to determine the expression pattern of each *ARF* during embryogenesis by studying *GFP* expression from 2 kb fragments of each promoter. Even though plasmodesmatal connections allow protein transport during embryogenesis (reviewed in (Kim and Zambryski, 2005)), ARFs seem not to be transported in general, which is why our strategy should reveal the maximal domain of activity for each of them. We found seven promoters (*ARF1, 2, 5, 6, 9, 13* and *18*) to be active in various patterns from 2-cell through globular stages, and an increased number of ARFs to be active during later stages. Five of these (*ARF1, 2, 6, 9* and *18*) are expressed both in suspensor and embryo cells, whereas the *ARF5/MP* promoter is embryo-specific and the *ARF13* promoter is exclusively expressed in the suspensor. Interestingly, it emerges from this expression analysis that every cell type of the globular stage embryo possesses a specific combination of co-expressed *ARF*s. Hence, if these ARFs are not identical in their biochemical properties (binding affinity to target sites, protein stability, affinity for Aux/IAA inhibitors, etc), auxin responses during embryogenesis are prepatterned by the expression domains of the *ARF*s.
Importantly, comprehensive expression mapping revealed that *ARF1, 2, 6, 9, 13* and *18* are co-expressed in the suspensor and raised the possibility that at least some if not all of these factors redundantly control suspensor cell fate.

To define the contribution of each of these six ARFs to suspensor and embryo development we set out to generate the hexuple mutant in **chapter 4**. Loss of the closely related ARFs 9, 13 and 18 in the *arf9-1 arf13-1 arf18-2* triple mutant did not result in any phenotypic aberrations. This could be caused by incomplete loss of the ARF functions in the T-DNA insertion lines, by redundancy with ARF1, 2 and 6 or by upregulation of additional redundantly acting ARFs in triple mutant suspensor cells.
Combining T-DNA insertion lines for *ARF1, 2* and *6* resulted in aberrant divisions around the suspensor-embryo junction and in addition led to enhanced phenotypic deviations previously described for the *arf1-5 arf2-8* double mutant (Ellis *et al.*, 2005). Hence, these 3 factors act redundantly in various processes including early embryogenesis. This is a surprising finding since ARF1 and 2 have been described as transcriptional repressors in protoplast assays, while ARF6 activated transcription in the same experimental set-up (Tiwari *et al.*, 2003). This result suggests that either the protoplast-based assays for transcriptional activity of ARFs are not representative for the *in vivo* function of those ARFs, or that for some biochemical function of the ARFs, their (repressive or activating) middle regions are not relevant. This could for example be the case if other factors associate with ARFs in higher-order transcription complexes.
To this point no hexuple mutant, carrying T-DNA insertions in *ARF1, 2, 6, 9, 13* and *18* is available. Nonetheless this line will be available in the near future and characterization of developmental phenotypes observed in it as well as in intermediate combinations will ultimately proof the redundant activity of all six factors.

