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A molecular network mediating adventitious root initiation in *Arabidopsis thaliana*

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Abstract

To adapt to the ever-changing rhizosphere conditions, land plants evolved a sophisticated root system. The genetic determinants of the root system establishment have been the targets of natural selection, resulting in a very complex but robust molecular networks and circuits. These networks provide the plant with precise cell-fate and developmental decisions. The plant root system consists of primary root, lateral roots and often adventitious roots (ARs). ARs derive from the aboveground organs in response to either intrinsic developmental cues or in response to the environmental ones. AR formation is a pre-requisite step for vegetative propagation, which is widely used to multiply elite genotypes in forestry and agriculture. The main focus of this study is to unravel the molecular networks controlling AR initiation (ARI) using the intact-etiolated Arabidopsis hypocotyl as a model system. Previous data from our laboratory showed that ARI in Arabidopsis is controlled by a crosstalk between the positive regulator auxin (IAA) and the negative regulator jasmonate (JA). First, combining genetic, biochemical and hormonomics approaches, we identified the auxin coreceptor complexes involved in ARI. We found that IAA is perceived by two F-box proteins (TRANSPORT INHIBITOR1/AUXIN-SIGNALLING F-BOX (TIR1) and its closest homolog AFB2 as well as three Auxin/Inodole-3-acetic acid (Aux/IAA) repressors (IAA6, IAA9 and IAA17). These coreceptor proteins possibly act in combinatorial manner to fine-tune the auxin signaling machinery during ARI. In addition, in a genetic screen, we also revealed that the COP9 SIGNALOSOME SUBUNIT 4 (CSN4) protein plays a central role in ARI by modulating the function of the auxin perception machinery. Next, *in silico* search for genes acting downstream of JA involved in ARI, we retrieved the recently characterized *DIOXYGENASE FOR AUXIN OXIDATION* (*DAO1*) and *DAO2* genes. The *DAOs* encode for enzymes that catalyze the conversion of free IAA into 2-oxindole-3-acetic acid (oxIAA), a rate-limiting step in auxin degradation. We found that the *DAO1* gene mediates a molecular circuit to stabilize the interaction between IAA and JA. Combining genetics, genome-wide transcriptome profiling, hormononics and cell biological approaches, we found that MYC2-mediated JA signaling controls the expression of the *ETHYLENE RESPONSE FACTOR 115 (ERF115)* gene, which is a repressor of ARI. Our genetic data revealed that *ERF115*-mediated ARI inhibition requires cytokinins (CKs). CKs have long been established as inhibitors of ARI. Altogether, ARI seems to be controlled by a complex molecular network guided by three hormonal pathways (IAA, JA and CK), in which JA-induced *ERF115* plays a role of “molecular switch”.

Sammanfattning

För att anpassa sig till de ständigt föränderliga förhållandena i rhizosfären så har landlevande växter utvecklat ett sofistikerat rotsystem. Genetiska determinanter för etablerandet av rotsystemet har varit särskilt utsatta för evolutionär selektion, vilket har resulterat i mycket komplexa men robusta molekylära nätverk och kretsar. Dessa nätverk förser växten med precisa cellöden och utvecklingsbeslut. Växters rotsystem består av en primärrot, laterala rötter och luftrötter (adventitious roots, AR). AR härstammar från vävnader ovan mark som en respons antingen på interna utvecklingssignaler eller signaler från omgivande miljö. AR-formering är en förutsättning för vegetativ propagering, en metod som vida används för att föröka framgångsrika genotyper inom skogsnäring och lantbruk. Denna avhandling har fokuserat på att utröna de molekylära nätverk som kontrollerar AR-initiering (ARI), med hjälp av intakta etiolerade hypokotyler i Arabidopsis som modellsystem. Tidigare studier från vår forskargrupp har visat att ARI i Arabidopsis kontrolleras av ett samspel mellan den positiva regleraren auxin (IAA) och den negativa regleraren jasmonat (JA). Först, med en kombination av genetiska, biokemiska och hormonomiska metoder, identifierade vi auxin-koreceptorkomplexen inblandade i ARI. Vi fann att IAA känns av via två F-boxproteiner (TRANSPORT INHIBITOR1/AUXIN-SIGNALLING F-BOX (TIR1) och dess närmaste homolog AFB2), samt tre Auxin/Indole-3-acetic acid (Aux/IAA)-repressorer (IAA6, IAA9 och IAA17). Dessa koreceptorproteiner agerar potentiellt i kombination för att finjustera auxinsignalmaskineriet under ARI. Dessutom påvisade vi, genom en genetisk screen, att COP9 SIGNALOSOME SUBUNIT 4 (CSN4)-proteinet spelar en central roll under ARI genom att modulera auxinavkänningsmaskineriets funktion. När vi sen genomförde sökningar *in silico* för att hitta gener involverade i ARI nedströms om JA, fann vi de nyligen karaktäriserade generna *DIOXYGENASE FOR AUXIN OXIDATION* (*DAO1*) och *DAO2*. DAO-generna kodar för enzymer som katalyserar omvandlingen av fritt IAA till 2-oxindole-3-acetic acid (oxIAA), ett begränsande enzymatiskt steg under auxindegradering. Vi fann att DAO1-genen medierar en molekylär krets för att stabilisera interaktionen mellan IAA och JA. Via en kombination av genetisk, helgenomisk transkriptomprofilering och cellbiologiska angreppssätt fann vi att MYC2-medierad JA-signalering kontrollerar uttrycket av genen *ETHYLENE RESPONSE FACTOR 115 (ERF115*), vilken fungerar som en repressor av ARI. Våra genetiska data blottlade att ERF115-medierad ARI-inhibering kräver cytokininer (CKs) CKs är sedan länge erkända inhibitorer av ARI. Sammantaget, ARI verkar kontrolleras av ett komplext molekylärt nätverk guidat av tre hormonella banor (IAA, JA och CK), för vilken JA-inducerad *ERF115* agerar som en ”molekylär switch”.

Abbreviations

AFB: auxin signaling F-box

AR: adventitious root

ARF: auxin response factor

ARR: Arabidopsis response regulator

Aux/IAA: auxin indole-3-acetic acid

AuxRE: auxin responsive element

COI1: coronatine insensitive1

Col-0: Columbia-0

CKX: cytokinin oxidase/dehydrogenase

CSN: Cop9 signalosome

DAO: dioxygenase for auxin oxidation

ERF: ethylene response factor

GFP: green fluorescent protein

GH3: Gretchen Hagen3

GUS: beta-glucuronidase

IAA: indole-3-acetic acid

IPT: isopentenyltransferases

JA: jasmonic acid

JA-Ile: jasmonoyl-isoleucine

JAZ: jasmonate zim domain

MeJA: methyl-ester jasmonate

NAA: naphthalene acetic acid

NINJA: novel interactor of jaz

OPDA: (+)-12-oxo-phytodienoic acid

PCR: polymerase chain reaction

RAP2.6L: related to apetala2.6-like

RNA: Ribonucleic acid

SCF: skp-cullin-f box

T-DNA: transfer DNA

TIR1: transport inhibitor1/auxin-signalling F-box

TPL: TOPLESS

TPR: TOPLESS related protein

List of papers

Paper I Abdellah Lakehal#, Salma Chaabouni#, Emilie Cavel, Rozenn Le Hir, Alok Ranjan, Zahra Raneshan, Ondřej Novák, Daniel I. Păcurar, Irene Perrone, François Jobert, Laurent Gutierrez, Laszlo Bakò and Catherine Bellini. A Molecular framework for the control of adventitious rooting by the TIR1/AFB2-Aux/IAA-dependent auxin signaling in Arabidopsis. **Molecular Plant (**doi.org/10.1016/j.molp.2019.09.001**)**. # These authors contributed equally to this work.

Paper II Daniel Ioan Pacurar, Monica Lacramioara Pacurar, **Abdellah Lakehal**#, Andrea Mariana Pacurar#, Alok Ranjan and Catherine Bellini. The Arabidopsis Cop9 signalosome subunit 4 (CSN4) is involved in adventitious root formation. **Scientific reports. 2017,** 7 (628), 1-12. # These authors contributed equally to this work.

Paper III Abdellah Lakehal#, Asma Dob#, Ondřej Novák and Catherine Bellini. A DAO1-Mediated Circuit Controls Auxin and Jasmonate Crosstalk Robustness during Adventitious Root Initiation in *Arabidopsis.* **International Journal of Molecular Sciences**. **2019**, 20 (18), 4428. # These authors contributed equally to this work.

Paper IV Abdellah Lakehal, Asma Dob, Rahneshan Zahra, Ondrej Novak, Sacha Escamez, Miroslav Strnad, Hannele Tuominen, Catherine Bellini. A Jasmonate-mediated molecular network provides cell-reprogramming decisions for organogensis in Arabidopsis. **(manuscript in preparation)**.

Additional publications that are not included in the thesis

Lakehal A*, Ranjan A, Bellini C. 2019 Multiple roles of jasmonates in shaping rhizotaxis: emerging integrators. In Jasmonate in Plant Biology: Methods and Protocols, Second Edition. Eds Antony Champion and Laurent Laplaze (in press)
* Corresponding author

Rahnshan Z, Nasibi F, **Lakehal A**, Bellini C, Unravelling salt stress responses in two pistachio (*Pistacia vera* L.) genotypes. 2018. **Acta Physiologia plantarum**. *40:172* doi.org/10.1007/s11738-018-2745-1.

Lakehal A* and Bellini C, Control of adventitious root formation: Insights into synergistic and antagonistic hormonal interactions. 2019. **Physiologia plantarum** 165: 90-100.
* Corresponding author

Introduction

Overview To survive and adapt to the ever-changing rhizosphere conditions, plants evolved sophisticated root system, which can precisely perceive and rapidly respond to the environmental changes. The ability of plants to react to their surrounding rhizosphere is governed by an intrinsic (epi) genetic competences, which allow their root system to continuously regenerate, branch out and expand towards water and nutrients as well as oxygen (reviewed in Rogers and Benfey, 2015; Orosa-Puente et al., 2018). The root system can also selectively absorb minerals and avoid toxic compounds by reinforcing their cell walls and/or managing their movement accordingly (Barberon et al., 2016; Galvan-Ampudia et al., 2013). Being sessile in a highly hostile environment, the genetic determinants of the root system establishment and its plasticity have been a target of natural selection, resulting in very robust genetic networks and circuits (reviewed in Motte and Beeckman, 2019). The root system usually consists of primary root (PR), lateral roots (LR) and often adventitious roots (AR). PR is established during embryogenesis, and more precisely, at the early steps of zygote divisions, a single cell called hypophysis is recruited to form the PR founder cells. The hypophysis divides asymmetrically to establish PR meristematic cells, which includes the quiescent center (QC) and columella stem cells (CSCs). After germination, the stem cell niche located at the root tip gives rise to all the cells that will form the PR through distinct and successive developmental phases (Reviewed in Petricka et al., 2012; Lakehal et al. 2019). LRs develop post embryonically along the PR, and their development begin with priming of the pericycle cells at the root basal meristem. Only specific subset of pericycle cells neighboring to the protoxylem tissues receives a discrete signal, which makes them primed to form LR founder cells. Some LR founder cells can then undergo successive formative cell divisions to form LR primordia (reviewed in Van Norman et al., 2013; Ötvös and Benková, 2017; Lakehal et al. 2019). ARs develop also post embryonically but unlike LRs they derive from the aboveground organs (such as leaves, hypocotyls, stems) either as part of the intrinsic developmental programs or as an adaptive strategy in response to environmental cues (Figure 1) (reviewed in Steffens and Rasmussen, 2016). Several plant species develop ARs as an output of a pre-determined intrinsic genetic information. For example, monocots such as rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum aestivum* L.) develop AR (also called crown roots) as part of their normal development (Figure 1B).

