Proteomic and mechanistic analysis of Auxin Response Factors in the Arabidopsis embryo

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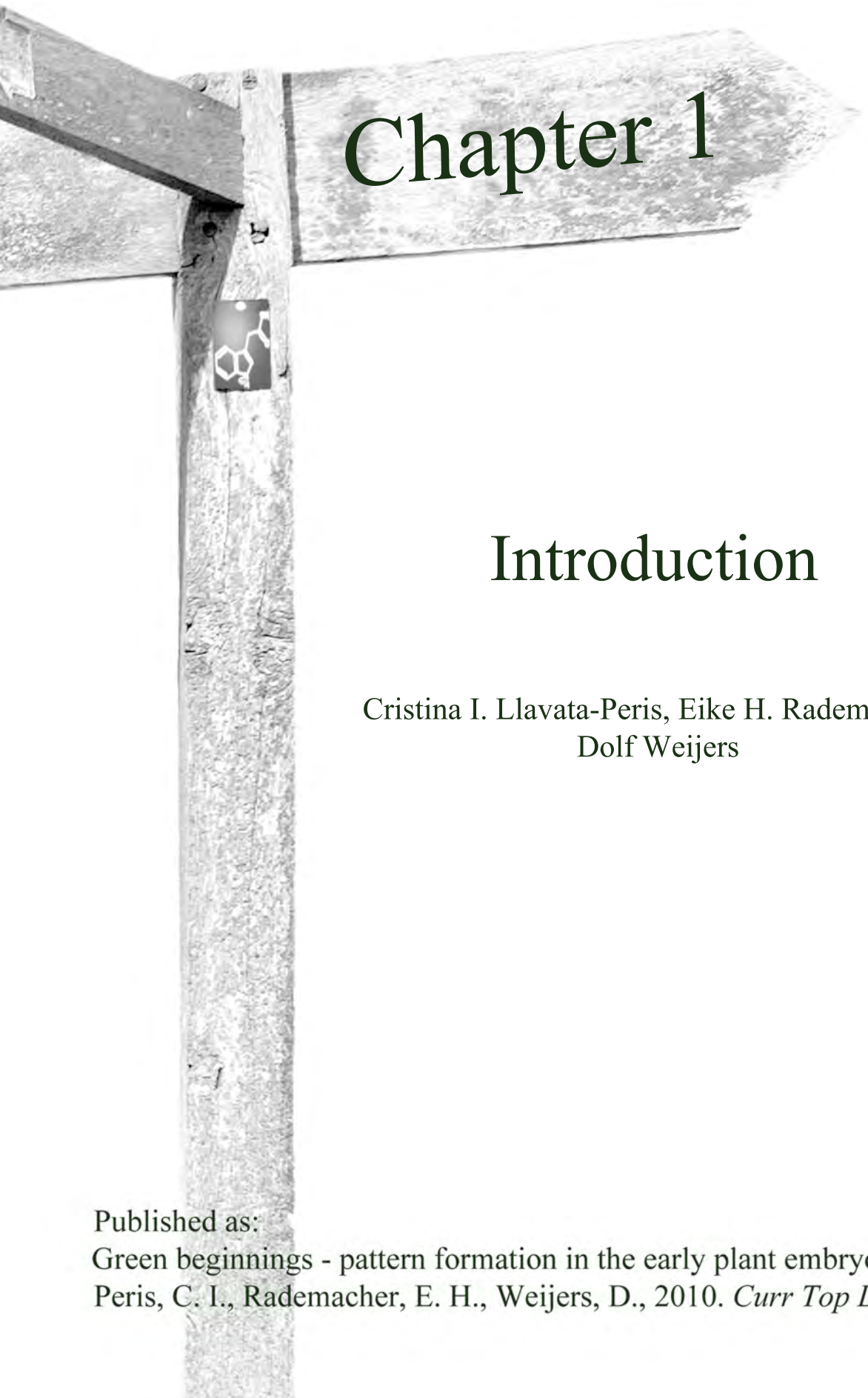

Chapter 1

Introduction

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Abstract

Embryogenesis in plants transforms the zygote into a relatively simple structure, the seedling, which contains all tissues and organs that later form the mature plant body. Despite a profound diversity in cell division patterns among plant species, embryogenesis yields remarkably homologous seedling architectures. In this review, we describe the formative events during plant embryogenesis and discuss the molecular mechanisms that regulate these processes, focusing on Arabidopsis. Even though only a relatively small number of factors are known that regulate each patterning step, a picture emerges where locally acting transcription factors and intercellular signaling contribute to the specification and spatio-temporal coordination of the various cell types in the embryo. Notably, several patterning processes are controlled by the plant hormone auxin. Most regulators that were identified in Arabidopsis have orthologs in other sequenced plant genomes, and several of these are expressed in similar patterns. Therefore, it appears that robust conserved mechanisms may underlie pattern formation in plant embryos.

1. Introduction–Seeds and Embryos

During evolution, plants as largely immobile organisms have developed sophisticated mechanisms to disperse their offspring and explore new habitats. One way to expand the populated area is to grow into the surrounding by means of vegetative propagation via specialized structures such as rhizomes or stolons. However, these mechanisms do not reach over great distances and fail to overcome relatively small obstacles.

The innovation that surpassed such restrictions and allowed plants to use wind, water, animals, gravity, and even ballistic mechanisms as means of dispersal is the seed. Furthermore, seeds permit the enclosed embryo to remain in a dormant state and survive long periods of harsh environmental conditions, thereby mastering not only spatial but also temporal restrictions to its habitat. Finally, when conditions are beneficial for germination, the seed provides the embryo with the nutrients that support the first steps of post-embryonic development. To fulfill all of these different requirements, seeds develop in very different shapes and sizes. Adaptations in seed morphology have enabled plants to occupy new ecological niches with the result that plants now dominate habitats on all different kinds of land, in both hot and cold climates (Kesseler and Stuppy, 2006).

Seed-bearing plants (spermatophytes) can be subdivided into two major groups: gymnosperms and angiosperms. The name-giving difference between these lies in the organization of the reproductive organs. Gymnosperms bear ovules on scales, which are usually in cone-like structures as, for example, in pine. Angiosperms instead have their reproductive organs arranged in flowers. Another important difference is that flowering plants feature a double fertilization step to produce the (typically) diploid embryo and the triploid extra-embryonic endosperm (Lersten, 2004). Despite these differences in seed anatomy and function, the development and basic body organization of the embryo are very similar among most higher plants (Cairney and Pullman, 2007; Johri et al., 1992). Accordingly, embryogenesis in both groups undergoes three common phases. Initially the fertilized egg cell (zygote) undergoes

elongation and after a few cell division rounds develops an apico-basal and radial axis. During the next phase, further cell divisions occur and the primordia of the fundamental organs for post-embryonic growth are established. A final phase of desiccation prepares the mature embryo for dormancy and equips the seed with storage products that aid the seedling in the process of germination (Bewley and Black, 1994).

Typically, seeds carry a single embryo that emerges as the product of fertilization of the egg cell (Fig. 1.1, box 1). However, the formation of poly-embryonic seeds has been observed in many taxa (e.g., Rutacea (Citrus) or Pinacea (Pinus); Batygina and Vinogradova, 2007 and Lakshmanan and Ambegaokar, 1984). Interestingly, the additional embryos within seeds do not always originate from cells that are derived from the zygote. More importantly, this is not even obligatory in mono-embryonic seeds. Hence, also unfertilized cells possess the potential to form embryos within a seed. Such asexual reproductive mechanisms of embryo formation have been summarized under the term apomixis (Fig. 1.1, box 2). We refer the reader to another review (Koltunow and Grossniklaus, 2003) that covers the various developmental origins of apomictic embryos. In addition to the naturally occurring origins of embryogenesis, male gametes (pollen grains) can give rise to viable but haploid embryos when cultured under certain conditions (Seguí-Simarro and Nuez, 2008; Fig. 1.1, box 3). Finally, somatic cells can be forced into an embryogenic pathway by treatment of cultured explants with auxin (2,4-D) (Toonen and de Vries, 1996; Fig. 1.1, box 4).

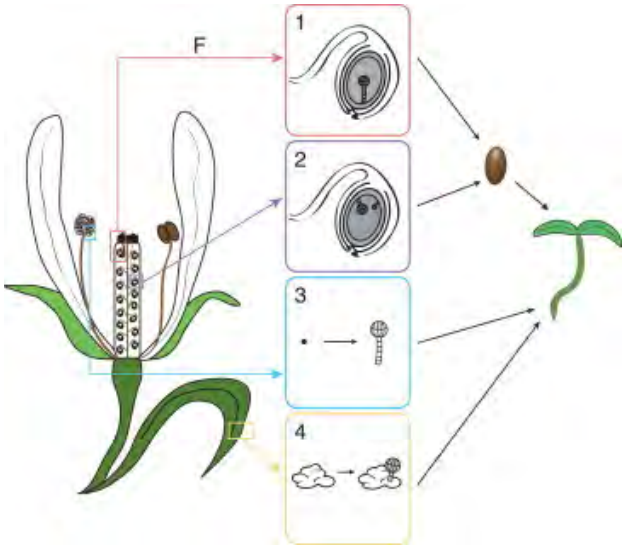

Figure 1.1. Origins of plant embryos. Embryogenesis normally occurs in the reproductive tissues within the flower. Shown here is a flower with subtending leaf. Zygotic embryogenesis (box 1) occurs when a pollen grain lands on the stigma of the carpel and fertilizes the egg cell in an ovule contained within the gynoecium. This ovule develops into a seed (brown oval) which, upon germination, generates the seedling (here dicotyledonous). Alternative modes of embryogenesis include the initiation of apomictic embryos within the ovule (box 2), microspore-derived embryos developing from pollen grains (box 3), and somatic embryos that are initiated from callus tissue derived from somatic cells (box 4). While only zygotic and apomictic embryogenesis pass through a seed stage, all these different modes of embryogenesis finally give rise to seedlings with the same body plan.

As transpires from the above, a wide variety of cells can generate embryos either naturally or after experimental treatment. However, despite the distinct origins of embryos and the often different patterns of cell divisions within the embryo, the outcome is always a seedling that consists of an apico-basal axis with apical shoot and root meristems, a radial axis that defines the respective tissues, and one or two cotyledons positioned in the immediate surrounding of the shoot apical meristem (SAM). It is therefore likely that a robust set of conserved genetically encoded instructions underlies pattern formation during embryogenesis in all species.

Molecular processes underlying pattern formation and development are best described in Arabidopsis thaliana. Due to the regular pattern of cell divisions during embryogenesis (Jürgens and Mayer, 1994), this species has also become the model for studying zygotic embryogenesis. In this review we will discuss embryogenesis in Arabidopsis to exemplify basic mechanisms that sculpt the organization of the plant body during embryogenesis. In the following sections, we will first describe the developmental landmarks during embryogenesis, and then discuss the molecular mechanisms that control pattern formation, followed by a perspective on the evolutionary conservation of the regulatory mechanisms that have been found in Arabidopsis.

2. Landmarks of Embryo Pattern Formation

In angiosperms such as Arabidopsis, the female gamete (egg cell) is positioned within the embryo sac, which in turn is embedded in the protective maternal tissue of the ovule inside the carpel (Fig. 1.2A, B). After deposition of a male microgametophyte (pollen grain) on the stigma of the carpel, a pollen tube extends toward the ovule. This tube eventually enters the ovule via its micropylar end and facilitates the delivery of two haploid sperm nuclei. One of these nuclei fertilizes the egg cell while the other fuses with the two nuclei of the central cell to give rise to the triploid endosperm (Lersten, 2004; Fig. 1.2C). Embryo and endosperm develop simultaneously, and several mutant studies suggest that their growth patterns are interdependent (reviewed in Berger et al., 2006). So far, however, there is no conclusive evidence that embryo pattern formation depends on the presence of the endosperm. Hence, consistent with the flexible origin of embryos, pattern formation in the embryo is driven by intrinsic factors rather than by environmental cues.
Embryo patterning follows a series of “landmarks” that each establishes part of the final organization. In the following, we briefly describe each such landmark.

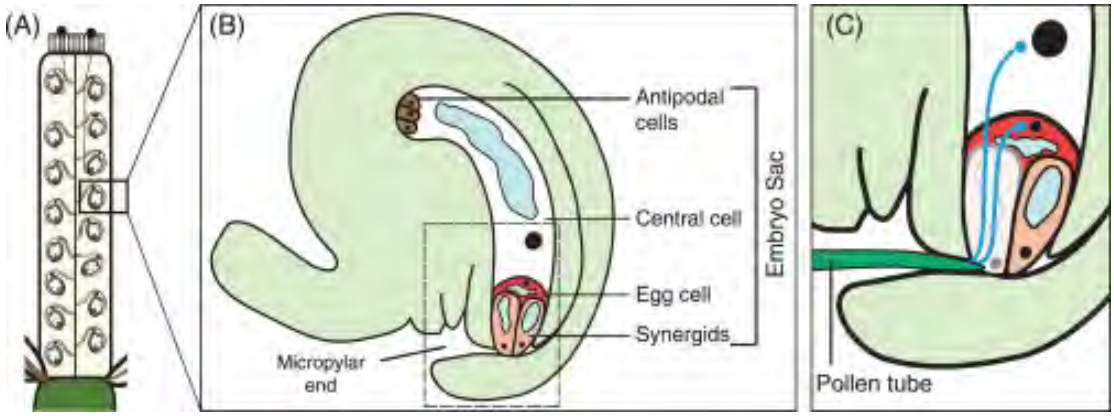

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Figure 1.2. Fertilization in Arabidopsis. Fertilization occurs within the gynoecium (A), where ovules (B) are attached to the placental tissues. The Arabidopsis ovule consists of a gametophyte (embryo sac) covered with somatic integument cell layers. The egg cell, surrounded by two synergid cells, is localized at the micropylar end. Additionally, the embryo sac houses a central cell nucleus and three antipodal cells. (C) Upon germination and growth, the pollen tube is attracted to the ovule by the synergid cells. The pollen tube then releases its generative nuclei in the degenerating synergid, upon which the nuclei fuse with the egg cell and central cell nuclei. (B, C) Drawn after Sundaresan and Alandete-Saez (2010).

2.1. Formation of the apico-basal axis

Embryo development occurs within the highly polarized environment of the ovule. Both the ovule and the embryo sac have a distinct polarized axis, and also the egg cell is intrinsically polar as judged from the localization of its organelles on the basal (e.g. vacuole) or apical end (e.g. nucleus) (Fig. 1.2C; Lersten, 2004). Fertilization induces stretching of the zygote, which is followed by an asymmetric division that gives rise to two daughter cells with different composition, shape, and developmental fate. The apical cell is small with a dense cytoplasm, while the basal cell is large and vacuolated. With three rounds of cell divisions, the apical cell generates a spherical proembryo consisting of a total of eight cells, while the basal cell only divides transversally and gives rise to a transient filamentous structure called the suspensor. This extra-embryonic suspensor connects the proembryo to maternal tissue and pushes it into the lumen of the ovule. Later during embryogenesis, only the uppermost suspensor cell, the hypophysis, becomes incorporated in the embryonic root meristem, as precursor of the quiescent center (QC) and central root cap cells (see below) (Fig. 1.3A).

At the eight-cell (octant) stage, four different domains can be distinguished along the apico-basal axis of the embryo; the proembryo consists of an upper and a lower tier of four cells each and is positioned on top of hypophysis and suspensor (Fig. 1.3A, B).

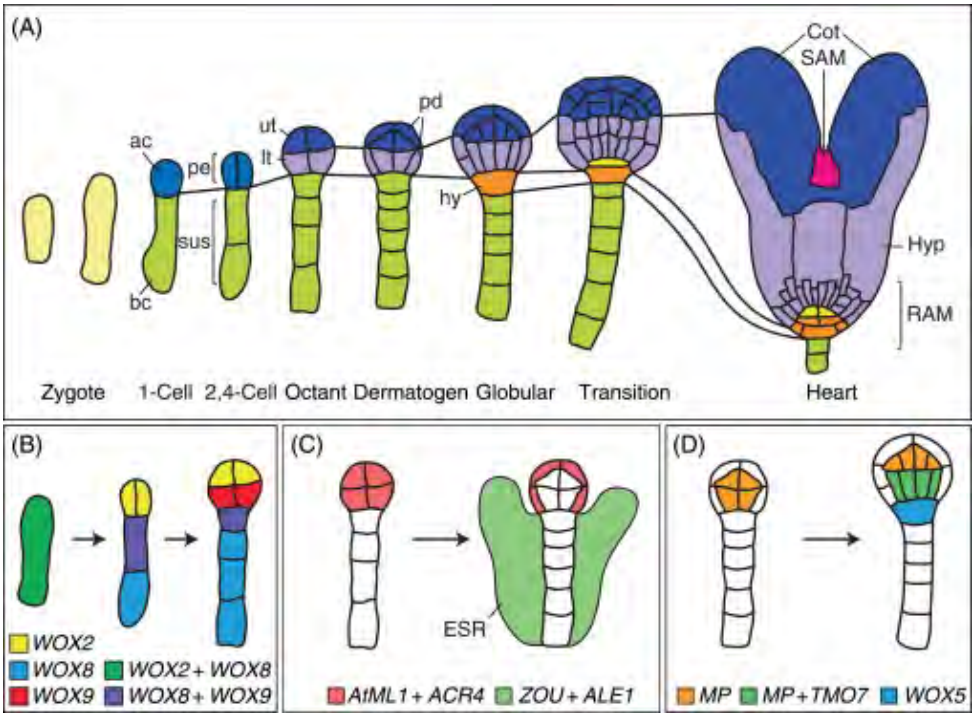

Figure 1.3. Embryo pattern formation and cell specification. (A) Stages of embryogenesis and cell lineages. The lines between embryos highlight the lineage relationships between different stages of embryogenesis. Stage nomenclature is given below each embryo. Colors represent clonally related regions. After fertilization the zygote elongates and undergoes an asymmetrical division generating a small apical cell (ac) and a larger basal cell (bc). From this point, two regions with different developing programs will be established, the embryonic proembryo (pe) and the extra-embryonic suspensor (sus). All cells in the four-celled proembryo divide to generate an upper tier (ut) and a lower tier (lt). Subsequently, a radial pattern is generated in the eight-cell proembryo when a layer of protoderm (pd) is established. At the globular stage, the uppermost cell of the suspensor is specified to become the hypophysis (hy). Through an asymmetrical division, the hypophysis gives rise to a lens-shaped cell from which the quiescent center will be generated and a basal cell from which stem cells and columella cells of the root tip will derive. Further divisions generate primordia for all seedling structures at the heart stage, including cotyledons (cot), shoot apical meristem (SAM), root apical meristem (RAM), and the hypocotyl (Hyp). (B–D) Molecular markers of cell fate decisions during pattern landmarks. Colors indicate the expression of genes as described in the box legends. (B) While WOX2 and WOX8 are coexpressed in the zygote, their mRNAs are found in different cells after two rounds of division. Combined with WOX9 expression, different combinations of WOX transcripts mark each cell. (C) In the octant stage, ATML1 and ACR4 are both expressed in the entire proembryo. Upon division, their transcripts are only found in the protoderm. ZOU and ALE1 transcripts are found in the embryo-surrounding endosperm region (ESR) and contribute to protoderm differentiation. (D) At the dermatogen stage, MP is expressed in all inner cells and remains so during two division rounds later. At the globular stage, the MP target TMO7 is expressed only in lower tier inner cells, while the WOX5 transcript is found in the hypophysis. (See Color Insert.)

2.2. Outside versus inside—radial pattern establishment

While the apico-basal axis of the embryo is preceded by polarity of the egg cell, the radial axis is established de novo after several rounds of cell division of the embryo.

All cells in the octant proembryo divide along a tangential plane, aligned along the apico-basal axis. This divides the proembryo in two different regions with different identities: an outer layer of eight cells, the protoderm, which is the precursor of the epidermis, and eight cells in the center of the proembryo, the inner cells, which are the precursors of ground and vascular tissues (Fig. 1.3A, C).

2.3. Stem cell niches—sources of all other cells

After several more rounds of cell division, the cells in the upper tier generate the SAM and the cotyledons (see below). Even though the shoot meristem can not be recognized by stereotypic cell divisions, gene expression markers for stem cells and for the organizing center (OC) become active in the area around the mid-globular stage (see below). The initiation of the root meristem, by contrast, is marked by a stereotypic asymmetric division of the hypophysis during the mid-globular stage. This initially extra-embryonic cell divides asymmetrically along the horizontal plane and generates two different cells: a small apical lens-shaped cell and a larger basal cell. Two vertical divisions of the apical cell form the QC. These cells control the undifferentiated state of the neighboring stem cells in the root apical meristem (RAM) (Jiang and Feldman, 2005). The descendants of the basal cell form the stem cells and the outer cell layers of the central root cap (columella) (Scheres et al., 1994; Fig. 1.3A, D).

With the specification of the SAM and RAM at this stage of embryogenesis, the sources for all cells of the post-embryonic body are established.

2.4. Initiation of cotyledons

The radial symmetry that characterizes the embryo until the late globular stage is broken with the initiation of the cotyledons. In the dicotyledonous Arabidopsis embryo, cotyledons are specified from two lateral zones at the apical domain of the proembryo. The function of these structures differs between plants that develop a single cotyledon and those that develop two. In the former case, the embryonic leaf remains under the soil and serves as a storage organ. In the latter case, the cotyledons serve first as storage organ and subsequently as the first photosynthetic organs.

The sum of the patterning landmarks as described above is a mature embryo with concentric tissue types arranged along an apico-basal axis that carries the meristems at either end. Most of the cell identity specification events can be readily observed by unique cell division planes or expansion (shape) changes. In each case, however, including the anatomically indistinguishable cell types, the cell fate is marked by a unique set of transcripts (Fig. 1.3B–D). Some of these merely mark a cell fate, while others encode proteins that control cell fate, division, or differentiation. In the following section, we will discuss the mechanisms that control pattern formation in Arabidopsis embryos.

3. Regulation of Embryo Pattern Formation

Patterning of the embryo requires both specification of individual cell identities and the coordination of cell specification between cells in space and time. Among the regulators of patterning, one therefore expects both intrinsic cell fate determinants, such as transcriptional regulators, and molecules that act as intercellular signals to coordinate the specification of cells on both long and short ranges. In the following, we will describe, separately for each developmental landmark, the key regulators that have emerged mostly from genetic studies. However, as the plant hormone auxin appeared as an important regulator in most patterning steps, we will first briefly describe the mechanism of action of this signal molecule.

3.1. Auxin biosynthesis, transport, and response

Auxins are indole-derived molecules that can control the division and elongation rate, as well as the identity or differentiation state of cells. Accumulation of and response to auxin have been shown to regulate many growth and developmental processes throughout embryonic and post-embryonic development. These include tropic growth responses, vascular development, leaf and flower initiation, root growth, and lateral root formation (Taiz and Zeiger, 2002). Auxin is synthesized from indole directly or from tryptophan through several intermediates that are not all known. Two types of biosynthetic enzymes have been shown to produce auxin pools that are required for normal development (Woodward and Bartel, 2005). These are the TAA1/TAR tryptophan aminotransferases that catalyze the conversion of tryptophan to indole-3 pyruvic acid (Stepanova et al., 2008 and Tao et al., 2008), and the YUCCA monooxygenases that convert tryptamine into N-hydroxyl tryptamine (Zhao et al., 2001; Fig. 1.4). Mutations in either of these pathways interfere with most if not all auxin-dependent processes, including those in the embryo. Auxin biosynthesis is thought to be localized in specific areas while auxin-dependent growth is observed also in tissues that do not appear to produce auxin (reviewed in Woodward and Bartel, 2005). Directional transport of auxin provides auxin also to these areas of the plant. To achieve this, cell membranes carry several auxin influx and efflux carriers. While influx carriers of the LAX family are not generally thought of as providing direction to transport (Petrasek and Friml, 2009), this is certainly the case for their counterparts, proteins of the PIN family of efflux carriers (Friml, 2003; Fig. 1.4). The polar subcellular location of these auxin transporters determines the direction of auxin flux. Therefore, the polar targeting of PIN proteins is an important control element in the regulation of auxin accumulation (Wisniewska et al., 2006).

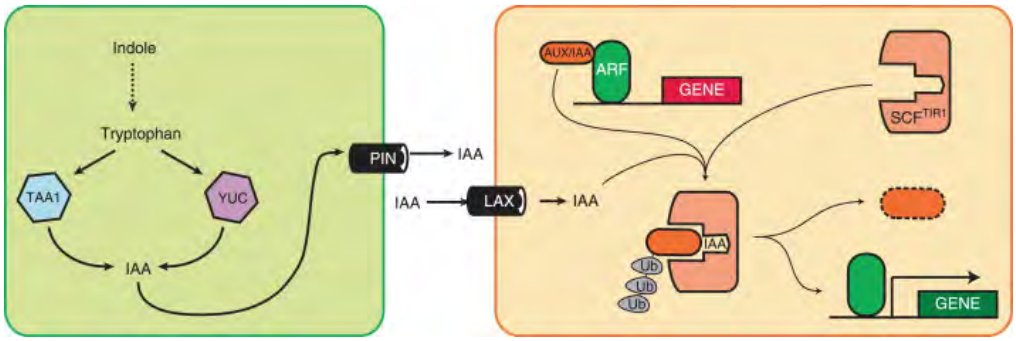

Figure 1.4. Auxin biosynthesis, transport, and response. Simplified schemes for the biosynthesis (left cell), transport (interface between cells), and response (right cell) of auxin. Note that many components have been omitted for clarity. Auxin is synthesized from indole either through the intermediate tryptophan (shown here) or independent of tryptophan (not shown). The TAA1 and YUCCA enzymes act in different branches to convert tryptophan into indole acetic acid (IAA). Auxin cannot exit cells freely, but its efflux is mediated by the rate-limiting PIN proteins, whose polar localization determines the direction of the flux. Auxin can enter cells by diffusion, yet uptake can be facilitated by the LAX influx carriers. Auxin directly binds to the SCF(TIR1) ubiquitin ligase and increases its affinity for the Aux/IAA proteins, targeting these for ubiquitin-mediated degradation. Under low auxin conditions, Aux/IAA proteins inhibit AUXIN RESPONSE FACTORS (ARFs) and DNA-binding transcription factors. Aux/IAA degradation releases the ARFs from inhibition and allows these to control gene expression.

Once accumulated, auxin is perceived by its receptor, the SCF-TIR1/AFB ubiquitin ligase. By binding to this enzyme complex, auxin facilitates the recognition of its substrates, transcriptional repressors of the Aux/IAA family, and their subsequent degradation by the proteasome (Chapman and Estelle, 2009; Fig. 1.4). Thus, auxin promotes degradation of transcriptional repressors. Without auxin, these Aux/IAA proteins bind to and inhibit another family of transcription factors, the AUXIN RESPONSE FACTORS (ARFs). Upon degradation of the Aux/IAA proteins, ARFs are released from inhibition and alter expression of their target genes (Fig. 1.4). Most of auxin's activity in controlling plant development can be accounted for by this short signaling pathway, as mutations in the auxin receptors TIR1/AFB cause defects indistinguishable from those of the auxin biosynthesis mutants (Cheng et al., 2007, Dharmasiri et al., 2005 and Stepanova et al., 2008). The activity status of auxin signaling can be indirectly visualized by the expression of an ARF-dependent gene expression reporter, DR5 (Ulmasov et al., 1997). When coupled to GFP, green fluorescence marks cells that show auxin-dependent gene expression (Ottenschlager et al., 2003 and Friml et al., 2003; Fig. 1.5). As discussed below, the landscape of auxin activity suggests that all patterning landmarks are closely mirrored by local auxin activity.

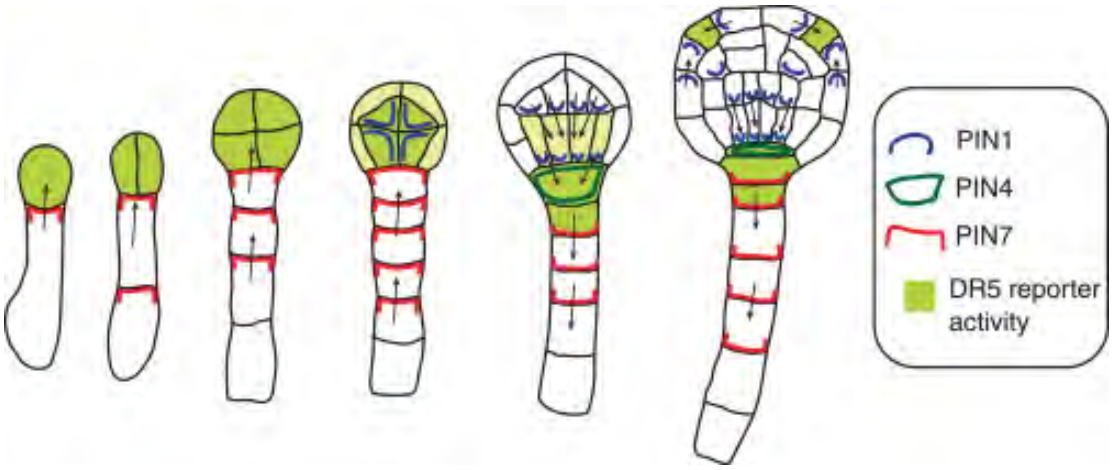

Figure 1.5. Auxin transport and response in Arabidopsis embryogenesis. Activity of the DR5–GFP reporter (green) and localization of PIN1 (blue lines), PIN4 (green lines), and PIN7 (red lines) during early embryogenesis. PIN7 is polar localized in the basal cell after zygote division and remains so until the globular stage (for stages, see Fig. 1.3). This is associated with DR5–GFP activity in the proembryo. PIN1 becomes polarly localized toward the basal end of the inner cells in the globular stage. This promotes auxin transport to the hypophysis (note DR5–GFP activity). Simultaneously, PIN7 polarity is switched to the basal end in suspensor cells and PIN4 is activated in the hypophysis. This constellation of PIN proteins and DR5–GFP activity is maintained at later stages. During the globular stage, PIN1 localization becomes polarized toward the flanks of the proembryo apex, eliciting new DR5–GFP activity maxima. Arrows indicate auxin flux direction as deduced from PIN polarity. (See Color Insert.)

3.2. Specification of the apico-basal axis

With the first division of the zygote, an apical and a basal cell fate are differentiated and the basic dualism of proembryo and suspensor development is precast. Genetic studies have not identified many factors that control this first event of embryogenesis, but recently, several components have been found to regulate aspects of zygote division as well as correct establishment and maintenance of the newly generated cell identities.

3.2.1. The first asymmetrical division–SSP/YDA pathway

Loss-of-function mutations in SHORT SUSPENSOR (SSP), YODA (YDA), MITOGEN ACTIVATED PROTEIN KINASES3 and 6 (MPK3/6), or GROUNDED (GRD) impair elongation of the zygote (Bayer et al., 2009, Lukowitz et al., 2004 and Wang et al., 2007). As a consequence, the basal cell is relatively small upon division of the zygote. Subsequently, the apical cell undergoes a normal pattern of divisions while aberrant division planes in the basal cell lineage produce a shorter and broadened cell file. This leads to a delayed establishment of the apico-basal axis perhaps due to the absence of signals from the malformed suspensor cells.

SSP encodes an interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase (Bayer et al., 2009), while YDA encodes a MAPKK Kinase (Lukowitz et al., 2004). Genetic studies are consistent with these proteins acting in a linear kinase pathway that also includes MAP Kinases 3 and 6 (Wang et al., 2007). Remarkably, SSP transcripts are delivered to the zygote and central cell by the pollen (Bayer et al., 2009). Hence, SSP accumulates only transiently in the zygote and provides a temporal cue for zygote development.

The output of the proposed SSP–YDA–MPK3/6 pathway in the zygote has not been identified so far. However, potential analogies to YDA activity in the zygote can be drawn from stomatal development. Here YDA and MPK3/6 negatively regulate the asymmetric division of meristemoid mother cells that give rise to a larger cell and the smaller meristemoid that becomes the guard mother cell (see chapter 9; Bergmann et al., 2004 and Wang et al., 2007). This signaling cascade is opposed by a set of bHLH transcription factors (SPEECHLESS (SPCH), INDUCER OF CBF EXPRESSION1 (ICE1), and SCREAM2 (SCRM2)) that promote initiation of asymmetric divisions to form meristemoids (Nadeau, 2009). The meristemoid-inhibiting YDA–MPK3/6 activity converges on the meristemoid-promoting ICE1/SCRM2/SPCH activities, as phosphorylation of SPCH by the YDA–MPK3/6 kinase cascade inhibits its activity (Lampard et al., 2008). Hence, signaling via YDA and MPK3/6 is translated into transcriptional activity by the phosphorylation status of a downstream transcription factor that is expressed in a cell-specific manner. A main open question is the identity and function of this direct phosphorylation target of the SSP–YDA–MPK3/6 cascade in the zygote.

3.2.2. The WOX2/8/9 pathway

Zygote division gives rise to two cells with dramatically different cell fates. How these cell fates are established and maintained is currently not well understood, nor is it known which intrinsic determinants, if any, segregate during zygote division. The best-studied candidates for regulators of apical and basal cell fate specification are transcription factors of the WUSCHEL-RELATED HOMEOBOX (WOX) family. WOX2 and WOX8/STIMPY-LIKE (STPL) mRNAs are coexpressed in the zygote, but upon division WOX2 transcripts are specific to the apical cell, while WOX8 and its close relative WOX9/STIMPY (STIP) are found only in the basal cell. Until the octant stage, activity of these three genes is further refined to form four domains along the apico-basal axis. Top-down these domains are characterized by WOX2 expression (apical tier), WOX9 expression (basal tier), coexpression of WOX8 and WOX9 (uppermost suspensor cell), and WOX8 expression (suspensor) (Fig. 1.3B). Whether WOX mRNAs are indeed segregating during zygote division remains to be demonstrated. Their function in specifying apical and basal cell fates, however, has

been established (Breuninger et al., 2008 and Haecker et al., 2004).

wox8 wox9 mutant embryos display normal zygote division but fail to correctly specify the apical and basal cell lineages. Subsequently, finger-like structures are formed by enlarged embryonic cells and, to a lesser extent, aberrant divisions in the suspensor cell file. Eventually, this leads to arrest of growth at around the heart stage phase of normal development. Analysis of several markers in wox8 wox9 embryos demonstrates that the basal lineage is not properly established. In addition to this, properties of the apical domain are also not fully developed, suggesting a non-cell-autonomous control of apical development by WOX8 and WOX9. This nonautonomy is partially mediated by promoting WOX2 expression in apical cells. wox2 mutant embryos display aberrant division planes during protoderm formation at the eight-cell stage of embryogenesis. However, this phenotype is enhanced to a higher frequency (30% vs. 80%), when closely related members of the WOX family (WOX1, WOX3) involved in further apical development are mutated as well. The lack of such phenotypes in wox1, wox3, and wox1 wox3 embryos suggests that WOX2 is the main regulator of embryonic shoot development. Furthermore, expression of WOX2 in wox8 wox9 embryos under control of the WOX9 promoter results in yda-like zygotic and embryonic phenotypes indicating that WOX2 expression is sufficient to establish various aspects of apical cell fates.

Activity of the SSP–YDA pathway as well as of WOX8 and WOX9 is crucial for the initial steps of embryo development. While the SSP–YDA pathway controls division of the zygote, the main outcome of WOX8 and WOX9 activity appears to be the establishment of basal cell fate. Consequently, zygotes in yda wox8 wox9 triple mutants divide in a yda-like manner producing two small daughter cells before they arrest (Breuninger et al., 2008). The absence of such an early developmental arrest in yda (Lukowitz et al., 2004) or wox8 wox9 (Breuninger et al., 2008) mutants suggests that these pathways act in a nonlinear manner and are necessary for the correct specification of apical and basal cell fates.

3.2.3. Auxin control of polar axis establishment

Several lines of evidence suggest an important role for auxin in establishing apical cell fate after zygote division. Transcriptional activation by auxin, as reported by the ARF-dependent DR5-GFP gene, is localized to the apical cell upon zygote division and remains active in the proembryo during the divisions that follow (Friml et al., 2003). This localized activity is likely the consequence of directional transport through PIN7, which is polarly localized in the basal cell toward the apical cell (Friml et al., 2003; Fig. 1.5). Pharmacological interference with PIN7 localization or a mutation in PIN7 impedes the apical auxin response and also interferes with normal subsequent divisions of the apical cell. Interestingly, mutations in ARF5/MONOPTEROS (MP) and IAA12/BODENLOS (BDL) show the same division defect, albeit at low frequencies (Berleth and Jurgens, 1993 and Hamann et al., 1999). Since MP and BDL are expressed in the apical lineage presumably already in the apical cell, this suggests that auxin is transported from the basal to the apical cell through PIN7 and elicits apical cell-specific characteristics through MP and BDL. Whether a similar auxin response is also involved in basal cell specification is not known, nor is it known what genes and processes are controlled by auxin in the apical cell or how auxin activity converges with SSP–YDA and/or WOX activities. Since PIN1 expression and DR5–GFP activity are disrupted in later stages of wox8 wox9 embryos (Breuninger et al., 2008), a plausible scenario would be that the SSP–YDA

and WOX modules act upstream of the auxin transport and response pathway, for example, by controlling the expression of PIN1, PIN7, MP, or BDL.

3.3. Establishment of the radial pattern

3.3.1. Delimiting territories—the epidermis cell fate

In the adult plant the epidermis will form the outermost boundary of the plant body to the surrounding environment and hence perform vital functions including the regulation of water status and gas exchange or the uptake of nutrients from the soil. Furthermore, the epidermis provides a means of mechanical restrictions that allow for directional growth. Hence, much attention has been given to epidermal structures of the adult plant such as stomata, trichomes, root hairs, the overlying cuticle, or the microtubule cytoskeleton of epidermal cells.

The fundamental question of how embryonic cells sense their outside position and are specified to become the founder cells of the epidermis remains mostly unanswered. The reason for this lies in the severe consequences of mutations in genes necessary for proper epidermal development (e.g., Johnson et al., 2005). A failure to specify the protoderm most likely leads to an early arrest of embryogenesis, a phenotype that is also caused by mutations in basic cellular/metabolic pathways. On the other hand, milder defects that allow for embryogenesis and germination of the seedling might disturb the establishment of the epidermis as barrier for water retention. Hence, the growing seedling will dehydrate quickly and subsequently might be overlooked in genetic screens (Tanaka et al., 2001 for examples).

Several genes have been identified that are involved in the formation of the embryonic cuticle and the physical separation of the embryo from the surrounding endosperm. Two of these genes, ZHOUPI (ZOU) and ABNORMAL LEAF SHAPE1 (ALE1), are predominantly expressed in the embryo-surrounding region (ESR) (Tanaka et al., 2001 and Yang et al., 2008; Fig. 1.3C). Genetic evidence suggests that the bHLH transcription factor ZOU controls expression of the secreted subtilisin-like serine protease ALE1. Morphological defects in ale1 seedlings are enhanced by mutations in either one of the two receptor-like kinases ABNORMAL LEAF SHAPE2 (ALE2) and ARABIDOPSIS CRINKLY4 (ACR4). ale2 acr4 double mutant seedlings not only show a lack of cuticle formation but also display a partial loss of epidermal cell fate specification (Tanaka et al., 2007). This implies that ALE1 might be involved in the generation of endosperm-derived signals that trigger ALE2- and ACR4-dependent signaling in the epidermis to maintain epidermal cell fate (Tanaka et al., 2007). Nonetheless, Brassica microspore embryos grown in culture develop a normal epidermal layer (Custers et al., 1997), suggesting that, while endosperm-derived signals may support protoderm specification or differentiation in zygotic embryos, this signaling is not required per se to form the epidermis.

Mutations in the above genes, however, appear not to affect the initial establishment of the protoderm but rather to interfere with the maintenance of epidermal cell fate or with differentiation at later stages of development. Protoderm formation is therefore likely controlled by factors intrinsic to the developing embryo. The homeodomain-GLABRA2 (HD-GL2) transcription factor ARABIDOPSIS THALIANA MERISTEM LAYER1 (AtML1) and its closest homolog PROTODERMAL FACTOR2 (PDF2) have been found to play a role in the maintenance of shoot epidermal cells. A lack of both gene functions results in a failure of cell

differentiation in the apical domain of the proembryo. Subsequently, embryos form a normal root and hypocotyl but develop a dome-shaped structure instead of proper cotyledons. Expression of both factors is found in all cells of the octant proembryo but is restricted to protodermal cells at later stages (Abe et al., 2003; Fig. 1.3C). Thorough inspection by deletion analysis revealed complex regulation of the AtML1 promoter through a number of different motifs (Takada and Jürgens, 2007). Interestingly, one of these motifs constitutes an L1 box which is also present in the PDF2 promoter and can be bound by both factors. Hence, AtML1 and PDF2 might positively regulate their own expression. Furthermore, a model emerges in which this positive feedback loop is confined to the outer cells by factors in the central domain of the proembryo that effectively repress the expression of AtML1 and PDF2 (Abe et al., 2003).

