



Characterization of adventitious root formation in *Populus* species and Norway spruce

Sanaria Abbas Jaafar Alallaq



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This thesis is dedicated to:

*The soul of my father, the man who taught me
perform all of life's tasks, no matter how big or small.*

My mother

My sisters and my brother

My husband and my two daughters

Who always encouraged me to go on every adventure

Especially this one

Their support and drive are what has raised me to be the person

Who I am today.

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Abstract

Adventitious root (AR) formation is a form of post-embryonic development and is a key adaptive trait in plants. *De novo* adventitious root regeneration represents an elegant evolutionary innovation that allows many plant species to multiply through vegetative propagation; it is widely used in forestry and horticulture to multiply elite genotypes. However, several tree species with high economic and ecological value are difficult to root, and the genetic and molecular bases underlying AR regeneration remain largely elusive. Recently our laboratory showed that jasmonate (JA) and cytokinins (CK) act cooperatively to repress AR initiation (ARI) in *Arabidopsis* hypocotyls, while auxin positively controls ARI by repressing this negative effect. With the recent availability of the reference genomes of *Populus* spp. and Norway spruce (*Picea abies*), the aim of this thesis is to explore the molecular and mechanistic foundations of AR formation in woody species and check whether or not there is conservation of the molecular mechanisms identified in *Arabidopsis*. First, physiological, molecular and hormonal approaches coupled with extensive anatomical analysis were combined to explore the role of light spectral quality in the control of ARI in *P. abies* de-rooted seedlings. We showed that constant red light (cRL) promotes ARI by reducing the content of the wound-induced phytohormones JA and JA-isoleucine and repressing the accumulation of the isopentyl-adenine-type cytokinins. These results suggest that the cooperative role of JA and CK signaling in the repression of ARI is evolutionarily conserved. Next we compared transcriptomic data from the cambium tissue of woody stem cuttings of the hybrid aspen T89, which is difficult-to-root, and from the hybrid poplar OP42, which is easy-to-root, under hydroponic conditions. The analyses revealed high transcriptional activity in OP42, with twice as many transcription factors differentially expressed in OP42 24 hours after cutting compared to T89. Although we did not observe significant differences in the expression of Auxin response factor (ARF) genes between the two genotypes, the production of transgenic plants downregulating or over-expressing *ARF6*, 8 or 17 confirmed that *PtARF6* and *PtARF8* positively and *PtARF17* negatively regulate AR development in transgenic hybrid aspen T89. Interestingly, the expression of *MYC2* orthologs as well as the expression of several genes involved in JA signaling increased more in T89 than in OP42, suggesting that JA could be a negative regulator of ARI in *Populus* spp. We also showed that overexpressing *PtMYC2* led to a reduced number of ARs in hybrid aspen T89 cuttings. In addition, many genes encoding ROS scavenging proteins such as peroxidases or GSTs were significantly differentially expressed in OP42 24 h after cutting but not in T89, which is interesting since peroxidase activity has often been positively correlated with ARI. In parallel to this research, we characterized the rooting phenotype of clones from the Swedish Aspen (SwAsp) collection using *in vitro* cuttings. We observed a significant variation in the

rooting ability as well as different root system establishment between the clones. We analyzed the expression of some genes known to be involved in AR development in selected clones with contrasting AR phenotypes but could not identify any correlation between gene expression and rooting phenotype. A transcriptomic analysis of selected clones, with contrasting AR phenotypes, could be a useful tool in the identification of marker genes, which can be used for future selection of the best rooting clones of *Populus* or other economically important trees in breeding programs.

Key words

Adventitious root, Conifers, *Picea abies*, auxin, cytokinins, jasmonate, red light, *Populus* spp., hybrid poplar, hybrid aspen, cambium, stem cuttings, *P. tremula*, Swedish Aspen (SwAsp) collection.

Sammanfattning

Adventivrötters (AR) bildning är ett post-embryonalt utvecklingsprogram och en viktigt anpassningsegenskap hos växter. De novo-generering av AR representerar en elegant evolutionär innovation som möjliggör vegetativ propagering hos många växtarter. Detta används frekvent inom skogsindustri och hortikultur för att föröka önskevärda genotyper. Flera arter av träd av högt ekonomiskt och ekologiskt intresse är däremot svåra att propagera, och de underliggande molekylära grunderna bakom ARs regenerering har i stort förblivit okända. Nyligen visade vårt forskningsgrupp att jasmonat (JA) och cytokiner (CK) agerar kooperativt för att hämma AR-initiering (ARI) i hypocotylen hos *Arabidopsis thaliana*, meda auxin kontrollerar AR-initiering positivt genom att hämma denna hämmande effect. I och med nylig tillgång till referensgenom i *Populus spp.* och gran (*Picea abies*), så ämnar denna anvhandling att undersöka den molekylära och mekanistiska grunden som ligger bakom AR-bildning i vedbildande arter, och att undersöka huruvida de molekylära mekanismer identifierade i *Arabidopsis* är evolutionärt konserverade. Först har jag kombinerat fysiologiska, molekylära och hormonella metoder tillsammans med extensiv anatomisk analys för att utforska rollen hos ljus spektralkvalitet för AR-initiering i avrotade *P. abies*-groddar. Vi visade att konstant rött ljus (cRL) främjar AR-initiering genom att minska halterna av de skadeinducerade hormonerna JA och JA-isoleucine, samt genom att hämma ackumulering av isopentyl-adenine-typer av cytokiner. Dessa resultat tyder på att den kooperativa rollen hos JA och CK-signalering för hämmande av AR-initiering är evolutionärt konserverad. Efter detta jämförde vi transkriptom-data från cambium-vävnad i vedstamsnitt hos hybridasp T89, som är svår att rota samt hybridasp OP42 som är enkel att rota, under hydroponiska förutsättningar. Analyserna visade hög transkriptionell aktivitet i OP42, med två gånger fler transkriptionsfaktorer differentiellt uttryckta i OP42 24 timmar efter snitt jämfört med T89. Även om vi inte observerade signifikanta skillnader i uttrycksnivåer hos auxin-responsfaktorer (ARF)-gener mellan de två genotyperna så såg vi att transgena växter med ned- eller uppreglering av *ARF6*, *8* eller *17* bekräftade att *PtARF6*, *PtARF8* positivt reglerar, samt *PtARF17* negativt reglerar AR-utveckling i transgena hybridasp. Intressant var att uttryck av *MYC2*-ortologer samt uttryck av flera gener involverade i JA-signalering ökade mer i T89 än i OP42. Detta indikerar att JA möjligen reglerar AR-initiering negativt i *Populus spp.* Vi visade också att överuttryck av *PtMYC2* ledde till reducerat antal AR i hybridasp-snitt. Dessutom observerade vi att flera gener som kodar för ROS-rensande proteiner som t.ex. peroxidaser eller GSTs uppvisade significant ändrade uttrycksnivåer i OP42 24 timmar efter snitt, vilket ej skedde i T89. Detta är intressant eftersom peroxidas-aktivitet ofta har visat sig positivt relaterat med AR-initiering. Parallelt med dessa undersökningar så karaktäriserade vi rotningsfenotyper hos kloner från Swedish Aspen (SwAsp)-

kollektionen hos *in vitro*-snitt. Vi observerade en significant variation i rotningsförmåga samt rotsystemsetablering mellan klonerna. Vi analyserade uttrycksnivåer av gener kända för att reglera AR-utveckling i utvalda kloner med kontrasterande AR-fenotyper, men kunde inte finna någon korrelation mellan genuttryck of rotningsfenotyp. Transkriptomanalys av utvalda kloner med kontrasterande AR-fenotyp skulle kunna utgöra ett användbart redskap för identification av markörgener, vilka kan användas för framtida selection av bästa rotningskloner i *Populus* eller andra ekonomiskt viktiga trädarter i förädlingsprogram.

Abbreviations

AR	Adventitious root
ARF	Auxin response factor
AOS	allene oxide synthase
AOC	allene oxide cyclase
BAP	6-benzylaminopurine
cWL	Constant white light
cRL	Constant red light
CK	Cytokinin
<i>cis</i> -OPDA	<i>cis</i> -12-oxo-phytodienoic acid
DAC	Days after cutting
DEG	differentially expressed genes
dnOPDA	dinor-oxo-phytodienoic acid
4,5ddh-JA	4,5-dihydrojasmonate
GO	gene ontology
IAA	Indole -3-acetic acid
IBA	Indole butyric acid
iPR	iP ribosides
iPRMP	iP riboside 5'-monophosphate
iP-types	isopentenyl-adenine-types
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucine
JAR1	jasmonate resistant1/GH311
JMT	JA carboxyl methyltransferase
LOX	lipoxygenase
LCM	Laser capture Microdissection
LED	Light emitting diodes
MeJA	methyl jasmonate
NAA	1-Naphtalene acetic acid
OPR3	OPDA reductase
OPC4	4-(3-oxo-2-(pent-2-enyl) cyclopentyl) butanoic acid

OPC6	6-(3-oxo-2-(pent-2-enyl) cyclopentyl) hexanoic acid
OPC8	8-(3-oxo-2-(pent-2-enyl) cyclopentyl) octanoic acid
12-OPDA	12-oxo-phytodienoic acid
PAT	Polar auxin transport
ROS	Reactive oxygen species
SwAsp	The Swedish Aspen collection
tnOPDA	tetranor-OPDA
18:3	α -linolenic acid
16:3	hexadecatrienoic acid

List of chapters

This thesis is a summary of the following three chapters (papers):

- I. Sanaria Alallaq, Alok Ranjan, Federica Brunoni, Ondřej Novák, Abdellah Lakehal, and Catherine Bellini. Red light controls adventitious root regeneration by modulating hormone homeostasis in *Picea abies* seedlings. 2020. *Frontiers Plant Science*. 11, 1–14. doi:10.3389/fpls.2020.586140.
- II. Alok Ranjan*, Irene Perrone*, Sanaria Alallaq*, Rajesh Singh, Federica Brunoni¹ Annegret Kohler, Francis Martin, Rishi Bhalerao, Valérie Legué, and Catherine Bellini. Genome wide comparative transcriptomic analysis of the cambium tissue from easy-to-root and difficult-to-root *Populus* genotypes.
* These authors contributed equally to this work. (manuscript).
- III. Sanaria Alallaq, Florencia Bannoud, and Catherine Bellini. Characterization of AR formation in aspen clones from the Swedish Aspen collection. (manuscript).

Additional publication not included in this PhD thesis

Abdellah Lakehal, Asma Dob, Zahra Rahnesan, Ondřej Novák, Sacha Escamez, Sanaria Alallaq, Miroslav Strand, Hannele Tuominen, and Catherine Bellini. 2020. ETHYLENE RESPONSE FACTOR 115 integrates jasmonate and cytokinin signaling machineries to repress adventitious rooting in *Arabidopsis*. *New Phytologist*. nph.16794. doi:10.1111/nph.16794.

1. General Introduction

Land plants play a vital role in everyday human activity. They provide us with food, oxygen, medicine, fuel, fibers materials for tools and shelter and they are also essential to the world's wildlife (White *et al.*, 2013). Plants have a unique feature of being able to reproduce in two ways: sexual reproduction through seeds and asexual propagation also called vegetative propagation. The latter is possible thanks to plants' ability to develop adventitious roots (ARs) from non-root tissues such as stems, leaves or hypocotyls (Bellini *et al.*, 2014; Steffens & Rasmussen, 2016). Adventitious root formation is a complex quantitative trait regulated by multiple endogenous factors such as phytohormones, phenolic compounds, polyamines or mechanisms related to the aging process, and environmental factors like light, temperature or nutrients (reviewed in Geiss *et al.*, 2018).

For many species, AR formation is intrinsically part of development and occurs post-embryonically. This is the case for monocots, for which ARs represent the main root system, but also for many naturally vegetatively propagated dicotyledonous plants like strawberries (*Fragaria* spp.), African violets (*Saintpaulia* spp.) or blackberries (*Rubus* spp.) (Figure 1A and B). Moreover, AR may be induced as a stress response to, for example, darkness, flooding or wounding (Figure 1C and D). These stress situations are not only caused by changes in the environment, but can be induced mechanically by wounding during tissue culture techniques (Figure 1C and D) (reviewed in Geiss *et al.*, 2018; Bellini *et al.*, 2014; Steffens & Rasmussen, 2016).

1.1. Why is it important to study adventitious rooting?

The importance of studying adventitious root formation lies in the fact that the ability of plants to undergo vegetative propagation from cuttings has been extensively used in breeding programs to multiply elite genotypes and fix interesting agronomic traits at relatively low cost (Stenvall, 2006; Mauriat *et al.*, 2014).

This process is economically important for forest trees such as *Populus* spp., *Pinus* spp., *Picea* spp. and *Eucalyptus* spp. as well as horticultural species. One major limitation in clonal propagation of woody species is the highly reduced or rapid loss of ability to form ARs in a number of genotypes (Ragonezi et al., 2010; Legué et al., 2014).

The ability to form adventitious roots varies between plant species, which are generally characterized as easy-to-root or difficult-to-root plants. The former have the ability to form ARs without any special treatment of the cuttings most of the time, while the latter require special treatments of either the mother plant or the cuttings, involving application of phytohormones and/or modifications to their environment (reviewed in Lovell & White, 1986).

In some woody plants, the rooting capacity may decrease after a phase change, from juvenile to mature. Researchers working with woody plants such as *Ficus pumila*, *Prunus avium* and *Eucalyptus grandis* have found that cuttings from young plants readily form ARs but when cuttings are taken from the same adult plant the ability has been lost (Davies et al., 1982; Dick & Leakey, 2006; Abu-Abied et al., 2014).

In conclusion, adventitious rooting is a key step in clonal propagation of economically important horticultural and woody species, therefore it is important to gain a better understanding of the mechanisms that regulate AR initiation (ARI) and development in order to improve its application.

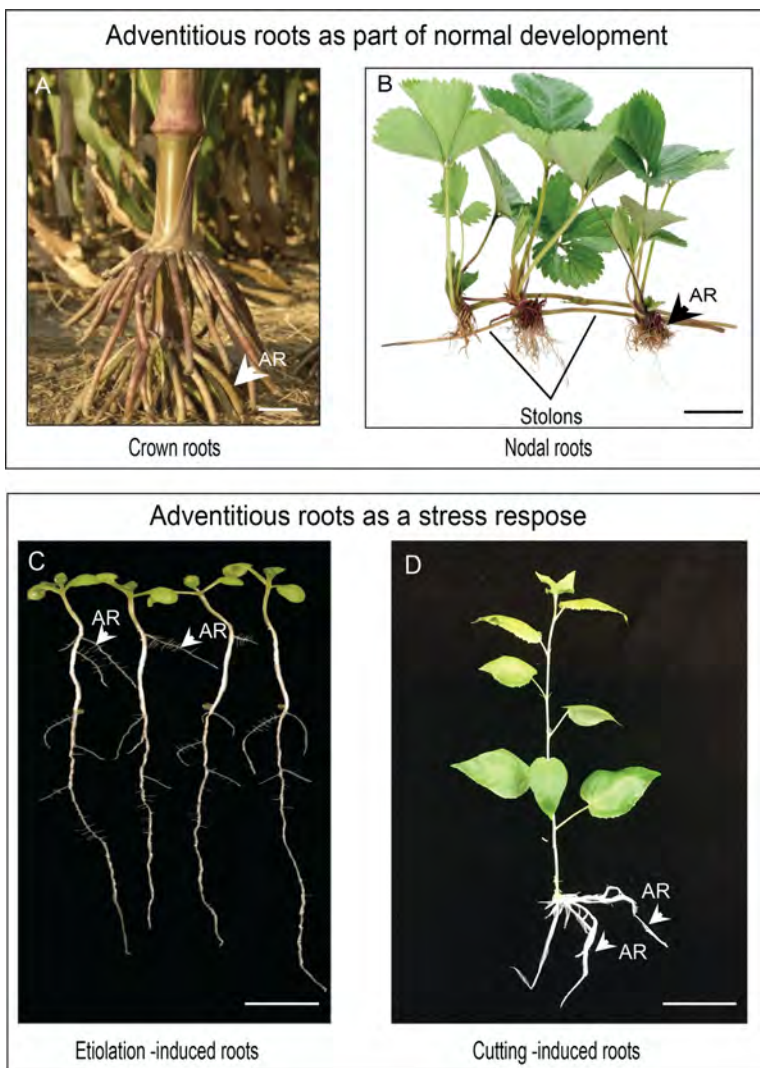


Figure 1: Examples of different types of adventitious roots.

This figure illustrates some examples of the developmental aspect of adventitious rooting.

(A) and (B) show types of AR that are intrinsically part of plant development.

(A) Crown roots as an example of AR in monocots (scale bar: 2cm) from <https://www.pinterest.com/pin/203295370653221607/>.

(B) Adventitious roots during vegetative propagation of strawberries (scale bar: 1cm) from <https://growingfruit.org/t/grin-usda-stolon/23666>.

(C) and (D) show stress-induced ARs.

(C) AR induced by dark-light transition in *Arabidopsis thaliana* (scale bar: 0.5 cm) from Gutierrez et al. (2009).

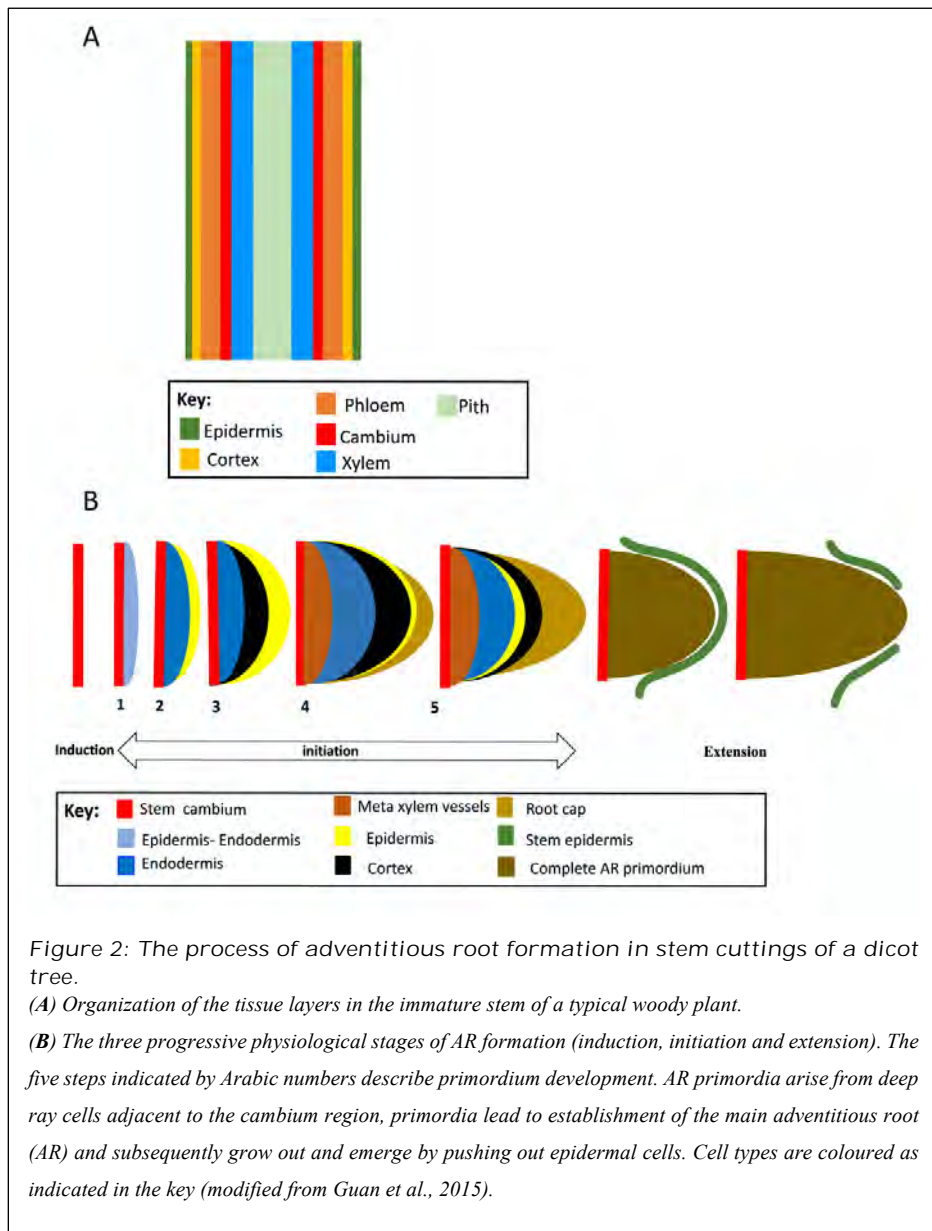
(D) AR induced by wounding in a *Populus mico*-cutting during in vitro vegetative propagation (scale bar: 0.5 cm) Photo: Sanaria Alallaq.