Our expression analysis for the *ARF* family revealed that expression of these factors is regulated in a cell type-specific manner. With the identification of the suspensor specific IAA10 – ARF13 auxin response machinery and the IAA12/BDL – ARF5/MP machinery in the proembryo two auxin responses relevant for proper embryogenesis have now been described. The localization of both machineries coincides with the separation of the apical and basal lineages after the first zygotic division. A major question is how these *ARF* expression patterns are controlled, and to what extent misexpression of *ARF*s in the early embryo upsets normal pattern formation. Two independent pathways have been shown to be involved in the separation of apical and basal cell fates. The SSP-YDA kinase pathway acts transiently before the division and hence might provide lineage dependent information (Bayer *et al.*, 2009), while members of the *WOX* clade of homeobox transcription factors (*WOX2, 8* and *9*) are expressed along the apico-basal axis and might confer positional cues (Haecker *et al.*, 2004).
In **chapter 5** we tested the connection of both pathways to either auxin response machinery by analyzing changes in the expression patterns of promoter-*GFP* lines in the respective mutant background. Loss of *SSP* activity did not cause any changes in the expression of "suspensor" *ARF*s, but along with the formation of smaller cells in the suspensor domain, expression of *ARF5/MP* expanded into the additional cells. Hence the SSP-YDA pathway does not act by promoting suspensor ARF activity but might rather restrict *ARF5/MP* expression to the apical lineage. From analyzing the suspensor defects in an *ssp mp* double mutant it should become clear whether *ARF5/MP* misexpression is causal to the *ssp* mutant phenotype. This might be the case, given the similar phenotypes induced by the *ssp* mutation and in lines that express *ARF5/MP* from the *ARF13* promoter (Chapter 2).
Expression of some, but not all suspensor *ARF*s was lost in *wox8 wox9* mutants. We found only *ARF9* and *ARF13* promoters to be down-regulated, while *ARF1, 2* and *6* promoters remained active in their normal expression patterns. These three genes therefore must be regulated by a mechanism distinct from the regulation of *ARF5/MP, ARF9* and *13* and independent of SSP / YDA or WOX8 / WOX9 activity.
Finally, expression analysis of *WOX* genes in *iaa10* lines with compromised activity of the suspensor auxin response machinery demonstrated that expression of early-acting *WOX* genes (*WOX2, 8* and *9*) is established independent of ARF activity. The localization of the later QC specific WOX5 on the other hand is depending on proper root pole establishment and consequently was misspecified in phenotypic *iaa10* embryos.

This thesis provides the coordinates of an embryo-wide auxin response matrix that is established from the very beginning of embryogenesis onwards and generates the cell type-specific responses to an auxin signal. The fact that cells are endowed with different response components puts forward the suggestion that auxin itself is not an instructive signal in embryogenesis but rather acts as a trigger to elicit a pre-defined response. How auxin stimuli are translated into the multitude of cellular responses has been a long-standing question. Our finding that distinct cell types express different sets of ARFs provides a relatively simple mechanism to create huge numbers of differing outputs in response to the simple trigger constituted by auxin. Despite the huge number of potential auxin responses the particular response of a single cell to an auxin stimulus seems to be destined by the present ARF set.

Such a binary output in response to auxin might not be sufficient to provide the flexibility needed in plant development. Yet, one can easily envision that by simply removing the activity of certain ARFs in a cell (by any posttranscriptional process) new responses are created. On top of this *ARF* expression patterns are not fixed to cell lineages and thus also initiation of expression of additional ARFs will be a way to adjust a cells auxin response during development.
However the translation of auxin stimuli into transcriptional changes by groups of ARFs controlled by a single Aux/IAA does not provide a mechanism to quantify auxin levels and hence does not allow for a graded response to it. In this scheme auxin merely is a trigger to coordinate developmental events in a spatio-temporal manner. Yet, graded auxin responses have been implied in root and leaf development and patterning of the female gametophyte (Ljung *et al.*, 2001; Grieneisen *et al.*, 2007; Pagnussat *et al.*, 2009). Such graded activity depending on the intensity of the auxin stimulus could only be achieved if various Aux/IAAs, with different binding properties to both the $SCF^{(TIR1/AFB)}$ complex and the ARFs, are present in the same cell. At this moment co-expression of Aux/IAAs as well as binding and dissociation constants for factors involved in auxin responses have not been reported, leaving gradual auxin responses speculative.

Unraveling the factors that control and set up the various expression patterns of ARFs found during embryogenesis will be a major task in describing morphogenesis. To this point we focused on the initiation of the auxin response matrix right after the zygotic division and found with the SSP-YDA and the WOX8 / 9 pathways two mechanisms that regulate expression of *ARF5/MP* or *ARF9* and *13*, while expression of *ARF1, 2* or *6* was unaffected in mutants of both processes.
Due to its transient activity the SSP-YDA pathway must provide lineage dependent information that is retained in cells of the basal lineage in the form of suppression of *ARF5/MP* expression. Contrary to this WOX8 and at early stages WOX9 are continuously active in the developing suspensor and hence might provide positional information. Loss of *ARF9* and *ARF13* expression in phenotypic *wox8 wox9* embryos demonstrates that this information is converted into a specific auxin response. Hence WOX8 and WOX9 activity is necessary to maintain proper suspensor cell identity. Interestingly the loss of *WOX8* and *WOX9* not only affects suspensor development but also has severe consequences for proper embryo development. This suggests that both *WOX* genes control an inductive signal from the basal to the apical lineage which promotes embryonic development. Potentially this involves downstream targets of ARF9 and ARF13; the nature of which is subject to ongoing work in our lab. It is tempting to envision that ARF9 and ARF13 control *PIN7* expression and other signals in a manner analogous to the ARF5/MP controlled hypophysis specification. In any event it will be interesting to see whether ARF9 and 13 contribute functionally to WOX8/9-induced processes. Restoration of the *wox8 wox9* double mutant by *ARF9* or *13* expressed from the *WOX9* promoter should resolve this issue.