A factor that has been shown to be involved in the activation of epidermis-specific genes such as AtML1 and ACR4 (Fig. 1.3C) is the calpain-like cysteine protease Arabidopsis thaliana DEFECTIVE KERNEL1 (AtDEK1). atdek1 embryos lack ACR4 and AtML1 expression and fail to develop an epidermis (Johnson et al., 2005). However, the initial periclinal divisions that form the protoderm at the octant stage can be observed in atdek1 embryos, and AtDEK1 is expressed ubiquitously until globular stage. This suggests that also AtDEK1 is a factor involved in epidermis maintenance rather than its specification (Johnson et al., 2005).

In conclusion, several signaling components have been identified that mediate the specification of outer and inner cells. Importantly, however, the nature of the information that is interpreted by the proembryo to define outer and inner is not at all known. This “signal” could be of chemical or physical nature, but in any event the normal epidermis specification in embryos grown in isolation suggests that specific external signals are probably not involved.

3.3.2. Definition of inner cell types

With the formation of the outer protodermal cell layer, an inner central domain is also set aside. These inner cells will give rise to the initials for ground tissue and provasculature, while protodermal cells are committed to only generate epidermis cells. On one hand the inner domain is characterized by the exclusion of epidermis specific factors such as AtML1 and PDF2 (see above) (Fig. 1.3C), while on the other hand these cells are also marked by the presence of specific transcripts, such as MP (Hardtke and Berleth, 1998; Fig. 1.3D) or PIN1 (Friml et al., 2003). No mutants have been described that impair specifically the establishment of inner cells. One reason could be that the upper and lower tier inner cells are specified through independent mechanisms. This is conceivable because markers for the shoot and root apical meristems are activated in the upper tier or lower tier inner cells around this stage (see below; WUS and TMO5/7; Mayer et al., 1998 and Schlereth et al., 2010). The only information on the mechanisms that might separate outer and inner cell fates comes from more pleiotropic mutants, such as mutations in RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1) and TOADSTOOL2 (TOAD2) (Nodine et al., 2007). The basal proembryo cell tier of rpk1 toad2 embryos contains no cells that differentiate into protoderm or ground tissue initials but consists entirely of cells that express the provascular marker SHORT-ROOT (SHR). Consistent with this phenotype, AtML1 is not expressed (Nodine et al., 2007). Unfortunately, no ligands or kinase substrates are known for these two receptor-like kinases.

3.4. Initiation of meristems

Stem cells are located at the ends of the apico-basal axis of the plant in the SAM and the RAM, respectively. Although the meristems differ in their anatomical characteristics, and the organs or cells that are produced, the basic organization is similar. In both, an organizing center (OC in the shoot meristem, QC in the root meristem) is surrounded by stem cells for various tissues. Strikingly, the factors that are involved in the maintenance of shoot and root meristem also show a degree of similarity (Lenhard and Laux, 2003, Sarkar et al., 2007 and Stahl et al., 2009). The initiation of the two meristems in the embryo, however, differs significantly.

3.4.1. The root meristem

The root meristem of Arabidopsis forms at the boundary of the apical and basal lineages. The proembryo contributes the stem cells for vascular, ground, and epidermal tissues, as well as the lateral root cap, while the QC and the columella root cap are derived from a former suspensor cell (Scheres et al., 1994). Cell identity specification therefore needs to be tightly coordinated between the neighboring proembryo and suspensor cells in order to generate the QC and its adjacent stem cells. From genetic studies, several factors have emerged that play a critical role in the specification of the uppermost suspensor cell as hypophysis, precursor of the QC and columella. Their activities mostly converge upon auxin transport or response. Indeed, pharmacological inhibition of auxin transport (Friml et al., 2003 and Hadfi et al., 1998), or genetic interference with auxin transport (pin1,3,4,7 mutant; Friml et al., 2003), synthesis (yucca1,4,10,11; Cheng et al., 2007; or taa1 tar1,2; Stepanova et al., 2008), or perception (tir1 afb1, 2, 3; Dharmasiri et al., 2005) prevents root formation.

As described above, the output of auxin activity is a change of gene expression through the ARF transcription factors. One of the 23 ARFs in Arabidopsis, ARF5/MP, is critically required for root formation. Mutants do not make a root and show abnormal hypophysis specification (Berleth and Jürgens, 1993) and cell division. Interestingly, MP is not expressed in the hypophysis itself, but in the adjacent proembryo cells, which suggests non-cell-autonomous control of hypophysis specification through secondary signals (Weijers et al., 2006). While PIN1 is present on all inner membranes of the proembryo until the 16-cell stage, immediately prior to hypophysis specification, PIN1 protein becomes polarly localized toward the future hypophysis (Fig. 1.5; Friml et al., 2003). This suggests that auxin is transported from the proembryo to the uppermost suspensor cell. Indeed, the DR5–GFP reporter is active in this cell, and this activation depends on MP activity. Furthermore, PIN1 expression is strongly reduced in mp mutants. Since treatment with external auxin does not restore root formation in the mp mutant, other signal(s) were proposed to act in parallel (Weijers et al., 2006). Recently, through the identification of MP target genes, a second MP-dependent cell–cell signal was identified. This is represented by the small bHLH transcription factor TARGET OF MONOPTEROS7 (TMO7). In addition, MP was shown to activate several other transcription factors, including the bHLH factor TMO5, which acts in the proembryo (Schlereth et al., 2010). In summary, MP coordinates root meristem formation by activating both cell-autonomous factors (e.g. TMO5) and mobile signals (auxin and TMO7).

Upon the initiation of the root meristem, the identity of this area is specified through a family of AP2-type transcription factors encoded by the PLETHORA (PLT) genes (Aida et al., 2004). PLT gene expression depends on MP, but probably not through

direct binding (Aida et al., 2004 and Schlereth et al., 2010). Mutations in PLT genes interfere with divisions of the hypophysis derivates, resulting in an absence of the QC at early heart stage (Galinha et al., 2007). Strikingly, when ectopically expressed, PLT proteins can convert shoot cells to root identity, supporting their role as root identity specifiers (Aida et al., 2004).

Regulators for later steps in root patterning have been identified (e.g., WOX5 and SCR; see section 4 below). For reasons of brevity, we will not discuss these here, but refer to excellent reviews on this subject (see chapter 3; Petricka and Benfey, 2008 and Ten Hove and Heidstra, 2008).

3.4.2. The shoot apical meristem

The SAM is initiated within the apical half of the proembryo and as such does not involve cell–cell signaling across the apico-basal boundary. Many factors are known to control aspects of shoot meristem function, but the vast majority of these acts in the homeostatic control of meristem size rather than the initiation of the meristem. The OC cells and the overlying stem cells mutually control the size of the other population, leading to a stable meristem size. The installation of the OC and stem cell area occurs during the mid-globular stage, while both are maintained throughout plant life. In this review, we will consider the mechanisms that are involved in positioning the OC and stem cells, and refer to chapter 4 and another review for discussions on meristem function (Tucker and Laux, 2007). The OC is marked by the WOX family member WUSCHEL, whose activity is critical for SAM formation (Mayer et al., 1998). WUS activity in the OC is later required to promote expression of the gene encoding the secreted peptide CLV3 in the stem cells (Schoof et al., 2000). In turn, CLV3 and the membrane receptor-like kinases CLV1 and CLV2 suppress WUS expression in the OC. WUS activity is supported by the vascular ARGONOUTE10/ZWILLE (ZLL) protein, which functions in a small RNA pathway. While ZLL, WUS, and CLV3 expression does not overlap in the mature SAM, WUS and ZLL are coexpressed in the upper tier inner cells, and CLV3 is not activated until the heart stage of embryogenesis (Tucker et al., 2008). How the coexpression state progresses into the state where ZLL and WUS are separated is not understood, and no factors that activate WUS locally have been identified, leaving the first step in SAM initiation elusive.

Several other factors act early in the embryo to specify the SAM. Among these are members of the class III HD-Zip transcription factors. These are expressed in the vascular tissues and extend to the future SAM area at the globular stage (Prigge et al., 2005). Simultaneous loss of multiple members prevents SAM formation, although it has not been studied exactly at what stage the first defects are manifested (Emery et al., 2003 and Prigge et al., 2005). Finally, the HD transcription factor SHOOT MERISTEMLESS (STM) accumulates in the entire SAM area at the late globular stage of embryogenesis (Long et al., 1996). STM is required for SAM formation, presumably by preventing differentiation (Barton and Poethig, 1993). The expression domains of STM and WUS are established independently (Endrizzi et al., 1996), but combined overexpression leads to functional ectopic meristem formation (Gallois et al., 2002), suggesting that both are required to generate a proper meristem.

In summary, several genes are required for meristem initiation and further elaboration toward a functional meristem, but the mechanisms that position the OC and stem cells have not been identified to date.

3.5. Cotyledon initiation

At the globular stage, a few cells at the flanks of the proembryo apex are selected to become cotyledons, after which cells start to proliferate at these sites (Fig. 1.2A). The correct establishment of cotyledons requires that, from a zone of competence, only these two sites are selected and, subsequently, that these cells acquire cotyledon identity. Like root initiation, also this process appears to be under heavy auxin control. Most mutations or treatments that affect auxin transport, biosynthesis, perception, or response cause changes in the pattern of cotyledon initiation (see Möller and Weijers, 2009 for summary). Strikingly, while auxin transport during root initiation is mediated by the redundant activity of multiple PIN proteins, PIN1 is the dominant family member in cotyledon initiation (Benkova et al., 2003 and Liu et al., 1993). Therefore, localization of PIN1 is particularly informative to define auxin streams that are required for cotyledon initiation. Ubiquitous presence of auxin in the proembryo apex causes the establishment of cotyledon identity around the entire circumference (Friml et al., 2003 and Weijers et al., 2005); hence, auxin transport acts to direct the accumulation of the positively acting signal auxin only to those sites where cotyledons need to be. Indeed, activity of the DR5–GFP reporter shows peaks of auxin response in the cotyledon initiation sites. PIN1 is expressed in the protoderm, its polarity facing the cotyledon initiation sites, and in the vasculature, where polarity is facing downwards (Benkova et al., 2003). The cotyledon initiation sites are marked by convergence of PIN1 in the adjacent membranes of neighboring cells (Fig. 1.5). How this convergence is regulated remains to be determined; at least this constellation explains the auxin accumulation points, as it does in mathematical simulations of the post-embryonic shoot apex (Jonsson et al., 2006). Consistent with the central role of PIN1 in defining auxin accumulation sites, mutations that interfere with correct PIN1 expression and polarity lead to altered cotyledon numbers or positioning (Friml et al., 2004 and Treml et al., 2005). The auxin accumulation is translated into cotyledon developmental programs through the ARFs MP and ARF7/non-phototropic hypocotyl4 (NPH4). Information on target genes of these transcription factors is scarce, but includes the AP2 transcription factor DRN (Cole et al., 2009), whose activity is required for proper cotyledon establishment (Chandler et al., 2007). Importantly, auxin does not only act through activating cotyledon initiation genes, but also negatively regulates the expression of CUP-SHAPED COTYLEDON (CUC) genes in the cotyledon positions. In mp and pid pin1 double mutants, these genes, which are normally expressed in the boundaries between SAM and cotyledons, expand into the cotyledon position, which correlates with reduced cotyledon initiation (Aida et al., 2002). Conversely, cuc mutants develop cotyledon fate around the entire apical circumference, presumably due to the absence of specified boundaries (Aida et al., 1997).

After selection of cells to become cotyledons, the developmental fate of these cells needs to be specified. Although several genes have been shown to be restricted to cotyledons (Fiers et al., 2004 and Long and Barton, 1998), very little is actually known about the identity of the initial specifying factors. This is in stark contrast to the number of genes that are known to act in the upper–lower axis definition in cotyledons (reviewed in Husbands et al., 2009). The only reported case of alterations in the identity of the cells in the position of cotyledons is when either an apolar PIN1 protein or a dominant-negative Rab GTPase was expressed ubiquitously in embryos. In these genotypes, root-like structures occasionally appear in the position of

cotyledons (Dhonukshe et al., 2008). Intriguingly, this is accompanied by the misexpression of the "root specifier gene" PLT1, whose ectopic expression has been shown to convert cells to root identity (Aida et al., 2004). PLT1 belongs to the same transcription factor family as ANT, which is normally expressed in the cotyledon primordia (Long and Barton, 1998). Therefore, although purely hypothetical at present, it is conceivable that the different PLT/AINTEGUMENTA-LIKE members define various organ identities after their initial specification.

4. Evolutionary Aspects of Plant Embryogenesis

During evolution, plant genes have diverged dramatically, and so have the patterns of cell division in embryos. However, despite often very different cell divisions, the overall body plan of plants has been conserved. The identification of molecular mechanisms in Arabidopsis now allows addressing whether conserved cellular and molecular mechanisms underlie pattern formation in phylogenetically unrelated plant species.

When considering the regulatory factors in pattern formation, it appears that most factors that are instrumental in Arabidopsis embryogenesis have putative orthologs in other genomes (reviewed in Nardmann and Werr, 2007). Obviously, there are only a handful of examples where the expression pattern of these orthologs has been studied, and the actual function has not been addressed for any except a few. However, the limited expression data available suggests that, to some extent, the position-specific expression of orthologs is conserved.

For example, orthologs to Arabidopsis WUS, WOX2, and WOX5 have been identified in maize (ZmWUS1, ZMWOX2, ZmWOX5; Nardmann et al., 2007) and rice (OsQHB; Kamiya et al., 2003b). Even though rice and maize embryos do not have the same shape or stereotypic cell division pattern as the Arabidopsis embryo (Fig. 1.6), in both cases, defined WUS and WOX5/QHB expression sites are found on either side of the vascular axis (Fig. 1.6). Also, in both monocot and dicot embryos, the endodermal layer surrounding the vascular axis expresses SCR or its rice ortholog OsSCR (Kamiya et al., 2003a). Therefore, the relative position of the WUS-marked SAM OC, the vascular axis, and the WOX5/QHB-marked root QC area is analogous in distant species, which suggests common underlying mechanisms. An open question is whether the establishment of this rather advanced constellation of expression domains (Fig. 1.6) follows steps similar to the ones found in Arabidopsis. Evidence suggests that this may at least in part be the case since the maize WOX2 ortholog ZmWOX2 is expressed specifically in the proembryo-like structure similar to its Arabidopsis counterpart (Nardmann et al., 2007). There are large collections of maize (Scanlon et al., 1994) and rice (Hong et al., 1995) embryo-defective mutants. Identifying causal genes and determining the expression of the conserved cell identity markers will undoubtedly shed light on the conservation of molecular mechanisms underlying plant embryo patterning.

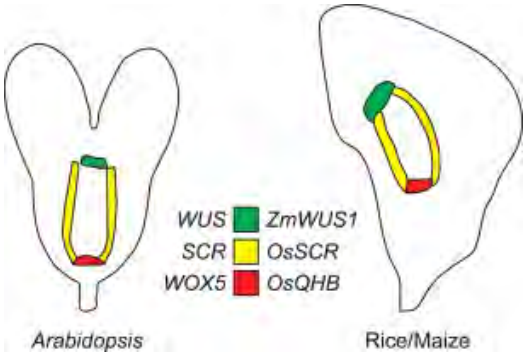

Figure 1.6. Conservation of plant embryo patterning. Comparison of the mRNA expression patterns of important Arabidopsis patterning regulators in monocotyledonous species. Embryos at approximately the same developmental stage are depicted. The rice/maize embryo does not have a "cotyledon primordium" that is as easily recognizable as the Arabidopsis cotyledon primordia but will initiate at the top left side. While the shape of Arabidopsis and maize/rice embryo is rather different, the relative position of WUS (or ZmWUS1), SCR (or OsSCR), and WOX5 (or OsQHB) is conserved and marks the shoot (WUS) to root (WOX5) axis.

At the very least, it appears that the profound role of auxin in embryo patterning in Arabidopsis is matched by similar roles in other species including Brassica (Hadfi et al., 1998), wheat (Fischer et al., 1997), maize, and the gymnosperm Picea abies (Hakman et al., 2009). The genomes of each of these species, and those of all other embryophytes analyzed so far, contain genes for auxin biosynthesis, transport, perception, and response (reviewed in Lau et al., 2008). Auxin has indeed also been detected in all land plants that were investigated (Cooke et al., 2002). Interference with polar PIN protein localization through treatment with auxin transport inhibitors, or with auxin, causes strong embryo patterning defects in all the above species (Fischer et al., 1997, Hadfi et al., 1998 and Hakman et al., 2009). Even though the exact defects depend on the species analyzed, this suggests that one common theme in the regulation of embryo patterning is the profound role of auxin.

The fact that very different cell division patterns can still give rise to similarly patterned overall plant structures suggests that cell division planes are not causally connected to patterning. This makes extrapolation of the mechanisms in Arabidopsis to species that have embryos with many more cells somewhat problematic. However, two fundamental issues emerge from the study of Arabidopsis embryogenesis. First, there is an early distinction between apical (embryonic) and basal (extra-embryonic) lineages. Second, the root initiates at the boundary between these two lineages. Observation of embryos from a wide array of plant species (Johri et al., 1992) shows that, even though the embryonic and extra-embryonic lineages are not always separated at the first division, the fundamental dualism is present in many plant species. Also, the site of root initiation relative to the embryo shape can vary substantially (see, e.g., Fig. 1.6). While the suspensor is easily recognizable in Arabidopsis, the shape and size are extremely variable in other species (Yeung and Meinke, 1993), which makes it difficult to define the suspensor without molecular markers. Nonetheless, in the rice embryo, in which the suspensor measures several cell diameters and is more continuous in shape with the proembryo (Nardmann et al., 2007), cells in the basal domain are more vacuolated similar to Arabidopsis suspensor cells. The QHB and ZmWOX5 expression regions are positioned in close association with these vacuolated cells (Kamiya et al., 2003b and Nardmann et al., 2007b), suggesting that a mechanism similar to the one in Arabidopsis could be involved in initiating the root meristem.

5. Concluding Remarks

Pattern formation in plant embryos involves several morphogenetic steps, during which cell type specification, asymmetric cell division, and cell–cell communication play critical roles. In this chapter, we have highlighted those patterning steps that we consider landmarks in embryo development and we have described the regulatory factors that are known to act in these processes. While critical regulators for each of these landmarks have been isolated mostly through genetics, it should be emphasized that with only a handful of factors for each patterning step, most of the factors involved remain to be identified. Yet, it emerges that one common theme in embryo patterning is the profound coordinating role of auxin. With its brief signal transduction pathway and a dedicated directional transport machinery, auxin is perfectly suited to coordinate cell fates both at long and at short ranges. A key open question is how specificity in its action is achieved such that the uppermost suspensor cell will be specified as hypophysis and the cells in the proembryo apex as cotyledon precursors. A further unifying theme in embryo patterning is the intersection between hormonal regulation and regional transcription factor activities. As an example, WOX transcription factors are required for proper auxin transport and response, while auxin activity in turn is required to specify the domain of WOX5 expression. The identification of the direct targets of the regionally acting transcription factors will soon provide insight into the wiring of the regulatory networks in both space and time.

Furthermore, phylogenetic and expression analysis of Arabidopsis regulators demonstrates that, despite profound differences in embryo anatomy, similar principles and mechanisms may underlie pattern formation across plant species. An important challenge will be the functional analysis of the role of these orthologs in other species, for example, through reverse genetics in model organisms.

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Chapter 2

Imaging of phenotypes, gene expression and protein localization during embryonic root formation in Arabidopsis

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Abstract

Plants grow elaborate architectures by repeatedly initiating new organs post-embryonically. The competence to do so depends on the activity of meristems, stem cell niches located at the tips of shoot and root. These meristems are first specified early during embryogenesis. Therefore, important insight into the activity of factors that are central to the establishment of stem cell niches in plants can be gained from studying early embryogenesis. However, embryos are not directly accessible to microscopic observation since they are embedded within the seed, which is itself enveloped by the fruit. Here we describe a suite of methods for the analysis of mutant phenotypes, fluorescent reporter gene expression and protein localization in Arabidopsis embryos, and show how these methods can be used to visualize key factors in embryonic root formation.

1. Introduction

During embryogenesis in plants one cell called the zygote undergoes a series of controlled divisions that will generate the basic body plan of the seedling, containing all tissue types. Therefore, a strong control of this process is of vital importance. One of the major developmental processes leading up to a fully formed embryo is the specification of an apical-basal axis and the formation of a radial pattern (1). To define these different regions, specific genes are activated to execute different genetic programs.

A critical step in the characterization of the function of selected genes during embryogenesis is the determination of their expression pattern at cellular resolution. A second important step is to understand the function of these genes. A straightforward method is the phenotypic analysis of knockout or overexpression lines.

Combining these two relatively simple approaches, elucidation of expression pattern, and assessment of mutant or misexpression phenotypes can give rich source of information, such as non-cell autonomous gene function.

In this chapter we discuss the use of two microscopic techniques in order to elucidate spatial and functional information of proteins expressed during *Arabidopsis* embryogenesis, with emphasis on the process of root initiation. In the first section we describe the analysis of knockout phenotypes in whole mount preparations of developing seeds using a microscope equipped with differential interference contrast (DIC) optics. The second section is devoted to studying the pattern of expression of genes using transcriptional or translational fusions to a fluorescent protein using confocal microscopy. The last section explains the use of immunofluorescence techniques for the visualization of low-abundant proteins using specific antibodies.

During embryogenesis, the root meristem is formed at the boundary between the proembryo and the suspensor. The suspensor is a transient filamentous structure of which only the uppermost cell, the hypophysis, contributes to the mature embryo by giving rise to the quiescent center and the columella stem cells (2). The correct

specification of the hypophysis strictly requires the action of the MONOPTEROS (MP) protein, as *mp* mutants show plants with no root and abnormal hypophysis (3). MP/ARF5 is a member of a family of transcription factors, the auxin response factors (ARF), whose activity is controlled by the plant hormone auxin. In fact the accumulation pattern of this protein shows that MP is not expressed in the hypophysis itself, but in the adjacent proembryo (4), which suggests a non-cell autonomous function in hypophysis specification through (a) secondary signal(s). Recently, several direct targets of MP were identified (5). Two of these, *TARGET OF MONOPTEROS5* (*TMO5*) and *TMO7*, encode transcription factors of the bHLH family. While both genes are expressed in the proembryo, the TMO7 protein accumulates in the hypophysis, consistent with being a mobile protein (5**).** At the same time during this process auxin is transported from the proembryo to the uppermost suspensor cell, which can be visualized by the activation of an ARF-dependent gene expression reporter, DR5 (6).

2. Materials

Phenotypic Analysis Using DIC Microscopy

1. Siliques from plants with a mutation in the gene of interest (usually heterozygous if mutation is embryo- or seedling-lethal).

2. Microscope equipped with DIC optics. In this case we use a Leica DMRB, but any alternative is possible.

3. CCD camera and computer with image-processing software.

4. Stereomicroscope.

5. Tweezers (e.g., Rubis Switzerland 5-SA).

6. Needle (e.g., Microlance; 3 0.6 × 25 mm).

7. Double-sided adhesive tape.

8. Microscope slides.

9. Microscope coverslips 20 × 20 mm.

10. Clearing/mounting solution: Chloral hydrate:water:glycerol (w/v/v) 8:3:1.

Analysis of Expression Patterns by Confocal Microscopy

1. Transgenic plants carrying a reporter construct (protein or promoter fused to the fluorescent protein of choice such as GFP).

2. Stereomicroscope.

3. Tweezers (e.g., Rubis Switzerland 5-SA).

4. Needle (e.g., Microlance; 3 0.6 × 25 mm).

5. Double-sided adhesive tape.

6. Microscope slides.

7. Microscope coverslips 24 × 50 mm.

8. Mounting solution: PBS with 4% paraformaldehyde (PFA), 5% glycerol, and FM4-64 (stock solution: 15.5 mM; working dilution: 4:1,000). The FM 4-64 is used as a counterstaining since it fluoresces intensely upon binding the plasma membrane.

9. Zeiss Axiovert 100 M (LSM510/Confocor 2) confocal microscope or any alternative that allows imaging of GFP and FM4-64 fluorescence.

Immunostaining of Embryos

1. 10× concentrated PBS buffer: 80 g NaCl, 2 g KCl, 14.4 g Na2HPO4, 2.4 g KH2PO4 in 1 l dH2O. Dilute 10 × PBS 10 times to get 1× PBS and adjust pH to 6.9 with HCl.

2. Fixative solution: 4% PFA in 1 × PBS, 0.1% Triton-X-100.
Fixative stock (10% PFA): Add 1 g of PFA in 10 ml dH2O. Heat in a water bath until PFA dissolves. Adjust pH to 6.9.

3. 3% driselase (Sigma-Aldrich, Cat. No. D9515) solution in 1× PBS. Vortex thoroughly, centrifuge for 2 min at maximum speed. Only use the supernatant.

4. Permeabilization solution: 20% DMSO, 3% NP40 in 1× PBS.

5. Blocking solution: 3% bovine serum albumin (BSA) in 1× PBS, sterile-filter before use.

6. Primary antibody dilution: α-GFP 1:600 in blocking solution. Dilution has to be chosen empirically; at the beginning try a range of different dilutions.

7. Different possibilities for primary antibody (examples):

- Anti-GFP from mouse, monoclonal (Roche, Cat. No. 11814460001).
- Anti-GFP from rabbit, polyclonal (Invitrogen, Cat. No. A6455).
- GFP booster ATTO 488 (ChromoTek, Cat. No. gba488 (this antibody is already coupled to a fluorescent dye; consequently no secondary antibody is necessary).

8. Secondary antibody solution: For example, Goat α-Rabbit-Alexa488, 1:600 (Invitrogen, Cat. No. A11008) in blocking solution. The choice of the secondary antibody depends on the first antibody used and the characteristics of the microscope that you are going to use for the analysis. Alternatives are Alexa546-coupled antibodies (red fluorescence) or anti-Mouse antibodies.

9. FM4-64 (stock solution: 15.5 mM; working dilution: 4:1,000).

10. Mounting solution: 90% glycerol, 10% 1 × PBS pH 8.5, 25 mg/ml 1,4-Diazabicyclo(2.2.2)octane (DABCO).

11. SuperFrost object slides (Thermo Scientific, Cat. No. J1800AHNZ).

12. PAP-pen (Sigma-Aldrich, Cat. No. Z377821).

13. Humid chamber (e.g., StainTray; Sigma-Aldrich, Cat. No. Z670146).

14. Oven set at 37°C.

15. Parafilm.

16. 20 × 20 mm coverslips.

17. 24 × 50 mm coverslips.

18. Stereomicroscope.

19. Tweezers (e.g., Rubis Switzerland 5-SA).

20. Needle (e.g., Microlance; 3 0.6 × 25 mm).

21. Double-sided adhesive tape.

22. Razor blade.

23. Eppendorf LoBind tubes (0.5 ml).

24. Zeiss Axiovert 100 M (LSM510/Confocor 2) confocal microscope or any alternative that allows imaging of GFP and FM4-64 fluorescence.

3. Methods

Differential Interference Contrast Microscopy for Analysis of Mutant Phenotypes

Direct visualization of plant embryos is not an easy task. Embryos are embedded in maternal tissue (the seed), which is enclosed in a protective structure, the silique. A better alternative for observing embryos therefore is the use of DIC microscopy also known as Nomarski microscopy. Using DIC it is possible to use whole mount preparation of isolated immature seeds without a need for staining. This means that developing seeds can be collected from the siliques, directly deposited in a drop of the mounting solution and with no further manipulation these can be observed by microscopy. DIC uses polarized light that strongly interacts with the sample, generating a high contrast with the background. The only prerequisite for the use of DIC microscopy is that the samples have to be transparent and not too thick. In *Arabidopsis thaliana*, the small size of developing seeds precludes the need to make histological sections. To render the opaque seeds translucent, a clearing solution is used that contains chloral hydrate and glycerol. This not only generates transparent samples but also creates an excellent refraction index for the use of DIC. Here we demonstrate this technique using a mutant of the auxin response factor MP/ARF5. This mutation produces an aberrant phenotype in the hypophysis (Fig. 1a, b).

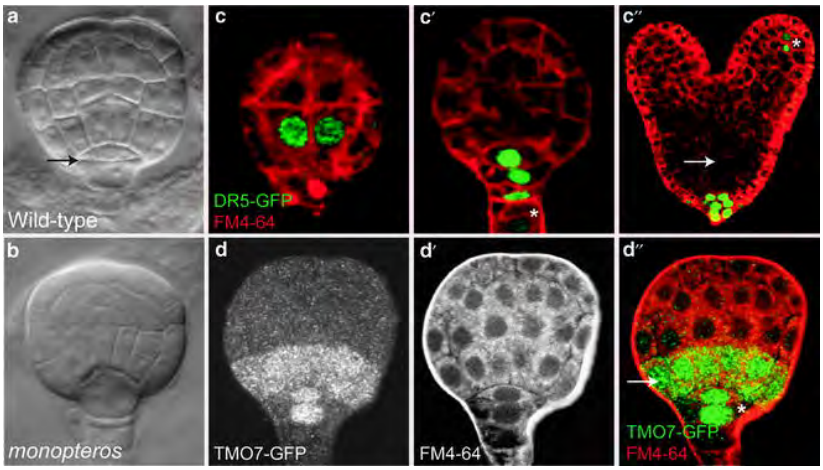

Figure 1. Imaging phenotypes, gene expression, and protein localization in *Arabidopsis* embryos. (**a**, **b**) Nomarski imaging of wild-type (**a**) and *monopteros* mutant (**b**) embryos at globular stage. Note that due to the high contrast of this imaging technique, cell walls and cell organelles such as vacuoles and nuclei are clearly visible. The arrow indicates the asymmetric division of the hypophysis cell in wild type. This division does not occur in the *monopteros* mutant embryo shown here. (**c**–**c″**) Expression of nuclear-localized triple *GFP* (*n3GFP*), driven from the auxin-responsive *DR5rev* promoter. Green nuclear signals highlight those cells that display active auxin response. Membranes are stained with the FM4-64 dye (*red*). Note that at the 16-cell stage (**c**) *DR5rev* is active in the inner cells in the lower half of the embryo. This activity is displaced to the hypophysis and subtending cells (also note weak signal indicated with an *asterisk*) at globular stage (**c′**). This maximum is maintained through the heart stage (**c″**), when additional expression peaks are established in the vascular cylinder (*arrow*), and in the cotyledon primordia (*asterisk*). (**d**–**d″**) Detection of TMO7-GFP fusion protein by indirect immunofluorescence in a globular stage embryo. Green TMO7-GFP signal (**d**) and *red* FM4-64 membrane staining (**d′**) are separately shown, as well as combined in a merged image (**d″**). TMO7-GFP is detected in cytosol and nuclei of the lower tier of the embryo (*arrow* in (**d″**)), and in the nuclei of the cells derived from hypophysis division (*asterisk* in (**d″**))

1. Collect siliques at the right stage of embryo development (see Note 1).

2. Stick the siliques to a piece of double-sided adhesive tape attached to a petri dish or a microscope slide. Open the silique along the septum using a hypodermic needle, separate the 2 valves of the silique, and make them stick to the tape. At this point the developing seeds are exposed. Be careful not to use a stereomicroscope with bottom-lighting, as this will heat up the sample quickly.

3. Carefully collect the developing seeds with the shaft of the needle (the opening should face away from the seeds) and deposit the seeds into a drop (~50 µl) of clearing/mounting solution on a slide (*see* Note 2). It is possible to collect seeds from 1 to 3 siliques per drop, but when embryos from the same stage need to be compared it is preferred to collect seeds from a single silique in individual drops. Carefully put a coverslip on top of the seeds. Be careful not to squash the seeds and add more clearing/mounting solution if necessary. Drain excess solution with a piece of filter paper if necessary. Leave at least 30 min at room temperature before observation. Alternatively, slides can be kept at 4°C for several days.

4. Observe the sample using a DIC microscope. An LCD camera is combined with the microscope and the software Mightex Capture is used for obtaining images. Make sure that the microscope settings are optimal.

Analysis of Expression Patterns Using Confocal Microscopy

Fluorescently tagged proteins are routinely used to monitor the presence, localization, abundance, transport, or degradation of a protein of interest. This technique makes use of the capacity of fluorescent proteins to absorb light at a particular wavelength and emit at a longer wavelength. Emitted photons can cover different regions of the spectra, representing different colors. The first fluorescent protein isolated was the green fluorescent protein GFP from the jellyfish *Aequorea victoria*. This protein has an excitation peak at 395 nm and a weaker excitation peak at 475 nm, and emits at 508 nm in the green range. Subsequently different mutants of the GFP protein have been created, with different chromophores and consequently with different spectra for absorption and emission.

The use of GFP includes the construction of transcriptional fusions that report the transcription pattern of a gene, and translational fusions that can be used to demonstrate protein localization. Here we show the use of transcriptional fusions as a reporter for the pattern of expression of the ARF-dependent gene expression reporter, *pDR5::GFP* (Fig. 1c–c″; ref. (4)).

1. Collect siliques at the right developmental stage of embryo (*see* Note 1).

2. Stick siliques to a piece of double-sided adhesive tape (on microscope slide or petri dish). Using a hypodermic needle open the silique along the septum, separate the 2 valves of the silique, and stick these to the tape. At this point the developing seeds are exposed. Be careful not to use a stereomicroscope with bottom-lighting, as this will heat up the sample quickly.

3. Carefully collect the developing seeds with the needle and deposit them in a drop (~50 µl) of mounting solution on a slide (*see* Note 3).

4. Place a 24 × 50 mm coverslip on top of the seeds.

5. Gently squash the developing seeds using the tip of a pencil in order to break the seed coat and release the embryo from the seed. This step requires practise. The efficacy of embryo extrusion can be checked under a microscope.

6. Samples can be observed using a confocal laser-scanning microscope; we use a Carl Zeiss LSM510. If you are using GFP (*see* Note 4) the settings that can be used are Argon laser line at 488 nm, band-pass filter ranging from 505 to 530 nm for the GFP signals, while for FM4-64 signals a long-pass filter of 650 nm can be used.

Immunostaining

In some cases the protein of interest may have a very low level of expression, and might not be visible with the confocal microscope when fused to a fluorescent protein reporter. A way to overcome this is the use of indirect immunofluorescence. Using a monoclonal or a polyclonal antiserum directed against the tag (in this case GFP) and a fluorescently labeled secondary antibody will increase the signal since multiple antibodies are now bound to a single tagged protein. A range of first and secondary antibodies is commercially available.
The essential steps in immunofluorescence are first of all tissue fixation and immobilization to slides. To facilitate antibody penetration, the cell wall is first degraded by a mix of fungal enzymes. Next, the cell membrane needs to be permeabilized using the detergent NP-40 and the solvent DMSO. After blocking the excess of non-target proteins with BSA, antibody incubation steps are performed. It is of vital importance to perform extensive washes with PBS buffer between different incubation steps to get rid of the different solutions. Also take care that the samples are always covered by liquid, as drying of the sample may affect the efficiency of the immunostaining process.
Here, we show the use of immunostaining to detect the TMO7 protein (5). Expression of this protein is too weak to be detected by direct observation. Immunofluorescence, however, allows detection of this protein (Fig. 1d–d″). The immunofluorescence protocol is adapted from the one described by ref. (8).

1. Prepare 0.5 ml Eppendorf LoBind tubes with 300–400 µl fixative solution (*see* Note 5). The use of LoBind tubes limits the loss of seeds by adhesion to the surface of the tube.

2. Isolate developing seeds from siliques (as in Subheadings 3.1 and 3.2) and transfer these to the Eppendorf tubes with the fixative solution (*see* Note 1). Keep tubes on ice while harvesting seeds. Note that during the procedure, there will be loss of embryos at several steps. Make sure to collect as many as possible, at least 5–10 siliques per developmental stage. Seeds of different stages can be harvested and processed separately to facilitate analysis of embryos of similar stage.

3. Incubate under vacuum for 1 h on ice.

4. Wash four times for 10 min with dH2O.

5. Use a glass Pasteur pipette to carefully transfer the seeds to SuperFrost object slides (*see* Note 6). Avoid air bubbles as this leads to excessive sticking of seeds to the glass wall. Use a needle to move seeds so that they are contained within a small area (<18 × 18 mm) and cover with a clean 18 × 18 mm coverslip.

6. Firmly squash the seeds with the tip of a pencil in order to extrude the embryos.

7. Freeze the slide in liquid nitrogen until bubbling stops. Lift the coverslip with a razor blade. If the coverslip is clean, the buffer containing seeds should (mostly) stick to the slide, not the coverslip.

8. Let the slides dry overnight at room temperature (RT).

9. The following morning place the slides in a humid chamber (*see* Note 7).

10. Use the PAP-pen to draw a circle around the plant material on the glass slides (*see* Note 8). Let the solvent evaporate.

11. Rehydrate the tissue with 1× PBS pH 6.9 for at least 5 min. In this and all following steps remove the solution by tilting the humid chamber. If necessary, drain excess liquid by holding a piece of filter paper next to the embryos.

12. Add 200 µl 3% Driselase solution per slide. Incubate for 30–45 min at 37°C in a humid chamber.

13. Wash 5–6× for 5 min with 1× PBS pH 6.9.

14. Add 200 µl Permeabilization solution per slide. Incubate for 1 h at RT.

15. Wash 5–6× for 5 min with 1× PBS pH 6.9.

16. Add 200 µl Blocking solution per slide and incubate for 2–3 h at 37°C in a humid chamber.

17. Add 100 µl 1st antibody solution per slide. Carefully cover the slides with a piece of Parafilm no bigger than the slide. Transfer to a humid chamber and incubate for 2 h at 37°C, and then overnight at 4°C (*see* Note 9).

18. Carefully remove Parafilm strips and wash 5–6 times for 5 min with 1× PBS pH 6.9.

19. Add 100 µl 2nd antibody solution per object slide. Cover the slides with Parafilm, transfer to the humid chamber, and incubate for 2 h at 37°C (*see* Note 10).

20. Wash 3× for 5 min with dH2O.

21. Add 200 µl 1% FM4-64 for 4 min.

22. Wash 3× for 10 min with dH2O.

23. Remove liquid and add a few drops of mounting solution.

24. Add a 24 × 50 mm coverslip and analyze directly with confocal microscope or store at 4°C in the dark (*see* Note 11).

4. Notes

1. For some studies it is important to choose the right developmental stage of the embryo. A way to identify the stage is to count the number of siliques from the first open flower down. As guidance, 3–4 siliques down from the first open flower usually contain globular stage embryos. You should also take into account the age of the plant. In older plants the developing seeds can be in a more advanced stage of development than expected.

2. The time needed for the clearing of the developing seeds is around half an hour, but it is also possible to prepare the slides and store them at 4°C overnight, observing them under the microscope the day after. Mild heating (30–40°C) will speed up clearing, but can also lead to enhanced tissue disruption.

3. At this point it is possible to look directly at the slides under the microscope, but it is also possible to store them at 4°C for a couple of days. If the slides are stored it is important to make sure that the seeds are covered in sufficient mounting solution.

4. A factor desirable in a fluorescent protein is a high brightness that can be easily detected by eye or using photodetectors when using a fluorescence microscope. The brightness depends on two factors: the efficiency in the absorption of the excitation light, called the extinction coefficient, and the ratio of photons emitted to photons absorbed, named the quantum yield. If you are doing long measurements where photobleaching (the photochemical destruction of the fluorophore) can pose a problem, the most advantageous situation is a molecule with high extinction coefficient because then it is possible to use lower excitation light levels, reducing the photobleaching. High quantum yield also means lower excitation light levels, resulting in higher brightness using the same intensity of excitation light. The enhanced versions of the GFP and YFP (eGFP and eYFP) have a similar quantum yield, but eYFP has a higher extinction coefficient, making it less susceptible to photobleaching. eCFP, the enhanced cyan form, is less susceptible to photobleaching but is less bright than eGFP and eYFP. For the choice of which fusion to use you can take into account these characteristics; it is also possible to try several different ones and choose for the one that gives better results.

5. The fixative solution should be freshly prepared or small aliquots may be stored at −20°C. PFA is a rapid fixative and a suspected carcinogen. It is recommended to wear protective gloves and work in a fume hood when handling the powder.

6. These slides possess a permanent positive charge that electrostatically binds the

fresh frozen tissue to the slides.