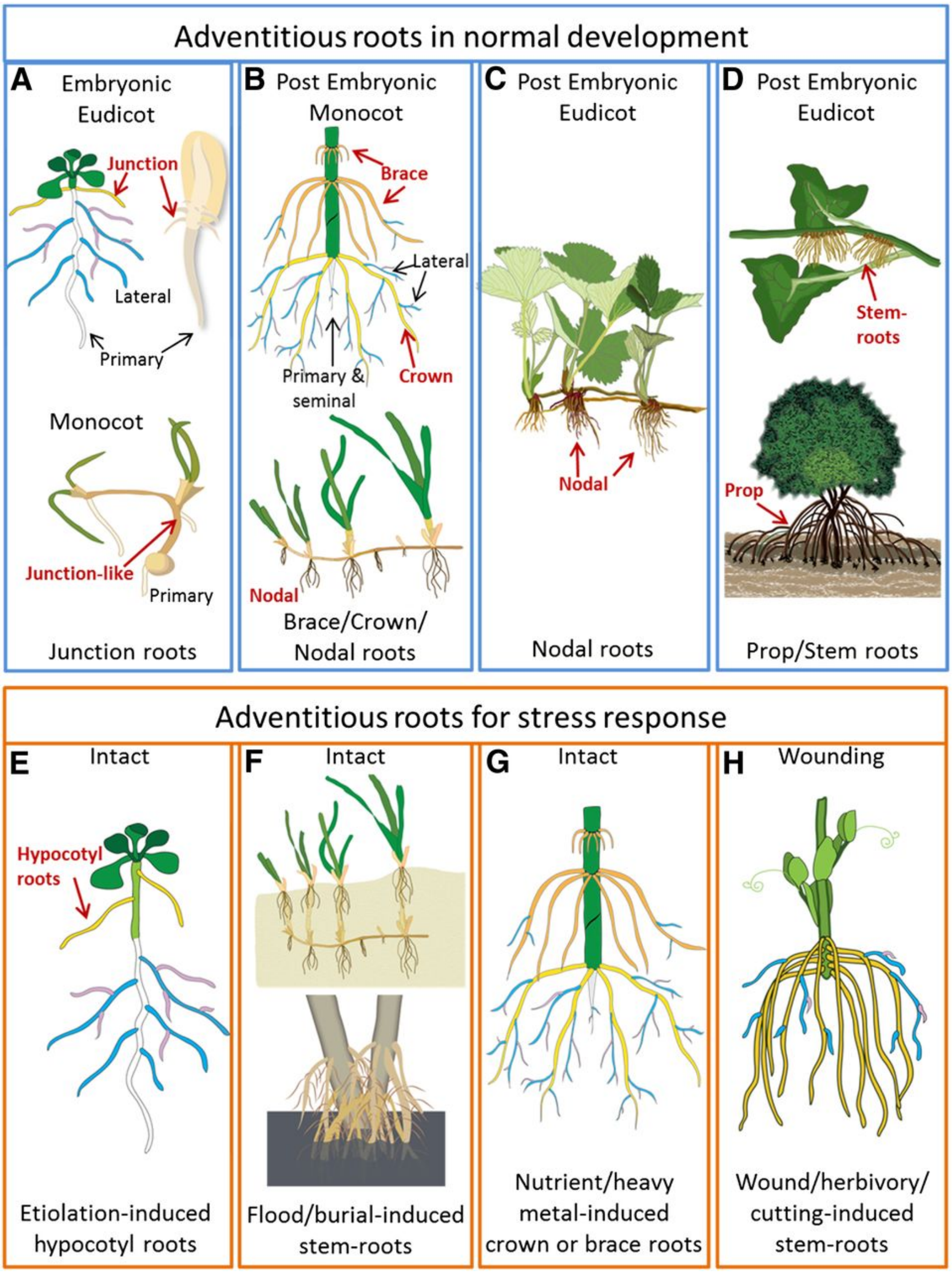

Figure 1: Developmental aspect of adventitious rooting. A-D: AR may develop in response endogenous cue as part of intrinsic developmental program. A. Although there is no consensus in the community, the roots forming from the hypocotyl-root junction are considered as AR. B. Crown roots and brace roots are types of AR that develop in monocots. C. Some dicots species such as strawberry or mangrove trees also develop AR throughout their normal development. E-H: AR may develop in response environmental cues as an adaptive strategy. E. intact etiolated hypocotyl develop AR. F. both monocots and dicots may develop AR in response to flooding to absorb oxygen. G. some species can develop AR from their stem to search for nutrient. H. several species develop AR from the excision site in response to wounding (Steffens and Rasmussen, 2016)

Plants can also develop AR in response to environmental cues such as etiolation, wounding, nutrient availability and flooding (Figure 1E-H). Several dicot species, for instance strawberry (*Fragaria × ananassa*) (Figure 1C) or blackberries (*Rubus spp*) also can develop AR during their lifespan to ensure their vegetative propagation (reviewed in Giess et al., 2009; Bellini et al., 2014). Notably, some species such as willow (*Salix faraglis)* or poplar (*Populus spp*) can develop preformed but dormant AR primordia in their stem, which can be activated in response to cutting or other inductive cues.

Adventitious rooting is an essential step for natural and artificial vegetative propagation, which is widely used to multiply long seed-to-seed cycle plants or elite genotype generated during breeding programs. The limiting step of vegetative propagation is that some species don't develop AR unless provided with phytohormones. This limitation causes enormous ecological and economic losses. For example, some genera of *Eucalyptus* or *Pinus* are difficult to root or completely recalcitrant to adventitious rooting and consequently fail to multiply (Rasmussen et al., 2009). The physiological and molecular bases encoding for recalcitrance are still elusive.

It is evident that phytohormones, such as auxin, are crucial signaling molecules involved in coordinating and establishing a proper root system by integrating several signaling pathways. This integration contributes in defining precise cell-fate decisions for different root system formation (Petricka et al., 2012; Lavenus et al., 2013; Pacurar et al., 2014b; Lakehal and Bellini, 2018).

Adventitious root initiation

Depending on the species and/or the inducible cue, AR development involves three consecutives but overlapping steps. First, cell fate specification and identity transition, in which a subset of cells perceive inductive cues (endogenous and/or exogenous) that activate unique downstream events to reprogram their (epi) genetic architecture. This reprogramming phase allows the targeted cells to acquire new cell-fate programs leading to the specification of AR founder cells. Second, initiation, in which the specified AR founder cells undergo successive formative cell divisions leading to the formation of dome-shaped primordia. Third, emergence and outgrowth, in which the formed primordia penetrate between the walls of the neighboring cell files and tissues to be in contact with the environment (reviewed in Bellini et al., 2014; Lakehal and Bellini, 2018). Very little is known about the mechanisms guiding and coordinating these steps.

Depending on both the species and organs, AR may initiate from various cell types and tissues with distinct anatomical and molecular identities. For example, in *Arabidopsis thaliana* (Arabidopsis), ARs initiate from xylem-pole pericycle cells in both intact and de-rooted hypocotyls (Sorin et al., 2005; Sukumar et al., 2013). Alternatively, they may initiate from procambium, and probably from the adjacent parenchyma cells in Arabidopsis leaf explants kept in on hormone-free B5 medium (Liu et al., 2014). Moreover, ARI may flow completely different mechanisms when the petiole is kept along with the leaf explant in Arabidopsis. In this system, AR initiation follows a two-step mechanism as in hormone-induced organogenesis systems (Bustillo-Avendaño et al., 2017). First, xylem and pericycle-like cells of the petiole undergo massive cell division forming a micro-callus, which subsequently undergoes a second step of cell specification, ensuring the transition from a callus identity to AR founder cell identity (Bustillo-Avendaño et al., 2017). These three examples reflect the developmental complexity of AR formation and shows that even in the same species AR formation can follow different pathways. Histological studies of stem cuttings from *Petunia hybrida* (petunia) (Ahkami et al., 2009), *Populus* (Rigal et al., 2012), or *Dianthus caryophyllus* L. (Agulló-Antón et al., 2014) revealed that the first cell division leading to primordia formation occurs in cambial tissues. It seems that AR form from the vasculature-associated cells possibly because of their competence to perceive the inductive cues as compared with other cell files. Nevertheless, in absence of distinctive markers that can clearly specify the exact cell files in these species, these data remain descriptive and require further follow up to understand the exact origin of ARI in the different species.

Role of auxin in AR formation

The word auxin includes the most abundant natural “auxin” indole-3-acetic acid (IAA) and other (natural or synthetic) auxinic compounds. Functional studies showed that IAA control most, if not all, growth and developmental processes including AR formation. IAA guides every step of AR formation starting from the early steps from cell reprogramming/identity transition until the formation of primordia, their emergence and elongation (Pacurar et al., 2014b; Lakehal and Bellini, 2018). These multifunctionalities are ensured by a dynamic control of the IAA spatiotemporal availability, allowing the generation of gradients (maxima or minima). The IAA gradients are generated through multiple mechanisms: *de novo* biosynthesis, transport and metabolism (conjugation and/or degradation) (reviewed in Ljung, 2013).

Role of IAA biosynthesis and homeostasis in AR formation

The role of IAA biosynthesis and homeostasis in adventitious rooting came primarily from mutant analysis. For example, the auxin overproducers *superoot1* (*sur1*) or *superoot 2* (*sur2*) spontaneously produce AR in their hypocotyls, and produce many more AR compared with the wild type (Boerjan, 1995; Delarue et al., 1998). Genetic screen for suppressors of the *sur2-1* mutant AR phenotype revealed that *ASA1/WEI2 (ANTHRANILATE SYNTHASE ALPHA 1/WEAK ETHYLENE INSENSITIVE2),* and *ASB1/WEI7 (ANTHRANILATE SYNTHASE β 1/WEAK ETHYLENE INSENSITIVE 7)* significantly contribute to AR formation as the loss-of-function mutations in these genes reduced the AR number in the *sur2-1* mutant (Pacurar et al., 2014a). *ASA1* and *ASB1* encode for the subunits α and β of the enzymes that catalyze the rate-limiting step in anthranilate biosynthesis. Therefore, the reduction of AR number in the suppressor mutants is due to the repression of the anthranilate-dependent IAA biosynthesis pathway. Similarly, the mature detached leaves of the Arabidopsis activation-tagged *yuc1-D* mutant which also overproduces auxin, developed more AR compared with those of the wild type (Liu et al., 2013). The *YUCCA1* gene encodes for the enzyme catalyzing the rate-limiting step in the tryptophan-dependent IAA biosynthesis (Zhao et al., 2001). Conversely, the leaf explants of the quadruple mutant *yuc1yuc2yuc4yuc6* produced very few AR compared with the wild type, and this defect was restored by adding 0.1 µM IAA confirming that the phenotype was due to a depletion in auxin (Chen et al., 2016). Interestingly, *YUC1* and *YUC4* genes were expressed rapidly in the mesophyll of the detached leaves suggesting that YUC1 and YUC4-dependent IAA biosynthesis plays a major role in cell fate-transition during leaf explant-derived AR (Chen et al., 2016). Similarly, inhibiting chemically the activity of the YUC enzymes with yucasin completely inhibited the AR formation, confirming the importance of the *de novo* IAA biosynthesis in ARI (Chen et al., 2016).

Role of IAA transport in AR formation

Directional, efflux-mediated, auxin transport plays an important role in establishing the IAA gradients during nearly all organogenesis programs (Benkova et al. 2003), including AR formation. Besides local IAA biosynthesis, IAA transport also mediates the accumulation of IAA at the wounding sites, this accumulation precedes any obvious anatomical changes leading to AR formation, indicating a causality correlation between IAA accumulation and AR formation (Ahkami et al., 2013; Sukumar et al., 2013; Zhang et al., 2019). Treating petunia-leafy cutting with naphthylphthalamic acid (NPA), which blocks the activity of the IAA efflux carrier PIN-FORMEDs (PINs), not only abolished the IAA accumulation but also severely inhibited AR formation (Ahkami et al., 2013). Unlike other developmental aspect, the role of IAA transport in AR formation is not well studied, except few reports that highlighted its importance during excision-derived AR. The *ATP-BINDING CASSETTE B19* (*ABCB19*) seems to be the main IAA transporter mediating IAA accumulation at the excision site in de-rooted etiolated Arabidopsis hypocotyls (Sukumar et al., 2013). Notably, *ABCB19* gene was rapidly upregulated after excision, especially in the epidermis and vascular tissues. In contrast to the overexpressing line (*35S:ABCB19*), the loss-of-function mutant *abcb19-1* produced fewer ARs compared with the wild-type (Sukumar et al., 2013). How wounding controls the expression of *ABCB19* gene awaits further investigation. Moreover, the PIN6 efflux carrier was also shown to be involved in AR formation (Simon et al., 2016). The role of PIN6 was revealed by mutant analysis, and in contrast to 35S:*PIN6* overexpressing line, the loss-of-function mutant *pin6* produced more ARs compared with the wild type (Simon et al., 2016).

Role of IAA signaling in AR formation

The IAA gradient generated in specific-cell types is perceived by co-receptor complexes consisting of TRANSPORT INHIBITOR1/AUXIN-SIGNALLING F-BOX (TIR1/AFB) receptors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005) and AUXIN/INDOLE-3- ACETIC ACID (Aux/IAA) repressors. Arabidopsis genome contains 6 TIR1/AFB paralogs (TIR1 and AFB1 to 5) (Parry et al., 2009) and 29 Aux/IAA paralogs (Overvoorde et al., 2005). Depending on the IAA amount in the nucleus (or even the type of auxinic compound), different combinations of co-receptors are formed to mediate specific signaling outputs, allowing IAA to provide distinct and specific information depending on the developmental context (Calderón Villalobos et al., 2012). The currently established model for auxin signaling suggests a simple pathway. In the resting state, which is marked by a low level of IAA in the cell, the Aux/IAA repressors physically bind to AUXIN RESPONSE FACTOR (ARF) family members and thereby inhibit their transcriptional activity. In the activation state, which is marked by an increased amount of IAA in the cell, the Aux/IAA repressors make a complex with one of the TIR1/AFB receptors. This interaction is mediated by IAA, which plays a role of "molecular glue" between the two proteins. Once a specific co-receptor is formed, the Aux/IAA are ubiquitylated and sent to the 26S proteasome for degradation (Gray et al., 2001) releasing the ARF transcription factors to activate or repress their downstream targets. The Arabidopsis genome contains 23 ARF paralogs (Okushima et al., 2005). The ARF proteins bind to the promoter of genes containing the auxin-response elements (AuxRE) TGTCTC to either activate or repress their transcription (reviewed in Guilfoyle and Hagen, 2007). High-resolution of crystal structures of ARF5 and ARF1 revealed unexpected mode of action of these transcription factors. It has been shown that ARF DNA-binding domains make homodimer complex on the AuxRE elements to activate the downstream target genes (Boer et al., 2014). Mutating conserved amino acids in the DNA-binding interface of *ARF5* did not affect the flooding of the corresponding protein but failed to complement the defects of the loss-of-function mutant *mp5* confirming that ARF homodimerization and heterodimerization through their DNA binding domain is crucial for their activity and the folding *per se* (Boer et al., 2014). Interestingly, the DNA-binding interface residues are highly conserved among several ARFs raising the possibility that all ARFs homodimerize and/or heterodimerize for their optimal activity (Boer et al., 2014).

