Auxin response and cell identity in the early Arabidopsis embryogenesis

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“Like auxin, money does
everything, but what it does depends
on who gives it to whom and under
what circumstances”
Leyser 2018

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Zusammenfassung der Doktorarbeit

In allen Entwicklungsprozessen werden orientierte Zellteilung und Zellschicksalsakquisition kombiniert, um Muster zu generieren. Ein wichtiger Koordinator dieser Entscheidungen in Pflanzen ist das Phytohormon Auxin. Es scheint fast alle Pflanzenentwicklungsprozesse zu koordinieren: von der Bildung der Wurzel- und Sprossmeristeme bis zur Fruchtentwicklung. Ein Prozess hat die wissenschaftliche Gemeinschaft besonders fasziniert: die Embryogenese. Wie aus einer einzelnen Zelle durch eine sehr streng kontrollierte Abfolge von Schritten die Körperorganisation der Pflanze in ihren Grundzügen entsteht. Der Arabidopsis-Embryo stellt dank seines sehr definierten Anfangs und seiner Regelmäßigkeit ein attraktives Modell zur Untersuchung der Embryogenese dar. Dies ermöglicht es Wissenschaftlern, die Entwicklung des Embryomusters in einer Einzelzellauflösung zu verfolgen.

Der polare Auxintransport ist grundlegend für die Bildung der apikal-basalen Achse und letztendlich für die Embryomusterbildung. Nach der asymmetrischen zygotischen Teilung entstehen zwei transkriptionell unterschiedliche Zellen. Die kleinere apikale Zelle variiert die Teilungsebene, um schließlich den eigentlichen Embryo zu bilden. Die größere Basalzelle teilt sich horizontal und alle außer ihrer obersten Zelle (Hypophyse) bleiben extraembryonal. In der apikalen Zelle reichert sich Auxin an und fördert die embryonale Identität. Diese Auxinantwort wird in Suspensorzellen blockiert; dennoch haben sie das Potenzial, embryonale Zellen zu werden, bis die apikal-basale Achse vollständig etabliert ist, das heißt bis zum Übergangsstadium. Trotz jahrzehntelanger Forschung ist nicht vollständig geklärt, wie Auxin diese Ereignisse reguliert, insbesondere in den sehr frühen Stadien der Embryonalentwicklung.

Diese Arbeit zielt darauf ab, unser Wissen über diesen komplexen Prozess zu erweitern, zum durch Einen durch die Analyse einer direkt an der Auxinantwort während der frühen Embryonalentwicklung beteiligten Gain-of-Function-Mutante und zum Andern durch die Untersuchung von Zellidentitätsänderungen in einer filamentösen Embryogenese.

Das erste Kapitel konzentriert sich auf die frühe Auxinantwort im Embryo. Hier beschreiben wir die Rolle des Aux/IAA IAA8 durch seinen Funktionsgewinn-Phänotyp. Aux/IAAs binden an Auxin-Response-Faktoren (ARFs) und unterdrücken die Transkription von Auxin-Response-Genen. In den IAA8-Mutantenembryonen beobachten wir abweichende Zellteilungen, beginnend mit einer horizontalen ersten apikalen Teilung. Darüber hinaus verursacht die IAA8-spezifische Blockierung der Auxin-Antwort in der apikalen Zelle dramatische Veränderungen des Zellschicksals.

Das zweite Kapitel beschreibt die Entstehung eines Embryos aus einer induzierten suspensorartigen Struktur. Dies wird durch eine zeitliche ektopische Aktivierung des YDA-Signalwegs (pS4:SSP) erreicht.

Dieser MAPK-Kinase-abhängige Weg kontrolliert die Zygotenpolarisation und fördert die Suspensoridentität während der ersten Schritte der Embryogenese. Unter Verwendung fluoreszierender Identitätsmarker beobachten wir, dass die Freisetzung von der SSP ektopische Expression den Übergang von frühen Basalzellen zu embryonalen Zellen auslöst und dies wahrscheinlich durch eine Auxin-Reaktion gefördert wird.

Kurz gesagt, das neue System zur Untersuchung des Zellschicksalserwerbs und der Charakterisierung einer Auxin-insensitiven Mutante erweitert unser Wissen darüber, wie Auxin die Zellteilung und Zelldifferenzierungsentscheidungen diktieren könnte, um das frühe Embryomuster zu bilden.

Thesis Abstract

In all developmental processes oriented cell division and cell fate acquisition are combined in order to generate patterns. A major coordinator of these decisions in plants is the phytohormone auxin. It seems to coordinate nearly all plant developmental processes: from the formation of the root and shoot meristems to fruit development. One case in particular has fascinated the scientific community: embryogenesis. How the basic organization of the plant body arises from a single cell in a very tightly controlled series of steps. The Arabidopsis embryo constitutes an attractive model to study embryogenesis thanks to its regularity. This allows scientists to track the development of the embryonic pattern in a single-cell resolution.

The auxin polar transport is fundamental for the apical-basal axis formation and ultimately for the embryo patterning. After the asymmetric zygotic division, two transcriptionally distinct cells are generated. The smaller apical cell will vary the division plane to ultimately form the embryo proper. The larger basal cell divides horizontally and all but its uppermost cell (hypophysis) will remain extraembryonic. In the apical cell auxin accumulates and promotes embryonic identity. This auxin response is blocked in suspensor cells; nevertheless, they have the potential to become embryonic cells until the apical-basal axis is fully established, that is, the transition stage. Despite decades of research, it is not fully understood how auxin regulates these events, especially at the very early stages of the embryonic development.

This work aims to widen our knowledge on this complex process through the study of a gain-of-function mutant directly involved in the auxin response during early embryo development and of cell identity changes in a filament-like embryo system.

The first chapter focuses on the early auxin response in the embryo. Here we describe the role of the Aux/IAA IAA8 through its gain-of-function phenotype. Aux/IAAs bind to auxin response factors (ARFs) and repress the transcription of auxin response genes. In the IAA8 mutant embryos we observe a horizontal division of the apical daughter cell followed by a series of aberrant cell divisions. Additionally, IAA8-specific blocking of the auxin response in the apical cell causes dramatic cell fate changes.

The second chapter describes the formation of an embryo from an induced suspensor-like structure. This is achieved by a temporal ectopic activation of the YDA pathway (pS4:SSP). This pathway controls zygote polarization and promotes suspensor identity during the first steps of embryogenesis. Using

fluorescent identity markers, we observe that the release of this activation triggers the transition from early basal cells to embryonic ones and this is likely promoted via an auxin response.

In brief, the new system to study cell fate acquisition and the characterization of an auxin-insensitive mutant further our knowledge on how auxin might dictate the cell division and cell differentiation decisions to form the early embryo pattern.

Publications

Manuscripts included in this thesis

Marina Ortega-Perez, Lisa Asseck, Helen Schäfer, Martina Kolb, Daniel Slane, Martin Bayer and Gerd Jürgens. A gain of function mutation in IAA8 affects cell division and differentiation in the early embryo. (To be submitted)

Kai Wang, Yingjing Miao, Marina Ortega-Perez, Houming Chen, Martin Bayer. A filament-like embryo system to study basal cell embryo transition. (To be submitted)

Additional publications not included in this thesis

Kai Wang, Houming Chen, Marina Ortega-Perez, Yingjing Miao, Yanfei Ma, Agnes Henschen, Jan U. Lohmann, Sascha Laubinger, Martin Bayer. Independent parental contributions initiate zygote polarization in *Arabidopsis thaliana*. *Current Biology* (2021) doi:10.1016/j.cub.2021.08.033.

Herud-Sikimic, Ole, Andre C. Stiel, Marina Ortega-Perez, Sooruban Shanmugaratnam, Birte Höcker, and Gerd Jürgens. 2020. "Design of a Biosensor for Direct Visualisation of Auxin." BioRxiv. https://doi.org/10.1101/2020.01.19.911735.

General Introduction

Development: about cell division and cell differentiation

Development is a constant balance between robustness and plasticity. Every developmental process must be robust enough to ensure that small disruptions (intrinsic or extrinsic) do not lead to a deleterious event. This robustness is partially achieved by redundancy, ensuring that a paralog can take over if the original protein fails. On the other hand plasticity, or the ability to adapt quickly to intrinsic fluctuations or environmental influences is also key to survival[1–3].

Framed between these two concepts, development consists of cell divisions and differentiation to form spatial patterns. Scientist have long been fascinated by how patterns evolved from a single cell. Regarding cell differentiation, numerous theories have been proposed over half a century ago; some examples are the Waddington landscape[4] or the French Flag model[5,6]. Briefly explained the Waddington landscape uses a metaphor (marbles rolling down a valley) to postulate how gene regulation shapes development (Figure 1.A). The French Flag model is based on a spatial gradient that dictates cell fate when a determined threshold is reached (Figure 1.B).

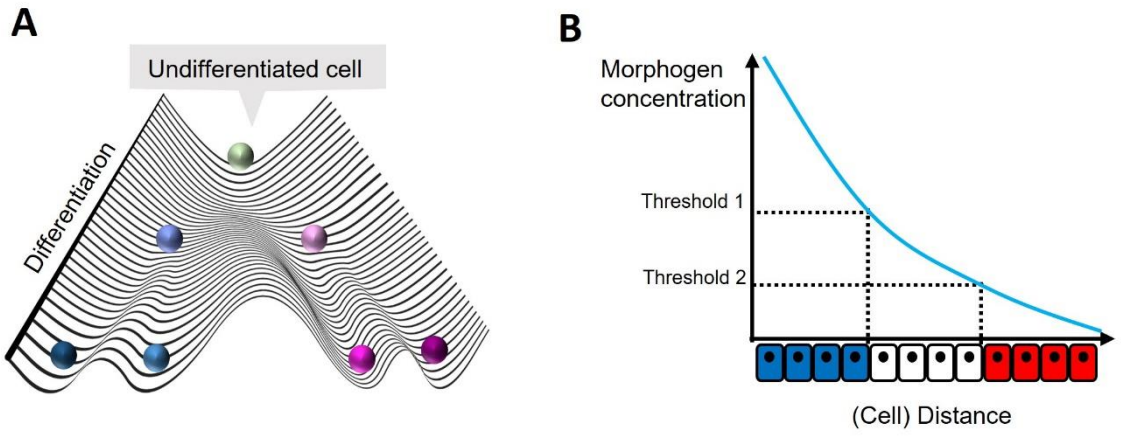

Figure 1. Differentiation theories from the 20th century. A) The Waddington landscape. An initially undifferentiated cell rolls down a system of valleys, choosing one path (identity) or another that will eventually lead it to a fully differentiated state (lowest marbles). **B)** The French Flag model. The X-axis represents cells with different identities (blue/white/red). At a given morphogen concentration threshold, the cell identity changes.

However, it is sometimes difficult to apply rigorously these postulated theories to actual developmental biology. Cell fate acquisition is a dynamic process (plasticity), and it is influenced by

fluctuations and stochasticity[7–9]. This process is more complex than the often imagined deterministic and binary switch scenarios.

Much was postulated in the nineteenth century regarding the second ingredient of development, cell division. Examples of cell division rules from this time applied to plants are the Rectangular Section[10] and the Principle of Minimal Area[11,12]. Errera illustrated his geometrical rule using soap bubbles and how they divide: the surface tension makes the dividing wall follow a minimal surface area configuration-the shorter cell wall rule. Recently, this rule was reported in the plant developmental process of embryogenesis[13] and completed with the concept of the cell shape as the major determinant for oriented cell division[14]. Almost 140 years ago, the importance of cell shape to dictate the division plane was already demonstrated in the field of animal research by the long-axis rule[15] and remains up to date[16,17]. All in all, it is important to remember that these are theoretical postulations and exceptions make the rule. Stochasticity and overriding via signaling molecules can influence the decision as well[18].

In summary, development is all about oriented cell division and cell differentiation, with the goal of creating patterns. Plants especially must modulate these two events very precisely due to their natural limitations: they have rigid cell walls which restrict their movement. One of the plant developmental events that has interested the research community is the process of how a “starter kit” of a plant arises from a single cell (zygote): embryogenesis. Although technically challenging, embryogenesis constitutes a nice model to investigate the fundamentals of plant developmental biology thanks to its very defined beginning and its relatively high degree of predictability.

Plant Embryogenesis

Embryogenesis is an attractive field of study because it provides an insight into the formation of all fundamental tissues. Plant embryos are not only the precursors of adult plants, essential organisms for our lives, but the embryos themselves are a part of our daily life too in the form of legumes, grains, coffee, nuts, etc.

Land plants are also named embryophytes due to their ability to form embryos. Although research on bryophytes like *Physcomitrium patens* (formerly *Physcomitrella*[19])[20] or *Marchantia polymorpha*[21] may facilitate our understanding of the molecular mechanisms underlaying embryogenesis, most of the embryo research focuses on seed plants, prominently on flowering plants. Among flowering plants, we can distinguish between monocots (like maize, rice, barley, or wheat) and eudicots (like tobacco plants or Arabidopsis). Generally speaking, monocot embryogenesis is fundamentally different from that of the eudicots: there is a single cotyledon, the embryo does not follow evident stereotypical divisions,

there is an extra axis (dorsoventral), and boundaries between suspensor and embryonic tissues are less clear[22–24]. Despite the agricultural interest in the study of embryogenesis in monocots (encompassing major crops), lack of an obvious pattern regularity and limited genetic resources have made scientist turn to the plant model Arabidopsis to shed light on this early developmental process. After all, the main regulatory genes of embryogenesis are most likely shared irrespective of embryo morphology[22,25].

Arabidopsis Embryogenesis

The Arabidopsis embryo is a very attractive model to study pattern formation due to the possibility to trace at single-cell resolution cell fate determination and divisions. It starts with the fertilization of an egg cell to form the so-called zygote. The zygote elongates and polarizes before asymmetrically dividing into two transcriptionally distinct cells[26]: the smaller apical cell and the larger basal one (Figure 2). This cell varies the division plane in different directions to form the apical cell lineage that results in the embryo proper. The basal cell divides horizontally to form the suspensor. Except the uppermost suspensor cell, the precursor of the hypophysis, the rest remains extraembryonic and are degraded eventually.

The zygotic division marks the initiation of the apical-basal axis of the embryo[26–28]. Recent studies have shown that early basal cells can acquire embryonic identity if the apical region of the embryo is disrupted[27,29,30]. In this work this is referred to as the suspensor-to-embryo transition. Published works indicate auxin as a potential trigger for this transition[29]. Interestingly, the suspensor cells lose their embryonic potential after the globular stage, the apical-basal axis is then fixed[31]. Hence, Arabidopsis seems to follow a two-step shoot-root polarity formation, a phenomenon also reported in other organisms like the brown alga *Fucus*[32].

Since this work focuses on very early embryonic development, this introduction will delve into the initial steps of embryo patterning formation: the zygote polarization and asymmetric division, the apical-basal axis formation, and the auxin mechanism. Notwithstanding, the road towards a mature embryo involves many steps that will not be described here (if interested these are reviewed in [33–35]).

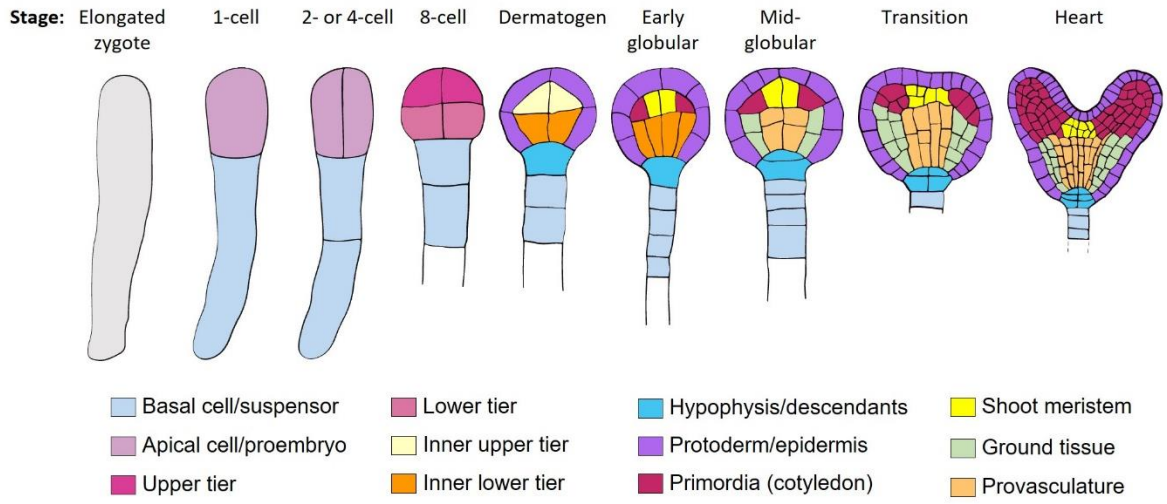

Figure 2. Arabidopsis embryogenesis stages. Starting from an elongated zygote to heart stage. The differentiated tissues are marked with varied colors (see figure legend**).** Modified after [34].

From a zygote to a one-cell embryo

Although cell division patterns vary widely among plant embryos, generally the first zygotic division is transverse and asymmetric[36–38]. The apical-basal size ratio varies considerably among species but, regardless of its size, the apical cell consistently acquires embryonic identity[39]. Genetically, this division in Arabidopsis has been studied based on mutants in which the zygotic division was altered like in *wrky2*[40], *yda*[41] or *mpk3-mpk6*[42]. These mutants allowed us to recognize two major pathways involved in this process: the YDA and the WRKY2/WOX pathways.

The YDA pathway is a Mitogen-activated-protein (MAP) kinase-dependent signaling pathway that can be activated in the zygote via two routes (Figure 3.A). The first route includes the receptor kinase ERECTA[43,44] and two membrane-associated kinases of the BRASSINOSTEROID SIGNALING KINASE (BSK) family: BSK1 and BSK2[45]. The ligand triggering the ERECTA activation remains unknown. The second additional activation route seems to be *Brassicaceae* specific and it is carried out by the membrane-associated pseudokinase SHORT SUSPENSOR (SSP)[46], a non-canonical BSK. Both signaling routes converge onto YDA (MAPKKK), continuing downstream through the MAPKKs MKK4 and MKK5[47], finally reaching the redundant MAPK pair MPK3 and MPK6[42]. The signaling pathway in the zygote culminates with the phosphorylation of the zinc finger transcription factor WRKY2[48]. In spite of the similarities shared by *wrky2* mutants[40] and the *yda* mutants[41], their phenotypes do not fully overlap. This suggests that the YDA cascade has additional targets in the zygote.

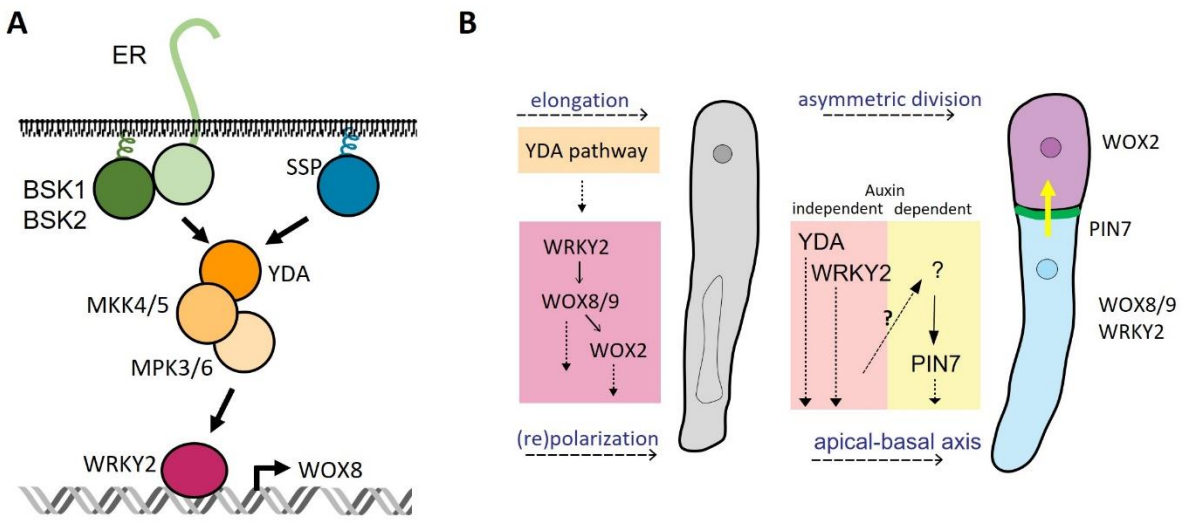

Figure 3. Early embryogenesis mechanism. A) YDA pathway. The two routes for YDA activation (ER-BSK/SSP) are illustrated. Modified after [44]. **B)** Asymmetric division of the zygote and formation of the apical-basal axis in Arabidopsis. At 1-cell stage, the apical cell expresses WOX2, while in the basal cell WOX8/9, and WRKY2 are present. PIN7 is expressed also in the basal cell but localized in the upper side. It directs auxin to the apical cell (yellow arrow).

Once phosphorylated, WRKY2 triggers the expression of WUSCHEL HOMEOBOX 8 and 9 (WOX8, WOX9)[40]. In the basal cell WOX8/9 regulate suspensor development and additionally embryo development in a non-cell autonomous manner with the activation of WOX2 in the apical cell (Figure 3.B)[49–51]. In accordance to this signaling model, in embryos expressing a constitutively active version of YDA (YDA-CA), the WOX8 marker expresses ectopically in the apical cells at early stages in addition to the basal expression and at later stages its suspensor expression is confined to the lower basal cells[52]. Hence the zygote polarization (YDA pathway) relates to the beginning of the embryo patterning (WOX pathway). However, the WOX pathway is not the only pathway dictating the pattern during embryogenesis. Auxin is also modulating the formation of the apical-basal axis and contributing to cell division and fate acquisition decisions.

Auxin in the early Arabidopsis embryogenesis

Auxins are a group of plant hormones initially identified as key regulators of growth (from Greek "auxein"; to grow/increase). The first clues of its action were already documented in the 19th century[53–55], but it took several decades to identify and isolate these molecules[56]. There are four types of endogenous auxins but the most common is Indole-3-Acetic Acid (IAA), generally simply referred to as auxin.

Auxin plays a role in an endless list of plant processes ranging from abiotic stress, through plant immunity to development and more[57]. Here we are going to describe its role in embryogenesis; however, the general molecular mechanism might apply to other plant processes as well.

The first evidence of auxin accumulation during embryogenesis occurs immediately after the zygotic division in the apical cell[28]. This was observed using a reporter (DR5:ER-GFP) based on a synthetic auxin-inducible promoter enriched with auxin response elements (AuxRE)[58]. The polar transport of this phytohormone (basal to apical flow) occurs through an auxin efflux carrier PIN7 situated in the apical end of the basal cell (Figure 3.B)[28].

At the 1-cell stage embryo, PIN7 pumps up the auxin synthesized in the maternal tissue[59]. How the polar localization of PIN7 is determined is still a matter of research. At the 8-cell stage another non-polarized auxin efflux carrier, PIN1, comes into play to redistribute the auxin in the apical cell lineage. At the dermatogen stage auxin is generated in the suspensor cells via the YUCCA (YUC) pathway[60] and an additional source arises in the proembryo apex[61]. The basal to apical flow of auxin reverts at the globular stage, when PIN7 repolarizes to the basal end in suspensor cells and the only left source of auxin is the protoderm. Loss-of-function mutants of PIN7 display aberrant embryonic divisions starting with horizontal positioning of the first apical division, but, in accordance with the model, recover at the globular stage. Nevertheless, higher order of PIN loss-of-function mutants end up in seedlings with severely affected apical regions (e.g: monocotyledon) and shorter primary roots[28].

Undoubtedly, the polar transport of auxin, or in other words, the auxin spatial gradient is fundamental for embryo patterning (see above for French flag model). But how do cells interpret this auxin gradient?

Auxin signaling machinery

The canonical auxin response pathway needs the interplay of AUXIN RESPONSE FACTORS (ARFs) and the auxin-coreceptor formed by the repressors AUXIN/INDOLE-3-ACETIC ACID (Aux/IAAs) and the TRANSPORT INHIBITOR RESPONSE 1 (TIR1). The F-box protein TIR1 is part of a bigger complex: Cullin RING-type E3 ubiquitin ligase $SCF^{TIR1/AFB1-5}$ [62], but for the sake of simplicity in this work, we will refer to it here just as TIR1.

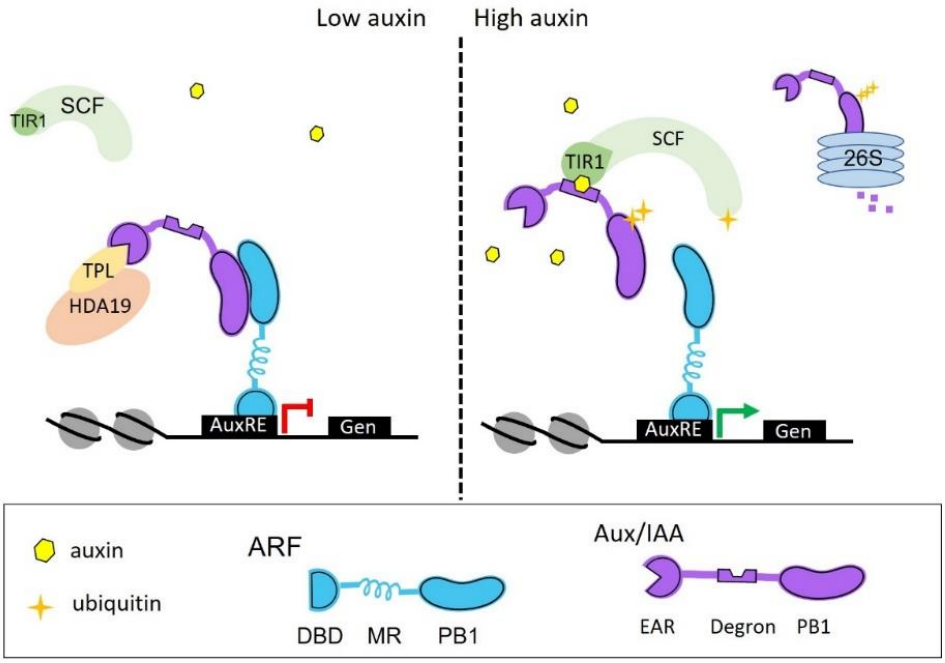

Figure 4. Simplified model of the ARF-Aux/IAA mechanism for auxin response. At low auxin concentrations (left), the auxin response genes are repressed. The Aux/IAAs bind the ARFs through their respective PB1 domains and recruit co-repressors like TOPLESS (TPL). These attract a histone deacetylase (HDA19) and compacts the chromatin. At higher auxin concentrations (right), the auxin glues the TIR1 (SCF complex) together with the Aux/IAA and promotes in this way its ubiquitination (orange stars), and degradation via the 26S proteasome. The ARFs can activate the transcription of auxin-responsive genes.