1.2. Anatomy and histology analysis of adventitious roots

For all plants, the primary root meristem is established during embryogenesis, but lateral and AR meristems are formed post-embryonically (Casson and Lindsey, 2003). While lateral roots are commonly formed from mature pericycle cells of the main roots, ARs develop from different tissues and consequently from different cell types. ARs are also formed after tissue culture regeneration of shoots with or without hormone applications. From the literature, it appears that there is a debate about the number, the nature and the terminology of the histology stages characterizing AR formation (Haissig, 1974; Lovell & White, 1986; Altamura, 1996; da Costa *et al.*, 2013; Guan *et al.*, 2015).

According to Kevers *et al.* (1997) this process can be distinguished by three phases (Figure 2B). The first phase is the induction phase, which precedes any anatomical event; the second, the initiation phase (cell divisions leading to the formation of internal root meristems); and the third, the expression or extension phase is characterized by the internal growth of root-primordia and root emergence. However, according to De Klerk *et al.* (1999) and Pijut *et al.*, (2011) a fourth stage exists and occurs before the induction phase and consists of cell dedifferentiation, it is followed by the induction phase during which no anatomical changes can be observed; thereafter, cells near the vascular bundles become meristematic and divide. This is followed by the development of dome-like root primordia, and finally root emergence. At the stage when the organized root primordium starts to differentiate and elongate, the vascular tissues also form and connect to the vascular system of the cutting.

Anatomical processes of AR formation have been analyzed in various species thanks to studies performed on cuttings, from vegetative portions of the plant, such as stems (rhizomes, tubers, corms, and bulbs), leaves or roots. It has been shown that ARs can arise from pericycle cells, parenchyma cells, cambium cells, or phloem initials. However, in all cases, the cells are located close to the vascular system.



For both herbaceous and woody plants there are two patterns of AR initiation. The indirect pattern consists of the formation of a callus, which is a mass of proliferating undifferentiated cells that often forms at the base of a cutting or after another type of mechanical damage, then root primordia initiate from the newly formed callus tissue. In contrast, in the case of the direct pattern, AR primordia form directly from the cells near the vascular system, without formation of a callus.

These two patterns of ARs can occur in the same species, but in general the indirect pattern is more often observed in difficult-to-root species while the direct pattern is characteristic of easy-to-root species (reviewed in Altamura, 1996; Guan *et al.*, 2015). The localization of AR initiation in tissues may vary from species to species.

The length of the developmental stages and the cellular origin of ARs have been shown to be species- and genotype-dependent (reviewed in Bellini *et al.*, 2014; Geiss *et al.*, 2018). This illustrates the complexity and the variability of the process and the consequent difficulties in identifying early events in complex structures such as stem cuttings.

1.3. Role of phytohormones in the control of adventitious root formation

1.3.1. The key role of auxin in the control of adventitious root formation

The plant hormone auxin or indole acetic acid (IAA) has been considered the master player in the initiation and development of ARs (Haissig, 1974; De Klerk *et al.*, 1999; Bellini *et al.*, 2014). Its exogenous application has a consistent effect across plant taxa in inducing root formation (Pacurar *et al.*, 2014b). Besides IAA, other types of auxins such as Indole-3-butyric acid (IBA), 1-naphthalen acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) have been used commercially to induce rooting from cuttings of many species because of their efficacy in stimulating ARs (De Klerk *et al.*, 1999; Pandey *et al.*, 2011).

In particular, IBA has been reported to be more effective than the other auxins in a wide range of species. This efficiency of IBA may be due to its stability upon light exposure and higher root-inducing capacity (Epstein & Ludwig-Müller, 1993; Ludwig-Müller *et al.*, 2005; Bellini *et al.*, 2014; Lakehal & Bellini, 2019). IBA is also used in combination with other auxins like 2,4-D or NAA, to stimulate more efficiently ARs in recalcitrant species of economic value (Oinam *et al.*, 2011; Pijut *et al.*, 2011). It is known that cuttings from many species have the ability to form ARs without using exogenous auxin, *e.g.* *Populus* spp. (Rigal *et al.*, 2012). In this context, wounding stimulated ARs at the base of cuttings through the accumulation of endogenous auxin via polar auxin transport (PAT) at the site of cutting (Rigal *et al.*, 2012). A high level of free IAA is required to induce ARs especially during the induction phase (Caboni *et al.*, 1997; Gaspar *et al.*, 2003). Bellamine and collaborators confirmed the important role of free IAA in the induction and expression phases at the base of *Populus tremula* × *Populus tremuloides* cuttings by using anti-auxins such as 2-phenoxy-2-methyl propionic acid (PBA) (Bellamine *et al.*, 1998). In *Eucalyptus globulus*, Negishi and collaborators found that the free IAA content was twice as high in easy-to-root cuttings compared to recalcitrant cuttings (Negishi *et al.*, 2011). The levels of auxin are tightly regulated (reviewed in Normanly *et al.*, 2010; Ljung, 2013) and the contributions of transport and biosynthesis to auxin homeostasis have been identified as being essential for AR formation (reviewed in Gonin *et al.*, 2019; Lakehal & Bellini, 2019).

In the model plant *Arabidopsis*, *superroot2-1* (*sur2-1*) overproduces auxin due to the accumulation of indole-3-acetaldoxime (IAOx), a common intermediate in the IAA and the indole-glucosinolate biosynthesis pathways (Barlier *et al.*, 2000; Mikkelsen *et al.*, 2004). This IAA-overproducing mutant develops an abnormally high number of ARs along the hypocotyl (Barlier *et al.*, 2000; Mikkelsen *et al.*, 2004). Similarly, the activation tagged *yuc1-D* mutant, which also overproduces auxin, spontaneously forms many ARs along the hypocotyl. The *YUCCA1* gene is reported to be directly involved in tryptophan-dependent auxin biosynthesis via the indole-pyruvic acid pathway (Zhao, 2001; Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011).

Pacurar and collaborators showed that the loss of function of several genes involved in auxin biosynthesis such as *ANTHRANILATE SYNTHASE ALPHA 1/WEAK ETHYLENE INSENSITIVE 2* (ASA1/WE12), *ANTHRANILATE SYNTHASE β 1/WEAK ETHYLENE INSENSITIVE 7* (ASB1/WE17) and *TRYPTOPHAN SYNTHASE BETA1* (TSB1) resulted in a reduced number of ARs in the *sur2-1* mutant background (Pacurar et al., 2014a). Chen et al. (2016) showed that the expression levels of both *YUC1* and *YUC4* increased in the mesophyll cells of leaf explants within four hours of cutting. The same authors showed that the double mutants *yuc1yuc4* and *yuc2yuc6* were partially unable to produce ARs, whereas the quadruple mutant *yuc1yuc2yuc4yuc6* was unable to produce ARs from leafy cuttings. All these results confirm the important role of auxin biosynthesis in adventitious root formation.

Polar auxin transport (PAT) plays an important role in the distribution of IAA and the establishment of IAA gradients (reviewed in Teale et al., 2006; Takahashi, 2013; Lakehal & Bellini, 2019). The surgical removal of the shoot apex, which is the major source of endogenous auxin, results in a reduction in endogenous IAA at the base of cuttings, causing a reduction in rooting (Liu & Reid, 1992). By using inhibitors of PAT, such as naphthylphthalamic acid (NPA) or 1,3,5 triiodobenzoic acid (TIBA), researchers observed a reduction in the development of ARs for many species, including *Helianthus annuus*, *Syringa vulgaris*, *Petunia hybrida* and *Oryza sativa* (Liu & Reid, 1992; Ford et al., 2002; Ahkami et al., 2013; Lin & Sauter, 2019). These experiments confirmed the importance of auxin biosynthesis at the shoot apex and the pivotal role of PAT in AR formation.

It is well known that IAA moves from cell to cell thanks to transporter proteins such as the influx carrier proteins AUXIN RESISTANT 1 (AUX1) and LIKE AUX1 (LAX), and the efflux carrier proteins such as PIN FORMED (PIN) or ATP BINDING CASSETTE B / MULTI DRUG RESISTANCE / P. GLYCOPROTEIN (ABCB/MDR/PGP) (reviewed in Takahashi, 2013). Li and collaborators found that cotyledon segments of mango (*Mangifera indica* L.) can form more ARs due to the increasing auxin concentration at the proximal cut surface via auxin influx carriers (Li et al., 2012). Sukumar et al. (2013) showed that excision of the root from *Arabidopsis* hypocotyls resulted in the stimulation of ARs at the base of the

cutting due to a 4-fold increase in auxin transport. The role of auxin polar transport was then confirmed by the characterization of mutants. Sukumar *et al.* (2013) showed that Arabidopsis mutants defective in IAA efflux transport (*pin1*, *pin3*, *pin7* and *abcb19*) had a significant reduction in ARI in de-rooted seedlings compared with the wild type. In addition, lines over expressing *ABCB19* had enhanced ARI in intact hypocotyls due to increased auxin transport (Sukumar *et al.*, 2013). Simon and collaborators demonstrated that the *PIN6* gene had a complex role in the control of auxin transport and homeostasis during AR and lateral root (LR) formation. They showed that the *pin6* knock-out mutant produced more ARs in both intact and de-rooted Arabidopsis seedlings compared to the wild type, while the *PIN6* overexpressing line developed fewer ARs compared to the wild type even after excision of the primary root (Simon *et al.*, 2016). In rice (*Oryza sativa* L.), Xu and collaborators found that the *OsPIN-FORMED1* (*OsPIN1*) gene was expressed in root primordia and AR emergence was significantly inhibited in the *OsPIN1* RNA-interference lines (Xu *et al.*, 2005). Similarly, Lin and Sauter (2019) found that the *OsPIN2* gene is expressed in epidermal cells above AR primordia and its activation controls AR emergence.

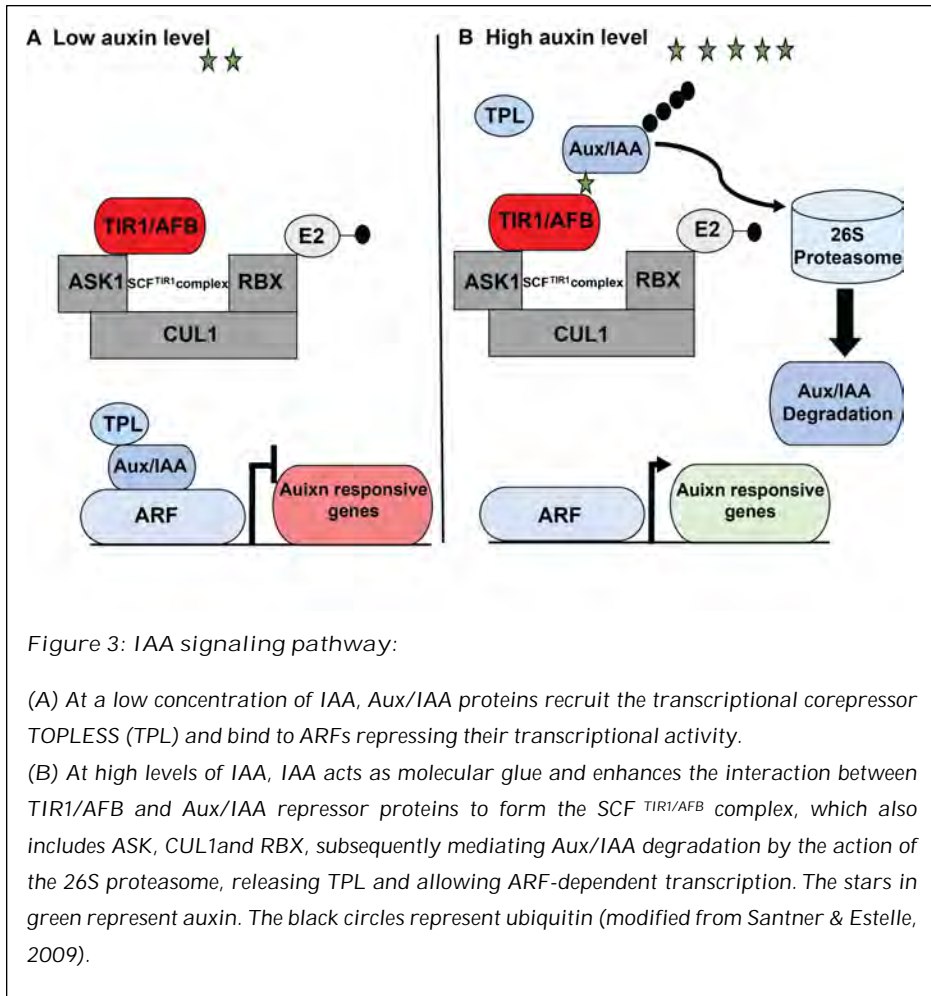
In addition to auxin polar transport, the homeostasis of auxin is controlled by conjugation with other molecules such as sugars, amino acids, or peptides or through degradation. In Arabidopsis, it has been shown that several members of the GRETCHEN HAGEN3 (GH3) family of acyl amido synthetases mediate conjugation of IAA with amino acids (Staswick *et al.*, 2005; Westfall *et al.*, 2010). Certain IAA conjugates can be hydrolyzed enzymatically and produce free IAA. This is the case when IAA is conjugated with amino acids such as alanine, leucine or phenylalanine (Kowalczyk & Sandberg, 2001; Le Clere *et al.*, 2002). In contrast, IAA conjugation with amino acids such as aspartate or glutamate produces intermediates in the oxidative degradation pathway of IAA (Östin *et al.*, 1998; Tam *et al.*, 2000). The degradation process of auxin is important for the maintenance of the auxin homeostasis in the plant (Pěnčík *et al.*, 2013; Peer *et al.*, 2013). Modification of this pathway can alter AR formation. Butler and Gallagher (2000) showed that, in stem cuttings of apple (*Malus domestica*), the expression of *ADVENTITIOUS ROOTING RELATED OXYGENASE 1* (*ARRO-1*)

was rapidly upregulated after IBA or IAA treatments to induce AR. This result suggested that this putative auxin oxidase gene could play a role in the regulation of auxin levels during AR formation in stem cuttings of apple.

In *Arabidopsis*, two genes that encode auxin oxidases have been identified (Mellor *et al.*, 2016; Porco *et al.*, 2016). *DIOXYGENASE FOR AUXIN OXIDATION 1* and 2 (*AtDAO1* and 2) act in concert with *GH3* genes to control auxin levels during plant growth and development (Mellor *et al.*, 2016; Porco *et al.*, 2016). Recently, Lakehal and collaborators (2019) showed that *AtDAO1* plays an essential role in auxin-jasmonate crosstalk during ARI in intact *Arabidopsis* hypocotyls (Lakehal *et al.*, 2019b).

The auxin signaling begins with the interaction between the endogenous IAA, which acts as a molecular glue between the TRANSPORT INHIBITOR1/AUXIN-SIGNALING F-BOX (TIR1/AFB) receptor proteins and the auxin-induced AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins that are transcriptional repressors in the auxin signaling pathway. Once a specific co-receptor complex is formed, the AUX/IAA proteins are ubiquitinated and targeted for degradation through the 26S proteasome machinery (Dharmasiri *et al.*, 2005; Parry *et al.*, 2009; Salehin *et al.*, 2015).

In the cell, when there is a low concentration of auxin, AUX/IAA repressors bind to members of the AUXIN RESPONSE FACTOR (ARF) transcription factor family and inhibit their transcriptional activity. At high concentrations of auxin, IAA acts as a molecular glue which triggers degradation of the Aux/IAA proteins, releasing the activity of ARFs which induce the expression of auxin-responsive genes (Figure 3) (Santner & Estelle, 2009).



In the model plant *Arabidopsis*, parts of the auxin signaling network controlling AR formation have been unraveled. Sorin and collaborators found that the *AUXIN RESPONSE FACTOR 17* (*ARF17*) gene, negatively regulates AR formation by repressing the expression of three auxin-inducible *GH3* genes (*GH3.3*, *GH3.5* and *GH3.6*) (Sorin *et al.*, 2005). Subsequently, Gutierrez and collaborators found that *ARF6* and *ARF8* transcription factors are positive regulators of ARI. They were shown to positively regulate the expression of *GH3.3*, *GH3.5* and *GH3.6* which, in the context of AR initiation, controls the homeostasis of jasmonate, which negatively controls ARI. Gutierrez *et al.*, (2009) also showed that the three

ARFs regulate each other's expression at the transcriptional level and at the posttranscriptional level by modulating the abundance of their respective regulatory microRNA. The microRNA *miR167* controls the transcript amount of the positive regulators *ARF6* and *ARF8*, while the negative regulator *ARF17* is regulated by *miR160* (Gutierrez et al., 2009, 2012). These transcription factors do not only act in Arabidopsis but probably also in other species. Recently it was shown that the expression of *ARF6* and *ARF8* genes increased in phloem parenchyma cells in black walnut (*Juglans nigra* L.) stem cuttings during the early stages of AR primordia formation whereas the expression of *ARF17* decreased (Stevens et al., 2018). Lakehal and collaborators showed that the F-box proteins TIR1 and AFB2 control JA homeostasis by promoting the degradation of at least three AUX/IAA (IAA6, 9 and 17) proteins that repress the transcriptional activity of *ARF6* and *ARF8* (Lakehal et al., 2019a). Overall, auxin-related genes play a central role in regulating AR formation (Pacurar et al., 2014b; Zhang et al., 2020).