The role of ARF6/ARF8 signaling module in AR formation seems to be evolutionary conserved

In the recent years, several laboratories, including ours, significantly contributed to dissecting the auxin signaling pathway involved in AR formation. IAA acts through three *ARF* transcriptional regulators during intact etiolated hypocotyl-derived ARI. ARF6 and ARF8 proteins are positive regulators, whereas ARF17 is a negative regulator (Sorin et al., 2005; Gutierrez et al., 2009, 2012). This signaling module acts in a complex manner involving transcriptional and post-transcriptional regulations of the corresponding *ARF* transcripts (Figure 2). Indeed, functional studies revealed that *ARF6/ARF8* transcripts are targets of microRNA miR167, whereas *ARF17* transcript is a target of miR160 (Figure 2) (Gutierrez et al., 2009). This complexity of regulation may partly reflect the plasticity and variability of the AR developmental phenotype (Gutierrez et al., 2009). Apparently, this signaling module seems to be evolutionary

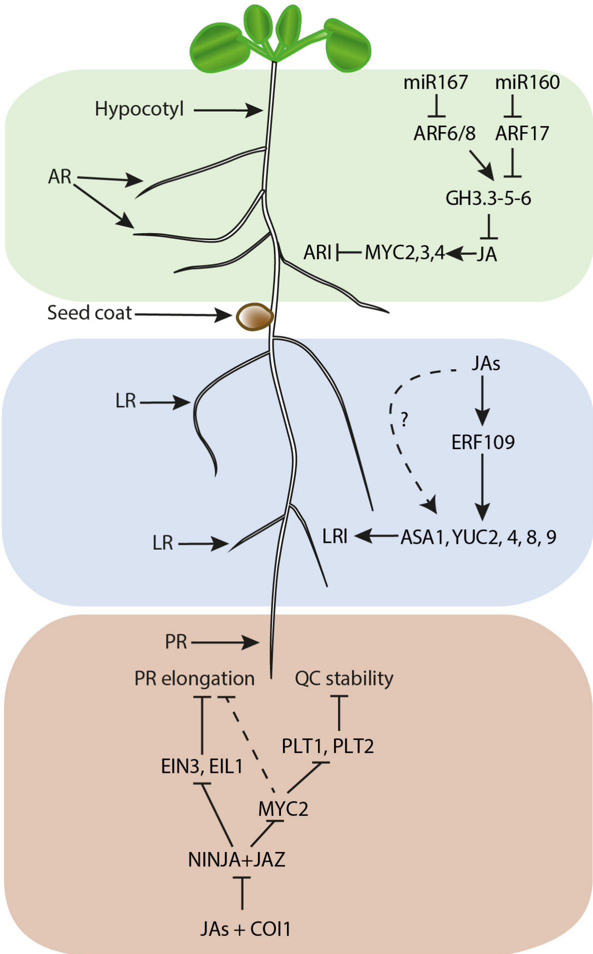

Figure 2 Integrative model for the role of jasmonates in root system establishment. In the context of ARI, ARF6/8 are positive regulator of ARI, whereas ARF17 is a negative regulator. ARF6/8 are targets of miR167, whereas ARF17 is a target of miR160. The three ARF control the expression of the auxin inducible GH3.3- 5-6 enzymes, which in turn conjugate JA with into an inactive form. *MYC2-3-4* -mediated JA signaling blocks ARI with a mechanism yet to be identified. In the context of LR formation, JA induce the expression of ERF109, which directly binds to the promoters of key genes in the L-Trp-derived IAA biosynthesis pathway, *ASA1* and *YUC2* and possibly other paralogs such as *YUC4*, *YUC8,* and *YUC9*. In the context of PR, JAs have a multifunctional role in inhibiting PR elongation. MYC2-mediated JA signaling destabilizes the stem cell niche and QC maintenance by directly repressing the expression of *PLT1* and *PLT2* genes. *PLT1* and *PTL2* are required for proper QC identity and stability. JAs also de-repress *EIN3* and *EIL1* by degrading their inhibitory JAZs proteins. *EIN3* and *EIL1* redundantly inhibit PR elongation. Lakehal et al., 2019 (in press)

conserved across taxa because the expression of *ARF6* and *ARF8* genes was induced in the difficult-to-root donor plants of *Eucalyptus globulus* when treated with far-red enriched light, and they were also induced at the cutting sites during vegetative propagation (Ruedell et al., 2015). Indeed, the far-red-enriched light promoted the AR formation in *Eucalyptus globulus* cuttings under these conditions (Ruedell et al., 2015). Moreover, *ARF6* and *ARF8* genes were specifically upregulated in the rooting-competent phloem parenchyma cells in black walnut cuttings (*Juglans nigra* L.), whereas the *ARF17* expression was downregulated at the same developmental stage (Stevens et al., 2018). Interestingly, this differential expression of the three *ARF* genes was observed before any anatomical changes leading to ARI, suggesting that these genes may control the ARI process (Stevens et al., 2018). Moreover, functional studies showed that *ARF6/ARF8*-mediated signaling module is possibly evolutionary-conserved among species because overexpressing miR167-resistant *ARF8* variant reportedly enhanced the adventitious rooting capacity in *Populus* (Cai et al., 2019). Unexpectedly, the miR160-resistant *ARF17* overexpressing lines reportedly produced more AR compared with the wild type, whereas the miR160a overexpressing lines produced fewer AR compared with the wild type (Cai et al., 2019). These data suggest that *ARF17* is rather a positive regulator of AR formation in *Populus*. We reason that in the absence of direct genetic evidence, which should be exclusively concluded by using either RNAi-mediated downregulation or CRISPR-Cas9 loss-of-function *arf17* mutants, these data remain preliminary and require further investigation. It would also be interesting to check whether ARF6/ARF8 act through the already identified pathway, in which JA pools are depleted through GH3.3 GH3.5 and GH3.6-mediated conjugation (Gutierrez et al., 2012).

LR and AR formation share some IAA-mediated signaling components

The AR and LR formation share several components from the auxin signaling machinery (Bellini et al., 2014). For example, the *ARF7* and *ARF19* genes, which are the main players in auxin mediated LR formation (Okushima et al., 2005; Wilmoth et al., 2005), also control AR formation. Indeed, the double loss-of-function mutant *arf7arf19* exhibited a reduced AR number compared with the wild type (Wilmoth et al., 2005). ARF7 and ARF19 directly control the expression of some of the *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) genes including *LBD16* and *LBD18* during LRI. The loss-of-function *lbd16* and *lbd18* also exhibited a reduced number of AR compared with the wild type (Lee et al., 2019), confirming that ARF7/19-LBD16/19 is a pleiotropic signaling module controlling both LRI and ARI, and possibly other *de novo* organogenesis or regeneration processes. These data indicate that the knowledge acquired in LR development or in other *de novo* developmental programs could be useful in dissecting the networks controlling ARI.

Role of IAA sensing in AR formation

Despite its importance in guiding and providing the exact molecular input during different developmental aspects, a detailed characterization of auxin sensing machinery is still missing. How IAA is perceived and what are the co-receptor complexes mediating the auxin signaling to control AR formation is still not known. Surprisingly, unlike in LR formation process, only descriptive data are available in the literature describing the role of some of the *Aux/IAA* genes using the gain-of-function mutants. For example, Bustillo-Avendaño et al., (2017) found that the gain-of-function *crane-2* or *solitary root-1* (*slr-1*) mutants were affected in vascular tissue regeneration and AR formation from leaf explants. The *crane-2* (Uehara et al., 2008) and *slr-1* (Fukaki et al., 2002) alleles harbor a mutation in the domain II of IAA18 and IAA14, respectively, making them resistant to IAA-mediated proteasome-dependent degradation. This type of mutations severely interferes with IAA signaling and subsequently lead to pleiotropic phenotypes. Therefore, we reason that they are not the optimum genetic tools to use because of their limitation in uncoupling the direct from indirect effects of the corresponding stabilized protein. In contrast, using auxin-sensitive loss-of-function alleles would be an optimum tool to dissect the IAA perception mechanisms. It was assumed that using loss-of-function mutants in the *Aux/IAA* gene family would not be informative because of the high functional redundancy and compensation among the same members of the family, as revealed by their initial characterization for obvious developmental defects, which indeed did not lead to obvious developmental phenotypes (Overvoorde et al., 2005). However, a careful characterization for subtle phenotypes revealed the role of certain *Aux/IAA* genes using loss-of-function mutants. For example, the loss-of-function mutant *short hypocotyl 2-31 (shy2-31/iaa3)* has a longer primary root due to an increase in the meristem activity compared with its wild-type counterpart (Dello Ioio et al., 2008). The same allele also exhibited a LR hydro-patterning defects (Orosa-Puente et al., 2018). In addition, the loss-of-function *iaa5-1, iaa6-1* and *iaa19-1* exhibited significantly reduced survival rate compared with the wild type in response to desiccation (Shani et al., 2017), and more importantly a decreased drought tolerance compared with the wild type (Salehin et al., 2019) suggesting their functional role in auxin-mediated responses to abiotic constraints. All these examples indicate that a careful characterization of single and multiple *iaa* mutants is needed and, by doing so, this will open up new perspectives on the role of the TIR1/AFB-Aux/IAA-dependent IAA sensing machinery in plant development and response to the environment. The role of the canonical TIR1/AFB-Aux/IAA-dependent auxin sensing machinery in ARI, which players are involved, and what are the mechanistic basis guiding this machinery during this process will be addressed in the first chapter of this thesis.

Mode of action of IAA signaling during AR formation

Accumulating evidence indicate that during LRI, IAA signaling triggers profound but coordinated transcriptional changes. These changes include the activation of the cell reprogramming and identity transition regulators, cell cycle machinery, and cell wall modifiers. In addition, it activates or inhibits other hormonal pathways (reviewed in Lavenus et al., 2013). However, the exact role of IAA signaling and its downstream targets during ARI are just starting to be mechanistically highlighted. ARF6/ARF8-mediated IAA signaling has been shown to control the expression of the auxin-inducible *GH3* genes (Figure 2) (Gutierrez et al., 2009, 2012), and this induction is likely direct as shown by chromatin immunoprecipitation (ChiP) experiments (Oh et al., 2014). The three GH3 enzymes have been shown to conjugate free JA with amino acids (Gutierrez et al., 2012). Indeed, the triple loss-of-function mutant *gh3.3gh3.5gh3.6*, which accumulated less JA and JA-Ile, produced very few ARs compared with the wild type (Figure 2) (Gutierrez et al., 2012). Another elegant work provided mechanistic insights on how IAA activates the cell fate transition regulator *WUSCHEL-RELATED HOMEOBOX11 (WOX11)* and its closely related paralog *WOX12* (Liu et al., 2014). The same authors observed that two days after cutting, the *WOX11* promoter activity increased at the wounding site of the leaf explants, which coincided with the IAA accumulation, as marked by the increased activity of the auxin responsive *DR5* promoter. These data suggest that *WOX11* is possibly transcriptionally regulated by wounding-induced IAA at the wounding site. Similarly, mutating an auxin-responsive-element (AuxRE) in the *WOX11* promoter abolished this induction (Liu et al., 2014). How IAA activates *WOX11* and which players from the IAA signaling machinery are involved in this activation is still not known yet. In contrast to the *35S:WOX11* or *35S:WOX12* overexpressing lines, the double mutant *wox11wox12* or the transgenic lines harboring the chimeric negative-dominant version of *WOX11* (*35S:WOX11:SRDX*) produced very few AR compared with the wild type. These data indicate that these genes control the leaf explant-derived ARI (Liu et al., 2014). WOX11, redundantly with WOX12, activates the expression of

LBD16 and *LBD29* genes (Liu et al., 2014). Indeed, overexpressing *35S:LBD29* could restore the AR defect of *35S:WOX11:SRDX* lines. These data indicate that *LBD29* acts downstream of *WOX11* during ARI (Liu et al., 2014).

A more recent study by Bustillo-Avendaño et al., (2017) used leaf explants including the petiole, as a model system and revealed that the expression of *WOX11* was not always associated with ARI sites but mainly with proliferating vascular tissues. This observation prompted the authors to suggest that *WOX11* is rather involved in callus formation (Bustillo-Avendaño et al., 2017). These discrepancies between the two studies are possibly due to the different experimental conditions.