7. The use of a humid chamber is recommended in order to keep the right humidity conditions and avoid drying out of samples during long incubation steps. There are commercial humid chambers that can be purchased, basically consisting of a container with holders where the slides can be temporarily fixed and a deposit on the bottom part that can be filled with water. The slides can be fixed to these supports, facilitating the exchange of liquids in washing steps by just tilting the box. In some cases this is not recommended because it can cause loss of sample, so we advice using a glass Pasteur pipette to carefully add or remove liquids.

8. This creates a hydrophobic barrier around the developing seeds, so the amount of antibody reagent per slide can be reduced. This also helps to minimize drying out of the slides.

9. Most commercially available antibodies directed against the GFP are also able to recognize YFP and CFP. If the antibodies are polyclonal, different epitopes of the same protein can be recognized. Hence, more than one antibody can bind to the GFP, leading to an increase of the signal. We have noticed that there can be large differences in results depending on the manufacturer and batch of the primary antibody. It is advisable to include negative and positive controls, and if possible test multiple antibodies.

10. There is a large range of commercially available secondary antibodies. For the immunofluorescence experiments we use an Alexa488-coupled antibody, which emits fluorescence in the green range. Other forms are available where the excitation and emission spectra cover the visible spectrum and extend into the infrared. Note that the same procedure can be used to detect multiple proteins (e.g., GFP and PIN1) (7). In this case, primary antibodies need to be from different host species, and secondary antibodies should have different fluorophores.

11. A problem that often occurs in immunostaining is the presence of fluorescence background. A good negative control is to incubate with only the secondary antibody.

Acknowledgments

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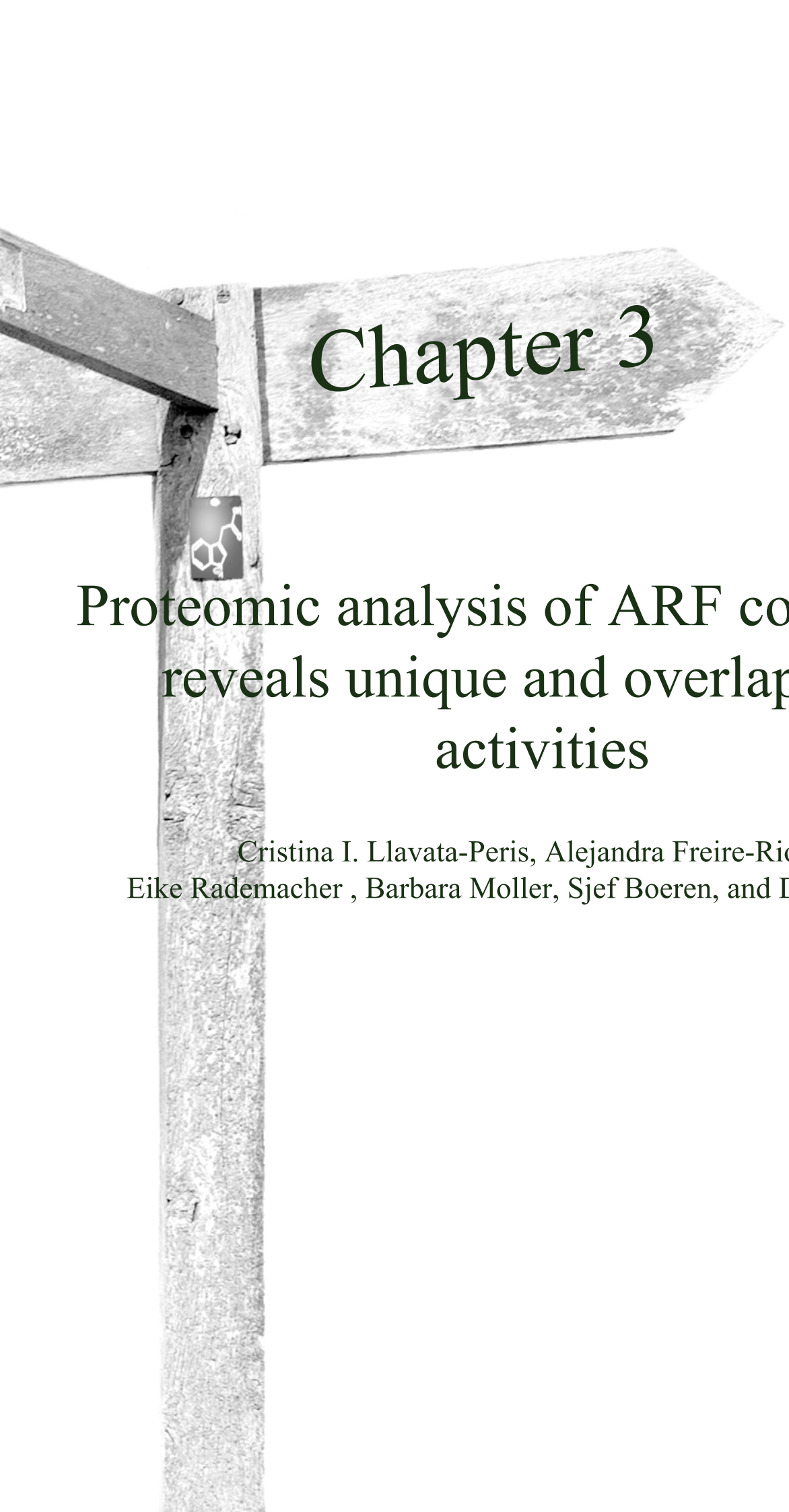

Chapter 3

Proteomic analysis of ARF complexes reveals unique and overlapping activities

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Eike Rademacher , Barbara Moller, Sjef Boeren, and Dolf Weijers

Abstract

Auxin controls many different processes during plant development. Yet, the pathway for gene regulation is relatively short, and involves two antagonistic families of transcription factors, the DNA-binding ARFs and their Aux/IAA inhibitors, as well as a ubiquitin ligase that targets Aux/IAA proteins. A relevant question to be solved is how specificity of auxin response is generated in the different cell types. We study the family of ARF transcription factors as source of signaling specificity. We use the suspensor of Arabidopsis embryo as a model since it expresses 6 phylogenetically different ARFs within a single cell type. We combine three different approaches, genetic analysis, *in vivo* protein-protein interactions using a dynamic FRET-based interaction assay, and mass-spectrometry based proteomics. We found that ARF6 and 9 are the main contributors to auxin response in suspensor cells. Furthermore we show that the ability to interact with Aux/IAA and to form homo- or heterodimers is different between ARF proteins. Finally we demonstrate that ARFs assemble in different protein complexes, and that components of these newly identified complexes contribute to normal auxin response. Collectively our data show that ARFs participate in both shared and divergent protein interactions, and hence provide a plausible explanation for the unique and redundant biological functions performed by these proteins.

Introduction

Auxin is a phytohormone that is crucial for many aspects of plant development. The processes in which this hormone has been implicated span from embryo development to flower transition, defense, tropic responses, and many other processes during plant life (Moller and Weijers, 2009; Nagpal et al., 2005; Ellis et al., 2005; Goetz et al., 2006). A key question in auxin biology is how the structurally simple tryptophan-like auxin Indole-3-Acetic Acid (IAA) molecule (Went, 1926) is able to elicit such diverse responses. Most of its activity in plant development appears to depend on auxin-dependent gene regulation, as components that interfere with this ability cause defects in most auxin-regulated processes (Chapman and Estelle, 2009). Auxin regulates the transcriptional activation or repression of genes through the *AUXIN RESPONSE FACTOR (ARF)* family of transcription factors (Tian et al., 2002; Okushima et al., 2005). Auxin activates these *ARF* transcription factors by promoting the degradation of members of a second family of transcription factors, the Aux/IAA proteins, which interact with and inhibit the ARFs (Kim et al., 1997; Tiwari et al., 2004). When cellular auxin levels rise, it binds both an Aux/IAA protein and the SCF(TIR1/AFB) ubiquitin ligase complex, thereby increasing the affinity for its Aux/IAA substrates, leading to their ubiquitination and degradation by the proteasome (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). This releases the ARFs from the inhibition by Aux/IAA proteins and allows them to control the transcription of their target genes.

Given this very brief pathway for auxin-dependent gene regulation, few scenarios can be envisioned to generate diversity and specificity. First, different TIR1/AFB – Aux/IAA co-receptor pairs have been shown to have unique auxin binding affinity (Parry et al., 2009; Calderon Villalobos et al., 2012). Therefore, this level may contribute to setting signaling thresholds in the cell. However, qualitative differences in output, as required for vastly different developmental contexts, will require further

diversification. We and others have previously shown that Aux/IAA proteins are generally interchangeable, and carry little qualitative specificity for the ARFs they inhibit (Weijers et al., 2005a; Muto et al., 2007). We therefore consider the 23-member ARF family a likely source of variation in output specificity. By analyzing patterns of activity of transcriptional reporters for all Arabidopsis *ARFs*, we recently demonstrated that there is a large degree of cell type-specificity. Both in embryo and root, a complex combinatorial pattern of expression could be observed (Rademacher et al., 2011b). As a consequence, each different tissue present in the embryo expresses a unique set of ARFs (Rademacher et al., 2011b), and these unique combinations could be an explanation for how different responses to auxin in the embryo can be generated. A requirement is that ARFs are to some degree functionally distinct. Indeed, mutant combinations and promoter-swap experiments support this prediction. Double mutants of *arf5/mp* and *arf6* show that these proteins act redundantly, while ARF1 and ARF5/MP appear to act antagonistically (Rademacher et al., 2011b). Likewise, the phylogenetically distinct ARF1, 2 and 6 redundantly control embryo development as a novel phenotype is observed in the triple mutant (Rademacher et al., 2011a). Finally, while ARF16 can only partly replace ARF5 (Weijers et al., 2005b), and ARF9 is unable to replace ARF5, misexpression of MP/ARF5 in the ARF13 domain (suspensor) even interferes with normal development (Rademacher et al., 2012). Hence, even in this limited set analyzed, ARFs appear to act redundantly, differently or even antagonistically.

Here we address the mechanisms underlying specificity and redundancy within a set of co-expressed ARFs. We use the Arabidopsis suspensor as a model since, based on our earlier observations; this cell type expresses 6 phylogenetically diverse ARFs (*ARF1, 2, 6, 9, 13, 18*). Of these, some are redundant (*ARF1, 2, 6*), while genetic interactions among the other 3 have not been addressed. Previously, it has been established that a bone fide auxin response acts in suspensor cells to control their normal development. The phenotypes observed in *arf1,2,6* mutants (Rademacher et al., 2011), or in *ARF9* RNAi lines (Rademacher et al., 2012), strongly resemble those found in *iaa10* gain of function mutants (Rademacher et al., 2012), as well as in auxin biosynthesis mutants.

We consider several levels at which ARFs may differ. First, the DNA binding specificity may vary among ARFs, an issue that has not been addressed. Second, ARFs may differ in their ability to interact with IAA10, or to form homo- or heterodimers. Such interactions have been shown or suggested, but the relevance of these interactions is not fully understood. Thirdly, ARFs may assemble into different protein complexes. Transcription factors can interact with other transcriptional regulators or other proteins to form transcription complexes (Payne et al., 2000; Broun, 2005; Ramsay and Glover, 2005; Girin et al., 2011). These, when different, may contribute to different functions of ARF complexes. Indeed, limited interactions of ARFs with other proteins have been observed. For example, ARF8 interacts *in vitro* with BIGPETAL, a bHLH transcription factor (Varaud et al., 2011); ARF5/MP interacts with the transcriptional co-regulator BRX (Scacchi et al., 2009), and several ARFs were shown to interact with the MYB77 transcription factor (Shin et al., 2007). Finally, ARF2 was shown to be directly phosphorylated and inhibited by the BIN2 protein kinase (Vert et al., 2008).
In this study, we have systematically analyzed the genetic interactions, as well as protein interactions among and between the 6 ARFs that are co-expressed in the

Arabidopsis suspensor. We combine this data with systematic mass-spectrometric identification of the transcription complexes in which these 6 ARFs act. Our data show that ARFs show both shared and divergent protein interactions, and these correlate with redundant and unique biological functions.

Results

Systematic genetic analysis reveals redundant and unique functions of suspensor ARFs

Of the 23-member *ARF* family in Arabidopsis (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002; Remington et al., 2004; Okushima et al., 2005), 7 genes are expressed at levels that can be detected by transcriptional reporters (promoter-SV40-3xGFP) in the globular stage embryo (Rademacher et al., 2011b). While MP/ARF5 is specific to the pro-embryo, ARF1,2,6,9,13 and 18 show divergent domains of expression that all overlap in the suspensor (Rademacher et al., 2011b). We previously demonstrated that ARF activity in the suspensor is important for the maintenance of extra-embryonic cell identity, and thus for preventing cells from adopting embryo identity. Stabilization of the suspensor-specific IAA10 protein, or misexpression of BDL/IAA12 induces suspensor proliferation and twin embryos (Rademacher et al., 2012). An unanswered question is whether these six ARFs contribute equally to the maintenance of suspensor identity. Furthermore, it has not been addressed whether there is a single ARF function in the suspensor to which these ARFs equally or differentially contribute, or if there are more independent subfunctions.

To address this problem, we have generated most of the possible double mutant combinations among the 6 suspensor-expressed ARFs included all double mutant combinations involving ARF9 (Fig. 1A). We particularly focused on ARF9 because several independent alleles showed stochastic suspensor defects, and an RNAi fragment that targeted *ARF9* and related ARFs showed *iaa10*-like defects (Rademacher et al., 2012). While combined loss of function in double mutant combinations was evident from the recapitulation or enhancement of known post-embryonic defects (*arf1 arf2*; Ellis et al., 2005), no double mutant except *arf6 arf9* showed embryo defects. These double mutants showed defects in suspensor division (Fig. 1C, F) in about 5% of the embryos. Even though this percentage is low, it can be considered significant as no such defects were observed in wild-type controls grown under identical conditions (N=275). These results suggest that, among the suspensor-expressed ARFs, ARF6 and ARF9 play a more prominent role than all others. However, as double mutants show low phenotype penetrance and defects are less severe than those of *aux/iaa* mutants, further redundancy with other suspensor-expressed ARFs is likely. To explore this possibility, we generated *arf6 arf9 arf13* and *arf6 arf9 arf18* triple mutants (Fig. 1D, G). In both cases, triple mutants showed only a small increase in the frequency of phenotypes (5- 9%), and qualitatively the phenotypes did not increase in severity (Fig. 1D, G). Hence, we conclude that there must be further redundancy with other ARFs expressed in the suspensor. Attempts to generate higher-order mutants failed due to highly pleiotropic and sterility defects (no shown), which precludes further analysis of redundancies.

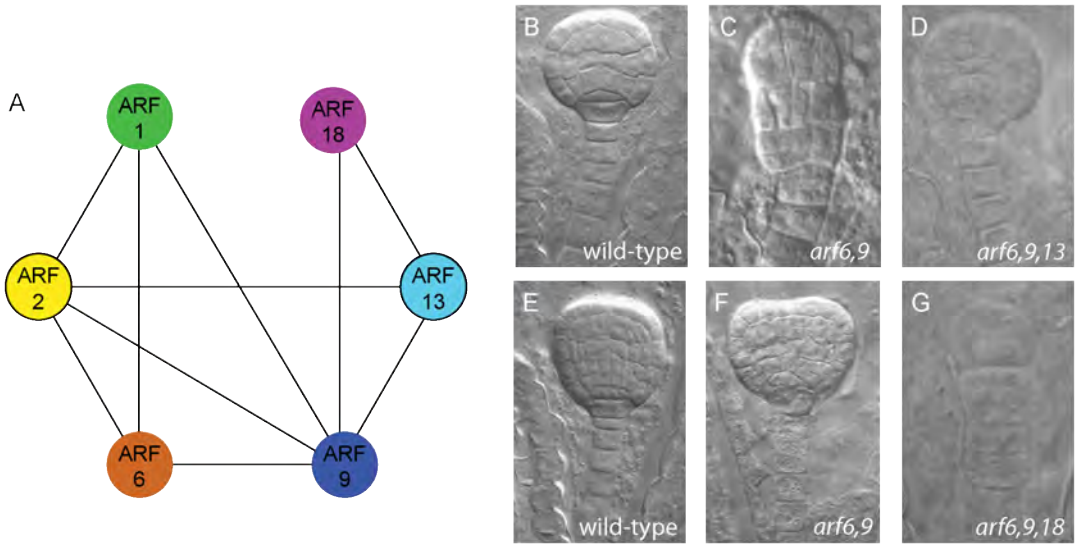

Figure 1. Genetic interactions among ARFs expressed in the suspensor.
(A) Scheme depicting the double mutants generated among ARFs expressed in the suspensor. Each line represents a double mutant. (B-G) Embryos at globular stage (B-D,G) and transition stage (E-F) of wild-type (B,E), *arf6,9* double mutant (C,F), *arf6,9,13* (D) and *arf6,9,18* (G) triple mutants.

Unique *in vivo* ARF-ARF interaction profiles

Redundancy among ARFs is complicated by the fact that these proteins have the capacity to form homo- and heterodimers (Ulmasov et al., 1999b). While the potential of homotypic (ARF-ARF) interaction is given from the presence of a dimerization domain (III/IV) at the C-terminus of most ARFs, most interactions have so far only been shown in yeast (Vernoux et al., 2011), and *in vivo* occurrence and biological significance are unclear.

To determine ARF-ARF interaction in an *in vivo* system, we adopted a protoplast-based FRET-FLIM assay. In this assay, dynamic interactions between CFP and YFP-tagged proteins can be visualized and quantified. We have previously used this assay to demonstrate ARF-Aux/IAA and bHLH-bHLH interactions (Rademacher et al., 2012; De Rybel et al., 2013). cDNAs of ARF1,2,6,9,13 and 18 were fused to CFP and YFP and expressed in leaf mesophyll protoplasts. As expected, CFP- and YFP-tagged ARF1,6,9 and 18 proteins localized to the nucleus of protoplasts (Fig. 2A-D). Interestingly, ARF18 additionally also accumulated in the cytosol, suggesting additional modes of regulation for this protein. Unfortunately, despite repeated attempts, we could not obtain detectable expression for CFP/YFP-tagged ARF2 or ARF13, hence these two proteins were omitted from the interaction assays.

Prior to measuring interactions, we used the ARF6-CFP / ETT/ARF3-YFP pair as a negative control. ETT/ARF3 is an atypical ARF since it has a diverged DNA-binding domain and lacks the C-terminal domains III/IV, and is therefore expected not to interact with other ARFs or Aux/IAAs. When co-expressed, the combination ARF6-CFP / ARF3-YFP resulted in a FRET efficiency of 3.0% and a Student's t-test p-value of 0.0001 (based on at least 20 cells). Hence, these values are considered to represent non-interaction, and based on this, a threshold of 5% FRET efficiency and p-value of <0.0001 were defined for interactions. Among all other combinations tested, we found a limited number of interactions that are summarized in Table 1 and depicted in Fig. 2E, F. Strikingly, the interaction profiles of the redundant ARF6 and ARF9 proteins showed both overlap and differences. While ARF6 and ARF9 both interacted

with ARF1, only ARF9 was able to interact with ARF18. Consistent with their independent interaction profiles and redundancy, ARF6 and ARF9 did not heterodimerize (Table 1, Fig. 2E). Finally, we found that while ARF1 and 6 can homodimerize, ARF9 cannot (Table1, Fig. 2E). These results indicate that ARFs have specific interactions with other ARFs, and also show that while homodimerization occurs *in vivo*, it is not a general property of all proteins. Hence, ARF-ARF interactions support both conservation and diversification of ARF function.

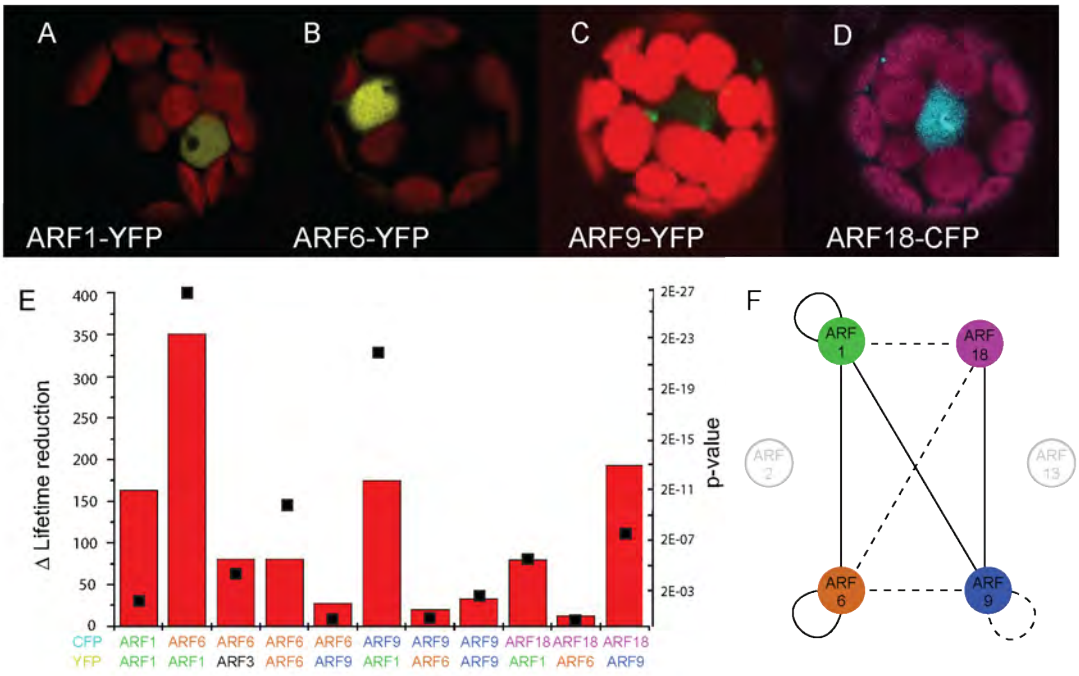

Figure 2. Specific pattern of interaction between ARF proteins expressed in the suspensor.
(A-D) Subcellular localization of ARF1-YFP (A), ARF6-YFP (B), ARF9-YFP (C) and ARF18-CFP (D) in mesophyl protoplasts. Chloroplast fluorescence is shown in red. (E) CFP lifetime reduction (grey bars, picoseconds, left Y-axis) and p-values (black dots, right Y-axis) for FRET interactions. ARF6CFP-ARF3YFP was used as negative control . (F) Scheme representing the network of interactions between ARFs as determined by FRET-FLIM. Continuous lines indicate interaction, dashed lines represent no interaction.

Table1. FRET-FLIM measurements. Average lifetime and FRET efficiency calculated for the different ARF proteins combinations. The pair ARF6CFP-ARF3YFP it was used as negative control.

	Average Lifetime (ps)	FRET Efficiency (%)	Δ LT reduction
ARF1CFP	2684.5		
ARF1CFP-ARF1YFP	2520.6	6.1	163.8394318
ARF6CFP	2690.82		
ARF6CFP-ARF1YFP	2339.02	13.07	351.800555
ARF6CFP-ARF3YFP	2609.21	3.03	81.60789322
ARF6CFP-ARF6YFP	2523.71	6.21	81.60789322
ARF6CFP-ARF9YFP	2662.99	1.03	27.8339072
ARF6CFP-IAA10YFP	2138.48	20.52	552.3436508
ARF9CFP	2704.8		
ARF9CFP-ARF1YFP	2529.04	6.49	175.7618765
ARF9CFP-ARF6YFP	2684.01	0.76	20.79784314
ARF9CFP-ARF9YFP	2671.32	1.23	33.47954604
ARF9CFP-IAA10YFP	2407.5	10.99	297.3005812
ARF18CFP	2685.89		
ARF18CFP-ARF1YFP	2605.84	2.98	80.04696581
ARF18CFP-ARF6YFP	2673.12	0.47	12.76716783
ARF18CFP-ARF9YFP	2492.14	7.21	193.7480769
ARF18CFP-IAA10	2607.9	2.9	77.99046823
IAA10CFP	2756.72		
IAA10CFP-ARF1YFP	2724.3	1.17	32.41857143

Differential ARF-IAA10 interactions suggest complex auxin-dependence

ARFs do not only have the possibility to homo- or heterodimerize with ARFs. The best-studied interaction partners of these molecules are the Aux/IAA proteins, transcription factors that bind ARFs and block their function (Kim et al., 1997; Tiwari et al., 2004). Previously, we identified the suspensor-specific IAA10 gene, and showed that gain-of-function phenotypes strongly resemble embryos in which suspensor ARF activity is reduced (Rademacher et al., 2012). An important question is which of the suspensor ARFs are targeted by IAA10. To address this question, we again adopted the protoplast-based FRET-FLIM assay to test interactions between ARF1,6,9 and 18 and IAA10.

Our results show that there is interaction between IAA10 and both ARF6 and ARF9 but not with ARF1 and ARF18 (see Fig. 3A, B, Table1). Consistent with their redundant action as revealed by genetic analysis, this result indicates that ARF6 and ARF9 proteins are good candidates to be controlled by Aux/IAA10. Interestingly, based on the inability of interacting with IAA10, ARF1 and 18 are not expected to be interchangeable (redundant) with ARF6 or ARF9, which is supported by the lack of phenotypic enhancement in *arf6,9,18* mutants compared with *arf6,9*.

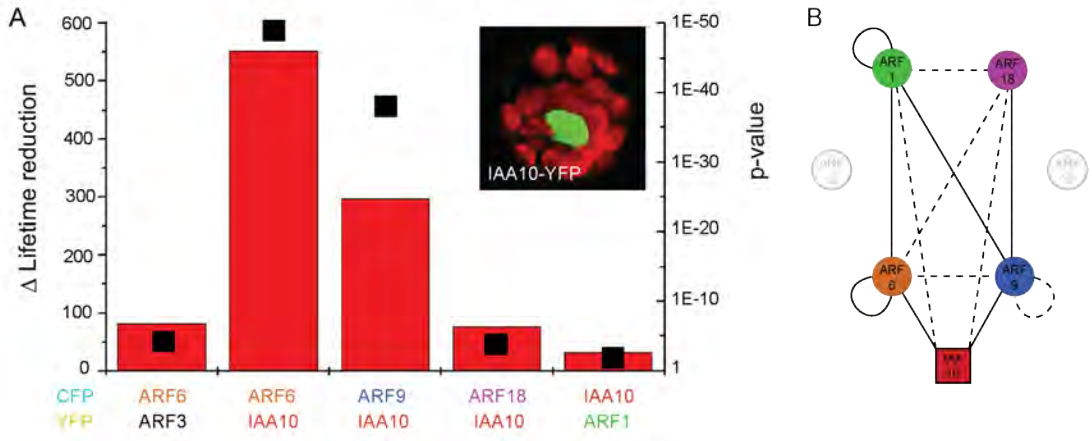

Figure 3: Specific IAA10-ARF interactions
(A) CFP lifetime reduction (grey bars, picoseconds, left Y-axis) and p-values (black dots, right Y-axis) for FRET interactions in protoplasts co-expressing CFP-tagged ARF6,9 and 18 with YFP-tagged iaa10, or YFP-tagged ARF1 and CFP-tagged iaa10. ARF6CFP-ARF3YFP was used as negative control. Inset shows expression of IAA10-YFP in protoplast (inset). (B) Scheme representing the network of interaction between ARFs and IAA10. Continuous lines indicate interaction, dashed lines represent no interaction.

Proteomic analysis reveals unique ARF transcription complexes

Genetic analysis, as well as ARF-ARF and ARF-Aux/IAA interaction profiles suggest that ARFs have partly redundant (e.g. ARF6 and ARF9), and distinct activities. To determine the molecular basis for functional diversification and conservation, we took a proteomics approach by identifying the protein complexes in which the different ARFs act.

First, we generated GFP-tagged versions of ARF1,2,6,9,13 and 18 to the C-terminus of each protein in a genomic fragment including the promoter (2 kb) and the coding sequence including all introns. As several splice versions of the ARF13 transcript exist, we generated several GFP fusions to each of the predicted C-termini, but were unable to detect expression in any of the lines. Therefore, ARF13 was omitted from the proteomics approach. Instead we included MP/ARF5, of which we previously showed that its activity is partly redundant with ARF6 and antagonistic to ARF1 (Rademacher et al., 2011). All these translational protein fusions encoded proteins that localized in the domain as predicted by their transcriptional fusions (Rademacher et al., 2011; Fig. 4). Importantly, all proteins localized to the nucleus, which is a prerequisite for acting as a transcription factor. Furthermore, functionality of the C-terminal ARF-GFP fusion proteins was tested in the case of MP/ARF5 by introducing the fusion protein into a strong *mp/arf5* allele (*mp*-B4149). The fusion protein completely restored normal development in this background. Unfortunately, no strong phenotypes are found in any of the other mutants, which preclude testing the functionality of the other ARF-GFP fusions. However, as all these proteins share the same overall domain architecture and all fusions were generated in the same way, we expect all these ARF-GFP proteins to be functional.

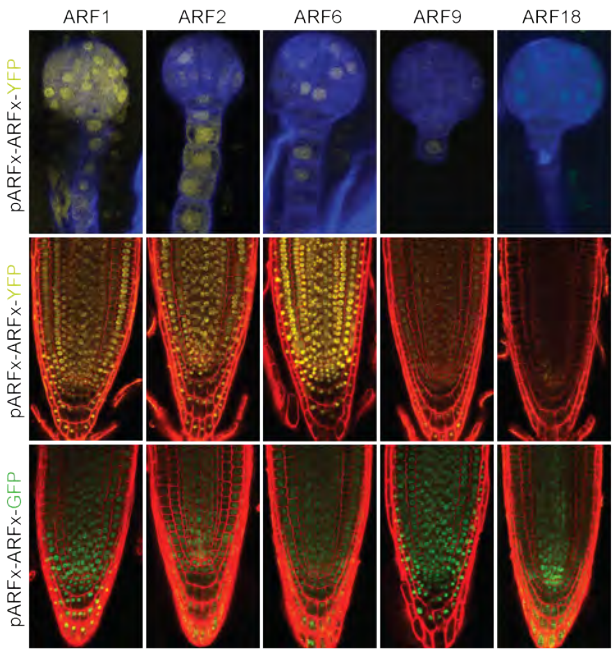

Figure 4: ARF protein accumulation patterns
Fluorescence of YFP or GFP in globular stage embryos and root tips of lines expressing C-terminal fusions of ARF1,2,6,9 and 18 to YFP or GFP from a 2 kb promoter fragment. While the intensity of signals varies between the ARFs, all show nuclear accumulation of the fusion protein.

To identify proteins that are part of complexes with ARFs, we extracted proteins and immunoprecipitated (IP) ARF-GFP complexes using an antibody directed against GFP. Following in-liquid Trypsin digestion, peptides were analyzed by nano-Liquid Chromatography – tandem Mass Spectrometry (nLC-MS/MS). To rank candidate proteins according to abundance in the immunocomplex and reproducibility of the identification, we used a label-free quantification method, that relies on comparing the peptide spectral counts in triplicate immunoprecipitations from each transgenic line and a non-transgenic control (De Rybel et al., 2013). With this analysis, each protein that is identified by at least 2 unique peptides is given a fold change (FC; ratio of abundance in transgenic line versus non-transgenic control) and a p-value (Student's T-test for difference of variance in two averages). We have previously used this method to identify nuclear bHLH protein complexes (De Rybel et al., 2013).
We performed three independent biological replicates for each transgenic line (ARF1, ARF2, MP/ARF5, ARF6, ARF9 and ARF18). For all but MP/ARF5, siliques were used for immunoprecipitation, while root tip tissue was used for MP/ARF5. The criteria to consider proteins as possible interactors of an ARF was a fold change of at least 10 times and a p-value smaller than 0.05. An overview of the proteins selected as candidates to form complexes with the ARFs can be found in table 2. Using this approach, specific sets of complexes were identified for each ARF. No two ARFs showed the exact same list of potential interactors, which shows that our method robustly and specifically identifies protein-specific complexes. As predicted by previous yeast-2-hybrid studies, and by our FRET-FLIM data, this mass spectrometry confirmed the *in vivo* occurrence of an ARF-ARF interaction. ARF1 was reliably detected in the ARF2-GFP immunocomplex (Table 2; Fig. 5). This result also shows that the interactions observed in this approach are potentially biologically meaningful.

Table 2. Putative interactors of ARF proteins identify with the IP-MS/MS experiments.
AGI= Arabidopsis Genome Initiative gene identification number.

RATIO	p-value	AGI	Gene name
82220.7309	2.87439E-07	AT1G59750	AUXIN RESPONSE FACTOR 1 (ARF1)
3697.54385	1.23441E-08	AT3G44740	Class II aaRS and biotin synthetases superfamily protein
945.241252	3.11958E-06	AT5G50340	ATP-dependent peptidases
273.047791	0.009562265	AT5G62010	AUXIN RESPONSE FACTOR 2 (ARF2)
125.551335	0.010081879	AT3G07050	NUCLEOSTEMIN-LIKE 1 (NSN1)
92.0407184	0.030818041	AT2G14820	NAKED PINS IN YUC MUTANTS 2 (NPY2)
83.0340714	5.1475E-05	AT1G79840	GLABRA 2 (GL2)
57.0075443	0.03731803	AT1G61520	LHCA3, PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 3
48.8261334	0.016869098	AT5G51280	DEAD-box protein abstrakt
46.0678206	0.009253462	AT3G17390	METHIONINE OVER-ACCUMULATOR 3 (MTO3)
25.5428881	0.048133524	AT3G49601	molecular_function unknown
23.5675444	0.019470977	AT5G56710	Ribosomal protein L31e family protein
19.610214	0.000228012	AT4G04950	MONOTHIOL GLUTAREDOXIN 17 (GRXS17)
14.0258116	0.021286117	AT1G05230	HOMEODOMAIN GLABROUS 2 (HDG2)
10.9381028	0.049266274	AT3G63400	Cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein
10.9051506	0.022930001	AT1G13440	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2 (GAPC2)
10.6516201	0.016978803	AT4G34200	EMBRYO SAC DEVELOPMENT ARREST 9 (EDA9)
10.1510196	0.021663799	AT4G19190	zinc knuckle (CCHC-type) family protein

>100 fold change
>50 fold change
>20 fold change
>10 fold change

p-value < 0.05
p-value > 0.05

RATIO	p-value	AGI	Gene names
3419.41385	1.97395E-07	AT1G59750	AUXIN RESPONSE FACTOR 1 (ARF1)
3411.91602	0.003059658	AT5G62010	AUXIN RESPONSE FACTOR 2 (ARF2)
102.285326	4.02707E-08	AT2G29550	TUBULIN BETA-7 CHAIN (TUB7)
71.9626255	1.81295E-06	AT1G63057	unknown protein
66.7627678	0.007275378	AT3G02310	SEPALLATA 2 (SEP2), AGAMOUS LIKE 2 (AGL2)
59.9202109	1.87289E-06	AT1G79840	GLABRA 2 (GL2)
47.8781102	0.000165	AT2G42830	AGAMOUS-LIKE 5 (AGL5), SHATTERPROOF 2 (SHP2)
39.3983167	0.009882843	AT1G05230	HOMEODOMAIN GLABROUS 2 (HDG2)
28.1284413	0.022922529	AT3G17390	METHIONINE OVER-ACCUMULATOR 3 (MTO3)

RATIO	p-value	AGI	Gene names
254.64393	0.12374253	AT2G34440	AGL29
227.01088	1.0278E-07	AT5G49400	zinc knuckle (CCHC-type) family protein
92.756282	0.03445879	AT4G01120	GBF2, bZIP (basic leucine zipper) transcription factor
67.37157	7.5889E-06	AT1G19850	AUXIN REPONSE FACTOR 5 (ARF5)
35.800575	0.03064855	AT5G15230	GASA4, Gibberellin-regulated protein 4
34.003216	0.00332254	AT1G48620	HON5
25.988826	0.44990208	AT4G08150	KNAT1, Homeobox protein knotted-1-like 1
21.446512	0.1514499	AT2G36930	C2H2-type zinc finger-containing protein
20.077979	0.00392455	AT5G62690	TUBB2

RATIO	p-value	AGI	Gene name
1362399.1	4.1179E-08	AT1G30330	AUXIN RESPONSE FACTOR 6 (ARF6)
10717.866	0.01289824	P42212	GFP
2357.3279	0.11650614	AT1G48625	F-box/kelch-repeat protein At1g48625
227.35334	0.08376514	AT4G28250	Expansin-B3;ATEXPB3
197.32116	0.08375186	AT1G09200	Histone H3.2
194.8371	0.1402292	AT4G26600	Putative uncharacterized protein AT4g26600
181.83103	0.11858203	AT3G28830	unknown protein
154.13881	0.12353743	AT2G48110	REF4

RATIO	p-value	AGI	Gene name
183487.08	5.6847E-05	AT4G23980	AUXIN RESPONSE FACTOR 9 (ARF9)
1763.7902	0.01559205	P42212	GFP
407.06678	3.9399E-09		GTP binding / GTPase
365.90162	0.04102688	AT3G46520	Actin-12;Actin-4;Actin-1/3
329.02552	0.02566423	AT1G47278	Putative uncharacterized protein (Fragment);unknown protein
268.15074	0.07274179	AT3G09940	MDHAR (MONODEHYDROASCORBATE REDUCTASE)
80.132772	0.1198182	AT5G21160	La domain-containing protein / proline-rich family protein
59.41477	0.14723457	AT4G39680	SAP domain-containing protein
20.642617	0.30955412	AT1G24020	MLP-like protein 423
14.886776	0.38422927	AT2G45950	Isoform 1 of SKP1-like protein

RATIO	p-value	AGI	Gene name
82350.328	0.00023753	Q9C5W9	AUXIN RESPONSE FACTOR 18 (ARF18)
1217.3289	0.02519121	P42212	GFP
435.90348	0.03921219	AT3G46520	Actin-12;Actin-4;Actin-1/3
308.44205	0.02979939	AT1G47278	Putative uncharacterized protein (Fragment);unknown protein
197.90708	0.09968121	AT3G09940	MDHAR (MONODEHYDROASCORBATE REDUCTASE)
105.98277	0.20395128	AT5G54740	lipid transfer protein (LTP) family protein
41.225799	0.23426548	AT1G24020	MLP-like protein 423
11.891008	0.41112462	AT2G45950	Isoform 1 of SKP1-like protein

Apart from this ARF-ARF interaction, the potential complex components could be classified in three categories. Firstly, several subunits of the MEDIATOR complex were recovered in the ARF6-GFP, ARF9-GFP and ARF18-GFP complexes (Table 3). MEDIATOR is a highly conserved complex found in almost all eukaryotes with an important function in the regulation of the transcription initiation (Boube et al., 2002; Bourbon, 2008; Malik and Roeder, 2010; Conaway and Conaway, 2011). The recovery of these subunits strongly suggests that the complexes isolated are active, chromatin-located transcription complexes rather than free nucleoplasmic proteins. The second category of interactors are other, non-ARF, transcription factors. In the case of both ARF1-GFP and ARF2-GFP complexes, the HD-Zip IV transcription factors GL2 (Di Cristina et al., 1996; Masucci et al., 1996) and HDG2 (Marks et al., 2009) were identified (Table2; Fig. 5). Again, the recovery of these transcription factors suggests the presence of ARF1-GFP and ARF2-GFP in active transcription complexes. Furthermore, the SEP2 (Pelaz et al., 2000, 2001) and SHP2 (Liljegren et al., 2000; Pinyopich et al., 2003) MADS-box transcription factors were recovered in the ARF2-GFP complex, while another MADS-box factor (AGL29) was found in the MP-GFP complex. Finally, a bZIP (AGL29) and a homeobox (KNAT1) transcription factor were found in the MP-GFP IP, which demonstrates that several ARFs engage in complexes with other transcription factors (Table2; Fig. 5).
A third category encompasses all other proteins. In this class, we found a variety of proteins including the BTB-domain protein NPY2 (Cheng et al., 2008), an F-box protein and the Histone3.2 isoform (Table2; Fig. 5).

Table 3. Mediator subunits found in IP-MS/MS experiments. AGI= Arabidopsis Genome Initiative gene identification number.

MEDIATOR	Ratio	p-value	AGI	Gene name
ARF6	154.138809	0.12353743	AT2G48110	REF4
	17.5764299	0.44042816	AT3G09180	MED27
	12.8389941	0.51006044	At1g55080	MED9
	9.96146011	0.51115255	At3g21350	MED6
	7.2475797	0.37390097	At1g16430	MED22
	6.08245634	0.37390097	At5g19910	MED31
ARF9	3.08932074	0.59149606	At5g19910	MED31
	1.75001794	0.16627009	At4g04780	MED21
	1.19056332	0.82099531	At3g21350	MED6
ARF18	118.401444	0.30084932	At3g21350	MED6

When comparing the proteins recovered in the different ARF-GFP complexes, it is notable that while some proteins are unique to a single ARF, others are shared between 2 or more complexes (Fig. 5). Strikingly, there is good accordance between ARF-ARF interactions as observed in FRET-FLIM (Fig. 2F) or by mass spectrometry (Fig. 5; Table 2) and the recovery of the same complex components. ARF1 and ARF2 have several common interactors (GL2, MTO3 and HDG2). Likewise, the interacting ARF9 and ARF18 proteins have at least four interactors in common (Actin-12, MDHAR, MLP-like protein and SKP1-like protein). In contrast, ARF6 that was shown not to interact with ARF9 and ARF18 in FRET FLIM experiments and not with ARF1 and ARF2 in the pull down experiments, has independent proteins that cannot be found in the IPs of the other ARFs (F-box/Kelch repeat, ATEXPB3, Histone H3.2).