Recently, it was shown that several members of the *WUSCHEL*-related *homeobox* (*WOX*) family, including *WOX11*, *WOX12* and *WOX5*, are induced by auxin and are involved in adventitious rooting in herbaceous and woody plants (Liu et al., 2014). For example, Liu and collaborators found that auxin accumulation activates the expression of *WUSCHEL RELATED HOMEBOX 11* and *12* genes (*WOX11* and *WOX12*) in leaf cuttings of Arabidopsis (Liu et al., 2014). *WOX11* responds to wounding-induced auxin accumulation in and surrounding the procambium. This gene, redundantly with its homolog *WOX12*, acts to control the transition of competent cells (procambium or its nearby parenchyma cells) into adventitious root founder cells by upregulating *BOUNDARY LATERAL DOMAIN 16* and *29* (*LBD16* and *LBD29*) genes at the base of leaf blade cuttings. In *Populus* cuttings, Xu et al., (2015b) found that the overexpression of *PeWOX11a* or *PeWOX11b* increased the number of ARs per cutting. Li and collaborators confirmed the involvement of *PeWOX11a/b* in adventitious rooting in hybrid poplar. They also showed that the overexpression of *PtoWOX5a* in the hybrid *P. alba* × *P. glandulosa*, increased the number of ARs but decreased their length (Li et al., 2018).

1.3.2. Role of other phytohormones in the control of adventitious root development

Several studies performed in different model plants and systems have reported the role of different classes of phytohormones as well as their interaction with each other and with the environment during AR development. Auxin appears as the central player which interacts with the other phytohormones in complex networks during the different stages of AR formation (reviewed in da Costa *et al.*, 2013; Bellini *et al.*, 2014b; Lakehal & Bellini, 2019).

1.3.2.1. Jasmonic acid has a controversial role in the control of adventitious root development

The plant hormone Jasmonic acid and its derivatives such as Methyl ester jasmonate (MeJA) or Jasmonoyl-isoleucine (JA-Ile), which is the active form, are collectively called jasmonates (JAs) and are oxylipin-derived hormones. JAs are very important molecules that regulate many genes involved in the control of many physiological processes in plant responses to biotic and abiotic stresses as well as plant growth and development (Wasternack & Strnad, 2018).

The biosynthesis of JAs has been extensively studied in many varieties of plants but mostly in the model plants *Arabidopsis thaliana* and *Lycopersicon esculentum* (tomato). In *Arabidopsis*, three pathways for the synthesis of JAs have been identified. They include the octadecane pathway starting from α -linolenic acid (18:3) and the hexadecane pathway starting from hexadecatrienoic acid (16:3) (Chini *et al.*, 2018; Ruan *et al.*, 2019). The biosynthesis of JA takes place in three cell compartments (Figure 4). In the chloroplast, the 13-LIPOXYGENASE (LOX) enzymes convert the α -linolenic acid (18:3) (α -LeA)(18:3) and the hexadecatrienoic acid (16:3) into 13-hydroperoxy-octadecatrienoic acid (13-HPOT), then then 13-HPOT is oxidized by the ALLENE OXIDE SYNTHETASE (AOS) enzyme to form the allene oxide which is then converted into 12-oxo-phytodienoic acid (12-OPDA) and its 16-carbon homolog the dinor-oxo-phytodienoic acid (dnOPDA) by the ALLENE OXIDE CYCLASE (AOC) enzymes. The 12-OPDA and the dnOPDA are converted to JA in the peroxisome by the 12-OXOPHYTODIENOIC ACID REDUCTASE 3 (OPR3) enzyme, giving rise to formation of the final JA (Feussner & Wasternack, 2002;

Gfeller *et al.*, 2010; Wasternack & Strnad, 2018). In the cytoplasm, JA is converted into active, inactive and partially active structures such as MeJA, JA-Ile, cis-jasmone (CJ) and 12-hydroxyjasmonic acid (12-OH-JA) by different chemical reactions (Reviewed in Ruan *et al.*, 2019) (Figure 4).

The conjugation of JA with the amino acid isoleucine (Ile) by the JASMONATE RESISTANT 1/GRETCHEN HAGEN3.11 (JAR1/GH3.11) enzyme produces the bioactive form jasmonoyl-isoleucine (JA-Ile). JAR1/GH3.11 belongs to group I of the auxin-inducible GH3 family (Staswick *et al.*, 2002; Staswick & Tiryaki, 2004). Interestingly, it was recently shown that three enzymes belonging to group II of the GH3 family contribute to the maintenance of JA homeostasis (Gutierrez *et al.*, 2012). Indeed, GH3.3, GH3.5 and GH3.6 enzymes conjugate free JA with other amino acids such as tryptophan, methionine or aspartate, thereby inactivating it. In this way they contribute to diminishing the JA pool in the intact hypocotyl of *Arabidopsis* and control AR initiation downstream of auxin (Gutierrez *et al.*, 2012).

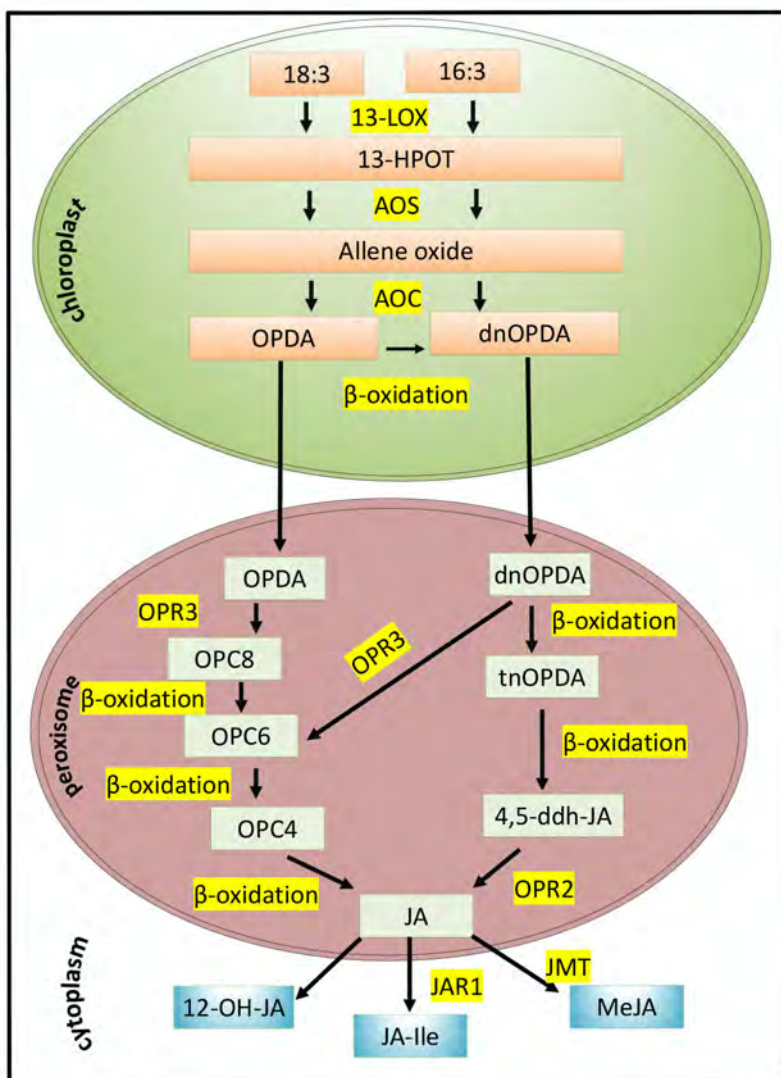


Figure 4: Jasmonate biosynthesis takes place in three different compartments of plant cells.

The first steps of JA biosynthesis occur in the chloroplast, where the LOX, AOS and AOC enzymes catalyze the production of 12-OPDA and dnOPDA, which are then transported to the peroxisome.

In the peroxisome the reduction of the cyclopentanone ring of OPDA is catalyzed by the peroxisomal enzyme OPR3. Three cycles of β -oxidation finally lead to the production of JA which is transported to the cytoplasm.

In the cytoplasm, JAR1 catalyzes the formation of the amino acid conjugate JA-Ile, which is the active form of jasmonate. JA is also metabolized into different structures by different chemical reactions such as MeJA and 12-OH-JA. (modified from Ruan et al. 2019).

Similar to auxin, the bioactive JA-Ile acts as a molecular glue or ligand necessary for the formation of the coreceptor complexes between JASMONATE ZIM DOMAIN (JAZ) transcriptional repressors and the Skp/Cullin/F-box CORONATINE INSENSITIVE 1 (SCF^{COI1}) receptor (Hoo & Howe, 2009; Pauwels & Goossens, 2011). In cells with sufficient bioactive JA-Ile, the JAZ repressor proteins bind to the COI1 receptor to form the Skp/Cullin/F-box CORONATINE INSENSITIVE1-JAZ complex (SCF^{COI1-JAZ}) (Sheard *et al.*, 2010). This results in the poly-ubiquitination and degradation of the JAZ repressor proteins through the 26S proteasome pathway, releasing the transcriptional activity of the master regulator MYC2/JASMONATE INSENSITIVE1 (MYC2/JIN1) and other MYC transcription factors to trigger the expression of JA-responsive genes (Figure 5). In contrast, when there is a low level of JA-Ile in the cells, JAZ repressor proteins

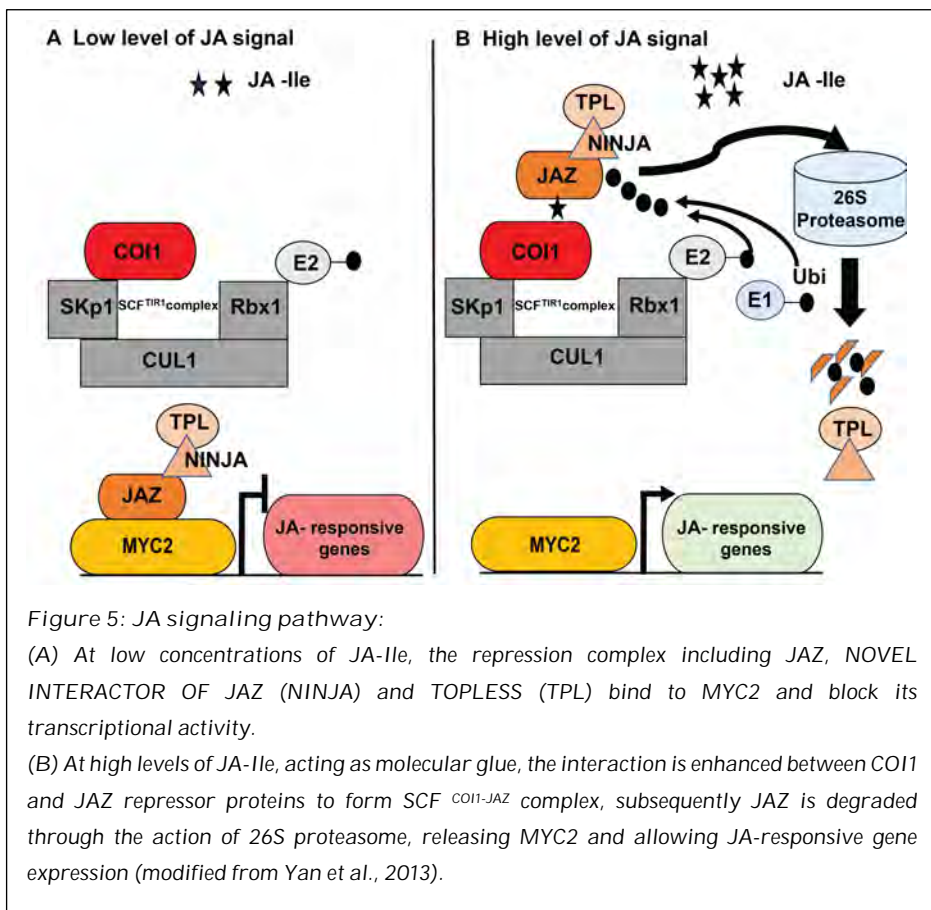


Figure 5: JA signaling pathway:

(A) At low concentrations of JA-Ile, the repression complex including JAZ, NOVEL INTERACTOR OF JAZ (NINJA) and TOPLESS (TPL) bind to MYC2 and block its transcriptional activity.

(B) At high levels of JA-Ile, acting as molecular glue, the interaction is enhanced between COI1 and JAZ repressor proteins to form SCF^{COI1-JAZ} complex, subsequently JAZ is degraded through the action of 26S proteasome, releasing MYC2 and allowing JA-responsive gene expression (modified from Yan *et al.*, 2013).

bind physically to transcription factors such as MYC2, MYC3 or MYC4, repressing their transcriptional activity (Figure 5) (Yan *et al.*, 2013).

JA plays a role in the control of primary root growth, lateral and adventitious root formation (Staswick *et al.*, 1992; Velloso *et al.*, 2007; Gutierrez *et al.*, 2012; Gasperini *et al.*, 2015; Fattorini *et al.*, 2018). However, it appears that the role of JA in the control of ARI depends on the species, the organ and the growth conditions (reviewed in Lakehal & Bellini, 2019).

It has been shown by several researchers that exogenous application of JA inhibits AR formation in various species. For example, Chen and collaborators found that exogenous application of MeJA inhibits AR formation in *Bupleurum kaioi* cuttings (Chen *et al.*, 2007). Lischweski *et al.* (2015) showed that leafy stem cuttings of petunia (*Petunia hybrida*) produced significantly fewer ARs compared to controls after treatment with JA, JA-Ile or OPDA. They also showed that exogenously applied JA repressed the positive effect of auxin (Lischweski *et al.*, 2015).

Gutierrez and collaborators showed that very low concentrations of JA significantly reduced the average number of ARs in Arabidopsis etiolated hypocotyls (Gutierrez *et al.*, 2012). Recently, Fattorini and collaborators found that expression of the negative regulator *ARF17* was very quickly induced by exogenously applied MeJA (10 μ M) (Fattorini *et al.*, 2018). All these findings support the hypothesis that JA is an inhibitor of AR formation. This hypothesis has been corroborated in Arabidopsis etiolated hypocotyls, by genetic approaches (Gutierrez *et al.*, 2012). For example, the loss-of-function mutants *coi1-16*, *myc2-1*, *myc2myc3myc4* and also the knockout mutant *jar1-12/gh3.11*, all altered JA signaling, resulting in the development of more ARs compared to the wild type, while the overexpressing *35S:MYC2* and *35S:JAR1* lines developed significantly fewer ARs than the wild type (Gutierrez *et al.*, 2012). These genetic data indicate that the COI1-dependent JA signaling pathway negatively regulates AR formation through MYC2, MYC3 and MYC4 transcription factors (Gutierrez *et al.*, 2012). More recently, Lakehal and collaborators found that the loss-of-function mutants *ninja-1* and *ninja-2* produced slightly fewer ARs than the wild type, and that the double mutants *ninja-1myc2-322B* and *ninja-2myc2-322B*, in which *myc2-322B* is a gain-of-function mutant, exhibit constitutively upregulated JA signaling with

a very strong reduction in the number of ARs compared to the wild type (Lakehal et al., 2020). Transcriptomic analysis of the *ninja-1myc2-322B* double mutant showed that many genes involved in JA biosynthesis as well as most JAZ genes were upregulated. Hormone quantification in this mutant confirmed that the levels of cis-OPDA, JA and JA-Ile were significantly higher compared to the wild type (Lakehal et al., 2020).

Nevertheless, some studies present the opposite theory, namely that JA is a positive regulator of ARI. Ahkami et al. (2009) found that excision of petunia cuttings led to rapid accumulation of JA at the wounding site as well as to an accumulation of soluble and insoluble carbohydrates, associated with increased transcriptional and metabolomic reprogramming at the base of the leafy stem cuttings, and an induction of AR formation (Ahkami et al., 2009). They concluded that JA could be the inducer of AR initiation in petunia cuttings. Lischweski and collaborators also proposed that JA acts as a positive regulator for ARI in petunia leafy stem cuttings. They showed that the downregulation by RNA interference of the *PhAOC* gene, involved in JA biosynthesis, reduced the number of ARs in the cuttings of the *AOC-RNAi* lines (Lischweski et al., 2015). A positive role for MeJA in promoting ARs in tobacco (*Nicotiana tabacum*) and Arabidopsis thin cell layers (TCLs) has also been shown (Fattorini et al., 2009, 2018). These authors observed the positive effect of MeJA only when the TCLs were cultured in a rooting medium containing a high (10 μ M) concentration of IBA and a low concentration of cytokinin (0.1 μ M kinetin) but they did not observe it when the TCLs were kept on hormone-free medium. Zhang and collaborators showed that leaf explants of Arabidopsis treated with coronatine-O-methyloxime (COR-MO) could not develop ARs (Zhang et al., 2019). The COR-MO acts as a JA-Ile competitive antagonist because it exhibits strong activity in preventing COI1-JAZ interaction (Monte et al., 2014), and this resulted in inhibition of the JA signaling machinery (Zhang et al., 2019).

In conclusion, the role of JA in the control of adventitious rooting could depend on the species and/or on the growth conditions. More investigation is needed to better understand the role of JAs during AR development.

1.3.2.2. The role of cytokinins in the control of adventitious root development

Cytokinins (CKs), a group of plant growth regulators, are involved in the regulation of many plant growth and development processes such as cell division, leaf senescence and caulogenesis, including adventitious shoot formation and rhizogenesis (lateral and adventitious root formation). CKs are mainly produced in the roots (Aloni *et al.*, 2005; Hwang & Sakakibara, 2006; Agulló-Antón *et al.*, 2014) although all organs can produce them (Hwang & Sakakibara, 2006; Chickarmane *et al.*, 2012; Kieber & Schaller, 2014). Trans-zeatin riboside (tZR) is considered the major form in the xylem sap, while iso-pentenyl-adenine (iP) type cytokinins are the major form found in the phloem sap (Corbesier *et al.*, 2003; Hirose *et al.*, 2008). Kudo and collaborators proposed a model for long-distance CK transport through the plant vascular system (Kudo *et al.*, 2010); in this model tZR is considered a long-distance messenger for shootward transport while iP is involved in rootward transport.

The role of CKs in AR formation has emerged from studies in various species and systems at different development stages of adventitious rooting. For example, trans-zeatin riboside present in the xylem sap acts as an inhibitor of AR formation in cucumber (*Cucumis sativus*) hypocotyls (Kuroha, 2002). Recently, Mao and collaborators found that the exogenous application of CK inhibited the development of adventitious root primordia in apple (*Malus domestica*) stem cuttings (Mao *et al.*, 2019), while Werner and collaborators showed that the overexpression of the CYTOKININ OXIDASE/DEHYDROGENASE (CKX1) gene involved in the degradation of CKs in tobacco (*Nicotiana tabacum*) and Arabidopsis reduced the endogenous cytokinin content and resulted in increased AR formation (Werner *et al.*, 2001, 2003). In line with this, Avalbaev *et al.* (2016) found that MeJA induced the accumulation of CKs by repressing the expression of the CKX1 gene (Avalbaev *et al.*, 2016). These data suggest a probable link between JA and CKs in the control of AR formation. Recently, Lakehal and collaborators confirmed this link between these two inhibitory hormones in intact Arabidopsis hypocotyls. They showed that CK signaling was induced by JA which, resulted in the repression of ARI (Lakehal *et al.*, 2020).

It has been reported that CKs modify the expression of auxin transporters

encoding genes such as *PIN1* and thus modulate the auxin distribution and gradient during LR formation (Laplaze et al., 2007; Růžicka et al., 2009). In *Arabidopsis*, Della Rovere et al. (2013) found that CKs regulate the expression of *PIN1* and *LAX3* in such a way that this could regulate the establishment of ARs (Della Rovere et al., 2013). In 2014, Agulló-Antón et al. showed that auxin negatively affected CK biosynthesis and/or transport in carnation (*Dianthus caryophyllus*) stem cuttings during the initial steps of adventitious rooting (Agulló-Antón et al., 2014).