In a low auxin concentration context, the ARFs are in a repressed state through the direct binding of Aux/IAAs (Figure 4. left)[63]. This might be physically preventing the association of ARFs with co-activators[64] and/or interrupting ARF-ARF interactions[65]. Aux/IAAs can reinforce the ARF inhibition attracting co-repressors like TOPLESS (TPL) and TOPLESS-RELATED (TPR) proteins[66,67]. These co-repressors are recognized by a histone deacetylase (HDA19) that eventually strengthens the repression by compacting the chromatin[31].

At higher auxin levels the landscape changes (Figure 4. right). Until recently, the F-box protein TIR1 was thought to act as the auxin receptor[68,69]. The auxin binding allowed it to recognize Aux/IAAs as substrate[70]. However, now we know that it is really about a co-receptor whose efficient auxin binding relies on the assembly of a TIR1-Aux/IAA complex[70,71]. Upon binding to TIR1, Aux/IAAs are withdrawn by polyubiquitination and subsequently degraded through the 26S proteasome[72]. Thus, the ARFs are relieved of their repression and can recruit chromatin-remodeling ATPases to reopen the locus and activate the transcription of auxin-responsive genes[64,73]. In this way, auxin is acting through the de-repression of auxin-responsive genes.

As mentioned above, the auxin signaling mechanism relies mainly on three components: ARFs, Aux/IAAs and TIR1. In this introduction, we will set aside the latter and focus on the ARFs and Aux/IAAs.

The Arabidopsis genome encodes 23 ARFs (ARF1-23) and 29 Aux/IAAs (Aux/IAA1-20 and Aux/IAA26-33)[74]. Extensive screening of loss-of-function mutants for both families without remarkable abnormal development, at least for most of them, indicates a high degree of redundancy[75,76]. Only 6 single loss-of-function ARF mutants present noticeable abnormal phenotypes: *arf2*[77,78], *arf3/ettin* (*ett*)[79], *arf5/monopteros* (*mp*)[80], *arf7/nonphototropic hypocotyl 4* (*nph4*)[75,81], *arf8*[82], and *arf19*[75,83]. These ARFs are involved in a long list of processes like plant aging, reproductive organogenesis, lateral root formation, embryogenesis, vasculature tissue formation, or gravitropism.

Regarding Aux/IAAs, Arabidopsis loss-of-function mutants have subtle abnormal phenotypes being the most prominent in *iaa3/shy2*[84], *iaa7/axr2*[85], and *iaa17/axr3*[86]. Research on lower plants like *Marchantia* or *Physcomitrium* might bypass the problem of lack of phenotype due to redundancy[21,87,88]. Thus, the characterization of more than 10 of the 29 Aux/IAAs in Arabidopsis has been done using gain-of-function mutants, in which the Aux/IAAs carry a point mutation that prevents their degradation[84–86,89–91]. The study of the mutant phenotype is the first step to clarify the function of these proteins. However, to fully understand how these proteins exert their function, scientists must also study their molecular structure.

Molecular structure of ARFs and Aux/IAAs

The study of the molecular structure of these protein family members has helped scientists elucidate their function in auxin perception and signaling. ARFs consist generally of three domains: a N-terminal DNA-binding domain (DBD), a middle region (MR) conferring activator or repressor activity, and C-terminal protein-protein interaction domain, Phox and Bem1p (PB1) (Figure 4).

The DBD domain is critical for ARF function because it recognizes the aforementioned AuxREs motifs on promoter regions. Additionally, it confers ARFs dimerization ability. This domain consists of three subdomains: a B3 subdomain involved in AuxRE recognition, an ARF dimerization subdomain (DD), and a Tudor-like subdomain of unknown function flanking the other two subdomains[92].

The DBD domain is followed by a middle region that mediates transcriptional activity. Middle regions rich in glutamine are attributed to transcriptional activation (activator ARFs: ARF5-8 and ARF19), while abundance of serines, prolines or threonines stands for transcriptional repression (remaining ARFs: ARF1-4, ARF9-18, and ARF20-23)[63,93]. It is important to note that the classical classification of ARFs into activators or repressors is based mostly on protoplast experiments with certain AuxREs motifs and should therefore be done with caution.

In their C-terminal region, ARFs have a protein-protein interaction domain resembling the structure of a type I/II Phox and Bem 1 domain; hence its name, PB1 domain[58,94]. Apart from its relevance for interactions, it has been postulated to be important for the DNA binding ability of these transcription factors[95].

The PB1 domain is also found in the C-terminal region of the Aux/IAA proteins (Figure 4). The Aux/IAA PB1 domains differ from the ARFs ones in a long insertion sequence that provides them with a more dynamic helix conformation[96]. Unlike in the ARF family, where some members lack this domain (ARF3, ARF13 and ARF17[97]), all members of the Aux/IAA family seem to have it. This supports the notion that the PB1 domain is necessary for the Aux/IAA repression function[98,99].

In protein interactions, the PB1 domains position in a front-to-back configuration determined by an electrostatic arrangement between a positive and a negative cluster[65,95,96,100–102]. This domain not only promotes the homodimerization ability of ARFs and Aux/IAAs, but also heterodimerization and multimerization[101]. The formation of heterogeneous chains of Aux/IAAs has been proposed as a way for the auxin signaling to gain specificity[101]. Whether multimerization of Aux/IAAs is fundamental for their repression activity is still a matter of debate, it might be for some Aux/IAAs[101], but not for all[95]. From an Aux/IAA standpoint, it has been postulated that oligomerization determines the binding affinity of the TPL/TPR co-repressors[103]. Additionally, Aux/IAAs PB1 domain seems to affect the efficiency of the TIR1-Aux/IAA interaction[104]. In brief, the PB1 function seems to be more complex and versatile than initially suggested.

Continuing with the Aux/IAA molecular structure, the PB1 domain in the C-terminal region follows the degron domain (Figure 4), required for the auxin-induced degradation of these repressors[105]. Classically, the auxin response relies on the degradation of the repressor via the TIR1 complex, thus the degron domain plays an essential role[106,107]. The core of this domain consists of 13 conserved amino acids[72,107]. A single point mutation in this core leads to stabilization of the Aux/IAA, generating auxin-insensitive mutants[89,91]. Auxin signaling specificity has been attributed in part to differences in Aux/IAAs degradation rates at varying auxin concentrations[71,108]. This is not only achieved by slight sequence variations in this domain, but also a higher diverging or even absent degron domains add flexibility to the auxin response[108–110].

Finally, in the N-terminal region of Aux/IAAs, there is the repressor domain (Figure 4). This is also known as the EAR domain thanks to its similarity to the ethylene response factor associated amphiphilic repression (EAR) motif[111,112]. Through this domain, Aux/IAAs interact with co-repressors TPL and TPR as mentioned above[67]. Non-canonical Aux/IAAs IAA32 and IAA33 lack this domain[112].

Accordingly, studies in the moss *Physcomitrium* suggest that this domain is not essential for the Aux/IAAs to exert their repression[99].

Conserved domains point inevitably to a functionality, but we cannot forget the relevance of intrinsic disordered or interdomain regions. These have been shown to be particularly important for dimerization[113], degradation[71,108,114] and a potential role with ARF or Aux/IAAs interaction cannot be discarded. These are also often the target of post-translational modifications[115].

Post-translational modifications may add another layer of complexity to the auxin signaling pathway in addition to the combinatorial possibilities between ARFs, Aux/IAAs and TIR1 degradation. Phosphorylation of ARFs[116] and Aux/IAAs[110,117] has been shown to affect auxin response destabilizing interactions and/or conferring higher auxin-resistance.

Regarding auxin response specificity achieved by the combination of ARFs and Aux/IAAs, some pairs have been reported over the years. The first ARF-Aux/IAA pair was reported almost 20 years ago in the context of embryonic development: MONOPTEROS (MP)/ARF5 and BODENLOS (BDL)/IAA12[89]. However, these are not the only ARF, and Aux/IAA expressed during embryogenesis.

ARFs and Aux/IAAs in the embryo: IAA8 as potential candidate

IAA12 was the first Aux/IAA identified to function during embryonic development. With horizontal first apical divisions and ultimately rootless seedlings, a gain-of-function mutant attributed to a point mutation in the degron domain led to this discovery[118]. This phenotype shared some similarities with *mp*[119] and three years later they were established as the first ARF-Aux/IAA module[89]. Since then, two other Aux/IAAs have been identified as players during embryogenesis: IAA10 (with ARF9 as partner)[120] and IAA18[121].

Intriguingly, transcriptomic data suggest that most likely other Aux/IAAs and ARFs also play a role in this early developmental process[122–125] According to this data, IAA8 is significantly upregulated 24 hap in the zygote compared to the egg cell expression[124]. At 2-cell stage IAA8 is also the most highly expressed Aux/IAA[125] (Figure 5). This makes IAA8 an interesting candidate for regulating the early embryonic pattern formation.

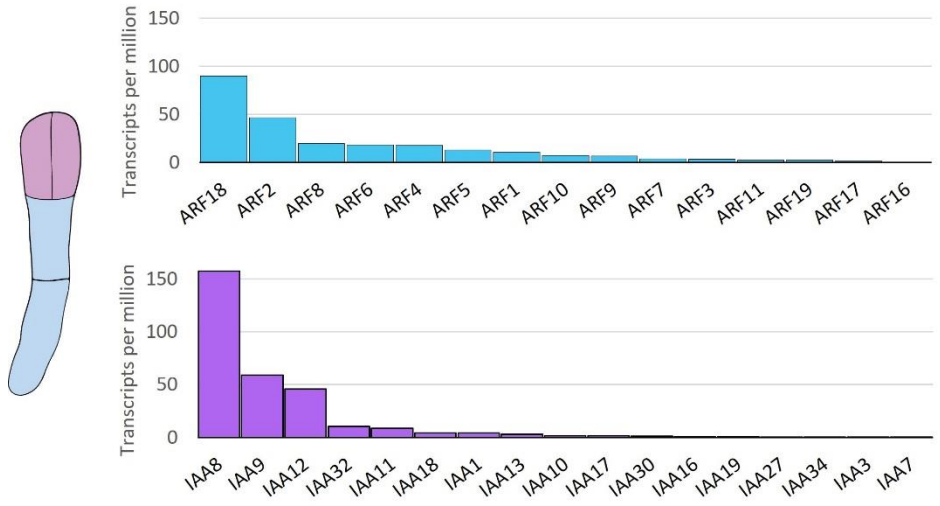

Figure 5. Expression of Aux/IAAs and ARFs at 2-cell stage in Arabidopsis. Graphic compiled from published RNA-seq data [125].

The function of IAA8 in Arabidopsis was first reported in lateral root formation[126]. The loss of function mutant *iaa8-1* has a subtle, almost wildtype[76], phenotype: delayed seed germination[127], and an increased number of lateral roots[126] and adventitious roots[128]. In addition, the double mutant *iaa8-1 iaa9-1* lacks leaf serrations[129]. On the other hand, gain-of-function IAA8 mutants have fewer lateral roots[91,126], twisted leaves, short primary inflorescence stems compensated with more lateral branches and a shorter primary root[91]. The plant reproduction organs are also affected: abnormal floral organs with dramatically decreased jasmonic acid levels leading to bent siliques with few seeds[91].

Where repression is needed, IAA8 exerts its function not only by increasing its expression, but also its stabilization via an unidentified post-translational modification[127]. This post-translational modification could be phosphorylation as IAA8 has been identified as a MAPK substrate[130]. A fundamental role of phosphorylation of Aux/IAAs to achieve a higher auxin-degradation resistance has been recently reported[110,117].

The search for IAA8 interactors has been conducted mainly using the PB1 domains of ARFs in two-hybrid assays[91,126,131]. However, discrepancies often arose between these publications, probably attributable to the different ARF regions used. Additionally, some were not confirmed when full length proteins were used[128].

Among the ARFs with more IAA8 interaction evidences using a two-hybrid approach are ARF5/MP[91,126,131,132], ARF6[91,131,132], ARF7[91,126,131,132], ARF8[91,131,132], and ARF19[91,126,131,133].

Some of these interactions correlate with similar mutant phenotypes observed with ARF loss of function mutants: affected lateral and adventitious roots development (*arf7/arf19*[75]) or reduced jasmonic acid level and abnormal floral organs (*arf6/arf8*[91,134]).

Given the high degree of freedom when it comes to ARF-Aux/IAA combinations[131], there must be additional mechanisms to achieve a specific outcome. One evident factor already mentioned is the Aux/IAA degradation rate. For example, IAA8 interacts with all TIR1/AFBs at very low auxin concentrations, while IAA12 needs a thousand times more auxin concentration and the interaction is restricted to TIR1 and AFB2[71]. Other layers adding to the complexity of auxin response could be the multimerization of Aux/IAAs[101], post-translational modifications (phosphorylation)[110,117] and a tightly controlled spatiotemporal expression[120]. The interpretation of the auxin signaling is therefore an intricate process.

Thesis scope

Auxin is a powerful molecule that participates in an immense amount of very diverse processes. How does this same signaling molecule translate into such varied outcomes? What are the links between the auxin pathway and other essential signaling pathways?

One of the main functions of auxin is the coordination of the developmental process, which translates into the coordination of cell differentiation and division. In embryonic development, auxin might exert this function with support of other essential pathways like the above-mentioned YDA-pathway or the WOX-pathway.

In this work, we study through a gain-of-function mutant how an auxin transcriptional repressor, IAA8, translates the auxin signal in the early steps of embryo patterning. Emphasis is partially set into potential differences with IAA12, also involved in embryogenesis. The IAA8 embryonic phenotype is analyzed with special attention to oriented cell divisions and cell fate changes. A brief look into the molecular mechanisms, e.g., ARF potential interactors and implication of the PB1 domain, is also provided.

Secondly, we study identity acquisition during the suspensor-to-embryo transition through an artificially induced embryogenesis. This involves the prolonged and transient activation of the YDA-pathway through overexpression of SSP. Not only embryonic and extraembryonic markers are studied but also auxin response.

CHAPTER 1

A gain of function mutation in IAA8 affects cell division and differentiation in the early embryo

Marina Ortega-Perez, Helen Schäfer, Lisa Asseck, Martina Kolb, Daniel Slane, Martin Bayer and Gerd Jürgens.

Declarations of contributions

This project was planned by me with the helpful input of Gerd Jürgens and Martin Bayer. I wrote the manuscript and made all main and supplemental figures and tables; Gerd Jürgens, Martin Bayer and Kai Wang reviewed it. Helen Schäfer screened several of the UAS:gIAA8D lines (used by me to assess the mutation percentage in the Supplemental Table 1), checked the expression of the shorter IAA8 promoter reporter line (data not shown), analyzed the embryos transactivated with the MEA promoter and cloned the ARF plant lines used for the pull-downs under the supervision of Daniel Slane. She also generated the pDRN driver line used in this study. The rest of the driver lines (except for pIAA8) were provided by Martin Bayer. The promotor activity domain scheme was modified from a template done by Daniel Slane. He and Martina Kolb generated the constructs used for the Dual-Luciferase assay. Regarding *in situ hybridization*, Martina Kolb worked towards the establishment of the method(s) and assisted me when needed. Lisa Asseck helped with the *in situ* crosses and carried out the removal of the antibody and addition of detection buffer in every repetition round. She also confirmed the knockout in the loss of function mutants and carried out the co-immunoprecipitations and westernblots with ARFs/IAAs plant lines and recombinant proteins. She cloned the ARF5 recombinant protein and generated the western blot photos that I modified for use in Supplemental Figure 9. The recombinant Aux/IAAs were designed and cloned by me. The rest of the experiments and their analysis were carried out by me.

Acknowledgements

We would like to thank Patric Sulz for making the initial steps into this project. The UAS:gIAA8D lines we have used are of his making. As well as the shorter promoter reporter line that Helen Schäfer checked (data not shown). Sarah Baumann generated the IAA12D-GFP plant line for Co-IP and mass-spectrometry. Jasmin Ehrismann and Steffen Lau assisted with experiments and discussions. Houming Chen generated CRISPR alleles of iaa8 and iaa9, not included in this publication (designed by me).

Extended Summary IAA8 Project

One of the most intriguing pattern formations is that of the embryo: embryogenesis. From a single cell (zygote) the cells divide and acquire different cell fates building in a few hours the basics of an adult organism. The Arabidopsis embryo constitutes an ideal model for the study of this process thanks to its predictable pattern of cell division and availability of genetic resources. The asymmetric division of the zygote generates a smaller apical cell and a larger basal one. The apical-basal axis is formed at this stage and will be irreversibly established at later stages (transition stage)[26,31]. The accumulation of auxin in the apical cell at this stage indicates a key role of this hormone in embryonic patterning[28]. This signaling is interpreted in the cell nucleus by auxin response factors (ARFs), their repressors Aux/IAAs and the ubiquitin ligase complex $SCF^{TIR1/AFB1-5}$ [63,70,72,107]. The presence of auxin triggers the degradation of Aux/IAAs and de-represses auxin response genes. In Arabidopsis 23 ARFs and 29 Aux/IAAs have been identified[74]. Unlike for some ARFs, loss-of-function Aux/IAA mutants result in almost wildtype phenotypes[75,76]. For this reason, the role of these repressors has been studied traditionally with gain-of function mutants[84–86,89–91]. These mutants carry a stabilizing mutation that prevents their auxin-mediated degradation. A few ARFs and Aux/IAAs have been reported to function during embryogenesis[118,120,121], the most remarkable of which being IAA12 and ARF5, which are essential for the hypophysis specification[89,98]. Nevertheless, transcriptional data at earlier stages suggest that other Aux/IAAs might be involved in earlier steps of the embryo formation[122–125]. One of these clearly upregulated Aux/IAAs during the first steps of embryogenesis is IAA8.

To assess the spatial-temporal expression of IAA8, we made use of reporter lines and *in situ* hybridization. IAA8 is expressed in the embryo from the zygote stage onwards in a seemingly ubiquitous manner. Its expression continues to mature embryo, focusing on the meristems and vascular tissue. Its expression in the developed root has also been observed.

To study IAA8 function, we checked the available loss-of-function alleles, including a double mutant (*iaa8-1 iaa9-1*), but no abnormal phenotypes were observed. Then we generated plant lines overexpressing IAA8D (a stabilized version of this Aux/IAA). Thanks to a transactivation system we were able to express IAA8D in different embryo domains and stages. We used the RPS5a promoter to compare the overexpression (OE) of IAA8D with the previously published IAA12D at early embryogenesis stages. Although zygotes of both overexpression mutants were slightly longer compared to wildtype, the earliest most remarkable phenotype was a horizontal division observed at the 2-cell stage in the apical cell. This first horizontal apical division was more frequent in IAA8D OE embryos than in the IAA12D ones. The percentage of aberrant divisions rose over the stages ultimately leading to non-germinating seeds.

The transactivation system allowed us to check the effect of IAA8D OE under different promoters. In general, using promoters active in the apical cell lineage resulted in severe mutant phenotypes, while suspensor-specific expression led to no significant differences with wildtype embryos. Interestingly, the domain-specific overexpression of IAA8D resulted sometimes in differences with the one of IAA12D. This possibly indicates major functional differences between these two Aux/IAAs. Furthermore, the embryonic IAA8D phenotype was most likely embryo-autonomous since overexpression in the endosperm did not cause a mutant phenotype.

We wondered if the wrong divisions observed were accompanied by a failure in cell fate determination. Our *in situ* hybridization approach with several early apical and basal markers confirmed an altered cell differentiation in IAA8D OE embryos. These altered cell identities were more frequent than the observed wrong divisions, and more prominent than in IAA12D OE embryos. Moreover, the aberrant cell fates were more frequent in IAA8D OE than IAA12D OE embryos, suggesting an IAA8 specific outcome.

Using the auxin-response reporter line DR5:ER-GFP we confirmed the lack of auxin response in IAA8D OE embryos at later stages. Furthermore, we used auxinole to block the auxin response in cultured ovules and observed the same horizontal apical division in addition to an oblique division. All together this indicates that the IAA8 gain-of-function phenotype is most probably caused by a specific disruption of the auxin response.

To achieve the specificity of the auxin response, IAA8 might be inhibiting one or more ARFs in the early embryo. Dual luciferase experiments in protoplasts with some ARFs pointed to ARF5, known to interact with IAA12, as potential candidate. We swapped the interaction domain (PB1) between IAA12 and IAA8 to evaluate the affinity for their respective ARFs and found out that this would not interchange their embryo mutant phenotypes. Thus, the affinity towards one ARF or another might not rely solely on the PB1 interaction domain. The interaction between recombinant IAA8D or IAA12D with plant derived ARF5 could not be confirmed neither with western blot nor with mass-spectrometry. Immunoprecipitation and subsequent mass-spectrometry with plant derived IAA8D and IAA12D is currently being done and expected to provide potential interactions soon.

In brief, an overexpression of IAA8D in the early embryo leads to an incorrect division plane in the apical cell and affects cell differentiation as well. This phenotype is most likely caused by an IAA8 specific block of the auxin response, and its specificity does not rely solely on the interaction domain with a certain ARF. Additional research is planned in the near future to further our understanding of this molecular mechanism.

CHAPTER 2

A filament-like embryo system to study basal cell-embryo transition

Kai Wang, Yingjing Miao, Marina Ortega-Perez, Houming Chen, Martin Bayer

Declarations of contributions

Kai Wang and Martin Bayer designed the project. Kai wrote the manuscript and made the figures. I, like the rest of the authors, contributed to the scientific ideas, discussion, and interpretation of the project. Houming and I revised the manuscript as well. Kai performed the main experiments. Yingjing Miao checked the *gWOX8Δ-nls-3xVenus* signals and the *DR5:GFP* signals. Houming documented the DR5:GFP signal too. I checked and documented the promoter activity of the pS4:NLS-GFP transgenic line for Figure 1. I also crossed and screened the embryonic identity markers MONOPTEROS (Figure 4) and PIN1 and HAN (not included in the manuscript). Additionally, I carried out a treatment with an auxin transport inhibitor (NPA) in ovule culture to check if the generation of the proembryo-like clusters would be repressed. However, no clear conclusions could be drawn from this experiment and was not included in the manuscript.

Extended Summary pS4:SSP Project

Most multicellular organisms develop from a single cell in a process known as embryogenesis. Unlike in the animal kingdom, plant embryos are initially connected to maternal tissues via a file-like structure called suspensor[135]. This structure not only positions the embryo in the endosperm but also provides nutrients and signaling molecules, for instance the phytohormone auxin[136,137]. Except for the upper most cell that will generate the root meristem[118], the rest of the suspensor is degraded at late stages of embryogenesis[136,138]. The suspensor arises from the basal cell, generated by the asymmetric division of the zygote. The other resulting cell, the smaller apical cell, will be the precursor of the embryo proper. In Arabidopsis this asymmetric division is mainly regulated via the YDA signaling pathway. This pathway is initiated by an unknown ligand binding the ERECTA receptor which triggers the phosphorylation cascade from the BSK1/BSK2 to YDA and continues through MKK4/5 and MPK3/6 [42,44,45,47]. In *Brassicaceae* an additional more direct activation of the YDA pathway can occur through the paternal SSP[46]. SSP bypasses the receptors and activates YDA directly. Recently it has been shown that until the transition stage the suspensor cells are capable of becoming embryonic cells if the apical cell lineage is disrupted[29,30]. Thus, the embryo proper seems to inhibit non-autonomously the suspensor-to-embryo transition[27,29]. TWIN genes might participate in the inhibition of transitions since loss-of-function mutants develop secondary suspensor-derived embryos despite a normal embryo on the top[139,140]. Furthermore, an increase in auxin response is observed in the suspensor cells about to transition, opening the door to speculate about a role of auxin in the process too[29,141,142]. Nevertheless, we are still far from understanding how the suspensor-to-embryo transition is regulated. Removal of the proembryo via laser ablation might be an elegant way to do it[29]; however, damage of the whole embryo and its technical challenging limitation could impede a proper analysis.

Here we developed an alternative system to study this process based on a transient prolonged activation of the YDA pathway. We confirmed that constitutive active YDA (YDA-CA) transgenic lines resulted in filament-like embryos[41] and decided to use a transactivation system to bypass the fertility defects associated with these lines. Transactivation of a constitutive active MPK6 (MPK6-CA) under the RPS5a promoter resulted in filament-like structures with eventually initial embryo-like structures. However, no viable seedlings were obtained; this YDA activation was still too strong for our purposes. Next, we tried to express SSP under a promoter only active at early embryogenesis stages, the S4 promoter: *$S4_{pro}$:SSP-YFP*. With this system we were able to report vertical divisions in the filament-like structure as early as 2 days after fertilization, being the embryo-like divisions more frequent 3 days after pollination. The resulting seeds were able to germinate and twin/triple-seedlings were observed. The ectopic expression of SSP led to a prolonged activation of the S4 promoter, indicating a potential

feedback loop. Crosses with cell identity fluorescent markers allowed us to determine that the cells in the filament-like structure resembled early basal cells. The embryonic identity acquisition was delayed and even where vertical divisions were observed, the signal of the embryonic marker was absent.