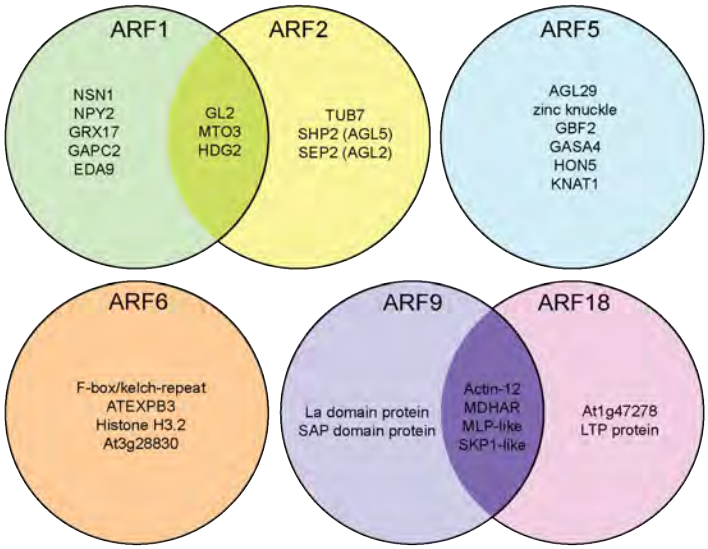

Figure 5: Unique and specific ARF protein interactions
Venn diagrams depicting the proteins identified in ARF-GFP protein complexes (threshold: fold change >10; p-value <0.05), and showing which proteins were found in more than one complex.

Proteomically defined ARF interactors mediate auxin response

Next, to determine if the newly identified ARF complex components are required for auxin response, we tested sensitivity to auxin in roots of a range of mutants (*gl2*, *hdg2*, *shp1 shp2*, *mel3/npy2*, *mel3/npy2 mel4*; Liljegren et al., 2000; Pinyopich et al., 2003; Cheng et al., 2008). For some genes, we used single mutants (*hdg2*, *gl2*, *mel3/npy2*), while for others (*shp1 shp2*, *mel3/npy2 mel4*) we used double mutants since redundancy with close relatives had been demonstrated (Liljegren et al., 2000; Pinyopich et al. 2003; Cheng et al., 2008). Homozygous mutants were grown on media containing 40 nM 2,4-D. This concentration inhibits wild-type root growth to approximately 40% of the growth rate observed without 2,4-D (Fig. 6), and changes in auxin sensitivity can be readily quantified using the degree of growth inhibition (Lincoln et al., 1990). Using this assay, we found that neither *hdg2* nor *mel3/npy2* mutants affected auxin sensitivity (Fig. 6). In contrast, both *gl2* and *shp1 shp2* double mutants were hypersensitive to auxin (Fig. 6). While growth in unsupplemented media was normal (Fig. 6), 40 nM 2,4-D completely shut down growth (Fig. 6).
These findings demonstrate that the ARF complex components GL2 and SHP2 are required for normal auxin sensitivity, and by extension, for ARF activity. ARF1 and 2 are considered to be repressors in certain contexts (Ulmasov et al., 1999a), and thus to act as negative regulators of auxin response. The auxin hypersensitivity of mutants in ARF1/2 complex components is in good agreement with such a negative function.
While growth of wild-type and hypersensitive mutants on media containing 40 nM 2,4-D ceased after 4 days (Fig. 6), we found that *mel3/npy2 mel4* double mutants continued to grow (Fig. 6). While there does not seem to be an effect of these mutations on growth rate in the presence of auxin (Fig. 6), the mutations do render roots less sensitive to the long-term inhibitory effect of auxin. Therefore, also the closely related MEL3/NPY2 and MEL4 proteins are required for normal auxin response. Taken together, several of the newly identified ARF complex components are required for normal responses to auxin, which further supports the validity of the mass spectrometric identification of active ARF complexes.

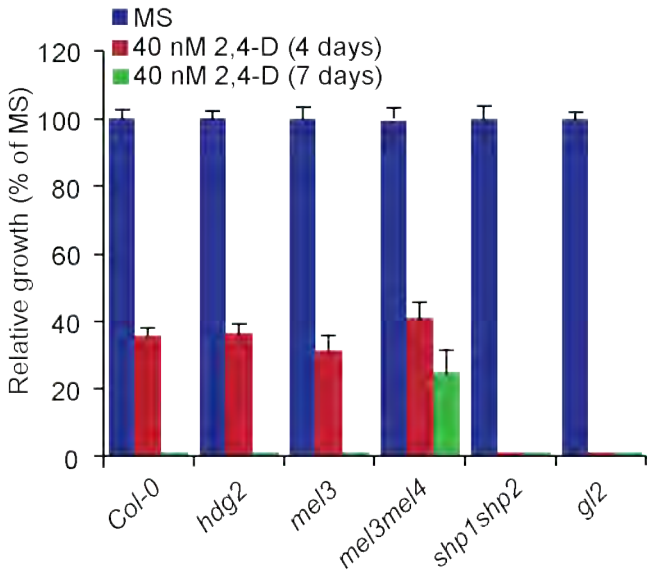

Figure 6: Novel ARF complex components mediate auxin responsive root growth
Relative root growth of wild type (Col-0) and homozygous mutant (*hdg2*, *mel3*, *mel3 mel4*, *shp1 shp2*, *gl2*) seedlings 4 days (red bars) and 7 days (green bars) after transfer onto media containing 40 nM 2,4-D. Growth was normalized to the length of roots grown on unsupplemented media (blue bars, represent 100% of growth).

Discussion

In this study, we address the problem of specificity in the auxin response pathway. Auxin triggers gene expression responses through a very brief pathway, in which interactions between components can contribute to the wide array of growth and developmental responses that that are subject to auxin regulation (Reviewed in Lokerse and Weijers, 2009). We consider the DNA-binding ARF transcription factors as a likely source of signaling specificity, and study the biochemical mechanisms underlying such specificity using a set of ARF proteins co-expressed in a single cell type of the Arabidopsis embryo (Rademacher et al., 2011). The 6 ARFs expressed in the suspensor are phylogenetically diverse, which allows the study of generic mechanisms of specificity representative for most, if not all, ARFs. We have used an approach in which we combined genetic analysis, *in vivo* protein-protein interaction assays and mass-spectrometry based proteomics to define properties and functions of individual ARF proteins. Our combined results provide an inventory of protein properties in the ARF family and give a plausible biochemical basis for both shared and divergent protein functions.

A systematic analysis of most possible double mutant combinations among the 6 suspensor-expressed ARFs revealed that contributions of these ARFs to normal suspensor development are not equal. Only *arf6,9* double mutants showed significant defects, while no other combination with *arf9* or *arf6* did. This suggests that ARF6 and 9 are the main contributors to auxin response in suspensor cells. It is unclear at present how this redundancy relates to the previously reported *arf1,2,6* redundancy (Rademacher et al., 2011). In both cases, the redundant ARFs are phylogenetically unrelated, and it is possible that ARF1/2 and 9 have similar functions that are uncovered in the mutant combination with *arf6*. Rigorous testing of further redundancies within this set of ARFs would require the generation of higher-order mutants. Unfortunately, in attempting to do so, we encountered strong fertility defects in all mutants that include *arf1,2* combinations (unpublished).

One complication when considering redundancy and divergent ARF functions, is that ARFs can in principle dimerize. Although ARF-ARF interactions have been shown *in vitro* (Ulmasov 1999b), in yeast (Vernoux et al., 2011), and in split-YFP assays (Vernoux et al., 2011), this property has not been addressed *in vivo* in a quantitiative manner. We used a dynamic FRET-based interaction assay (Kremers et al., 2006) to determine interactions among suspensor-expressed ARFs in a protoplast system. There are striking differences between the ARFs tested in their propensity to homodimerize, or to form heterodimers with other ARFs. Although the relevance of such interactions for biological function remain to be demonstrated, our data show that the potential complexity of the interaction network is limited by the intrinsic specificities of ARF-ARF interactions. We further tested interactions of suspensor-expressed ARFs with IAA10, and found that again, there was intrinsic specificity of interactions. IAA10 interacts with ARF6 and 9, but not with ARF1 and ARF18. Interestingly, the *arf6,9* double mutant show phenotypes similar to the *iaa10* gain-of-function mutant, which supports a redundant ARF6/9 function that correlates with both proteins being targeted by IAA10. Thus, both specific ARF-ARF and ARF-Aux/IAA interactions limit the complexity of the auxin network. Recently, a systematic yeast-based ARF-Aux/IAA interactome was reported (Vernoux et al., 2011). ARFs were categorized as ARFa (Q-rich middle region; activators) and ARFr (non Q-rich middle region; repressors), and all possible interactions (ARFa-ARFa; ARFa-Aux/IAA; ARFa-ARFr; ARFr-ARFr; ARFr-Aux/IAA; Aux/IAA-Aux/IAA) were tested. It appeared that some types of interactions were more prevalent than others and, in this study, the general topology of the interaction network was simplified to contain only ARFa-Aux/IAA and Aux/IAA-Aux/IAA. Modeling this network suggested that ARFr proteins are required for generating robustness to fluctuating auxin concentrations. While some of the *in vivo* interactions we identified are consistent with this simplified topology, others are not. For example, ARF9 has a non Q-rich middle region, yet is redundant with ARF6 (an ARFa), and interacts with IAA10. Likewise, we find several ARF-ARF interactions by FRET-FLIM (and MS; ARF1-ARF2) that are not part of this simplified scheme. It will be interesting to see how the inclusion of genetically supported “atypical” interactions will complicate or sophisticate models for auxin response.

An aspect in auxin biology that is under-explored is the mechanism by which ARFs regulate gene expression. While some interactors have been identified previously (BIN2-ARF2/9; MYB77-ARF7; BPE-ARF8; BRX-MP; Vert et al., 2008; Shin et al 2007; Varaud et al, 2011; Scacchi et al.,2010), no systematic comparison of *in vivo* ARF complexes had been performed. We used C-terminally GFP-tagged ARFs to identify such protein complexes by mass spectrometry. Interestingly, for each of the ARFs tested, we found evidence that the complex identified represents an active transcription complex (see below for discussion). Furthermore, even though some ARFs are highly redundant (e.g. ARF1 and 2), no two ARFs had the same interaction profile. In general, functional diversification of the ARFs is therefore well reflected by biochemical interactions with non-ARF proteins. Since MS-based protein complex identification is not saturating, and low-abundance interactions will easily be missed, it can not be excluded that there is more overlap between ARF interaction profiles than seen here. Nonetheless, both the limited overlap and the differences are very informative, and provide a starting point for future, more mechanistic studies on specificity in auxin response. For example, while double mutant analysis did not show a genetic interaction between ARF9 and 18, there is substantial overlap between complex components. It is well possible that ARF9 and 18 are indeed redundant, but

that a third ARF masks these activities in the *arf9,18* double mutant. One such candidate is ARF13, for which no interaction profiles could be collected.
Beyond providing testable predictions about the ARF interaction network, our study also identifies new components in auxin responsive gene regulation. Strikingly, we find two subfamilies of well-characterized transcription factors to associate with *ARF1* and/or *ARF2*. *GL2* and *HDG2* belong to the HD-Zip class IV family, and are known to control root hair (*GL2*) and trichome (*HDG2*) development (Masucci et al., 1996; Marks et al., 2009). A function in auxin response was unsuspected, but surpisingly *gl2* mutants showed hypersensitivity to auxin in root growth. Similarly, the flower and fruit developmental regulators *SEP2* and *SHP2* of the MADS transcription factor family (Pelaz et al., 2000; Pinyopich et al., 2003) were found in complex with ARF2. While roles in auxin response were not previously reported, *shp1 shp2* mutants showed clear hypersensitivity. The mechanistic basis for the requirement of the *GL2* and *SHP* transcription factors in *ARF1/2* activity remain to be investigated through the analysis of (common) target genes, and the effect of *gl2* and *shp1 shp2* mutations on auxin-responsive gene expression. However, this result shows that new, unexpected mediators of auxin response can be identified through proteomics approaches.
Finally, while this study does not give definite general answers about the mechanisms of ARF action, it does add several layers of information that help define ARF protein properties. An important question in this context is whether the classical separation of ARFs into Q-rich activator and non Q-rich repressor categories is supported by interaction profiles. In our analysis we failed to find unifying principles at genetic- or protein-interaction levels that correlate with the phylogenetic separation or Q-richness of ARFs. Even though there is no doubt that ARFs have different activities on well-defined minimal promoters in transcription assays (Tiwari 2003), based on our findings we propose a more subtle classification of ARFs that is informed by biological activity, and protein interactions. We anticipate that detailed investigation of the mechanisms of ARF activity based on protein interactions will help to generate a more general understanding of conserved and divergent ARF activity as a basis for specificity on auxin response.

Material and Methods

Plant material, growth, transformation and selection of lines
References and order numbers of all T-DNA lines used can be found in table 4
Seeds were sterilized by incubation for 10 min with EtOH and bleach (70%-30%) followed by 2 steps of rinsing with 70% EtOH and a final washing step with 100% EtOH. Seeds were plated on 0.5x Murashige & Skoog medium containing 1% (w/v) sucrose, 0.8% (w/v) agar and supplemented if necessary for selection of transgenes by 25 mg/L Kanamycin, 15 mg/L Phosphinothricin or 50 mg/L Hygromycin.
Wild-type plants are all Columbia (Col-0) ecotype (for all the controls and transformation of transgenes).
Seedlings were grown on plates for 1-2 weeks on continuous light at 22 degree and transfered to soil and grown under standard conditions at 23°C in a 16h light/8h dark cycle.
Plants were transformed by floral dip method as described by Clough and Bent, 1998; using the *Agrobacterium* strain GV3101 (pSoup).

Table 4. Insertion mutant lines used in this study and primers used for genotyping.

Gene	Locus	Allele	Stock #		Primer for genotyping
ARF1	AT1G59750	*arf1-5*	SALK_079046	LP	CAGACACTCCTTCCTCAGTGC
				RP	GCCCTCTGTATTCCCATTTTC
ARF2	AT5G62000	*arf2-8*	SALK_108995	RP	ATGAAGATTTTGCGAACCATG
				LP	TTACACAGATTTGCTCTCCGG
ARF6	AT1G30330	*arf6-1*	CS24606	RP	ATCATTCATCATCTGATCAAAATGCAGG
				LP	ACGCTTTGATGGCCAACC
ARF9	AT4G23980	*arf9-1*	SAIL_881_H05	RP	TGAGGAAAACAATTATTGTTGTGC
				LP	AAGTCGACTTGTTGCGTTGAC
ARF13	AT1G34170	*arf13-2*	SALK_138188	RP	ACGAGGTTTCCACTTTCCATC
				LP	GCAAGCAACAAAATTAGATCTTTC
ARF18	AT3G61830	*arf18-2*	GABI_513E07	RP	TCTTCCTCCAAAGATACTTTGTCG
				LP	ATCCCCATTCTCACCCCTG

Molecular Cloning

All the DNA amplifications were performed using Phusion Flash polymerase (Finnzymes), DNA purification was achieved by using the High Pure PCR Product Purification Kit (Roche). Plasmids were amplified by culturing transformed *Eschericha coli* XL1-Blue strains (Stratagene) in LB medium at 37 ºC. For extraction of DNA from cell cultures we used the GeneJet Plasmid Miniprep Kit (Fermentas)

For the translational fusions of the ARFs with GFP, the respective fragments were amplified from BAC clones or genomic DNA and subcloned into pBluescript SK (-). These constructs were confirmed by restriction analysis and sequenced to check for any mutation. The correct fragments were then cloned into binary pGreenII vectors (Hellens et al., 2000) carrying eGFP and the desired marker gene for plant selection and then used for plant transformation.

The cloning to generate FRET-FLIM and YFP constructs was performed using a set of Ligation-Independent Cloning vectors according to procedures described previously (De Rybel et al., 2011).

All primers used for gene and cDNA amplification are listed in table 5

Table 5. Primers used for cloning. Red letters indicates restriction sites used for conventional cloning.

Gene	Locus	DNA	Construct	Cloning	Primer	
ARF1	AT1G59750	Genomic	GFP	conventional	FW	*TCAA*GGGCCC**AGATTATTTCATCTTAGG**
					RV	*AAGT*ACGCGT**TCTTGATCCCGCCATAGATG**
ARF2	AT5G62000	Genomic	GFP	conventional	FW	*TCAA*GGGCCC**AGTTTACGTGTTGTTAA**
					RV	*AAGT*ACGCGT**AGAGTTCCCAGCGCTGGACA**
ARF6	AT1G30330	Genomic	GFP	conventional	FW	*TCAA*GGGCCC**TCACTAAATTCACCCAT**
					RV	*AAGT*ACGCGT**GTAGTTGAATGAACCCCCAA**
ARF9	AT4G23980	Genomic	GFP	conventional	FW	*TCAA*GGGCCC**AGATGGTGGTGGTGGGT**
					RV	*AAGT*ACGCGT**GTTGGAATGATTATCTGTTT**
ARF13	AT1G34170	Genomic	GFP	conventional	FW	*TCAA*GGGCCC**AGAGTCAAAAAAATGATTTT**
					RV	*AAGT*ACGCGT**GAGGTTGGGTTTGGTCAAAC**
ARF18	AT3G61830	Genomic	GFP	conventional	FW	*TCAA*GGGCCC**CGTGGCTGACGGAAAAAAA**
					RV	*AAGT*ACGCGT**CCCCCTACTACGATTTTCGA**
ARF1	AT1G59750	Genomic	YFP	LIC	FW	TAGTTGGAATGGGTTCGAA**AGATTATTTCATCTTAGGTGGACTG**
					RV	*TTATGGAGTTGGGTTCGAAC***TCTTGATCCCGCCATAGATG**
ARF2	AT5G62000	Genomic	YFP	LIC	FW	TAGTTGGAATGGGTTCGAA**AGTTTACGTGTTGTTAAAAA**
					RV	TTATGGAGTTGGGTTCGAAC**AGAGTTCCCAGCGCTGGACA**
ARF6	AT1G30330	Genomic	YFP	LIC	FW	TAGTTGGAATGGGTTCGAA**TCACTAAATTCACCCATAAT**
					RV	TTATGGAGTTGGGTTCGAAC**GTAGTTGAATGAACCCCCAA**
ARF9	AT4G23980	Genomic	YFP	LIC	FW	TAGTTGGAATGGGTTCGAA**AGATGGTGGTGGTGGGTTTT**
					RV	TTATGGAGTTGGGTTCGAAC**GTTGGAATGATTATCTGTTT**
ARF13	AT1G34170	Genomic	YFP	LIC	FW	TAGTTGGAATGGGTTCGAA**AGAGTCAAAAAAATGATTTTGTT**
					RV	TTATGGAGTTGGGTTCGAAC**GTTATCTGTGACGTTTGGTG**
ARF18	AT3G61830	Genomic	YFP	LIC	FW	TAGTTGGAATGGGTTCGAA**CGTCTCCGCTTTGCAAGGAT**
					RV	TTATGGAGTTGGGTTCGAAC**CCCCCTACTACGATTTTCGA**
ARF1	AT1G59750	cDNA	FRET-FLIM	LIC	FW	TAGTTGGAATAGGTTCATGGCAGCTTCCAATCATTC
					RV	TTATGGAGTTGGGTTCTCTTGATCCCGCCATAGATGATGAT
ARF2	AT5G62000	cDNA	FRET-FLIM	LIC	FW	TAGTTGGAATAGGTTCATGGCGAGTTCGGAGGTTTC
					RV	TTATGGAGTTGGGTTCAGAGTTCCCAGCGCTGGACAATGAAGG
ARF6	AT1G30330	cDNA	FRET-FLIM	LIC	FW	TAGTTGGAATAGGTTCATGAGATTATCTTCAGCTGG
					RV	TTATGGAGTTGGGTTCGTAGTTGAATGAACCCCCAACTGAT
ARF9	AT4G23980	cDNA	FRET-FLIM	LIC	FW	TAGTTGGAATAGGTTCATGGCAAATCGCGGAGGTGA
					RV	TTATGGAGTTGGGTTCGTTGGAATGATTATCTGTTTTGGA
ARF18	AT3G61830	cDNA	FRET-FLIM	LIC	FW	TAGTTGGAATAGGTTCATGGCGAGTGTTGAAGGTGA
					RV	TTATGGAGTTGGGTTCCCCCCTACTACGATTTTCGAATGATT

Microscopy

The DIC and fluorescence microscopy was performed according to procedures explained in chapter 2.

The imaging of fluorescent reporters in embryos, roots and protoplast was conducted using Zeiss LSM510 confocal laser scanning microscope or on a Leica SP5-II system (HyD detector). The settings used were: tdTomato excitation at 561 nm and detection at 568-600 nm; YFP excitation at 514 nm and detection at 525-550 nm; GFP excitation at 488 nm and detection at 500-535nm. Embryo phenotypes were analyzed using chloral hydrate cleared preparations (chloral hydrate, water and glycerol, 8:3:1) using a Leica DMR microscope equipped with differential interference contrast (DIC) optics. Protein accumulation was analyzed in embryos and roots of homozygous T3 lines. For counterstaining it was used water containing 1 µM FM4-64 (Invitrogen).

FRET-FLIM

Transfections were performed as described (Russinova et al., 2004) using Arabidopsis (Columbia wild-type) mesophyll protoplasts, obtained by sandwich tape method (Wu et al., 2009).

FRET-FLIM analysis in Arabidopsis leaf mesophyll protoplasts was performed as described previously (Rademacher et al., 2011)

IP-MS/MS and analysis of data

Immunoprecipitation experiments were performed as described previously (Zwiewka et al., 2011) for each experiment we used 3g of siliques and/or root tips of translational fusions of ARF1, -2, -5, -6, -9, -18 to GFP in a Col-0 background. The MP-GFP IP was performed in the mp-B4149 background (Weijers et al, 2005).

For each sample we used three biological replicates to be compared with three non-transgenic Col-0 samples. Anti-GFP coupled magnetic beads (Milteny Biotech) were added to the total protein extracts in order to isolate interacting proteins.
Nano-LC-MS/MS and statistical analysis using MaxQuant and Perseus software was performed as described previously (Hubner et al., 2010; Lu et al., 2011; De Rybel et al, 2013).

Auxin sensitivity assay
Root inhibition assay was performed as described in Lincoln et al., 1990.
4-day old seedlings were transferred onto fresh MS medium supplemented with 40nM of 2,4D for 4 days. The length of new primary root was measured using ImageJ software. The percentage of root growth inhibition was calculated relative to the growth on MS with no auxin.

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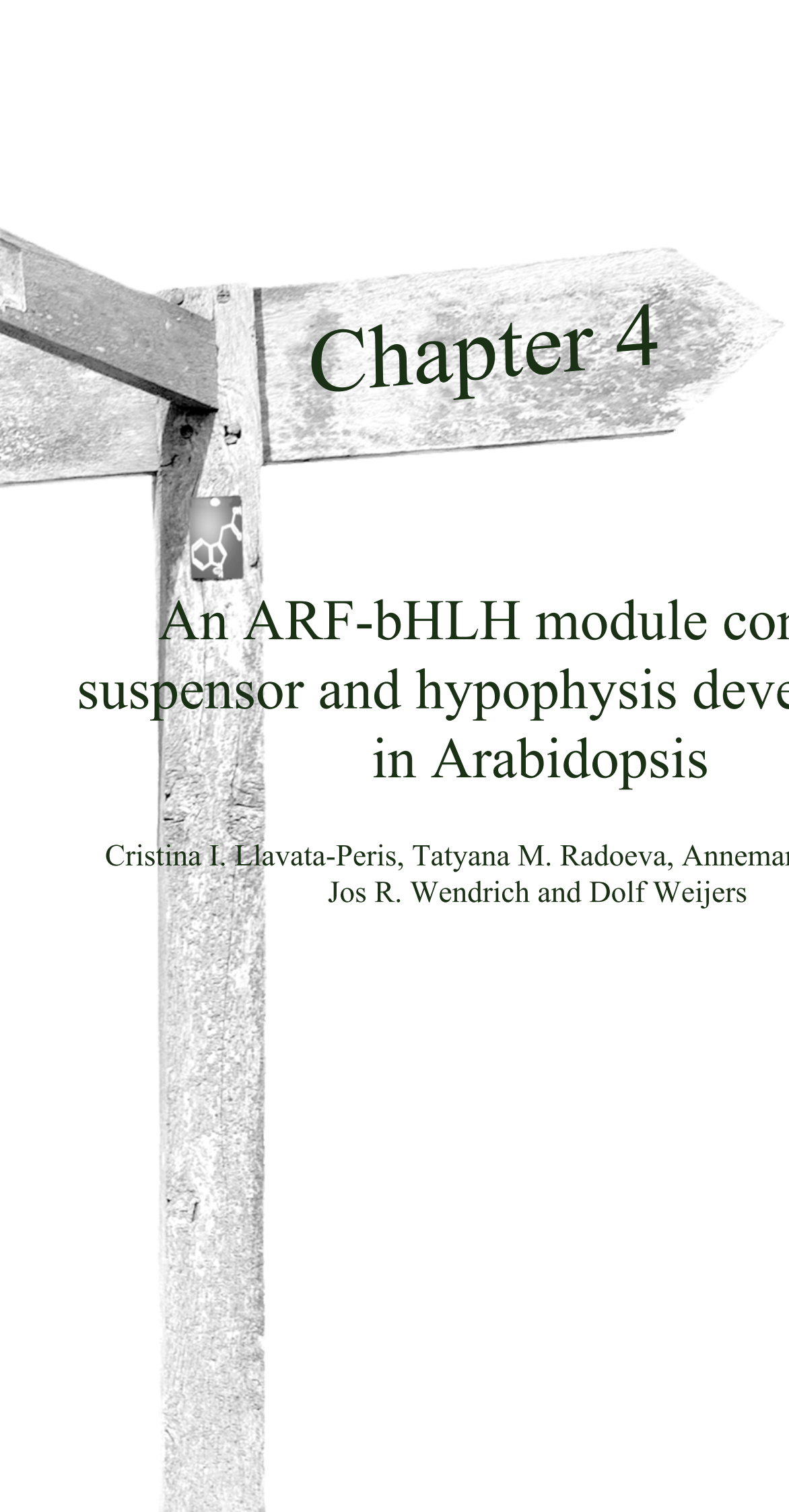

Chapter 4

An ARF-bHLH module controls suspensor and hypophysis development in Arabidopsis

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Abstract

Auxin controls many aspects of plant development through changes in gene activity. These transcriptional effects are mediated by ARF transcription factors. An important question in plant biology is which genes are controlled by ARFs to direct specific developmental auxin output. Among the genes misregulated upon inhibition of ARF activity in extra-embryonic suspensor cells, we identified specific subsets of the basic Helix-Loop-Helix (bHLH) transcription factor family. Here we describe the functional dissection of the contribution of 4 of these bHLH proteins to auxin-dependent suspensor and hypophysis development. All 4 genes are expressed in embryos in patterns consistent with being activated or repressed by ARFs in the suspensor. We confirm that several of these *bHLH* genes are rapidly up- or downregulated upon auxin treatment, and that their expression is disturbed in *arf* mutant backgrounds. Loss- and gain of function phenotypes uncover a function of these genes in mediating auxin activity in suspensor and hypophysis development. This work identifies *bHLH* genes as functionally relevant target genes of ARF transcription factors acting in the suspensor, which is an important step towards understanding how local responses to auxin are generated. Interestingly, this novel auxin-ARF-bHLH module acts in parallel to the previously established auxin-ARF5/MP-TMO module and hence defines such modules as a conserved element in auxin-dependent developmental processes.

Introduction

Auxin plays a key role in the generation of multiple developmental and physiological responses in plants (Moller and Weijers, 2009; Nagpal et al., 2005; Ellis et al., 2005; Goetz et al., 2006). While the final outcome of the many functions of auxin differs greatly between processes (reviewed in Berleth and Sachs, 2001), these processes share a common, simple response pathway. Auxin is sensed in the nucleus by TIR1/AFB ubiquitin ligase complexes (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). As a consequence of auxin binding, the Aux/IAA proteins are targeted for degradation, releasing their inhibition of DNA-binding ARF transcription factors (dos Santos Maraschin et al, 2009). Once activated, ARFs can regulate transcription of genes that eventually direct the developmental or physiological response.

A key question in plant biology is how auxin activity is locally translated into precise responses. To address this question, an important step is to identify the genes that are regulated by ARF transcription factors in single cell types. Identification of ARF targets can be achieved by interfering with their normal activity through misexpression of a non-degradable aux/iaa protein and studying which genes are misregulated (e.g. Schlereth et al., 2010). Recently, we have established the early Arabidopsis embryo as a model for studying different auxin responses in a simple developmental context. Seven *ARF* genes are expressed in different, partially overlapping patterns in the early embryo (Rademacher et al., 2011), and some of these converge on redundant activities (Rademacher et al., 2012). Interestingly, inhibition of ARFs in suspensor cells leads to a loss of suspensor identity and acquisition of embryo fate (Rademacher et al., 2012). Previously, a transcriptomics approach was used to identify genes that are either up- or downregulated while suspensor cells switch to embryo identity due to local expression of the ARF inhibitor bodenlos/iaa12 (Lokerse 2011). In this dataset, many genes were up- or down-regulated under the

expression of the GAL4 dependent UAS promoter in the suspensor specific M0171 background; among these, members of the YUCCA, bHLH, B3 superfamily, AP2/EREB, MYB, AUX/IAA and F-box gene families were over-represented. Particularly the regulation of *bHLH* genes by ARF transcription factors in the suspensor is interesting since it has previously been shown that two different *bHLH* genes, *TMO5* and *TMO7*, are direct targets of *ARF5/MP* in root formation (Schlereth et al., 2010; De Rybel et al., 2013).

The bHLH family of genes is conserved in all eukaryotes, and represents one of the largest families of transcription factors (Riechmann et al., 2000; Ledent and Vervoort, 2001). In the plant kingdom, *bHLH* genes are present from algae to flowering plants. This retention during evolution allowed gene diversification, and the establishment of functionally different bHLH subfamilies. Indeed, bHLHs are involved in a large number of different processes from regulation of female reproductive tract, (Gremski et al., 2007; Heisler et al., 2001; Crawford and Yanofsky, 2011), embryogenesis (Chandler et al., 2009; Schlereth et al., 2010), stomatal development, (reviewed by Pillitteri and Torii, 2007), root hair growth (Yi et al., 2010), fruit opening (Sorefan et al., 2009), light and hormone signaling (Halliday et al., 1999; Fairchild et al., 2000; Huq and Quail, 2002; Khanna et al., 2004; Oh et al., 2004), wound and drought response (Smolen et al., 2002; Chinnusamy et al., 2003) to shoot branching (Komatsu et al., 2001). The Arabidopsis genome contains 167 bHLHs (Bailey et al., 2003; Li et al., 2006), all but 3 "orphans" are classified into 28 subfamilies based on sequences in the bHLH domains. The *bHLH* genes that are misregulated in embryos that misexpress bdl in suspensor cells, belong to two distinct subfamilies: *bHLH49*, *-63*, *-60* belong to subfamily 25 and *bHLH100* belongs to subfamily 12. Particularly in the case of subfamily 25 genes, the high level of sequence homology (25%-61%), and their co-regulation upon suspensor-specific auxin response inhibition (Lokerse 2011) suggests functional redundancy. It has been shown that *bHLH* genes belonging to the same subfamily often are involved in the same biological functions, as in the case of *GL3*, *EGL3* and *TT8*, that redundantly control anthocyanin biosynthesis (Nesi et al., 2000; Zhang et al., 2003), *HEC1*, *2* and *3* that have an overlapping function in the control of stigma development (Gremski et al., 2007) and *TMO5* and closely related genes that redundantly control vascular tissue development (De Rybel et al., 2013).

Here we analyze the functions of subfamily 12 and 25 *bHLH* genes in relation to suspensor-specific auxin responses. We show that *bHLH100* and members within bHLH subfamily 25 redundantly control suspensor and hypophysis development. Furthermore, we show the requirement of proper auxin signaling for the regulation of these genes. Our results show how auxin controls different developmental outputs through distinct auxin-ARF-bHLH modules.

Results

Dynamic, auxin-dependent bHLH gene expression during embryogenesis

Several *bHLH* genes were found to be either up- or downregulated upon suspensor-specific bdl misexpression (Lokerse unpublished). These belong to two different clades in the bHLH family, *bHLH49, -60, -63* belong to the subfamily 25 (Carretero-Paulet et al., 2010) (Fig. 1) and were upregulated in M0171>>bdl embryo (Lokerse unpublished). In contrast, *bHLH100* (subfamily 12) was downregulated (Fig. 1). To verify if these bHLH genes are involved in normal embryo development we first determined their gene expression and protein accumulation patterns in embryos.

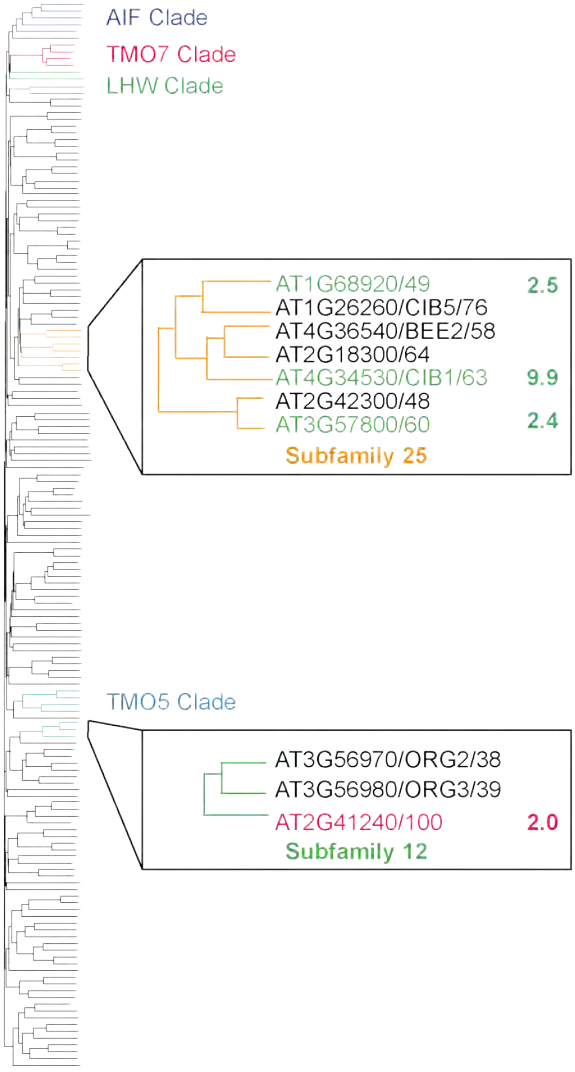


Figure 1: Phylogenetic tree of bHLH family.
Protein-based neighbor-joining tree (BLOSUM62) of all 167 Arabidopsis bHLH proteins. Full protein sequences were used to generate an alignment. Subfamilies 25 and 12 are highlighted, and the genes studied here are marked in green (subfamily 25) or red (subfamily 12). The fold-change in a microarray where the activity of ARFs is inhibited in the suspensor (M171>>bdl) is indicated in green (upregulated) or red (downregulated). Note that both subclades cluster distinctly from the TMO5, TMO7, LHW and AIF clades.

Transcriptional fusions of 2kb upstream fragments of these four *bHLH* genes to nuclear 3xGFP (n3GFP; Takada and Jürgens, 2007) showed highly dynamic and unique expression patterns during embryogenesis and root development, which are depicted in Fig. 2. *pbHLH49-n3xGFP* is expressed in all cells until the 8-cell stage (not shown). From early globular stage onward, the reporter becomes restricted to the suspensor and lower half of the proembryo (Fig. 2A). From heart stage onward, the expression vanishes from the future QC and columella initials (Fig. 2A). At this stage, expression is more pronounced in vascular cells (Fig. 2A). *pbHLH60-n3xGFP* is expressed only in the suspensor at the 8-cell stage (Fig. 2B). Expression later expands to the whole embryo (Fig. 2B) and becomes specific for the protoderm around heart stage (Fig. 2B). *pbHLH63-n3xGFP* has a very restricted pattern of expression, which is present in the protoderm of the embryo and becomes activated only at early heart stage (Fig. 2C). *pbHLH100-n3xGFP* is specific for the suspensor until the 16-cell stage (Fig. 2D). From the early globular stage onward, expression expands to the protoderm of the proembryo (Fig. 2D). Hence, transcriptional fusions show dynamic expression of all 4 *bHLH* genes.

These bHLH genes were identified on the basis of their misregulation upon suspensor-specific inhibition of ARF transcription factors. Hence, it would be expected that genes that were upregulated in the microarray should normally be expressed in the pro-embryo. This is indeed the case for subfamily 25. Although expression is found in the suspensor at early stages, eventually each gene is expressed in specific regions of the pro-embryo. Conversely, genes downregulated in the M0171>>bdl experiment are expected to be normally expressed in the suspensor. This is indeed the case for *bHLH100* (Fig. 2D). Therefore, while dynamic, the expression patterns of all 4 bHLH genes are consistent with their misexpression in M0171>>bdl embryos.

To determine if these transcriptional patterns reflect the ultimate accumulation of the bHLH proteins, or whether post-transcriptional regulation also contributes to gene regulation, we generated protein fusions with YFP or td-Tomato using genomic fragments. These fusions showed the same pattern of expression for all the genes, indicating that protein accumulation in the embryo is mainly controlled through transcriptional regulation, and that proteins are not mobile. In all cases bHLH-YFP and bHLH-tdTomato fusion proteins accumulate in the nucleus (Shown for bHLH49-YFP in Fig. 2E), in agreement with their predicted function as transcription factors.

To define the spatio-temporal control of these *bHLH* genes by the auxin response system, gene expression was determined in embryos in which a stabilized version of IAA10 is expressed in the suspensor (*pARF13*-iaa10; Rademacher et al., 2012). This stabilized version of IAA10 leads to suspensor proliferation and the change of the suspensor identity towards an embryonic identity (Rademacher et al., 2012). For this analysis we used the *pbHLH49-n3xGFP* reporter, as it shows a distinct, pro-embryo-specific pattern in wild-type at late globular stage (Fig. 3A). *bHLH49* expression in pARF13-iaa10 embryos was weaker in the suspensor region during late globular stage (Fig. 3B). This demonstrates that normal auxin response is important for the wild-type *bHLH49* expression pattern.