It is well known that the interaction between auxin and cytokinin plays a key role during plant organogenesis. There are several reports showing that auxin/cytokinin concentration ratio is a critical and important factor in regulating the cell fate acquisition in *in vitro* systems (De Klerk et al., 2001; Falasca et al., 2004; Kareem et al., 2016). In apple microcuttings, low CK levels are required at the early induction stage of AR formation in order to trigger cell divisions. But at later stages, CKs become inhibitors of AR formation (De Klerk et al., 1999; De Klerk, 2002). Histological analysis has determined that cytokinins inhibit the differentiation of AR primordia, mostly during the early stage of their development (Bollmark & Eliasson, 1986). In *Populus* cuttings, Ramirez-Carvajal et al. (2009) showed that the type-B CK response regulator (*PtRR13*) negatively controls the formation of AR primordia (Ramírez-Carvajal et al., 2009). More recently, Bustillo-Avendaño et al. (2018), confirmed the dual role of CKs in *de novo* organogenesis processes in *Arabidopsis* leaf explants including the petiole. They found that CKs could be positive regulators of cell division in the vasculature during the first stage of ARI but negative regulators of root primordia initiation (Bustillo-Avendaño et al., 2018). Hormone quantification at the base of cuttings from different species showed that auxin and cytokinin have opposite content levels during the 48 hours after cutting. Auxin levels are always high during the early stages (induction stage) whereas CK contents are low (Maldiney et al., 1986; Bollmark et al., 1988; Berthon et al., 1989; Kevers et al., 1997; De Klerk et al., 1999; Dong et al., 2012). All these results confirm that auxin and cytokinin play antagonistic roles during AR formation.

1.3.2.3. The role of salicylic acid during adventitious root development

Salicylic acid (SA), is a stress-related hormone which has been reported to be a positive regulator for AR formation in different species. *Arabidopsis* mutants defective in SA biosynthesis *eds5-1* and *eds5-2* developed fewer ARs compared to the wild type (Gutierrez *et al.*, 2012), and treatment of mung bean hypocotyl cuttings with SA significantly increased AR numbers in a dose- and time-dependent manner (Yang *et al.*, 2013). Yang and collaborators suggested that SA promotes AR formation by stimulating the differentiation of cells at the origin of a new apical meristem. They observed that, after 48 hours of SA treatment, explants developed more root primordia than the control hypocotyls treated with water only (Yang *et al.*, 2013). Agulló-Antón and collaborators (2014) analyzed the endogenous content of SA at the base of carnation stem cuttings, treated or untreated with auxin. They observed that endogenous SA levels were high after the excision and dropped during cold storage and rehydration, both in non-treated and auxin-treated cuttings. Once the cuttings were transferred to rooting conditions, with or without auxin treatment, the SA level remained constant in non-treated cuttings whereas it was highly induced 12 hours after transfer to rooting conditions in auxin-treated cuttings. The SA level rapidly came back to the steady state level 12 hours later (Agulló-Antón *et al.*, 2014). Recently, Pasternak *et al.* (2019), showed that exogenous SA promoted AR formation but inhibited primary and lateral root growth in a dose-dependent manner in *Arabidopsis*. They showed that the different tested concentrations of SA could activate auxin synthesis in a similar way, but affected auxin transport in a concentration-dependent manner (Pasternak *et al.*, 2019). All these findings indicate that SA plays a positive role in AR formation and interacts with auxin at different levels.

1.3.2.4. The role of abscisic acid during adventitious root development

Absciscic acid (ABA) is another class of stress-related hormone but in contrast to SA, it has been shown to negatively regulate AR formation. For example, the ABA-deficient tomato (*Lycopersicon esculentum*) mutant *notabilis* (*not*) developed

prolific adventitious roots (Thompson *et al.*, 2004). Still in tomato, McAdam *et al.* (2016) suggested that the shoot-derived ABA inhibited the development of both ARs and LR through ethylene- and auxin-mediated pathways (McAdam *et al.*, 2016). In flooded rice plants, ABA also negatively affected AR emergence, probably via the altered balance between ethylene (ET) and gibberellic acid (GA) (Steffens *et al.*, 2006). In a recent study, Vaičiukynė and collaborators (2019) showed that exogenous ABA application to aspen cuttings significantly reduced the number of ARs per explant (Vaičiukynė *et al.*, 2019).

1.3.2.5. The role of ethylene during adventitious root development

Ethylene (ET) is also a stress-related hormone that has been shown to have a positive effect on AR formation in a variety of plants such as apple, rice, tomato, sunflower, petunia and mung bean (reviewed in Lakehal & Bellini, 2019; Gonin *et al.*, 2019). In tomato, Negi and collaborators found that the *Never ripe* (*Nr*) mutant, which is insensitive to ethylene and delayed in ripening, developed fewer ARs than the wild type (Negi *et al.*, 2010).

Transcriptomic analyses that have been performed with petunia cuttings suggest that ethylene plays the role of a stimulator of AR formation (Druege *et al.*, 2014). Veloccia *et al.* (2016) showed that ET enhanced the formation of ARs when combined with IBA in dark-grown *Arabidopsis thaliana* seedlings. It was suggested that ET would enhance the conversion of IBA into active free IAA (Veloccia *et al.*, 2016). Recently Bai and collaborators showed that IBA stimulated ET production during AR development in stem cuttings of apple (*Malus domestica*) (Bai *et al.*, 2020). These data suggest that ET acts in synergy with auxin in promoting AR formation; however, it interacts not only with auxin but also with other phytohormones during AR formation. For example, in deep water rice (*Oryza sativa*), AR development is induced when the plants are submerged. The addition of paclobutrazol, an inhibitor of GA biosynthesis, inhibits root emergence, demonstrating that it depends on GA activity (Steffens *et al.*, 2006). On the other hand, root growth rate depends on GA concentration and exogenous ABA acts as a potent inhibitor possibly of GA but also of ethylene signaling. On its own, GA is inefficient in promoting AR but acts in synergy with the ET which accumulates when the plants are submerged. These results indicate that root

emergence and elongation are distinct phases of AR growth that are regulated through different networking between ethylene, GA and ABA signaling pathways (Steffens *et al.*, 2006). Ethylene has also been shown to stimulate rooting of hypocotyls of difficult-to-root Norway spruce cuttings by accelerating the breakdown of CKs (Bollmark & Eliasson, 1990b). All these findings suggest that ET is a positive regulator of AR formation, acting either in synergy with other positive regulators or by stimulating the degradation of repressors.

1.3.2.6. Role of gibberellins (GAs), strigolactones (SLs) and brassinosteroids (BRs) in the control of adventitious root development

The roles of GA, SLs and BRs in AR formation are still not clearly understood, but some studies have shown that they participate in this process in different species. For example, in stem cuttings of tobacco (*Nicotiana tabacum*), Niu *et al.* (2013) found that the exogenous application of GAs reduced the number of ARs (Niu *et al.*, 2013). Similarly, in stem cuttings of the hybrid aspen clone T89 (*P. tremula* × *P. tremuloides*) and in etiolated *Arabidopsis* hypocotyls, Mauriat *et al.* (2014) found that GA treatment negatively affected AR formation, suggesting that the inhibitory effect of GAs is mediated by the perturbation of polar auxin (more precisely auxin efflux in *Populus* and both efflux and influx in *Arabidopsis*), and is independent of the JA signaling pathway and SL biosynthesis and signaling pathways (Mauriat *et al.*, 2014). Recently, Moriconi and collaborators showed that GAs appear to be involved in inhibition of AR development in barley (*Hordeum vulgare*) (Moriconi *et al.*, 2019). In contrast, in deep water rice, Steffens *et al.* (2006) found that GAs promote AR formation *via* interaction with ET signaling (Steffens *et al.*, 2006). This interaction between GAs and ET may be specific to flooded species but this is still uncertain and awaiting more investigation (Bellini *et al.*, 2014).

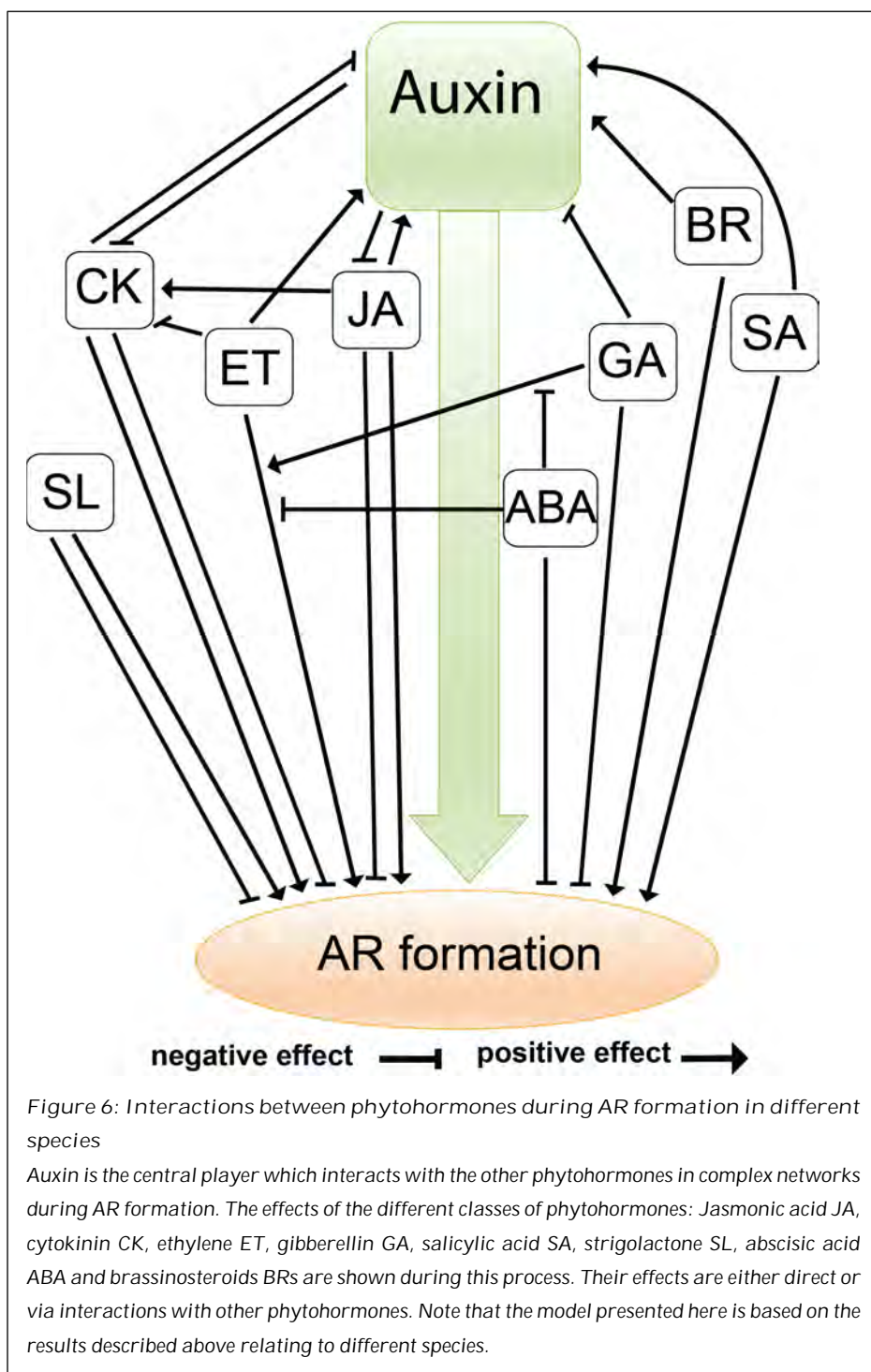
Strigolactones (SLs) repress AR formation in *Arabidopsis* and pea (*Pisum sativum*) (Rasmussen *et al.*, 2012). The cited authors found that AR formation was enhanced in the SL-deficient and SL-response mutants in both species. In addition, SLs repress AR formation independently from IAA, ET and CK

pathways (Rasmussen *et al.*, 2012, 2017). Kohlen and collaborators showed that SLs inhibit AR formation in tomato (*Solanum lycopersicum*). They found that *CAROTENOID CLEAVAGE DIOXYGNASE 8 (SICCD8)* knock-down transgenic lines with different levels of strigolactone reduction produced more ARs compared to control plants (Kohlen *et al.*, 2012). Despite their negative effect, SLs were shown to promote AR formation in rice through modulating auxin transport (Sun *et al.*, 2015). This discrepancy in the effect of SLs suggests that their role in control AR formation may be species-specific and requires further investigation.

Brassinosteroids (BRs) have been shown to have a positive effect on AR formation in most of the published works that describe the effects of exogenously supplied BRs during AR development. For example, in a recent study, Uzunoğlu & Gökbayrak reported positive effects of BRs on rooting of hard-to-root grape (*Vitis* spp.) cuttings (Uzunoğlu & Gökbayrak, 2018). The stimulation of AR formation by the application of BRs was also observed in geranium (*Pelargonium* sp.), *Coleus* (*Plectranthus forskohlii*) stem cuttings (Swamy & Seeta Ram Rao, 2006, 2010) and Norway spruce adult cuttings (Ronsch *et al.*, 1993).

In *Arabidopsis thaliana* hypocotyls, Maharjan *et al.* (2014) showed that exogenously applied BRs stimulated ARI in the hypocotyl of the *gulliver1/sur2-7* mutant, a weaker allele than the auxin overproducer mutant *sur2-1*, which accumulates less auxin and therefore does not normally develop ARs. Maharjan *et al.* (2014) observed that BR treatment stimulates auxin biosynthesis. These data suggest that the positive effect of BRs during AR formation is probably dependent on auxin biosynthesis (Maharjan *et al.*, 2014).

In conclusion, all the results described above demonstrate the complexity of the interactions between the phytohormones that control AR formation (Figure 6). Further detailed investigation is required to clarify the discrepancy in the effects on adventitious rooting of some important hormones.



1.4. Environmental factors influencing adventitious root formation

It is known that AR formation is controlled by many endogenous and environmental factors including nutrients, temperature and light conditions (reviewed in Bellini *et al.*, 2014; Geiss *et al.*, 2018).

1.4.1. Mineral nutrition

Mineral nutrients classified as macronutrients (e.g., nitrogen, phosphorus, potassium, magnesium, calcium and sulfur) and micronutrients (e.g., iron, boron, copper, chloride, molybdenum, manganese and zinc), are essential for plant growth and have specific functions in plant metabolism. These nutrients are considered to be key factors determining root morphogenesis (Bellini *et al.*, 2014; Geiss *et al.*, 2018). The adventitious rooting process and mineral nutrition are intimately related (reviewed in Bellini *et al.*, 2014). For example, both number and length of ARs are positively correlated with the initial total nitrogen (N) concentration in the cuttings of pelargonium (*Pelargonium × hortorum*) (Druege *et al.*, 2004), *Chrysanthemum indicum* (Druege *et al.*, 2000), *Euphorbia pulcherrima* (Zerche & Druege, 2009) and petunia (Zerche *et al.*, 2016). The effect of external nitrogen application in favoring AR formation by cuttings has also been shown for *Eucalyptus globulus* (Schwambach *et al.*, 2005, 2015) and petunia (Hilo *et al.*, 2017). In a recent study, Yang and collaborators (2019) found that limitation of nitrogen in cuttings of petunia inhibited AR formation. They suggested that the nitrogen limitation in these cuttings attenuated auxin signaling by modifying the expression levels of specific ARFs, *GH3* and *SAUR* genes, thereby suppressing the auxin dose–response of ARI (Yang *et al.*, 2019). Besides nitrogen, other minerals such as phosphorus, potassium, magnesium, calcium, iron and manganese also influence rooting of cuttings (reviewed in Li *et al.*, 2009; Bellini *et al.*, 2014; Geiss *et al.*, 2018; Druege *et al.*, 2019; Gonin *et al.*, 2019).

1.4.2. Temperature

Temperature is another environmental factor that can impact many aspects of the adventitious rooting process starting from the growth rate of the donor plant up to root development, including root initiation, growth, orientation and rooting time (Kristiansen *et al.*, 2005). Temperature may influence adventitious rooting capacity by interacting with several aspects such as, water and nutrient uptake, enzymatic activity and phytohormone responses (reviewed in De Almeida *et al.*, 2017; Geiss *et al.*, 2018). For example, Da Rocha Corrêa & Fett-Neto (2004) showed that subjecting the donor plants of *Eucalyptus saligna* cuttings, an easy-to-root species, to a moderate heat shock at 40 °C increased the root density and the root length in the cuttings thus obtained. In contrast, in the case of the difficult-to-root *E. globulus* cuttings, lower temperatures were more effective with the best rooting response observed with day/night cycles of 30 °C /20°C. In pelargonium cuttings, Druege & Kadner (2008) found that lowering the air temperature during cutting cultivation under low light, increased sugar levels in the cuttings as well as repressed leaf senescence and contributed to improved rooting at the base of the cuttings.

Based on the findings summarized above, we can conclude that there is an interaction network between environmental factors and AR formation and these factors seem to be very important parameters when considering rooting in vegetative propagation practices. Hereafter, we will discuss the effect of light, which is considered the most significant environmental factor.

1.4.3. Light: an environmental cue that controls adventitious root development

Among environmental factors light is, perhaps, the most important one that controls the photo-biological processes in plants (Alabadí & Blázquez, 2009). Plants have the ability to perceive different light signals, which regulate different aspects of development during their life cycle, for example seed germination, shade avoidance, de-etiolation, phototropism and flowering (Figure 7) (Quail, 2002a; Schepens *et al.*, 2004; Fittinghoff, 2008; Alabadí & Blázquez, 2009; Kozai *et al.*, 2016; Paik & Huq, 2019) . Plants have at least five different classes of

photoreceptors which are responsible for perceiving different light qualities and intensities (Figure 7). In Arabidopsis, five phytochromes (PHYA, PHYB, PHYC, PHYD and PHYE) have been identified that detect and respond to red light (RL) or far red (FR) light (600-750 nm). Blue/UV-A light (320-500 nm) is perceived by the cryptochromes CRY1, CRY2 and CRY3, the phototropins PHOT1 and PHOT2, and the F-box containing Flavin binding proteins such as the three LOV domain proteins, ZETLUPE (ZTL), KELCH REPEAT F-BOX1 (FKF1) and LOV KELCH protein2 (LKP2). Finally, UV-B (280-320nm) is perceived by UVB-RESISTANCE 8 (UVR8) (Schepens *et al.*, 2004; Bae & Choi, 2008; Xu *et al.*, 2015a; Paik & Huq, 2019) (Figure 7). Recent advances in plant photoreceptor research have identified novel roles of the receptors other than photoperception. For example, PHYB has been shown to act as a thermosensor and to integrate light and temperature signaling pathways (Jung *et al.*, 2016; Legris *et al.*, 2016). This supports the suggestion that photoreceptors are involved not only in light perception but also in the perception of a wide range of environmental cues suggesting a role as “multisensors”.