WOX11 is an evolutionary conserved gene and its function in adventitious rooting seems to be conserved among taxa. The rice genome contains at least 11 *WOX* genes (Zhao et al., 2009). Combined mRNA, in situ hybridization and *OsWOX11* promoter activity in serial cross sections revealed that *OsWOX11* was mainly expressed in the emerging primordia of shoot-born crown roots but not at earlier stages, suggesting that *OsWOX11* is rather involved in the emergence of the primordia (Zhao et al., 2009). Indeed, the loss-of-function mutant *wox11* or RNAi-downregulated *WOX11* lines exhibited a significant delay in crown-roots emergence. In contrast, the overexpressing *pUBQ:OsWOX11* lines not only exhibited an enhanced crown roots growth rate but also resulted in ectopic emergence (Zhao et al., 2009). Interestingly, OsWOX11 protein interacts physically with the ETHYLENE RESPONSE FACTOR3 (OsERF3) to promote crown root emergence (Zhao et al., 2015). Both genes *WOX11* and *ERF3* are rapidly induced by different types of auxins (IAA, NAA and 2-4 D), and to a lesser extent by cytokinins (6-BA) suggesting that *OsWOX11* plays an important role in meditating the crosstalk between IAA, CK and possibly ethylene during crown root formation (Zhao et al., 2015). Indeed, it has been shown that WOX11 directly binds to the promoter of the *RESPONSE REGULATOR2* type-A (*OsRR2),* and thereby represses its expression. In addition, transcriptome analysis of *wox11* mutant and wild-type root types revealed several differentially expressed genes from the auxin and cytokinin signaling pathways (Jiang et al., 2017). The *Populus* genome contains two *WOX11* paralogs: *PeWOX11a* and *PeWOX11b*, and overexpressing the corresponding coding sequence (*35S:PeWOX11)* not only increased the rooting rate, but also enhanced the number AR per explant (Xu et al., 2015).

In conclusion, Although, its function seems to be species- and/or context-dependent, *WOX11* appears to be an evolutionary conserved gene, which is required for proper AR formation. It possibly converges the IAA, CK and ethylene signaling pathways to ensure a coherent developmental input for ARI.

Role of IBA in AR formation

Besides IAA, other types of auxinic compounds such as 2,4-dimethylphenylalanine (2,4-D), Indole-3-butryic acid (IBA), 1-Naphthaleneacetic acid (NAA) have long been used as promoting agents for adventitious rooting during vegetative propagation and hormone-induced *de novo* organogenesis practices. Notably, IBA is the most used auxin for adventitious rooting likely due to its effectiveness and stability upon light exposure. Although its presence as a natural auxin in Arabidopsis and other species is still debatable, several reports indicate that IBA is produced by the plants at very low level (reviewed in Frick and Strader, 2017). Genetic screens for mutants resistant to IBA but sensitive to IAA shed light on genes controlling the conversion of IBA to IAA. The conversion of IBA to IAA occurs in the peroxisome, and is mediated by several enzymes including the short-chain dehydrogenase/reductase indole-3-butyric acid response 1 (IBR1), the acyl-coA dehydrogenase/oxidase-like (IBR3) and the enoyl coA hydratase (IBR10) as well as enoyl-COA hydratase2 (ECH2) (reviewed in Frick and Strader, 2017. Indeed, the double mutant, *ech2 ibr10*, in which IBA to IAA conversion is impaired, exhibit very few LR (Strader et al., 2011) and AR compared with the wild type (Fattorini et al., 2017). The conversion of IBA to IAA seems to be genotype-dependent because different elm *(Ulmus americana*) cultivars differentially responded to IBA, and the adventitious rooting capacity was correlated with their ability to convert IBA to IAA (Kreiser et al., 2016). However, in the absence of functional studies, these data remain preliminary.

Overview about jasmonates (JA)

Jasmonic acid (JA) and its derivatives, collectively called jasmonates, are oxylipin-derived plant hormones. The pioneer studies in the JA field has been performed in 1899 by Hesse and Muller (1899). They extracted the first compound jasmone from jasmine flowers (*Jasminium grandiflorum)*. Later, Demole et al., (1962) extracted and characterized the structure of the first JA derivative, which was methyl jasmonate (MeJA). Since then, JA research attracted the attention of several laboratories, which contributed enormously in understanding the complex pathways of its biosynthesis, metabolism, transport, signaling, evolution and its biological role (reviewed in Wasternack and Hause, 2013).

JA biosynthesis and homeostasis

The first step of JA biosynthesis occurs in the chloroplast, a-linolenic acid (18:3) (a-LeA) is oxygenated by 13-LIPOXYGENASES (LOXs) enzymes to produce in 13(S)-hydroperoxy-octadecatrienoic acid (13-HODE), which is further catalyzed by ALLENE OXIDE SYNTHETASE (AOS) and ALLENE OXIDE CYCLASEs (AOCs) to produce (+)-12-oxo-phytodienoic acid (OPDA) (reviewed in Wasternack and Hause, 2013). Very recently, a proteomics study of the chloroplast outer- envelope identified a putative OPDA exporter, which was named JASSY (Guan et al., 2019). JASSY exports OPDA from the chloroplast to the cytosol for further conversion. The loss-of-function mutant *jassy* exhibited a number of JA-deficient phenotypes including increased susceptibility to the necrotrophic pathogen *Botrytis cinerea* and insensitivity to wounding-induced gene expression (Guan et al., 2019). Indeed, *jassy* did not accumulate free JA upon mechanical wounding. OPDA is reduced by 12-OXOPHYTODIENOIC ACID REDUCTASE 3 (OPR3) in the peroxisome to form JA (Guan et al., 2019).

A new route for JA biosynthesis was also recently discovered. A careful characterization of the loss-of-function mutant *opr3-3*, which was still producing small amount of JA and jasmonoyl isoleucine (JA-Ile) upon mechanical wounding (Chini et al., 2018). These data suggested the presence of an OPR3-independent JA biosynthesis cascade. Analytical chemistry combined with physiological and genetic approaches revealed that the cytoplasmic OPR2 enzyme mediates a new branch of JA biosynthesis by reducing 4,5-didehydrojasmonate to synthesize JA (Chini et al., 2018). Although OPR2 seems to be the main catalyzer of 4,5-ddh-JA, *opr2* knockout mutant did not show any obvious JA deficient-like phenotypes (Chini et al., 2018), thus, further experiments are needed to unravel the biological relevance of OPR2-dependent JA biosynthesis pathway during plant growth, development and in response to the environment. This discovery also raised interesting questions about the evolution of the JA biosynthesis pathways because the genome of the ancestral land plants such as the bryophyte *Marchantia polymorpha*, which produces neither JA nor its bioactive form JA-Ile, contains OPR-like genes (Monte et al., 2018). The

Mp*ORP*-like genes are phylogenetically more similar to the Arabidopsis *OPR2* than *OPR3* (Monte et al., 2018). Thus, it would be interesting to functionally characterize these genes in *M. polymorpha* to get more insights about the significance and relevance of the *OPR2* dependent pathway. It may also give insights about the co-evolution of receptor-ligand partners of JA signaling, and may also give hints for the evolution of other hormonal sensing machineries. JA is conjugated into the bioactive form jasmonoyl-L-isoleucine (JA-Ile) by the cytoplasmic-localized JASMONATE RESISTANT1 (JAR1/GH3.11) enzyme, which belongs to group I of GRETCHEN HAGEN3 (GH3) protein family. Strikingly, other members of group II of the GH3 family, the auxin inducible GH3.3, GH3.5 and GH3.6, can also conjugate JA with amino acids but into inactive forms. The three enzymes catalyze the conjugation of free JA to JA-Trp, JA-Met and JA-Asp (Gutierrez et al., 2012).

JA translocation

JAs mediate systemic and long-distance transcriptional reprogramming and defense responses. A rapid signal can be transmitted from wounded to unwounded tissue to trigger the downstream events (Toyota et al., 2018). Several studies suggested that JA and JA-Ile are mobile molecules and can be actively transported. A simple grafting experiment confirmed this hypothesis and revealed that OPDA and its derivatives, but not JA-Ile, are translocated from wounded shoots to unwounded roots, and this movement has a strong physiological relevance (Gasperini et al., 2015; Schulze et al., 2019). For example, repetitive mechanical wounding in Arabidopsis cotyledons inhibited PR expansion in wild type whereas it did not have any significant effect in the loss-of-function mutant *aos* suggesting that JA produced upon mechanical wounding in the cotyledons moves rootward to inhibit PR expansion (Gasperini et al., 2015). Interestingly, the cytokinin perception mutant (*wooden leg*) or the transgenic line overexpressing the *CTYOKININ OXIDASE* 7 (*CKX7*) gene involved in CK degradation (*35S:CKX7* line), which are defective in phloem development in the root, exhibited a strong insensitivity to wounding-induced *JAZ10* expression in the root when they were wounded in the shoot (Schulze et al., 2019). Although a direct evidence is still missing, these data suggest that OPDA and its derivatives likely move rootward through a functional phloem tissue (Schulze et al., 2019). The first cellular JA transporter has recently been identified in a screen using the yeast cells (*Saccharomyces cerevisiae* strain YPH499) as heterologous system. Yeast cells expressing one of the putative transporters *JASMONATE TRANSPORTER1/ATP-BINDING CASSETTE G16 (JAT1/ABCG16)* were tolerant to exogenously applied JA in term of cell-growth inhibition (Li et al., 2017). It has been shown that the JA-inducible JAT1/ABCG16, which was localized both at the plasma membrane and at the nuclear envelope, mediated both JA efflux from the cytosol to the apoplast and JA-Ile influx into the nucleus. Ensuring a dual role, JAT1/ABCG16 seems to be a gate keeper-like and provides a rate-limiting step in controlling the amount of JA-Ile entering to the nucleus (Li et al., 2017). *JAT1/ABCG16* seems to modulate the JA signaling machinery in response to a rapid changing of JA pools. JA and JA-Ile pools are rapidly created and redistributed in response to environmental and endogenous cues. The JAT1/ABCG16 is a half-size transporter meaning that it should form a homodimer or a heterodimer to be functional (Ji et al., 2014). Therefore, finding the partner of these transporter and a careful biochemical and physiological characterization of the protein complexes are required to unravel its specificity and biological relevance of the opposite dual role.

JA signaling

Pharmacological, genetic and pull-down approaches confirmed that (+)-7-iso-JA-L-Ile (JA-Ile) is the most bioactive form of JAs compared with the other isomers (Fonseca et al., 2009). JA-Ile is a molecular glue (or ligand) that facilitates the formation of the coreceptor complexes between JASMONATE ZIM DOMAIN (JAZ) repressors and the Skp/Cullin/F-box CORONATINE INSENSITIVE 1 (SCF^{COI1}) receptor (Figure 3). JA-Ile mediates transcriptional regulation (activation or repression) *via* a very simple signaling pathway. At the active state marked by the presence of sufficient amount of JA-Ile in the cell, the JAZ repressors physically bind to the COI1 receptor to form the $SCF^{COI1\text{-}JAZ}$ complex. Once this complex is formed, the JAZ proteins are ubiquitylated and targeted to the 26S proteasome machinery for degradation (Figure 3). At the resting state, which is marked by the presence of low amount of JA-Ile, the JAZs interact physically with several transcription factors including the master regulator MYC2/JASMONATE INSENTIVE1 (MYC2/JIN1), and subsequently impair their transcriptional activity (Xie et al., 1998; Thines et al., 2007; Chini et al., 2007; Sheard et al., 2010). JAZ repressors can also recruit the co-repressor TOPLESS (TPL) or TPL-related proteins *via* the co-adaptor NOVEL INTERACTOR of JAZ (NINJA) to repress the activity of several TF through chromatin remodeling (Figure 3) (Pauwels et al., 2010). Although few reports suggested the presence of NINJA-dependent and NINJA independent JA signaling pathways, the mechanistic and genetic as well as spatiotemporal regulation of these signaling cascades is missing. Besides JA-Ile, its hydroxylated metabolite 12-hydroxy-JA-Ile (12OH-JA-Ile) has recently been proposed as an endogenous bioactive form of jasmonate. 12OH-JA-Ile triggers a range of JA associated transcriptional and physiological responses in a COI1-dependent manner (Poudel et al., 2019).