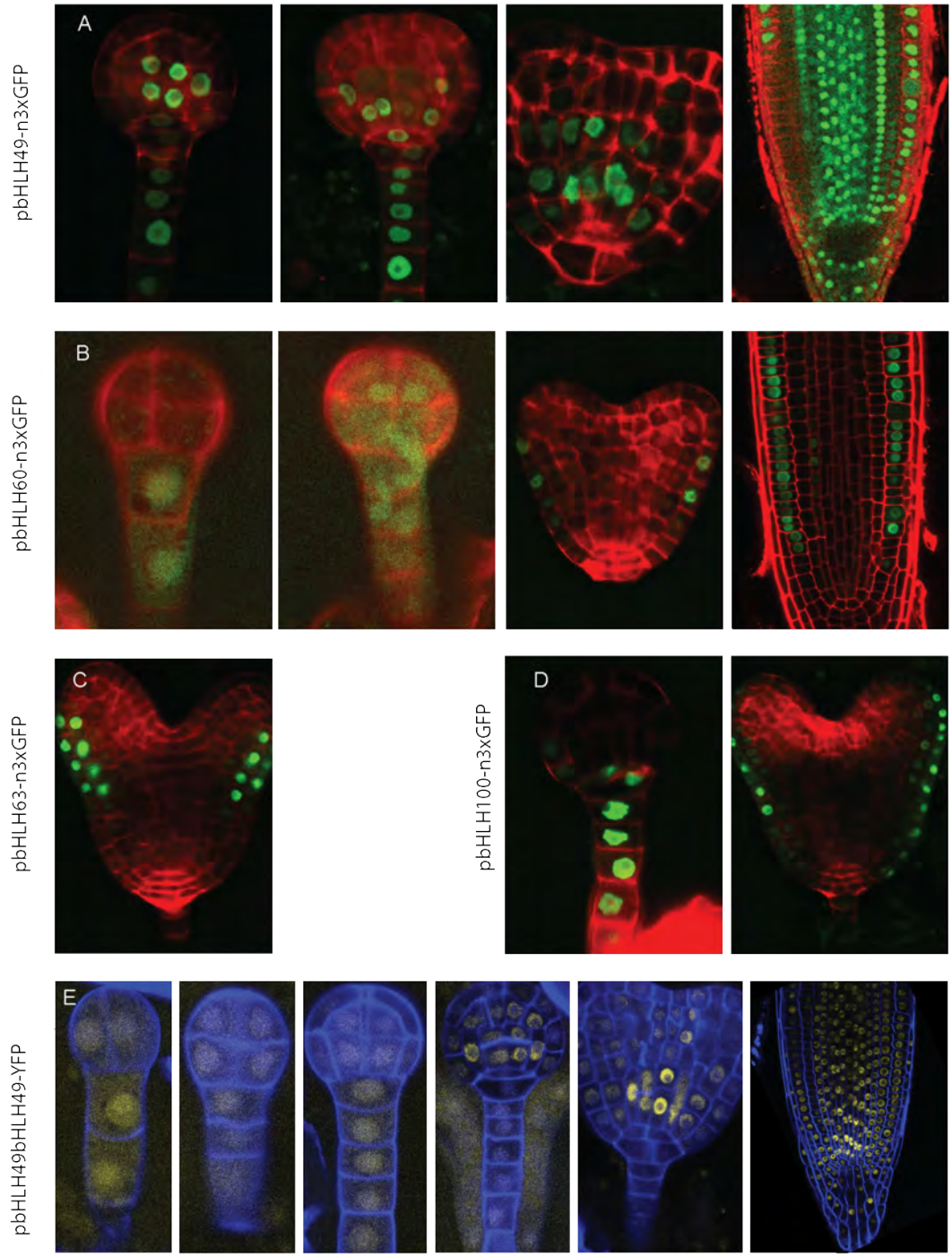


Figure 2: Dynamic pattern of expression of bHLH49, -60, -63, -100 in embryo and root.
Expression of transcriptional fusions of 2 kb promoters of bHLH49 (A), bHLH60 (B), bHLH63 (C), bHLH100 (D) to nuclear 3xGFP throughout embryogenesis and in the root tip. (E) Fluorescence of a translational pbHLH49-bHLH49-sYFP fusion protein throughout embryogensis and in the root tip.

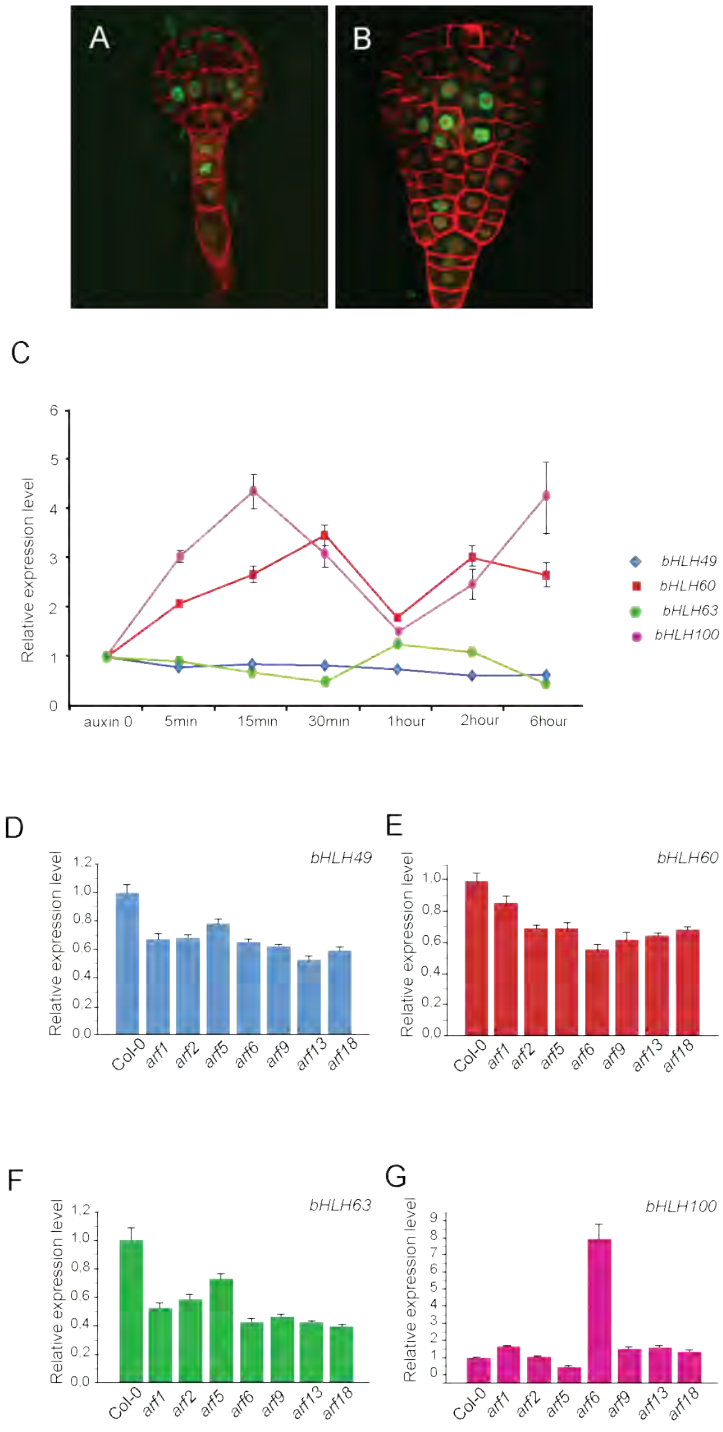


Figure 3: Auxin controls expression of bHLHs through different ARFs.
(A,B) Expression of pbHLH60-n3GFP in wild-type (A) and pARF13-iaa10 (B) embryos of comparable stage. (C) Transcript levels of bHLH49, bHLH60, bHLH63 and bHLH100 in wild-type seedlings treated with 1 µM 2,4-D for 0, 5, 10, 15, 30 minutes, 1, 2 and 6 hours. Expression was determined by qRT-PCR and normalized to reference genes. Expression at 0 minutes was set to 1. (D-G) expression of bHLH49 (D), bHLH60 (E), bHLH63 (F) and bHLH100 (G) transcripts in seedlings of wild-type (Col-0) and various *arf* mutants as determined by qRT-PCR. Normalized expression in Col-0 was set to 1.

Auxin directly controls bHLH expression

These *bHLH* genes were selected based on a change in expression upon auxin response inhibition in the suspensor (Lokerse, 2011). However, as auxin response inhibition causes dramatic changes in cell fates and patterning (Rademacher et al, 2012), these changes in bHLH gene expression may not be a direct consequence of auxin/ARF regulation. To verify the auxin control of these genes different approaches were taken. First we conducted a time course of exogenous auxin treatment to determine if the expression of the genes was rapidly altered upon auxin treatment. Expression of the various bHLH genes was quantified by qRT-PCR. In this assay, *bHLH49* was significantly downregulated already after 5 minutes of auxin treatment, and expression continued to decrease until 6 hours after incubation (Fig. 3C). In contrast, both *bHLH60* and *100* showed a very significant and rapid increase of transcript levels, already 5 minutes after treatment. In both cases, transcript levels decreased again after 15 (*bHLH100*) and 30 (*bHLH60*) minutes. *bHLH63* expression was not significantly affected by auxin treatment (Fig. 3C). Given this rapid response, it is very likely that *bHLH49*, *-60* and *-100* are immediate ARF targets. As a first step in determining whether this is indeed the case, we used qRT-PCR to quantify transcript levels in a range of *arf* mutants. 7 ARFs are expressed in the globular stage embryo, and we reasoned that these might be the regulators of bHLH gene expression. Even though most single mutants in these ARFs do not show phenotypes, quantitative changes in bHLH expression may occur in the absence of phenotypic consequences. When compared to wild-type seedlings, we found that expression of all 4 *bHLH* genes was affected in most of the single *arf* mutants (Fig. 3D-G). This result is unexpected, and suggests that, while no specific ARF-bHLH connections can be drawn, these 7 ARFs collectively contribute to the proper regulation of these 4 *bHLH* genes. A striking result was that *bHLH100* expression is dramatically increased in the *arf6* mutant background (Fig. 3G), suggesting a significant negative regulation of *bHLH100* by *ARF6*.

To determine if auxin regulation impacts bHLH protein accumulation, we treated protein translational fusions with auxin. Since bHLH63 and 100 cannot be readily detected in root tips, we restricted this analysis to bHLH49-tdTomato. In contrast to the transcript, which is rapidly downregulated by auxin treatment (Fig. 3C), bHLH49 protein accumulated to higher levels, already 3 hours after incubation (Fig. 4B). Hence, bHLH49 is under dual auxin control, where transcript and protein are oppositely affected. This result strengthens the regulation of bHLH49 by auxin, but also reveal complex combination of both transcriptional and post-transcriptional or post-translational control.

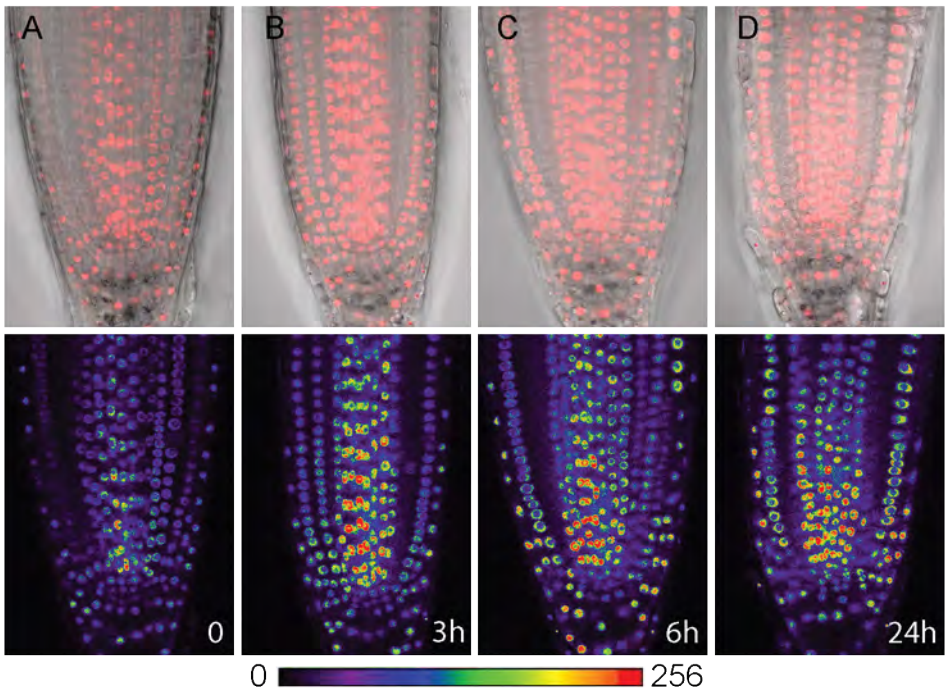
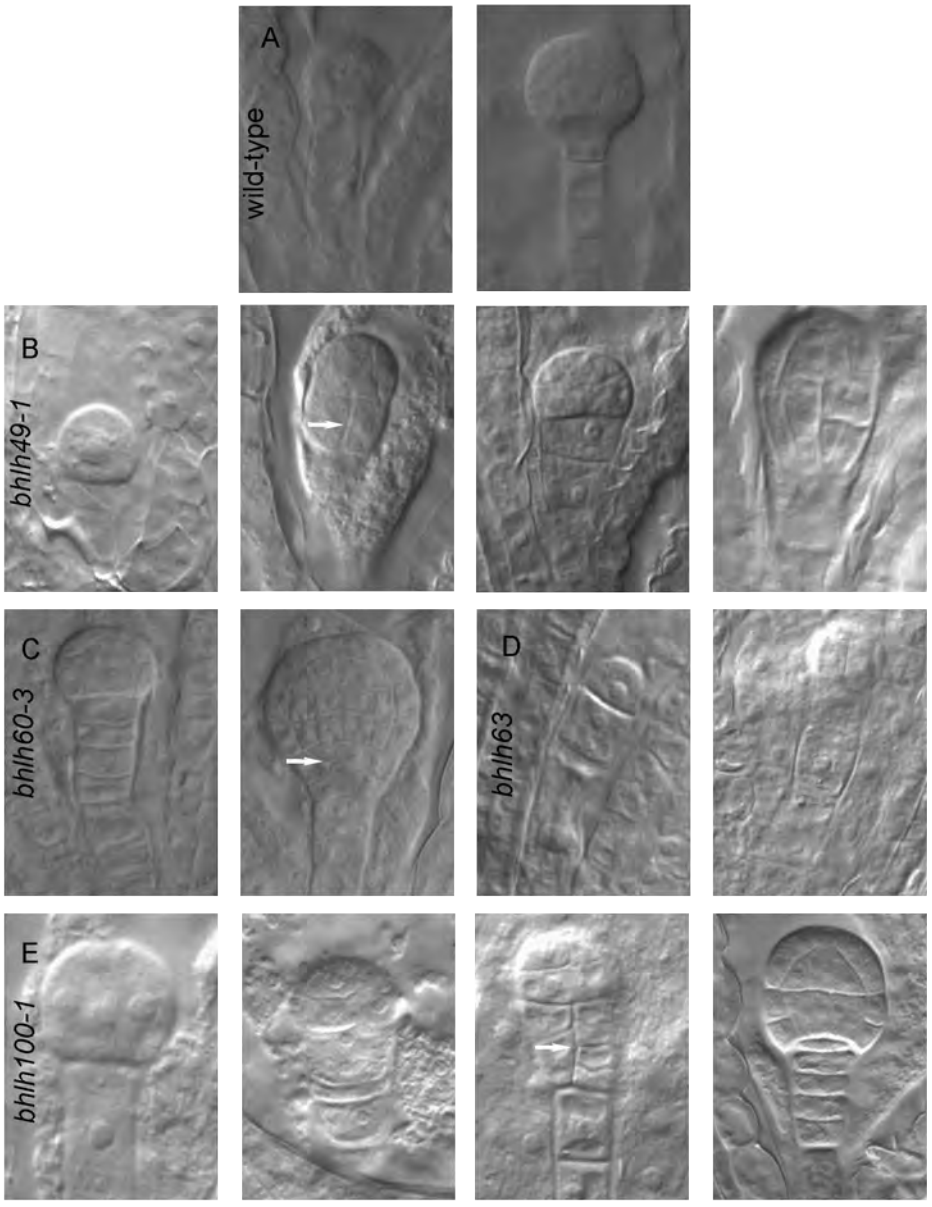


Figure 4: Auxin stabilizes bHLH49 protein
(A-D) Fluorescence of tdTomato (upper panel) and false color intensity plots (lower panel) of root tips of a pbHLH49-bHLH49-tdTomato line treated with 1 µM 2,4-D for 0 (A), 3 (B), 6 (C) and 12 (D) hours. Pixel intensity values in the false color plots are shown in the scale bar.

bHLH genes contribute to suspensor and hypophysis development

To better understand the function of these genes, in the tissues where they are expressed, we systematically analyzed the consequences of mutating or misexpressing the *bHLH* genes. To address consequences of gene knockout, we collected insertion lines from public collections (Table 1). Two independent *bhlh49* alleles were identified, also 2 alleles were recovered for *bhlh100* and one for *bhlh63* and *bhlh60*. We next used qRT-PCR to quantify transcript levels in homozygous insertion lines. All lines showed downregulation of the bHLH transcript and can hence be considered bona fide knockout or knockdown alleles (Fig. 5G). We subsequently analyzed embryo development in these lines with reduced function of the *bHLH* genes. Strikingly, all lines showed defects during embryogenesis, and in all cases, phenotypes were specific to the hypophysis and/or suspensor (Fig. 5B-E). The penetrance of phenotypes, and the exact cellular defects differed between lines, but all showed highly significant phenotypes (14.6% in *bhlh49-1* [N=137]; 18.8% in *bhlh49-2* [N=148]; 13.20% in *bhlh60* [N=115]; 12.17% in *bhlh63* [N=265]; 6.78% in *bhlh100-1*; [N=135]; 21.78% in *bhlh100-2*; [N=120]; 0% in wild-type [N=141]) (Fig. 5F). The most common defects observed are longer suspensor, proliferation in the hypophysis region and defects in the first divisions of the pro-embryo (Fig. 5B-E). Thus, while the gene expression patterns of all 4 *bHLH* genes differ, as does their dependence on auxin and ARFs, all 4 appear to contribute to normal suspensor and hypophysis development.



F

	% PHENOTYPES	N
bhlh49-1	14.6	137
bhlh49-2	18.8	148
bhlh60	13.20	115
bhlh63	12.17	265
bhlh100-1	6.78	135
bhlh100-2	21.78	120

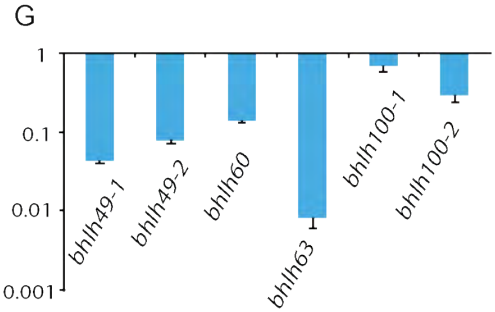
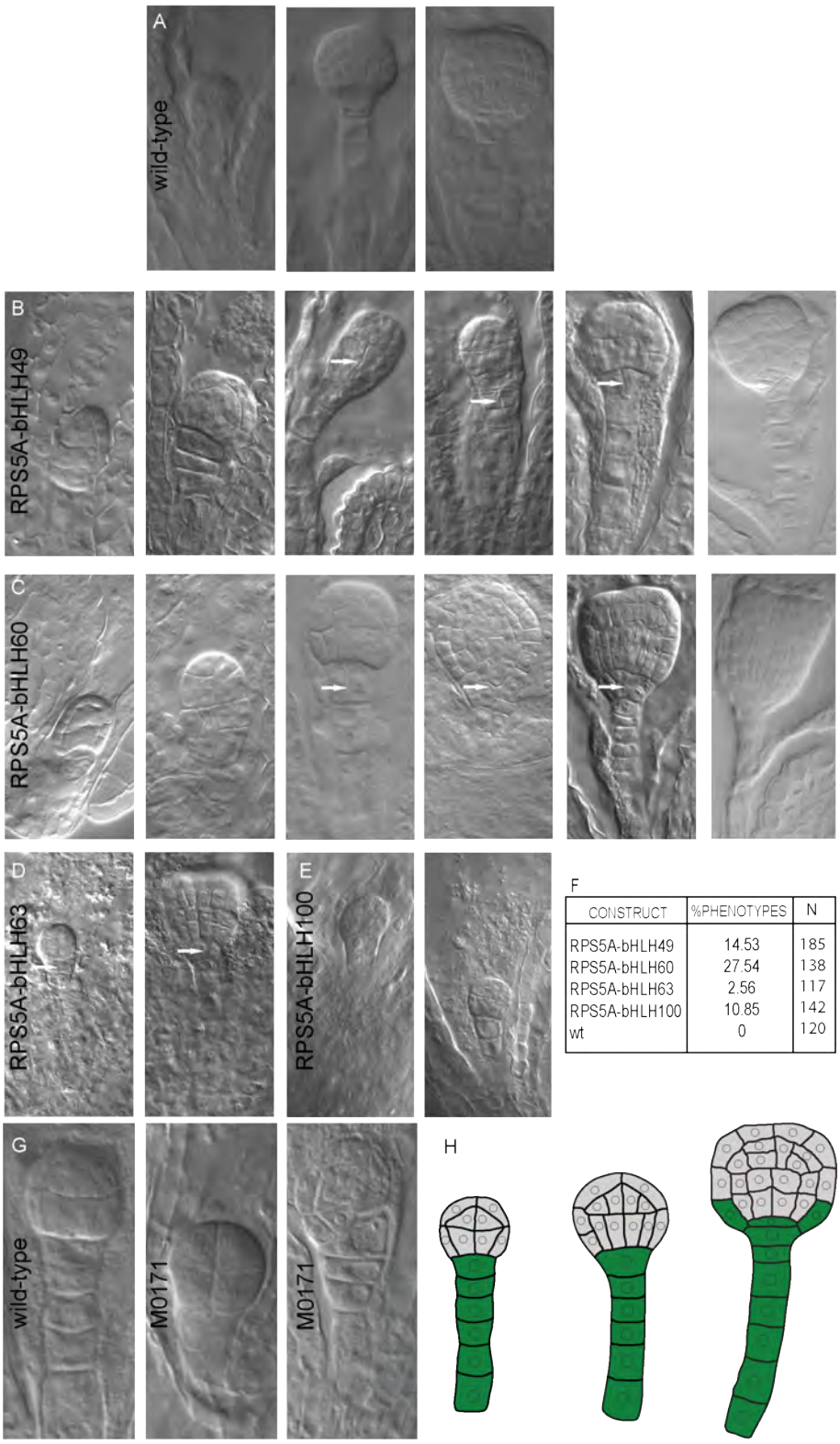


Figure 5: bHLH genes are required for normal embryo development
(A-E) Embryos of wild-type (A), *bhlh49* (B), *bhlh60* (C), *bhlh63* (D) and *bhlh100* (E) at preglobular (left panel) and early-globular (right panel) stages. Note the aberrant division planes in hypophysis and suspensor cells (arrowheads). (F) Frequencies with which representative phenotypes were observed in the alleles studied. (G) Transcript levels of the bHLH genes in respective mutants as determined by qRT-PCR on homozygous seedlings. Expression was normalized using reference genes and the expression level in wild-type was set to 1.

Expression of these 4 *bHLH* genes is controlled by auxin response in the embryo, and changes during the suspensor to embryo transformation. The loss-of-function phenotype of *bhlh100*, the only gene downregulated in M0171>>bdl embryos, is consistent with the gene contributing to the bdl-induced defects. To determine whether ectopic activation of the bHLHs that are upregulated in M0171>>bdl embryos (*bHLH49*, *-60*, *-63*) may also contribute to bdl-induced defects, we used a misexpression approach. Each of the bHLH cDNAs was misexpressed using the strong *RPS5A* promoter that drives ubiquitous expression in embryo and suspensor (Weijers et al., 2001). Embryo phenotypes were then scored in 4 independent lines for each construct. The phenotypes in this case can be classified in three different kinds, but in all cases affected mainly the suspensor (Fig. 6A-E). Based on phenotype penetrance, *bHLH49* and *-60* were much more potent to induce defects (15% and 28% respectively; N=185 and N=138), than *bHLH63* (2.6% N=117); wild-type 0%; N=120). These results show that misexpression of mainly *bHLH49* and *60* induce bdl-like phenotypes, and may indeed contribute to the bdl-induced defects. To determine if ectopic expression in suspensor cells is causal to these defects, we took advantage of the GAL4-*UAS* missexpression system. We used the M0171 driver line that expresses GAL4 in suspensor cells (schematically shown in Fig. 6H; Schlereth et al., 2010) to drive the expression of *bHLH* genes. M0171>>bHLH63 expression induced suspensor proliferation (Fig. 6G; 6.75 %; N=76). This suggests that ectopic expression of this gene contributes to the suspensor defects upon auxin response inhibition.



CONSTRUCT	%PHENOTYPES	N
RPS5A-bHLH49	14.53	185
RPS5A-bHLH60	27.54	138
RPS5A-bHLH63	2.56	117
RPS5A-bHLH100	10.85	142
wt	0	120

Figure 6: bHLHs misexpression causes defects in suspensor development.
(A-E) Embryos of wild-type (A) pRPS5A-bHLH49 (B), pRPS5A-bHLH60 (C), pRPS5A-bHLH63 (D) and pRPS5A-bHLH100 (E) lines. Embryos af different stages are shown, with younger stages on the left. Note aberrant division planes (arrowheads) (F) Percentages with which the representative phenotypes were observed in various transgenic lines. (G) Phenotypes in lines that misexpress bHLH63 specifically in the suspensor using the M0171 GAL4 driver line. (H) Schematic representation of the expression pattern of the M0171 driver line.

bHLHs mediate auxin-dependent growth

To confirm the function of bHLH subfamily 25 and 12 in auxin signaling we tested the auxin sensitivity in roots of loss-of-function mutants (*bhlh49-1, bhlh49-2, bhlh63, bhlh100-1, bhlh100-2*) and misexpressors (*pRPS5A-bHLH49, pRPS5A-bHLH60, pRPS5A-bHLH63, pRPS5A-bHLH100*). Plants were grown on media supplemented with 40nM 2,4D. In wild-type, this concentration inhibits root growth to 45% of that on unsupplemented media (Fig. 7A). Changes in the level of root inhibition can be quantified and used as a readout of auxin sensitivity (Lincoln et al., 1990). The results obtained show a slight reduction in auxin sensitivity in all the loss-of-function and misexpressor lines tested with the exception of *pRPS5A-bHLH63* and *pRPS5A-bHLH60(not in figure)*. These results confirm that bHLHs are required for normal auxin response. The effects are subtle, although it is well possible that redundancy among subclade 25 genes obscures the full extent of involvement in auxin-dependent growth.

To determine if these *bHLH* genes are not only output of auxin response, but perhaps also feedback on auxin activity, we analyzed the expression of an auxin-dependent reporter, *DR5-GFP* (Ottenschläger et al., 2005), in the *pRPS5A-bHLH49* overexpressor background (Fig. 7B-G). The results show that the pattern of *DR5-GFP* is unaffected in embryos of *pRPS5A-bHLH49* even despite clear embryo defects (indicated by arrowheads in Fig. 7E-G), which renders the possibility of a feedback loop between bHLHs and auxin responses unlikely.

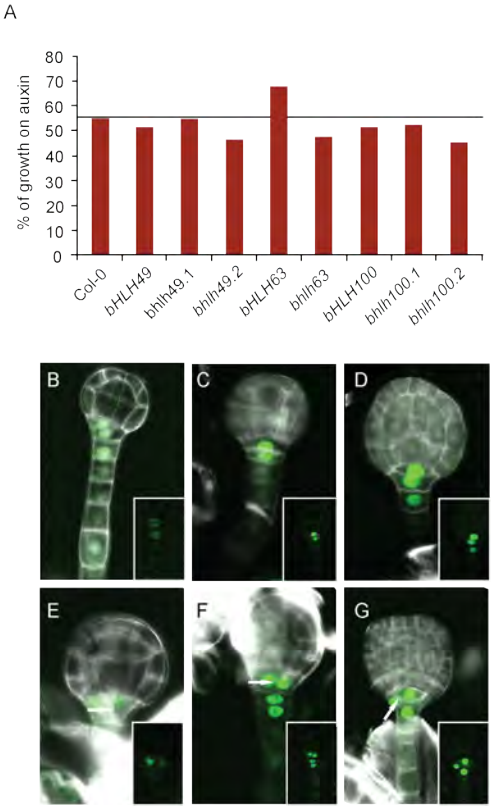


Figure 7: bHLH genes mediate auxin dependent growth.

(A) Auxin sensitivity of wild-type (Col-0), bhlh mutants and bHLH RPS5A misexpression lines (capital letters). Root growth 4 days after transfer to plates containing 40 nM 2,4D is expressed as a percentage of growth on unsupplemented control plates. (B-G) Expression of the auxin response reporter pDR5-n3GFP in wild-type (B-D) and RPS5A-bHLH49 (E-G) embryos. Note that GFP signals are comparable between the two genotypes despite aberrant cell divisions in the RPS5A-bHLH49 embryos (arrowheads). Insets in (B-G) show only the GFP channel.

Discussion

In our work we discover new roles for the subfamily 25 and 12 of bHLHs, by showing their importance for embryo development, in particular for correct suspensor and hypophysis development. Furthermore, we demonstrate that these genes are part of the genetic network that is controlled by auxin to regulate embryo development

A key question in plant biology is how the activity of generic hormones such as auxin is locally translated into developmental responses. Previously, we have shown that the activity of the ARF5/MP transcription factor is in part relayed by the TMO7/bHLH134 (Schlereth et al., 2010) and the TMO5/bHLH32 (De Rybel et al., 2013) proteins. Hence, in this case, auxin-ARF-bHLH modules allow auxin-dependent developmental responses. The goal of the present study was to determine if a very different auxin response, mediated by a cohort of ARFs (ARF1,2,6,9,13,18) in suspensor cells, is likewise executed through ARF-bHLH modules. In a previous transcriptomics study, we had identified a set of *bHLH* genes whose expression is altered in embryos upon inhibition of ARF activity in suspensor cells (Lokerse 2011). Here we demonstrate that 4 of these genes show dynamic expression patterns that are largely consistent with their regulation during auxin-dependent suspensor-embryo transformation (SET): *bHLH49, -60* and *-63* are all embryo-enriched at globular stage and are upregulated during SET, while *bHLH100* is suspensor-enriched and downregulated during SET (Fig. 2). Both the rapid regulation of bHLH gene transcripts by exogenous auxin and deviations in expression in *arf* mutants suggest that these *bHLH* genes are indeed regulated by auxin-ARF activity (Fig. 3C; D-G). Moreover, both loss- (Fig. 5) and gain-of-function (Fig. 6) phenotypes are consistent with mediating auxin activity in embryo and suspensor development. One complication here is that the number of possible phenotypes is limited when only considering cell division pattern. Therefore, the future use of cell type-specific reporter or marker genes should help clarifying mutant phenotypes in gain- and loss-of-function lines. In any event, it is striking that most lines in which bHLH levels are altered show significant phenotypes in the suspensor/hypophysis area. Even though the hypophysis may be relatively sensitive to perturbation, defects are rarely observed in wild-type, and previous characterization of over 100 different insertion lines failed to identify such defects. Hence, we conclude that regulated bHLH activity is critical for normal suspensor and hypophysis development.
With the identification of auxin-ARF-dependent activity of these bHLH genes in suspensor development, we have found a second, independent ARF-bHLH module that acts in parallel to the ARF5/MP-TMO5/7 module in the pro-embryo (Schlereth et al., 2010; De Rybel et al., 2013). Both the ARF and bHLH families have expanded during land plant evolution (Carretero-Paulet et al., 2010; Pires and Dolan, 2010), and it is therefore conceivable that evolutionary old ARF-bHLH modules have diversified to mediate auxin-dependent developmental processes. It will be interesting to determine if the ARF-bHLH module is indeed a general principle in plant development.

Finally, with separate auxin-ARF-bHLH modules operating in different cell types of the embryo, it is interesting to consider how these might interact. We have previously shown that ARF5/MP activates TMO7, which then moves to the adjacent uppermost suspensor cell (Schlereth et al., 2010). TMO7 contributes to hypophysis division, a

process that also requires local auxin response (Schlereth et al., 2010). Very recently, a potential connection between these two ARF-bHLH modules has emerged. TMO7 belongs to a bHLH subfamily known as the paclobutrazol-resistant (PRE) family helix-loop-helix (HLH) factors, which can promote cell elongation by interacting antagonistically with another subfamily of HLH factors (called IBH1 and IBH-LIKE, or AIF; Bai et al., 2012; Ikeda et al., 2012). In a yeast 2-hybrid screen, Ikeda and colleagues recently identified subfamily 25 bHLH proteins bHLH49 and -63 as direct interactors of IBH1. Based on misexpression and mutant analysis, they proposed a model in which subfamily 25 bHLHs promotes cell elongation during post-embryonic development, while IBH1/AIF bHLH proteins inhibit the subfamily 25 bHLHs. PRE bHLH proteins sequester IBH1/AIF proteins and thereby activate subfamily 25 proteins (Fig. 8). There is a striking parallel to the network we have identified in the embryo. If the same model applies to TMO7 and subfamily 25 bHLH genes, one would expect that TMO7 indirectly promotes activity of subfamily 25 bHLH proteins. Indeed, phenotypes in TMO7 RNAi lines (Schlereth et al., 2010) are very similar to those found in *bhlh49* mutants, which is consistent with this model. Our future efforts should reveal whether indeed the two ARF-bHLH modules are interlinked, and importantly, what downstream processes they control to drive hypophysis and suspensor development.

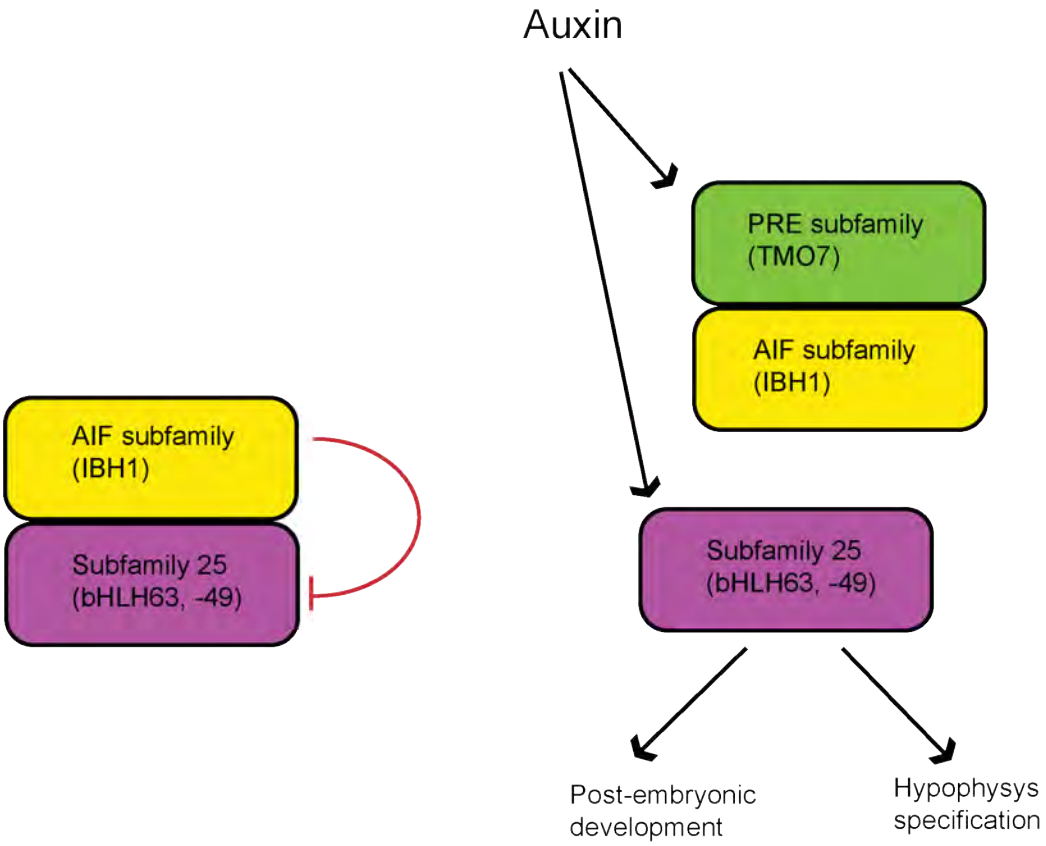


Figure 8: Interplay between 3 subfamilies of bHLH for the control of developmental responses.

Material and methods

Plant material, growth conditions and transformation

All seeds of *Arabidopsis thaliana* are ecotype Columbia with the exception of the line M0171 line, which is in the C24 ecotype (Schlereth et al., 2010)

Seeds were sterilized in 25% bleach/75% ethanol solution for 10 minutes, and washed twice with 70% ethanol and once with 100% ethanol. Dried seeds were sown on plates containing MS (0.5x Murashige and Skoog salts, 1% (w/v) sucrose, and 0.8% (w/v) agar) and supplemented with 25 mg/L kanamycin or 15 mg/L phosphinothricin for appropriate antibiotic selection. After incubation at 4°C for 24 hrs, the plants were grown under standard long day conditions (16/8 hrs light/dark) at 22°C. Seedlings were transferred to soil at an age of at least 10 days.

Plants were transformed using a standard floral dipping method, as described by Clough and Bent, 1998.

Details about the insertion lines used are in table 1.The insertion mutants were obtained from the Nottingham Arabidopsis stock centre and primers used for genotyping are listed in table 1.

Table 1. Insertion lines used in this study.

Gene	Locus	Allele	NASC	Stock #	Internal #	Primer for genotyping	
bHLH49	AT1G68920	*bhlh49-1*	N661158	SALK_135188C	6.1	LP	TTTCCGTCGTAACAACGAATC
						RP	CTAGTACCGGGTTGCAACAAG
bHLH49	AT1G68920	*bhlh49-2*	N663212	SALK_087424C	6.2	LP	TAACACAGGGCAATGGAAAAG
						RP	TGCTGAAAACTCATCTTTCGC
bHLH60	AT3G57800	*bhlh60-1*	N862886	SAIL_1219_E01	60-3	LP	AAAAGCAGCAAGAGAATGACG
						RP	TCAAGCAACCGATAGCCATAG
bHLH063	AT4G34530	*bhlh63-2*	N878510	SAIL_1211_F11-LP	23.2	LP	TAAATTTGGGATTAGTCCCGC
						RP	ACAAGATCACAGGCAAAGCAG
bHLH100	AT2G41240	*bhlh100-1*	N664325	SALK_150637C	75.1	LP	TTGGTCGGTGTAAACGAGATC
						RP	TTGTGGTAGAAAAATGTGATTGC
bHLH100	AT2G41240	*bhlh100-2*	N656649	SALK_074568C	75.2	LP	TCTCAATAGTCCACGTCCACC
						RP	AATGCTTGTGAAACTGTTGCC

Table 2. Primers used to generate the constructs used in this study.

Gene	Locus	DNA	Construct	Primer	
bHLH49	AT1G68920	Genomic	TdTo/YFP	FW	TAGTTGGAATGGGTTCGAAGAAGTTATTAGCATTAACTG
				RV	TTATGGAGTTGGGTTCGAACTGGCTCAACCTTCATATTTG
bHLH60	AT3G57800	Genomic	TdTo/YFP	FW	TAGTTGGAATGGGTTCGAATTTTTGTAAATATAGAAGAGAAAATAG
				RV	TTATGGAGTTGGGTTCGAACCAGCTCCATTTTGACCTGAT
bHLH63	AT4G34530	Genomic	TdTo/YFP	FW	TAGTTGGAATGGGTTCGAAATTAAAAATGATAAGTGTCA
				RV	TTATGGAGTTGGGTTCGAACAACTCCTAAATTGCCATAGA
bHLH100	AT2G41240	Genomic	TdTo/YFP	FW	TAGTTGGAATGGGTTCGAAAACCGAAGTGTTGTACTGTT
				RV	TTATGGAGTTGGGTTCGAACTGTAAACGAGTGTCCACATT
bHLH49	AT1G68920	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGGATTTAAGTGCGAAAGA
				RV	AGTATGGAGTTGGGTTCTCATGGCTCAACCTTCATAT
bHLH60	AT3G57800	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGGATCTGACTGGAGGATT
				RV	AGTATGGAGTTGGGTTCTTACAGCTCCATTTTGACCT
bHLH63	AT4G34530	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGAATGGAGCTATAGGAGG
				RV	AGTATGGAGTTGGGTTCTCAAACTCCTAAATTGCCAT
bHLH100	AT2G41240	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGTGTGCACTTGTCCCTCC
				RV	TTATGGAGTTGGGTTCGAATCATGGCTCAACCTTCATATTTG
bHLH63	AT4G34530	cDNA	UAS	FW	TAGTTGGAATGGGTTCGAAATGAATGGAGCTATAGGAGGTGACC
				RV	TTATGGAGTTGGGTTCGAACTCAAACTCCTAAATTGCCAT

Cloning

All cloning was performed using the LIC cloning system and the vectors described in De Rybel et al. (2011). For transcriptional fusions of *bHLH49*, *-60*, *-63*, *-100*, to n3GFP 2 Kb fragments upstream of the ATG were amplified from genomic DNA

using Phusion Flash polymerase (Finnzymes) and annealed into vector pPLV04 (Lokerse, 2011). For translational fusions to sYFP (pPLV17) and tdTomato (pPLV22), the same promoter fragment was amplified together with the genomic coding sequence excluding the stop codon. To generate misexpression lines using *pRPS5A*, the coding sequences of all genes were amplified from cDNA clones and annealed into pPLV28. All constructs were verified by sequencing. Primers used are listed in Table 2.

qRT-PCR analysis

qRT-PCR analysis was performed as described previously (De Rybel et al., 2010). RNA was extracted using an RNeasy kit (Qiagen). Using 1 µg of total RNA it was synthesize Poly(dT) cDNA using an iScript cDNA Synthesis Kit (Biorad). Analysis was performed using a CFX384 Real Time PCR detection system (BioRad) with iQ SYBR Green Supermix (BioRad). Primer pairs were designed with Beacon Designer 7.0 (Premier Biosoft International). All reactions were performed in triplicate. Data were analyzed using the software qBase (Hellemans et al., 2007). Gene expression levels were normalized using as internal control *EEF1α4* and *CDKA1;1*. Primer sequences are listed in Table 3.