Phytochromes are present in the form of two interconvertible isoforms – the biologically inactive form Pr and the biologically active Pfr – in response to FR and R light respectively (Sager *et al.*, 1988; Galvão & Fankhauser, 2015). The active Pfr forms of phytochromes translocate from the cytoplasm to the nucleus, where they interact directly with a class of basic helix-loop-helix (bHLH) transcription factors called *PHYTOCHROME INTERACTING FACTORS* (PIFs) to trigger a transcription cascade that leads to light-regulated gene expression. Among the transcription regulators that control light signaling pathways, PIFs have been characterized as key players in transducing light signals perceived by phytochromes (Sakamoto & Nagatani, 1996; reviewed in Quail, 2002b; Leivar & Monte, 2014; Paik *et al.*, 2017).

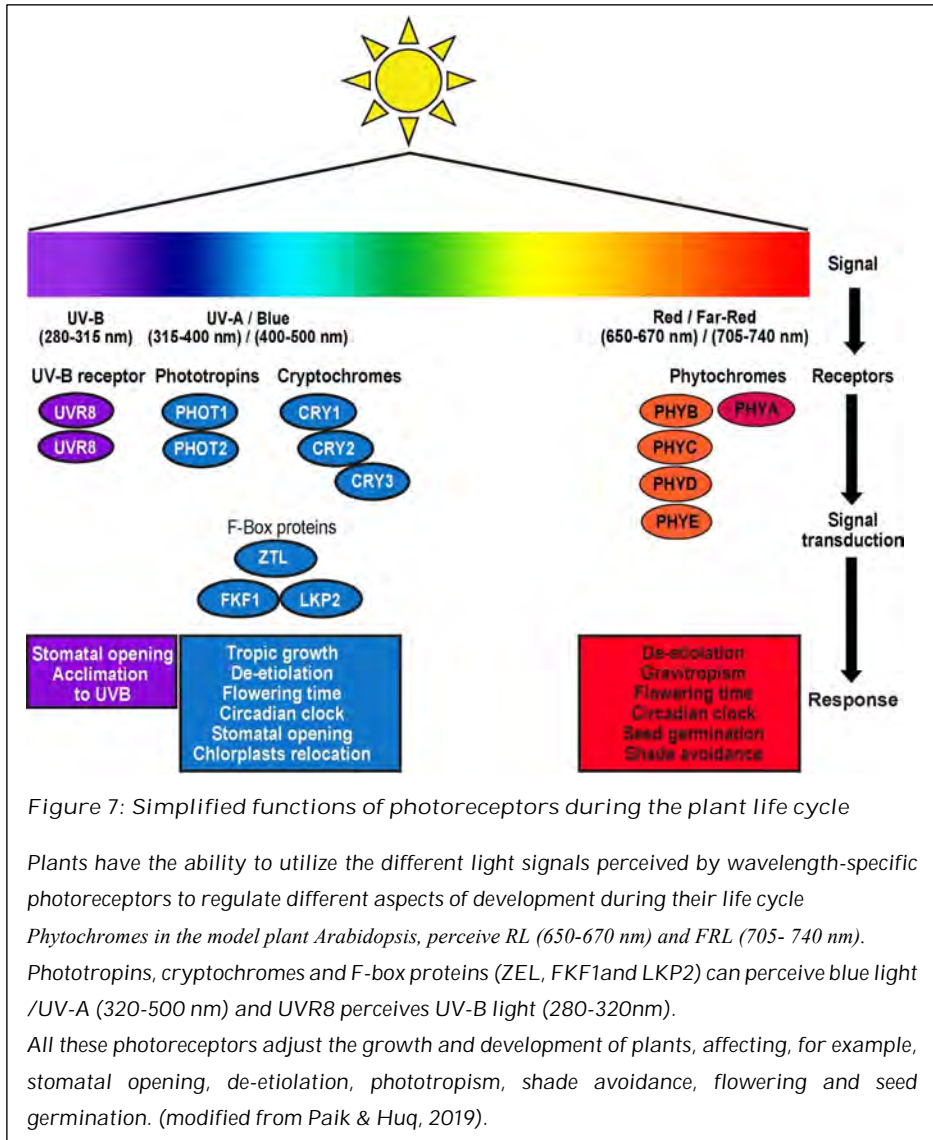


Figure 7: Simplified functions of photoreceptors during the plant life cycle

Plants have the ability to utilize the different light signals perceived by wavelength-specific photoreceptors to regulate different aspects of development during their life cycle

Phytochromes in the model plant Arabidopsis, perceive RL (650-670 nm) and FRL (705- 740 nm).

Phototropins, cryptochromes and F-box proteins (ZEL, FKF1 and LKP2) can perceive blue light /UV-A (320-500 nm) and UVR8 perceives UV-B light (280-320nm).

All these photoreceptors adjust the growth and development of plants, affecting, for example, stomatal opening, de-etiolation, phototropism, shade avoidance, flowering and seed germination. (modified from Paik & Huq, 2019).

It has been demonstrated that light and phytohormone signaling pathways interact during plant growth and development (Lau & Deng, 2010). For example, the active PIFs stimulate auxin biosynthesis and promote auxin signaling responses *via* direct interaction with Auxin Response Factors (ARFs) (reviewed in Küpers *et al.*, 2020). A low R:FR ratio and high ambient temperature has been shown to stimulate the expression of *YUCCA* genes through PIF4, PIF5 and PIF7, confirmed by an accumulation of auxin in the shoot and in the elongating hypocotyl of *Arabidopsis* seedlings (Franklin *et al.*, 2011; Hornitschek *et al.*, 2012). Liu and collaborators reported that after etiolated *Arabidopsis* and tomato seedlings were exposed to light for different lengths of time, both IAA biosynthesis and transport increased in the hypocotyl sections (Liu *et al.*, 2011). In sunflower *Helianthus annuus*, a low R:FR light ratio increased auxin biosynthesis (Kurepin *et al.*, 2007).

Light also controls auxin polar transport through the PIN form (PIN) proteins. For example, in *Arabidopsis* seedlings, a low R:FR light ratio leads to redistribution of the PIN3 protein in the endodermis of the hypocotyl from a downward apical distribution towards a more lateral outward distribution (Keuskamp *et al.*, 2010). This results in the redirection of the auxin flow towards the hypocotyl epidermis, which triggers the elongation of the whole hypocotyl (Keuskamp *et al.*, 2010). Similarly, the phototropism of the hypocotyl induced by a unilateral blue light is due to enhanced auxin signaling on the side of the hypocotyl which does not receive light (Ding *et al.*, 2011). The asymmetry in the auxin distribution is attenuated in the *pin3* and *phot1* mutants, and it has been shown that PIN1, PIN3, PIN4 and PIN7 are required for normal phototropic bending (Haga & Sakai, 2012; Willige *et al.*, 2013).

Light also interacts with JA signaling. Recently it has been established that JA, plays an important role in the inhibition of hypocotyl growth regulated by PHYA and PHYB. The expression of JA biosynthesis genes and the abundance of the JAR1 protein that catalyzes the production of the active form JA-Ile are regulated by PHYA (Hsieh & Okamoto, 2014). In shade, for example under dense canopies, the reduction of the R/FR light ratio promotes plant growth in order to outcompete neighboring plants but has a negative effect on JA signaling. In *Arabidopsis*, R and B lights stabilize the JA-related transcription factors MYC2,

MYC3, and MYC4 through the activation of the corresponding photoreceptors while darkness destabilizes them (Chico *et al.*, 2014). The positive effect of blue light on MYC2 regulation has also been observed in Norway spruce (*Picea abies*), in which it has been shown that MYC2 gene expression is upregulated under blue light (OuYang *et al.*, 2015). In contrast to MYCs, low R/FR light ratio stabilizes seven of the 10 JAZ repressors and reduces their degradation by JA. The fact that FR-enriched light has the opposite effect on the stability of MYCs and JAZ proteins might explain, on the molecular level, why canopy shade represses JA-mediated defenses, facilitating reallocation of resources from defense to growth (Chico *et al.*, 2014).

Light signaling pathways also interact with CK signaling. Dobisova and collaborators demonstrated that both light quality and quantity could control spatiotemporal specificity of *CYTOKININ INDEPENDENT1* (*CKI1*) expression in etiolated seedlings of *Arabidopsis* via *PHYA*-mediated signaling (Dobisova *et al.*, 2017). Light can also stimulate an increase in endogenous cytokinin content in several species (Köhler *et al.*, 1980; Qamaruddin & Tillberg, 1989; Zubo *et al.*, 2008). Oh and collaborators demonstrated that ARF6 interacts with both PIF4 and brassinosteroid-signaling transcription factor BZR1. These three transcription factors act together in the regulation of gene expression and cell elongation (Oh *et al.*, 2014).

All these results suggest that there is a link between light and phytohormone signaling, involving IAA, JA, CKs and BRs, and promoting or inhibiting AR formation.

1.4.3.1. The role of light in adventitious root formation

Light is a very important parameter in optimizing rooting conditions for many types of cuttings during vegetative propagation (Bellini *et al.*, 2014). It has been accepted that rooting of cuttings is influenced by different aspects of light: quality, intensity and duration of exposure or photoperiod (Antonopoulou *et al.*, 2004; Poudel *et al.*, 2008; Iacona & Muleo, 2010; Ragonezi *et al.*, 2010a; Wu & Lin, 2012; Daud *et al.*, 2013; Hoffman *et al.*, 2016; Christiaens *et al.*, 2019). For example, in *Pinus sylvestris*, Niemi *et al.*, (2005) found that light sources with

different wavelengths could significantly affect AR and mycorrhiza formation. Hoffman and collaborators (2016) showed that *Populus deltoides* microcuttings, grown in a medium containing IBA, performed best under a wide spectrum fluorescent light regime (Hoffman et al., 2016). Adventitious root formation is affected by interactions between phytohormones and light (Bellini et al., 2014; De Almeida et al., 2017). For instance in the model plant *Arabidopsis*, Sorin and collaborators found that the light hypersensitive *ago1* mutant had a low capacity to form ARs, probably because of a combination of disturbed auxin homeostasis and general upregulation of light responses (Sorin et al., 2005).

It has been reported that the expression of the positive regulators of adventitious rooting *ARF6* and *ARF8* are induced by FR light in donor plants as well as in microcuttings of the difficult-to-root *Eucalyptus globulus* (de Almeida et al., 2015; Ruedell et al., 2015). This probably contributed to improving rooting of these recalcitrant species, which had lower endogenous IAA levels and higher transcript levels of the rooting inhibitory genes *TPL*, *IAA12/BODENLOS* and the cytokinin-related gene *ARR1* (de Almeida et al., 2015; Ruedell et al., 2015). In *Protea cynaroides*, which is considered a difficult-to-propagate ornamental plant, root formation is induced by a reduction in the endogenous concentration of 3,4-dihydroxybenzoic acid and ferulic acids when the plants are kept under R light (Wu & Lin, 2012). Recently, we found that R light promotes ARI in de-rooted Norway spruce seedlings by repressing the accumulation of the wound-induced phytohormones JA and CK (Alallaq et al., 2020).

In a recent report, Christiaens and collaborators reported that low FR light improved rooting of *Chrysanthemum morifolium* cuttings as well as decreasing the inhibiting effect of the auxin transport inhibitor NPA, which confirms that phytochromes play an important role in AR formation via their interaction with auxin (Christiaens et al., 2019).

The effect of light on rooting is often species-dependent. For example, BL retards AR formation in several species such as *Prunus serotina* (Fuernkranz et al. 1990), *Morinda citrifolia* (Baque et al. 2010) and *Betula pendula* (Pinker et al. 1989) while it has a positive effect on other species such as sweet basil (*Ocimum basilicum*) (Lim & Eom, 2013) and cherry (*Prunus avium* × *P. cerasus*) rootstock (Iacona & Muleo, 2010). In a recent study, Gil et al. (2020) reported that BL and

NAA treatment significantly improved rooting of single leaf-bud cuttings of *Chrysanthemum*. The authors found that the abundance of *LATERAL ORGAN BOUNDARIES DOMAIN 1 (LBD1)* transcripts was higher after blue light treatment, suggesting that the rapid induction of *CmLBD1* may play a critical role in initiating AR formation (Gil et al., 2020).

On the other hand, previous studies have reported that low light intensity could increase soluble sugars at the base of cuttings from many species, causing increased rooting (Druege et al., 2004; Druege & Kadner, 2008; Husen, 2008; Klopotek et al., 2010; Tombesi et al., 2015). It has been reported in the literature that the level of light irradiance on stock plants and cuttings may influence AR formation positively or negatively and this influence is species-dependent (Strömquist & Eliasson, 1979; Eliasson & Brunes, 1980; Pinker et al., 1989; Fuernkranz et al., 1990; Palanisamy & Kumar, 1997; Rapaka et al., 2005; Ragonezi et al., 2010a; Libao et al., 2020). For instance, Bollmark & Eliasson found that high irradiance significantly reduced the rooting of *Picea abies* cuttings compared with cuttings grown under low light intensity. They concluded that the high light levels promoted the accumulation of endogenous cytokinin which, in turn, reduced the rooting ability of the cuttings (Bollmark & Eliasson, 1990a). In a recent study, Libao et al. (2020) found that high light intensity accelerated AR development of lotus (*Nelumbo nucifera*) cuttings. They observed that the plants under high light intensity had higher IAA content compared with those under low light or in darkness. They suggested that AR formation is significantly affected by light and directly regulated by auxin (Libao et al., 2020). Based on the results discussed above, we conclude that there is a complex network between light quality and / or quantity and adventitious rooting among different plant species.

1.5. Adventitious rooting in trees

Paper pulp, timber production and energy feedstocks are mostly obtained from plantation forestry. One way of increasing the yield of tree plantations is to use vegetative propagation technology. Rooting of stem cuttings used for transplant production is considered an advanced technique in vegetative propagation, aimed

at maintaining the desired traits of elite plants as well as producing large numbers of these valuable plant species in a cost-efficient way (De Klerk et al., 1999; Preece, 2003; Leakey, 2004; Pijut et al., 2011). Adventitious rooting is an essential step in the vegetative propagation of many different commercial tree species such as Poplar, Pine, Norway spruce and Eucalyptus (De Klerk, 2002; Geiss et al., 2018). Therefore, a deep understanding of the molecular mechanisms underlying adventitious root formation in trees should open new avenues to enhance the efficiency of vegetative propagation of economically important plants (Bellini et al., 2014; Legué et al., 2014). In the last few decades, good progress has been made in the study of molecular and genetic aspects of AR development in *Arabidopsis* and other model species but the underlying molecular mechanisms in woody species such as Poplar and Norway spruce are largely unexplored and still need more investigation (Bellini et al., 2014).

The rapid development of sequencing technologies has allowed the identification of differentially expressed genes in relation to developmental events, including the molecular and genetic aspects that control AR formation in woody species (reviewed in Legué et al., 2014). More specifically, in *Populus* spp., Ramírez-Carvajal et al. (2009) showed that during the first 48 h after excision, in the stem of the hybrid aspen *Populus tremula* × *Populus alba*, an important remodeling of the expression of genes encoding Aux/IAA and ARF proteins occurred. In this research, they also demonstrated that the cytokinin type-B response regulator *PtRR13*, which acts downstream of CKs, is a negative regulator of AR primordia formation in intact plants. The reduction in the CK content in cuttings reduces the effect of the *PtRR13* gene and allows the expression of ethylene and auxin signaling pathways that coordinate to induce AR formation (Ramírez-Carvajal et al., 2009). Rigal and collaborators described the transcriptional profiles at the base of stem cuttings of *P. trichocarpa* during different stages of AR formation. They showed that several genes from the APETALA2-domain-containing transcription factor family including *PtAINTEGUMENTA-Like1* (*PtAIL1*), (*PtAIL9*), *PtPLETHORA1.1* (*PtPLT1.1*) and *PtBABYBOOM* (*PtBBM*) were highly expressed at the base of the cuttings during stage one (primordium organization) and stage two (primordium differentiation) of AR development (Rigal et al., 2012). Their study demonstrated that *PtAIL1* transcription factor is a key

regulator of AR development in poplar. Based on their transcriptomic data analysis, several members of the ARF family were also found to be specifically expressed during rooting formation.

Recently, Shu *et al.* (2019) showed changes in gene expression in cuttings of clone 84K of the hybrid *Populus alba* × *P. glandulosa*. They showed that genes involved in hormone signaling were significantly differentially expressed during the induction phase *i.e.* the first 24h after stem excision. This finding is consistent with all observations in *Populus* spp. described above. Shu *et al.* (2019) also reported that *PagF-BOX-LIKE1* (*PagFBL1*), the hybrid poplar homolog of the Arabidopsis auxin receptor *TIR1*, is a key regulator in the auxin signaling pathway, which regulates adventitious rooting through its interaction with Aux/IAA28 in clone 84K. Based on their transcriptomic data analysis, several members of the ARF and GH3 gene families were found to be specifically expressed during AR initiation, suggesting that the corresponding signaling module identified in Arabidopsis (Gutierrez *et al.*, 2012) is evolutionarily conserved in woody plant species (Shu *et al.*, 2019). Indeed, Ruedell and collaborators found that, in *Eucalyptus globulus*, the expression of *ARF6* and *ARF8* genes was induced when the donor plant of this difficult-to-root species was treated with FR enriched light. Their expression was also induced at the cutting site of microcuttings. This increase in *ARF6* and *8* expression was associated with a significant improvement in rooting ability (Ruedell *et al.*, 2015). Recently, Stevens and collaborators observed that *ARF6*, *ARF8* and *ARF17* were differentially expressed in the rooting competent phloem parenchyma cells of black walnut (*Juglans nigra* L.) cuttings during the early stages of AR primordia formation. They found that the *ARF17* expression decreased concomitantly with the increase in *ARF6* and *ARF8* expression, suggesting that these genes have a similar function as in Arabidopsis in controlling AR1 in black walnut (Stevens *et al.*, 2018). In the hybrid poplar *P. deltoides* × *P. euramericana*, clone 'Nanlin 895', Cai and collaborators showed that the microRNA miR167, which targets *ARF6* and *ARF8*, is a negative regulator of AR formation, which is consistent with the results for Arabidopsis presented by Gutierrez *et al.* (2009). They found that overexpression of the microRNA resistant *PeARF8.1^{mut}* enhanced adventitious rooting ability in poplar (Cai *et al.*, 2019). These data confirmed that *ARF6/ARF8*

mediated signaling module plays an important regulatory role among species. In another recent functional study in hybrid poplar clone 'Nanlin 895', Liu et al. (2020) found that overexpressing the microRNA *PemiR160a*, which targets *PeARF17*, had a negative effect on AR formation in the hybrid poplar. This suggested that *PeARF17* could be a positive regulator of AR initiation in contrast to its effect in *Arabidopsis* (Gutierrez et al., 2009). This was confirmed by the overexpression of *PeARF17* which promoted ARI in the cuttings of the hybrid poplar (Liu et al., 2020). These results suggested that *ARF17* function in controlling ARI could be species-dependent.