The MEDIATOR25 is an integrative hub for JA signaling

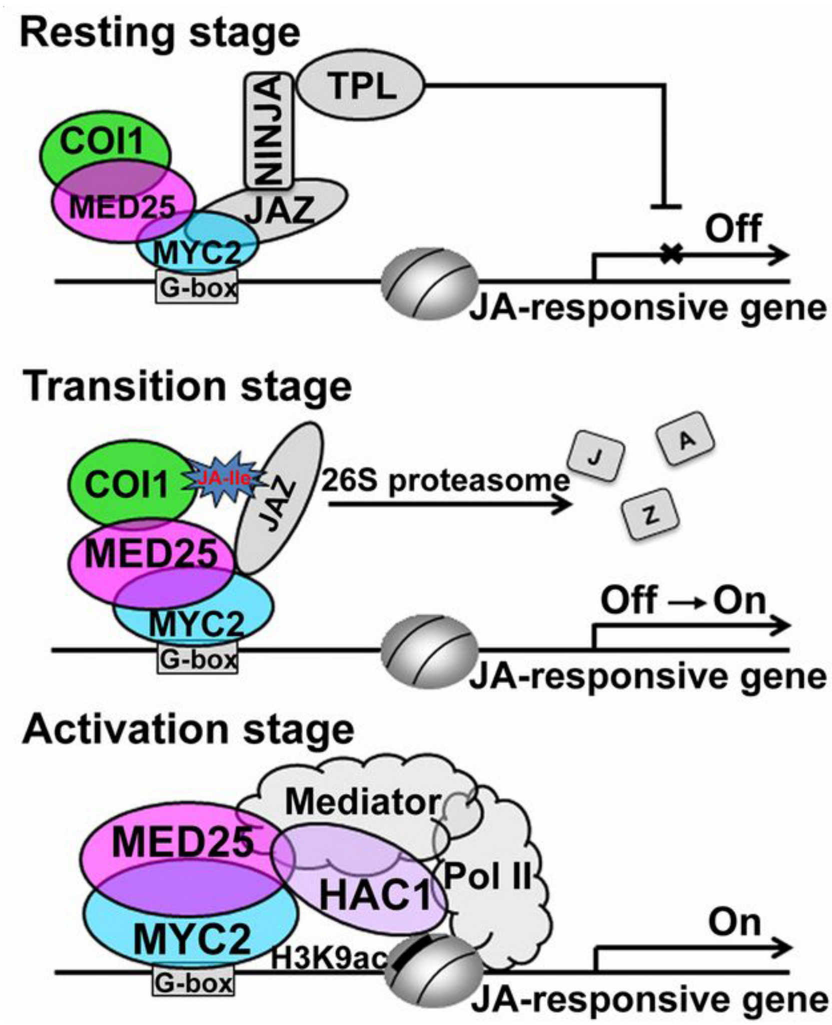

Figure 3: JA signaling pathway: At the resting stage, the repression machinery consisting of JAZ, NINJA and TPL block the transcriptional activity of MYCs by either physical interaction through JAZ or through TPL-mediated chromatin remolding. NINJA serves as co-adaptor for JAZ-TPL interaction. JAZs also compete with MED25 for MYC2 accessibility. The COI1 receptor binds to MED25 in the promoters of JA-responsive genes to facilitate their activation once the nucellus receive sufficient amount of JA-Ile. At the transition stage, marked by sufficient amount of JA-Ile in the nucleus, JA-Ile, as molecular glue, facilitates the interaction between COI1 and JAZ, and subsequently the JAZ are ubiquitylated and targeted to 26S proteasome for degradation. At the activation stage: MED25 binds into the de-repressed MYC2. Other components form the transcriptional machinery are recruited to trigger the transcription of the JA-responsive genes. (An et al., 2017)

Accumulating evidence revealed the importance of MEDIATOR25 (MED25) subunit in modulating JA signaling (Chen et al., 2012). The mediator complex is an evolutionary conserved multi-protein consisting of 29 subunits in Arabidopsis. Pulldown and gene expression assays suggest that MED25 plays multiple roles in JA signaling both during the resting and activation states. At the resting state, it physically brings COI1 to the promoter of MYC-target genes to facilitate the degradation of its competitors JAZs upon the presence of JA-Ile (An et al., 2017). JAZs were proposed to block MYC3 transcriptional activity by competing with MED25 (Zhang et al., 2015). At the activation state, once the JAZs are degraded, MED25 physically binds to MYC transcription factors to facilitate the formation of the transcriptional pre-initiation complex and the recruitment of the RNA polymerase II, which triggers the expression of JA-responsive genes (Figure 3) (An et al., 2017). In addition, extensive chromatin immuno-precipitation experiments showed that MYC2 and MED25 co-occupy several G-box-containing JA enhancers near MYC2 target genes (Wang et al., 2019). MED25 also connects the JA enhancers to the promoters of the MYC2 target genes through chromatin looping during JA-dependent transcriptional regulations (Wang et al., 2019). Targeted genomic deletions using CRISPR-Cas9 technology on the putative JA enhancer near *MYC2* gene resulted in *myc2 enhancer* (*me2*) allele mutants, which exhibited a significant reduction in the expression of *MYC2* gene, and consequently were affected in the expression levels of the downstream targets of MYC2 in response to MeJA, indicating the importance of these regions in JA signaling and response (Wang et al., 2019). Based on these data, one would expect that deleting these regions near MYC2 might interfere with MYC2-mediated JA responses. However, in contrast to the loss-of-function mutant *myc2*, the allele *me2* was completely sensitive (or even slightly hypersensitive) to MeJA-mediated root inhibition compared with the wild type (Wang et al., 2019). These data raise the question about the physiological and biological significance of these putative enhancers. Further research is needed to understand the role of JA enhancers during plant growth, development and in response to the environment.

Apparently, *MED25* functions as an integrative hub for *MYC* mediated JA signaling. Indeed, the loss-of-function mutant *med25* exhibits profound defects in JA-mediated responses including gene expression and pathogen defense, reminiscent to JA deficient mutants (Chen et al., 2012). Interestingly, the role of *MED25* in JA signaling seems to be conserved among other plant species (Liu et al., 2019). For example, pull down assays showed that *Solanum lycopersicum* (tomato) *Sl*MED25 physically binds to its partner *Sl*MYC2. Transgenic lines with reduced *SlMED25* expression (antisense) lines exhibited a downregulation of the wounding and JA-responsive genes (Liu et al., 2019).

It is worthy to mention that the exact physiological and biological relevance of MED25 is still poorly understood. How specific is the role of this subunit in JA signaling, and what are the contributions of other subunits from the MEDIATOR complex in regulating specifically JA signaling machinery but not a general transcriptional regulation, remains to be determined.

Role of JA in plant development

JA has always been associated with plant fertility, defense and immunity but recent reports demonstrated its importance in plant development. Treatments with exogenous JAs affect seeds germination, hypocotyl elongation, primary root expansion, lateral and adventitious rooting, and leaf growth and senescence (reviewed in Wasternack and Hause, 2013). Besides its important role in flower development, JA also controls flowering time. A knockout mutant *coi1-2* exhibited an early flowering phenotype compared with its wild-type counterparts. It was proposed that upon stress, COI1-dependent JA signaling repress *FLOWERING LOCUS T* gene expression, a key gene for flowering time, which lead to a delay in flowering (Zhai et al., 2015).

Accumulating evidences revealed that JA signaling integrates multiple hormonal pathways to control primary and lateral root development. For example, *MYC2* mediated JA signaling directly represses the expression of the auxin inducible *PLETHORA1* (*PLT1*) and *PLT2* genes to control the quiescent center (QC) stability in the PR (Figure 2) (Chen et al., 2011). JA also inhibits PR elongation by mediating the degradation of JAZ proteins, which physically interact with the ethylene-stabilized *ETHYLENE INSENSITIVE 3* (*EIN3*) and its closest paralog *EIN3-LIKE1* (*EIL1*) transcription factors (Figure 2) (Zhu et al., 2011). *EIN3* redundantly with *EIL3* negatively regulate PR elongation. Exogenously applied MeJA induces the expression of *ETHYLENE RESPONSE FACTOR1* (*ERF1)* in an *EIN3* and *COI1* dependent manner to control the elongation zone of the PR (Zhu et al., 2011; Mao et al., 2016).

It has long been established that JA promotes LR formation by inducing IAA biosynthesis. *MYC2* mediated JA signaling directly activates the expression of *ERF109* transcription factor, which in turn binds to the promoter sequence of *ASA1* and *YUC2* to activate L-Tryptophan-dependent IAA biosynthesis pathway (Figure 2) (Cai et al., 2014). Indeed, in contrast to the loss-of-function mutant *erf109,* transgenic lines overexpressing *ERF109 (35S:ERF109)* exhibited IAA reminiscent phenotypes including higher LR density compared with the wild type (Figure 2) (Cai et al., 2014). Together, these examples indicate that JA is a potential integrator that coordinates different hormonal signaling networks to provide a coherent developmental input for root system architecture.

Role of JA in AR formation

A continuous application of physiological concentrations of 12-oxo-phytodienoic acid (OPDA), JA or methyl-Jasmonate (MeJA) inhibited AR formation in *Bupleurum kaoi* (Chen et al., 2007)*, Petunia hybrida* (Petunia) leafy cuttings (Lischweski et al., 2015) and Arabidopsis leaf explant (Zhang et al., 2019) as well as etiolated hypocotyl (Gutierrez et al., 2012) indicating a negative role of JAs in ARI. In line with these observations, genetic approaches using Arabidopsis etiolated hypocotyl as a model system strongly supported the inhibitory role of JAs (Gutierrez et al., 2012). For example, the weak allele *coi1-16,* which harbors a point mutation substituting a phenylalanine 245 by a leucine in the coding sequence of the *COI1* gene (Ellis and Turner, 2002), produced significantly more AR compared with the wild type (Gutierrez et al., 2012). In contrast to the loss of function *myc2*, the overexpressing line *35S:MYC2* produced significantly fewer AR compared with the wild type (Gutierrez et al., 2012). These data clearly indicate that *COI1* dependent *MYC2* mediated JA signaling pathway inhibits ARI in Arabidopsis hypocotyl. Despite the clear physiological and genetic evidence supporting the negative role of JA in ARI in Arabidopsis etiolated hypocotyl, its role in other model systems has always been debatable. Fattorini et al., (2009) used hormone-induced organogenesis system to investigate the role of MeJA in AR formation. These authors reported that MeJA in combination with high dose of the natural auxin, Indole-3-butyric acid (IBA, 10 μM) and cytokinin, Kinetin (Kin, 0,1 μM) promoted AR formation in Arabidopsis seedlings and stem thin cell layers when grown in a continuous darkness (Fattorini et al., 2009; Fattorini et al., 2018), but the genetic and mechanistic bases of these observations are still inexistent. These data raise interesting questions and hypothesis about the role of hormonal interaction in controlling AR formation when combined all together. However, we reason that using continuous and arbitrary hormonal concentrations is not an optimum approach to investigate the role of JAs because AR development consists of several and dynamic steps, and each step may require a precise molecular signature. JA response is regulated by precise physiological concentrations of the bioactive form JA-Ile. These physiological concentrations are generated and fine-tuned by multiple levels of regulations including biosynthesis, metabolism and transport. Therefore, using continuous and arbitrary concentrations may trigger unexpected and pleiotropic responses, which might not reflect the physiological and natural conditions. In natural conditions, plants reprogram their cells using very sophisticated and robust intrinsic signaling networks to regenerate and form new roots. In fact, one should consider all these facts while interpreting hormone-induced organogenesis data (reviewed in Lakehal and Bellini, 2018).

The rapid and transient wounding-induced JA accumulation at the base of petunia leafy cuttings and Arabidopsis leaf explants was correlated with a positive role of JA in promoting ARI. It has been proposed that JA accumulation induces a profound transcriptional and metabolomic reprogramming leading to AR formation at wounding site (Ahkami et al., 2009). Interestingly, RNAi silencing of the single copy gene *PhAOC* reportedly reduced the number of AR in petunia leafy cutting, suggesting that AOC-mediated JA biosynthesis is required for AR formation (Lischweski et al., 2015). In line with this, treating Arabidopsis leaf explant with coronatine-O-methyloxime (COR-MO) inhibited AR formation (Zhang et al., 2019). COR-MO is a JA-Ile competitive antagonist that inhibits the formation of JAZ-COI1 coreceptor complexes, and thereby blocks JA signaling machinery (Monte et al., 2014). Similarly, the loss of function *coi1-2* mutant produced significantly fewer AR compared with the wild type suggesting a positive role of JA signaling in AR formation (Zhang et al., 2019). It has long been known that JA activates IAA biosynthesis and transport (Sun et al., 2009). Exogenously applied MeJA in Arabidopsis seedlings induces the expression of *ERF109* transcription factor, which in turn binds directly to the promotor of *ASA1* and *YUC2* genes to activate their transcription, and subsequently promotes IAA production (Cai et al., 2014). Interestingly, transcriptome analysis revealed that *ERF109* was rapidly and transiently induced at the wounding site of Arabidopsis leaf explant suggesting a link between wounding-induced JA and IAA biosynthesis during

ARI (Zhang et al., 2019). Indeed, treating leaf explants with anthranilate or overexpressing *ASA1* gene (*35S:ASA1*) complemented the AR defect in the loss-of-function mutant *coi1-2* indicating that IAA biosynthesis acts downstream of COI1-dependent JA signaling (Zhang et al., 2019). The same authors suggested that wounding-induced JA is required only for the early events of AR formation, and when applied continuously it inhibits this process by unknown mechanism. Zhang et al., (2019) speculated that the constitutively JA-induced *ERF109* might perturb AR formation. Because ERF109 protein was found to interact with JAZ9 *in vitro*, the same authors assumed that 2 hours after wounding, ERF109 protein is sequestered by JAZ9 repressor to avoid JA hypersensitivity phenotype that would lead to AR inhibition. This assumption is surprising because constitutive overexpressing *ERF109* lines exhibited auxin-reminiscent phenotypes (Cai et al., 2014). If a constitutive *ERF109* activation negatively affected AR, one would also expect that the constitutive expression of its downstream target *ASA1* would negatively affect AR number. In contrast, the overexpressing *35S:ASA1* lines produced more AR compared with the wild type (Zhang et al., 2019). In addition, more research is needed to clarify the physiological and mechanistic bases of the negative effect of continuous JA application. More importantly, mechanical wounding triggers substantial signaling and response changes including a rapid cytosolic calcium dynamics and hormonal accumulation (ethylene, JA, IAA and CK) (Ikeuchi et al., 2017; Toyota et al., 2018) and a number of these changes are overlapping and possibly regulating each other at different levels to control ARI. In fact, unraveling the mechanistic and physiological bases controlling these tight regulations is needed. Besides, several questions remain to be answered. What is (are) the specific and direct mediator(s) of mechanical wounding involved in the control of ARI? How do the hormonal changes orchestrate each other's signaling and response during ARI? How is the JA peak restored to the steady state level rapidly after wounding? Is there any correlation between the increase of IAA and the decrease of JA at the wounding site? Which one is the cause or consequence of the other? The chicken and egg causality dilemma should be raised in this context. Although uncoupling and specifying the causes from consequences during mechanical wounding would be challenging, answering these questions would provide significant insights about the role of JA in ARI.