Table 3. Primers used for qRT-PCR

Gene	Locus	Forward Primer	Reverse Primer
bHLH49	AT1G68920	CCTCTACCTCATGGATTCATGC	GGATCACATTTTGCAAATCACC
bHLH60	AT3G57800	CAGTCATTTGATTCAGAGAG	ATCCTACTAAATTCTACATTGTC
bHLH63	AT4G34530	CCAATCCACAACATAACAATG	CTCTTTGATACATCAACTAATGGG
bHLH100	AT2G41240	CCGACCAAAACAGTAAGTCAG	ACACCACTCAAGACATTCCC
CDKA	AT3G48750	ATTGCGTATTGCCACTCTCATAGG	TCCTGACAGGGATACCGAATGC
EEF	AT1G30230	CTGGAGGTTTTGAGGCTGGTAT	CCAAGGGTGAAAGCAAGAAGA

Microscopic analysis

Differential interference contrast (DIC) microscopy, fluorescence and confocal microscopy were performed as described in Chapter 2 (Llavata-Peris et al., 2013).
Embryo phenotypes were analyzed using a clearing solution of choral hydrate, water and glycerol 8:3:1 on a Leica DMR microscope equipped with DIC optics.
Confocal imaging was performed on a Zeiss LSM510 or on a Leica SP5-II system (HyD detector). The settings used were: tdTomato excitation at 561 nm and detection at 568-600 nm; YFP excitation at 514 nm and detection at 525-550 nm; GFP excitation at 488 nm and detection at 500-535 nm.

Auxin sensitivity assay

The auxin sensitivity assay was performed as described in Lincoln et al. (1990) with minor modifications.
Plants growing 4 days on MS medium were transferred to plates supplemented with 40nM 2,4-D or to control plates lacking 2,4-D. After 4 days, root growth was measured on 2,4-D containing and control plates, and the degree of inhibition in mutants and transgenic lines was compared to wild-type.

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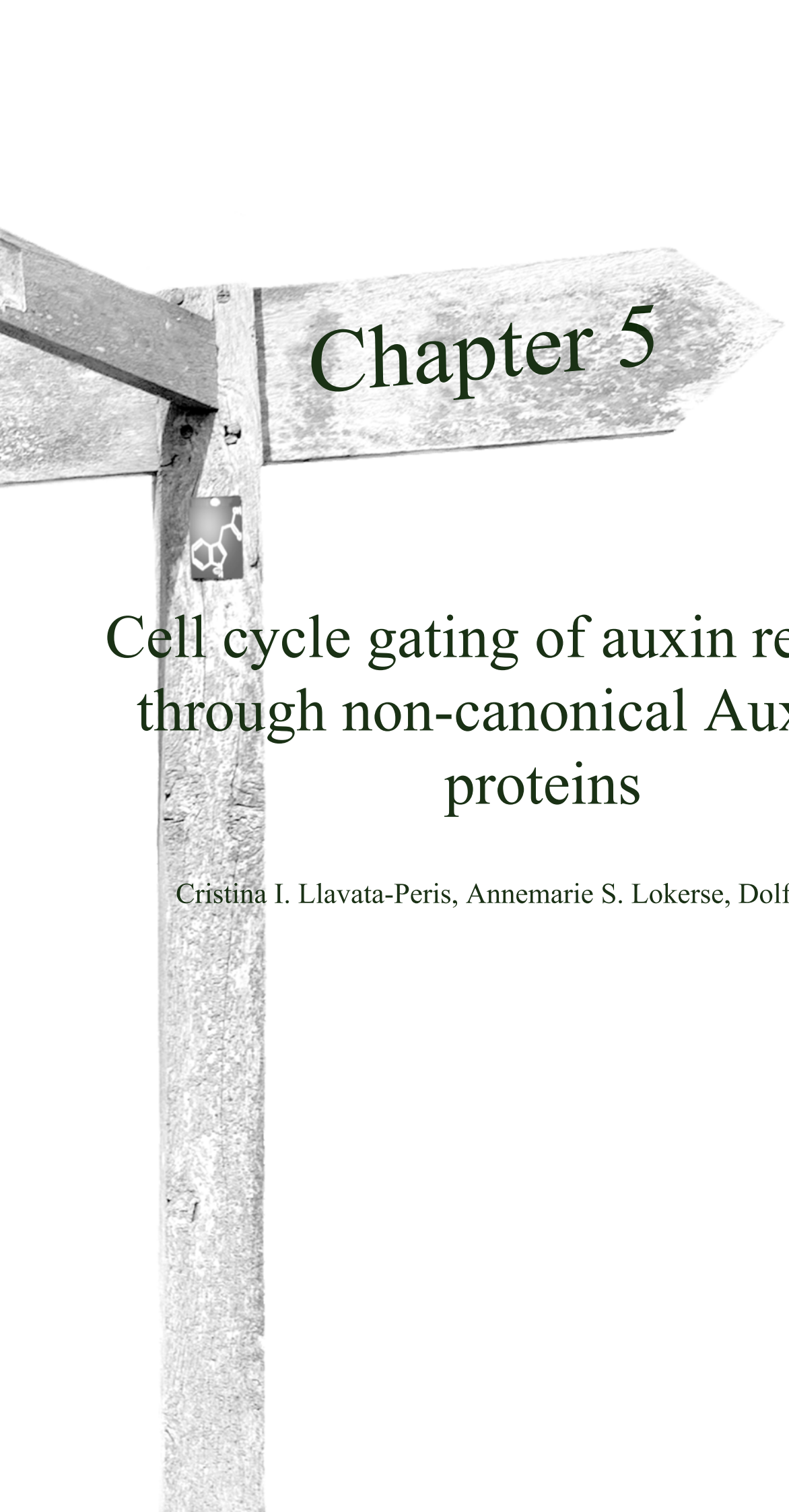

Chapter 5

Cell cycle gating of auxin response through non-canonical Aux/IAA proteins

Cristina I. Llavata-Peris, Annemarie S. Lokerse, Dolf Weijers

Abstract

Auxin triggers many developmental responses by regulating gene activity. Among the genes regulated by auxin are many whose activity feeds back on auxin levels or response. We previously identified several unconventional members of the family of Aux/IAA inhibitors as part of the genes regulated by auxin in young embryos. These non-canonical Aux/IAA proteins (ncIAA) lack the motif required for auxin-dependent degradation, yet are found in most sequenced plant genomes. Their apparent regulation by auxin represents a paradox, in that scenario auxin activity would render cells insensitive to auxin. Here we study the regulation and activity of ncIAA proteins. We demonstrate that the expression of these genes is rapidly upregulated by auxin, and that this requires ARF transcription factors. We confirm that ncIAA proteins are not subject to auxin-dependent degradation. However, both gain- and loss-of-function data show that ncIAAs do inhibit auxin-dependent gene expression, growth and development. When exploring stimuli and compounds that may trigger ncIAA protein degradation, we found that no known hormone was able to do so. Instead, we found that ncIAA protein disappears during cell division, and in addition, ncIAA transcripts are strongly regulated during cell cycle progression. Hence, we conclude that ncIAA proteins are targeted for degradation by a cell cycle-dependent process. Our findings provide the first indication of cell cycle gating of auxin reponses and links two main regulatory processes that quantitatively and qualitatively control plant growth.

Introduction

Developmental responses to the plant hormone auxin are controlled by a family of transcription factors, the AUXIN RESPONSE FACTORS (ARFs), that direct hormone-dependent transcription. In recent years, genome-wide transcript profiling has identified a large set of auxin-dependent genes (Paponov et al., 2008; Sawa et al., 2002; Pufky et al., 2003; Himanen et al., 2004; Redman et al., 2004), and in-depth studies have demonstrated the direct control of several of these by ARFs (e.g. Schlereth et al., 2010). A major question in auxin biology is how specific sets of ARF target genes translate local auxin accumulation to developmental output. As described in Chapter 4, a previous transcriptomics approach has identified a suite of novel, potential developmental mediators of auxin activity in the early embryo. However, in this micro-array study (Lokerse, 2011), as in many others (Overvoorde et al., 2005), a second category of auxin-dependent genes surfaced. This category comprises genes that can collectively be defined as feedback regulators that act to modulate auxin levels or responsiveness following an initial auxin pulse (Reviewed in Benjamins and Scheres, 2008). Members of several gene families involved in auxin biosynthesis (YUCCA; Zhao et al., 2001), conjugation (GH3; Staswick et al., 2005), transport (PIN; Galweiler et al., 1998, PID; Christensen et al., 2000) and response (Aux/IAA; Abel et al., 1995, ARF; Ulmasov et al., 1997a) were found to be strongly misregulated upon interfering with ARF activity in suspensor cells (Lokerse, unpublished). Strikingly, the misregulation of these genes was highly convergent: negative signaling regulators and positive regulators of efflux were downregulated, while biosynthesis genes were induced (Lokerse unpublished). It follows that auxin pathways are connected in such a way that “normal” activity is re-established following a change in auxin activity. While many individual examples of feedback

regulation have been shown (reviewed in Reed, 2001), the relevance of these events is not at all clear. Intuitively, one could predict excessive auxin response if such feedback is compromised, but neither such hyper-responsiveness nor a developmental consequence has so far been shown. In this chapter we focus on a group of genes that is part of the feedback regulation system of auxin, belonging to an unusual subfamily of Aux/IAA proteins that lacks the domain II that is important for auxin-dependent degradation. For that reason we will refer to them as non canonical Aux/IAA (ncIAA; Sato and Yamamoto, 2008).
The ncIAA subfamily constitutes 7 members (IAA20, -30, 29, -31, -32, -33, and -34) in Arabidopsis. With the exception of IAA29, all lack the domain important for auxin dependent degradation. Although the information available about mechanism of their function, stability of proteins and auxin dependence is fragmentary, auxin-related phenotypes have been observed in overexpression lines for some of these genes (Sato and Yamamoto, 2008). Furthermore, based on limited expression studies, the expression of some of these genes is auxin inducible (Sato and Yamamoto, 2008), but at least IAA20 does not appear to be degraded in response to auxin (Dreher et al., 2006). Interestingly IAA20 and IAA30 were strongly downregulated upon suspensor-specific ARF inhibition (Lokerse 2011). Hence, these proteins, that are deeply conserved in plant genomes (Wang et al., 2010; Jain et al., 2006; Audran-Delalande et al., 2012), pose an interesting paradox in auxin biology. Their auxin-responsive transcription would in principle be expected to permanently suppress auxin response. In an attempt to solve this paradox, we here consider the function and regulation of ncIAA proteins in relation to embryo and root development.

In this chapter we focus on three members of the ncIAA gene family, IAA20, IAA30 and IAA33; all expressed in both embryo and root. We confirmed a dynamic pattern of expression that in all cases follows auxin activity. Indeed, both gain- and loss-of-function studies showed that normal ncIAA expression is required for proper auxin-dependent developmental responses. Furthermore, we show that ncIAA proteins are inert to most protein-destabilizing signals and we made a striking finding when studying the cell cycle control of the protein stability. We discovered two levels of cell cycle regulation, both protein stability and gene expression are controlled by this process. This finding sheds new light on the relation between auxin activity and the cell cycle and represents the first evidence of cell cycle gating auxin responses through components of the auxin feedback loop system.

Results

Dynamic expression of ncIAA genes
Based on overall protein homology the non canonical AUX/IAA (ncIAA) cluster together in the phylogenetic tree of the AUX/IAA proteins (Fig. 1A). The maintenance of ncIAAs as a separate clade is supported by the clustering of rice ncIAA proteins with the Arabidopsis ncIAAs (Supplemental Fig.1). This evolutionary conservation suggests an ancient and perhaps important function in auxin biology. The Arabidopsis ncIAA clade consists of seven members that share a common protein domain topology. One exception is IAA29, that has a similar domain organization as the canonical AUX/IAAs. IAA20, -30, -31, -32, -33, -34, all lack domain II required for auxin dependent degradation. IAA33 also lacks domain I, known to be important for the repression activity of Aux/IAA proteins (Dreher et al., 2006). To initially determine the potential role of Arabidopsis ncIAAs in auxin activity and

development, we studied the pattern of expression of the whole subfamily throughout embryogenesis and in the postembryonic root using transcriptional fusions of 2 kb promoter fragments to a nuclear 3xGFP reporter (Takada and Jürgens, 2007). Expression of four out of the six ncIAAs could be detected during embryogenesis. *IAA30* is the earliest expressed, from the four-cell stage onward (not shown). *IAA20*, is activated slighty later, and become expressed in the lower tier of the embryo including the suspensor and hypophysis at globular stage (Fig. 1B). *IAA33* is expressed everywhere in the embryo until late globular stage (Fig. 1F). During later stages, *IAA20* and *IAA30* expression becomes broader and is also present in vascular cells (Fig. 1B,D), while *IAA33* remains localized in the hypophysis region (Fig. 1F). *IAA31* is activated only at later stages, and is restricted to the cotyledon primordia (not shown). The expression in the embryo suggests that *IAA20, -30* , and *-33* may have a function related to embryonic root initiation and *IAA31* to cotyledon formation.

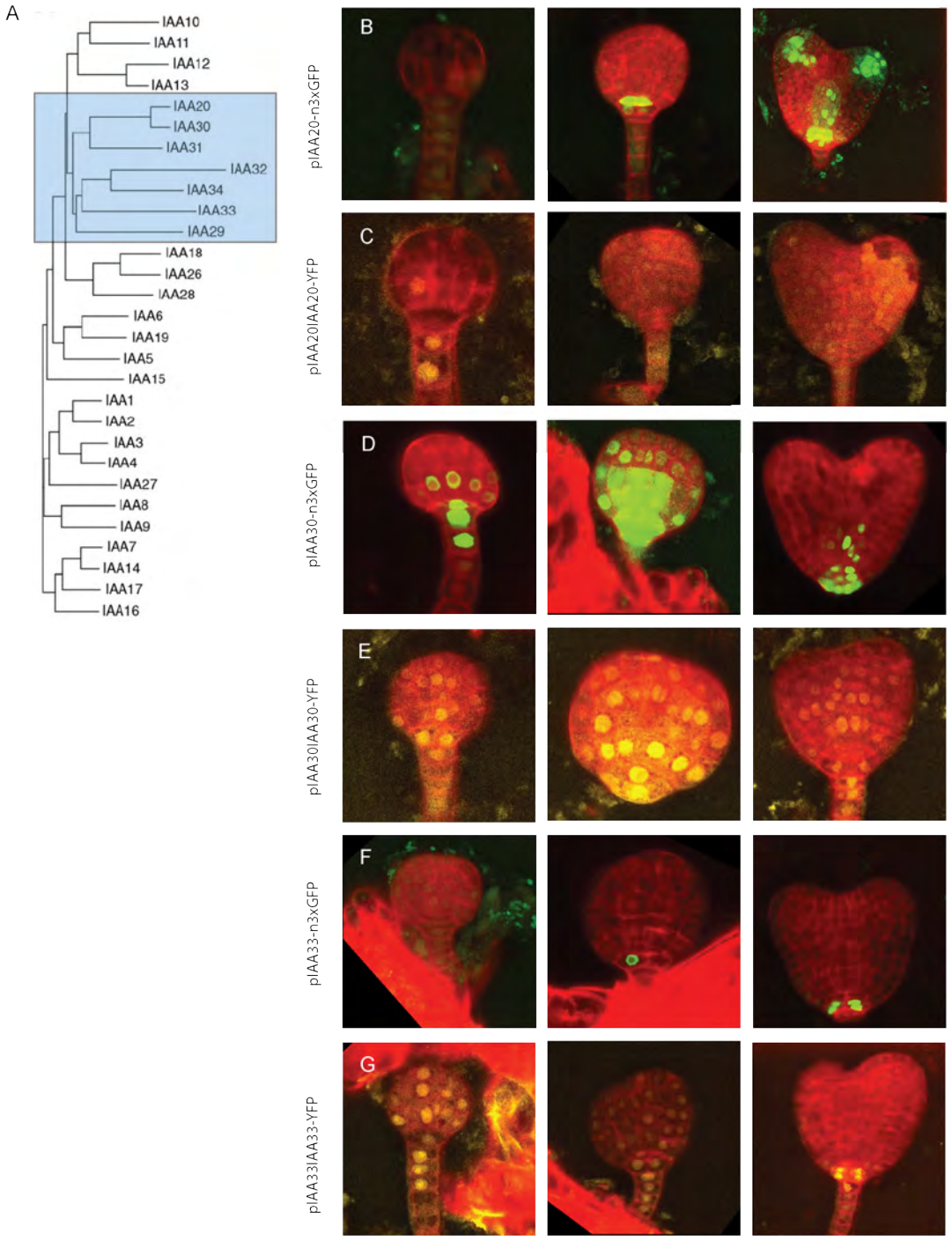

Figure 1. ncIAA are expressed dynamically thoughout embryogenesis.
(A) Phylogenetic tree of Arabidopsis Aux/IAA proteins using neighbor-joining tree (BLOSUM62). The ncIAA clade is highlighted in blue. (B-G) Gene expression (B,D,F; promoter-nuclear 3xGFP fusions) and protein localization (C,E,G; protein-YFP fusions) of IAA20 (B,C), IAA30 (D,E) and IAA33 (F,G) in preglobular (left), globular (middle) and heart (right) stage embryos.

Post-embryonic expression in the root was consistent with this local activity, since *IAA20*, *-30* and *-33* were detected (Fig. 2A-C), while *IAA31* could not be detected (not shown).
IAA20 and *-30* are expressed in the quiescent center (QC), columella and in young vasculature cells (Fig. 2A,B). In addition, *IAA20* and *IAA30* are expressed during lateral root development (Fig. 2G,H) being *IAA30* expressed during the earliest stages of lateral root formation (Fig. 2H). *IAA33* is also expressed in QC and columella, and is additionally detected in root hair cell files (Fig. 2C,I). In all three cases, the expression in the vasculature corresponds to the xylem cells (inset Fig. 2A,B,C). In conclusion, the ncIAA genes are all dynamically expressed, and their activation correlates with developmental events.
All these patterns of gene activity define sites of protein accumulation, as translational fusions of IAA20, -30 and -33 to sYFP showed identical patterns in both embryo (Fig. 1C,E,G) and root tips (Fig. 2D,E,F). Hence, all these ncIAA genes are dynamically expressed, and this leads to the local accumulation of detectable levels of protein. The latter is in contrast to canonical Aux/IAA proteins, which accumulate to extremely low levels (e.g. IAA12/BDL: Weijers et al., 2006).

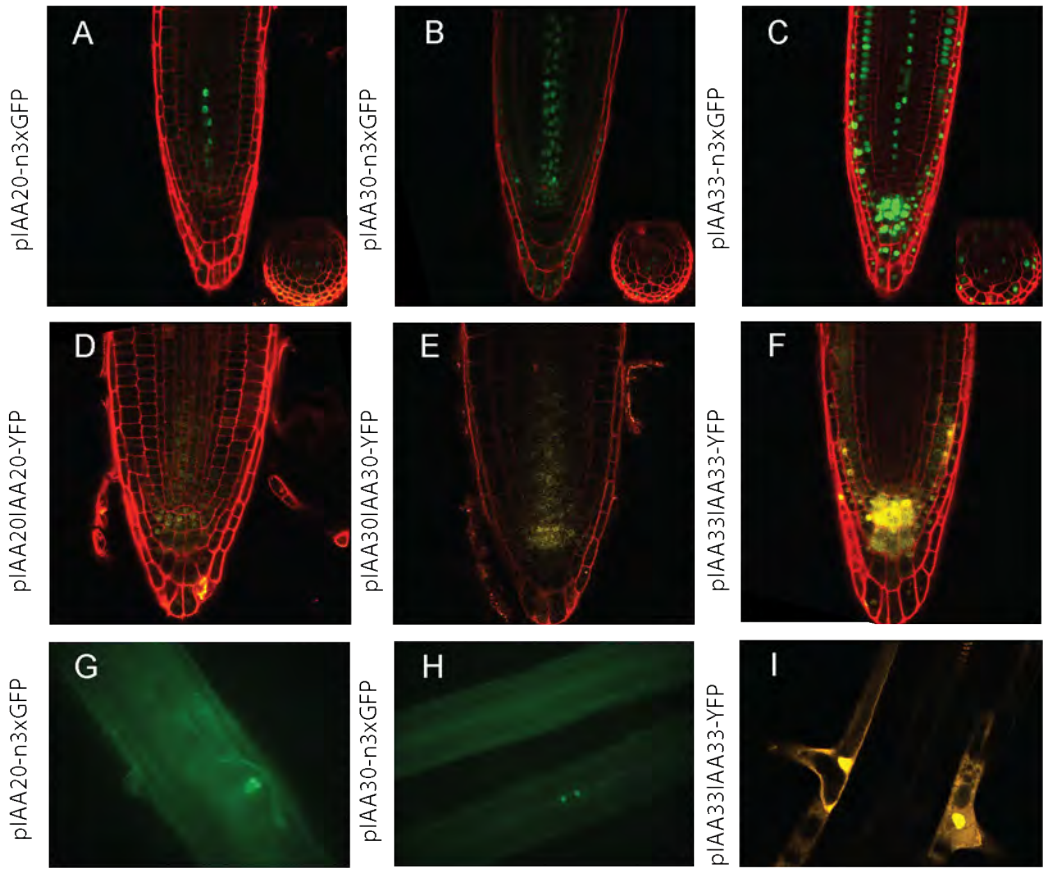

Figure 2: ncIAA expression in roots.
Expression pattern of IAA20 (A,D,G), IAA30 (B,E,H) and IAA33 (C,F,I) as determined using transcriptional fusions of promoters with nuclear 3xGFP (A-C,G,H), or by translational fusions YFP (D-F,I) in post-embryonic roots. Insets in (A-C) show a cross-section of the meristem. (G,H) Details of lateral root iniation sites. (I) Detail of epidermis showing IAA33-YFP protein accumulation in root hair cells.

Auxin-dependent expression of ncIAA genes

All cells in which ncIAA reporters are expressed are known to display elevated auxin activity (or "auxin maxima"), based on the expression of generic auxin-dependent reporters such as DR5-GFP (Ottenschläger et al., 2005). This strong spatial correlation suggests that ncIAA genes may respond to auxin activity. To test if indeed ncIAA genes are an immediate output of auxin activity, we determined mRNA expression of these genes using qRT-PCR in seedlings incubated with exogenous auxin in a time course. As *IAA31* expression was undetectable in roots, we focused the analysis on *IAA20, -30* and *-33*. While both the amplitude and kinetics of induction varied for the three genes, all were induced by auxin (Fig. 3A). *IAA20* expression is induced after 30 minutes and reaches eight-fold induction after two hours of incubation. *IAA30* and *IAA33* were both about 2.5-fold induced after respectively 30 minutes and six hours of auxin incubation (Fig. 3A). Hence, these three ncIAAs appear to be output of auxin action, but since neither is very rapidly upregulated, it is unclear if this is a direct transcriptional response. To test this directly, we repeated the auxin treatment in the presence of the drug cycloheximide (CHX). CHX blocks proteins synthesis, and only direct transcriptional regulation should occur in its presence (Franco et al., 1990). Auxin induction of ncIAA expression still occurred despite the presence of CHX (Fig. 3B), which strongly suggests that ncIAA gene activation is a direct transcriptional auxin response.

Primary transcriptional responses to auxin are mediated by ARF transcription factors (Ulmasov et al., 1999). Indeed, *IAA20* and *IAA30* were initially identified because their expression was downregulated in embryos in which ARF activity had been inhibited by misexpression of *iaa12/bdl* (Lokerse 2011). To test if indeed ncIAA transcription is regulated by ARFs, we analyzed their expression in *arf* knock out mutants. We included *arf1, -2, -6, -9, -13,* and *-18* in this analysis as these ARFs are all expressed in the suspensor (Rademacher et al., 2011), in which *IAA20, -30* and *-33* are all expressed (Fig. 1B,D,F). Interestingly, steady-state expression levels of all three ncIAAs was altered in several *arf* mutants. Notably, while *IAA20* and *IAA33* transcripts were downregulated in *arf2* and *arf6* mutants (Fig. 3C), *IAA30* was markedly upregulated in *arf2*, *arf6* and *arf9* mutants (Fig. 3C). This demonstrates that ARF activity is required for normal ncIAA expression. Finally, we tested if the response to exogenous auxin also requires ARF activity by repeating the auxin time series in all mutant *arf* backgrounds. As expected, the timing and level of auxin-dependent ncIAA transcription relative to both wild-type control and untreated mutant control was altered in several *arf* mutants (Fig. 3D). These data collectively show that ncIAAs are direct, ARF-dependent targets of auxin-responsive transcription.

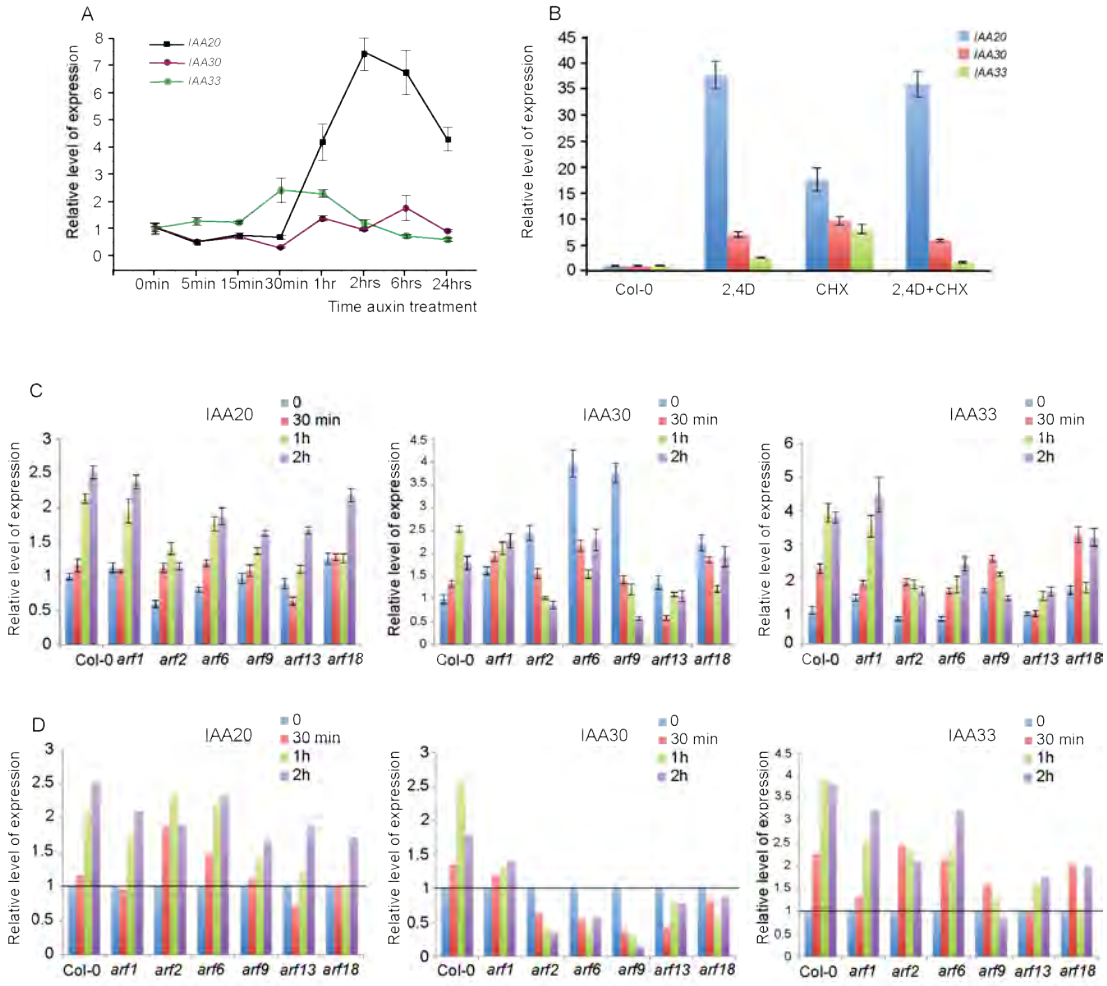

Figure3. *ncIAA* genes are direct targets of auxin response.
Expression levels of ncIAA transcripts in seedlings as determined by qRT-PCR. (A) Time-course of auxin treatment (1 µM 2,4-D) in wild-type seedlings. Expression of *IAA20*, *-30* and *-33* was normalized using reference genes and mock-treated controls. Level at timepoint 0 was set to 1. (B) Expression of IAA20, -30 and -33 in wild-type seedlings treated with 1 µM 2,4-D in the presence or absence of 50 µM cycloheximide (CHX). Expression was normalized to reference genes and the untreated control was set at 1. (C) Expression of IAA20, IAA30 and IAA33 in time-courses of auxin treatment (1 µM 2,4-D) during 0, 30, 60 or 120 minutes in wild-type and a series of arf mutants. Expression levels was normalized using reference genes and set to 1 in the untreated wild-type. (D) Same as (C), but expression level normalized to the untreated respective *arf* mutant.

NC-Aux/IAA proteins act as inhibitors of auxin response.

It is unclear what role the ncIAA proteins would play in auxin response. Canonical Aux/IAA proteins act by inhibiting the nuclear, DNA-binding ARF transcription factors (Ulmasov et al., 1997b). To determine if ncIAAs may act similarly, we first determined their subcellular localization in lines expressing translational fusions to sYFP. A significant fraction of the proteins could be detected in the nuclei of cells in the previously defined ncIAA expression domain (Fig. 1C,E,G; Fig. 2D,E,F). However, in all three cases the accumulation is not limited to the nucleus but also found in the cytoplasm. This pattern of subcellular localization is observed in cells of both embryo and root (Fig. 1C,E,G; Fig. 2D,E,F). In addition, this localization pattern is not due to the YFP protein, as fusions to tdTomato showed the same pattern (Fig. 4C). We tested if this accumulation pattern is the consequence of active shuttling between cytoplasm and nucleus. To this end we used the drug leptomycin in order to

interfere with nuclear export. Even after 16 hours of incubation, pIAA33IAA33-YFP protein is still detected in both cytoplasm and nucleus at levels comparable with control treatments (Fig. 4B). Hence, the cytosolic localization does not appear to be the consequence of active shuttling. To test if the cytosolic fraction is a product of the nuclear fraction, as for example the result of (partial) degradation, we generated plants in which IAA33 was fused to both tdTomato and an SV40 nuclear localization signal (NLS). This NLS efficiently targets proteins to the nucleus (see e.g. SV40-3xGFP in Fig. 1). In these lines, IAA33 protein was solely detectable in nuclei (Fig. 4D), which excludes the option that the cytosolic fraction is a product of nuclear IAA33.

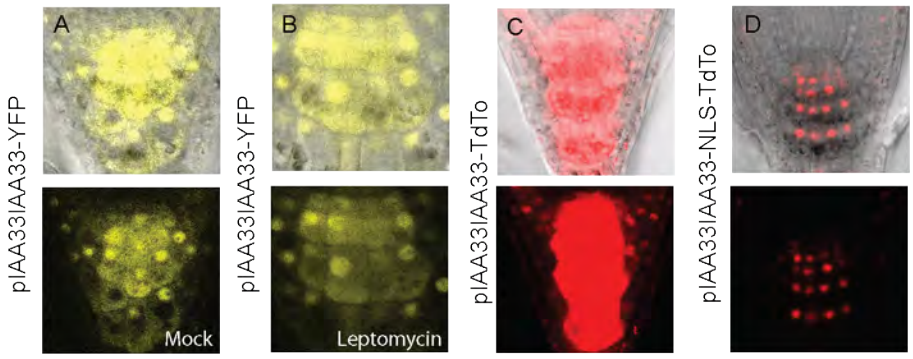

Figure 4. Nuclear and cytosolic ncIAA protein localization
Subcellular localization of IAA33:YFP (A,B), IAA33-tdTomato (C) and IAA33-NLS-tdTomato (D) in collumela cells of roots tips. (A) Control-treated root and (B) root treated with 30 nM leptomycin during 16 hours. The lower panels show the fluorescent channel without trasmitted light.

Given that a major fraction of ncIAA proteins localizes to nuclei, and because their potential to bind ARFs was suggested by yeast 2-hybrid assays (Vernoux et al., 2011), it is plausible that ncIAAs inhibit ARF activity. To test this hypothesis, we generated gain- and loss-of-function lines to see if changes in the levels of these proteins induce auxin-related defects. Overexpression of wild-type canonical Aux/IAA proteins does not induce defects (Yan et al., 2013; Rogg et al., 2001), but mutants in which the domain II is mutated cause pleitropic auxin-deficiency phenotypes (Timpte et al., 1994; Tian et al., 2002; Park et al., 2002). Since ncIAAs are predicted not to be subject to auxin-dependent degradation (Dreher et al., 2006), one could expect such phenotypes upon misexpression of the wild-type proteins. IAA20, 30 and 33 cDNAs were misexpressed using the strong RPS5A promoter. This promoter is expressed broadly during embryogenesis and post-embryonically restricted to meristems (Weijers et al., 2001). During embryogenesis, only weak phenotypes in the planes of division in the hypophysis were observed in the misexpression lines (Fig. 5B,C). In contrast, strong auxin-related phenotypes were found during post-embryonic development. RPS5A-IAA20 plants showed a severe dwarf phenotype (Fig. 5D), similar to mutants that are impaired in auxin signaling (Okushima et al., 2005; Zhang et al., 2007; Tian et al., 2002) (Fig. 5D).

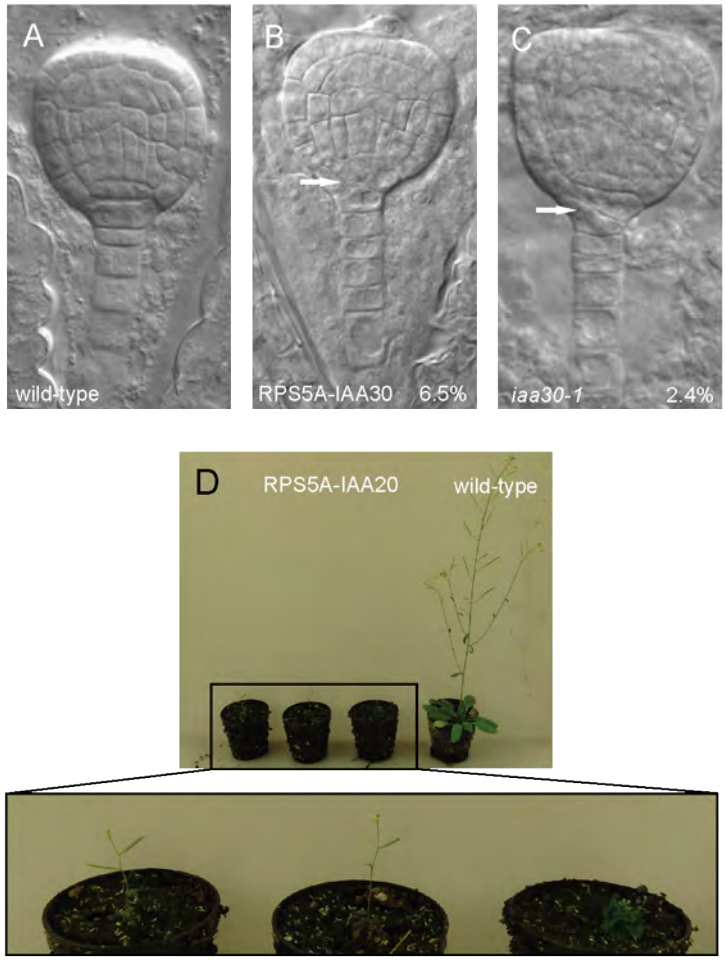

Figure 5: Developmental defects upon altering ncIAA levels
(A-C) transition-stage wild-type (A), RPS5A-IAA30 (B) and *iaa30-1* (C) embryos. The percentage of defective embryos is indicated (6.5% N=218 and 2.4%,N=167 respectively, defects in hypophysis division are indicated with arrowheads). (D) wild-type (right) and RPS5A-IAA20 plants showing a strong dwarf phenotype.

In both IAA20 and IAA30 misexpressors, anomalies in lateral root development were observed. Defects were obvious by macroscopic observation, as both misexpressors had far fewer lateral roots (Fig. 6A). Indeed, when examining the distribution of lateral root primordia along the root of 5-day old seedlings, in some lines up to 50 % of roots of the misexpressor lines showed no lateral roots at all (Fig. 6 A,B). Also, while in those roots that had lateral root primordia, there was a shift towards younger primordia stages in the misexpressors (shown for IAA30, Fig. 6C) compare to wild-type plants (Fig. 6C).
Such a phenotype can be due to defects in the initiation of lateral roots or arrest of development prior to its emergence from the main root. To discriminate these options, we investigated lateral root development using a root-bending assay. In this assay, 3-day old seedlings are turned 90 degrees, upon which a new lateral root initiates on the outside of the root bend (Ditengou et al., 2008). As the change of gravity vector causes synchronous initiation, this allows the comparison of the progression of lateral root development after bending between genotypes. Since *IAA30* is expressed during early lateral root intiation (Fig. 2H), we focused the analysis on this gene, using overexpressor lines and insertion lines (Fig. 6F). After 16 hours, all wild-type plants

initiated lateral roots in the region of bending while a fraction of the overexpression roots failed to initiate primordia (Fig. 6D). After 48 hours, all stages can be observed in wild-type plants with the highest percentage in stage three (Fig. 6E). IAA30 misexpressor lines also have a distribution of stages, but these are shifted to earlier stages. These results show that increased levels of IAA30 protein inhibits both lateral root initiation and progression of lateral root development. This phenotype strongly resembles gain-of-function *iaa14/slr* (Fukaki et al., 2002) and *arf7 arf19* double mutants (Okushima et al., 2005), and suggests that this ncIAA inhibits auxin-dependent lateral root initiation.

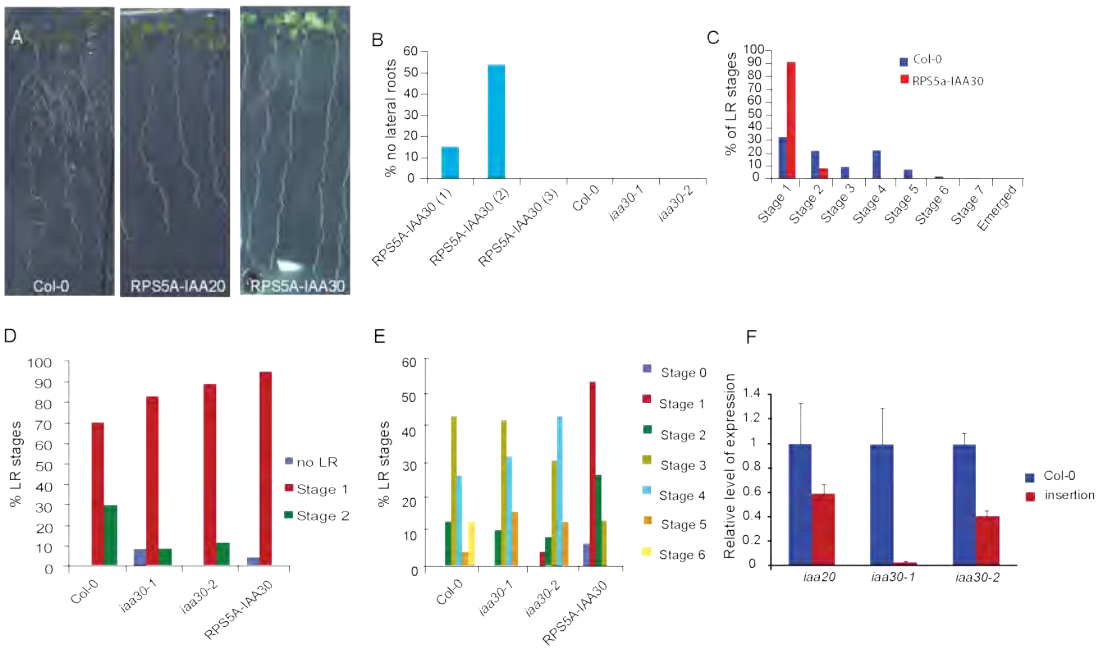

Figure 6: IAA30 regulates lateral root development.
(A) Wild-type, RPS5A-IAA20 and RPS5A-IAA30 seedlings. (B) Percentage of 5-day old seedlings without emerged lateral roots in wild-type, iaa30 mutants (iaa30-1,-2) and 3 independent RPS5A-IAA30 lines (N=20). (C) Distrubution of lateral root stages in roots of 5-day old wild-type and RPS5A-IAA30 seedlings (N=20). (D,E) Distribution of lateral root stages at 16 (D) or 48 (E) hours after manual bending of the root in wild-type (Col-0), two iaa30 alleles and RPS5A-IAA30 (N=30).