Since auxin might be involved in the suspensor-to-embryo transition, we checked in our system the auxin response using the DR5:ER-GFP reporter. Embryos with a recognizable wildtype apical region showed an auxin response maximum shifted to basal cells, indicating a possible initiation of a secondary proembryo-like structure. Auxin might serve as impulse for the suspensor-to-embryo transition.

With our prolonged and transient activation of the YDA pathway we were able to generate filamentous-like embryos with a delayed embryonic identity acquisition. These mutant embryos often resulted in several proembryo-like structures which ultimately lead to twin/triple seedlings. With fluorescence markers we were able to determine that these filamentous cells retained early basal identity for a longer period. Furthermore, the acquisition of the embryonic identity in the apical regions was delayed. At some point, maybe due to an auxin accumulation along this structure, an auxin response maximum might trigger the formation of an embryonic cluster. Thus, this system allowed us to study how the suspensor-to-embryo transition occurs.

Discussion

A single cell can give rise to a whole new organism through the process known as embryogenesis. Although scientists have been fascinated by Arabidopsis embryogenesis for over almost 60 years[143,144], there is still much to decipher. Our knowledge is still not sufficient to fully explain how the cells make the decisions to differentiate and divide to go from a zygote to a mature embryo with all basic tissues. The fact that after decades of research our understanding is not sufficient, already tells us how tight and intricate the modulation of the embryonic pattern formation is.

In this work we tried to disentangle how this complex process works in the plant model Arabidopsis from two perspectives:

- From a direct auxin response inhibition perspective: effect of IAA8 on early cell divisions and fate determination.
- From a filament induced embryogenesis perspective: cell identity disruptions caused by embryonic prolonged activation of the YDA pathway.

Auxin and cell divisions in the very early embryo

Traditionally, the first implication of auxin in embryogenesis occurs after the asymmetrical division of the zygote, in the apical cell that is to divide vertically. Auxin accumulates in this cell[28], and it presumably modulates the direction of the division plane, although direct evidence is still lacking[13]. This has been inferred from disruption of the polar auxin transport[28] or direct blocking of the auxin response[80,118] which results in transverse divisions in the apical cell. The auxin response is interpreted by the cells via a system that involves Aux/IAA proteins[145]. IAA8 (and its homolog IAA9) are the Aux/IAAs expressed most highly during early embryogenesis[122,124], but their function in this process has remained unclear; a conundrum begging an explanation. IAA8 is expressed during zygotic activation[124], thus, making it an interesting candidate to study the early steps of the zygote towards an embryo and the auxin implication.

Here, we report that the expression of a degradation-impaired IAA8 version under the RPS5a promoter leads to aberrant divisions in the Arabidopsis embryo (Appx. 1 Figure 1). Furthermore, the frequency is higher compared to the previously reported RPS5a>>IAA12D, constituting a first solid evidence of an auxin transcriptional response occurring in this context. This is the first prominent mutant phenotype of IAA8D overexpression; nevertheless, we also observed significant differences one step earlier, during zygote elongation. The apical and basal cells of these gain-of-function embryos are longer than

expected in wildtype, but the asymmetrical division remains unaffected (Appx. 1 Supplemental Figure 3). The same phenomenon has been reported recently for constitutively active YDA (YDA-CA)[44]. Interestingly, phosphorylation sites for MPK have been reported for IAA8[130], specifically for all but the splice variants 1 to 3[146]. Furthermore, prolonged activation of the YDA pathway leads to a similar RPS5a>>IAA8D mutant phenotype (Appx. 2 Figure 1). All this could hint towards a connection between the embryonic YDA pathway and a transcriptional auxin response via IAA8. Regarding the affected zygote elongation of our gain-of-function mutant, it could be interesting to follow up this line of research to see if auxin plays indeed a role, perhaps indirect or subtle, in embryo patterning earlier than postulated.

Back to the 2-cell stage mutant phenotype, we tried to decipher if the transverse division of the apical cell caused by IAA8D can truly be attributed to a disruption in the auxin response. At later stages, the DR5:ER-GFP signal observed in the hypophysis (Appx. 1 Figure 5 and Appx. 2 Figure 5) is not present in IAA8D embryos, a strong indication that IAA8 functions by inhibiting the auxin response. Nonetheless, the lack of signal in our DR5 reporter line at 2-cell stage, led us to test this observation at early embryonic stages in a slightly indirect way using auxinole (AXO). Auxinole prevents the formation of the auxin receptor complex between TIR1 and Aux/IAAs[147], which ultimately results in a constitutive repression state of the auxin response, at least via this TIR1-Aux/IAA pathway. Embryos cultured with AXO-treatment exhibited significantly more aberrant divisions at 1-cell stage, these including not only in the horizontal but also in an oblique orientation (Appx. 1 Figure 5). The fact that the apical cells also divided in an oblique direction is compelling, since this has not been observed neither in our auxin-insensitive mutant nor in BDL embryos[118]. Other mutants like *gnom* (*gn*)[148] (responsible for vesicle trafficking of PIN1[149]) or WOX2 (*Houming, personal communication*) do show an obliquely oriented division additional to the transverse one. It is tempting to speculate that auxin might be able modulate this division not only in an Aux/IAA-dependent way but also through an as of yet unknown mechanism. Nevertheless, the transverse division observed in our IAA8 gain-of-function phenotype is most probably caused by auxin disruption. Furthermore, it is not a general blocking of the auxin response, nor a general Aux/IAA repression, since the phenotype differs from RPS5a>>IAA12D. Given its nature as Aux/IAA, IAA8 might be doing this together with a specific ARF in this early embryonic context.

Mechanisms of the Aux/IAA-ARF response in the apical cell

Pairs of ARF and Aux/IAAs in embryogenesis have been reported already twenty years ago. The best characterized is the ARF5-IAA12 module, also known as MONOPTEROS (MP) and BODENLOS (BDL)[89]. More recently, the pair of ARF9 and IAA10 has also been reported in embryonic development[120]. The

ARF-Aux/IAA interaction is taking place supposedly through the PB1 domain of both ARF and Aux/IAA[63] (for more information refer to introduction section). Since the module ARF5-IAA12 is widely studied and IAA12D overexpression leads to a different mutant readout than IAA8D (Appx. 1 Figure 2, Figure 3, and Figure 4), we used it to study potential IAA8 interactors.

Firstly, some dual luciferase experiments pointed towards an interaction with ARF5 (Appx 1 Supplemental Figure 8). Our cell fate experiments (discussed in depth later in this section) suggested again ARF5 as potential candidate since DRN and PIN7 have been shown to be direct targets of this ARF[150,151]. No other clear direct interactors came to light with the *in situ* experiments, but a probable interconnection with other pathways (YDA/WRKY2) was implied.

Secondly, and most disconcerting, was the result of exchanging the PB1 domains of IAA12 and IAA8, referred to as 8-8D-12 and 12-12D-8 (EAR-Degron (D: stabilized)-PB1 domains). In theory, this would exchange the ARF interactors, accordingly, the specific auxin response blocked and ultimately would be reflected in a swap of gain-of-function phenotypes. Much to our surprise, the mutant phenotypes remained unchanged (Appx. 1 Figure 6). We did observe a slight strengthening of the typical IAA8 gain-of-function phenotype in the 8-8D-12 lines: more filament-like structures were documented than with RPS5a>>IAA8D (Appx 1 Figure 6) and consistently with the reported more elongated zygotes with intact polarity, extreme lengthy basal cells were observed too. This observation is perhaps attributable to the use of the coding sequence for the swap construct instead of the genomic one and consequently a more rapid translation of the protein. Another explanation could be a stronger stabilization of IAA8 conferred by the PB1 domain (discussed in more detail below)[104]. In 12-12D-8 lines, the transverse divisions were very seldom, and an acute angle was sometimes observed at 8-cell stage, in accordance with the RPS5a>>IAA12D phenotype. These observations open several possibilities: 1) the co-repressors recruited via the EAR domain[67,112] might play a more influential role than previously postulated in determining the outcome of the auxin repression. A yeast-two-hybrid analysis showed that IAA8 only interacts with one of the 5 TPL/TPR proteins[66], and IAA12 could have an affinity for another one. However, in our mass-spectrometry data after pull-down with recombinant Aux/IAAs several TPL/TPR proteins were identified indistinctly for both Aux/IAAs, indicating no specific preference for co-repressor (Appx. 1 Supplemental Figure 8). Another possibility could be that the oligomerization through the PB1 domain affects the TPL/TPR binding affinity as suggested previously[103]. Hence, exchanging the PB1 domains, might influence the oligomerization setup and eventually the repression. 2) IAA8 and IAA12 are targeted differently from the TIR1 complex in the same auxin level environment, which in turn determines their degradation rate and the liberation of the repression. In our experiment, both Aux/IAAs carry a point mutation in the degron domain which stabilizes the protein; however, their degradation rate might still differ due to differences in the

domain itself[108] or attributed to the other domains or interdomain regions[104,114]. It has been shown that exchanging the PB1 domain between Aux/IAAs affects the TIR1 interaction in both ways, positively or negatively[104]. Nevertheless, in our case the PB1 swap does not result in an appreciable change of phenotype; thus, the TIR1-dependent degradation might not be as influential for the mutant readout or the PB1 domains of IAA12 and IAA8 confer similar TIR1 affinity. 3) Other regions apart from the strict PB1 domain determine the interaction with ARFs directly or indirectly. As it happens with the TIR1 interaction[104], intrinsically disordered regions or even the other domains could be modulating allosterically the interactions traditionally attributed to the PB1 domain[152]. Not only the direct Aux/IAA-ARF interaction could be affected, but maybe the ability of these Aux/IAAs to multimerize and form chains, which have been postulated to confer the auxin signaling specificity[101]. Compared with the ARF domains and functions, little is known about Aux/IAAs, leaving us with a wide angle for speculation. Are Aux/IAAs also binding DNA and positioning themselves close to their ARF interactors in this way? Are other domains or interdomain regions contributing in an allosteric manner to the interaction specificity of the PB1 domain? Undoubtedly, the PB1 domain is necessary for repression function of Aux/IAAs[98,99], but perhaps not sufficient for the specificity of the ARF-Aux/IAA interaction.

With the PB1 swap experiment we tested at the same time potential differences on splice-variants of IAA8. The relevance of alternative splicing in an embryonic context has been reported recently[26,153]. We used the splice variants 1 and 4 in our experiment. The splice variant 1 together with 3 are the most prominent in the embryo *(Nodine, personal communication)*, while variant 4, although regarded as the representative gene model in TAIR, seems to be absent[154]. To the naked eye, the embryonic phenotype of both variants appears the same and there were no statistical differences on frequency (Appx. 1 Figure 6). Hence, alternative splicing might not play an important role in the function of IAA8 in the early embryo.

Auxin and cell fate determination in the early embryo

Cell division is essential for developmental processes as is cell differentiation. Often erroneously thought to be inseparable, cell division does not necessarily govern cell fate determination. These divisions are fundamental to the creation of embryonic structures, but their orientation plane does not always dictate the identity pattern. For instance, some mutants display aberrant embryonic divisions, but their apical-basal axis remains intact[155–157].

For this reason, we should not only check the orientation of the cell division plane but also their cell fate via their transcriptomic profile. In fact, more transcriptionally abnormal cells were observed in our

study than incorrectly divided ones (Appx. 1 Figure 4) in our experiments with the gain-of-function IAA8, consistent with to previous reports[158].

We used *in situ* hybridization (Chapter 1) and fluorescent markers (Chapter 2) to assess the cell identity. When the YDA cascade was ectopically activated (pS4:SSP-YFP), the resulting filamentous structures retained early basal cell identity (Appx. 2 Figure 4). The embryo-like clusters did not acquire embryonic identity. This suggest that the cells are proliferating but maintaining a basal stem cell identity. In this mutant, the auxin response was altered (Appx. 2 Figure 5): expression of the DR5 reporter was sometimes weak in the putative hypophysis and stronger at the boundary of the suspensor-like cells and the embryonic-like cluster. Interestingly, this could imply that an auxin response precedes the acquisition of the embryonic identity, since these clusters did not resemble embryonic cells yet, but the auxin is accumulating in the putative boundary between the suspensor and future embryo. In a similar way, the blocking of the auxin response through the overexpression of IAA8D results in frequent abnormal cell fate changes (Appx. 1 Figure 4). Apical/embryonic markers tended to extend to the basal cells and/or disappear or randomly located in embryo-looking cells. It is intriguing to see that the overexpression of IAA8D does not trigger a binary switch: apical cells do not turn systematically into suspensor ones, but rather the cell differentiation seems to fluctuate and occur stochastically. For example, the HAN marker is often incorrectly found in the suspensor but at the same time some apical markers continue to express it while others do not (Appx. 1 Figure 4 A). In the same way, the WOX8 marker is sometimes mislocated in apical cells, but not homogeneously: often only one of the two apical cells had WOX8 identity (Appx. 1 Figure 4 E). This event was also observed with the pS4:SSP mutant (*Kai Wang, personal communication*) and has been reported recently for YDA-CA too[52].

At this point, we cannot ignore the multiple hints for a potential connection between the YDA pathway and the IAA8 mediated auxin response. As mentioned before, they do not only share similar effects in aberrant cell divisions (and in zygote elongation when compared with YDA-CA), but the overactivation of the YDA cascade seems to delay the auxin response and affect cell identity in a IAA8-like tendency. Furthermore, similar WOX8 mispatterning (restricted to the lowest basal cell) (Appx. 1 Figure 4 E) was observed in YDA/-related mutants like *wrky2*[40], *hsp90*^{RNAi} and YDA-CA[52]**.** A possible link could be the presence of MAPK phosphorylation sites in IAA8[130,146]. This is a matter that should be researched in the coming years.

From both, RPS5a>>IAA8D and pS4:SSP experiments we can conclude that auxin indeed modulates the cell differentiation during early embryogenesis, but in absence of this signal the differentiation process still occurs in an apparently initial stochastic decision which then triggers a more coordinated differentiation of surrounding cells. Thus, identity acquisition might not be as deterministic as the scientific community expects. Embryonic or suspensor identity might appear in one cell that for an

unkown reason (maybe attributable to stochasticity) escapes the SSP/IAA8 repression. Furthermore, cell differentiation is not a binary switch since some cells might be in a middle stage between both identities; this explains the variation in marker signal strength observed in both experimental set ups. A single cell transcriptomic analysis would give us a more thorough overview of what is actually happening in the cell, but often these techniques rely on cell fate markers to sort them[123,159], which might be altered in mutants as in our case RPS5a>>IAA8D or pS4:SSP. At a 1-cell stage embryo, manual dissection[160] or laser capture microdissection[25,161] are definitely challenging techniques, especially if the morphology is slightly different from wildtype, but it could be a very informative experiment to carry out in the future.

Putting things together: let us speculate

First of all, it is worth mentioning that the IAA8 work presented here, and conclusions drawn from it are based on a gain-of-function phenotype. From a genetically traditional point of view, the loss-of-function phenotype would be a better suited strategy to address the function of IAA8. However, without being the exception to the norm[76], loss-of-function *iaa8-1* or *iaa8-1 iaa9-1* do not present major abnormalities in embryo development. This indicates a high redundancy among Aux/IAAs, which is to be expected if we consider the high degree of robustness that an essential developmental process should have (more about this in the Introduction section).

Once our limitations are clear, we can speculate based on our findings how IAA8 is acting in the early embryo. We know it probably governs oriented cell divisions and cell differentiation. We could image it acting in the basal cell, where auxin concentration is very low, to prevent it from changing to an apical fate. This would be akin to the situation of stem cell maintenance in the shoot meristem reported for the MP-mediated auxin signaling[162]. Previously reported, stem cells are resistant to auxin-dependent differentiation, but still require a low auxin concentration to maintain their status[163]. This would be in accordance with the low auxin concentration in the basal cell during embryogenesis. In the basal cell, IAA8 is strongly repressive, while maybe in the apical cell, although present, its repression is less strong due to a higher auxin concentration and in consequence a higher degradation rate. This does not mean that IAA8 is expressed in the apical cell without reason (Appx. 1 Figure 1), but in this context IAA8 promotes (maybe indirectly by slowly liberating the repression sites when getting degraded) cell differentiation towards an apical/embryonic fate. Two opposite functions, cell differentiation and stem cell maintenance, for the same protein at different auxin concentrations have been previously postulated for MP[162,164,165]. MP, and ultimately auxin, act in opposite ways in different cells changing from repressing to activating the very same target[150,162]. We can envision this easier with

the French flag model (explained in the general introduction of this thesis) (Figure 6 A): at a low auxin concentration IAA8 maintains the basal cell in an undifferentiated stage, while in the apical its rapid degradation due to higher auxin concentration promotes the differentiation towards embryonic cells. This model can also help us illustrate what might be happening in the SSP artificially induced embryogenesis too. Where the extra embryo starts developing is perhaps dictated by a presumable difference in auxin concentration along the filament. It would be very informative to block the auxin transportation in this mutant (for example via an NPA treatment) and observe where the cells start the reprogramming towards an embryo (if at all).

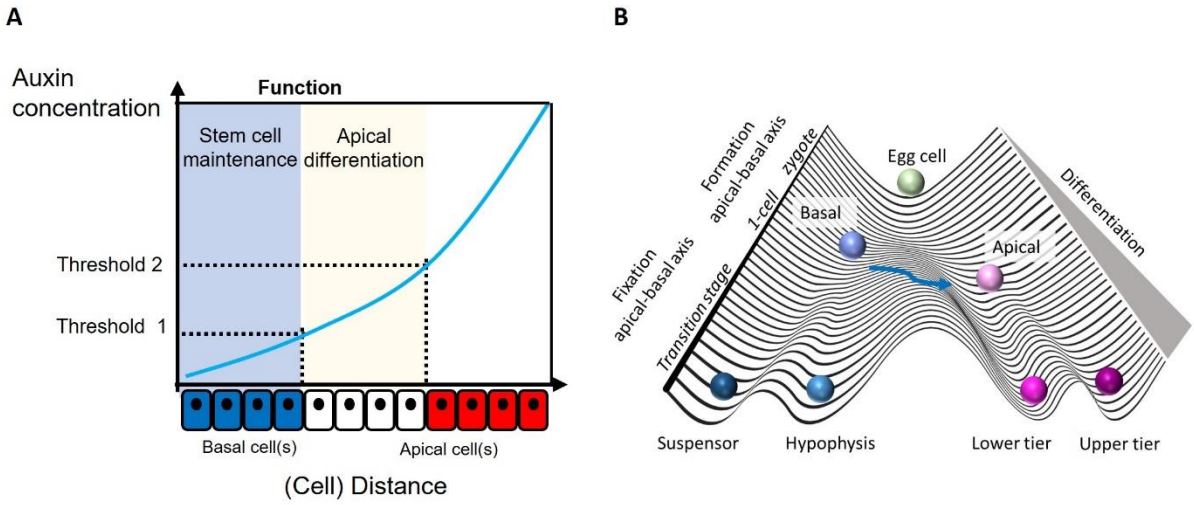

Figure 6. Speculations on how cell differentiation is regulated in the early Arabidopsis embryo. A) French flag model for differentiation adapted to auxin and the early embryo stage. IAA8 might be functioning in the basal cell maintaining early suspensor identity, nevertheless expression data suggests it is also present in the apical cell. It can be that when a certain auxin concentration threshold is reached, in the apical cell, IAA8's function in this cell type might differ from the one in the basal lineage. **B)** Adapted Waddington landscape. When the egg cell is fertilized the marble starts rolling down and differentiating. At 1-cell stage the apical-basal axis is formed. The basal cell (blue) stays in a more undifferentiated state than the apical one (pink); therefore, it has still embryonic potential (represented by blue arrow); the apical cell does not have the ability to revert to an undifferentiated, more basal cell-like, state (arrow only in one direction). At early stages, IAA8 might contribute to the undifferentiated basal cell maintenance, preventing its switch to an embryonic fate. However, at the transition stage, the basal lineage loses this ability and can only lead to differentiated suspensor cells. The apical-basal axis is established.

An adapted Waddington landscape (Figure 6 B) can facilitate the explanation of differentiation in the bigger picture of (early) embryogenesis. Embryogenesis starts with the fertilization of the egg cell (the marble starts running down the differentiation valley) and divides asymmetrically into transcriptionally distinct apical and basal cells: the apical-basal axis is formed[26]. From this stage on (and maybe earlier, albeit more subtly), auxin acts as attractor in this valley, shaping its slopes and esplanades, giving

directionality to the differentiating cells. The pink marble (apical cell) goes down the valley faster, thus not having the ability to dedifferentiate and become suspensor. On the other hand, the blue marble (basal cell) is slower and until transition stage it can jump into the apical lineage valley if a generation of a new embryo is needed (blue arrow)[29–31]. IAA8 overexpression in the suspensor does not cause major disruptions maybe because here its repression is already strong. Thus, it could be contributing to delaying the blue marble/basal cell into an undifferentiating stage at early stages. A prolonged undifferentiating stage is also observed when SSP is ectopically expressed. This, together with other hints mentioned above, could indicate a link between the YDA pathway and IAA8 in the basal cell. An interesting experiment could be to copy the pS4:SSP set up for IAA8 (pS4>>IAA8D) and see if we get a similar phenotype, this would also allow us to liberate the embryo from IAA8 repression at some point and observe its implications in the embryonic development.

If auxin is acting towards differentiation, and this signal is disrupted, the fate directionality is lost, the marble starts fluctuating between the slopes or rather the valley has flattened and where it lands in the end is seemingly random. Regarding cell division, it might rely on a slightly different regulation, since often normally divided cells still show cell-fate failures. Furthermore, disruption of the auxin signaling in the apical cell does not lead to an equally frequent transverse, vertical or oblique first apical division expected from a random decision. Additionally, if auxin directs the division plane in the embryo, why do we not observe a vertical division in the suspensor too when we disrupt this signal? One possible explanation could be inferred from the above-mentioned dual auxin function: a disruption in the stem cell maintenance might not be the same as a disruption in the apical differentiation. Additionally, recent studies suggest the geometrical rule of the shortest cell wall, more precisely, the cell shape to be a major determinant of the orientation of the cell division[13,14]. In normal circumstances auxin overrides this rule, therefore when auxin is disrupted the cell division is not completely random, but it can still be moderately dictated by cell morphology.

Concluding remarks

In brief, in the first chapter of this thesis we have demonstrated that IAA8 plays an intrinsic role in the early embryo patterning by modulating the cell division plane in the apical cell and cell fate acquisition. The IAA8 gain-of-function embryos display aberrant divisions starting with the first apical division and show as well aberrant cell fate differentiation. In the second chapter, we have made use of an artificially induced embryogenesis to study identity acquisition. We have observed how the prolonged activation of the YDA pathway leads to filamentous structures and its release triggers the embryonic transition. It seems that in an undifferentiated cell cluster one cell accumulates auxin and this triggers an identity switch and from then on, the cells start creating a pattern of differentiation to form the embryo.

The similarities between the two processes described in Chapters 1 and 2 suggest an interplay of auxin signaling with other signaling pathways established earlier in the developing embryo (possibly the YDA pathway). We hope that further experiments will reveal the IAA8 molecular mechanism of action. Additionally, future investigations are necessary to reveal how after the zygotic division auxin directs the cell division and fate acquisition decisions. Is auxin the real executor of these decisions or is it just giving a certain directionality? Does auxin reinforce an earlier signal provided by YDA/WRKY2 or does auxin generate it *de novo*? This complex developmental process has occupied scientist for over half a century; nevertheless, our findings and the fundamental questions that this work has given rise to promise an exciting field of study for future research.

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Appendix

A gain of function mutation in IAA8 affects cell division and differentiation in the early embryo

Marina Ortega-Perez, Helen Schäfer, Lisa Asseck, Martina Kolb, Daniel Slane, Martin Bayer and Gerd Jürgens.

Abstract

During embryogenesis, the signaling molecule auxin controls oriented cell division and cell differentiation. In the cell nucleus, Aux/IAA repressors together with another co-receptor are responsible for perceiving the auxin levels and trigger the auxin response through the de-repression of auxin response factors (ARFs). The role of some Aux/IAAs in the embryo patterning has been described before; however, the significance of the most abundant protein of this family in this context, namely IAA8, remained unclear. Here we show that IAA8 ubiquitous overexpression leads to a horizontal first apical division and ultimately to non-germinating seeds. Using basal and apical identity markers, we were able to detect cell fate failures in these mutant embryos. Comparison with IAA12 overexpressing embryos and auxinole treated embryos allowed us to confirm that this mutant phenotype is specific for the auxin response of IAA8. Initial steps towards the elucidation of its molecular mechanism (potential ARF interactors) are presented. Our findings reveal that IAA8 contributes to the decisions of cell division and differentiation in the early Arabidopsis development.