Other transcription factors which are evolutionarily conserved have been shown to play a role in AR development among taxa. For example several members of the *WUSCHEL-related homeobox* (*WOX*) family, including *WOX11*, *WOX12* and *WOX5* which are induced by wounding and auxin have been shown to play a role in AR development in *Arabidopsis* and *Populus* spp. (Liu et al., 2014; Xu et al., 2015b; Hu & Xu, 2016; Bustillo-Avendaño et al., 2018). In the hybrid poplar *P. deltoides* × *P. euramericana* (clone 'Nanlin 895') stem cuttings, the overexpression of *PeWOX11a* or *PeWOX11b* increased the number of ARs per explant (Xu et al., 2015b) and the overexpression of *PtoWOX5a* in the hybrid poplar *P. alba* × *P. glandulosa* increased the number of ARs per cutting but had a negative effect on AR length (Li et al., 2018). Recently, Li et al. (2020) showed that *PtoWUSa*, another member of the *WOX* family, could be involved in AR development in poplar through regulating polar auxin transport in ARs (Li et al., 2020).

As an adaptative response, AR formation was recently associated with salt stress (Zhang et al., 2020). Zhang and collaborators demonstrated that a salt-responsive gene module negatively regulated AR formation in poplar. In this module, the expression of the transcription factor *bZIP53* is induced by salt stress and exhibits transactivation activity. Its overexpression in poplar lines inhibited AR growth. The *bZIP53* transcription factor directly regulates the expression of the *IAA4-1* and *IAA4-2* genes, which negatively regulate AR development in poplar (Zhang et al., 2020).

In order to explore the molecular mechanisms that control AR development in conifers, a simple and synchronized experimental model system, based on the ability of hypocotyl cuttings from young *Pinus* spp. seedlings to develop ARs after a pulse-treatment with an optimal dose of different auxin compounds, has been used (Grönroos & Arnold, 1987; Diaz-Sala et al., 1996; Lindroth et al., 2001a). Histology analysis performed with *P. taeda*, *P. contorta* and *P. radiata* showed that similar anatomical modifications could be observed during ARI in hypocotyl cuttings from these three species (Grönroos, 1987; Diaz-Sala et al., 1996; Lindroth et al., 2001b,a; Ricci et al., 2008). The cambial region of the hypocotyl, which is located centrifugal to the resin canal at the xylem poles, has the ability to form ARs and exhibit rapid cell division as well as re-orientation of division planes to organize the root meristem when submitted to exogenous auxin (Abarca & Díaz-Sala, 2009a; Díaz-Sala, 2014; Pizarro & Díaz-Sala, 2019).

Pinus contorta was first used as a model species to study the molecular basis of AR development in conifers (Lindroth et al., 2001a,b; Brinker et al., 2004). It was shown that the transcript level of *PcCDC2*, which encodes a cyclin-dependent kinase of the PSTAIRE class, increased linearly during the first 12 days of IBA-induced AR development in *P. contorta* hypocotyl cuttings as compared to the control (Lindroth et al., 2001a). S-adenosylmethionine synthase (SAMS) activity, which is required for the methylation of several substances, increased four-fold at day three after IBA-induced AR development in hypocotyl cuttings of *P. contorta* as compared to the control (Lindroth et al., 2001b). A transcriptomic analysis during AR formation in *P. contorta* hypocotyl cuttings using an array of expressed sequence tags (ESTs) from *P. taeda*, revealed the timing of the molecular events that occur during auxin-induced AR development (Brinker et al., 2004). The cited authors found that during the first three days after auxin treatment, the genes involved in protein synthesis were upregulated, while the expression of genes related to protein degradation decreased; they reported the opposite expression trend when ARs formed and elongated. During this period they also observed the downregulation of cell wall synthesis genes and the upregulation of genes involved in weakening cell walls and cell adhesion. The opposite expression trend for cell wall-remodeling genes was observed from day

3 to day 12 in the root primordia, root meristem and root formation phases (Brinker et al., 2004).

During the first 24 hours of auxin-induced AR development in *P. taeda* hypocotyl cuttings, Hutchison et al. (1999) reported high expression of α -EXPANSIN genes, which are responsible for cell wall loosening (Hutchison et al., 1999). These results confirmed that modifications of the cell wall occur during the formation of AR primordia in conifers.

In conifers, as in other species, local auxin gradients are required for ARI (Díaz-Sala et al., 1996; Brunoni et al., 2014, 2019; Pizarro & Díaz-Sala, 2019). Brinker and collaborators showed that active auxin transport was reduced at the beginning of AR development in *P. contorta* hypocotyls, but during root meristem differentiation this process was induced in order to activate the auxin response machinery (Brinker et al., 2004). The cited authors suggested that AR formation was regulated by exogenous auxin supply, which stimulates the activation of competent cells and endogenous auxin that stimulates the establishment of the new meristem.

Very few genes with a well-defined function in AR formation have been identified in conifers. For example, the nodulin-like (5NG4) from *Pinus taeda* (Busov et al., 2004), the *SHORT-ROOT* (*PrSHR*) gene from *Pinus radiata* (Solé et al., 2008) and the *SCARECROW-LIKE* (*SCR-L* or *SCL*) genes from *Pinus radiata* (Sánchez et al., 2007) are considered to be associated with AR development in hypocotyl cuttings. All these genes are induced in the presence of exogenous auxin which is needed for *Pinus* hypocotyl cuttings to develop ARs.

SHR and *SCL* belong to the *GRAS* family of transcription factors, which was named after the first three genes that were identified *GIBBELLIC ACID INSENSITIVE* (*GAI*), *REPRESSOR OF GAI* (*RGA*) and *SCARECROW* (*SCR*). It has been reported in *P. radiata*, that several members of the gene family are involved in the establishment of AR meristem (Sánchez et al., 2007; Solé et al., 2008; Abarca et al., 2014). *GRAS* proteins are a family of putative transcription factors acting in relation to several aspects of plant growth and development such as meristem maintenance and development and signal transduction (Stuurman et al., 2002; Bolle, 2004; Sánchez et al., 2007).

During the initial stages of AR development, the expression patterns of both *PrSCL1* and *PrSHR* overlap. After cell division, the expression of both genes increases and is specifically localized in the cambial region (Sánchez *et al.*, 2007; Solé *et al.*, 2008). This pattern overlaps with the asymmetric distribution of auxin which has been reported. Several pine *GRAS* genes are expressed during embryo development and during the initial stages of AR formation. This could reflect an embryo type competence for adventitious organogenesis in cuttings (Abarca *et al.*, 2014; Brunoni *et al.*, 2019).

Based on all results discussed above, we can conclude that further investigations examining the functional genes that are involved in AR formation of tree species will improve our understanding of the molecular mechanisms that control this important process in economically important trees species and will be particularly valuable when devising strategies for large-scale vegetative propagation.

2. Aim of Thesis

The broadest aim of this thesis is to go a step further in the understanding of the adventitious root formation and translate the knowledge acquired with *Arabidopsis* to woody species. Specifically, this work was supported by the recent availability of the reference genomes of *Populus* spp. and Norway spruce (*Picea abies*) to explore the molecular and mechanistic foundations of AR formation in woody species and check whether or not there is conservation of the molecular mechanisms identified in *Arabidopsis*.

The thesis is organized into three chapters (papers) that focus on different set of research questions.

Chapter I.

The objective here was to unravel the role of light spectral quality in the control of ARI in *P. abies* de-rooted seedlings.

Chapter II.

The goal here was to understand why some genotypes can root easily and others not?

In an attempt to answer this question:

- ❖ We compared the transcriptome data from cambium tissues obtained immediately after cutting and 24 h later by Laser Capture Microdissection (LCM) from *P. trichocarpa* × *P. maximowiczii* (clone OP42) which we defined as easy-to-root from woody stem cuttings and the hybrid aspen *P. tremula* × *P. tremuloides* (clone T89) which we qualified as difficult-to-root from woody stem cuttings, under hydroponic conditions.
- ❖ We investigated the role of four transcriptional factors, ARF6, ARF8, ARF17 and MYC2 in hybrid aspen.
- ❖ We did a comparative study between T89 and Op42 in term of adventitious rooting, under hydroponic and *in vitro* conditions.

Chapter III.

The aim of this chapter was to unravel the mechanisms controlling adventitious root formation of aspen clones from the Swedish Aspen (SwAsp) collection using *in vitro* cuttings (A comparative study).

3. Results and discussion

Paper I: Red light controls adventitious root regeneration by modulating hormone homeostasis in *Picea abies* seedlings

Rhizogenesis can be affected by light at different stages and in several ways (reviewed in De Almeida et al., 2017), and light has been reported to influence rooting either positively or negatively in a species-dependent manner (Strömquist & Eliasson, 1979; Bollmark & Eliasson, 1990; Poudel et al., 2008; Gutierrez et al., 2009a; Iacona & Muleo, 2010; Wu & Lin, 2012; Daud et al., 2013; Christiaens et al., 2019). Different light quality treatments using Light Emitting Diode (LED) technology appear very promising for improving rooting of cuttings, but their use with forest trees including conifers is still limited (Christiaens et al., 2016; De Almeida et al., 2017).

Very little is known about the exact mode of action of light in the control of AR formation. In Paper I, we combined physiological, molecular and hormone-based approaches coupled with extensive anatomical analysis to explore the role of light spectral quality in AR regeneration in conifers using *Picea abies* (Norway spruce) de-rooted seedlings as our model system. We took advantage of the robust experimental system involving hypocotyl cuttings from young Norway spruce (*Picea abies*) seedlings (Paper I, Figure S1A, B, C).

Auxin is not sufficient to stimulate adventitious rooting in Norway spruce hypocotyl cuttings kept under white light

First, we tested the response of N. spruce hypocotyl cuttings to three different white light (WL) regimes: 1) In a growth chamber with long-day conditions with 16 h of light at 75 $\mu\text{mol} / \text{m}^2/\text{s}$ (400 to 700 nm); 20°C day temperature and 18°C night temperature; light was provided by Cool White TL-D tubes (Paper I, Figure. S2 A). 2) In a Percival growth cabinet with long-day conditions with 16h light at 69 $\mu\text{mol} / \text{m}^2/\text{s}$ (400 to 700 nm); light was provided by Cool White fluorescent tubes (Paper I, Figure S2B). 3) In a Percival growth cabinet (E-30NL/floraLED) under constant white light (cWL) and constant temperature

(20°C); light, at 75 $\mu\text{mol /m}^2\text{/s}$ (400 to 700 nm) was provided by six CLF *floraLED* modules (Paper I, Figure S2 C). Under all these conditions, the hypocotyl cuttings were kept in hormone-free (HF) distilled water and none of them developed any ARs. Therefore, we tried to stimulate rooting with exogenous application of three different auxins: IAA (Indole Acetic Acid), NAA (1-Naphtalene Acetic Acid) and IBA (Indole Butyric Acid) at two concentrations (1 or 5 μM). The three auxin compounds were chosen because of their different features in terms of stability, metabolism and transport, but they all trigger the auxin signaling machinery. None of the auxins were able to stimulate the development of ARs under WL conditions (Paper I, Figure 1A, B, C). These data are in line with previous work (Bollmark & Eliasson, 1990a) which showed that Norway spruce hypocotyl cuttings grown under high WL irradiance were unable to develop ARs. Our results suggest that WL represses the formation of ARs on Norway spruce hypocotyl cuttings and that exogenous auxin is not sufficient to release this inhibitory effect.

Constant-red light promotes adventitious rooting in Norway spruce seedlings

Several studies have addressed the positive effect of red light (RL) in the control of adventitious rooting in different species (Poudel *et al.*, 2008; Baque *et al.*, 2010; Daud *et al.*, 2013), but the exact molecular mechanisms underlying its effect have remained largely elusive. Hence, we wondered whether RL could promote ARI in Norway spruce de-rooted seedlings. We grew three-week-old de-rooted Norway spruce seedlings, in distilled water, under either constant white light (cWL) (Paper I, Figure S2C) or constant red light (cRL) (Paper I, Figure S2D). ARs were counted at different time points for a period of 30 days (Paper I, Figure 1D). In contrast to cWL, the de-rooted seedlings grown under cRL produced ARs at the base of the hypocotyls. Under these conditions, ARs started to emerge after 15 days and continued to increase over time, reaching an average of 2.5 roots/per cutting (Paper I, Figure 1D). These data indicate that cRL promotes ARI even in the absence of exogenously applied auxin, possibly by repressing the negative regulators of ARI, whereas cWL inhibited this process. All

these results are line with previous reports showing the positive effect of RL on AR formation in different species (Wu & Lin, 2012; Daud *et al.*, 2013; Poudel *et al.*, 2008).

Light quality affects hormone content during the early stages of AR formation

Recently we showed that JA and CK cooperatively repress AR initiation (ARI) in *Arabidopsis* hypocotyls (Lakehal *et al.*, 2020). Although the relationship between light quality and hormone signaling is complex (reviewed in Lau & Deng, 2010; De Almeida *et al.*, 2017), we hypothesized that the light may perturb the balance between hormones or their signaling pathways during ARI in Norway spruce hypocotyls. To test this hypothesis, we quantified the endogenous content (precursors, active molecules and their conjugates) of different hormones known to either inhibit or induce AR development, including Jasmonates, CKs and IAA, at the base of de-rooted hypocotyls, during the early events of ARI (Paper I, Figure 2A-G). Our data showed that under cWL the endogenous IAA content increased after cutting and was higher than in seedlings kept under cRL. These results suggested that the inability to initiate ARs in cWL could not be explained by a reduction in the auxin content, which is in line with the fact that exogenous applications of IAA cannot stimulate ARI under these conditions (Paper I, Figure 1A, B, C). In contrast, under cWL, we observed an accumulation of isopentyl-adenine-type (iP-type) cytokinins, including the precursors iP riboside 5'-monophosphate (iPRMP) and the iP ribosides (iPR), leading to an increased CK content (Paper I, Figure 2E, F; Supplemental data set 2). Our data were in agreement with previous reports showing that a putative cytokinin accumulated in Norway spruce seedlings grown under a high WL irradiance (270 $\mu\text{E}/\text{m}^2/\text{s}$), and thought to inhibit rooting of cuttings (Bollmark & Eliasson, 1990a).

Interestingly, we found that under cRL conditions, although the free IAA content was significantly reduced compared to that under cWL 24 h after cutting and continued decreasing over time (Fig. 2 A), seedlings developed ARs. This could

be explained by a significant reduction in the endogenous level of JA, JA-Ile and CKs compared to the situation under cWL (Fig. 2 A-G). These results suggest that the positive effect of cRL on ARI was not the result of modification of IAA homeostasis; rather it was a consequence of a decrease in the content of the negative regulators JA, JA-Ile, and CKs.

Jasmonate and Cytokinin repress IAA- and cRL-induced adventitious root initiation

In an attempt to better understand how auxin, JA and CK interact during ARI in Norway spruce hypocotyl cuttings were kept under cRL in either a distilled water control or distilled water complemented with IBA or NAA, JA, 6-Benzylaminopurine (BAP), or a combination of these hormones.

Our results revealed that all tested concentrations of IBA and NAA significantly increased, in a dose dependent manner, the number of ARs per cutting compared with the control (Paper I, Figure A, B). Notably, IBA appeared to be the most efficient auxin, which is in line with several reports describing IBA as the most effective auxin within a wide range of species (Reviewed in Geiss *et al.*, 2018; Stevens *et al.*, 2018).

We then tested the effect of exogenously applied JA and BAP at different concentrations. We showed that both JA and BAP inhibited ARI in a concentration-dependent manner (Paper I, Figure 3C, D). In order to check whether or not JA and/or CK repress the positive effect of auxin under cRL, hypocotyl cuttings were treated with either IBA + JA or IBA + BAP. Our results showed that both JA and BAP repressed the positive effect of IBA (Paper I, Figure 3E, F). These results indicate that JA and BAP act downstream of auxin signaling to repress ARI in Norway spruce hypocotyl cuttings. Our results are in line with those of Gutierrez *et al.* (2012) and Lakehal *et al.* (2020), who showed that JA and CK act downstream of auxin signaling in intact *Arabidopsis* hypocotyls.

In order to check whether JA and BAP have an additive or synergistic effect, hypocotyl cuttings were treated with JA + BAP, under cRL. We did not observe any additive or synergistic effect (Paper I, Figure 3G), suggesting that JA and

CK act in the same pathway. These results are in line with our recent data on intact *Arabidopsis* hypocotyls, which showed that CK signaling is induced by JA to repress AR formation (Lakehal *et al.*, 2020).

Constant RL downregulates JA signaling in de-rooted Norway spruce hypocotyls

The fact that JA and JA-Ile contents were reduced in hypocotyls kept under cRL compared to cWL (Paper I, Figure 2B) prompted us to check whether the expression of genes involved in JA biosynthesis or JA signaling was affected at the base of the hypocotyls kept under cRL. We searched the Norway spruce genome for putative orthologs of the *Arabidopsis* key genes in JA signaling or biosynthesis. We identified eleven *CORONATINE INSENSITIVE 1* *AtCOI1* related genes, (Xie *et al.*, 1998) (Paper I, Figure S3A), five putative orthologs of *AtMYC2* transcription factor (Lorenzo *et al.*, 2004) (Paper I, Figure S3B), respectively one and three putative orthologs of *JASMONATE ZIM-DOMAIN3* (*AtJAZ3*) and *AtJAZ10* transcriptional repressors (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007) (Paper I, Figure S3C), and two putative orthologs of *ALLENE OXYDE CYCLASE* (*AtAOC*), which encodes a key enzyme in the JA biosynthesis pathway (Stenzel *et al.*, 2012) (Paper I, Figure S3D). We confirmed that the expression of *PaMYC2-like* (MA_15962g0010), *PaJAZ3-like* (MA_6326g0010), *PaJAZ10-like* (MA_10229741g0010) and *PaAOC-like* (MA_56386g0010) was induced by exogenously applied JA (Paper I, Figure 4A).

Next, we analyzed the expression of these genes, together with that of *PaCOI1-like* (MA_108477g0010), at the base of hypocotyl cuttings kept under cWL or cRL and at several time points after cutting (Paper I, Figure 4B). In cRL, the relative amount of transcripts of *PaMYC2*, *PaJAZ3*, and *PaAOC* was slightly reduced compared to that observed in cWL.

These data are in agreement with the reduced content of JA-Ile in the hypocotyl cuttings kept under cRL compared to cWL (Paper I, Figure 2F and Supplemental data set 2). We also showed that the expression of *PaMYC2-like*, *PaJAZ10-like*, and *PaCOI1-like* was slightly upregulated 24h after cutting

under cRL compared to cWL (Paper I, Figure 4B), but at 48 h and 72 h, the expression of *PaMYC2-like*, *PaJAZ10-like* and *PaJAZ3-like* was downregulated (Paper I, Figure 4) further confirming that JA signaling is repressed under cRL compared to cWL. This repression is possibly due to the reduction in JA and JA-Ile.

We concluded that when de-rooted hypocotyls are kept under cRL, the JA and JA-Ile endogenous contents decrease faster than under cWL. This results in the downregulation of JA signaling. This is in line with our previous results showing that the reduction in JA and JA-Ile contents and the downregulation of the JA signaling pathway contribute to improving ARI in intact *Arabidopsis* hypocotyls (Gutierrez *et al.*, 2012, Lakehal *et al.*, 2020).

Anatomical characterization of the rooting stages in Norway spruce hypocotyls in the presence or absence of auxin or jasmonate

In order to identify the cells or tissues at the origin of ARs that could be the targets for hormone treatments such as auxin or JA, we analyzed and compared the anatomy of hypocotyl cuttings kept under cRL, in hormone-free distilled water or in the presence of IBA or JA, at time 0 (immediately after cutting), 3, 5, 10, 13 and 15 days after cutting (Paper I Figure. 5 and 6).