Expression of stabilized *aux/iaa*s in the suspensor renders these cells insensitive to auxin stimuli. Consequently suspensor cells start to proliferate and in some cases secondary embryogenesis can be observed.
Induction of proliferation of the suspensor has been reported for a variety of treatments affecting the development of the primary embryo. One general cue to this change in suspensor

development is the death of the primary embryo caused by for example toxin expression (Weijers *et al.*, 2003) or embryo lethal mutations as in *sus* or *rasp* mutants (Schwartz *et al.*, 1994; Yadegari *et al.*, 1994). The formation of a secondary embryo however has been the exceptional case in only a handful of mutants; in particular these are *twn1, twn2, lec1 fus3* and *amp1*((Vernon and Meinke, 1994; Lotan *et al.*, 1998; Vernon *et al.*, 2001; Vidaurre *et al.*, 2007). The role of *TWIN1* and *TWIN2* in embryogenesis is unclear at the moment, but research on *LEC1*, *FUS3* and *AMP1* implicates these genes to be involved in a network that is involved in controlling auxin biosynthesis and ARF activity (Suzuki and McCarty, 2008). Unfortunately the role of these factors in early embryogenesis has not caught much attention and hence the influence of these genes on morphogenesis still needs to be investigated in greater detail. Even though the connections at present are sketchy at best, it is remarkable that suspensor-derived embryogenesis may converge upon auxin signaling, particularly since auxin (2,4-D) treatment is a classical inductive treatment in somatic embryogenesis (Luo and Koop, 1997). Whether similar cellular and gene expression programs are common to all these cases is a major outstanding question.

Outlook

Several independent pathways control the establishment and maintenance of cell fates during embryogenesis. Here, we described to which extent auxin response machineries are involved in morphogenesis and defined the novel suspensor specific machinery in greater detail. Our finding that specific sets of ARFs define the cellular response to auxin not only provides an elegant explanation for how the magnitude of different processes is controlled by auxin but also provides multiple entry points for the regulation of other processes. The impact of two such processes on auxin response in the suspensor has been shown with the regulation of *ARF5/MP, ARF9* and *13* promoters in *ssp* and *wox8 wox9* mutants. Certainly further signals are integrated into cell specification during patterning of the embryo and further dissection of the connections in the relevant networks will be a fruitful task in future projects.

Appendix

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List of Publications

Rademacher, E. H. and Weijers, D. (2007). Got Root? – Initiation of the Embryonic Root Meristem. *Int. J. Plant Dev. Biol.* **1(1)**, 122-126.

de Smet, I., Lau, S., Vanneste, S., Benjamins, R., **Rademacher, E. H.**, Schlereth, A., Voß, U., de Rybel, B., Vassileva, V., Naudts, M., Levesque M. P., Ehrismann, J. S., Inzé, D., Luschnig, C., Benfey, P. N., Weijers, D., Jürgens, G. and Beeckman, T.; Successive auxin response modules control de novo organogenesis in the Arabidopsis root; *PNAS.*
under review

Rademacher, E. H. *, Möller, B.*, Lokerse, A. S.* and Weijers, D.; A cellular expression map of the Arabidopsis AUXIN RESPONSE FACTOR family reveals cell type-specific auxin responses.
manuscript in preparation
* contributed equally

Rademacher, E. H., Lokerse, A. S., Schlereth A., Kientz, M., Jürgens, G. and Weijers, D.; Distinct auxin response machineries control separate cell fates in the early plant embryo.
manuscript in preparation