Introduction

One of the most complex pattern formations is the rise of a whole miniature organism from a single cell: embryogenesis. Plant embryogenesis is undoubtedly a thoroughly researched field, since not only adult plants are essential for humans, but also plant embryos in form of legumes, coffee, etc. Although the formation of the embryo in monocots like rice, wheat or maize is for sure of great agricultural importance, their irregularity and lack of genetic resources made scientist turn to Arabidopsis as plant embryogenesis model[1,2]. In Arabidopsis, the zygote (fertilized egg cell) elongates and divides asymmetrically to form two transcriptionally distinct cells[3]: a smaller apical cell and a larger basal one. At this stage, maternal auxin is transported from the basal cell to the apical one via the auxin efflux carrier PIN7[4,5]. This polar auxin transportation forms an auxin spatial gradient[4], vital to the formation of the apical-basal axis and ultimately for the embryo patterning. The apical cell then turns the division plane to a vertical one and generates a 2-cell embryo, whereas the basal cell keeps dividing horizontally and forms the suspensor. Several auxin-related mutants have been shown to follow an incorrect apical division at this stage, generating a transverse division. Especially the gain-of-function mutant phenotype of BODENLOS (BDL)/IAA12, and the loss-of-function mutant phenotype of its interaction partner MONOPTEROS (MP)/ARF5[6–8], has captured the attention of researchers. IAA12 was the first Aux/IAA described to play a role in the translation of the auxin signaling in the Arabidopsis embryo[7]. Aux/IAAs are transcriptional repressors that impede the activation of auxin responsive genes by auxin response factors (ARFs)[9]. To interpret the auxin signal, cells need in addition to Aux/IAAs and ARFs, the Cullin RING-type E3 ubiquitin ligase $SCF^{TIR1/AFB1-5}$ complex[10]; here referred to as TIR1 for simplicity. At low auxin concentrations, Aux/IAAs exert their repression by binding ARFs through their interaction domain at the C-terminal, the PB1 domain[9]. They can increase their repression by recruiting co-repressors like TOPLESS (TPL) or TOPLESS-RELATED PROTEINS (TPR)[11,12] and these attract the histone deacetylase 19 to compact the chromatin[13]. When the auxin level increases, TIR1 and Aux/IAAs form an auxin receptor complex triggering the ubiquitination and proteasome 26S degradation of Aux/IAAs[14–16]. Thus, ARFs are liberated from their repression and activate the transcription of auxin responsive genes. This is a simplified model since a lot more factors might be involved in the interpretation of the auxin signaling like post-translational modifications[17–19] or Aux/IAA multimerization[20]. One layer of complexity is added via the combinatorial possibilities between ARFs and Aux/IAAs[21]. The Arabidopsis genome encodes a total of 23 ARFs and 29 Aux/IAAs[22]. The role of these Aux/IAAs has been inferred from gain-of-function mutants in which a point mutation in the degron domain stabilizes the Aux/IAA, increasing their resistance to auxin-dependent degradation[6,16,23–27]. Aux/IAA loss-of-function mutants resulted in no or very subtle abnormal

phenotypes probably due to high redundance[28,29]. Three of these 29 Aux/IAAs, namely IAA12, IAA10 and IAA18, have been identified during embryogenesis as players in pattern formation at early to mid-globular stage[6,30,31]. Nevertheless, there is enough high-quality transcriptomic data to suspect that other Aux/IAAs are involved during earlier steps of the Arabidopsis embryogenesis[32–35]. This data points out IAA8 and its homolog IAA9 as potential candidates since their expression in the zygote, 1-cell and 2-cell stage are the highest among Aux/IAAs[32–35].

Here we make use of a gain-of-function IAA8 mutant to address its role during the very early steps of embryogenesis. Aberrant cell divisions and cell fate changes are among the most prominent traits of this mutant; thus, indicating a participation in embryo patterning at early developmental stages.

Results

IAA8 expresses ubiquitously in the embryo

To better asses the functionality of IAA8 during the early embryogenesis we studied its endogenous expression. Firstly, we checked IAA8 mRNA with *in situ* hybridization confirming expression from very early stages of embryogenesis onwards (zygote to heart stage) (Figure 1 A-D). IAA8 expression was detectable ubiquitously in the embryo proper, however no signal was detected in suspensor cells. In order to confirm this expression pattern, we generated several reporter lines. Two protein fusion lines with a long promoter (~5kb upstream of the 2nd ATG), with either IAA8 wildtype or the stabilized version (IAA8D) fused to a super folder GFP were generated. None of them resulted in detectable signal, even though a gain-of-function phenotype was noticeable for the stabilized version of IAA8 (Supplemental Figure 1). For this reason, we decided to check the expression avoiding the use of the coding region, despite the fact that in this way we were renouncing to *cis*-elements that might determine the wildtype expression pattern of IAA8. A shorter promoter reporter line (1763 bp upstream of the 1st ATG) showed signal only from dermatogen stage onwards (data not shown). Hence, we used the long promoter described above and amplified the signal with a GAL4:UAS system to drive NLS:tdtomato expression. With this setting we were able to detect strong expression at 1-cell stage (Figure E) in both basal and apical cell. The expression remained stronger in the most basal cell at 8- to 16-cell stage while also increasing in the apical cells (Figure F). IAA8 was expressed not only in embryonic cells but also in the chalazal end and weaker in endosperm and integuments (Supplemental Figure 1). In mature embryos the IAA8 expression was noticeable in the root meristem and cotyledons. Post embryonic expression in the roots (vascular tissue and QC center) was documented as well.

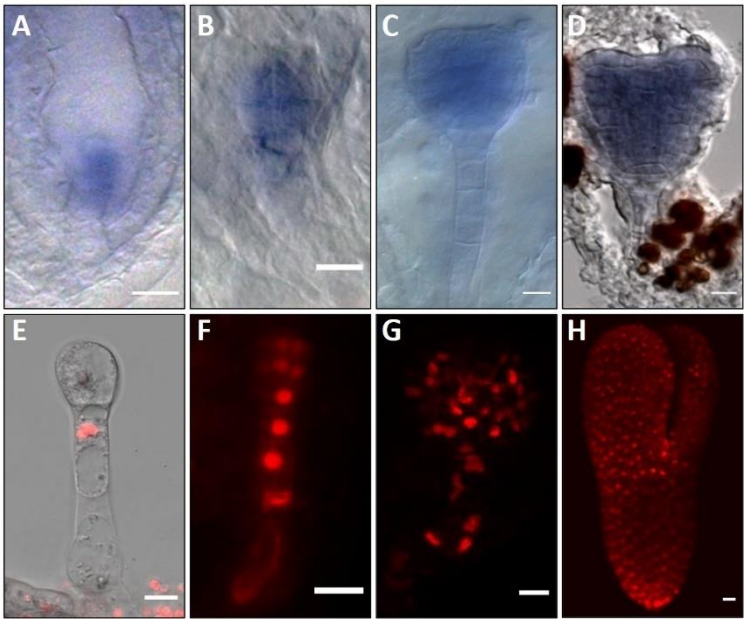


Figure 1. IAA8 endogenous expression during embryogenesis. A-D) *In situ* hybridization of IAA8 mRNA at different stages: zygote **(A)**, 8-cell **(B)**, early globular **(C)** and heart stage **(D)**. A-C whole mounts; D section; scale bars 10 µm. **E-H)** Confocal images of the reporter line pIAA8_5kb:GAL4:UAS:NLS-tom_2.5kbIAA8UTR. Stages: one cell **(E)**, 16-cell **(F)**, globular **(G)** and torpedo **(H)**. Scale bars 10 µm.

IAA8 loss of function shows no phenotype

We examined first the loss of function alleles *iaa8-1* and *iaa8-1 iaa9-1*[28]. Genotyping and real time PCR confirmed the lack of these genes (data not shown). No post-embryonic phenotype was observed, and the embryos followed all the wildtype pattern of division.

IAA8D overexpression postembryonic phenotype

An attempt to generate stable lines using the 5kb promoter region resulted in strong post-embryonic phenotypes when IAA8 carried the stabilizing mutation (Supplemental Figure 2). Some of the T1 seedlings were monocot (36% n=22) and all of them had a shorter primary root. The shooting time of these seedlings was severely affected. No primary shoot was generated, and the secondary ones presented abnormal inflorescences either ending in a pin-like structure or with few flower buds. The flower organs showed mutant phenotypes with altered petal number or unusually big petals. The few siliques that developed were curved and mostly seedless. Interestingly the same construct with a wildtype IAA8 caused no abnormalities (n=44): seedlings, adult plants and embryo development were unaffected.

In both versions, wildtype IAA8 or IAA8D, the protein was fused to super folder GFP. However, no GFP signal was detected either in the microscopy screening nor in western blots analysis. Hence, we assumed that there is a strong negative selection and in the survival lines the IAA8-GFP levels are below the detection limit. Nevertheless, enough to cause strong post-embryonic phenotypes with the stabilized IAA8.

IAA8D apical overexpression leads to aberrant embryonic divisions

Due to difficulties generating stable IAA8 overexpression mutants, we used a two-component system to check the embryonic phenotype. The RPS5a promoter was used previously as a strong embryonic promoter to trigger the expression of IAA12D[36]. For this reason, we used a driver line with this promoter and used IAA12D for comparison in most of our experiments.

First, we checked for effects in zygote elongation and polarity, using 1-cell stage embryos from reciprocal crosses using the driver line (RPS5a::GAL4-VP16) and Col-0/UAS:lines (UAS:gIAA8D and UAS:cdsIAA12D). The zygote length seemed to be affected by the overexpression of Aux/IAAs (Figure 2.A and B). When pollinating with the driver line, the zygote length of IAA8D OE (overexpression) embryos was significantly higher than wildtype and IAA12D OE embryos. When the UAS:lines were used as pollen donor, the effect in the zygote length was more prominent. In this case, all three crosses differed from each other. However, the zygote polarity (apical/basal cell length ratio) was not significantly different among the three samples (Supplemental Figure 3).

Second, we examined the embryonic divisions at different developmental stages. Several UAS:gIAA8D lines were checked 48 hap (Table S 1) and one of them was chosen for comparison with IAA12D overexpression. Both RPS5a>>IAA8D and >>IAA12D, were delayed compared to Col-0 (Figure 2.H). The first aberrant division with IAA8D OE embryos appeared at the 2-cell stage as a horizontal first apical division. IAA12D OE embryos did not present this kind of phenotype. Overexpression of IAA12D lead to the known oblique division at 8-cell stage, whereas IAA8D OE embryos did not follow this pattern. The mutant phenotype of IAA8D OE embryos 72 hap (hours after pollination) was present in nearly 100% of the ovules and varied slightly (Supplemental Figure 4). All IAA8D OE embryos were misshapen at mature stage, all the seeds had a raisin-like form, and none were able to germinate.

To determine whether this mutant phenotype was due to apical or basal IAA8D OE, we tested several driver lines with specific domain expression (Supplemental Figure 5 and Figure 3). Generally, overexpression in the apical cell (RPS5a>>, DRN>>, PDF1>>) led to severe wrong divisions and produced 100% non-germinating seeds (Figure 2, and Figure 3. A, B and G). On the other hand, when the overexpression took place in suspensor cells (M0171>>, YUC1>>, ARF13>> (data not shown)), the

embryo pattern and subsequent seedlings developed as in wildtype (Figure 3. D, E and G). The IAA8D overexpression under the YUC1 promoter led indeed to some seedlings with monocot or fused cotyledons probably due to the apical expression at late stages of this promoter; nevertheless, the root in these seedlings was unaffected. Unlike pYUC1>>IAA8D, >>IAA12D overexpression caused 100% non-germinating seeds. In general, noticeable differences in the abnormal phenotype were observed between IAA8D and IAA12D embryos. For instance, transactivation in the embryonic vascular tissue (Q0990>>) caused significantly more frequent wrong divisions in IAA8D embryos than in IAA12D and the seedling phenotype differed from each other as well (Figure 3. C and G).

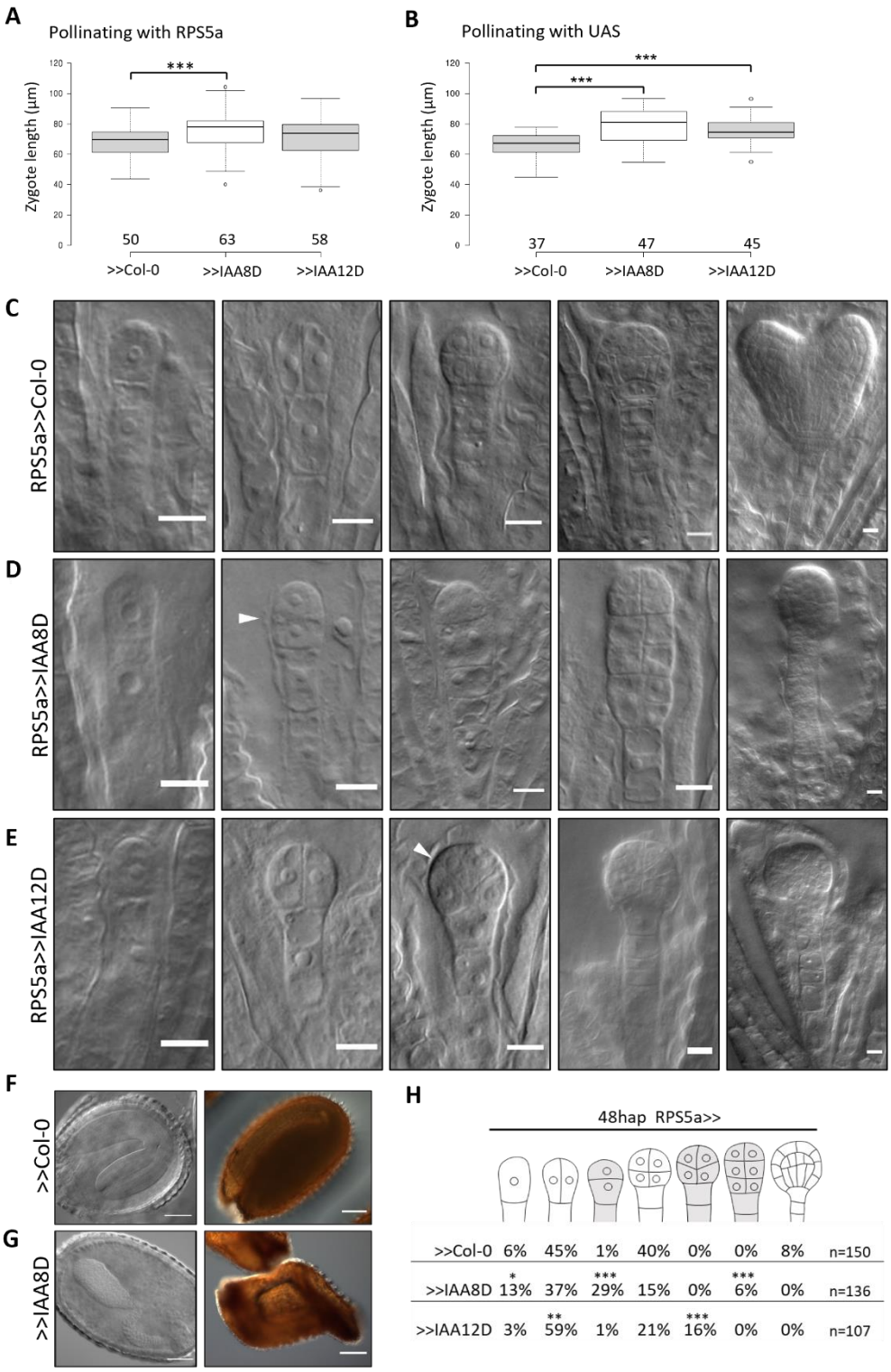
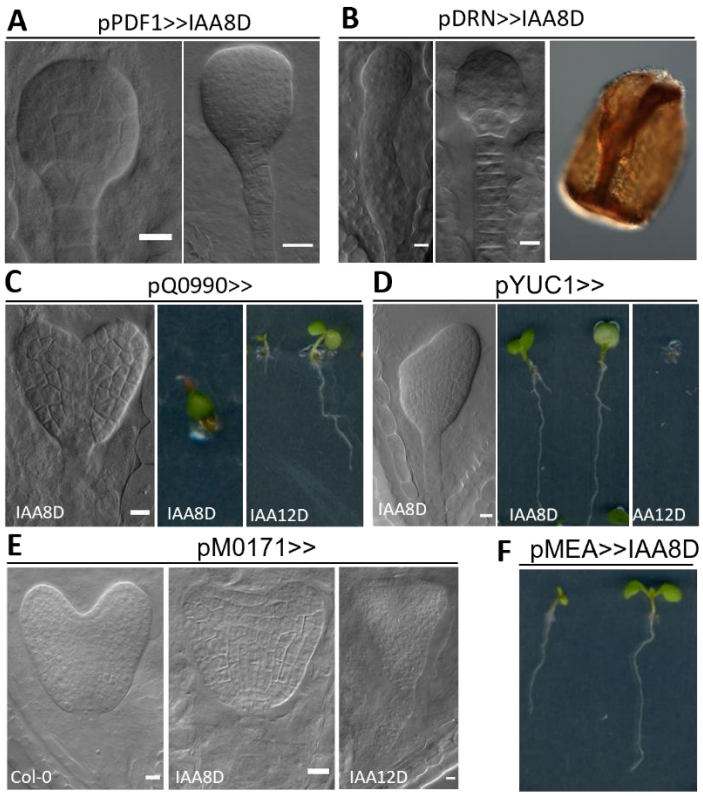


Figure 2. Embryonic phenotype of IAA8D overexpression under the RPS5a promoter compared to wildtype and IAA12D. A-B) Zygote length (apical+basal cell length) when pollinating with the driver line **(A)** or with the UAS lines **(B)**. Numbers above the X-axis represent the number of embryos measured for each line. One way ANOVA with post hoc Tukey; ***p<0.01. Measurements carried out under blind conditions. **C-E)** Embryos from 1-cell to heart stage from RPS5a>>Col-0 **(C)**, >>IAA8D **(D)** and >>IAA12 **(E)**. Scale bars 10 µm. **F-G)** Late embryo phenotype and seeds. Scale bars 100 µm. **H)** Table summarizing the

phenotypes observed 48hap. Embryo schemes with abnormal divisions are shaded in grey. Z-test using Col-0 as reference; * p<0.1; **p<0.05; ***p<0.01

Overexpression with M0171 resulted also in remarkable difference between the overexpression of these two Aux/IAAs (Figure 3. E and G). While >>IAA8D remained wildtype, severely affected embryos were produced in >>IAA12D. In brief, we can say that IAA12D OE in the suspensor does have a stronger effect than IAA8D overexpressed in this domain.

The aberrant divisions caused by IAA8D overexpression are probably an embryo-autonomous effect since overexpression under the MEA promoter resulted in no significant differences with >>Col-0 (Figure 3. F and G). No aberrant divisions in the endosperm were observed.



G

Mutant % (n=total)

pPDF1>>

	72 hap	96hap	Seeds
Col-0	0% (52)	0% (19)	WT (30)
IAA8D	15% (40)	56% (25)	no germ. (30)

pDRN>>

	48 hap	72 hap	Seeds
Col-0	0% (29)	0% (18)	WT (10)
IAA8D	0% (37)	62% (52)	no germ. (10)

pQ0990>>

	96 hap	Seeds
Col-0	0% (30)	WT (16)
IAA8D	100% (41)	100% (15)
IAA12D	10% (21)	14% (15) ≠IAA8

pYUC1>>

	72 hap	96 hap	Seeds
Col-0	0% (29)	0% (22)	WT (11)
IAA8D	0% (43)	7% (28)	81% (21)
IAA12D			100% (12) ≠IAA8

pM0171>>

	48 hap	120 hap	Seeds
Col-0	0% (27)	4% (48)	WT (25)
IAA8D	0% (38)	1% (117)	WT (22)
IAA12D		32% (77)	39% (18)

pMEA>>

	48 hap	Seeds
Col-0	10% (21)	WT (12)
IAA8D	10% (30)	17% (23)

Figure 3. Domain specific transactivation. **A-F)** Images of cleared embryos (DIC), seeds or seedlings overexpressing IAA8, IAA12D or Col-0 under different promoters. Scale bars 10 µm. **G)** The tables show the percentage of abnormal embryos/seedlings; the n (total number) is indicated between brackets. Shade in pink denotes significant differences with Col-0 population and yellow among the UAS:IAA lines. Fisher's exact test; p<0.01.

IAA8D overexpression leads to cell fate changes

The abnormal embryonic cell divisions caused by the overexpression of IAA8D under the RPS5a promoter were prominent; we wanted then to see if these wrong orientations reflected an underlying defect such as a cell fate failure. Hence, we conducted *in situ* hybridization with sections of

RPS5a>>IAA8D ovules at 48 hap, when the first morphological defects appeared (see above). For comparison we used RPS5a>>Col-0 and >>IAA12D material (Figure 4). We checked the identity pattern of several apical markers such as HAN, DRN, MP, WOX2 and HDA3 and basal ones like PIN7, WOX8 and MEE26.

The known apical markers WOX2, DRN and especially HAN were consistently apical in wildtype embryos. This apical pattern differed significantly from IAA8D embryos in which expression was extended to suspensor cells at frequencies of 42% (HAN), 45% (DRN) and 71% (WOX2) or was even solely basal (3% WOX2) (Figure 4.A-D). For IAA12D OE embryos these tendencies towards basal expression were notably less pronounced, ranging from 9% (HAN) to 13% (DRN). Published apical markers MP and HDA3 did not show consistent apical signal in our test materials (Supplemental Figure 6).

The previously reported suspensor markers WOX8 and PIN7 were used to test basal identity. Although WOX8 was detected sometimes apically in wildtype embryos, this percentage increased considerably in IAA8D and IAA12D embryos. Additionally, a second misallocation of the signal was categorized with this probe referred here to as abnormal basal. This classification means that the signal was basal but not in all suspensor cells like expected in wildtype expression. IAA12D and especially IAA8D embryos showed often basal expression confined to the lowest basal cell (Figure 4.E). The basal marker PIN7 was always found in suspensor cells in wildtype material. The signal was despite optimization of probe and *in situ* procedure very weak. Interestingly the same probe was easy to detect in IAA8D material maybe due to a possibly stronger expression and/or an easier *in situ* signal apical detection. The PIN7 expression in IAA8D embryos was extended to the apical regions in 71% of the embryos. This phenomenon was remarkably weaker in IAA12D embryos (17%).

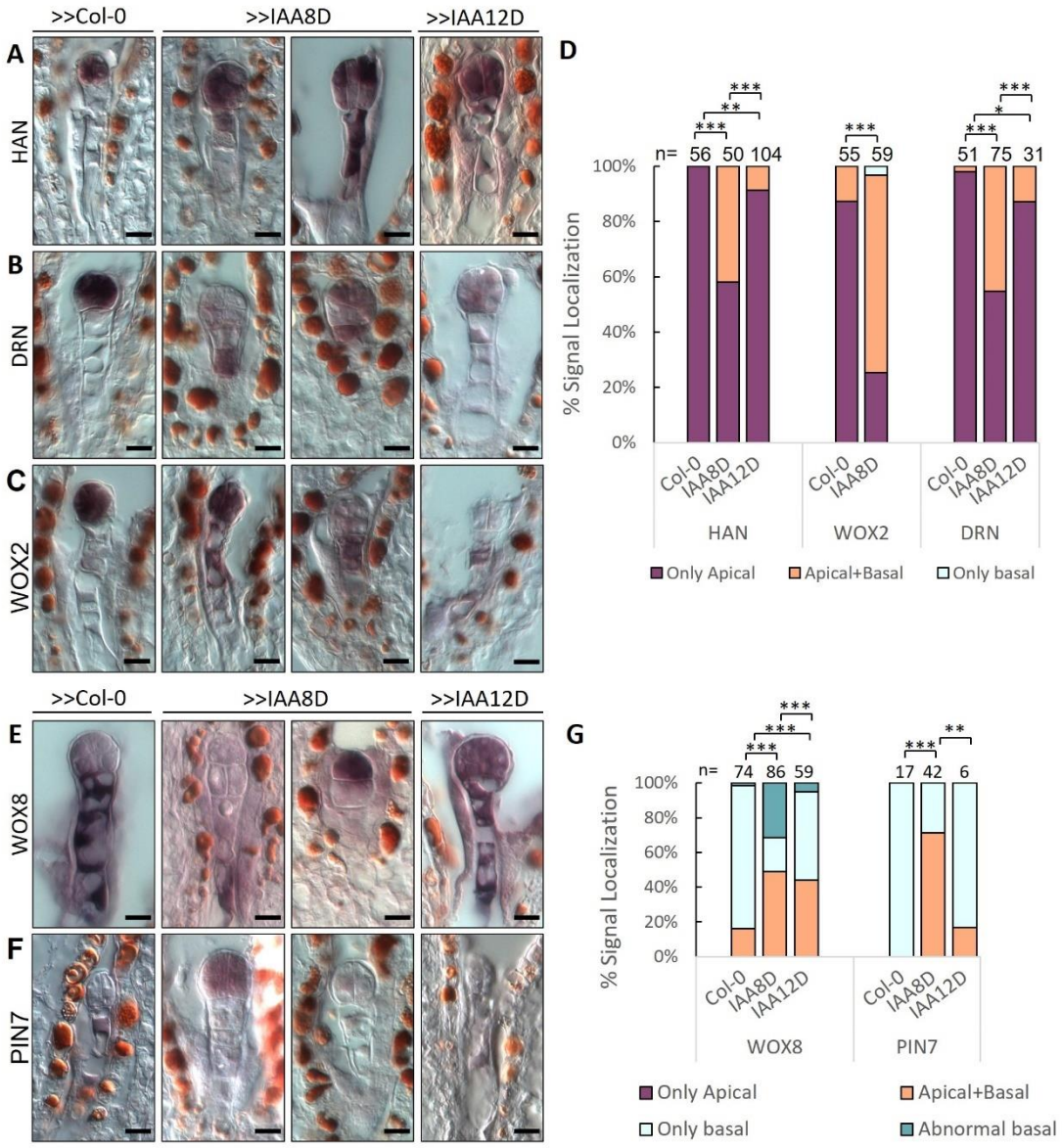


Figure 4. ***In situ*** **hybridization against different apical (A-C) and basal markers (E-F)**. RPS5a>>GAL4 was used for transactivation. All embryos collected 48 hap. Scale bars 10 µm for all images. **D** and **G** summarize the percentages of signal location for each material and probe. N numbers appear above the bars in the charts. Fisher´s exact test; * p<0.1; **p<0.05; ***p<0.01.

Some published apical markers like MP and HDA3 and the basal marker MEE26 did not show clear-cut expression patterns in our laboratory conditions (Supplemental Figure 6). The patterns of MP and HDA3 did not differ between Col-0, IAA8D and IAA12D embryos. The basal MEE26 pattern for IAA8D was moderately changed towards expression in the lowest basal cell, like WOX8 in some IAA8D embryos.