In hormone-free distilled water, the induction phase takes place during the two first days after cutting. During this period no anatomical modification was observed (Figure 8A, B). From 72 h to 312 h after cutting, cell division occurred, followed by the organization of AR clusters of dividing cells (Figure 8C-F). All these events represented the initiation stage. Fifteen days after cutting, emerging AR primordia and elongating ARs were observed (Figure 8G). In order to understand when exogenous JA inhibits the ARI process, we compared the anatomy of hypocotyls kept under cRL, in hormone-free distilled water or in the presence of IBA or JA, at different time points after cutting (Paper I Figure 5 and 6).

This detailed histological and anatomical analysis allowed us to demonstrate that exogenous auxin accelerated AR development compared to that in HF medium, while exogenously applied JA repressed the very early stages of ARI since no

divisions could be observed in JA-treated hypocotyls.

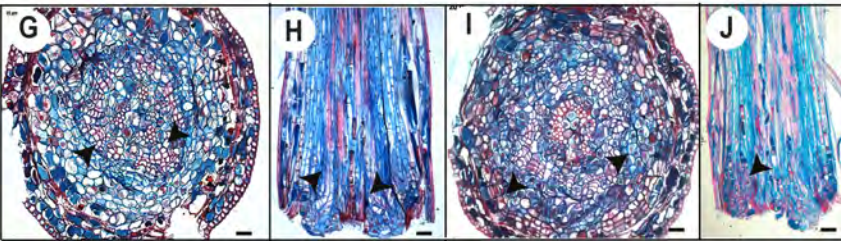
In summary, in paper I we demonstrated that cRL induces AR regeneration by both repressing JA biosynthesis and signaling and inhibiting wounding-induced CK accumulations. Underlying this interaction, our data suggest that (at least) part of the signaling module governing ARI in *Arabidopsis* seems to be evolutionarily conserved.

Time 0

48h after cutting

72h after cutting

120h after cutting



312h after cutting

360h after cutting

Figure 8: Histological events during adventitious root formation in Norway spruce hypocotyls under cRL

(A–B) Anatomical structure at the base of the hypocotyl of seedlings kept in hormone-free distilled water at time zero (immediately after cutting): (A) cross section (B) longitudinal section. e, epidermis; Co, cortex; Ph, phloem; en, endodermis; c, cambium region; P, pith; x, xylem.

(C) 48 h after cutting no anatomical change could be observed.

(D) 72 h after cutting, a few cells in the cambial zone and in the adjacent phloem re-acquire meristematic features, with dense cytoplasm and large nuclei; Inset shows a higher magnification.

(E–F) 120 h after cutting, periclinal and anticlinal divisions can be clearly observed in the cambial zone and in the outermost layers of the phloem region (arrows) in cross section (E) and in longitudinal section (F), showing that these cells are organized into vertical files externally but adjacent to the vascular cylinder.

(G–H) 240 h after cutting, radial rows of trachea elements (Te) around the xylem are observed (arrows) in cross section (G) and longitudinal section (H).

(I–J) 312 h after cutting: clusters of dividing cells (meristemoid structures Me) are observed at the periphery of the trachea elements (arrows) in cross section (I) and longitudinal section (J).

(K–L) 360 h after cutting, AR primordium (ARP) and emerging AR (arrows) in longitudinal section (K) and cross section (L). e, epidermis; Co, cortex; Ph, phloem; en, endodermis; c, cambium region; P, pith; x, xylem.

Additional results linked to paper I

Constant blue light (cBL) inhibits ARI in de-rooted Norway spruce hypocotyls

To investigate why Norway spruce seedlings did not produce ARs under white light conditions, we also tested their responses to constant blue light (cBL) (Figure 9 A, B) and observed that under these conditions de-rooted seedlings were still unable to regenerate AR (Figure 11A). These results raised the possibility that in cWL, the blue light component of the spectrum had a negative effect on ARI. To test this hypothesis, hypocotyl cuttings were kept under cWL but a yellow filter was added to remove most of the BL component compared to regular WL (Figure 9C, D). Under these conditions 83% of the cuttings developed at least 1 AR (Figure 11A). These results confirmed that the BL component of the WL spectrum had an inhibitory effect on ARI.

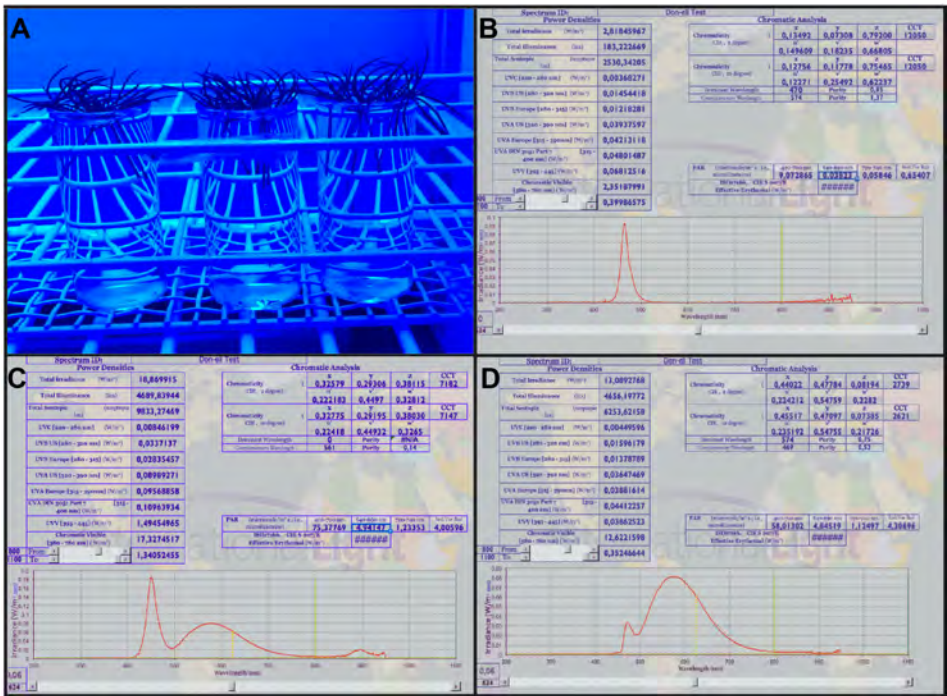


Figure 9: Blue light represses adventitious root initiation in Norway spruce de-rooted seedlings

(A) Hypocotyl cuttings transferred to 24 ml vials filled with distilled water and placed in monochromatic cabinets equipped with blue LEDs (460 nm, $/9 \mu\text{mol}/\text{m}^2/\text{s}$).

(B) Spectral emission curves for continuous blue light LED (cBL- 460 nm, $/9 \mu\text{mol}/\text{m}^2/\text{s}$).

(C) Spectral emission curves for continuous white light LED

(D) Spectral emission curves for continuous white light + yellow filter. The yellow filter was designed to absorb most of the blue light component from the WL spectrum.

cBlue Light has the same effect as cRed Light on hormone homeostasis

We wondered whether cBL repressed ARI by modifying hormone homeostasis; therefore we quantified the endogenous content of different hormones known to either inhibit ARI, such as JA, ABA and CKs (Steffens *et al.*, 2006; Ramírez-Carvajal *et al.*, 2009; Gutierrez *et al.*, 2012) or promote ARI, such as IAA and SA (Gutierrez *et al.*, 2012) (Figure 10). Interestingly, cBL had the same effect as cRL on the homeostasis of these hormones, therefore we concluded that cBL did not inhibit ARI by inducing an accumulation of the repressing hormones JA, CKs and ABA as under cWL. Other hormones such as gibberellins or strigolactones have been shown to inhibit ARI (reviewed in the introduction), therefore more investigation is needed to definitely exclude a hormone effect downstream of cBL and allow us to understand the role of blue light.

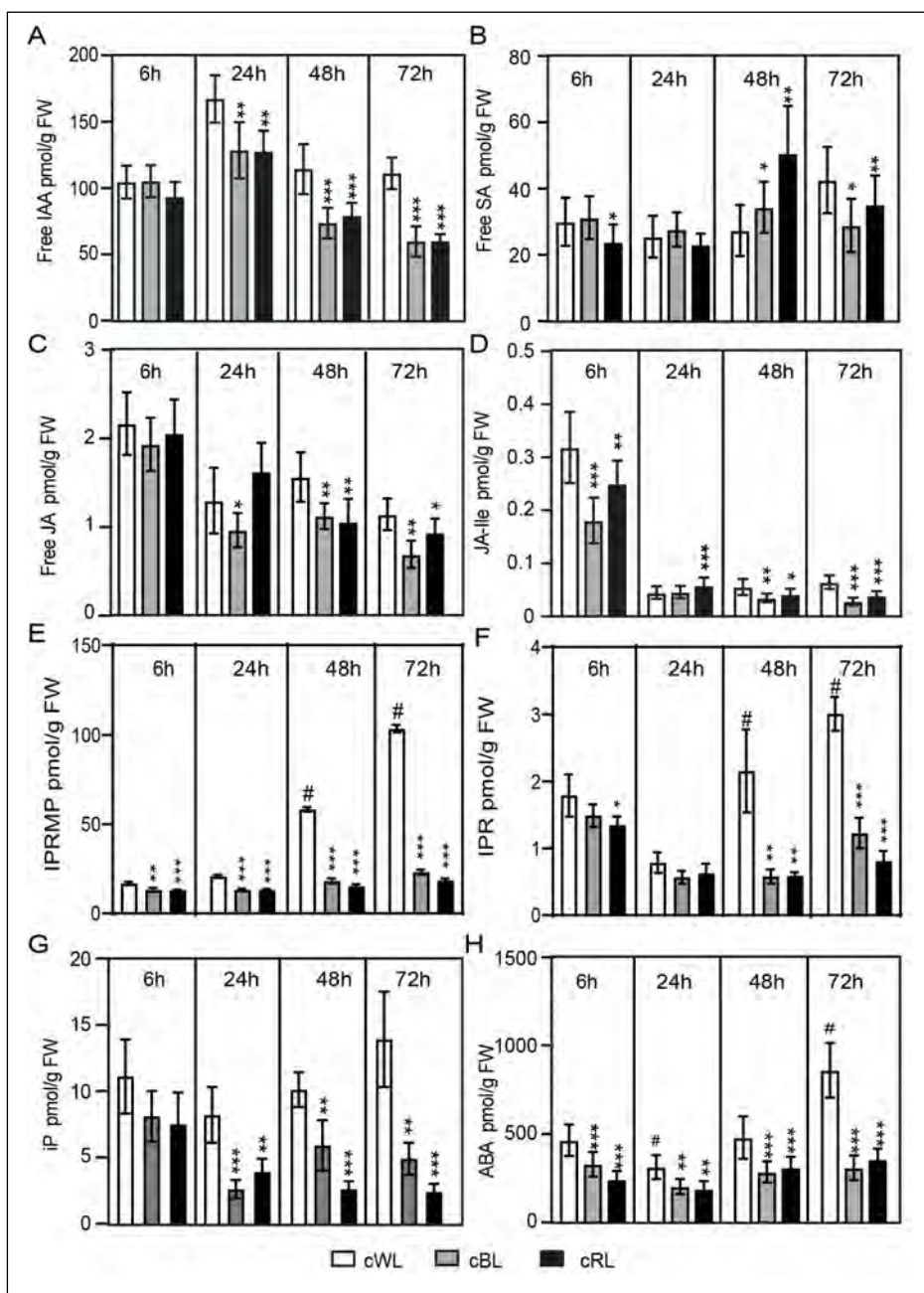


Figure 10: cBL has the same effect as cRL on hormone homeostasis but not as cWL

Three-week-old Norway spruce seedlings were grown under long-day conditions as described in Alallaq et al., 2020. De-rooted seedlings were then kept in constant cWL, cBL or cRL for 6, 24, 48 and 72 hours. For each time point under each condition 5 mm hypocotyls were taken and pooled for hormone quantification. Five independent biological replicates were collected for analysis of free IAA, SA and free JA and JA-Ile contents as well as ABA content, and an additional five independent biological replicates were collected for quantification of cytokinins.

Values are the means with standard deviation (SD) of five biological replicates. Asterisks indicate statistically significant differences under light conditions cRL versus cWL or cBL versus cWL in a t-test; *, **, and *** correspond to p-values of ($0.05 > p > 0.01$, $0.01 > p > 0.001$, and $p < 0.001$ respectively; $n = 5$); A hash sign indicates statistically significant difference at 24 h, 48 h or 72 h versus 6 h in t-test; $p < 0.001$, $n = 5$; FW, fresh weight.

IBA but not IAA or NAA can stimulate ARI under cBL

We then checked whether it was possible to induce AR development under cBL with exogenously applied auxin. We tested 1 μ M and 5 μ M of IAA, IBA or NAA and observed a significant positive effect only with IBA (Figure 11 B, C, D). The establishment of local auxin gradients is necessary to achieve the proper development of organs, at the correct location and time. The IBA is an auxin precursor that is converted to IAA in a peroxisomal β -oxidation process (Strader & Bartel, 2011). In Arabidopsis, altered IBA-to-IAA conversion leads to multiple plant defects, indicating that IBA contributes to auxin homeostasis in critical ways. Like IAA, IBA and its conjugates are transported in plants, but through different carriers, most of which still need to be identified (reviewed in Damodaran and Strader, 2019). In addition, blue light has been shown to affect the polarization of the PIN3 protein, thereby modifying local auxin distribution (Zhang et al., 2013). Therefore, one hypothesis is that cBL may affect the local IAA transport, preventing the induction of AR, but not IBA transport, which requires different transporters. This, of course, requires additional investigation to confirm or disprove it.

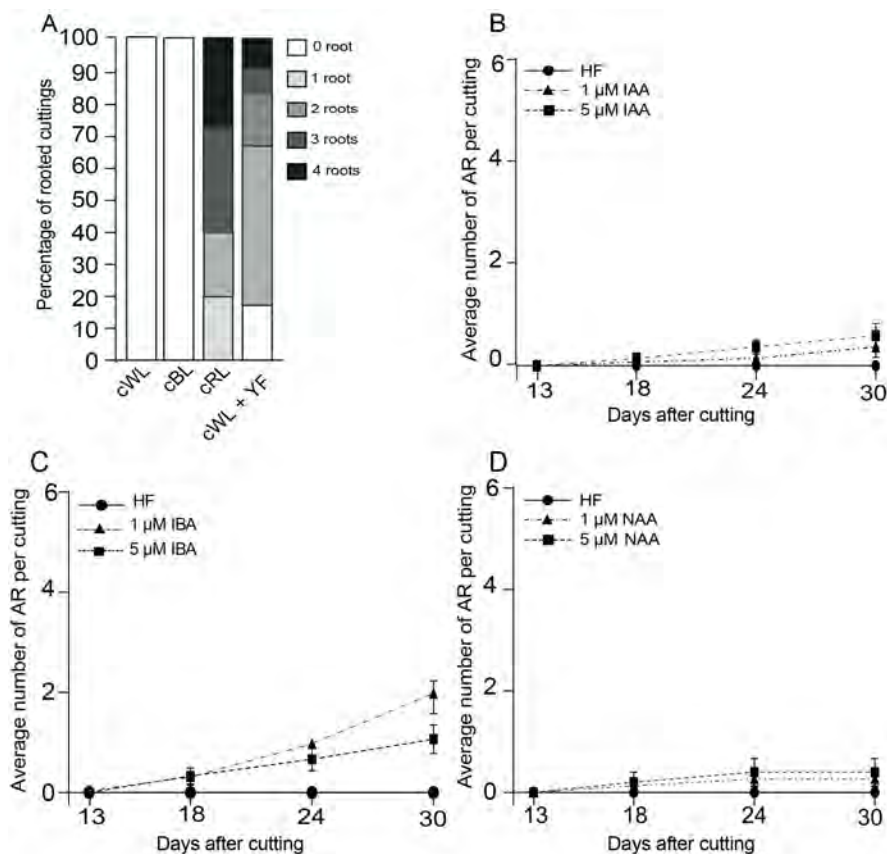


Figure 11: The effect of light on ARI in de-rooted Norway spruce hypocotyls

(A) The effects of all white light spectrum on ARI in de-rooted Norway spruce hypocotyls:

Percentage of rooted hypocotyl cuttings kept under cWL, cWL with a yellow filter (YF), constant red light (cRL) or constant blue light (cBL).

(B to D): IBA but not IAA or NAA can induce ARI in cBL

Three-week old Norway spruce seedlings were de-rooted and kept under cBL for 30 days in hormone free (HF) distilled water, or in presence of 1 or 5 μM IAA (B) or in presence of 1 or 5 μM IBA (C) or in presence of 1 or 5 μM NAA (D)

(B) Exogenously applied IAA concentration did not have any significant effect compared to the HF condition (Repeated measures ANOVA; $P = 0,073$).

(C) Exogenously applied IBA significantly increased the average number of ARs over time (Repeated measures ANOVA; $P < 0.0001$); and with increasing concentration compared to control HF conditions (Repeated measures ANOVA; $P < 0.0001$).

(D) Exogenously applied NAA concentration had no significant effect compared to the HF treatment (Repeated measures ANOVA; $P = 0,073$).

Paper II: Genome wide comparative transcriptomic analysis of the cambium tissue from easy-to-root and difficult-to-root *Populus* genotypes

Vegetative propagation from stem cuttings relies on the ability to produce adventitious roots (ARs), which is a postembryonic organogenesis process induced from differentiated cells other than those specified to develop roots. The rooting capacity of cuttings varies from individuals within species, populations, or even clones (Abarca & Díaz-Sala, 2009a,b). During the last ten years significant advances in the identification of genes involved the AR process both in model and tree species have been made, nevertheless we still have a long way to go to understand why some genotypes can root easily and others not. In Paper II we attempted to answer this question. We analyzed the transcriptome of cambium tissues obtained immediately after cutting and 24 h later, by Laser Capture Microdissection (LCM). We compared tissues taken from *P. trichocarpa* × *P. maximowiczii* (clone OP42), which we defined as easy-to-root from woody stem cuttings and the hybrid aspen *P. tremula* × *P. tremuloides* (clone T89) which we qualified as difficult-to-root from woody stem cuttings. OP42 is one of the most widely used poplar clones worldwide and also in Northern Europe (Taerwe *et al.*, 2015) and can be easily propagated from dormant stem cuttings. In contrast the hybrid aspen T89 cannot be propagated *via* dormant stem cuttings but can be easily propagated *in vitro* and is very amenable to genetic transformation (Nilsson *et al.*, 1992). The analysis of the transcriptomic data sets identified several transcription factors; these are putative regulatory hubs in the cambium and could be involved in adventitious root formation.