To test if indeed ncIAA proteins inhibit auxin response, we tested auxin sensitivity in a root growth assay. We analyzed root growth of plants misexpressing IAA20 or IAA30 after 5 days of transfer to media containing 40 nM 2,4-D, and compared this to growth on non-supplemented media. The results showed that, while root growth in the absence of auxin is not significantly affected (with the exception of IAA20, Fig. 7A), both IAA20 and IAA30 misexpressors are less sensitive to auxin-dependent root growth inhibition (Fig. 7B), consistent with a function in inhibiting auxin response. To test if ncIAA proteins also contribute to auxin response in otherwise wild-type plants, we analysed auxin-dependent root growth in insertion lines for *iaa20* and *iaa30*. While neither of these lines showed obvious phenotypes, presumably do to genetic redundancy, *iaa20* mutant seedlings were slightly more sensitive to auxin (Fig. 7B), which implies that this ncIAA is required for restricting auxin response.
To investigate if ncIAA inhibit auxin response at the level of ARF-dependent gene regulation, we tested the expression of the auxin-responsive, canonical *IAA19* gene by qRT-PCR in auxin-treated ncIAA misexpressor lines, as well as in *iaa20*, *iaa30* and *iaa33* loss-of-function mutants. In line with the phenotypic effects and auxin

sensitivity assays, auxin-induction of *IAA19* expression was significantly reduced in all three misexpression lines (Fig. 7C). Importantly, bot *iaa30* and *iaa33* mutants showed an exaggerated auxin responsive *IAA19* expression after 30 minutes of treatment (Fig. 7C). Hence, the ncIAA proteins act as inhibitors of transcriptional auxin response.

Finally, to determine if ncIAAs also inhibit the expression of auxin-dependent gene expression during normal development, we analyzed the expression of the pIAA2-GFP reporter in RPS5A-IAA30 embryos. *IAA2* is often used as an auxin response marker (Swarup et al., 2001; Shibasaki et al., 2009; Frank et al., 2000), and the gene is expressed at major sites of auxin response in the wild-type embryo (Fig. 7D). In RPS5A-IAA30 embryos, however, the signal was strongly downregulated in the hypophysis (Fig. 7D), supporting the function of ncIAAs in inhbiting auxin-dependent gene regulation. No upregulation could be observed in the *iaa30* mutant (Fig. 7D), which could mean that there is genetic redundancy with other ncIAA genes during embryogenesis.

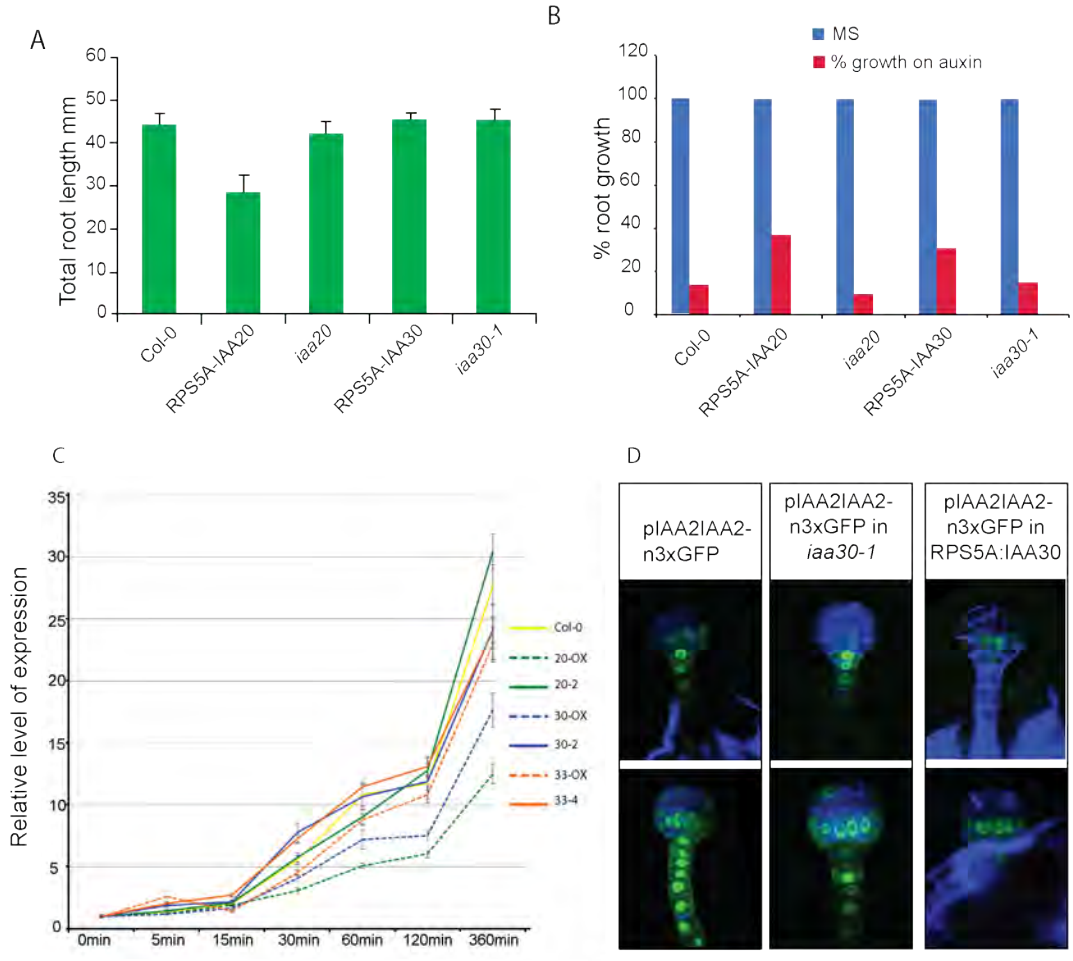

Figure 7. ncIAAs modulate auxin response

(A) Root length of 5-day old seedlings of wild-type (Col-0), iaa20 and iaa30 mutants, and RPS5A-IAA20 and RPS5A-IAA30 lines (N=20). (B) Relative root length of wild-type (Col-0), iaa20 and iaa30 mutants, and RPS5A-IAA20 and RPS5A-IAA30 lines after 5 days of growth on media containing 40 nM 2,4-D, normalized to the length of roots grown on control media (100%; N=20). (C) Expression of IAA19 as determined by qRT-PCR in a time-course auxin treatment (1 µM 2,4-D) in wild-type (Col-0), iaa20 (20-2), iaa30 (30-2) and iaa33 (33-4) mutants, and RPS5A-IAA20 (20-OX), RPS5A-IAA30 (30-OX) and RPS5A-IAA33 (33-OX) seedlings. Expression was normalized using reference genes and the level in untreated wild-type was set to 1. (D) Expression of pIAA2-n3xGFP in wil-type (left panel), iaa30 mutant (middle) and RPS5A-IAA30 (right) embryos.

ncIAA proteins are not auxin-labile

The ncIAA proteins, like canonical Aux/IAAs, act as inhibitors of auxin response following their transcriptional induction by ARF transcription factors. However, unlike the canonical Aux/IAAs, ncIAAs lack a well-conserved domain II that mediates auxin-dependent protein degradation (Ramos et al., 2001). To test if these proteins are subject to auxin regulation, we monitored the accumulation of fusions of IAA20, 30 and 33 to tdTomato in root tips during incubation with auxin.

In the cases of IAA20 and IAA30, rather than triggering degradation, the incubation with auxin increased the level of protein accumulation (Fig. 8A), starting already after 3 hours of incubation, and is enhanced steadily until 6 hours. In contrast, IAA33 protein accumulation is nearly unaffected during the first 3 hours, and decreases in the next 3 hours (Fig. 8A). This is in strong contrast to the short half-life (minutes) as reported for canonical Aux/IAAs (Abel et al., 1994). This shows that ncIAA proteins are very stable, and that their degradation is not triggered by auxin. To test protein stability, roots were incubated with cycloheximide (CHX) to block *de novo* synthesis of proteins. Strikingly, after 12 hours there is still appreciable protein accumulation (Fig. 8B), confirming the high protein stability.

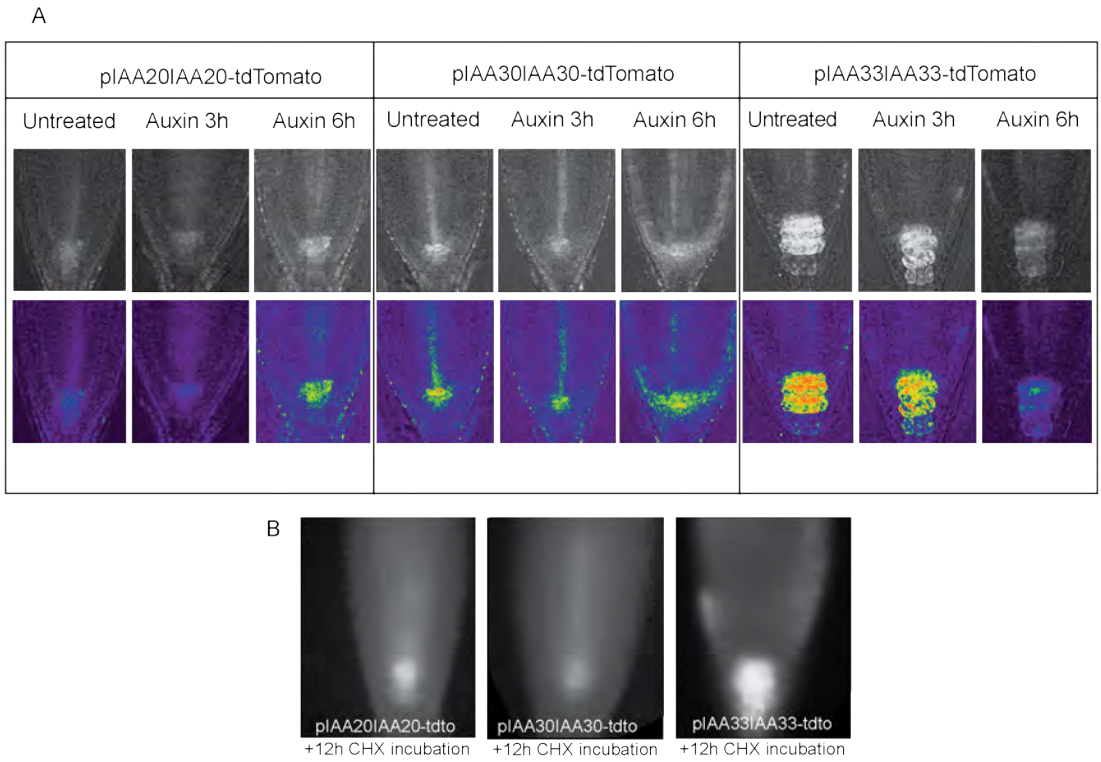

Figure 8. ncIAA proteins are not auxin-labile

Accumulation of IAA20-tdTomato, IAA30-tdTomato and IAA33-tdTomato proteins as determined by fluorescence intensity in root tips. Seedlings were treated with 1 µM 2,4-D for 3 or 6 hours, or without auxin. Upper panel shows fluorescence signal and lower panel shows false color intensity scale. (B) Accumulation of IAA20-tdTomato, IAA30-tdTomato and IAA33-tdTomato proteins after 12 hours of cycloheximide (CHX) incubation (B).

Regulation of ncIAA protein stability

The high stability of ncIAA proteins is paradoxical, as the induction by auxin would rapidly suppress auxin response without the possibility to re-establish responsiveness. To determine whether other triggers might promote ncIAA degradation, we first tested a range of plant hormones. The stability of several proteins is regulated by hormones other that auxin. For example, EIN3 stability is promoted by ethylene (Guo and Ecker, 2003), and ARR1 by cytokinin (Sakai et al., 2001), while jasmonic acid promotes degradation of JAZ proteins (reviewed by Chini et al., 2007), gibberelic acid triggers DELLA protein degradation (Dill et al., 2001) and brassinosteroids control BES/BZR degradation (He et al., 2002; Yin et al., 2002).

To explore the possibility of nc-IAA being regulated by other hormones we determined the effect of a 16-hour incubation with a range of hormones (abscissic acid [ABA], brassinolide [BL], jasmonic acid [JA], kinetin [a cytokinin] and gibberelic acid [GA3]) on the level of fusion protein accumulation. We did not detect a reduction in protein level in any of the hormone treatments (Fig. 9). The only noticeable change was an increase of IAA30 protein in the case of ABA (Fig. 9) and of IAA33 protein in the case of BL (Fig. 9). However, the long time of hormone incubation makes it likely that these are secondary effects, perhaps due to increased auxin levels. In conclusion, no other hormone appears to trigger ncIAA degradation.

Another possibility of regulation for proteins is the circadian clock as in the case of TOC protein (Millar et al., 1995a). To determine if time of day determines ncIAA levels,we performed a time course of 12 hours where the accumulation of the protein in the root was analyzed every three hours. There were no significant changes between the different time points (data not shown), which indicates that the circadian clock does not play a central role as ncIAA stability regulator.

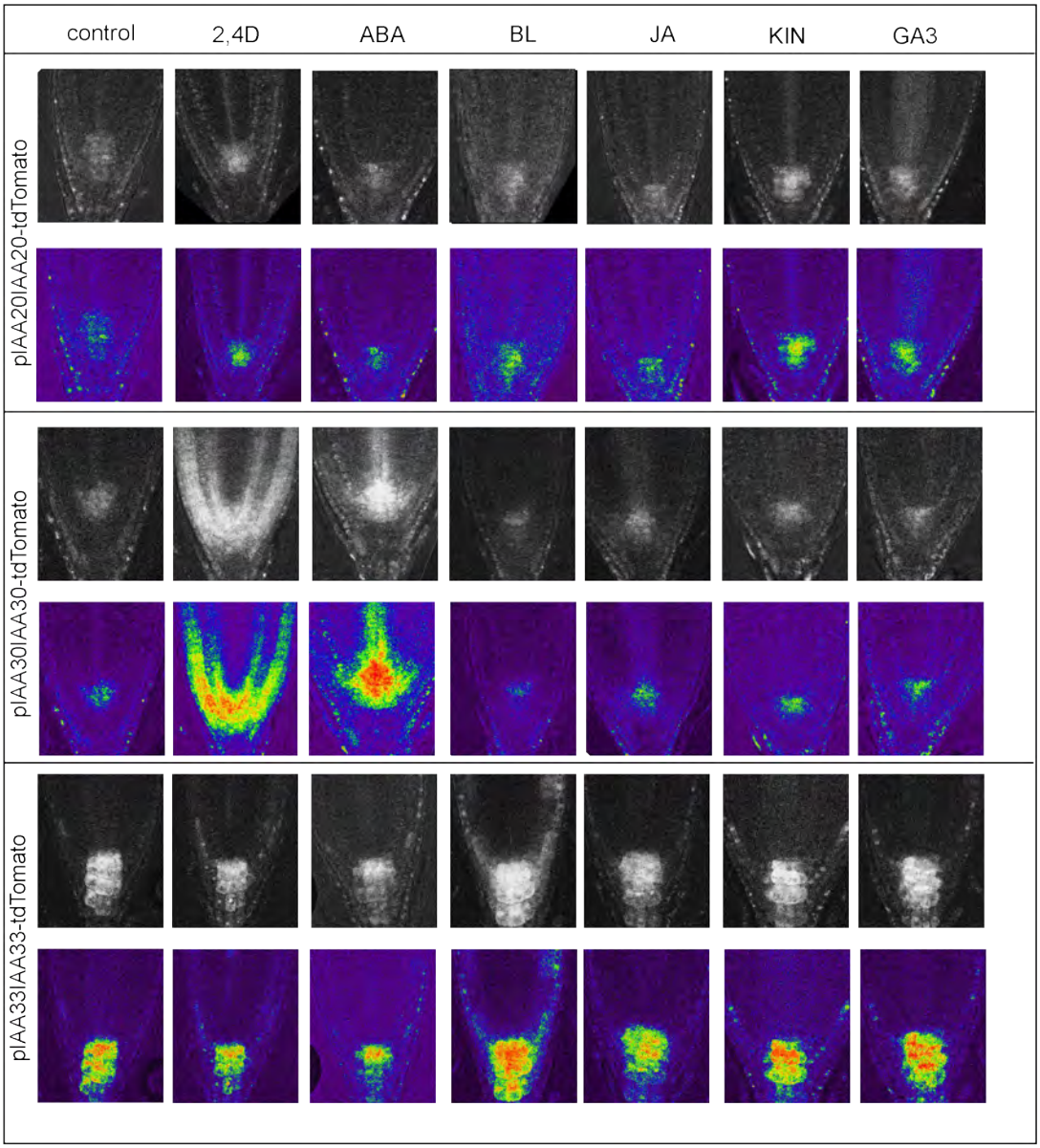

Figure 9: Hormones do not control ncIAA stability
Accumulation of IAA20-tdTomato, IAA30-tdTomato and IAA33-tdTomato proteins in root tips after 16 hours of incubation in unsupplemented control media, or in media containing 1 µM auxin (2,4-D), 1 µM Abscissic Acid (ABA), 1 µM brassinolide (BL), 1 µM Jasmonic Acid (JA), 1 µM kinetin (KIN) or 1 µM Giberrellic Acid (GA3). Upper panel shows fluorescence signal and lower panel shows false color intensity scale.

We next considered cell cycle as a potential regulator of ncIAA stability. There is very profound control of protein stability during the cell cycle, with Cyclins as a clear example of labile proteins (Planchais et al., 2004). In addition, several other protein types are actively degraded to connect cellular functions to cell cycle progression (del Pozo et al., 2002). To determine if the cell cycle might contribute to ncIAA stability, we quantified the level of IAA33-tdTomato protein in epidermal cells. When cells divide, the nuclear envelope breaks down and any protein present in the nucleus diffuses throughout the cytosol. We quantified fluorescent intensity in confocal sections of IAA33-tdTomato roots in cells in interphase, as well as in cells undergoing cytokinesis (evident by loss of nuclear membrane). Strikingly, the

pIAA33IAA33-tdTomato signal is reduced almost to background level during cytokinesis (Fig. 10A,C). To determine if this is specific to IAA33, or a more general property of nuclear proteins during cytokinesis, we used the nuclear pARF6ARF6-GFP protein (Fig. 10B). In the case of pARF6ARF6-GFP the overall fluorescent signal decreases slightly during cytokinesis, but the level is still comparable to the nuclear intensity in interphase cells (Fig. 10C). This result strongly suggests that pIAA33IAA33-tdTomato protein is degraded during cytokinesis, and suggests a role for the cell cycle in ncIAA protein stability.

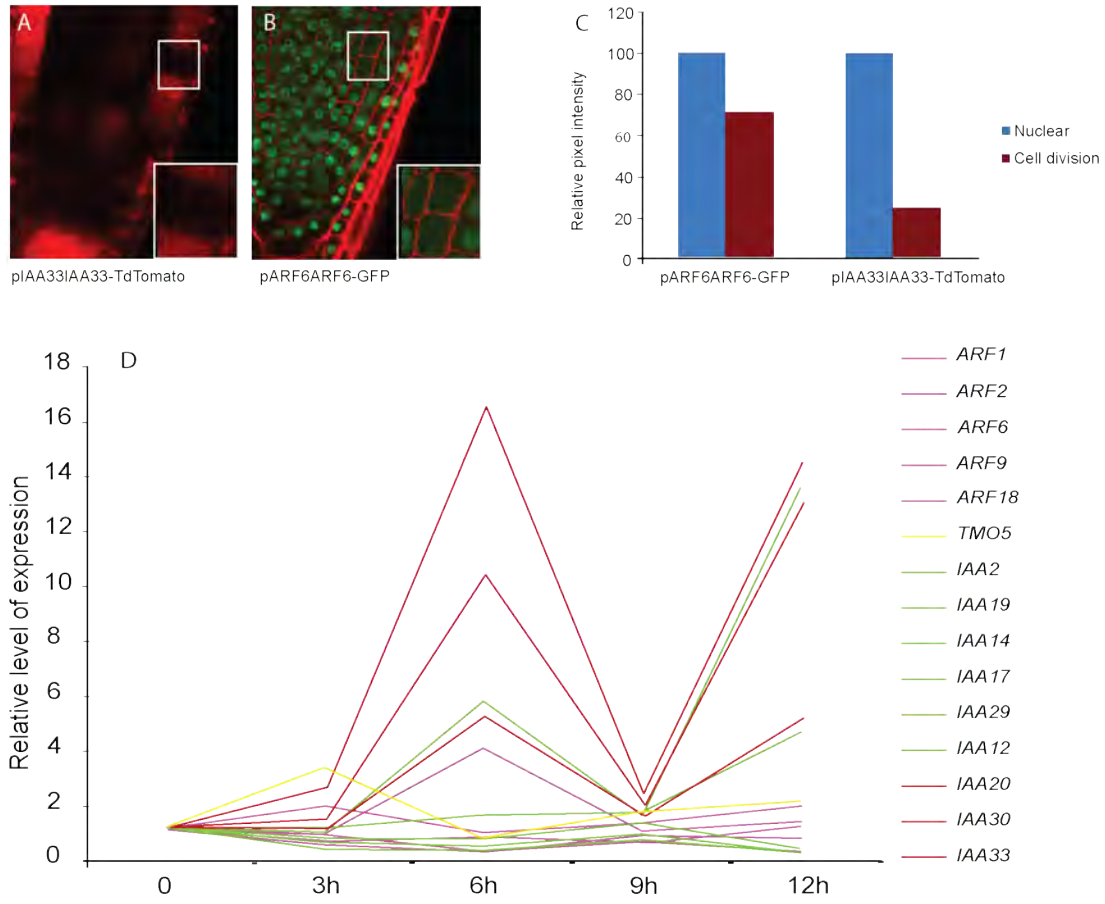

Figure 10: Cell-cycle regulation of ncIAA protein stability and gene expression
(A,B) Protein accumulation in pIAA33IAA33-tdTomato (A) or pARF6ARF6-GFP roots. Inset shows cell undergoing cytokinesis. (C) Quantification of the protein levels in dividing cells calculated as average of pixel intensity inside cells from images such as (A) and (B) (N=12). (C). Expression level of ARF1,2,6,9,18 (purple), canonical IAA2,12,14,17,19,29 (green), TMO5 (yellow) and non-canonical IAA20,30,33 (red) transcripts in roots at 0, 3, 6, 9 and 12 hours after transfer to hydroxyurea (HU)-containing media. Expression was determined using qRT-PCR and normalized using reference genes. The expression level at 0 hours after transfer was set to 1.

Cell cycle gating of auxin response

If cell cycle progression triggers degradation of ncIAA proteins, these proteins could be part of a mechanism that connects the cell cycle to auxin response. Whether such a connection exists has not been explored, but one prediction would be that auxin activity, or auxin responsiveness should depend on cell cycle progression. To determine if cell cycle-regulation of auxin response is plausible, we examined the expression of several components of auxin response in roots at various timepoints after releasing a cell cycle block (Fig. 10D). Cell cycle synchronization of root cells can be achieved by use of the drug hydroxyurea (HU; Cools et al., 2010). After

transfer of seedlings to media containing HU, there is an initial lag phase where all the cells reach the G1-S transition, while after 6 hours all cells progress synchronously to S phase (Cools et al., 2010). We tested mRNA expression of several *ARFs*, the ARF5 target *TMO5* (Schlereth et al., 2010), the auxin-responsive canonical *Aux/IAA* genes, as well as the *ncIAA* genes themselves by qRT-PCR at 0, 3, 6, 9 and 12 hours after transfer to HU.
Of the 5 *ARFs* tested (*ARF1, 2, 6, 9, 18*), only one showed fluctuation in this experiment. *ARF18* was 4-fold more highly expressed 6 hours post transfer (HPT) compared to the start of the experiment (Fig. 10D). This suggests that ARF levels are relatively inert to cell cycle regulation. Likewise, 2 out of 6 canonical Aux/IAA transcripts tested (*IAA2, 12, 14, 17, 19, 29*) changed significantly during cell cycle progression. *IAA29* was induced both at 6 and 12 HPT, while *IAA2* was upregulated at 12 HPT. Also the direct ARF target *TMO5* (Schlereth et al., 2010) was mildly modulated during the cell cycle. Strikingly however, all 3 ncIAA transcripts tested (*IAA20, 30* and *33*) were dramatically regulated by cell cycle progression. Expression peaked at 6 and 12 HPT (Fig. 10D) and reached levels that were more than 16-fold higher than at the start of the experiment.
While the precise timing of expression during the cell cycle, and the mechanisms for regulation remain to be investigated, these results provide the first evidence that auxin response is subject to cell cycle regulation. The apparent control of both ncIAA transcripts and protein stability by cell cycle suggests that these factors may link the cell cycle progression to auxin response.

Discussion

In this chapter we show the first indication of cell cycle control of auxin responses through a novel class of auxin signaling regulators, the non-canonical Aux/IAA proteins (ncIAA). This is an important discovery, as it demonstrates a link between auxin signaling and cell cycle control and shows for the first time a mechanistic relation between these two processes that profoundly control plant growth and development.

We demonstrate that ncIAA proteins perform a biological function important for correct plant development, using a mechanism that is similar to that of the canonical Aux/IAA proteins. ncIAA genes are rapidly induced in the presence of auxin, and ncIAA proteins can inhibit the activity of ARFs. However, there is a fundamental difference between ncIAAs and the canonical Aux/IAAs; while auxin triggers degradation of the latter, ncIAAs are not subject to this regulation. This finding sketches a scenario in which the synthesis of ncIAA proteins is triggered where auxin accumulates, leading to protein accumulation in the nucleus and cytoplasm. Without further regulation, this ncIAA protein pool will accumulate and irreversibly suppress auxin response. If however, we include cell cycle as protein stability regulator in this scenario, the result is that cells accumulate ncIAA after the first auxin response, leading to a block in auxin responses until the next cell division occurs. During cytokinesis, ncIAA are targeted for degradation and this is predicted to re-establish auxin responsiveness in the two daughter cells (Fig. 11). This model represents a one-shot triggering of auxin responses, and an off-switch mechanism to be insensitive to the presence of auxin in cells until the next cell division occurs.

This discovery expands the current knowledge about the relation between cell-cycle and auxin signaling, in providing a convergence point between these two processes. To date there was only sparse information about the connection between them; it was known that several genes important for cell cycle progression are under transcriptional control of auxin (Fuerst et al., 1996; Richard et al., 2002; Cruz-Ramirez et al, 2012). On the other hand, auxin responses have been shown to be dependent on correct cell cycle progression; mutants in the *HOBBIT* gene, encoding a subunit of the Anaphase Promoting Complex (APC) responsible for the degradation of cell cycle proteins, show a reduction in auxin responses (Blilou et al., 2002), maybe due to the persistence of ncIAA in this mutant background. Our results help to draw a more complete picture about the significance of the relation between cell cycle and auxin signaling.

One open question still is to better understand the exact mechanism of function of these proteins. In yeast-two-hybrid (Vernoux et al., 2011), it has been shown that ncIAA proteins have the potential to interact with both ARFs and canonical Aux/IAAs. Interestingly, the profile of interaction appears to be specific as in the case of the canonical Aux/IAA, IAA10 (Chapter 3, this thesis). Another observation that reinforces the notion of specific interactions comes from the study of lateral root development in ncIAA overexpressing lines; while IAA20 and IAA30 both suppress lateral root development, IAA33 does not have any effect on the number or distribution of lateral roots. As defects similar to the ones found in IAA20/30 misexpression lines have been observed in *arf7 arf19* mutants (Okushima et al., 2005), the mechanism of ncIAA action likely involves inhibition of ARFs. Whether additional modes of action occur should become clear from unbiased identification of *in vivo* interaction partners.

Another striking point is that although the misexpression of ncIAA proteins induces strong postembryonic phenotypes, embryo patterning appears relatively inert to these proteins. Overexpression of wild-type canonical Aux/IAA proteins are unable to induce defects in any of the developmental contexts studied (Yan et al., 2013; Rogg et al., 2001). This result can been interpreted to indicate that the auxin-TIR1 degradation pathway is efficient, and not limiting. In this context, it is interesting that misexpression of wild-type ncIAA proteins are at all able to induce defects. As these genes are expressed at moderately high levels during development, this suggests that there is differential capacity of cells to degrade ncIAA proteins. By extension, this would suggest that embryos have a high capacity to degrade ncIAA proteins. This can be a consequence of the quick proliferation of cells during embryogenesis, leading to rapid clearance of ncIAA protein, even if it is present in a higher amount due to the use of overexpression lines. The identification of a “degron” in ncIAA proteins that mediates cell cycle-dependent degradation should help resolve this question.

Future studies should reveal more specific details about the relationship between cell cycle and auxin signaling. It will be important to understand during which cell cycle phase ncIAA proteins are degraded, if the response to auxin varies per cell cycle phase, and if manipulations of ncIAA levels alter this cell cycle “gating”. Finally, it will be important to determine if cell cycle regulation of ncIAA proteins is specific to Arabidopsis, or whether it is a more widespread capacity.

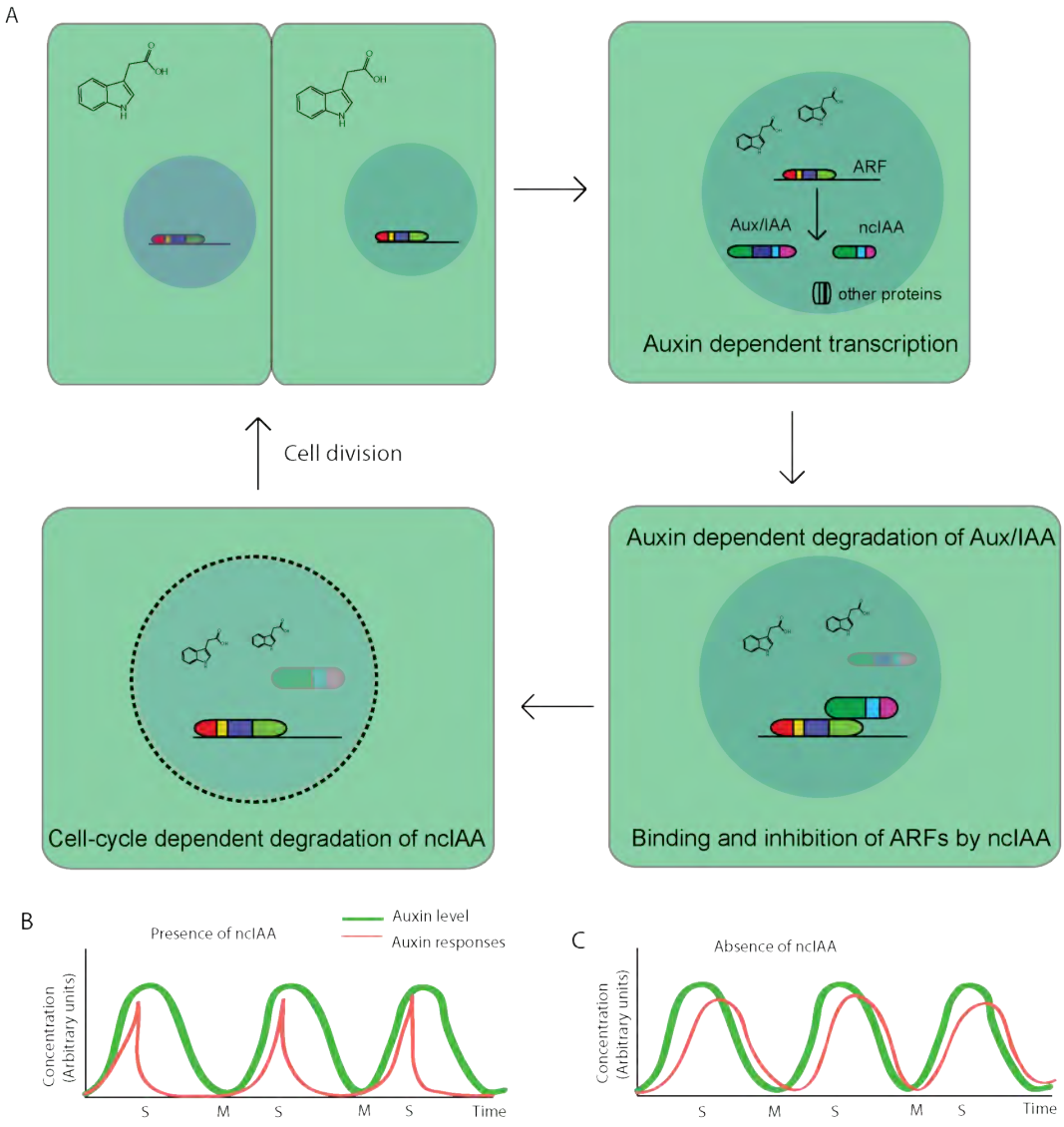

Figure 11: Cell cycle regulation of auxin responses.
(A) Graphic representation of one cycle of division of a hypothetical plant cell in presence of auxin. After cell division auxin accumulates in the nucleus allowing the auxin dependent transcription of genes. Among these genes are present ncIAA that when translated will bind and inhibit ARFs. Since ncIAA are not degraded by auxin cells will be able to respond to auxin only once. When the cell divides the ncIAA will be targeted for degradation, and the new cells will be able to generate another pulse of auxin-dependent gene transcription.
(B,C) Hypothetical graphic representation of the auxin responses produced in a cell in the presence and absence of ncIAA.

Material and methods

Plant material, growth conditions and transformation

All seeds of *Arabidopsis thaliana* are ecotype Columbia. Seeds were sterilized in 25% bleach/75% ethanol solution for 10 minutes, and washed twice with 70% ethanol and once with 100% ethanol. Dried seeds were sown on plates containing MS (0.5x Murashige and Skoog salts, 1% (w/v) sucrose, and 0.8% (w/v) agar) and supplemented with 25 mg/L kanamycin or 15 mg/L phosphinothricin for appropriate

antibiotic selection. For assays of synchronization and interference with nuclear transport we used Hydroxyurea (Sigma; concentration 2mM) and leptomycin (Sigma; concentration 1mM).
After incubation at 4°C overnight, the plants were grown under standard long day conditions (16/8 hrs light/dark) at 23°C. Seedlings were transferred to soil at an age of at least 10 days.
Plants were transformed using a standard floral dipping method, as described by Clough and Bent, 1998.
Details about the insertion lines used are in Table 1. The insertion mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and primers used for genotyping are listed in Table 1.

Table 1. Insertion lines used in this study and primers used for genotyping

Gene	Locus	Allele	Stock #	Internal #	Primers for genotyping	
IAA20	AT2G46990	*iaa20-1*	ET8075	48.2	LP	CGTCACGATAACCTTTCATGC
					RP	CCTATGAAATAGTGATGGGCG
IAA30	AT3G62100	*iaa30-1*	SALK_065384C	51.1	LP	CGGAACAATTGTAATATCTCCG
					RP	AGGGAGAAGCTCATCGTCTTC
IAA30	AT3G62100	*iaa30-2*	SM_3.23645	51.2	LP	CAGCAGTGTTCCAAAACAATTC
					RP	AGGGAGAAGCTCATCGTCTTC
IAA33	AT5G57420	*iaa33-1*	SAIL_713_F02	94.3	LP	CGTGAATCCAATTGTTCATTG
					RP	GCCAGTTAAAGATCACGAAACC
IAA33	AT5G57420	*iaa33-2*	WiscDsLoxHs021_05H	94.4	LP	ATCGGCTGTTACAAAACATCG
					RP	TTGAGTTCCATGTAAGCGTCC

Cloning

All cloning was performed using the LIC cloning system and the vectors described in De Rybel et al. 2011
For transcriptional fusions of IAA20, -30, and -33 2 kb fragments upstream of the ATG were amplified from genomic DNA using Phusion Flash polymerase (Finzymes) and annealed into pPLV4 (Lokerse 2011).
For translational fusions to the YFP and TdTomato, the same promoter fragment was amplified together with the genomic coding sequence excluding the stop codon and annealed into pPLV16, pPLV23. To generate misexpression lines using pRPS5A the coding sequences of all genes were amplified from cDNA clones and annealed into pPLV28. All constructs were verified by sequencing. Primers used are listed in Table 2.

Table 2. Primers used for amplifying genomic DNA and cDNA

Gene	Locus	DNA	Construct	Primers	
IAA20	AT1G68920	Genomic	TdTo	FW	TAGTTGGAATGGGTTCGAATGTGTGTATCTTTCCAATAAAATGCAA
				RV	TTATGGAGTTGGGTTCGAACGTAGTGGTAATTAGCTCTTG
IAA30	AT3G57800	Genomic	TdTo	FW	TAGTTGGAATGGGTTCGAAGCTTCCCTTCTCTCATTTTC
				RV	TTATGGAGTTGGGTTCGAACGTAGTGATAAGCTCTTGAGA
IAA33	AT4G34530	Genomic	TdTo	FW	TAGTTGGAATGGGTTCGAACCTATTAAGGCTTTATTGAG
				RV	TTATGGAGTTGGGTTCGAACCTCGTTTCTTTTAACTTGTC
IAA20	AT2G41240	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGGGAAGAGGGAGAAGTTCATCG
				RV	AGTATGGAGTTGGGTTCTCAGTAGTGGTAATTAGCTC
IAA30	AT1G68920	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGGGAAGAGGGAGAAGCTC
				RV	AGTATGGAGTTGGGTTCTCAGTAGTGATAAGCTCTTG
IAA33	AT3G57800	cDNA	RPS5a	FW	TAGTTGGAATAGGTTCATGAATAGTTTCGAGCCACA
				RV	AGTATGGAGTTGGGTTCTCACTCGTTTCTTTTAACTT

Microscopy

The differential interference contrast (DIC) and fluorescence microscopy was performed according to procedures explained in Chapter 2 (Llavata-Peris et al., 2013). The imaging of fluorescent reporters and protein accumulation in embryos and roots was conducted using Leica SP5-II system (HyD detector). The settings used were: tdTomato excitation at 561 nm and detection at 568-600 nm; YFP excitation at 514 nm and detection at 525-550 nm; GFP excitation at 488 nm and detection at 500-535nm.

Embryo phenotypes were analyzed using a Leica DMR microscope equipped with DIC optics. Samples were prepare using chloral hydrate solution (chloral hydrate, water and glycerol, 8:3:1). Homozygous T3 lines were used in embryos and root preparations. For counterstaining we used 1 µM FM4-64 (Invitrogen).

qRT-PCR analysis

Primer pairs were designed using Beacon Designer 7.0 (Premier Biosoft International). qRT-PCR analysis was performed as described previously (De Rybel (De Rybel et al., 2010). RNA was extracted using RNeasy kit (Qiagen). Poly(dT) cDNA was generated from 1 µg of total RNA with an iScript cDNA Synthesis Kit (Biorad) and analyzed on a CFX384 Real Time PCR detection system (BioRad) with iQ SYBR Green Supermix (BioRad) according to the manufacturer's instructions. All reactions were performed in triplicate. Data were analyzed using the software qBase (Hellemans et al., 2007). Gene expression levels were normalized using as internal control *EEF1α4* and *CDKA1;1*. Primer sequences are listed in Table 3.

Table 3. Primers used for qRT-PCR

Gene	Locus	Forward Primer	Reverse Primer
IAA20	AT1G68920	TCGGTGCGGCAGAGGAAATGAT	TTCTCCCAATAGGAACTCCTTCCATG
IAA30	AT3G57800	CTGTGGAGGAGGAAGAAGAGAATGA	TCAGCCCAGAGAATGGATGC
IAA33	AT4G34530	AGATCATTCCAAGGCTTT	CTTATGCGTTGACATATAGAA
ARF1	AT1G59750	GCAGTCAATGCAAGGATG	CCCGCCATAGATGATGAT
ARF2	AT5G62000	AACAACTTGAATGATGCT	GATTATTAGTCTGGAATGGT
ARF6	AT1G30330	CCATCTTCCAACAATGTC	ATACCGTTACCGAGATTC
ARF9	AT4G23980	AATAACTCTTACTCTGTGTCAA	GTTGTCTCAGGCTTCTTC
ARF18	AT3G61830	CAACCGATGGATACTTGT	CTTCTTCTGCTCACTCAT
IAA2	AT3G23030	GCTATGTCTTGGATTACCC	CACTGTTGTTGTTCTTACG
IAA19	AT3G15540	GTGGTGACGCTGAGAAGGTT	CGTGGTCGAAGCTTCCTTAC
IAA14	AT4G14550	TCAGAAGAGCGGCGAAGC	GACATCACCAACGAGCATCC
IAA17	AT1G04250	TCTTCCCGGTGGAGATACAG	TTGATTTTTGGCAGGAAACC
IAA29	AT4G32280	ACAACATATCTAATCCAACAAC	TTCATATTCGGTAAACATAGTG
IAA12	AT1G04550	TTGGGTCTAAACGCTCTGCT	AAGCCCTGAACTTTCGGATT
CYCA3	AT1G47220	GATTCCGACAATCGAAAC	CGTCAATTAGAGGTAAAGTCAAG
CDKA	AT3G48750	ATTGCGTATTGCCACTCTCATAGG	TCCTGACAGGGATACCGAATGC
EEF	AT1G30230	CTGGAGGTTTTGAGGCTGGTAT	CCAAGGGTGAAAGCAAGAAGA

Root bending assay

The root bending assay to induce synchronous lateral root initiation was performed as described in Ditengou et al., 2008.