The IAA8 phenotype is caused by auxin response disruption

We wondered if the morphological and transcriptional changes caused by IAA8D overexpression were indeed caused by a block in auxin response in the apical cell. We crossed our UAS:IAA8D with a RPS5a:GAL4-DR5:ER-GFP to check the auxin response. Imaging of solely the driver line DR5:ER-GFP signal at early stages (1-cell to 8-cell stage) was not possible in our laboratory conditions. Therefore, later stages (dermatogen to heart stage) had to be used to assess the DR5 activation in IAA8D OE embryos (Figure 5.A). Wildtype embryos transactivated with the marker driver line showed GFP signal in the hypophysis as early as dermatogen stage, getting significantly stronger at heart stage. In our IAA8D OE embryos the DR5 signal was completely absent. To visualize the DR5 signal at an earlier stage (2-cell stage), closer to the appearance of the first wrong divisions, we tried to conduct *in situ* hybridization with a GFP specific probe. Despite optimization of probe and staining, no signal was detected in the driver line (data not shown). Hence, we discarded this method to check auxin disruption at the 2-cell stage. We came then across an alternative that, although indirectly, could help answering our question: auxinole (AXO), a synthetic antagonist blocking the formation of the TIR1 auxin receptor AUX/IAA complex[37]. An initial test with DR5:ER-GFP seedlings treated with 100 µM AXO resulted in a reduction of DR5 signal in the root tip and a reduction of the number of lateral roots by 98% (Supplemental Figure 7), confirming its effectivity to block the auxin response. We treated cultured ovules 24 and 48 hap with 100 µM AXO or DMSO (mock treatment) and checked for abnormal embryonic divisions (Figure 5.B). At early stages AXO caused around 10% of aberrant apical divisions. These divisions were not only horizontal as expected for auxin disruption in the apical cell inferred from previously reported auxin mutants like *pin7*[4] or BDL[6], but also oblique (Figure 5.B). The frequency of wrong divisions was significantly higher than in the mock treatment. We often observed cell death in the AXO-treated ovules, therefore we decided to check at later stages when the ovules would have a higher survival rate in culture (Figure 5.C). Mutant divisions were more prominent in this experiment, not only restricted to the hypophysis division but giving rise to disorganized embryos. It seems that auxinole may cause a wide range of effects during embryogenesis in addition to the horizontal apical division at 1-cell stage. In contrast, the effect observed with IAA8D overexpression at very early stages appears to be more specific. To achieve this specific auxin response in the embryo, which also differs from IAA12, IAA8 probably acts together with an early embryonic transcription factor. Given that IAA8 is an Aux/IAA, it might be a specific ARF-Aux/IAA module.

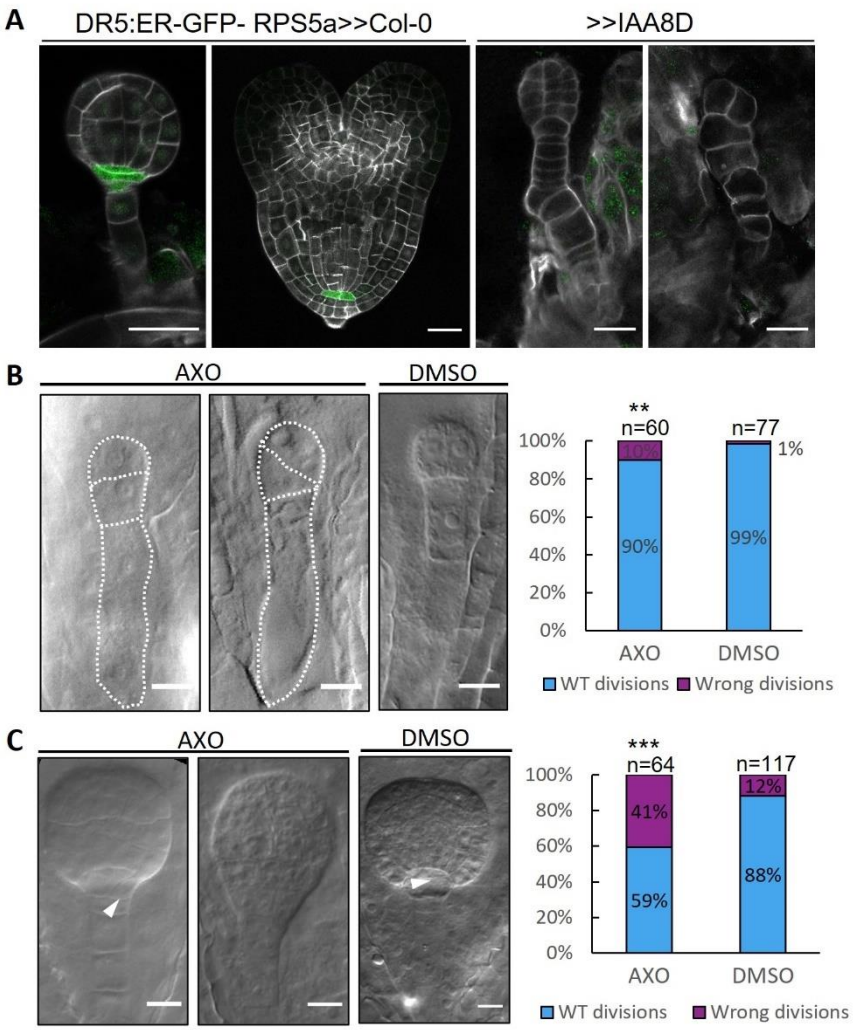


Figure 5. Auxin response in the RPS5a>>IAA8D mutant and auxinole treatment. A) DR5 activity (ER localized GFP signal in green) in wildtype and transactivated embryos. Scale bars 20 µm. Cell walls stained with renaissance. **B)** DIC images of cleared embryos (48hap) treated with AXO or DMSO. Scale bars 10 µm. Dash-line marks division in AXO treated embryos. Chi-square test; **p<0.05. **C)** DIC images of cleared embryos (96 hap) treated with AXO or DMSO. White arrows mark hypophysis division. Col-0 embryos; scale bars 10 µm. Chi-square test; ***p<0.01.

Identifying the molecular mechanism of IAA8

We started looking for ARF interactors with preliminary trials using the dual luciferase assay in protoplasts with the DR5 promoter. These experiments gave us a first hint of possible ARF interactors. Both IAA8 and IAA12 were able to inhibit DR5 activation by ARF5 and ARF6 (Supplemental Figure 8). No effect on DR5 activity was observed when repressor ARFs like ARF13 and ARF18 were used (data not shown). We decided to rescue the IAA8D OE phenotype with ARF5 overexpression. Preliminary data pointed towards rescue; however, difficulties with silencing of the UAS:lines and segregation distortion impeded further experiments in this direction (data not shown). An interaction with ARFs is

likely, however, we cannot exclude other potential interactors of another nature. For this reason, we started checking interactors using pull-down and mass-spectrometry assays. First, we used recombinant proteins of IAA8 and IAA12. We overexpressed recombinant IAA8D-myc and IAA12D-myc, conducted an immunoprecipitation after mixing with Arabidopsis seedling extract and ultimately a mass-spectrometry to identify binding interactors. Here we detected the known Aux/IAA interactors, TPR2 and TPR3, but no ARFs. We realized that when recombinant Aux/IAAs were used for the pull-down with plant-extracted ARF5-HA, no interaction was observed (Supplemental Figure 9), whereas the reciprocal pull-down, that is, recombinant ARF with plant Aux/IAA-GFP resulted in signal in the western blot. This may suggest that plant-specific post-translational modifications are needED for Aux/IAAs to interact with ARFs *in planta* and Aux/IAAs were therefore not detected in our preliminary assay with recombinant Aux/IAAs.

Since the Aux/IAA-ARF interaction occurs through the PB1 domain, a swap between the PB1 domains of IAA8 and IAA12 would exchange their interactors. In theory, this exchange would lead to a swap in the phenotype since Aux/IAAs act by inhibiting ARFs which in turn will switch auxin responsive genes on or off. With this in mind, we made protein chimeras exchanging the PB1 domain: UAS:8-8D-12 and UAS:12-12D-8. To avoid problems generating stable mutants we continued using the transactivation system. For this experiment we tested two different splice variants of IAA8 (IAA8.1 and IAA8.4). We crossed several T2 plant lines with the RPS5a driver line and check around 48 hap. We classified the embryos into categories: WT divisions, IAA8D-like divisions, IAA12D-like or neither the typical IAA8 or IAA12 divisions but also not wildtype (non-wildtype nor IAA8/12) (Figure 6). The mutant embryos of the UAS:8-8D-12 lines showed often typical IAA8, this is, horizontal apical divisions or file-like embryos. None of them had the typical oblique divisions of IAA12D OE. In the embryos overexpressing 12-12D-8 the aberrant divisions were significantly fewer, similarly to the IAA12D phenotype at 48 hap. Furthermore, no typical IAA8D-like divisions were detected. Taking these results together, it seems that the exchange of the PB1 domains does not exchange the mutant phenotypes.

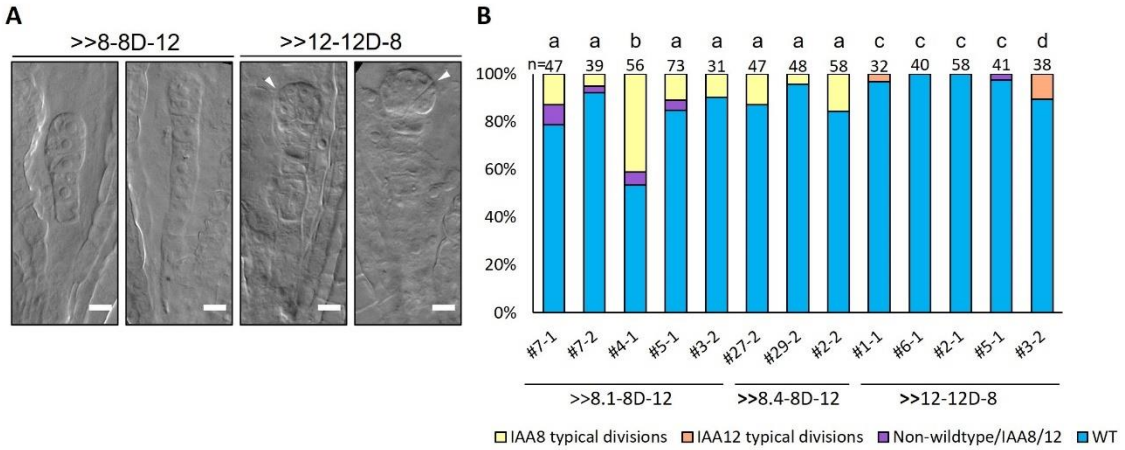


Figure 6. PB1 domain swap constructs transactivated with RPS5a. **A)** DIC cleared embryos ca. 55hap. White arrows indicate oblique divisions. Scale bars 10 µm. **B)** Percentage corresponding to the type of divisions observed. IAA8 typical divisions (yellow), IAA12 typical divisions (orange), non-wildtype but also not classifiable as IAA8 or IAA12 divisions (purple) and wildtype divisions (blue). Letters above bars indicate different populations. Fisher´s exact test $p<0.01$. T2 plants, zygosity unknown for these lines.

To confirm the lack of specificity in the interaction of the sole PB1 domain we overexpressed under RPS5a the respective PB1 domains of IAA8 or IAA12 fused to GFP and checked for aberrant embryonic divisions (Supplemental Figure 10). In accordance with the previous results, embryos expressing one or the other of these two transgenes showed very similar phenotypes. These results indicate that the Aux/IAA PB1 domain alone may not confer enough specificity for the interaction with ARFs.

Discussion

The roles of some Aux/IAAs have been described in Arabidopsis embryogenesis, like IAA12[7], IAA10[30] or IAA18[31]. However, based on transcriptome data these are not among the ones strongest expressed during early embryo development[32,34]. IAA8 and its homolog IAA9 stand out due to high expression at this stage. This fact led us to wonder if IAA8 would indeed have a function in the embryo formation. We first assessed its spatio-temporal expression with a reporter line and an *in situ* hybridization approach. We confirmed that IAA8 is expressed widely (apical and basal cell lineages, no specific pattern found in the proembryo) and from very early stages onwards (Figure 1). Interestingly, the expression domain detected with the reporter line differed from the mRNA spatial detection in the suspensor. A possible explanation could be the previously reported potential cell-to-cell mobile nature of IAA8's mRNA[38]. Another factor that might contribute to a weaker signal in the basal lineage is the their cell nature: highly vacuolated which reduces the density of the cytoplasm. In brief, IAA8 is

expressed from zygote stage onwards until the mature embryo, and this expression seems ubiquitous at least until heart stage.

Once its endogenous expression was determined, we moved on to its functional characterization. The loss-of-function *iaa8-1 iaa9-1* single or double mutant did not have any abnormalities during embryogenesis. The lack of a strong mutant phenotype is a common observation among loss-of-function Aux/IAAs mutants in Arabidopsis [24,25,28,39], being evasive or very subtle despite higher order mutants. This indicates a high redundancy in this family of repressors, adding robustness to developmental process of embryogenesis[40]. For this reason, we decided to generate a gain-of-function mutant by expressing an undegradable IAA8 version (IAA8D). Due to difficulties generating stable viable IAA8D mutants, we used a two-component system GAL4-UAS. Ubiquitous and strong IAA8D overexpression from the zygote stage onwards (promoter RPS5a>>) resulted in early mutant phenotypes, for instance, significant differences in zygote length and most remarkably horizontal first apical divisions (Figure 2.D). The zygote polarity (apical/basal ratio) was unaltered (Supplemental Figure 3). Although both IAA8D and IAA12D overexpression seem to slightly delay the embryonic development, the frequency of transverse first divisions in IAA8D OE embryos was markedly higher than in the previously characterized IAA12D OE embryos (Figure 2.H). Furthermore, the IAA12D typical acute angle at 8-cell stage was not detected in IAA8D mutants. Our transactivation system allowed us to test a range of spatio-temporal domains for overexpression effects (Figure 3). Overexpression in the apical cell linage (promoters: DRN, PDF1, Q0990) resulted in aberrant divisions and disorganized embryos that produced in most of the cases 100% non-germinating seeds (RPS5a, DRN, PDF1) or severely affected seedlings (Q0990). Additionally, we tested suspensor specific promoters (M0171, ARF13, YUC1 partially) which did not result in remarkable mutant phenotypes. From these findings, we can conclude that IAA8D OE in the apical cell lineage has a strong mutant outcome, whereas IAA8D OE in the suspensor cells does not cause major embryonic disruptions.

Next, we tested if these division anomalies correlated with failure in cell fate determination. We conducted *in situ* hybridization with several apical and basal embryonic markers (Figure 4 and Supplemental Figure 6). At the same developmental stage unusual cell fate changes (sometimes over 70%) occurred at a higher frequency than the aberrant wrong cell divisions (30% at 48 hap) in IAA8D OE embryos. The apical cell identity in these mutant embryos was often shifted to the suspensor region and the signal of basal markers was also found often in the apical cell lineage. Still, it is important to mention that this was not an absolute switch from basal to apical identity or vice versa, since also frequently, apical markers were found in apical regions but in a what seems to be a random pattern. Although it is not obvious what these observations really mean, one possible interpretation could be

the following: the cell fate determination might become a noisy/slightly random event if the essential cell fate directionality signal is lost, in our case the auxin response.

Other auxin-related mutants (like bdl[7] or PIN7[4]) have been shown to divide aberrantly at the 2-cell stage embryo. Nevertheless, it remains unclear if this anomaly is the product of a specific auxin block in the apical cell. To address this, we checked the DR5 signal at 1-cell stage[4] in our IAA8D OE embryos, but the reporter line itself was below the detection limit at this stage. At later stages the DR5 signal observed in the control embryos was not present in our mutant (Figure 5.A). Then we used a synthetic antagonist for the action of the TIR1 auxin receptor, namely auxinole (AXO)[37], to specifically block the auxin response and check if we could reproduce the transverse apical division. In fact, significantly more frequent horizontal divisions were observed in AXO-treated than in solvent-treated embryos (Figure 5.B). In this experiment we did not only observe this expected division orientation but also a less frequently reported one, an oblique division in the apical cell. Intriguingly, we did not encounter this type of cell division in our IAA8D mutants. Thus, it could be that division orientation of the apical cell is auxin regulated but not only through an Aux/IAA-dependent pathway. This would give rise to oblique and horizontal divisions when the whole auxin response is blocked, but only to the second type when the Aux/IAAs response is compromised. In conclusion, the abnormal cell divisions of IAA8D embryos are probably the result of a specific disruption in auxin response.

To achieve the specificity of its response, IAA8 possibly acts through a determined ARF-Aux/IAA pair, as has been shown for other members of this protein family like ARF5 and IAA12[6] or ARF9 and IAA10[30] in the embryo. The ARF-Aux/IAA interaction is mediated via the PB1 domain of both types of protein[9]; hence, we thought exchanging the PB1 domains of IAA8 and IAA12 would lead to a swap in ARF interactor, and ultimately an exchange of the mutant phenotype. Contrary to this expectation, the swap of the PB1 domains did not change the outcome (Figure 6). These results could imply that the PB1 domain alone is not enough to give a certain degree of specificity in the interaction with ARFs. Further domain swaps between the Aux/IAAs and ARF domains should be done to widen our knowledge in this direction. Regarding the IAA8-ARF module, dual luciferase and preliminary rescue experiments pointed towards an interaction with ARF5; nonetheless we decided to widen our search for other ARFs and other kinds of interactors using pull-down and mass-spectrometry approaches.

Despite potentially sharing ARF5 as interactor, IAA8's implication in embryogenesis differs noticeably from IAA12. On the one hand, the frequency of transverse apical divisions at 1-cell stage was remarkably higher in IAA8D OE than in IAA12D OE embryos. Furthermore, the acute angle at 8-cell stage characteristic of IAA12 was never found with IAA8. On the other hand, although the same driver line was used, the cell fate changes with IAA8 were more prominent; a fact that could be interpreted as a specific outcome of IAA8. Additionally, the domain-specific overexpression results point out

differences between these Aux/IAAs as well. Most striking was the divergence in phenotypes obtained with the suspensor promoter M0171>>, whereas IAA8D embryos remained completely wildtype, the abnormal development in IAA12D ovules was very much noticeable.

Overall, our findings indicate a functional role of IAA8 in the early Arabidopsis embryogenesis: possibly involved in zygote elongation, orientation of the division plane and cell differentiation in an auxin-dependent context. This phenotype is specific to the accumulation of IAA8 in the apical cell and distinct from the one of IAA12. It is a question of future research to find out the molecular mechanisms through which IAA8 acts in the early embryo. Further work is certainly required to find its interaction partners and disentangle the complexity of IAA8's function in embryogenesis.

Materials and Methods

Plant conditions and crosses

All generated plant mutants were in *Arabidopsis thaliana* Col-0 background. The GAL4-GFP enhancer-trap line (M0171) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were surface sterilized briefly with 70% EtOH and 100% EtOH and sowed on half-strength Murashige and Skoog (MS)-containing 0.8% agar and 10 g/l sucrose. The stratification step was done overnight at 4°C in dark, then the plates were transferred to a plant chamber with 16h/8h light/dark cycle at 23°C. After 7-days the seedlings were transplanted to soil, watered once with Confidor WG70 200µg/l (Bayer CropScience) and continued growing under the same long day conditions.

For crosses the flowers were emasculated and pollinated the next day. Driver lines were used as pollen donor unless indicated.

The crosses for the analysis of the zygote polarity and elongation where done reciprocally and their screening and measurements in the microscope were done under blind conditions.

Plasmid construction

Most of the constructs were cloned using the InFusion method (Takara). The primers were designed using the online tool from the manufacturer and PCR fragments were amplified by the Hifi polymerase or the PrimeSTAR GXL. Templates were either amplified via PCR or digested with restriction enzymes. Usually fragments and templates were treated with the Cloning Enhancer (Takara) following the manufacturer's directions prior to the InFusion reaction and transformation into *Stellar* cells. Ready-to-dip clones were fully sequenced before transforming into plant material.

The long promoter line of IAA8 comprised 5kb upstream of the 2nd ATG, GAL4 fused protein and UAS:NLS-tdtomato with 2.5 kb of IAA8 3´UTR. A modified pCAMBIA vector (pMB3300) was used as backbone.

The UAS lines were done in the pGREEN backbone with basta resistance. For IAA8 the whole genomic sequence was used (starting at the first ATG; splicevariant 4) including 2238 bp of the 3´UTR, whereas for IAA12 the coding sequence and the tNOS terminator was used. The stabilizing mutation in the IAA8 degron domain was a change of Pro_{170} to Ser_{170}.

For the SWAP and UAS:GFP-PB1 constructs, the cds fragments from IAA8D (splice variants 4 and 1) and IAA12D were amplified directly from pJIT plasmids. The Aux/IAAs protein sequences were aligned to identify the PB1 domains. For IAA12 PB1, 354 bp starting from the stop codon were used; for IAA8 372 bp. This PB1 fragments were used for both SWAP constructs and UAS:GFP-PB1 constructs. The backbone (pBAY bar UAS-tnos) was kindly provided by Martin Bayer and linearized with via PCR.

RNA *in situ* hybridization

The probes were amplified directly from cDNA from seedlings except for WOX8 (from plasmid) using the primers listed in the Table S 2. They were transcribed *in vitro* and labelled with up to a 1:3 ratio of UTP: Digoxigenin-11-UTP (Roche) using the T7 Polymerase (Thermo Scientific). Proper labelling was checked with a dot blot using DIG-labeled Control RNA (Roche) as reference. For the cell fate check (Figure 4) and late IAA8 expression (Figure 1D), the *in situ* hybridization was done using sections. The UAS:lines were emasculated and pollinated the next day with the driver line RPS5a. Two days after pollination (for the cell fate changes experiments) the siliques were fixed, embedded and the *in situ* hybridization was performed as described previously[41]. For the early IAA8 expression pattern (Figure 1 A-C), the ovule material of the *tt-8* mutant[42] was used for the whole-mount *in situ* hybridization method followed with minor changes as published[43].

Ovule culture and auxinole treatment

The flowers were emasculated and pollinated the next day (self-pollination, Col-0). Several timepoints were tested to check the viability of the ovules. 24 hours after pollination (hap) was the timepoint with the least pollination/developmental stage variability. For the early stage AXO experiment, siliques 18 hap were prepared with a syringe needle under the binocular as previously reported[44]. The prepared ovules were transferred into a well-plate with NT5 medium and 100 µM AXO or the equivalent DMSO[45]. The plate was covered with paper tissue to reduce AXO degradation and stored in the plant chamber

under long day conditions. At 48hap the ovules were transferred to a microscope slide with Hoyer's solution for microscopy. For the late stage AXO experiment, the same procedure was followed starting the ovule culture 48hap and prolonging the AXO incubation 12 hours more than described above.

Microscopy

Clearing of embryos was done using Hoyer's mounting solution (chloral hydrate, water, and glycerol (ratio w/v/v: 8:3:1) and 10% gum arabic) or Chloralhydrate. For Confocal images embryos were treated with Renaissance 2200[46] staining for 1 min prior to mounting for microscopy. The *in situ* material was mounted in 15% glycerol. For widefield, DIC and epifluorescence images the Zeiss Axio Imager Z.1 (AxioVision software; AxioCam HR camera; Plan-APOCHROMAT ×40 and x10; GFP filters Zeiss 38HE and Zeiss 09; tdtomato filter AHF F36-504) was used. Image processing was done ultimately with ImageJ (Fiji). The confocal laser scanning microscope used was a Leica TCS SP8 (LAS X software; objectives HC PL APO 40x/1.10 W Corr CS2 and HC PL APO 10x/0.40 CS).