Despite the fact that the role of JAs in AR formation may be species and organ-dependent, we propose the following hypothesis for the initiation of AR at the base of stem cuttings: JA and JA-Ile rapidly and transiently accumulate at the base of cuttings forming one of the earliest wounding-associated hormonal peaks (Ahkami et al., 2009). The JA peak is possibly involved in healing the wound and protect the wounding site from pathogen attacks (Ikeuchi et al., 2017). This peak is always followed by a peak of auxin (Ahkami et al., 2013; Druege et al., 2014). Auxin accumulation at wounding site is the result of combined processes including biosynthesis transport, and metabolism. Auxin directly activates the expression of GH3.3, GH3.5 and GH3.6 enzymes, along with other cell reprogramming events. The three GH3 enzymes conjugate JA to amino acids and consequently deplete the JA pool (Gutierrez et al., 2012). This “de-repression mechanism" triggers the formative cell divisions thereby allowing ARI. JAs are known to strongly inhibit cell division by repressing the transcription of several cell cycle genes, including B-type cyclin-dependent kinases (Pauwels et al., 2008). Therefore, a continuous JA treatment or a constitutively active JA signaling would inhibit AR formation likely by interfering with the expression of the cell cycle progression genes needed for pericycle cell division. JA might also repress the expression of genes coding for key factors required for cell fate decisions.

Role of CKs in AR formation

Cytokinins are adenine-derived phytohormones that were called CKs because of their ability to promote cell division (cytokinesis). The role of CKs in adventitious rooting initially comes from physiological studies. For example, the *trans*-zeatin riboside was proven to be the major inhibitor of AR formation in the cucumber hypocotyls (Kuroha et al. 2002). Similarly, treating apple stem cutting with synthetic CK (6-BA) inhibited the development of the AR primordia (Mao et al., 2019). Data from several reports using hormone-induced organogenesis systems also led to a consensus conclusion that the balance between CK and IAA is a critical determinant for organogenesis (Kareem et al., 2016). It is assumed that CKs are required at the first stages of adventitious root initiation as they trigger the cell cycle machinery, but they inhibit this process at later stages. Nevertheless, the exact molecular mechanisms mediating the effect of CK in AR formation is not well studied.

CKs accumulate at the wounding site after excision and this accumulation is due to an increase of *de novo* biosynthesis, as indicated by the upregulation of key genes involved in the biosynthesis pathway (Bustillo-Avendaño et al., 2017). Extensive transcriptome analysis of the Arabidopsis hypocotyl explants revealed that several genes involved in the CK metabolism were differentially expressed upon wounding (Ikeuchi et al., 2017). In line with this, the active base (*trans* Zeatin) accumulated at the wounding site after 12 h after excision (Ikeuchi et al., 2017). This accumulation induced the CK signaling and response machineries, as indicated by an increase of the CK-responsive *TCSn* and *ARR5* promoter activity. The *TCSn* (two-component signaling sensor) is a CK responsive synthetic promoter, which contains concatemerized binding motifs for activated type-B Response Regulator (RR) (Zürcher et al., 2013). The CK accumulation at the wounding site of leaf explants that include the petiole is thought to play a dual role in *de novo* organogenesis processes (Bustillo-Avendaño et al., 2017). First, it promotes the vascular-tissue proliferation resulting in the formation of microcalli, and second, it inhibits ARI derived from the microcalli (Bustillo-Avendaño et al., 2017). This complex regulation implies that ARI in this system follows a two-step mechanism resembling those observed during hormone-induced organogenesis. The direct evidence for this hypothesis comes from the analysis of several mutants affected in CK perception or signaling pathways (Bustillo-Avendaño et al., 2017). The *wooden leg* mutant, which harbors a mutation in the gene encoding the CK receptor ARABIDOPSIS HISTIDINE KINASE4 or the triple loss-of-function mutant *ahp1ahp2ahp3*, affected in the expression of the ARABIDOPSIS HIS PHOSPHOTRANSFER proteins exhibited a reduced regeneration capacity and callus formation compared with the wild type. Similarly, the triple loss-of-function mutant in the *type B ARABIDOPSIS RESPONSE REGULATOR (B-ARR)* genes was unable to properly regenerate vascular tissue or form a callus compared with the wild type (Bustillo-Avendaño et al., 2017). Notably, the leaf explants that were able to regenerate produced significantly more AR, which were indeed initiated from the preformed microcalli, confirming the dual role of CK (Bustillo-Avendaño et al., 2017). This type of regulation has previously been proposed as “an indirect” ARI in difficult-to-root species such as of *Pinus* and *Eucalyptus* (Rasmussen et al., 2009). This is very interesting observation and research is required to confirm that indeed there is a two-step cell identity transition. The fast-developing of single cell omics and cell-lineage tracing techniques will make it possible to verify whether the proliferating vascular cells have a callus identity. Extensive and dynamic analysis using the same approaches will also reveal the players guiding the cell-identity transitions from a callus to AR founder cells.

Interestingly, IAA exogenously applied to the triple loss-of function mutant *arr1arr10arr12* not only restored the regeneration capacity but significantly increased the AR number compared with the wild type, indicating that CK and IAA interact during this process (Bustillo-Avendaño et al., 2017). Accordingly, downregulation of *ARR1, ARR10* and *ARR12*, using oestradiol-inducible artificial microRNA, resulted in the production of ARs rather than shoots by root explants incubated in CK-rich shoot induction medium (Meng et al., 2017). ARR1 directly binds to the promoter of *YUCA4* gene and repress its expression indicating that CK and IAA antagonistically regulates ARI (Meng et al., 2017). Mechanical wounding appears to locally enhance both JA and CK biosynthesis and signaling in Arabidopsis (Ikeuchi et al., 2017). In addition. JA repress the expression of the *CYTOKININ OXIDASE/DEHYDROGENASE (CKX1)* gene (Avalbaev et al., 2016) and the *CKX1* overexpressing line (*35S:CKX1*) produced more AR (Werner et al., 2003). In fact, it would be interesting to functionally check whether JA represses ARI through enhancing the CK accumulation.

The accumulating evidence of the mode of action of CK in AR formation come mostly from the model species Arabidopsis. Translating this knowledge into woody species is needed to understand the negative role of CK in stem cutting in trees. Ramirez-Carvajal et al. (Ramirez-Carvajal et al., 2009) found that the expression of some of the *RESPONSE REGULATOR* type A and type B decreased at the excision site of *Populus* stem cuttings. This downregulation coincides with the AR primordia formation suggesting a negative role of the RR transcription factor in this process. Indeed, overexpressing the active variant of *PtRR13* reportedly inhibited AR formation reaffirming the negative role of CK in AR formation (Ramirez-Carvajal et al., 2009).

Results and discussion

Paper I: A Molecular framework for adventitious rooting controlled by TIR1/AFB2-Aux/IAA-dependent auxin signaling in Arabidopsis

TIR1 and AFB2 auxin receptors act synergistically to control ARI

We previously showed that ARI is controlled by three transcription factors from the AUXIN RESPONSE FACTOR family. ARF6 and ARF8 are positive regulators and ARF17 is a negative regulator (Gutierrez et al., 2009, 2012). The three transcription factors control the expression of the auxin-inducible genes *GH3.3*, *GH3.5* and *GH3.6*. The three GH3 enzymes conjugate the negative regulator JA with amino acids (Gutierrez et al., 2012). How auxin is perceived to activate the ARF6/ARF8 signaling module and which Aux/IAA-TIR/AFB coreceptor(s) is (are) involved in this process were not known yet. In **paper I**, we first investigated the role of *TIR1/AFB* gene family members in ARI. We characterized the available single and multiple mutants in this family (Parry et al., 2009). Among the single mutants only *tir1-1* and *afb2-3* produced fewer AR compared with the wild type, and the double mutant *tir1-1afb2-3* exhibited a stronger reduction in AR **(paper I, Figure 1A)**, suggesting a synergistic or additive effect of the two mutations. We also examined the lateral root density in these mutants and found that *tir1-1* and *afb2-3* produced fewer LRs compared with the wild type. Similarly, the double mutant *tir1-1afb2-3* produced fewer LR compared with single mutants **(paper I, Figure S2A, S2B, S2C)**. These data are in line with previous reports showing that only TIR1 and AFB2 control LR formation (Parry et al., 2009; Xuan et al., 2015). In conclusion, TIR1 and AFB2 act synergistically or additively to control both LRI and ARI.

Characterization of the Expression pattern of the *TIR1/AFB* genes during ARI

Next, we checked the expression pattern of TIR1 and AFB2 proteins during the early events of ARI using translational fusion lines (Parry et al., 2009). Interestingly, TIR1 and AFB2 exhibited an overlapping expression domain in the hypocotyls and both were slightly expressed in the vasculature at T0 and T9 **(paper I, Figure 1B)**. They were strongly expressed in the ARI primordia at T72 **(paper I, Figure 1B)**, suggesting that these genes are also involved in the AR emergence. The absence of an obvious defect in AR number in the mutants of the remaining gene members (*afb1-3, afb3-2, afb4-8, afb5-5* and *afb4-8afb5-5*) raised an obvious question about their presence in the hypocotyls. Therefore, we also checked the expression of other members of the family. Using translational fusion lines, we found that AFB1 and AFB3 were highly and ubiquitously expressed throughout the different stages of ARI, whereas AFB5 was also expressed in the hypocotyl but to a lesser extent. In our conditions, AFB4 was not detected in the seedlings **(paper I, Figure S3A, S3B, S3C)**. This is in line with previous report showing a very weak expression of the AFB4 transcripts in different organs and throughout several developmental stages, as indicated by the qRT-PCR data (Prigge et al., 2016).

TIR1 and AFB2 proteins control ARI

The strong expression of AFB1 and AFB3 in the hypocotyl prompted us to check their role in ARI using multiple mutants to rule out any possible functional redundancy masking their roles. We counted AR number in the double mutant *afb2-3afb1-3* and *afb2-3afb3-4*, and found they retained the phenotype of the single mutant *afb2-3* (**paper I, Figure 1A)**. We also counted AR in the triple mutant *tir1-1afb1-1afb3-4* and found that it retained the phenotype of the single mutant *tir1-1* **(paper I, Figure 1A).** These data constitute a strong genetic evidence for the specificity of TIR1 and AFB2 in ARI. This is in line with previous reports demonstrating that TIR1 and AFB2 are the major players in auxin sensing machinery as indicated by their major involvement in auxin-mediated proteasome-dependent Aux/IAA degradation (Parry et al., 2009; Havens et al., 2012).

TIR1 controls both JA biosynthesis and conjugation, whereas AFB2 preferentially controls JA conjugation during ARI

To check whether TIR1 and AFB2 act upstream of the ARF6/ARF8 signaling module, we quantified the relative transcript amount of *GH3.3*, *GH3.5* and *GH3.6* during the early events of ARI in *tir1-1*, *afb2-3* and the double mutant *tir1-1afb2-3.* As expected, we found that the three *GH3* genes were downregulated in *afb2-3* and *tir1-1afb2-3* compared with the wild type throughout all the time points tested **(paper I, Figure 1C)**. Surprisingly, the expression of these genes was not significantly altered in *tir1-1* compared with the wild type, but they were significantly more downregulated in the double mutant *tir1-1afb2-3* than the single *afb2-3* mutant suggesting a synergistic effect of the *tir1-1* mutation on the expression of the *GH3* genes **(paper I, Figure**

1C). We assumed that TIR1, besides its redundant function in JA conjugation, might have another role in controlling ARI by regulating other auxin-mediated signaling cascades, which converge synergistically with the phenotype of *afb2-3* mutation. To test this assumption, we quantified JA and JA-Ile in *tir1-1*, *afb2-3* and in *tir1-1afb2-3.* Interestingly, we found that *tir1-1* and *afb2-3* produced more JA at T0 and T9 and more JA-Ile at all time points, whereas the double mutant produced more JA-Ile only at T9 **(paper I, Figure 2C, D)**. Unexpectedly, *tir1-1* produced the same amount of JA and JA-Ile as *afb2-3* **(paper I, Figure 2C, D)**, suggesting that TIR1 controls JA homoeostasis during ARI. It has previously been shown that TIR1-dependent auxin signaling negatively controls JA biosynthesis during male organ development in Arabidopsis due to the repression of the expression of key genes in JA biosynthesis pathway, through a pathway yet to be identified (Cecchetti et al., 2013). This result prompted us to check the expression of several genes involved in JA biosynthesis (*LOX2, AOC1-4*, *OPCL1* and *OPR3*) in the single mutant *tir1-1*, *afb2-3* and the double mutant *tir1-1afb2-3*. Indeed, some of the tested genes in JA biosynthesis were downregulated in *tir1-1* or *tir1-1afb2-3* compared with the wild type, whereas they were not significantly different in *afb2-3* compared with the wild type **(paper I, Figure 3A-C)**. We concluded that TIR1, besides its function in JA biosynthesis, synergistically controls JA pool through ARF6/ARF8-GH3-mediated conjugation. The auxin signaling module that mediates the action of TIR1 and how TIR1 acquired this dual role are not known yet and await further investigation.