Synchronization assay

Synchronization of roots was performed as described in Cools et al., 2010. Seedlings were plated on sterilized membrane and grown for 5-6 days. Next, the membrane was transferred to MS plates containing 2mM HU until harvesting. Root tips were cut and collected for posterior RNA extraction.

Auxin sensitivity assay

Auxin sensitivity assay was performed as described in the previous chapter.

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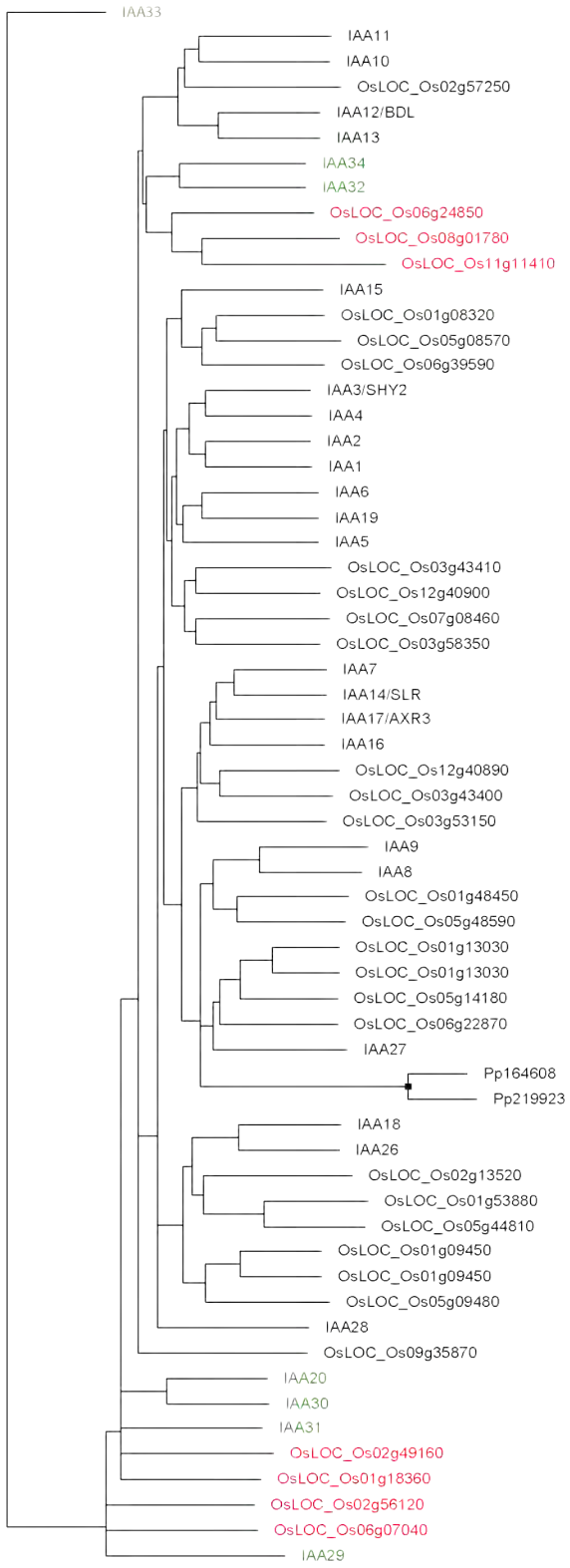
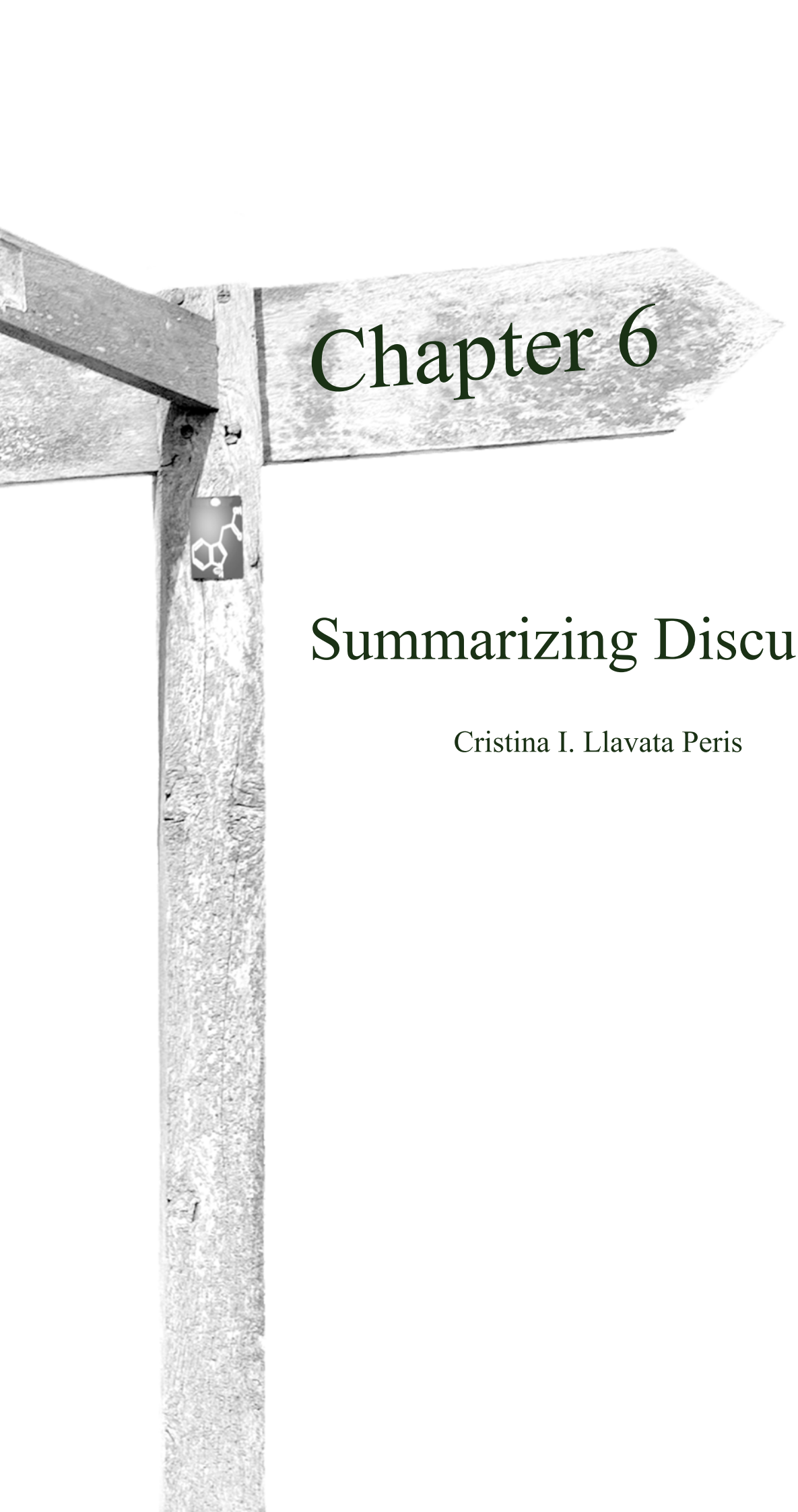
Supplementary information



Suplemental Figure1: *ncIAAs* genes from rice and Arabidopsis cluster together in a phylogenetical tree. (A) Phylogenetic tree of Arabidopsis and rice Aux/IAA proteins using neighbor-joining tree (BLOSUM62) method. The ncIAA clade is highlighted in green, closest rice homologs are highlighted in red.



Chapter 6

Summarizing Discussion

Cristina I. Llavata Peris

Embryogenesis in plants is a process that generates a relatively simple structure, the seedling, which possesses the precursors of all the basic tissues of the mature plant, and has stem cell systems (meristems) that generate all plant organs post-embryonically. As the specification of cell types, and the establishment of the spatial pattern in which they occur, is highly predictable in the early embryo, this offers a good model to study fundamental aspects of plant development. In **Chapter 1** we review the different factors involved in cell identity, pattern formation and developmental landmarks acquisition during embryogenesis. Both locally acting transcription factors and intercellular signals are responsible for specifying the different cell types present in the embryo. These distinct identities are reflected in unique cell division patterns. Although there is a big diversity in the cell division patterns among different plant species, the result of embryogenesis in all cases produces similar seedlings architectures, therefore seems that robust conserved mechanisms may underlie pattern formation in plant embryos, in fact it has been shown that many of the regulatory mechanism found in Arabidopsis are evolutionary conserved throughout plant species.

Some of the critical regulators of embryo development have been identified, but the specific factors involved in each patterning step and the molecular and cellular mechanisms of action are largely unknown. In part, progress in early embryogenesis research is and has been hampered by the poor accessibility of embryos in seed and fruit structures. Hence, continued development and improvement of methods for visualization of embryos and their cellular and molecular characteristics is crucial in addition to the identification of new regulators. In order to characterize the function of putative regulators of embryogenesis, one needs to determine the gene expression and protein localization pattern at the cellular level, as well as phenotypic consequences of misexpression or downregulation of the gene. In **Chapter 2** we described three different methods to obtain spatial and functional information about proteins expressed during embryogenesis. The first method is the use of Differential Interference Contrast microscopy (DIC), also known as Nomarski microscopy, for the facile analysis of mutant phenotypes of embryos within the seed. We also explain the use of confocal microscopy for the study of gene expression and protein localization. Finally we describe the use of indirect immunofluorescence to detect proteins that are lowly expressed.

In the past years, the plant hormone auxin has surfaced as a critical regulator of most of the patterning steps during embryogenesis (reviewed in Moller and Weijers, 2009; Peris et al., 2010). Responses to auxin are generated through the activation or repression of genes by a family of transcription factors, the AUXIN RESPONSE FACTORs (ARFs; Ulmasov et al., 1999). Auxin promotes the degradation of a second family of transcription factors, the Aux/IAAs, which binds and inhibit ARFs (Kim et al., 1997; Tiwari et al., 2004). When auxin enters the cell, it binds both Aux/IAA proteins and the SCF(TIR1/AFB) ubiquitin ligase complex, increasing the affinity for the Aux/IAA substrates and triggering their ubiquitination and degradation by the proteasome (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). In this way ARFs control the transcription of their target genes. It is striking that such a brief pathway mediates all the different developmental processes controlled by auxin, and a key question is at which level the multitude of individual responses to auxin are generated. Several possibilities can be envisaged to generate this diversity; most notably proteins can diverge at the level of interaction affinities (protein-protein or

protein-DNA), interaction specificities and stability. At the level of protein-protein interaction, it has been shown that different Aux/IAAs have distinct affinities for TIR1 and other AFB proteins (Prigge et al., 2010). In addition it has been shown that there is limited specificity in ARF-Aux/IAA interactions in a yeast 2-hybrid assay (Vernoux et al., 2011). This could explain some of the specificity in auxin responses, but a more comprehensive study of the network of protein *in planta* interactions will be needed. In **Chapter 3** we study protein interactions among and with members of the ARF protein family as a source of specificity in auxin response output. We consider three different levels at which the interaction profiles of these proteins can differ. First, ARFs can have different interaction affinities for different Aux/IAA. Second, ARFs can interact with other transcriptional regulators and form different protein complexes. And thirdly ARFs can be specific in the binding and regulation of DNA sequences.

In our work we use the suspensor of *Arabidopsis thaliana* as a model since this cell type expresses 6 phylogenetically distinct ARF genes (*ARF1, 2, 6, 9, 13, 18;* Remington et al., 2004) that have both overlapping and antagonistic functions (Rademacher et al., 2011). While analyzing homo and heterodimerization among these ARFs, we found that there is specificity that distinguishes the ARFs. ARF9 interacts with ARF1 and ARF18 but not with ARF6, while ARF6 interacts with ARF1 but not with ARF9 and ARF18. Furthermore, not all the ARFs tested have the possibility to homodimerize. This depicts a scenario where specificity is an important characteristic since only some combinations among proteins are possible. This is also true when we study the specificity of ARFs for Aux/IAA. When tested the interaction with a suspensor expressed Aux/IAA, IAA10, we found that only ARF6 and ARF9 can interact with IAA10, further supporting the idea that there is specificity not only in interactions among ARFs but also between ARFs and Aux/IAAs. In addition, the ability of both ARF6 and ARF9 to interact with IAA10 is an indication of a redundant function performed by these proteins, being both targeted by this Aux/IAA. A non-degradable version of IAA10 induces a strong phenotype in the suspensor, causing proliferation of the tissue, loss of suspensor identity, and acquisition of embryo identity (Rademacher et al., 2012). Since IAA10 interacts with ARF9 and ARF6 this suggest that the function of IAA10 is at least partially performed by ARF6 and ARF9, pointing to them as the main contributors to auxin response in the suspensor. This idea is further supported by the analysis of double mutant combinations among the ARFs. Only *arf6 arf9* double mutant show significant defects in the suspensor while no other combination with *arf9* and *arf6* do. However, as the penetrance of phenotypes observe are low even in this double mutant, there likely are other ARF proteins that redundantly perform the same function together with ARF6 and ARF9. In the future, targeted knockout of other ARFs in suspensor cells should help addressing this question. In any event, ARF-ARF and ARF-Aux/IAA interaction profiles now help rationalizing the shared and opposing activities of the different ARF proteins.

When we study the second level at which ARFs may differ, protein complex formation, we found striking differences in the protein interaction profiles of the ARF proteins studied. In some cases several proteins found to interact with more than one ARF, pointing out the fact that these proteins might redundantly participate together in some regulation process. However, no two ARFs show the same interaction profile, which demonstrates functional diversification at the level of protein complex formation in the ARF family.

Using proteomics, we also find new candidate regulators of auxin-dependent gene regulation. These genes belong to other families of transcription factors. We found that *GL2* and *HDG2* interact with *ARF1* and *ARF2*. These genes belong to the HD-Zip class IV family and were previously shown to control root hair and trichome development (Masucci and Schiefelbein, 1996; Marks et al., 2009). We also identify two proteins belonging to the MADS transcription factor family, SEP2 and SHP2, as interactors of ARF2. These proteins were found previously to control flower and fruit development (Pelaz et al., 2000; Pinyopich et al., 2003). Interestingly, none of these proteins were expected to have a function in the auxin response, but our studies showed that the *gl2* mutant as well as the *shp1 shp2* double mutant display hypersensitivity to auxin in root growth. The identification of these proteins as new regulators of auxin response highlights a more general issue. Most components of auxin biology have been identified based on genetic screens for altered auxin response (Leyser et al., 1993; Gray et al., 1999), by transcriptional responses to auxin (Abel and Theologis, 1996). Here, we use a completely independent approach, where proteins are associated to auxin response due to their biochemical property of interacting with ARF proteins *in vivo*. This allows identifying developmental effectors or mediators of more specific responses than genetic screens for auxin-response mutants that are more likely to identify general components in the signaling pathway. Interesting future questions relate to how these new components regulate auxin response. For example, are there common targets genes of HD-ZIP, MADS and ARF factors, and do the proteins cooperate to control these?

It is becoming clear that one should be open-minded to discover new components of developmental processes, particularly when new factors are already known to have a function in a very different context. In analogy, SHP1 and SHP2 were recently described to have another function that is very different from the one that their identification was based upon. Many genes are expressed in an oscillating pattern along the primary root, and this oscillation is thought to relate to lateral root initiation (Moreno-Risueno et al., 2010). Among the transcription factors that oscillate in this process, the MADS-box protein family was over-represented, among them SHP1 and SHP2. Likewise, several ARF genes, including *ARF2* and *ARF7*, also oscillate. The *arf7 shp1* double mutant indeed has fewer lateral roots. These results suggest that these genes function together in lateral root development. This is an independent indication of functional interaction between ARF and MADS families of transcription factors and it will be interesting to see if the SHP1-ARF7 interaction also involves protein complex formation.

Our study of the mechanisms of ARF activity based on protein interactions will help to generate a mechanistic understanding of conserved and divergent ARF activity. ARFs were initially classified into “activator” and “repressor” types based on amino acid enrichment in their middle regions (Ulmasov et al., 1999). However, this classification is not absolute (Lokerse and Weijers, 2009), and there is currently no explanation for why, for example, Q-richness is correlated with “activator” activity. With the protein interactions identified here, we now have a set of testable predictions about functional redundancy and variation based on biochemical properties that can be the basis for future studies.

By the characterization of genes that are regulated by ARFs in **Chapters 4 and 5,** we study how specific responses to auxin are generated. Interference with the normal ARF activity by the use of a non-degradable version of Aux/IAA leads to the loss of the suspensor identity and the acquisition of an embryo fate (Rademacher et al.,

2012). A transcriptomics approach in this background had previously identified genes that are either up- or downregulated (Lokerse, 2011). The most representative genes found were transcription factors, auxin-regulated genes and zygote-enriched genes. Among the transcription factors identified, several were members of the bHLH family. We choose them for a more detailed analysis, because two other bHLH genes, *TMO7/bHLH134* (Schlereth et al., 2010) and *TMO5/bHLH32* (De Rybel et al., 2013), are direct targets of *ARF5/MP*, and are involved in specific developmental responses to auxin. The goal of the study was to determine if a different auxin response, mediated by ARFs expressed in the suspensor, is likewise executed by an ARF-bHLH module (**chapter 4**). The bHLHs studied in this chapter belong to two different subfamilies (12 and 25). These genes show dynamic patterns of expression during embryogenesis and a rapid regulation of the gene transcripts upon auxin incubation. Moreover, their transcription is disturbed in *arf* mutant backgrounds. Consistent with a function as direct ARF output, both loss- and gain of function plants showed strong phenotypes in the suspensor. Hence, with these *bHLH* genes we have identified a layer downstream of ARFs in suspensor development, and the identification of the targets of these bHLH factors will help to better understand how cell fates, shapes and division planes are acquired in different tissues during embryogenesis.

In addition to providing insight into the regulation of suspensor development, the identification of these bHLH genes is biologically meaningful output of ARFs acting in the suspensor, which demonstrates the existence of separate auxin-ARF-bHLH modules in development. Both ARFs and bHLHs appeared early in plant evolution and they have expanded since the first land species of plants (Carretero-Paulet et al., 2010; Pires and Dolan, 2010). This suggest that the ARF-bHLH module can be an old mechanism to mediate auxin-dependent development and that the number of modules has increased and diversified along with the evolution of plants to cover all the different developmental outputs that appeared with the evolving of more sophisticated structures and processes controlled by plants. This raises the interesting question of how widespread the occurrence of such modules is, and whether other ARF-bHLH connections mediate other auxin-dependent developmental processes. Both auxin and bHLHs are involve in a myriad of developmental steps, many of them regulated by genes belonging to both families, like the regulation of female reproductive tract (Gremski et al., 2007; Heisler et al., 2001; Crawford and Yanofsky, 2011), root hair growth (Yi et al., 2010), fruit opening (Sorefan et al., 2009), light signaling (Halliday and Fankhauser, 2003; Fairchild et al., 2000), wound and drought response (Smolen et al., 2002; Chinnusamy et al., 2003) and shoot branching (Komatsu et al., 2001), among others. Future studies should reveal whether similar direct ARF-bHLH regulation underlies these processes.

Strikingly, compromised activity of either the MP-TMO7 (Schlereth et al., 2010) or the “suspensor” ARF – bHLH subfamily 25 module leads to very similar defects in the uppermost suspensor cell (hypophysis). Indeed, TMO7 protein moves from the pro-embryo to the hypophysis (Schlereth et al., 2010), but if and how TMO7 activity relates to that of bHLH subfamily 25 is an important question. Very recently, it was shown that members of the TMO7 subclade (PRE family) positively regulate subfamily 25 members by sequestering AIF/IBH bHLH proteins that otherwise bind to and inhibit subfamily 25 bHLH proteins (Bai et al., 2012; Ikeda et al., 2012). In these studies, this tri-antagonistic system was shown to operate in post-embryonic control of cell elongation, in part through the direct regulation of *EXPANSIN* genes by

subfamily 25 proteins (Bai et al., 2012; Ikeda et al., 2012). However, given the similar phenotypes in *TMO7* RNAi lines (Schlereth et al., 2010) and in the *bhlh49* mutant, as well as the movement of TMO7 to the hypophysis (Schlereth et al., 2010), a similar network may operate in the embryo. An important next step will be to effectively demonstrate this link and what processes they control to lead to correct hypophysis development. The proposed role of subfamily 25 genes in promoting cell elongation (Bai et al., 2012; Ikeda et al., 2012) may in fact also explain the phenotypes in these, as well as *TMO7* mutants. The hypophysis undergoes peculiar shape changes prior to asymmetric division, and modification in the expansion rate or direction may directly or indirectly influence the plane of cell division (Lukowitz et al., 2004). It will be important to confirm overlapping pattern of expression of the three clades in the embryo. If all the genes are expressed in a similar pattern of expression the next step could be to interfere with component of one of the subfamilies and analyze the effect in the correct specification of the suspensor.

In the same transcriptomic study, where the two subfamilies of bHLHs were identified, two members of the Aux/IAA family of transcription factors were also found (Lokerse, 2011). These two genes, *IAA20* and *IAA30*, encode proteins that belong to a structurally different group of Aux/IAAs, as they lack the domain important for the auxin-mediated degradation (Dreher et al., 2006). In **Chapter 5** we study this subfamily of non-canonical Aux/IAA (ncIAA) proteins in more detail, and make a striking finding. On one hand these genes behave as the canonical Aux/IAA do in that their transcription is directly activated by ARFs and their misexpression causes phenotypes that agree inhibition of ARFs. However, we demonstrate that, unlike the canonical Aux/IAAs, their protein stability is not regulated by auxin. Hence, the presence of auxin will trigger the transcription of *ncIAA* genes, leading to the accumulation of ncIAA protein that will inhibit ARFs and suppress further auxin response. The insensitivity to auxin-dependent degradation is paradoxical, as cells would never recover from this inhibition. By systematically screening for stimuli and signals that can promote ncIAA protein degradation, we identify the cell cycle as a prime candidate. We found that not only protein stability, but also *ncIAA* gene expression are strongly modulated during cell cycle progression. This finding links the cell cycle, whose regulation is vital for controlled cell proliferation and differentiation, and the developmental regulator auxin that is involved in almost any plant developmental process. This also suggests that auxin response is “gated” by the cell cycle: cells can respond to auxin after cell division but after an initial auxin response ncIAA protein will accumulate and block further auxin response until the next cell division. Even though it remains to be demonstrated that auxin response is in fact gated by the cell cycle in the way proposed here, our findings suggest an elegant mechanism for how auxin response is limited. For “continuous” auxin responses that quantitatively drive e.g. cell elongation in the presence of auxin, this mechanism may not be relevant. However, one can envisage that developmental outputs that require auxin to act as a trigger for e.g. cell fate change, it will be beneficial to have a mechanism that will block further auxin responses after an initial trigger. The model presented here (Fig. 1) is based on the premise that there is enough ncIAA to block all the auxin responses, that the ncIAA-ARF interactions are stable and that there is limited specificity among ARFs and ncIAAs. These parameters will need to be defined.

Our data about the connection between cell cycle and auxin adds to the limited knowledge that exists about the link between these two processes. It has been found

that several genes necessary for cell cycle progression are controlled by auxin (Fuerst et al., 1996; Richard et al., 2002; Cruz-Ramirez et al., 2012). In addition, it was reported that mutants in the *HOBBIT* gene, encoding a subunit of the Anaphase Promoting Complex (APC) that targets cell cycle proteins for degradation, show a reduction in auxin responses (Blilou et al., 2002). While these findings provide sparse links, the identification of the specific genes, through which cells can link the progression of cell cycle with the generation of auxin responses, is an important advance in our understanding of auxin processes. Future studies should reveal what the point of convergence is, for example by identifying the factors required for cell cycle-dependent degradation of ncIAA proteins.

In summary, by identifying interaction partners of ARF transcription factors, and by showing that these mediate ARF activity during plant development, the work in this thesis offers a plausible explanation for how specific auxin responses are generated. Our work also identified a point of convergence between auxin response and the cell cycle. Finally, this work lays the foundation for future mechanistic studies towards the biochemical basis of divergence in ARF activity.

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Summary

In Chapter 1 we have reviewed how cell identities are established during embryogenesis of *Arabidopsis thaliana*, and how the interplay between transcription factors and cell communication contribute to the specification of the different cell types in a spatio-temporally coordinated manner. We pay special attention to the central role of the plant hormone auxin. Though most knowledge has been gained in Arabidopsis, we discuss the potential conservation of regulatory pathways in other species. This suggests the existence of a robust shared mechanism underlying embryogenesis, conserved in seed plants during evolution.

Embryogenesis offers a compact and predictable model for studying the activity and mechanisms of factors that are central to the establishment of cell identities in plants. However, the embedding in seeds and fruits present technical challenges. **In Chapter 2** we show a set of microscopy techniques necessary for the study of phenotypes, pattern of expression, and protein localization in Arabidopsis embryo. We explain the use of Differential Interference Contrast Microscopy (DIC), also known as Nomarski microscopy, the use of confocal microscopy and finally we describe the use of indirect immunofluorescence to detect proteins that are lowly expressed.

A key question in auxin biology is how auxin, a relatively simple molecule is able to elicit many different developmental responses. **In Chapter 3** we describe how the family of DNA-binding ARF transcription factors act as a source of variation in auxin output specificity. We consider three levels at which ARFs may differ: differential interactions with Aux/IAA inhibitors or with other ARFs, assembly into different protein complexes and binding to different genomic target sites. We use genetic analysis, *in vivo* protein-protein interactions using a dynamic FRET-based interaction assay and mass-spectrometry based proteomics, and demonstrate both shared and unique protein interactions among the ARFs proteins. These results identify new regulators of auxin response and offer a plausible explanation for the unique and overlapping functions in auxin-dependent plant development.

In **Chapter 4** we study two subfamilies of bHLHs whose expression was strongly misregulated in a transcript profiling experiment in which auxin response in the suspensor had been blocked. We show that expression of these genes is auxin-, and ARF-dependent. Based on loss- and gain-of-function phenotypes, we conclude that these bHLH genes act downstream of auxin to regulate normal suspensor development. Strikingly, this work identifies a novel auxin-ARF-bHLH module that operates in parallel to the previously known auxin-ARF5/MP-TMO5/TMO7 module in adjacent embryo cells. Hence, we conclude that such modules may represent a universal element in auxin-dependent plant development.

Auxin activity involves feedback control at several level, including direct feedback on ARF activity by their Aux/IAA inhibitors, that are also ARF transcriptional targets. In **Chapter 5** we describe a special subfamily within the Aux/IAA family, the non-canonical aux/IAAs (ncIAA). Two genes belonging to this subfamily were strongly downregulated in the suspensor of embryos in which ARF activity was blocked. We found that these proteins have a function similar to that of the canonical Aux/IAAs:

they bind and inhibit ARFs. Furthermore, their transcript levels are quickly upregulated in the presence of auxin, but unlike the other members of the subfamily, proteins are not targeted for degradation by auxin. When studying the factors that control the stability of ncIAA we made a striking finding. Cell cycle regulates the protein stability but also the timing of gene expression. This is an important indication of the relation between two processes that profoundly regulate plant growth and development: cell cycle control and auxin signaling.

Starting from the identification of interaction partners of ARF transcription factors, and a characterization of the genes they regulate, our work offers explanations for how specific auxin responses are generated.. Furthermore we demonstrate a link between the cell cycle and auxin responses. Taken together, this represents a stepping stone point for the future study of mechanisms of divergence in ARF activity.

Samenvatting

In **Hoofdstuk 1** wordt beschouwd hoe unieke cel-identiteiten worden vastgelegd tijdens de embryogenese van *Arabidopsis thaliana*. Daarnaast wordt besproken hoe het samenspel van de werking van transcriptiefactoren en cel-cel communicatie de gecontroleerde aanleg van verschillende celtypes in ruimte en tijd controleert. Hierbij wordt nadruk gelegd op het plantenhormoon auxine. Hoewel verreweg de meeste kennis is verkregen middels de studie aan Arabidopsis, bespreken we de mogelijkheid dat soortgelijke mechanismen de embryogenese van andere plantensoorten controleren. We concluderen dat er waarschijnlijk zeer robuuste gemeenschappelijke mechanismen werkzaam zijn die in de evolutie van zaadplanten tot vergelijkbare lichaamspatronen heeft geleid.

Embryogenese biedt een aantal grote voordelen bij het bestuderen van de werkingsmechanismen van factoren die de aanleg van cel-identiteit sturen. Twee hiervan zijn dat het embryo klein en compact is, en dat het patroon zeer voorspelbaar is. Doordat embryo's in zaden en vruchten verborgen zitten, zijn er echter ook technische uitdagingen. In **Hoofdstuk 2** wordt een aantal microscopische technieken uiteengezet die in dit proefschrift gebruikt worden om mutante fenotypes, genexpressie-patronen en eiwit-lokalisatie te visualiseren in het Arabidopsis embryo. In het bijzonder gaat het hierbij om Differentieel Interferentie Contrast (DIC), ook wel Nomarski-microscopie genoemd, om confocale microscopie alsmede indirecte immuunfluorescentie voor de detectie van laag-abundante eiwitten.

Een centrale vraag in de auxine biologie is hoe een relatief eenvoudig molecuul in staat is om een grote hoeveelheid verschillende ontwikkelingsprocessen aan te sturen. In **Hoofdstuk 3** wordt beschreven hoe de familie van DNA-bindende ARF transcriptiefactoren als een bron van variatie fungeert in het bepalen van specifieke auxine "output". De ARFs kunnen op verschillende niveaus bijdragen aan het genereren van specificiteit: differentiële interacties met Aux/IAA inhibitors of andere ARFs, assemblage in verschillende eiwitcomplexen en binding aan verschillende genomische targets. In dit hoofdstuk wordt een combinatie van genetische analyse, in vivo eiwit-eiwit interactie met behulp van een dynamische FRET-gebaseerde interactie-assay en massa spectrometrie-gebaseerde proteomics gebruikt. Deze combinatie van aanpakken laat het bestaan zien van zowel overlappende als unieke interacties tussen en met de ARF eiwitten. Deze analyse heeft geleid tot de identificatie van nieuwe regulatoren van auxine activiteit en biedt een plausibele verklaring voor de unieke en overlappende functies in auxine-afhankelijke plantenontwikkeling.

In **Hoofdstuk 4** worden de functies van twee subfamilies van bHLH transcriptiefactoren beschreven, waarvan de expressie sterk veranderd was in een genoomwijde expressie analyse waarin auxine respons in de suspensor was geblokkeerd. Er wordt laten zien dat de expressie van deze bHLH genen auxine- en ARF-afhankelijk is. Op basis van fenotypes ter gevolge van verhoogde en verlaagde genactiviteit is te concluderen dat deze bHLH genen downstream van auxine de normale suspensor-ontwikkeling reguleren. Hiermee wordt een nieuwe auxine-ARF-bHLH module geïdentificeerd. Deze module werkt in parallel met de auxine-ARF5/MP-TMO5/TMO7 module die in de naastliggende embryo-cellen actief is. We concluderen dat deze modules wellicht een universeel element in de auxine-gestuurde plantenontwikkeling zijn.

In de activiteit van het hormoon auxine zijn op verschillende niveaus van terugkoppelingsmechanismen actief. Een voorbeeld hiervan is de activering van de Aux/IAA eiwitten door de ARF transcriptiefactoren. Op hun beurt binden Aux/IAA eiwitten de ARFs en remmen zij de ARF activiteit. In **Hoofdstuk 5** wordt de functie van een subfamilie van zogenaamde non-canonical Aux/IAA eiwitten (ncIAA) bestudeerd. Twee genen behorend tot deze subfamilie waren eerder geïdentificeerd op basis van gereduceerde expressie in embryo's waar in suspensorcellen ARF activiteit was geblokkeerd. Hier wordt laten zien dat deze twee ncIAA eiwitten functioneel vergelijkbaar zijn met reguliere Aux/IAA eiwitten: ze binden en remmen ARFs. Daarnaast wordt ook de transcriptie van deze genen door auxine gestimuleerd, maar in tegenstelling tot de reguliere Aux/IAA eiwitten worden ncIAA eiwitten niet afgebroken in aanwezigheid van auxine. Door te bestuderen onder welke omstandigheden ncIAA eiwitten worden afgebroken is er een verrassende ontdekking gedaan: Zowel de stabiliteit van ncIAA eiwitten als hun genexpressie wordt gestuurd door de celcyclus. Deze vinding suggereert een koppeling tussen auxine respons en de celcyclus, twee mechanismen die beide een zeer ingrijpende rol in de plantenontwikkeling spelen.

Door interactiepartners en target genen te bestuderen van de ARF transcriptiefactoren biedt dit onderzoek verklaringen voor het ontstaan van specifieke reacties op een algemeen hormoon. Daarnaast wordt en nieuwe link tussen auxine en de celcyclus laten zien. Dit onderzoek biedt daarmee vele aanknopingspunten voor toekomstig onderzoek naar de mechanismen achter de verschillen in ARF activiteit.

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When one starts something new in an unknown place one can feel a little bit scared or lost. Luckily Carolina was there from the very beginning. Carol, you made me feel welcome and you showed me the many secrets that Wageningen hides. And I should thank Alice, Saiko and Joakim to put truth into the saying: "All’s well that ends well".

The PhD journey included some special safaris that I enjoyed enormously. For the company of the parrot: Ale, Stefi, Giulia, Norma, Christoph and Marco; guys, you really know how to have fun and that is a contagious thing. You are the coolest people that I have ever met. Droef, Gent, International, oldies, nerds, heroes, dancers... I hope that we never stop increasing this list.

And the same goes for my friends in Valencia and abroad: Fus, Anita, Mariam, Luis, Ana, Virginia, Miguel, Kristina, Katja, Dorothee and Tamara; you make Europe seem small with each country as our playgrounds. I know you for many many years, but still, you always have the power to make me smile when we meet.

A special thank goes to Caperune for putting the soundtrack to the PhD experience and to my "Wageningenian" love story.
Johan, we are together in the same journey for more than four years now, I don't know if it was destiny or chance that we met but I hope that we still have a long way together.

I also feel very lucky with my family. Although it seems that this life train is taking me far from you all, I know that you are always there. The nice moments each time that I make a quick stop in Valencia and the continuous support that I get from you make me feel like I never departed. I don’t know what will happen next, but I know that you will always be the station that I can call home.

Curriculum Vitae

Cristina Isabel Llavata Peris was born on the 24th of July 1981 in Valencia, Spain. She completed her secondary education at “Istituto Lluis Vives” in Valencia in 1999. She started studying Biology at the University of Valencia in 1999 and obtained a M.Sc degree in 2004. After she moved to Italy to study Biotechnology at University of Rome “La Sapienza”, performing her master thesis in the group of Sabrina Sabatini.
In March 2009 she started a four-year PhD project in the group of Prof. dr Dolf Weijers at Wageningen University resulting in this thesis.
Following her PhD she intends to continue a career in plant science.

Publications

Peris C.I., Rademacher E.H., Weijers D. (2010). Green beginnings - pattern formation in the early plant embryo. *Curr Top Dev Biol.* **91,** 1-27

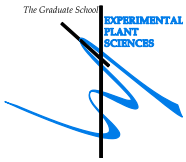
De Rybel B.*, van den Berg W.*, Lokerse A.S., Liao C.-Y., van Mourik H., Möller B., **Llavata-Peris C.I.**, and Weijers D. (2011). A versatile set of Ligation-Independent Cloning vectors for functional studies in plants. *Plant Physiol.* **156,** 1292-1299.
(* equal contribution)

Rademacher, E.H.*, Möller, B.*, Lokerse, A.S.*, **Llavata-Peris, C.I**., van den Berg, W., and Weijers, D. (2011). A cellular expression map of the Arabidopsis AUXIN RESPONSE FACTOR gene family. *Plant J.* **68,** 597-606.
(* equal contribution)

Rademacher, E.H.*, Lokerse, A.S.*, Schlereth, A., **Llavata-Peris, C.I.**, Bayer, M., Kientz, M., Freire Rios, A., Borst, J.W., Lukowitz, W., Jürgens, G., and Weijers, D. (2012). Different auxin response machineries control distinct cell fates in the early plant embryo. *Dev. Cell* **22,** 211-222.
(* equal contribution)

Llavata-Peris, C.I., Lokerse, A., Möller, B., De Rybel, B., and Weijers, D. (2013). Imaging of Phenotypes, Gene Expression and Protein Localization During Embryonic Root Formation in Arabidopsis. *Meth. Mol. Biol.* **959,** 137-148.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Cristina Isabel Llavata Peris
Date: 4 October 2013
Group: Biochemistry, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Proteomic and meganistic dissection of AUXIN RESPONSE FACTOR action in the suspensor	Sep 03, 2009
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Green beginnings - Pattern Formation in the Early Plant Embryo; IN: Current Topics in Developmental Biology (Vol. 91, 2010, pp. 1-27); Elsevier Inc.	Dec 2009
Imaging of Phenotypes, Gene Expression, and Protein Localization During Embryonic Root Formation in Arabidopsis; IN: Methods Molecular Biology (Vol. 959, 2013, pp 137-148), Plant Organogenesis: Methods and Protocols, Ive De Smet (ed.); Springer - New York	May 2011
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Utrecht University	Jun 01, 2010
ExPectationS Career Day, Wageningen University	Nov 19, 2010
ExPectationS Career Day, Wageningen University	Nov 18, 2011
EPS PhD student day, Wageningen University	May 20, 2011
► **EPS Theme Symposia**	
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden University	Jan 20, 2011
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen University	Jan 28, 2010
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 26-27, 2012
► **Seminars (series), workshops and symposia**	
Invited Seminars Biochemistry (Sabrina Sabatini, Dorus Gadella, Bruno Mueller, Klaus Harter)	Mar-Dec 2009
Meeting Siren	Mar 08, 2010
Invited Seminars Biochemistry (Roland Boer, Teva Vernoux, Richard Smith)	Apr-Oct 2010
Joint meeting groups Dolf Weijers and Ben Scheres (Utrecht University)	Feb 11, 2011
Meeting Siren	Oct 10, 2011
Invited Seminars Biochemisrty (Masahiko Futurani, Jürgen Kleine-Vehn, Michael Nodine)	Apr-May 2012
Meeting Siren	May 09, 2012
► **Seminar plus**	
► **International symposia and congresses**	
Molecular Aspect of Plant Development	Feb 23-26, 2010
Early Embryogenesis in Plants	May 08-10, 2012
► **Presentations**	
Oral: Meeting Siren	Mar 08, 2010
Oral: Meeting Siren	Oct 10, 2011
Oral: Meeting Siren	May 09, 2012
► **IAB interview**	Feb 17, 2011
► **Excursions**	
Scientific PhD Excursion to Barcelona (Spain) organized by Sacco de Vries	Apr 21-25, 2009
Subtotal Scientific Exposure	*13.6 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Postgraduate course 'Statistical analysis of ~omics data'	Dec 13-17, 2010
Highthroughput Screening of Candidate Genes	Jul 18-22, 2011
Live-Embryofluorescence Imaging	Jun 15-17, 2012
► **Journal club**	
Participate in weekly journal club (Dolf Weijers group)	2009-2013
► **Individual research training**	
Secondment at Malcom Bennet lab (Nottingham, UK) 2 weeks	2012
Subtotal In-Depth Studies	*9.9 credits**

4) Personal development	date
► **Skill training courses**	
WGS course Competence Assessment	Jan 2010
WGS course Project and Time Management	Jan 11,25 & Feb 22, 2011
WGS Course Moral Dilemmas in Daily Scientific Practices	Feb 15-18, 2011
Language courses: Dutch I	2010
Sharpen your communication	Mar 09-10, 2010
Language courses: Dutch II	2010
Language courses: Dutch III	2011
SIREN workshop-Reaching your dream job	Jul 25-26, 2012
► **Organisation of PhD students day, course or conference**	
Organization of workshop Proteomic Identification of protein complexes	Oct 07-13, 2010
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*11.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.1**

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 ECTS credits

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

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