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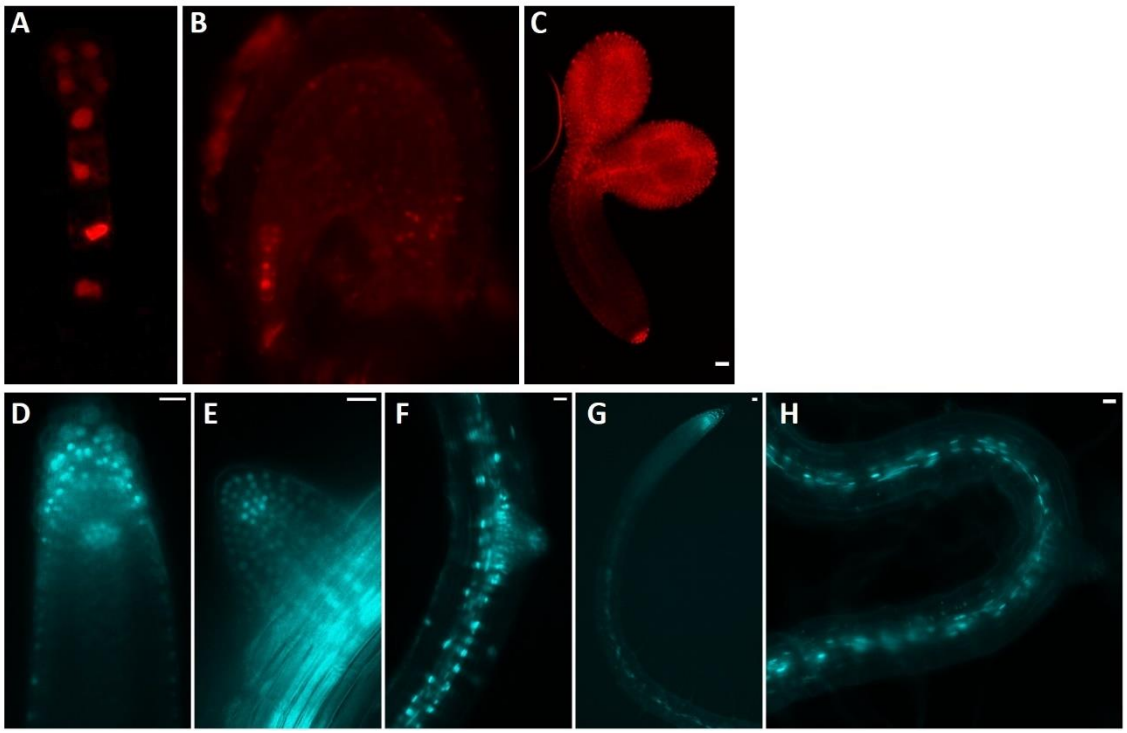
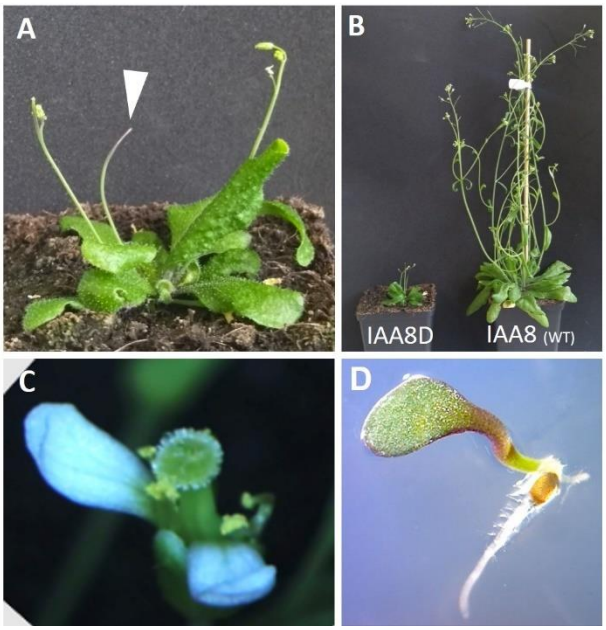
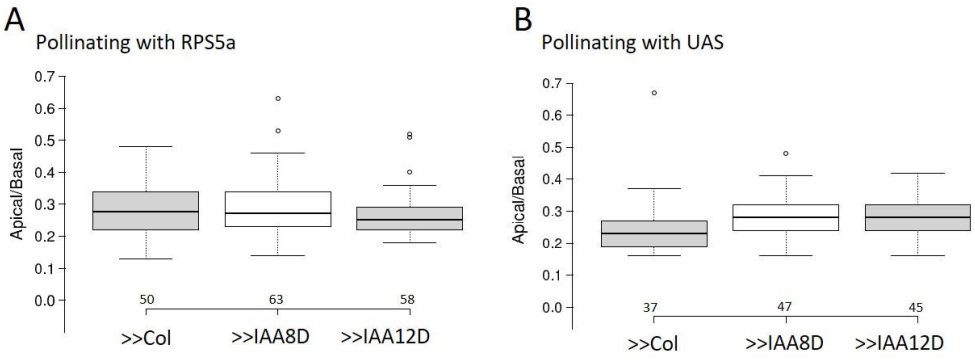
Supplemental Information



Supplemental Figure 1. A-B) Embryonic expression of pIAA8_5kb:GAL4:UAS:NLS-tom_2.5kbIAA8UTR. **A)** 8-cell stage with prominent expression in the suspensor cells. **B)** Ovule overview with an 8-cell stage embryo with a strong expression in the second lowest basal cell. **C)** Mature embryo with strongest expression in the cotyledons, vasculature tissue and root tip. **D-H)** Post embryonic expression with pIAA8_5kb:GAL4:UAS:NLS-tom_2.5kbIAA8UTR **(D-F)** or pIAA8_2kb:3xVenus:tNOS **(G-H)**. The expression is strong in the root tip **(D)** especially at the root cap and quiescent center, in lateral roots **(E)** and in the vascular tissue **(F** and **H)**. The signal is strong in the apical meristem, weakens significantly through the basal meristem and reappears in the differentiation zone. Scale bars 20 μm.



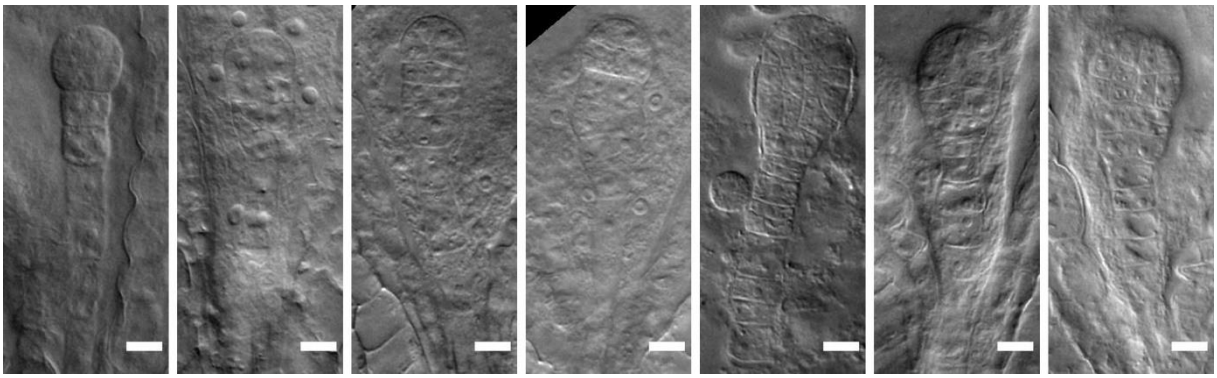
Supplemental Figure 2. Post embryonic phenotype of the IAA8D overexpression under its own promoter (5kb). **A)** Rosette of a T1 plant with the stabilized version of IAA8. The primary shoot is missing, and the inflorescences are severely affected. White arrowhead indicates a pin-like inflorescence. **B)** Comparison of a T1 plant overexpressing the stabilized IAA8 (left) and the wildtype version of IAA8 (right). 7 weeks-old plants. The wildtype version had to be cut back once to be able to compare with the delayed stabilized plants. **C)** Flower overexpressing IAA8D, the number of petals is often affected. The stamens are defective as well. No pollen is attached to the stigma. **D)** 10-days-old monocot seedling (T1 IAA8D). 36% monocot (8/22), 100% short primary root. WT version n=44, all normal.



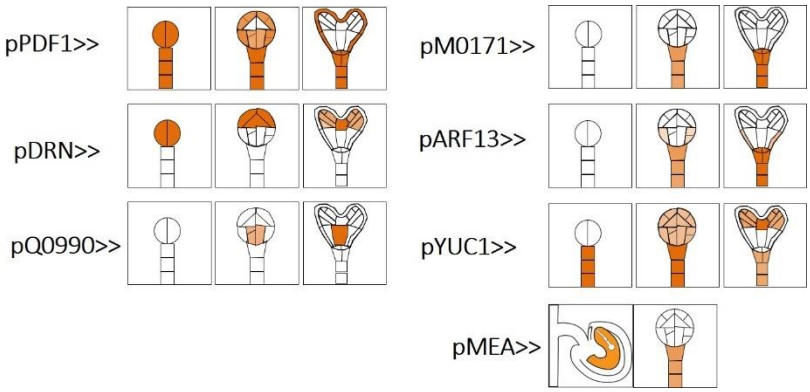
Supplemental Figure 3. The zygote polarity is not affected in the overexpression mutants. **A)** Pollination with the driver line RPS5a>>VP16-GAL4; **B)** Pollination with the UAS:lines or Col-0. The zygote polarity is indicated as apical/basal ratio. One-way ANOVA test showed no significance for either of the experiments.

Table S 1. Cell divisions at 48 hap in embryos expressing the driver line RPS5a::VP16-GAL4. The aberrant divisions for the IAA8D OE embryos range between 15 to 32 %. The number of embryos checked (n) is indicated in the last row.

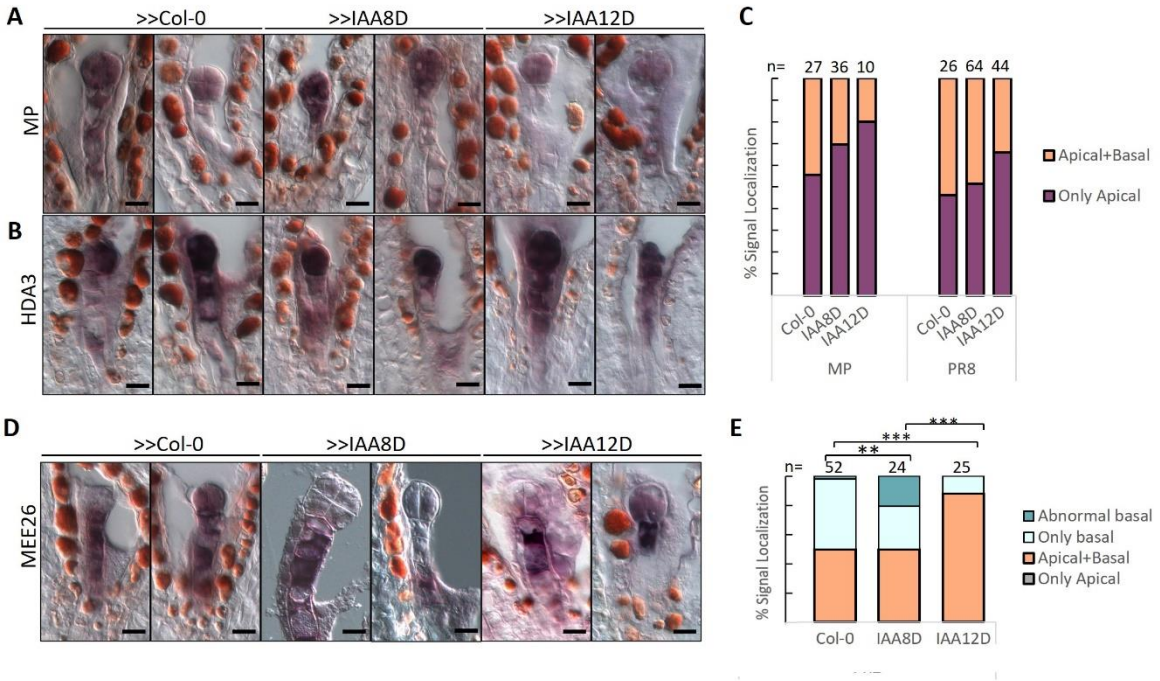
		>>IAA8D						>>IAA12D
	>>Col-0	#2-5	#6-6	#3-1	#9-4	#P1	#P2	#O1
Wildtype Embryos	99%	68%	85%	68%	83%	86%	65%	83%
1-cell	6%	18%	15%	15%	26%	7%	13%	3%
2-cell	45%	36%	39%	40%	40%	53%	37%	59%
8-cell	40%	14%	28%	10%	17%	23%	16%	21%
Globular	8%	0%	3%	3%	0%	3%	0%	0%
Aberrant divisions	1%	32%	15%	32%	17%	14%	35%	17%
2-cell mutant	1%	19%	13%	17%	11%	10%	29%	1%
>8-cell mutant	0%	14%	2%	15%	6%	4%	6%	16%
n=	150	74	93	72	98	139	136	107



Supplemental Figure 4. Variations of RPS5a>>IAA8D embryo phenotype at 72 hap. The embryos can be categorized into 4 groups: double vertical file of cells (23%), generally disorganized embryo (71%), filamentous-like (4%) and wildtype (2%). N=184.



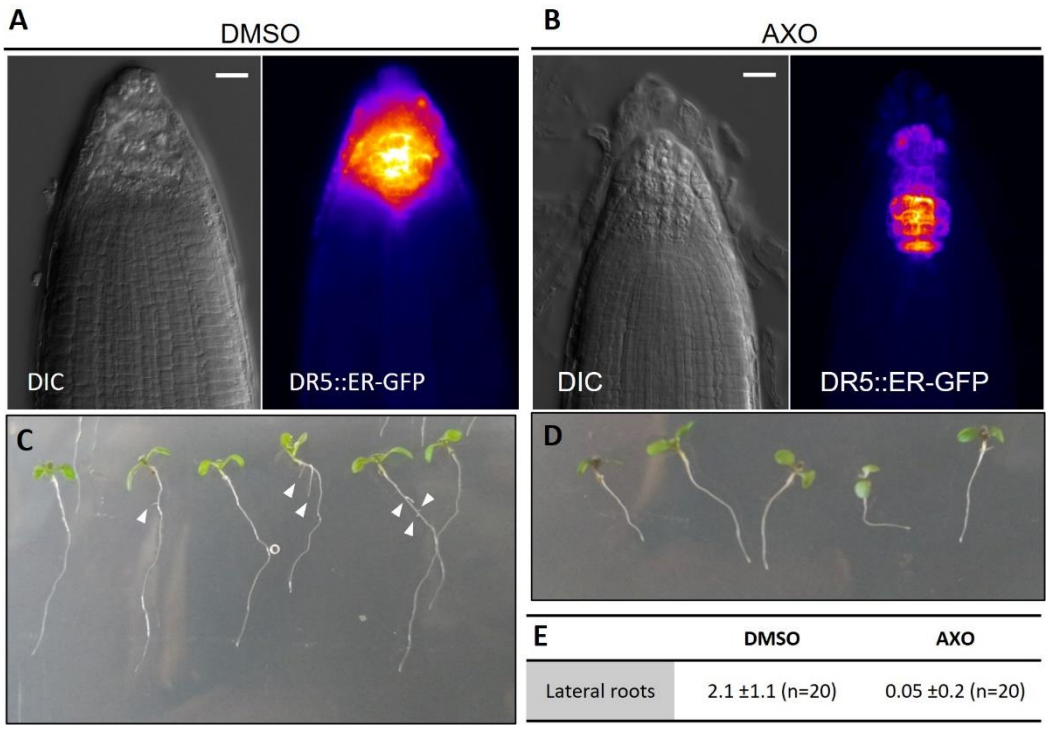
Supplemental Figure 5. Promoters used for domain specific expression. Expression domain is colored in orange with the intensity referring to the strength of the expression.



Supplemental Figure 6. *In situ* hybridization with sections using different probes MP **(A)**, PR8/HDA3 **(B)** and PR2/MEE26 **(D)**. Scale bars 10 µm for all images. **C** and **E)** Percentages of signal location for apical (C) and basal (E) markers. No significant differences were observed with the apical markers. For the basal marker the proportions were significantly different; Fisher´s exact test; ** p <0.05; *** p <0.01.

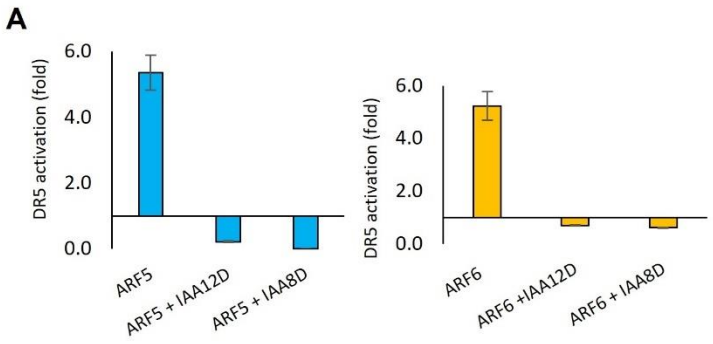
Table S 2. Oligonucleotides used for probe amplification prior to in vitro transcription. The antisense primers contain a T7-promoter sequence (underlined) added to the 5´end. For some genes more than one probe was used for detection as a mix of 1:1 ratio.

Probe	Primer pair 5´->3´	Modified from (if applicable)
IAA8	GCTTTTGTTAGCTTTCTCTGG TAATACGACTCACTATAGGGTCAAACCCGCTCTTTG	-
PR2	AACAGACACAAAAGCATATAAGCA TAATACGACTCACTATAGGGTATAAAAAAACAATATTAATTAG	-
PR8	TCTTACATTTTCAGCTGCT TAATACGACTCACTATAGGGTGGTGAAAACGTGAATCTAAAAT	33
WOX2	CATGCAAACCATCGTCTTAAAACC TAATACGACTCACTATAGGGCATAAAATTTATAATTTCATTAAACCTTCG	47
WOX8	TACACCATCATCATGTCCTCCT TAATACGACTCACTATAGGGTATCCATAGCACCATAACATTTGC	47
DRN	TTTTTCCAACAAGAAATCTTCGCC TAATACGACTCACTATAGGGCCAACATTGGGAAAGGTAGCAAC	48
PIN7	CGCTTCTTCTCTTTCTTTTCG TAATACGACTCACTATAGGGTCCACAGCAGAGCTAAACCC	49
HAN	TACACTTAGGGTTTTCAAACCAG TAATACGACTCACTATAGGGCTGGTGCTTCTTCTTGCATCT	49
	GGTTCAAACCGACCAGTACG TAATACGACTCACTATAGGGCGAGAGACGATGTTGATGTTTAT	
MP	CATCCGGAACCAAAGAAAGAAACTGTG TAATACGACTCACTATAGGGTCTTGTACCTGACTGGTCTTTCAACAGC	50
	AGTTCGAGATCTTCATGAGAATACTTGG TAATACGACTCACTATAGGGGAATCAGGAACACGTATAAGTGGC	
	ACTCAGTTGAACGGTCTC TAATACGACTCACTATAGGGTCTCCCGACTGACCCGGTT	
GFP	CCACCTACGGCAAGCTGAC TAATACGACTCACTATAGGGCCAGGATGTTGCC	-



E	DMSO	AXO
Lateral roots	2.1 ±1.1 (n=20)	0.05 ±0.2 (n=20)

Supplemental Figure 7. **A-B)** Root tip of a DMSO-treated **(A)** or an auxinole-treated **(B)** DR5::ER-GFP seedling. On the left DIC image; on the right GFP signal with Fire LUT. Scale bars 20 µm. **C)** Mock-treated seedlings developing lateral roots (white arrowheads). **D)** Auxinole-treated seedlings without lateral roots. **E)** Table summarizing the number of lateral roots (mean and standard deviation, n =20).



B

IAA8 sample	
Rank	≥4 fold change genes
1	IAA8, AT2g22670
2	TPL, At1g16510
3	TPR1, At1g75340
4	TPR4, At3g16670
5	TPR2, At3g17700

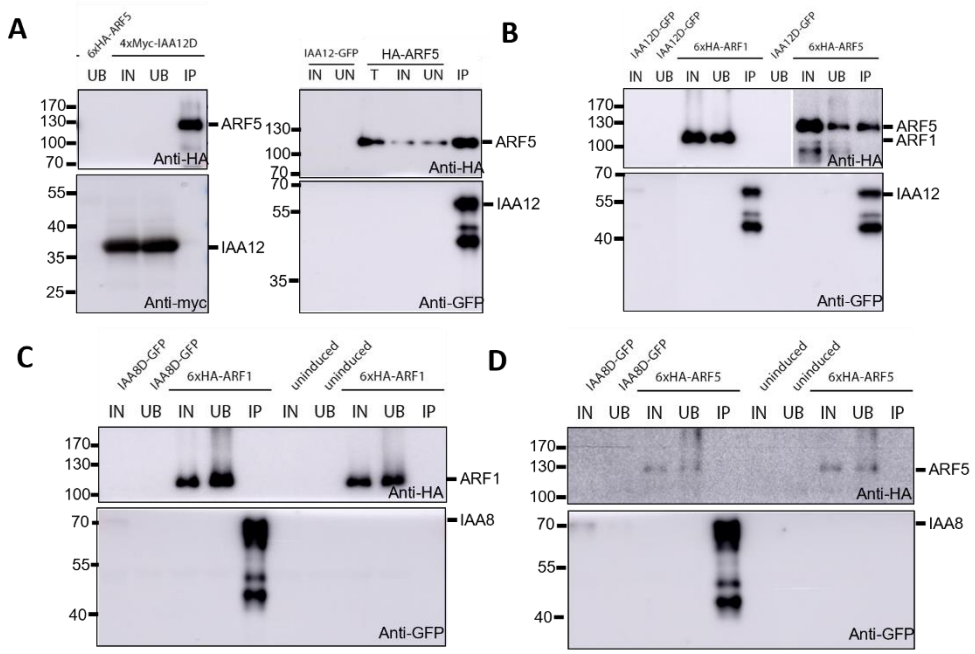
C

IAA9 sample	
Rank	> 4 fold change genes
1	IAA9, AT5g65670
2	TPR2, At3g17700
3	TPR4, At3g16670
4	TPL, At1g16510
5	TPR1, At1g75340
6	Unch. Calcium-dependent lipid-binding family protein, At1g48090
7	IAA12, At1g03830

D

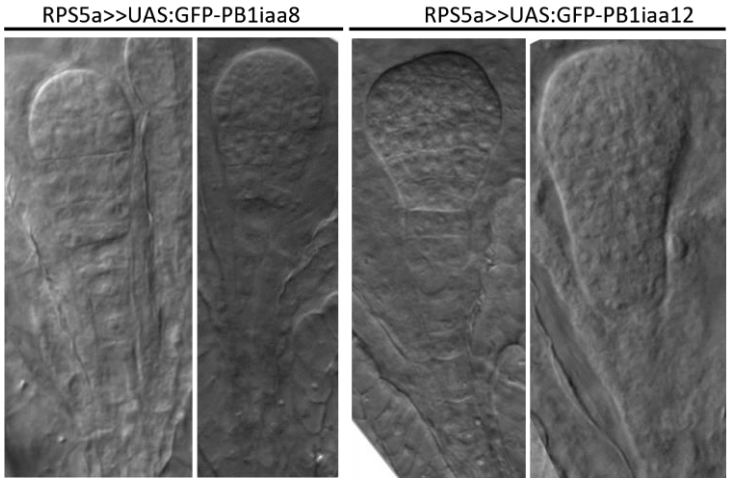
IAA12 sample	
Rank	>4 fold change genes
1	IAA12, At1g03830
2	TPR4, At3g16670
3	TPL, At1g16510
4	PMRT15 (methyltransferase), At4g35660
5	IAA9, AT5g65670
6	TPR1, At1g75340
7	Unch. Calcium-dependent lipid-binding family protein, At1g48090

Supplemental Figure 8. A) Dual luciferase experiments using the DR5 promoter. The activation of DR5 by ARF5 and ARF6 was repressed to lower than basal levels by co-expression of IAA12 or IAA8. **B-D)** Mass-spectrometry analysis after pull-down with recombinant Aux/IAAs and seedlings extract. Tables present >4-fold peptides compared to control sample (plant extract) for the IAA8D **(B)**, IAA9D **(C)** and IAA12D **(D)** samples.



Supplemental Figure 9. Westernblots of the immunoprecipitations/co-immunoprecipitations. **A)** Recombinant 4xMyc-IAA12D (31.25 kDA) does not interact with plant extracted 6xHA-ARF5 (107.51 kDa) (left blot, anti-HA beads used for the IP), but the opposite combination of recombinant HA-ARF5 (100.89 kDA) with plant extracted IAA12D-GFP (54.82 kDa) does result

in an interaction (right blot, anti-GFP beads used for the IP). **B)** IAA12D-GFP interacts with ARF5 but not with ARF1 (83.01 kDa) (all plant extracted, anti-GFP beads used for the IP). **C-D)** Plant extracted IAA8D-GFP does not interact neither with 6xHA-ARF1 nor with 6xHA-ARF5, uninduced IAA8 was used as control. Anti-GFP beads used for the IP. All transgenes in plant were expressed under the RPS5a promoter. The Aux/IAAs in plant were induced overnight with DEX; the ARFs in plant are constantly expressed. The antibodies used are referred in the lower right corner of each gel as anti-GFP, anti-myc or anti-HA. UN: unbound, IN: input, IP: immunoprecipitated, T:total.



Supplemental Figure 10. Embryonic phenotype of embryos overexpressing GFP fused to the PB1 domain of IAA8 or IAA12 under the RPS5a promoter 72 hap. Both constructs apparently generate indistinguishable phenotypes.

Supplemental Material & Methods

Recombinant Aux/IAAs and co-immunoprecipitation (Co-IP)

In the pCOLI backbone, the cds of IAA12 and IAA8 (splice variant 1) were tagged at the N-terminus with 4x myc; for ARF5 the HA tag was used. Transformation, IPTG induction and harvesting was done as previously described with minor modifications[51]. After protein extraction, myc magnetic beads (Chromotek) were incubated with the supernatant 1 h at 4°C. The samples were then washed twice in extraction buffer and incubated 2 hours at 4°C with plant extraction of 7-days-old seedlings. The beads were washed once, resuspended in 1xLaemmli 200 mM DTT in MiliQ water, and boiled 6 min at 95°C prior to mass-spectrometry (Proteome Centrum Tübingen).

Plant extracted proteins for Co-IP and western blot

The Aux/IAAs IAA8D (genomic) and IAA12D (cds) were cloned in the pBay bar backbone with RPS5a:GAL4-VP16-GR_UAS:NLS-(Aux/IAA) with a GFP at the C-terminus. 7-days-old seedlings were induced overnight with 25 µM DEX on ½ MS plates. The seedlings were manually harvested and ground with pestle and mortar using liquid nitrogen. As control uninduced seedlings were used. The ARF lines were generated with the pGREEN backbone and 6xHA at the N-terminus of ARF5 or ARF1 under the

RPS5a promoter. No DEX induction was needed for these plant lines. The ground seedlings were incubated in 3 ml lysis buffer (50 mM TRIS-HCL pH 7.5; 150 mM NaCl; 1% Triton X-100; 4x protease inhibitor cocktail) at 4°C for 30 min. Then centrifuged at 8000 rpm for 5 min. The Aux/IAAs samples were then incubated with anti-GFP magnetic beads for 2 hours at 4°C, washed twice and then incubated with the ARF extraction for another 2 hours. After two times washing, the beads were resuspended in 1xLaemmli+ 100 mM DTT and boiled 5 min prior SDS-PAGE/Western blot. The western blot approach was carried out according to published protocols with minor modifications[52].

AXO plate treatment of seedlings

Col-0 5-day-old seedlings were transferred to 100 µM AXO (or mock plates) and incubated one and half days under long day conditions.