Llavata Peris, C., **Rademacher, E. H.** and Weijers D. (2010). Pattern formation in plant embryogenesis; *Curr. Top. Dev. Biol.*
invited review in preparation

Acknowledgments

The results presented in this thesis are the outcome of a four year long journey to the sites of ARF activity during embryogenesis in *Arabidopsis thaliana*. Just like every trip in this world it would have been only half as interesting without all the people I met along the way. Many I met on occasions such as the EPS meetings, international conferences, at the MPI in Cologne, at the ZMBP in Tübingen or here in Wageningen. Unknowingly they all contributed to the success of this thesis by raising interesting questions, stimulating me to think further than auxin and helping to overcome the pitfalls every PhD student faces from time to time. To name them all would go beyond the scope of these few pages which is why I will limit myself to the ones in my immediate surrounding.

The laboratory of Biochemistry at Wageningen University provided an open space for ideas and experiments and I would like to express my gratitude to Prof. Dr. Sacco C. de Vries for his willingness to clear the way for this project and guide my progress.

With the beginning of my thesis I became a founding member of the just then established group of Dr. Dolf Weijers. In the following years I not only greatly benefited from the thoughtful leadership of Dolf but also had the unique chance to be part of a new research group developing out of one man's passionate research interest.
To date, Barbara Möller, Annemarie S. Lokerse, Cristina Llavata Peris and Willy van den Berg joined the group and made it a truly exciting working place. I'm glad to have been part of this team and to know that these great people will continue the work on embryonic auxin responses.

Much of the bits and pieces of good laboratory practise I learned from Dr. Jasmin Ehrismann and Dr. Alexandra Schlereth while working on my diploma thesis in Tübingen. The respective laboratory routines provided me with a vital starting ground and still live on in the daily work here in Wageningen.

Not much GFP would I have seen without the support of Dr. Jan-Willem Borst and Boudewijn van Veen who maintained the usability of the microspectroscopic equipment at the Micro-Spectroscopy Centre and hence enabled me to see the light at the end of the tube.

A great deal of "work behind the scenes" was taken over by Dr. Cathy Albrecht and Laura van Egmond, I much appreciate not having had to deal with all the ordering and bureaucracy. I also would like to thank the former and present members of the biochemistry lab for the good times I had at "Lab-Uitjes", "Borrels" and BBQs and the insights I got into techniques like mass-spectroscopy and NMR.

Throughout most of my time in Wageningen I had the joy to find a cheerful companion in Dr. Isabella A. Nougalli Tonaco. I will always remember the great times we had.

Finally I'd like to thank the two biologists that were most influential on my career – my dear parents. Through your patient endurance and interest in nature you taught me to see the miracles of life.