Aux/IAA6, 9 and 17 redundantly to repress the transcriptional activity of ARF6 and ARF8 during ARI

Auxin response output is generated by a precise co-perception mechanism, which is controlled by specific combinatorial co-receptor complexes between TIR1/AFBs and Aux/IAA proteins (Calderón Villalobos et al., 2012). In this paper, we also aimed to identify which Aux/IAA repressor(s) inhibit the transcriptional activity of ARF6 and ARF8 during ARI. Because the Aux/IAA family consist of 29 members (Overvoorde et al., 2005), and most of them have been shown to interact with ARF6 and ARF8 in a yeast-two-hybrid assays (Vernoux et al., 2011), we combined several approaches to reach our aim. First, we characterized the available loss-of-function *iaa* mutants for AR defect. We found that *iaa5*, *iaa6*, *iaa7*, *iaa8*, *iaa9* and *iaa17* produced significantly more AR compared with the wild type **(paper I, Figure 4A)**, suggesting that the corresponding proteins physically interact and inhibit the transcriptional activity of ARF6 and ARF8. To verify this, we tested the protein-protein interaction of these Aux/IAAs with ARF6 and/or ARF8 using Co-immunoprecipitation (CoIP). We found that only IAA6, IAA9 and IAA17 interact with ARF6 and ARF8 **(paper I, Figure 5A-H)**. We confirmed these data by an independent approach using bimolecular fluorescence complementation (BiFC) technique **(paper I, Figure 5I-P)**. The triple mutant *iaa6 iaa9 iaa17* showed an additive effect in terms of AR **(paper I, Figure 4C)**. To confirm that Aux/IAA6 and IAA9 and IAA17 act through ARF6 and ARF8 signaling module, we quantified the relative transcript amount of *GH3.3*, *GH3.5* and *GH3.6* during the early event of ARI in the single mutants *iaa6*, *iaa9* and *iaa17.* Interestingly, we found that the three *GH3* genes were significantly up-regulated in the three loss-of-function mutants compared with the wild type. Indeed, the loss-of-function mutant *iaa4,* which was not affected ARI, retained the same expression levels for the three *GH3*s as the wild type **(paper I, Figure 7A)**. We also checked whether the three genes have overlapping expression patterns with *ARF6* and *ARF8* (Gutierrez et al., 2009, 2012) by checking their respective promoter activity using transgenic lines expressing transcriptional fusions. We found that the three promoters were active in the hypocotyl, as indicated by the β-glucuronidase (GUS) activity. We also observed that light repress the expression of *IAA6* and *IAA17* **(paper I, Figure 4D-H)**.

We concluded that IAA6, IAA9 and IAA17 physically bind to ARF6 and ARF8 and thereby repress their transcriptional activity.

ARF6, ARF8 and ARF17 are short-lived proteins and their degradation is proteasome dependent

The transcriptional machinery translates the input received from the endogenous and/or exogenous cues into precise developmental output or response to the environment. Transcriptional responses are energy-demanding and therefore, their tight regulation is needed to avoid any undesirable responses. We previously showed that ARF6, ARF8 and ARF17 regulate each other at the transcriptional and posttranscriptional levels to ensure a correct balance between their relative transcript amount as AR formation is the result of these tight regulations (Gutierrez et al., 2009). During the CoIP experiments performed to analyze the interaction between ARF6, ARF8 and the Aux/IAA proteins we observed that the ARF proteins were highly unstable. Therefore, we wondered whether these proteins were also regulated at the posttranslational level. Previously it had been shown that ARF1 was a relatively short-lived transcription factor and that its degradation was proteasome-dependent (Vernoux et al., 2011). We therefore checked the degradation rate of ARF6, ARF8 and ARF17 in a protoplast system expressing the corresponding tagged proteins. We found that the three proteins were unstable, and that their degradation could be blocked by adding the proteasome inhibitor MG132 **(paper I, Figure 6A-E)**. We then confirmed this observation *in planta* using stable transgenic lines. **(paper I, Figure 6F)**.

In conclusion, we propose that AR initiation in the Arabidopsis hypocotyl depends on a specific sensing module comprising two F-box proteins (TIR1 and AFB2) and three Aux/IAA proteins (IAA6, IAA9 and IAA17) acting to modulate the transcriptional activity of ARF6 and ARF8. This signaling module acts upstream of JA homeostasis **(paper I Figure 7)**. Importantly, besides the transcriptional and post-transcriptional regulation, ARF6, ARF8 and ARF17 are also regulated by the proteasome. However, further research is required to check the biological significance of the post-translational regulation. It would also be important to check which cues control this regulation and whether it is IAA-dependent.

Paper II The Arabidopsis Cop9 signalosome subunit 4 (CNS4) is involved in adventitious root formation

Identification of a viable allele of *csn4* as a suppressor of the *sur2-1* AR phenotype

To identify novel players acting downstream of auxin involved in ARI, a forward genetic screen for suppressors of the *superroot2-1* mutant was adopted (Pacurar et al., 2014a). The mutant *sur2-1* is an overproducer of IAA, and consequently it forms a high AR number compared with the wild type (Delarue et al., 1998). We isolated a suppressor mutant in which AR phenotype was restored to the wild type level (**paper II, Figure 2A, B, C**). Using combined map-based-cloning and whole genome sequencing, we identified a point mutation substituting Alanine 302 to Valine in the locus of the subunit 4 of the constitutive photomorphogenesis 9 (COP9) signalosome (CSN) complex, as the potential causal mutation (**paper II, Figure 1A, B)**. Genetic complementation and allelic test confirmed that the suppressor mutant was indeed a weak allele mutant in the *CSN4* gene (**paper II, Figure 2A, B, C)**.

Auxin sensing and response are perturbed in the mutant *cns4-2035*

The CSN is an evolutionarily conserved multiprotein complex, consisting of eight subunits, named CSN1-CSN8 (reviewed in Wei and Deng, 2003). The CSN regulates the function of the cullin-RING E3-ligase (CRL) activity by cleaving the RELATED TO UBIQUITIN (RUB) peptide from the CULLIN subunit (reviewed in Mergner and Schwechheimer, 2014). The CRLs are also evolutionarily conserved multiprotein complexes involved in a plethora of physiological and developmental processes as well as response to environment. In Arabidopsis, CRLs comprise several subclasses including the Skp1-Cullin1-F-box (SCF) complex (reviewed in Hua and Vierstra, 2011). The SCF complex is central hub of several hormonal sensing machineries including auxin and JA (reviewed in Williams et al., 2019), suggesting that CSN complex may play a crucial role in regulating the signaling machineries of these two hormones during ARI. To test whether the novel weak allele *csn4-2035* was affected in auxin signaling, we performed an auxin-dependent root inhibition assay. We measured primary root elongation rates in light-grown seedlings in the absence or presence of different doses of the synthetic auxin 2,4-D. We found that the mutant *csn4-2035* was relatively resistant to 2-4-D compared with its wild type counterparts in term of inhibition of primary root elongation (**paper II, Figure 2I and Figure S1)**. This data indicated that the auxin perception machinery was perturbed in this mutant. Because our previous data showed that the etiolated hypocotyl-derived ARI correlates with transcript amount of the auxin-responsive genes *GH3.3*, *GH3.5* and *GH.6* (Pacurar et al., 2014a), we quantified the relative transcript amount of these three genes in the double mutant *sur2-1 csn4-2035* (2035) and found that the genes were severely downregulated in the suppressor mutant 2035 compared with *sur2-1* indicating that AR suppression was likely due perturbation of ARF6- and ARF8-mediated auxin signaling (**paper II, Figure 3A)**. Furthermore, we showed that *csn4-2035* allele contains the same CSN4 protein amount as its wild type counterpart (**paper II, Figure 3B)** suggesting the changing Alanin302 to Valine did not affect the amount of the protein *per se* but significantly affected the CSN complex activity. A high-resolution structural study of the whole human-CSN complex shed light into the function of each subunit and their role in sensing and mediating the removal of NEDD8 from the neddylated-CRL proteins (Lingaraju et al., 2014). Interestingly, the crystal structure highlighted the role of PCI domain of CSN4 in sensing and facilitating the binding of the neddylated-CRL proteins to CSN (Lingaraju et al., 2014). Our data showed the mutant *csn4-2035* accumulated high amount of NED8-CULLIN1 protein compared with the wild type (**paper II, Figure 3C)**. We also used the loss-of-function *csn4-1* mutant, which accumulated only NED8-CULLIN, as a control further confirming that CSN4 is involved in removing RUB/NEDD8 from CULLIN1. However, further biochemical and structural studies are needed to unravel the function of CSN4 in the de-neddylation of different SCF complexes and specify their potential role in each hormonal signaling pathway.

CSN subunits play differential roles in AR and LR formation

To get insight about the role of each subunit in ARI and potentially in LR, we counted the number AR and LR in the available viable single *csn* mutants (*csn1-10, csn2-5, csn3-3, csn5a-1, csn5a-2 and csn5b*). We also checked their potential role in suppressing *sur2-1* phenotype. All the tested mutants produced fewer AR and LR compared with the wild type and suppressed more or less the *sur2-1* phenotype. In contrast, the mutant *csn2-5* retained the same AR and LR number as its wild type counterpart. Surprisingly, the *csn5b* mutant produced significantly more AR compared with the wild type and enhanced slightly the AR number in *sur2-1* mutant background, suggesting its negative role during ARI. The LR density was not affected in this mutant (**paper II, Figure 5A, B, C, D)**. Although thorough characterization is needed using more independent viable weak alleles, these data suggest that there is a functional specificity of each subunit of the CSN complex.

Paper III A DAO1-mediated circuit controls auxin and jasmonate crosstalk robustness during adventitious root initiation in *Arabidopsis*

Jasmonate induces the expression of *DAO1* and *DAO2* genes in a COI1-dependent manner

To identify novel players acting downstream of JA with a potential role in ARI, we adopted a candidate gene approach. We searched in the publicly available JA-related transcriptomic dataset (**paper III, Figure S1;**Winter et al., 2007). Among several potential candidates, we focused our attention on key players in the auxin catabolism the *DIOXYGENASE FOR AUXIN OXIDATION 1* (*DAO1*) and its closely related paralog genes. The DAO proteins catalyze the conversion of free IAA into 2-oxindole-3-acetic acid (oxIAA), a rate-limiting step in auxin degradation (Zhao et al., 2013; Mellor et al., 2016; Zhang et al., 2016; Porco et al., 2016). *DAO1* was also rapidly induced at the wounding site in the Arabidopsis leaf explant during ARI(Zhang et al., 2019), suggesting that these genes may play important role on wounding-JA-IAA interactions during this process or possibly other developmental processes. We confirmed that JA induces *DAO1* and *DAO2* in a dose- and time-dependent manner and showed that this induction requires a functional COI1-dependent signaling machinery (**paper III, Figure 1A, B, C)**. We observed that the promoters of both *DAO1* and *DAO2* contain at least one G-box motif (**paper III, Figure 1D;** Godoy et al., 2011), suggesting that they may be direct targets of MYC2 transcriptions factor. However, JA induced the expression of *DAO1* and *DAO2* the wild type and loss of function *jin1-2/myc2* in a similar manner (**paper III, Figure 1E, F)**. These data suggest that either these genes are not regulated by *MYC2* or that *MYC2* acts redundantly with its closely related paralogs *MYC3* and *MYC4*. Therefore, checking the expressing of these genes in higher order mutants is needed before drawing any conclusion.

JA induces the expression of *DAO1* and *DAO2* genes independently of TIR1/AFB-dependent auxin signaling

JA is known to interact with IAA at different levels including its transports, signaling and biosynthesis (reviewed in Lakehal and Bellini, 2018). JA is known to promote *de novo* IAA production by inducing the key genes in the tryptophan-dependent IAA biosynthesis pathway. Recently, Mellor et al., (2016) demonstrated that *DAO1* is slightly induced by exogenously applied IAA. These results prompted us to check whether JA effect on *DAO* expression is dependent on auxin signaling. It is worth to mention that in our experimental conditions exogenously applied IAA did not (or very slightly) induce the expression of *DAO1* and *DAO2* (**paper III, Figure 2A)**. Next, we chemically blocked *TIR1/AFB*-dependent IAA signaling using auxinole. The auxinole is a potent auxin antagonist that inhibits the function of the TIR1/AFB receptors (Hayashi et al., 2012). Interestingly, we found that JA still induces these genes even in the presence of auxinole (**paper III, Figure 2B)**, indicating that JA-mediated *DAO* expression does not require a functional TIR1/AFB-dependent IAA signaling.

DAO1, but not *DAO2,* plays a major role in IAA catabolism during adventitious root initiation

We investigated the biological relevance of *DAO1* and *DAO2* genes in ARI in the etiolated hypocotyls and found that the loss-of-function mutant *dao1-1* produced more AR than the wild type, whereas *dao2-1* mutant retained wild-type phenotype (**paper III, Figure 3A)**. To investigate the genetic link between *DAO1* and *DAO2*, we generated a double mutant *dao1-1doa2C* using CRISPR-Cas9 technology (**paper III, Figure S2A and S2B)**. Interestingly, the *dao1-1doa2C* double mutant exhibited the same AR number as the single mutant *dao1-1* suggesting a minor role for DAO2 in ARI (**paper III, Figure 3B)**. We further checked AR responsiveness of *dao1-1* and *dao1-1dao2C* mutants to exogenously applied IAA. One μM IAA did not stimulate AR production in the wild type, whereas it dramatically stimulated it in the single mutant *dao1-1* and the double mutant *dao1-1dao2C* in a similar manner (**paper III, Figure 3D, E, F)**. These data further confirmed that DAO1 is the major player of IAA homoeostasis during ARI. We found that *DAO1* gene is ubiquitously expressed throughout all seedling including the etiolated hypocotyls, as revealed by the promoter activity of *DAO1* fused with the coding sequence of the ß-glucuronidase reporter gene (**paper III, Figure 3G)**.