Dual-Luciferase experiments with protoplasts

The dual-luciferase assays were generally performed as published before[53]. We used 6 µg for transfection of the reporter construct (LucTrap vector with DR5:Firefly Luciferase), 6 µg of the effector (ARF or Aux/IAA cds in pJIT60) and 2 µg of the co-reporter construct with the Renilla Luciferase (pGL4.70 2x35S::hRLuc) for normalization. Arabidopsis root protoplasts were transfected, incubated overnight and pelleted. The bioluminescence assay was carried out with the Dual-Luciferase® Reporter Assay (Promega) and the Infinite F200 plate reader (Tecan).

A filament-like embryo system to study basal cell-embryo transition

Kai Wang, Yingjing Miao, Marina Ortega-Perez, Houming Chen, Martin Bayer

Abstract

During embryogenesis of flowering plants, the embryo proper is connected with the mother tissue through the suspensor. Although the suspensor is eventually degraded during seed maturation, it is indispensable for delivery of nutrition and phytohormones during early embryogenesis. The differentiation to produce the two functionally different structures takes place very early. The suspensor contains most of the cells derived from the basal daughter cell after the asymmetric zygote division while descendants of the apical daughter cell mainly contribute to the embryo proper. However, what mechanism regulates the distinct differentiations and whether the differentiation directions are alterable are largely unknown. The Brassicaceae embryogenesis follows a very stereotypical development process. A filamentous suspensor is generated by horizontal divisions of the basal daughter cell, and the clear boundary between the suspensor and the embryo proper is formed, thus providing an advantageous model to study the mechanism of this distinct differentiations. Several researches have indicated that the suspensor has the potential to transit into an embryo in Arabidopsis and *Brassica napus*. The function of Auxin is emphasized in regulating the suspensor-to-embryo(suspensor-embryo) transition. Here, through activating YDA pathway in early embryogenesis, we generated filament-like embryos in Arabidopsis to study the suspensor-embryo transition. The development of twin proembryos derived from these filaments was easy to follow, and twin healthy seedlings were finally generated. Through using transgenic marker lines, we indicated that the filamentous embryo was in an undifferentiated status. Strong auxin response was not observed in early filamentous embryos. Later, we observed maximum auxin response in the suspensor region and vertically divided suspensor cells. Taken together, we developed an elegant system that can be used to study the mechanism of embryonic transition. Our preliminary results implies that it is important to have an early inhibition of auxin response in the whole embryo and a later activation of auxin response in the suspensor for the suspensor-embryo transition.

Keywords

YDA signaling, SSP, MPK6, cell lineage, filament-like embryo, twin embryos, embryonic transition, cell identity, auxin response

Introduction

Embryogenesis is the initiating step of the development of multicellular organisms. Different from animal embryogenesis, embryos of flowering plants have a file of cells connected with the mother tissue, called suspensor, most part of which is degraded during embryo maturation[1]. Considered to hold and provide nutrition and phytohormone for the above embryo proper, suspensor structures are quite diverse among plant kingdom[2]. As both the suspensor and the embryo proper are formed form the zygote, an important question arisen is how the different differentiation directions are determined. In many plant species, the embryogenesis is variable and the boundary between embryonic and suspensor tissue is difficult to distinguish[3]. However, in *Brassicaceae* embryogenesis follows a very stereotypical development process. After asymmetric division of the zygote, the cell fates of two daughter cells are determined. The small apical cell develops into the spherical embryo proper while the large basal cell divides horizontally to form the filament-like suspensor. Except the uppermost suspensor cell that contributes to the hypophysis, the suspensor is degraded in the end[3]. Thus, *Brassicaceae* provide a practical tool to study the mechanism of distinct differentiations of embryos.

In Arabidopsis, zygotic polarity is regulated by a Mitogen-Activated Protein Kinase (MAPK) cascade: YODA(YDA, MKKK4)-MKK4/5-MPK3/6[4]. The *yda*, *mkk4 mkk5* and *mpk3 mpk6* mutants show symmetric zygote division, and the basal daughter cell adopts embryo-like development, leading to embryos without the suspensor [5–7]. In transgenic lines that express a constitutively active version of YDA (yda-CA), the first division becomes more asymmetric and filament-like embryos without embryo proper are formed, which results in embryonic lethality[5]. Thus, the YDA cascade promotes suspensor differentiation and inhibits proembryo differentiation. The BR-signaling kinase 1 (BSK1), BSK2 and SHORT SUSPENSOR (SSP/BSK12) function upstream of the YDA cascade. Different from BSK1/2, SSP is a constitutive input for YDA signaling with an unusual paternal-of-origin effect[8,9]. The YDA cascade regulates zygote polarity by phosphorylating the transcription factor WRKY2, which in turn activates *WOX8* expression. During embryogenesis, *WOX8* is expressed from the zygote stage onward and marks suspensor identity [10,11].

Recent transcriptome data of the developing zygotes and isolated cell lineages of the 1-cell embryo and the 32-cell embryo showed that the basal cell and early suspensor cells have quite close transcriptional states in Arabidopsis. However, their transcriptional states are much different from that of the zygote, suggesting that the basal cell lineage has differentiated from the zygote [12]. However, suspensor cells are still capable to become an embryo. *Liu et al.* showed that when the young proembryo was removed by laser ablation, a new embryo proper was developed from the remnant top suspensor cell. This potential was possessed only by early basal cell lineage as suspensor cells no

longer own the ability after the globular stage [13]. Thus, the embryonic potential of the early basal cell lineage might be suppressed by the normal embryo proper[14]. Consistent with this theory, secondary embryos were formed from suspensor cells when the original embryo proper was abnormal in *sus* mutants[15]. However, secondary embryos are still developed from suspensor cells in some mutants like *twn1* and *twn2* although the embryo proper appears normal[16,17].

The development of the apical daughter cell is regulated by auxin, which is initially transported from the basal cell by PIN7[18]. Compared to the basal cell, the apical cell shows stronger DR5 response[18]. When the apical auxin response was suppressed by direct inhibition or decreased auxin influx, the apical cell divided horizontally and gave rise to an abnormal embryo proper[19,20]. Recent data suggests that auxin transported from surrounding maternal tissue is responsible for auxin response in the apical cell[21]. Later on, strong auxin response is mainly taking place in the hypophysis, which is vital for the root initiation[19,20,22]. Collective observations imply that local auxin response may be also involved in the suspensor-embryo transition. In the laser ablation system, strong DR5:GFP signal was observed in the remnant top suspensor cell after the removal of early embryo proper[13]. Similarly, in some knock-out lines where the embryo-like proliferation in the suspensor was observed, maximum DR5:GFP response was found in suspensor cells before the initiation of secondary embryos [23,24]. Thus, it is tempting to presume that increased auxin response in suspensor cells promotes the transition. However, other studies showed that inhibiting auxin response in suspensor cells promotes this transition. BODENLOS/INDOLE-3-ACETIC ACID INDUCIBLE 12 (BDL, IAA12) is an AUX/IAA protein that inhibits auxin response. When the stabilized *bdl*, a dominant-active version of *BDL*[19,25], was trans-activated in the whole embryo (*RPS5A>>bdl*) or only in the early suspensor (*M0171>>bdl*), embryo-like proliferation was observed in the suspensor, suggesting that the suspensor-embryo transition is inhibited by local auxin response [26,27]. However, compared to *M0171>>bdl*, twin seedlings were more frequently generated in *RPS5A>>bdl*[26,27]. As the formation of the embryo proper on top was also abnormal in *RPS5A>>bdl*[26,28], the suspensor-embryo transition might be further facilitated by the abnormal embryo proper.

Removing the embryo proper through the laser ablation system is an elegant tool to study the transition of suspensor cells without the above embryo proper. However, the thick covering ovule tissue reduced the precision during ablation[13]. The microspore-derived embryo in *Brassica napus* provides another strategy to address the transition. When cultured with a special media, the microspore of *Brassica napus* can divide horizontally into a suspensor-like filament and then generates proembryos on top, in the middle or at several positions of the long filament[29]. This suspensor-like filament system will provide tempting information regarding the suspensor-embryo transition. In Arabidopsis filament-like embryo can also be induced by activating the YDA pathway[5]. To check what

mechanism triggers/inhibits the embryonic transition of the early basal cell lineage, we generated filament-like embryos in Arabidopsis through activating the embryonic YDA pathway in different degrees. By following the embryonic development of these transgenic lines, we detected their ability to produce multiple embryos and multiple seedlings, and chose pS4:SSP-YFP lines for further analysis. By using different fluorescent marker lines, we found that the early filament embryos resemble the basal cell lineage. Strong auxin response was not observed in filament-like embryos. Obvious auxin response was observed in the putative hypophysis and tips of cotyledon primordia, suggesting the normal embryonic development of the apical embryo. In contrast, maximum auxin response was detected in suspensors cells. Maximum auxin response in the vertically divided suspensor cell implied the initiation of suspensor-embryo transition. Taken together, we developed an easy method to study the suspensor-embryo transition. Our results suggest that an early inhibition of auxin response in the whole embryo and a later activation of auxin response in the basal cells are both important for the suspensor-embryo transition.

Results

Continuously activating the embryonic YDA signaling leads to filament-like embryos

First, we checked the embryogenesis of *yda-CA* transgenic lines. Although long filament-like embryos were formed[5], the *yda-CA* lines showed severe stomata defect, arrested flower development, abnormal silique shape and reduced ovule formation (not shown), consistent with previous observation[30,31]. These defects together contribute to a severe loss of fertility. To avoid the fertility defect, we tried to activate the YDA pathway specifically in the embryo sack. Replacing Tyr 144 by Cys in MPK6 (MPK6-CA) caused strong activation of MPK6 which functions downstream of YDA [6,7,32]. Therefore, we tried to trans-activate MPK6-CA by the *RPS5A* promoter (*RPS5A*$_{pro}$*>>MPK6-CA*) in the embryo using the GAL4-UAS two-component expression system [33,34].

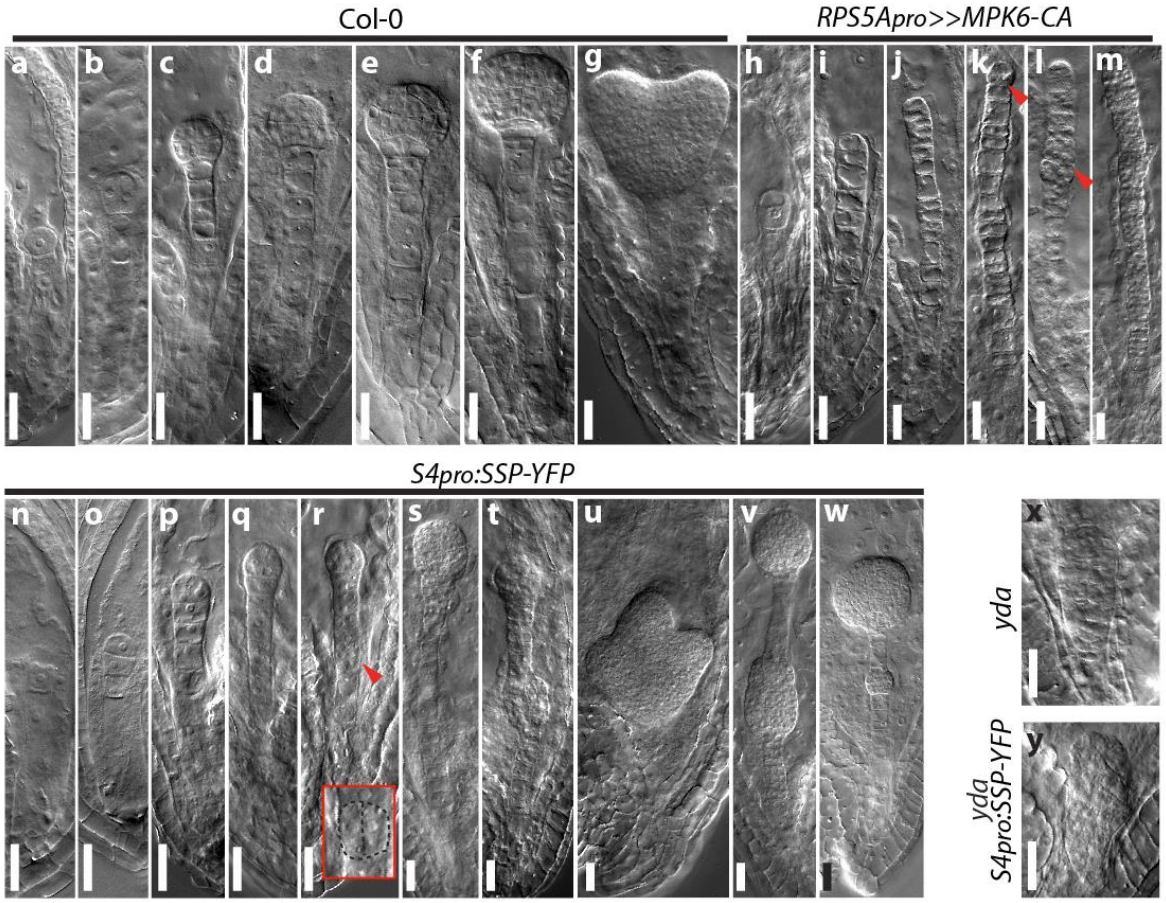


Figure 1. Embryonic development of Col-0, *RPS5A$_{pro}$>>MPK6-CA* and *S4$_{pro}$:SSP-YFP* in early embryonic stages. **a**-**g**, The embryonic development of Col-0 in 1 DAP at the 1-cell stage (**a**) or the 2-cell stage (**b**), 2 DAP at the 8-cell stage (**c**), 2.5 DAP at the 32-cell stage (**d**) or the early globular stage (**e**), 3 DAP at the later globular stage (**f**), and 4 DAP at the heart stage (**g**). **h**-**m**, The embryonic development of *RPS5A$_{pro}$>>MPK6-CA* in 1 DAP (**h**), 2 DAP (**i**), 2.5 DAP (**j**), 3 DAP (**k** and **l**) and 4 DAP (**m**). **k** and **l**, Filament-like embryos with the first vertical division either in the uppermost apical cell (**k**, arrowhead) or in middle cells (**l**, arrowhead). **n**-**w**, The embryonic development of *S4$_{pro}$:SSP-YFP* in 1 DAP (**n** and **o**), 1.5 DAP (**p**), 2 DAP (**q** and **r**), 3 DAP (**s** and **t**) and 4 DAP (**u**-**w**). **r**, the 2-DAP embryo with an additional vertical division within basal cells (arrowhead and the enlarged image with dished lines). **s**, the 3-DAP embryo containing an embryo-like cell cluster on top and a very long basal part. **t**, the 3-DAP embryo containing twin embryo-like cell clusters . **u**-**w**, 4-DAP embryos containing triple cotyledon primordia (**u**) or twin embryo-like cell clusters (**v** and **w**). **x**, the *yda* early embryo. **y**, the *yda S4$_{pro}$:SSP-YFP* early embryo. DAP: day after pollination. Scale bar represents 20 μm in all panels.

After hand pollination, long filament-like embryos were formed in *RPS5a>>MPK6-CA* at early stages, indicating a strong activation of the YDA pathway (Figure 1a-l). During the growth of filaments, the first vertical division is mainly observed on the top (Figure 1k). However, the embryo-like development was also sometime observed in the middle of filaments (Figure 1l). This observation resembles the microspore system that the embryonic fate is not always first delivered to the apical[29]. The long filaments developed into thick sticks and several embryo-like structures are generated eventually (Figure 1m, 2b and 2h), implying that MPK6 inhibition of embryonic differentiation was partly overcome in the end. However, these structures cannot develop into intact seedlings after germination (Figure 2n), indicating that the normal embryogenesis was severely retarded by continuous activation of the YDA pathway.

Lines and crosses	Vertical division of basal cells at 2 DAP	twin embryos at 3DAP	twin embryos at 4DAP	multiple embryos at 5 DAP	Single seedling with >2 cotyledons primordia	multiple seedlings
$S4_{pro}$:SSP-YFP #3	0% (0/82)	14% (14/100)	21.01% (25/119)	27.97% (40/143)	19.18% (14/73)	9.59% (7/73)
$S4_{pro}$:SSP-YFP #10	0% (0/56)	6.54% (7/107)	7.89% (6/76)	23.96% (46/192)	10.64% (20/188)	8.51% (16/188)
$S4_{pro}$:SSP-YFP #14	1.47% (3/204)	5.08% (6/118)	5.02% (11/219)	19.67% (71/361)	25.74% (35/136)	7.35% (10/136)
$S4_{pro}$:SSP-YFP #14 x Col-0	-	-	-	22.60% (33/146)	-	-
Col-0 x *$S4_{pro}$:SSP-YFP #14*	-	-	-	3.16% (3/95)	-	-

Table 1. The ratio of multiple embryos and seedlings in different lines and crosses
The numbers are listed below the ratio. The crosses are given as female x male. DAP: day after pollination. ">2" means more than two. "-"means the ratio was not calculated.

Specifically enhancing YDA signaling at early stages promotes twin embryos formation

Since continuous activation of the embryonic YDA pathway caused a strong embryonic defect, enhancing the YDA signaling only at early stages might be a better approach to facilitate embryonic transition from filament-like embryos. SSP is a strong input to the embryonic YDA pathway[8,9]. The *At3g10100* promoter is mainly active during early embryogenesis[35]. so we use this promoter (here after called S4 promoter) to express *SSP* fused with a YFP (*$S4_{pro}$:SSP-YFP*) in the *ssp-2* mutant background. In this way, we expected to stimulate YDA signal at early stages and shut it down at late stages. Three lines with strong phenotype were chosen for analysis. 2 days after pollination (DAP), *$S4_{pro}$:SSP-YFP* embryos developed into filaments (Figure 1n-r). Occasionally, a vertical division was observed in basal cells of line *#14* (Figure 1r and Table 1). 3 DAP, a long filamentous basal part with an proembryo-like cluster was generated (Figure 1s). In addition, ovules containing twin embryo-like cluster were occasionally observed (Figure 1t and Table 1). 4 DAP, twin clusters were constantly expanding (Figure 1v,w) and embryos containing 3 cotyledon primordia were observed (Figure 1u). Ovules containing twin/triple embryos were more frequently observed at 5 DAP, indicating that filament-derived secondary embryos were generated continuously (Table 1). These tri-cotyledon embryos and multiple embryos underwent normal embryogenesis although the development was strongly delayed compared to wild type (Figure 2). Interestingly, twin embryos can also develop in opposite orientations (Figure 2e, k). After seed germination, we often observed tri-cotyledon seedlings, twin-root seedling and twin/triple seedlings (Figure 2 and Table 1).

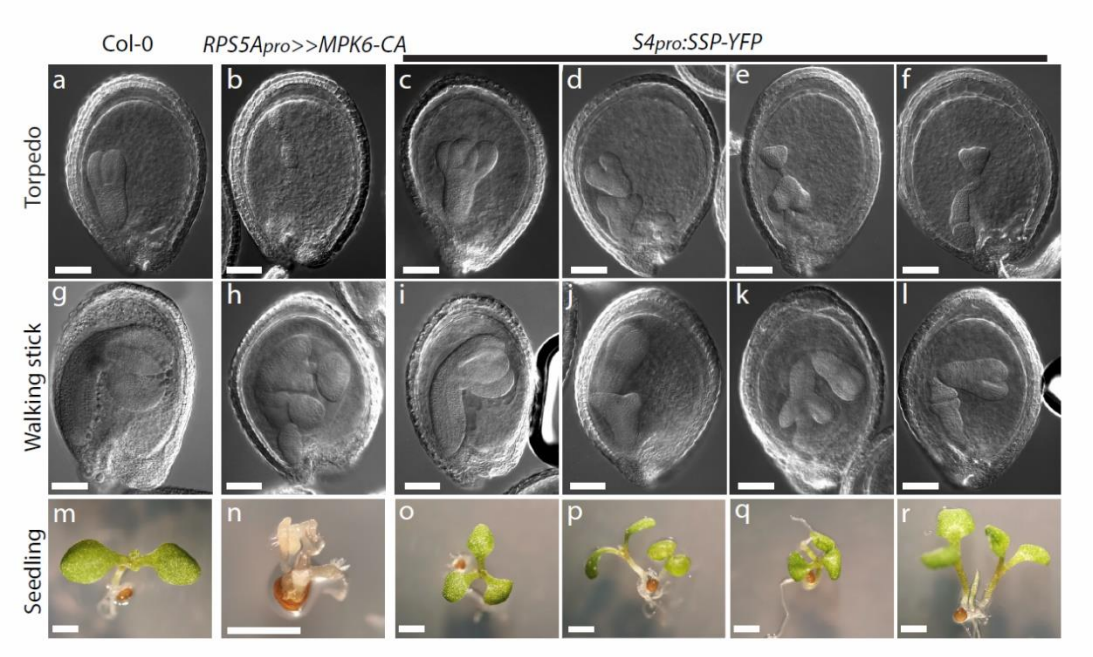


Figure 2. Late embryonic phenotypes and seedling phenotypes of Col-0, *RPS5A$_{pro}$>>MPK6-CA* and *S4$_{pro}$:SSP-YFP.* **a**-**l**, ovules of late embryonic stages in Col-0, *RPS5A$_{pro}$>>MPK6-CA* and *S4$_{pro}$:SSP-YFP* stained with Hoyer's solution. **a** and **g**, Col-0 embryos at the torpedo stage (**a**) and the walking-stick stage (**g**). **b** and **h**, *RPS5A$_{pro}$>>MPK6-CA* embryos at the torpedo stage (**b**) and the walking-stick stage (**h**). **c**-**f** and **i**-**l**, *S4pro:SSP-YFP* embryos of the torpedo (**c**-**f**) stage and walking-stick stage (**i**-**l**) with triple cotyledons (**c** and **i**), twin embryos growing in either the normal orientation (**d** and **j**) or reverse orientations (**e** and **k**), and triple embryos (**f** and **l**). **m**-**r**, 5 DAP seedlings of Col-0 (**m**), *RPS5A$_{pro}$>>MPK6-CA* (**n**), and *S4$_{pro}$:SSP-YFP* (**o**-**r**) containing tri-cotyledons (**o**), twin seedlings (**p**), twin roots (**q**) or triple seedlings (**r**). DAP: days after germination. The scale bars represent 100 μm in **a**-**l** and 1 mm in **m**-**r**.

Endogenous SSP is only shortly active in the zygote[8]. We then wonder whether the filament-like embryos were indeed caused by an activation of the YDA pathway. In *yda/+* mutants, a quarter embryos show severe development defect[5]. In *S4$_{pro}$:SSP-YFP yda-11/+* , 26.73% (54/202) embryos showed severe elongation defect, indicating that the embryonic phenotype in *S4$_{pro}$:SSP-YFP* were primarily induced by activating the YDA pathway. In contrast to *pRPS5A$_{pro}$>>MPK6-CA* seedlings, *S4$_{pro}$:SSP-YFP* seedlings grew and flowered normally in soil (not shown). Thus, the influence of *S4$_{pro}$:SSP-YFP* transgene was only restricted to the embryo. These transgenic lines provide a good model to study the embryonic transitions of the basal cells.

SSP-YFP transgene changed the S4 promoter activity

As the S4 promoter is mainly active in early embryos and *SSP-YFP* inhibition of embryonic differentiation was removed during cell proliferation, we wonder how long and where *SSP-YFP* was expressed. In *S4$_{pro}$:nGFP*, nuclear-localized GFP was observed in the zygote and early embryos. The signal became weaker from the globular stage onward and disappeared after the heart stage (Figure 3a-e). Similar to *S4$_{pro}$:nGFP*, membrane-localized YFP signal was also detected in the whole *S4$_{pro}$*:SSP-YFP embryo during early stages (Figure 3f-i). However, the signal was still obvious in the center of

expanding cell clusters while it was attenuated in other cells (Figure 3j-k). YFP signal was barely detected in heart embryos and torpedo embryos (Figure 3i-m). As the S4 promoter is more active in early embryos, these cells with higher SSP-YFP signal (Figure 3j-k) may be still in the early stage while surrounding cells had differentiated further. Taken together, the activity of S4 promoter is also influenced by the expression of SSP-YFP, implying that cell identities might have been altered in these embryo-like clusters.