Curriculum Vitae

Eike Hendrik Rademacher was born on the 9th of June 1980 in Göttingen, Germany.
After finishing his final secondary school examinations (Abitur) at Gymnasium im Paul-von-Denis-Schulzentrum, Schifferstadt in 1999, he did his national service and subsequently began in 2000 to study biology with specialization in genetics, plant physiology and biomathematics at the Eberhard Karls University of Tübingen.
From 2001 on he actively participated in the scientific work at the laboratory of Prof. Dr. G. Jürgens by working as student assistant in several research projects. In 2005 his growing interest in the morphogenesis of plants eventually resulted in the successful completion of a diploma thesis under the supervision of Dr. D. Weijers.
When Dr. D. Weijers was appointed assistant professor at the laboratory of Biochemistry at Wageningen University in January 2006 Eike took the opportunity to follow up on the fascinating implications of his diploma thesis and also joined the laboratory of Biochemistry to carry out a PhD thesis. The many findings of this work resulted in the thesis at hand.
In parallel to the above mentioned activities Eike has been enrolled in a program of economical studies at the FernUniversität in Hagen. Here he has specialized in operations research and organization/planning. He expects to complete these studies with a diploma degree in March 2010.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Eike H. Rademacher
Date: 10 November 2009
Group: Laboratory of Biochemistry, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Identification of novel ARF and Aux/IAA functions in *Arabidopsis* embryogenesis	04 Apr 2006
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Intl. J Plant Dev Biol.: Got Root? Initiation of the embryonic root meristem	09 Mar 2007
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*4.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day, Wageningen University	19 Sep 2006
EPS PhD Student Day, Wageningen University	13 Sep 2007
1st Joint Retreat of PhD students in Exp. Plant Sciences, Wageningen, NL	02-03 Oct 2008
► **EPS theme symposia**	
EPS Theme 1 symposia 'Developmental Biology of Plants', Leiden University	12 May 2006
EPS Theme 1 symposia 'Developmental Biology of Plants', Wageningen University	11 Oct 2007
EPS Theme 4 symposia 'Genome Plasticity', Wageningen University	12 Dec 2008
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Experimental Plant Sciences, Lunteren, The Netherlands	03-04 Apr 2006
ALW Meeting Experimental Plant Sciences, Lunteren, The Netherlands	02-03 Apr 2007
ALW Meeting Experimental Plant Sciences, Lunteren, The Netherlands	07-08 Apr 2008
ALW Meeting Experimental Plant Sciences, Lunteren, The Netherlands	06-07 Apr 2009
Seminars (series), workshops and symposia	
EPS symposium on Intracellular Signaling, Amsterdam, NL	02 Feb 2006
NVBMB spring symposium 2007, Wageningen, NL	18 May 2007
Seminar series "Frontiers in Plant Development" (estimated two per year)	2006-2009
Seminar series "Plant Cell Biology" (estimated two per year)	2006-2009
European Flying Seminar (estimated two per year)	2006-2009
Seminar series "Biochemistry" (estimated four per year)	2008-2009
► **Seminar plus**	
Hiroo Fukuda (U of Tokyo, J)	26 Nov 2007
Richard Vierstra (U of Wisconsin - Madison, USA)	14 Apr 2008
Zhenbiao Yang (U of California - Riverside, USA)	13 Jun 2008
► **International symposia and congresses**	
19th IPGSA Meeting, Puerto Vallarta, MEX	21-25 Jul 2007
Symposium on Plant Cell Biology, MPIZ Cologne, D	05-06 Sep 2007
9th International EMBL PhD Symposium, EMBL Heidelberg, D	25-27 Oct 2007
5th Symposium of SFB446: Mechanisms of Cell Behaviour, ZMBP Tübingen, D	02-03 May 2008
Conférence J. Monod: Fine tuning of plant signaling pathways, Roscoff, F	04-08 Jun 2008
Symposium of SFB572: Pattern formation & cell type specification, Cologne, D	03-05 Jun 2009
20th International Conference on Arabidopsis Research, Edinburgh, GB	30 Jun-04 Jul 2009
► **Presentations**	
Oral: ALW Meeting EPS, Lunteren, The Netherlands	02 Apr 2007
Poster: ALW Meeting EPS, Lunteren, The Netherlands	07 Apr 2008
Poster: 19th IPGSA Meeting, Puerto Vallarta, MEX	21-25 Jul 2007
Oral: Conf. J. Monod: Fine tuning of plant signaling pathways, Roscoff, F	04 -08 Jun 2008
Poster: 20th International Conference on Arabidopsis Research, Edinburgh, GB	30 Jun-04 Jul 2009
► **International Advisory Board interview** (G. Coupland, MPIZ-Cologne, D)	05 Dec 2008
► **Excursions**	
Subtotal Scientific Exposure	*20.3 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
In depth EPS course Proteomics	27 Feb-02 Mar 2006
Summer School: Signalling in Plant Development and Plant Defence	19-21 Jun 2006
► **Journal club**	
Journal club Biochemistry / Plant development	Weekly 2006-2009
► **Individual research training**	
Subtotal In-Depth Studies	*5.2 credits**

4) Personal development	date
► **Skill training courses**	
PhD Competence Assessment	12-28 Sep 2006
WGS Course: Project and Time Management	31 Oct-12 Dec 2006
Centa Dutch Courses; level A1 + level A2	2006-2007
BCFevent 2009	28 May 2009
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*5.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.1**

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 credits

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

A

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