The *dao1-1* mutant produces less JA and JA-Ile in the etiolated hypocotyls

To get mechanistic insight about the role of DAO1 in ARI, we performed hormone profiling in *dao1-1* mutant and the wild type at the early stages of ARI. Our data confirmed that although *dao1-1* produces reduced amount of OxIAA, it retains the same amount of free IAA as the wild type (**paper III, Figure 4A, Figure S3A)**. This possibly due to enhanced GH3-mediated conjugation of IAA with amino acids. Indeed *doa1-1* produced huge amount of the IAAsp and IAGlu as compared with the wild type (**paper III, Figure S3B, S3C** and Mellor et al., 2016; Zhang et al., 2016; Porco et al., 2016). As indicated above, besides their role in conjugating IAA, the GH3.3, GH3.5 and GH3.6 proteins also conjugate JA with amino acids (Gutierrez et al., 2012). We hypothesized that the AR defect in *dao1-1* cannot be explained exclusively by an IAA accumulation but it is possibly due to another signaling molecular network. As obvious candidate, we first quantified JA and JA-Ile content in the mutant *dao1-1* and the wild type at the early stages of ARI. Interestingly, we found that the mutant *dao1-1* accumulated significantly less JA and JA-Ile at the time point tested (**paper III, Figure S3A 4B, C)**, suggesting that the AR defect is due JA and JA-Ile deficiency. Our data was in line with previous report revealing that the fertility defect of the rice *dao* mutant was linked to JA and JA-Ile deficiency (Zhao et al., 2013). This deficiency was due to a downregulation of the JA biosynthesis pathways, as revealed by the relative transcript amounts of the key genes in the JA biosynthesis (Zhao et al., 2013). To verify the possibility that the mutant *dao1-1* is affected in JA biosynthesis, we quantified the JA precursor *cis*-OPDA. We found that the mutant *dao1-1* retained the same amount of this precursor as the wild type (**paper III, Figure 4D)**. We also found that the expression levels of the *ALLENE OXIDE CYCLASE2* (*AOC2*) and *OXOPHYTODIENOATE-REDUCTASE3 (OPR3)* genes were not affected the mutant *dao1-1* compared with the wild type (**paper III, Figure 4E)**. Next, we checked the expression levels of the *GH3.3*, *GH3.5* and *GH3.6* genes. Interestingly, we found that *GH3.5* and *GH3.6* were slightly upregulated at T9, nine hours after transferring the seedling to the light, in the mutant *dao1-1* compared with the wild type (**paper III, Figure 4F)**. These data may partly explain the decrease of JA in the mutant *dao1-1,* however further experiments are needed to get clear explanation about the exact mechanism leading to JA and JA-Ile reduction in the mutant *dao1-1.* It would be interesting to check the expression of other JA metabolism or degradation pathways. Interestingly, phylogenetically-related 2-oxoglutarate–dependent Fe (II) dioxygenase were shown to oxidase JA (Caarls et al., 2017; Smirnova et al., 2017). Thus, checking the expression of these genes would give more insight.

Manuscript IV A Jasmonate-mediated molecular network provides cell-autonomous decisions for adventitious rooting in Arabidopsis

NINJA-dependent and NINJA-independent JA signaling repress AR initiation

As stated in the introduction, stress-induced JA phytohormone has been implicated in many developmental programs including PR, LR and AR formation as well as regeneration (reviewed in Wasternack and Hause, 2013). We have previously shown that COI1-dependent MYC2-mediated JA signaling inhibits ARI in the intact etiolated hypocotyls (Gutierrez et al., 2012). To dissect the role of JA signaling components and get more mechanistic insights about JA action during ARI, we adopted a genetic approach. Our data showed that mutations in JAZ repressors, which are a part of the JA-nuclear sensing machinery, exhibited a very weak phenotype. The quintuple mutant *jazQ (jaz1 jaz3jaz4jaz9jaz10)* (Campos et al., 2016), which harbor five null mutations in the closely-related paralogs exhibited a slight reduction in AR as compared with the wild type, whereas the quadruple mutant *jaz7jaz8jaz10jaz13* retained the same number of AR as the wild type (**paper IV, Figure 1B)**. These data confirm the high functional redundancy among the members of the JAZ family. Next, we explored the role of the single copy gene *NINJA* (Pauwels et al., 2010) and found that two loss-of-function alleles *ninja-1* and *ninja-2* exhibited a slight but significant reduction in the number of AR compared with the wild type (**paper IV, Figure 1C)**. We reasoned that the mild phenotype of *ninja* mutants could be due to a NINJA-independent repression of MYC2, MYC3 or MYC4. To test this hypothesis, we genetically combined *ninja* mutants with the gain-of-function mutants *myc2-322B* or *atr2D* harboring mutations in the *MYC2* and *MYC3* genes respectively. These mutations prevent MYC2 and MYC3 proteins to bind to some JAZ repressors (Smolen et al., 2002; Gasperini et al., 2015). Interestingly, the double mutants *ninja-1myc2-322B* or *ninja-1atr2D* exhibited a dramatic decrease in AR number compared with the wild type (**paper IV, Figure 1C and 1E)**. These data suggest that JA signaling controls AR in a NINJA-dependent and NINJA-independent ways.

***NINJA* and *MYC2* are expressed in the etiolated hypocotyl**

To check the spatiotemporal expression of *NINJA* and *MYC2* during ARI, we used transgenic lines expressing the transcriptional fusions *pNINJA:GUS* and *pMYC2:GUS* (Gasperini et al., 2015). Seedlings were grown in ARI conditions and stained at different time points; etiolated in the dark (T0), or after shifting to the light for 9h or 24h (T9L and T24L, respectively) including their dark controls (T9D and T24D). The two promoters were constitutively active throughout the ARI process (**paper IV, Figure 1F)**

NINJA-dependent JA signaling controls ARI in the xylem-pole pericycle cells

Because we and others have previously shown that the first event of hypocotyl-derived adventitious rooting takes place in the xylem pole pericycle (XPP) cells (Sorin et al., 2005; Sukumar et al., 2013), we checked whether NINJA-dependent JA signaling affect ARI at this cell type. Hence, we used cell type-specific functional complementation approach by using two xylem pole pericycle specific promoters. We used *ProXPP* (Andersen et al., 2018) and *ProGATA23* (De Rybel et al., 2010) promoters to drive the expression of translational fusion of NINJA and mCITRIN reporter protein and transformed the respective construct into *ninja-1myc2-322B* double mutant background. Interestingly, we found that at least two independent lines from each construct were partially restoring the AR defect of the double mutant *ninja-1myc2-322B* as compared with the wild type (**paper IV, Figure 2E, 2F and 2G)**. These data suggest that NINJA-dependent JA signaling might act in cell autonomous manner to inhibit the early event of xylem pole pericycle cell reprogramming leading to ARI.

The double mutant *ninja-1 myc2-322B* exhibited an elevated and constitutive JA-response signature

We have previously observed that CYCB1;1:GUS, which is a marker cell division, was expressed in the hypocotyl as early as 48h after moving the seedlings to light marking the first xylem pole pericycle divisions (Sorin et al., 2005). These considerations led us to conclude that the first events guiding the cell reprogramming in the hypocotyl likely occur between T0 and T24. To get mechanistic insights into the role of MYC2-dependent JA signaling in the early event of ARI, we carried out a comparative transcriptomic analysis between *ninja-1myc2-322B* and the wild type using RNA-Seq. Because ARI is dynamic developmental process, we performed RNA-Seq in a time course experiment (T0, T9 and T24) (**paper IV, Figure 3A)**. Pairwise comparison using NOIseq parameters (fold change ≥ 2 and probability ≥ 0,8) revealed 530 DEGs at T0, 671 DEGs at T9 and 579 DEGs at T24 (**paper IV, Figure 3B)**. Interestingly, most of the *JAZ* genes, which are makers of JA response, were highly upregulated in *ninja-1myc2-332B* compared with the wild type in different time points (T0, T9 and T24) confirming that this double mutant exhibits an enhanced constitutive JA signaling (**paper IV, Figure 3C)**. We also found that the double mutant *ninja-1myc2-322B* produced more *cis*-OPDA, JA and JA-Ile compared with the wild (**paper IV, Figure 4B, 4C and 4D).**

JA signaling controls the transcriptional regulation of the stress related *ERF114* and *ERF115* transcription factors.

We found that two members of the group X of the *ETHYLENE RESPONSE FACTOR* gene family (*ERF114* and *ERF115*) were up regulated in the double mutant *ninja-1myc2-322B* compared with the wild type (**paper IV, Figure 5A 5B and 5C)**. We validated the *ERF115* expression data by qPCR (**paper IV, Figure 5C)**. The ERF114 and ERF115 transcription factors have been implicated in several developmental processes including brassinosteroid-mediated stem cell maintenance, JA-mediated root regeneration, wound-induced root regeneration and callus formation (Kong et al., 2018; Heyman et al., 2013, 2016). All these processes require tight and precise cues leading to cell reprogramming and identity transitions. These considerations prompted us to investigate the role *ERF115* in the ARI process. We first checked the spatiotemporal expression of *ERF115* using transgenic lines expressing the transcriptional fusion *pERF115:GUS* (Heyman et al., 2013). The *ERF115* genes was highly expressed in the vascular tissue of the hypocotyl and to a lesser extent in the root (**paper IV, Figure 5D)**. After shifting the seedling to light for 24h (T24L) the GUS signal was dramatically reduced in the hypocotyl compared with the seedling which were kept in dark conditions (T24D) (**paper IV, Figure 5D)**. we also confirmed the light-dependent downregulation of *ERF115* by qPCR (**paper IV, Figure 5E)**. This data suggests that likely *ERF115* is involved JA-mediated ARI.

The *ERF115* gene represses hypocotyl-derived AR initiation

To test whether *ERF115* is involved in ARI, we checked the phenotype of loss-of-function *erf115* and found that it retained the same phenotype as the wild type (**paper IV, sup data)** possibly because the high functional redundancy among the *ERF* gene family. Arabidopsis genome contains 122 paralogs (Nakano et al., 2006). To overcome this redundancy, we phenotyped the triple mutant *rap2.6l-1erf114Cerf115* and the negative-dominant *pERF115:ERF115:SRDX* line, Interestingly, both *rap2.6l-1erf114Cerf115* and *pERF115:ERF115:SRDX* produced more ARs compared with the wild type , whereas the overexpressing line *35S:ERF115* phenocopied *ninja-1myc2-322B* double mutant by producing extremely few AR (**paper IV, Figure 6A, 6B, 6C)**.

We found that 35S:ERF115 produced more CK active bases (iP, *c*Z and *t*Z) (**paper IV, Figure 7C)**. Our data also suggest that ERF115-mediated ARI inhibition requires cytokinins as revealed by a genetic evidence (**paper IV, Figure 7F)**. The multiple mutants (*35S:ERF115 35S:CKX1*) or (*35S:ERF115 ipt3ipt5ipt7)* produced the same AR as the wild type. These data indicate that depleting the CKs in the *35S:ERF115* background is sufficient the restore its AR defect (**paper IV, Figure 7F)**.

Conclusions and perspectives

Adventitious root initiation is a post-embryonic developmental program controlled by several endogenous and exogenous cues. In this study, we shed more light on the important components of the signaling networks modulating ARI. It is established that IAA and CK interact with each other at different levels to control most, if not all, organogenesis programs including ARI but the mechanism guiding this interaction and how their interaction lead to unique and specific developmental outputs are still poorly understood. JA and its derivatives including the bioactive conjugate JA-Ile also emerge as central integrators of several signaling and hormonal pathways to control a number of developmental programs such as tissue repair, regeneration or ARI.

Combining several approaches, we found the IAA receptors involved in ARI, which are acting upstream of JA. We also identified a novel molecular circuit mediated JA-induced *DAO1*, an auxin degradation gene acting downstream of JA signaling to control the auxin pools. We also found that JA signaling rely on CKs to inhibits ARI. Although more research is needed, we attempt to conclude that JA plays the role of a modulator or molecular switch between the IAA and CK to control ARI.

The next challenging step is to check whether the knowledge gained using Arabidopsis as model will be translated to other species. It would also be interesting to study the *evo devo* aspect of the identified pathway using the tree species such *Populus* or conifers such as *Pinus* or *Pecia abies* since their genome sequences are available. Interestingly, recent reports, based on transcriptome data, suggested that this pathway is likely to be an evolutionary conserved. The availably of the fast-developing gene editing techniques such as CRISPR-cas9 will allow to conduct functional studies to verify these claims.

Unraveling the genetic and molecular bases controlling any plant developmental program is primordial step towards understanding its complexity, which in turn will greatly help in finding solutions for the limiting steps during plant breeding. Besides its crucial role in vegetative propagation as well as adaptation in response the environmental constraint, adventitious rooting could also be a great model system to study *de novo* organogenesis in response to the environmental cues.

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