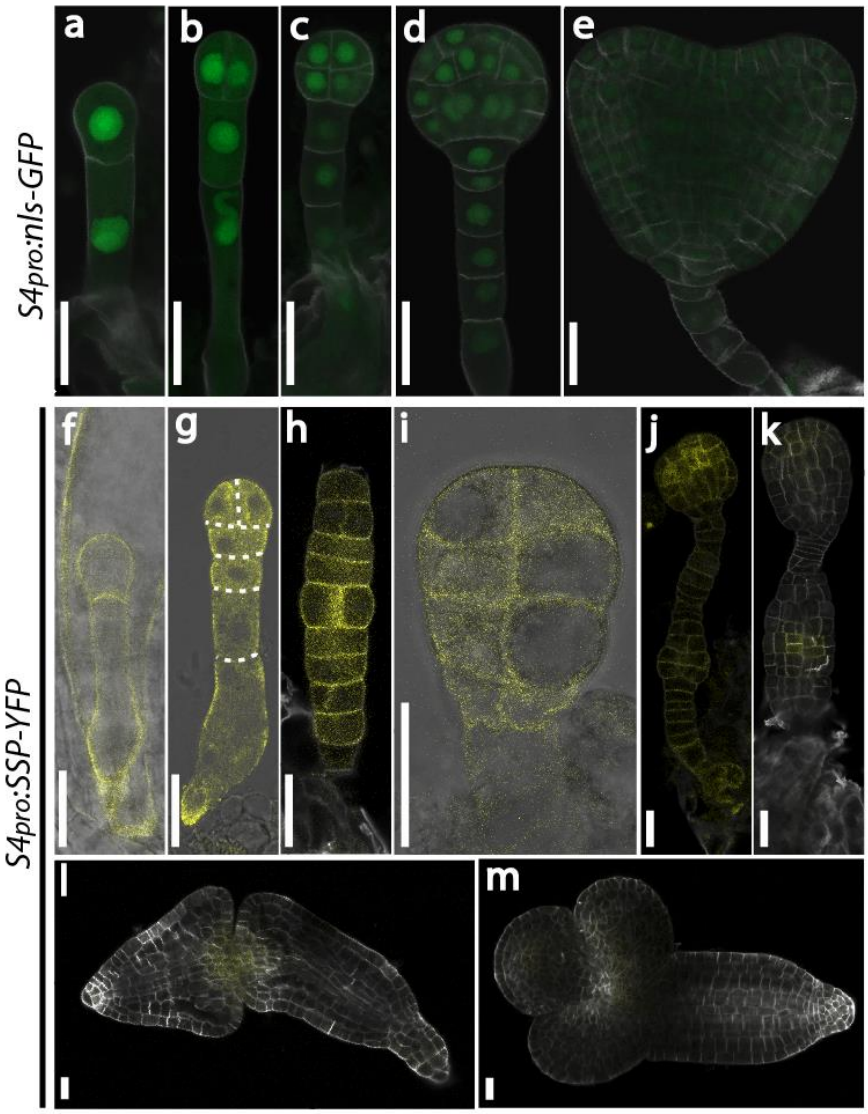


Figure 3. Expression of *SSP-YFP* in *S4$_{pro}$:SSP-YFP* embryos. **a**-**e**, GFP signals in *S4$_{pro}$:nls-GFP* 1-cell embryo (**a**), 2-cell embryo (**b**), 8-cell embryo (**c**), globular embryo (**d**) and heart embryo (**e**). **f**-**m**, membrane-localized YFP signals in *S4$_{pro}$:SSP-YFP* 1-cell embryo (**f**), early embryo showing first vertical division (**g**), filament-like embryo (**h**), apical cells of an early embryo (**i**), embryos containing twin proembryo-like early clusters (**j**) or further-proliferated clusters (**k**), twin embryos at the heart stage (**l**), and the torpedo embryo containing three cotyledon primordia (**m**). The very apical cells of the embryo in **h** were lost during sampling. Large vacuoles were observed in apical cells (**i**). Dashed lines in **g** indicate cell walls. Scale bar represents 20 μm in all panels.

The filamentous embryo has the identity of early basal cells

To check cells identities of *S4$_{pro}$:SSP-YFP* early embryos, different promoter marker lines were crossed with *S4$_{pro}$:SSP-YFP*. From the reciprocal crosses experiment between *S4$_{pro}$:SSP-YFP* and Col-0, we found that higher frequency of twin embryos were observed when using *S4$_{pro}$:SSP-YFP* as mother, which was equivalent to self-crossed *S4$_{pro}$:SSP-YFP* (Table 1), suggesting a strong maternal contribution to YDA activation in our transgenic system. Therefore, *S4$_{pro}$:SSP-YFP* was always used in the maternal side to cross with fluorescent markers lines. F1 embryos were checked directly from crosses.

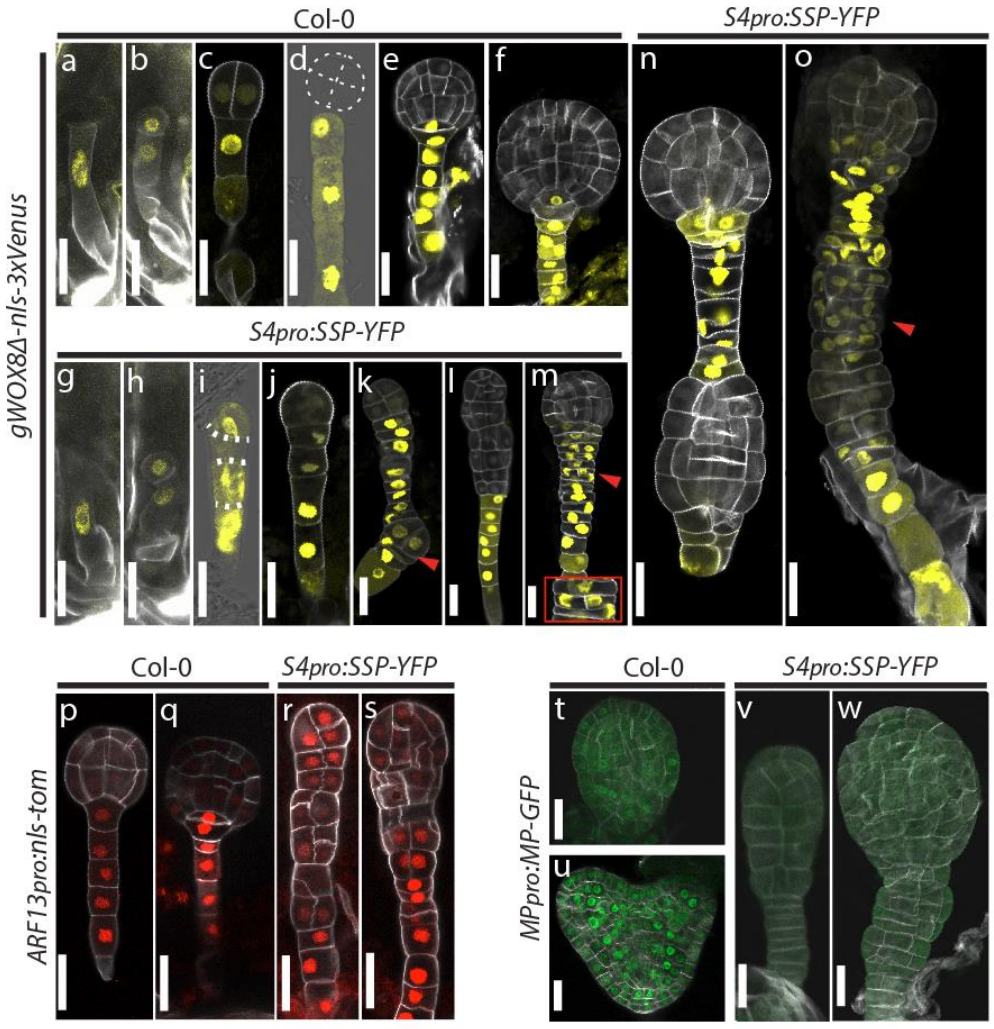


Figure 4. The expression patterns of fluorescent markers in Col-0 and *S4$_{pro}$:SSP-YFP* lines. a-f, the *gWOX8Δ-nls-3xVenus* signal in Col-0 zygote (**a**), 1-cell embryo (**b**), 2-cell embryo (**c**), 8-cell embryo (**d**), early globular embryo (**e**) and late globular embryo (**f**). **g-o**, the *gWOX8Δ-nls-3xVenus* signal in *S4$_{pro}$:SSP-YFP* zygote (**g**), 1-cell embryo (**h**) and embryos of different stages and shapes (**i-o**). **i**, the early embryo with a horizontal division of the apical daughter cell of the zygote. **j**, the filament-like embryo with the first vertical division of the top apical cell. **k**, the filamentous embryo showing the first vertical division of basal cells (arrowhead). **l**, the embryo containing an apical stick-like cell cluster. **m**, the early embryo containing a proembryo-like cluster on top and the first vertical division of basal cells (arrowhead and the enlarged box). **n** and **o**, embryos containing twin proembryo-like cell clusters. **p-s**, the *ARF13$_{pro}$:nls-Tom* signal in Col-0 32-cell embryo (**p**) and globular embryo (**q**), and in the *S4$_{pro}$:SSP-YFP* filament-like embryo (**r**) and the embryo with a proliferated apical cluster(**s**). **t-w**, the *MP$_{pro}$:MP-GFP* signal in Col-0 globular embryo (**t**) and heart embryo (**u**), and in *S4$_{pro}$:SSP-YFP* embryos in early stage (**v**) or a bit late stage (**w**) . Dashed lines in **d** and **i** indicate the cell wall. Scale bar represents 20 µm in all panels.

gWOX8Δ-NLS-3xVenus was used as marker for suspensor identity[10]. In wild-type background, Venus signal was detected in the elongating zygote and both daughter cells (Figure 4a,b). At the 2-cell stage, weak Venus signal was still detected in the apical cells (Figure 4c). From the 8-cell stage onward, Venus signal was only observed in suspensor cells (Figure 4d-f). In contrast, obvious YFP signal was observed in apical cells of filamentous embryos in *S4$_{pro}$:SSP-YFP* (Figure 4i-k), suggesting that the embryonic identity had not been acquired yet. *ARF13* is also specifically expressed in the suspensor[10,36]. In *ARF13$_{pro}$:nls-Tom*, ARF13 promoter was fused with a nuclear localization signal sequence and Tomato RFP sequence. Specific Tomato signal was observed in the wild-type suspensor (Figure 4p,q), whereas strong Tomato signal was detected in the whole early filamentous embryo in *S4$_{pro}$:SSP-YFP* although some apical cells divided vertically (Figure 4r). Combined together, these results suggest the filamentous embryo in *S4$_{pro}$:SSP-YFP* is a file of cells with most likely basal cell identity.

The expanding cell clusters did not fully obtain proembryo identity

In *S4$_{pro}$:SSP-YFP*, proembryo-like clusters were generated from the filamentous embryos (Figure 1). While strong *gWOX8Δ-NLS-3xVenus* and *ARF13$_{pro}$:nls-Tom* signals were observed in the basal part of these embryos, the apical clusters did not show strong fluorescent signals despite the abnormal shapes (Figure 4i-n). These observations imply that these clusters might have obtained embryonic identity. Sometimes, when basal cells divided vertically, the *gWOX8Δ-NLS-3xVenus* signal in daughter cells became weaker (Figure 4k). These cells may undergo proliferation to generate another embryo-like cluster (Figure 4n). In addition, we also sometime observed strong Venus signal in these daughter cells (Figure 4m). It seems plausible that these daughter cells may proliferate into cell clusters partly with the suspensor identity (Figure 4o). Some cells in this basal cluster may eventually obtain the embryonic fate as *gWOX8Δ-NLS-3xVenus* signal in these cells was weaker than the others (Figure 4o).

Although these proembryo-like clusters showed weak suspensor identity, whether these clusters really gained the embryonic identity was not clear. *MONOPTEROS/Auxin Response Factor 5 (MP/ARF5)* is specifically expressed in the embryo proper[36]. We make the *MP$_{pro}$:MP-nGFP* line where the MP promoter was fused with the MP genomic sequence and a nuclear-localized GFP sequence. In wild type, despite a weak gain-of function phenotype during embryogenesis, the MP-GFP signal was specifically detected in the embryo proper (Figure 4t,u). However, the MP-GFP signal was not observed in *S4$_{pro}$:SSP-YFP* embryo-like clusters in the early stage or a bit late stage (Figure 4v,w). Taken together, although the proembryo-like clusters in *S4$_{pro}$:SSP-YFP* have weak suspensor identity, they have not fully acquired the embryonic identity yet. The embryonic identity in these clusters could be directly suppressed by YDA activation since SSP-YFP signal was detected in cell clusters (Figure 3j,k).

Importantly, although the SSP-YFP signal was strong in early basal cells, these cells can divide vertically and then generate clusters, suggesting that these basal cells have overcome SSP inhibition. How can these cells override SSP inhibition to divide vertically?

Altered Auxin response in *$S4_{pro}$:SSP-YFP*

Auxin response is crucial for embryonic development of the apical daughter cell after zygote division. When auxin response is blocked, the apical daughter cell divides horizontally like the basal daughter cell and the embryonic development is deregulated. Several studies have linked auxin response with suspensor-embryo transition. To check whether auxin response is deregulated in *$S4_{pro}$:SSP-YFP*, we checked the DR5:GFP signals in Col-0 and *$S4_{pro}$:SSP-YFP*. 4 DAP, maximum DR5 response was observed in wild-type hypophysis (Figure 5a), whereas GFP signal was not observed in filament-like embryos in *$S4_{pro}$:SSP-YFP* (Figure 5b), suggesting that the YDA signal has a primary inhibition on auxin response. Intriguingly, in proembryo-like clusters of *$S4_{pro}$:SSP-YFP* embryos, weak DR5:GFP signal was still detected in the putative hypophysis region (Figure 5c,d), suggesting that the apical-basal axis was partly established. Strong DR5:GFP signal was observed in the hypophysis of wild type and *$S4_{pro}$:SSP-YFP* embryos at 5 DAP (Figure 5e-g). Obvious DR5:GFP signal was also detected in cotyledon primordia of these embryos though the signal in *$S4_{pro}$:SSP-YFP* was weaker than in wild type. These results demonstrate that embryogenesis of the proembryo-like clusters was delayed, yet not fully blocked. This is consistent with the observation that healthy tri-cotyledon seedlings and twin seedlings were generated eventually. As the SSP-YFP signal was very weak during the heart stage (Figure 3l), the direct inhibition of the SSP-YFP on cell differentiation might has been erased from this stage onward. Strikingly, the maximum auxin response in *$S4_{pro}$:SSP-YFP* embryos had shifted to basal cells at 3 DAP (Figure 5c). At this stage, we also directly observed the maximum DR5:GFP signal in the dividing basal cells (Figure 5d), reminiscent of the auxin response in wild-type apical daughter cell after zygote division. It seems plausible that the increased auxin response in basal cells serves as an momentum for the embryonic transition.

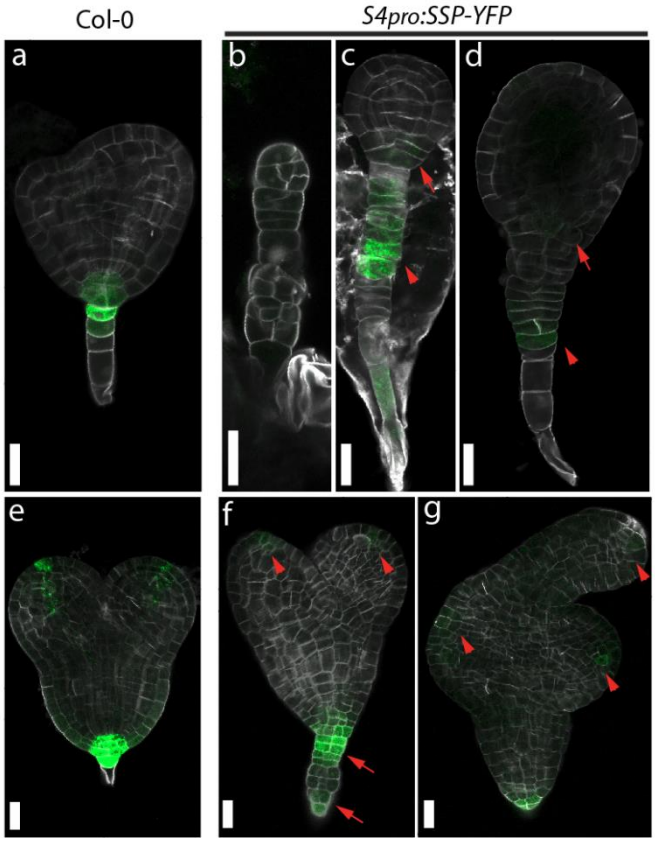


Figure 5. DR5 response in Col-0 and *S4$_{pro}$:SSP-YFP* embryos. a-g, *DR5:GFP* signal in Col-0 embryos (**a** and **e**) and *S4$_{pro}$:SSP-YFP* embryos (**b**-**d**, **f**, **g**) at 4 DAP (**a**-**d**) and 5 DAP (**e**-**g**). **b**-**d**, *S4$_{pro}$:SSP-YFP* 4-DAP embryos with a filamentous structure (**b**), a long basal part (**c**)or the first vertical division in basal cells (**d**). Arrows in **c** and **d** indicate the DR5:GFP signal in putative hypophysis. Arrowheads in **c** and **d** indicate the maximum auxin response in basal cells. **f** and **g**, heart stage *S4$_{pro}$:SSP-YFP* embryos containing two (**f**) or three (**g**) cotyledon primordia. Arrowheads in **f** and **g** show the DR5:GFP signal in tips of cotyledon primordia. Strong DR5 signal was also observed in the basal part of twin clusters (**f**, arrow). Scale bar represents 20 µm in all panels.

Discussion

Enhancing the YDA signal in early embryos promotes the generation of twin seedlings

In this study, we tried three different strategies to activate the embryonic YDA pathway, aiming to produce filament-like embryos. In *yda-CA*, although filamentous embryos were produced[5,37], the development is hard to follow because of strong fertility defects. Thus, we tried to specifically enhance the YDA signal in the embryo by the GAL4-UAS system. Similar to *yda-CA*, long filament-like embryos were formed in *RPS5A$_{pro}$>>MPK6-CA* (Figure 1). However, the embryonic development was severely delayed or blocked because of continuously activation of the YDA signaling (Figure 1 and Figure 2). Although multiple embryo-like clusters were formed, mature embryos were barely observed, making it hard to depict the developmental process (Figure 2). Finally, by enhancing YDA signaling at early embryonic stages, we developed an approach to convert filament-like embryos into twin embryos and

finally into healthy twin seedlings (Figure 1 and Figure 2). Reciprocal crosses between wild type and *S4$_{pro}$:SSP-YFP* indicates that the maternal expressed *SSP-YFP* played a major role in the YDA activation in our system. The endogenous SSP regulates embryogenesis with a paternal effect[8]. Our result implies that *SSP* promoter sequence is vital for the paternal manner of SSP.

Basal cells in filamentous embryos have embryonic potential

In *RPS5A$_{pro}$>>MPK6-CA*, first embryo-like proliferation can be initiated in the middle of filamentous embryos (Figure 1), suggesting that the first embryonic transition is not solely possessed by top apical cells. Different from *RPS5A$_{pro}$>>MPK6-CA*, filament-like embryos was shorter in *S4$_{pro}$:SSP-YFP*. Besides, first embryo-like proliferation was only initiated from the apical part and were formed earlier in *S4$_{pro}$:SSP-YFP*, suggesting that the level of activation on the YDA signaling was weaker in *S4$_{pro}$:SSP-YFP*. By crossing *S4$_{pro}$:SSP-YFP* with early suspensor marker lines *gWOX8Δ-NLS-3xVenus* and *ARF13$_{pro}$:nls-Tom*, we found that the filamentous have the identity of early basal cells (Figure 4). Second proembryo-like clusters were initiated from these cells (Figure 1), suggesting that these cells have embryonic potential. Even 5 DAP, new embryo-like clusters were still initiated from the basal part (Table 1), indicating that this potential can endure for quite long time. That explains why only 7%~10% twin seedlings were formed eventually while 20%~30% twin embryos were generated (Table 1). According to *Liu et al.,* when proembryo was removed after the globular stage, suspensor cells did not have the embryonic potential[13]. Thus we prefer to call these basal cells along *S4$_{pro}$:SSP-YFP* filamentous embryo "basal lineage ground cells (BLGCs)" that can either develop into a suspensor or convert into an embryo.

Embryonic transition and feedback in our system

Although the *S4* promoter is mainly active at early stage, we observed longer SSP-YFP signals when using *S4* promoter (Figure 3). In wild type, *SSP* transcripts were only detected in the pollen and zygote[8,38]. Although 225bp *SSP* 5' UTR was fused with the S4 promoter in our construct, this is unlikely the reason of prolonged expression. As activated YDA signaling prevented differentiation in the beginning, the BLGCs will strongly activate the *S4* promoter activity. Then increased SSP-YFP expression activated the YDA signaling, which in turn promoted the generation of more BLGCs. In proembryo-like clusters, SSP-YFP is mainly localized in the center (Figure 3). Since the *S4* promoter is more active at early stages, it seems plausible that these cells containing stronger SSP-YFP signal were still in very early embryonic stages, which also contribute to prolonged SSP-YFP expression. Accordingly,

surrounding cells containing lower SSP-YFP signal may have differentiated further. However, although *gWOX8Δ-NLS-3xVenus* and *ARF13$_{pro}$:nls-Tom* patterns partly mimic that of wild type in these clusters, *MP$_{pro}$:MP-GFP* was not observed (Figure 4), suggesting that the proembryo identity was only partly acquired in these clusters. That might explain why triple cotyledon primordia were frequently formed (Figure 1). As *S4$_{pro}$:SSP-YFP* seems to establish a positive feedback loop, activating YDA pathway at early stages without a possible feedback onto the promoter would be an even better method to study the BLGCs-embryo transition. Expressing SSP-YFP with a strong promoter that is solely active in the egg cell and/or zygote would be fascinating.

The initiation of embryonic transition from basal lineage ground cells

We showed that enhanced YDA signaling inhibits embryonic development primarily. During filament elongation, some BLGCs finally adopted vertical division and tried to proliferate into an embryo. Thus, these cells have at least partly overcome the inhibition of YDA signaling. Interestingly, this violation is not negatively correlated with SSP-YFP signal strength since vertically divided BLGCs still showed strong SSP-YFP signal like other BLGCs (Figure 3). This suggests that some BLGCs may gained signals for the vertical division, although the following transition processes were still strongly suppressed by YDA signaling in early stages. Auxin response in apical daughter cells is vital for its vertical division. Thus, we wonder whether auxin response in BLGCs is different from that of wild-type suspensor cells. As most vertical division of BLGCs were observed from 3 DAP to 4 DAP, we checked the DR5:GFP activity in *S4$_{pro}$:SSP-YFP* mainly at that stages. Twin embryos were observed when auxin response was suppressed by expressing stabilized *bdl*[26,27]. Similarly, auxin response was presumably suppressed in the very early stage in our system. Before the initiation of second embryo, DR5 activity was very weak in the putative hypophysis of the top cell cluster, also reflecting a retarded auxin response (Figure 5). Strikingly, we observed strong DR5 activity in BLGCs before and right after their vertical division (Figure 5). This observation is consistent with other researches [13,39], strongly suggesting the role of auxin response in BLGC-embryo transition.

It was proposed that the embryonic potential of suspensor cells might be suppressed by the normal embryo proper[14]. As the embryonic identity of the apical cluster was acquired quite late, its inhibition on BLGCs, if exists, might not occur in the early stage so that BLGC-embryo transition can happen. This might be related to auxin contribution. In our system when the above embryo proper is abnormal, auxin transported from the bottom and/or embryo proper may converged in some BLGCs, resulting in a strong auxin response to diminish YDA signaling inhibition. Similar regulation could happen in wild

type as YDA signaling regulates suspensor development and vertical division was also observed in *yda* basal cells[5].

Materials and Methods

Plant Materials and Growth Conditions

The *yda-11* (SALKseq_078777) and *ssp-2* (SALK_051462) mutants were described previously[8] (See Methods in Chapter II). Seeds used in this study were sterilized with 70% ethanol, and then transferred to half-strength Murashige and Skoog (½ MS) medium containing 1% (w/v) sucrose and 1% (w/v) agar (pH = 5.7)[40] at 4°C in dark for 2 days. Then seeds and seedlings were geminated and grown under long-day conditions as described previously[41].

Plasmid constructions and transgenic lines

To make *$S4_{pro}$:SSP-YFP*, 1750 bp sequence upstream of *At3g10100* start codon was fused with *SSP* genomic sequence in pBay-bar vector. A Citrine YFP was introduced in between SSP kinase domain and SSP TPR domain as described before[8]. To make *UAS_{pro}:MPK6-CA*, 3 times UAS sequence was fused in pGreenII vector with MPK6 CDS containing a Y144>C mutation. In *$ARF13_{pro}$:nls-Tom*, 2 kb sequence upstream of *ARF13* start codon was fused with *Tomato RFP* and a nuclear localization sequence in pGreenII vector. *gWOX8Δ-NLS-3xVenus* was kindly provided by Thomas Laux[11]. Transgenic lines were generated by floral dip using *Agrobacterium tumefaciens* GV3101[42]. *$S4_{pro}$:SSP-YFP* was transformed into ssp-2. *UAS_{pro}:MPK6-CA* and *$ARF13_{pro}$:nls-Tom* were transformed into Col-0. Transgenic seeds were screen on ½ MS containing 50 mg/L phosphinothricin. *$S4_{pro}$:SSP-YFP #14* was crossed with *yda11/+* to get *$S4_{pro}$:SSP-YFP yda/+*. *$RPS5A_{pro}$:GAL4-VP16* [43], *DR5:GFP:ER* [43], *MP_{pro}:MP-GFP* and *$S4_{pro}$:nls-GFP* [35] were described before.

DIC microscopy

For differential interference contrast (DIC) imaging, ovules were hand-pollinated and incubated in Hoyer´s solution[5] at room temperature (incubating 1 day for ovules collected 1 DAP (day after pollination), 2 days for ovules collected 2 DAP, and so on). For embryos of *$RPS5A_{pro}$>>MPK6-CA*, *UAS_{pro}:MPK6-CA* was crosses with *$RPS5A_{pro}$:GAL4-VP16* and F1 embryos were collected. To count the ratio of horizontal division of the apical daughter cell, 1.5-DAP ovules were collected for imaging. DIC images were taken with a Zeiss Axio Imager and AxioVision 4 software.

Confocal microscopy

A Zeiss LSM 780 NLO microscope with ZEN 2.0 software and a Leica TCS SP8 microscope with LAS X software were used for confocal microscopy. Embryos were dissected from ovules using a Zeiss Stemi

2000 binocular and shape needles. For cell wall imaging, dissected embryos were incubated in SCRI Renaissance 2200 (SR2200) staining solution for 5-10 min, and confocal images were obtained with 405 nm excitation wavelength and 415-475 nm detection wavelength[44]. GFP images were obtained with 488 nm excitation wavelength and 496-533 nm detection wavelength. For YFP imaging, 514 nm laser wavelength was used for excitation, wavelength between 526 nm and 553 nm was recorded. For RFP imaging, 561 nm excitation wavelength and 580-633 nm detection wavelength were used.

Seedling phenotyping

Seedlings were grown on ½ MS medium for 7 days before counting different phenotypes. Seedling images were taken with a Zeiss Stemi 2000 binocular equipped with a camera.

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