Hybrid aspen and hybrid poplar show different patterns of adventitious root formation

In order to better understand why some genotypes, readily develop ARs and others do not, we compared the rooting efficiency of cuttings from the poplar clone OP42 (*P. trichocarpa* × *P. maximowiczii*) and the hybrid aspen clone T89 (*P. tremula* × *P. tremuloides*) from juvenile plants kept *in vitro* (Paper II,

Figure 1 and Supplemental Figure 1A, B, D) and from stem cuttings of plants grown in the green house for three months (Paper II, Figure 2, and Supplemental Figure 1C, E). No significant difference in the average number of ARs per cutting between the two genotypes was observed when the plants were juvenile and kept *in vitro* (Paper II, Figure 1A). However, we observed a difference in the anatomy of AR formation (Paper II, Figure 1B to I). In the case of T89, ARs developed all around the base of the cuttings in a crown-like arrangement (Paper II, Figure 1B-E), while in OP42 ARs developed a few millimeters above the base of the cuttings and along the stem (Paper II, Figure 1 F-I, O, Q). When cuttings were taken from three-month-old plants grown in the greenhouse (Paper II, Supplementary Figure 1C) and kept in a hydroponic culture system as described in Merret *et al.* (2010) and Rigal *et al.* (2012) (Paper II, Supplemental Figure 1E), T89 cuttings were unable to develop ARs (Paper II, Figure 2A, B) while 100 % of OP42 cuttings rooted (Paper II, Figure 2A, C). We also compared the anatomy and the timing of the rooting process in juvenile cuttings of these two genotypes under *in vitro* conditions (Figure 12).

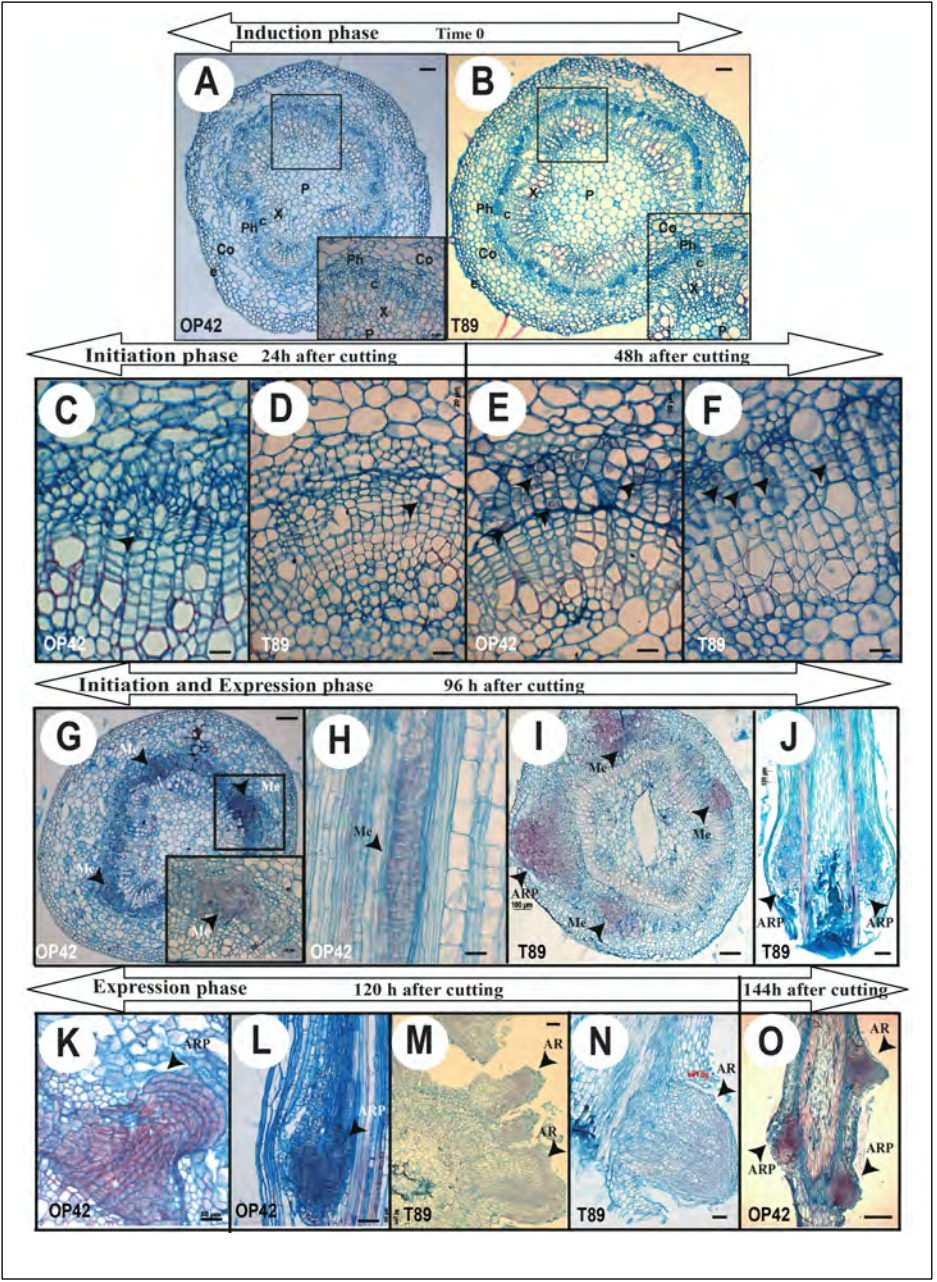


Figure 12: The anatomy of AR formation in juvenile cuttings of OP42 and T89 under in vitro conditions

(A-B): At time 0 (immediately after cutting), the stems of microcuttings showed typical collateral vascular bundles. The cells of the cambium did not show any type of activity: Cross section of OP42 (A) and cross section of T89 (B). e, epidermis; Co, cortex; Ph, phloem; c, cambium region; P, pith; x, xylem.

(C-D): 24h after cutting, the cambium became active, certain cells in the cambial zone and adjacent phloem became more densely stained in their cytoplasm and nuclei appeared more frequently (arrows). At the same time, the first mitotic divisions were observed. Cross section of OP42 (C) and cross section of T89 (D).

(E-F): 48h after cutting, most cells in the cambium showed signs of meristematic activity, with a more dense cytoplasm and with nuclei containing large prominent nucleoli (arrows). Cross section of OP42 (E) and cross section of T89 (F).

(G-H): 96h after cutting, first root meristematic cells (Me - arrows). Cross section of OP42 (G) and longitudinal section of OP42 (H).

(I-J): 96h after cutting, dome-like primordia and AR primordium (ARP) started to appear (arrows). Cross section of T89 (I) and longitudinal section of T89 (J).

(K-L): 120h after cutting, AR primordium (ARP) started to appear (arrows) and they penetrated the stem cortex. Cross section of OP42 (K) and longitudinal section of OP42 (L).

(M-N): 120h after cutting, the ARs have emerged (arrows). Cross section of T89 (M) and longitudinal section of T89 (N).

(O) 144h after cutting, the ARs have emerged (arrows), longitudinal section of OP42.

Transcriptomic profile and functional classification of DEGs from cambium tissue between OP42 (easy-to-root) and T89 (difficult-to-root) poplar genotypes

In order to better understand this extreme difference in rooting performance, we analyzed the transcriptome of the cambium from OP42 (easy-to-root) and T89 (difficult-to-root) cuttings from three-month-old plants grown in the greenhouse (Paper II, Supplemental Figure 2A). We collected homogenous and specific cambium tissue from the basal 5 mm of stem cuttings at time T0 (immediately after cutting) (Paper II, Supplemental Figure 2B) and T1 (24 h after transfer into hydroponic conditions) (Paper II, Supplemental Figure 2C). For this we performed Laser capture Microdissection (LCM) (Paper II, Supplemental

Figure 2D-I). The RNA-seq reads were mapped to the *P. trichocarpa* reference genome (Paper II, Supplemental Data set 1, sheet1) and 17,997 genes were classified as expressed in all biological replicates in both genotypes at both time points (Paper II, Supplemental Data set1, sheet2). Interestingly, there were more variations in OP42 after 24 h in hydroponic conditions than in T89 (Paper II, Figure 3). According to the gene ontology (GO) enrichment analysis (Paper II, Supplemental data set 3, sheets 4 and 5), differentially expressed genes (DEGs) in T89 were mostly involved in biological processes and molecular functions related to carbohydrate catabolism or redox mechanisms, regulation of transcription, response to abiotic stresses, cation binding, nucleic acid binding activity, or electron carrier activity (Paper II, Supplemental data set 3 sheet 4 and 5). Interestingly, 74.6 % of DEGs in OP42, were exclusively upregulated in OP42 at T1 (Paper II, Figure 3B), suggesting that very specific events might occur in OP42 during the 24 h timeframe. These upregulated genes were involved in cellular components, biological processes or molecular functions related to transcription regulation, translation and post translation regulation (Paper II, Supplemental Data set 3, sheet 4). When the two genotypes were compared to each other, 25 % of the DEGs were differentially expressed between OP42 and T89 at T0 (Paper II, Figure 3A, Supplemental Data set 2) and 14% 24 hours after transfer into hydroponic conditions (Paper II, Figure 3A, Supplemental Data set 2, sheets 5 to 7). The DEGs differing between T89 and OP42 are mostly involved in cellular and chemical homeostasis, photosynthesis, dioxygenase activity and protein synthesis (Paper II, Supplemental data set 3, sheet 4 and 5).

Genes related to cambium or vascular tissues behaved similarly in both genotypes

In order to confirm the quality and the specificity of the data set, we selected a list of 40 *Arabidopsis* genes described as being expressed in the cambium or vascular tissues and checked the expression of their putative *Populus* orthologs (Paper II, Supplementary Figure 3C and Supplemental Data set 3, sheet 1). All were found to be expressed and mostly behaved in a similar way in the two

genotypes, showing a slight upregulation or downregulation in OP42 and T89 between T0 and T1 (Paper II, Supplementary Figure 3C and Supplemental Data set 3, sheet 1).

Genes encoding ROS scavenging proteins are mostly up regulated in OP42 compared to T89

Reactive oxygen species (ROS) are signaling molecules involved in the response to biotic and abiotic stress as well as many aspects of plant development, including AR formation, as shown by recent studies (reviewed in Nag *et al.*, 2013; Li *et al.*, 2017; Velada *et al.*, 2018). Forty-three differentially expressed genes encoding ROS scavenging proteins, 33 of which belong to the GLUTATHIONE S-TRANSFERASE superfamily (GSTs) and 10 to the PEROXIDASE superfamily (Paper II, Supplemental Data set 3 sheet 3) were found. Approximately half of these genes were up regulated at T1 compared to T0 in both genotypes, but on average the size of the change was significantly higher in OP42 than in T89 (Paper II, Supplemental Figure 4; Supplemental Data set 3, sheet 3). Strikingly, 32 out of 43 genes were significantly upregulated in OP42 compared to T89 at T1 and 21 of those were also upregulated in OP42 at T0 (Paper II, Supplemental Figure 4; Supplemental Data set 3, sheet 3) Several studies support an important role for H₂O₂, a type of ROS, as a positive downstream component of auxin signaling during AR formation in cuttings (Li *et al.*, 2009; Liao *et al.*, 2009; Huang *et al.*, 2011). In addition, changes in peroxidase activity and peroxidase isoform patterns have been proposed as biochemical markers of the successive adventitious rooting phases (Gaspar *et al.*, 1992; Ludwig-Müller, 2003) and recently Zhang *et al.* (2019) showed that several genes encoding peroxidases were differentially expressed during the AR formation process in cuttings from the poplar clone “NL895” (*Populus euramericana*). Our results suggest that a higher expression of genes encoding ROS scavenging proteins in OP42 cambium could contribute to its greater ability to root compared to T89.

Cambium from the easy-to-root OP42 shows increased transcriptional activity after cutting compared to the difficult-to-root T89

The different stages of adventitious root initiation in *Populus* are associated with substantial remodeling of the transcriptome (Ramírez-Carvajal et al., 2009; Rigal et al., 2012) therefore we focused our analysis on the expression of transcription factors (TFs). From the 58 families of transcription factors identified in *Populus*, 49 were represented in the DEG list (Paper II, Table 1, Supplemental data set 2, Supplemental data set 3, sheet 2) and most of the variations were observed in OP42 (Paper II, Table 1). Twenty-four hours after cutting, 210 and 209 TFs were up or down regulated respectively in OP42, while in T89 there were only 89 upregulated and 43 downregulated (Paper II, Table 1). Among the 49 families of TFs, the most represented DEGs belong to the *ARF*, *bHLH*, *ERF*, *LBD*, *MYB*, *MYB-related*, *NAC* and *WRKY* families. *AUXIN RESPONSE FACTOR* genes are TFs that regulate the expression of auxin response genes. In *Arabidopsis*, the importance of this family in the adventitious rooting process has been demonstrated (Gutierrez et al., 2009). In our data set, although some of these were more downregulated in OP42 24 h after cutting compared to T89, they mostly behaved in a similar way in both genotypes (Paper II, Supplemental Figure 5; Supplemental Data set 3, sheet 4) and did not allow discrimination between T89 and OP42. *AtMYC2*, a member of the *bHLH* family master regulator in the jasmonate signaling pathway, was shown to be a negative regulator of AR formation in *Arabidopsis* (Lakehal et al., 2020). In *Populus* there are six putative orthologs of *AtMYC2*, three of them (Potri.001G083500, Potri.001G142200, and Potri.003G092200) were upregulated in both T89 and OP42, but more so in T89, while Potri.003G147300 was exclusively upregulated in T89, which led to a significant increase in *PtMYC2* expression in T89 compared to OP42 (Paper II, Supplemental Figure 6A; Supplemental Data set 3, sheet 2). The potential upregulation of JA signaling in T89 was corroborated by a greater change in the expression of JA biosynthesis genes and that of several jasmonate-inducible *JAZ* genes 24 h after cutting in T89 compared to OP42 (Paper II, Supplemental Figure 6; Supplemental Data set 3, sheet 4).

PtARF6, *PtARF8* positively control AR development in *Populus* while *PtARF17* is a negative regulator

Because the expression of *ARF* genes was not significantly different between the two genotypes, we wondered whether *PtARF6*, *PtARF8* and *PtARF17* that control AR initiation in *Arabidopsis* (Gutierrez et al 2009) play a role in the control of AR in *Populus*. The *P. trichocarpa* genome contains four *PtARF6* (*PtARF6.1*, *PtARF6.2*, *PtARF6.3*, *PtARF6.4*), two *PtARF8* (*PtARF8.1*, *PtARF8.2*) and two *PtARF17* genes (*PtARF17.1*, *PtARF17.2*). The *PtARF6.1*, *PtARF6.2*, *PtARF6.3* and *PtARF6.4* group together with *AtARF6* with high bootstrap, suggesting that these are the most likely candidates to be the functional orthologs of *AtARF6*; *PtARF8.1/2* and *PtARF17.1/2* group with *AtARF8* and *AtARF17* respectively (Paper II, Supplemental Figure 6 A). We retrieved the expression patterns of *PtARF6* (*PtARF6.1/2/3/4*), *PtARF8* (*PtARF8.1/2*) and *PtARF17* (*PtARF17.1/2*) genes from the AspWood database (<http://aspwood.popgenie.org>; Sundell et al., 2017). AspWood provides high-resolution *in silico* transcript expression profiling of the genes expressed over the phloem, cambium and other zones of developing wood in *Poplar*. We observed that *PtARF6.1/2/3/4* and *PtARF8.1/2* are highly expressed in the in phloem-cambium region while *PtARF17.1/2* genes exhibit limited expression (Paper II, Supplemental Figure 6 B-D).

In order to assess the role of *PtARFs* in ARI we overexpressed *PtARF6.4* and *PtARF8.2* under the promotion of the cambium-specific gene *PtHB3* and produced downregulated RNAi lines for *PtARF6.3/4*, *PtARF8.1/2* and *PtARF17.1/2* genes in the hybrid aspen clone T89 (Paper II, Figure 4). Transgenic lines downregulated for the expression of *PtARF17.1/2* produced significantly more ARs than the control (Paper II, Figure 5), suggesting that *PtARF17.1/2* are negative regulators of ARI in hybrid aspen. In contrast, the lines downregulated for *PtARF6.3/4* and *PtARF8.1/2* produced fewer ARs than the control, while lines expressing *PtHB3:ARF6.3/4* and *PtHB3:ARF8.1/2* produced significantly more ARs than the control (Paper II, Figure 5), indicating that *PtARF6.3/4* and *PtARF8.1/2* are positive regulators of ARI in T89. These results

confirm that *PtARF6*, *PtARF8* and *PtARF17* have similar functions in the control of AR1 in hybrid aspen as in Arabidopsis.

PtMYC2 is a negative regulator of adventitious root development in hybrid aspen

We observed higher upregulation of the JA signaling pathway in T89 compared to OP42, therefore we wondered whether *PtMYC2* had a similar function in the regulation of AR1 in *Populus* as in Arabidopsis. We produced hybrid aspen T89 lines overexpressing *PtMYC2* (Potri.003G092200). Two lines with the highest expression level of *PtMYC2* were selected (Paper II, Figure 6A) and analyzed for their AR development phenotype. In both lines the average number of ARs per cutting was significantly reduced, suggesting that *PtMYC2* is also a negative regulator of AR development in hybrid aspen (Paper II, Figure 6C). The difference in the expression of genes involved in the JA signaling pathway in OP42 and T89 prompted us to check their behavior in the presence of exogenously applied JA. Stem cuttings from one-month *in vitro* grown plants of both T89 and OP42 were harvested and transferred to a medium without JA or with 5, 10 or 20 μ M. Adventitious roots were counted during the rooting period 15 after cutting (Paper II, Figure 6 C and D). Although both genotypes behaved in the same way in the absence of JA, T89 was much more sensitive to exogenously applied JA than OP42. In T89 AR development was significantly repressed by 5 μ M of JA whilst it required 20 μ M of JA to obtain the same level of repression in OP42 (Paper II, Figure 6 C and D).

In summary, in paper II: we demonstrated that T89 was unable to produce roots from woody stem cuttings whereas OP42 rapidly produced ARs under hydroponic growth conditions. However, under *in vitro* conditions, both genotypes were able to develop ARs. The comparative histological study of T89 and OP42 under *in vitro* conditions confirmed that, in both genotypes, AR formation is initiated in the cambium region. Transcriptomic profiling of cambium tissue of woody stem cuttings of both genotypes under hydroponic conditions identified several transcription factors; these may act as regulatory hubs in the cambium and could

be involved in adventitious root formation. We found that MYC2 negatively regulates adventitious rooting in hybrid aspen.

The role of three transcriptional factors, ARF6 and ARF8, known in *Arabidopsis* to act as positive regulators in AR development, plus ARF17, known to act as a negative regulator, was confirmed in transgenic hybrid aspen, suggesting that at least this part of the regulatory interaction is conserved in hybrid aspen.

Paper III: Characterization of adventitious root formation in aspen clones from the Swedish Aspen collection

As mentioned above, adventitious rooting is a major step in vegetative propagation and represents a switch of dedifferentiation, acquisition of a new cell fate and redifferentiation into a new developmental organ. Rooting capacity differs between cuttings depending on the genotype and the developmental and physiological state, and parameters characteristic of these different stages may be used as markers of rooting ability (De Klerk, 1996). In an attempt to identify useful markers that could be used for future selection of the best rooting clones for large scale propagation during breeding programs, we combined phenotypic and molecular approaches to unravel the mechanisms controlling adventitious root formation of aspen clones from the Swedish Aspen (SwAsp) collection using *in vitro* cuttings. The SwAsp collection (Luquez *et al.*, 2008) comprises 116 aspen individuals collected from 12 sites spanning the entire range in Sweden. These clones contain high levels of genetic variation (Ingvarsson, 2005), have no significant population structure and are suitable for high-resolution association mapping (Ingvarsson, 2008). Ninety- six clones were transferred to *in vitro* conditions and these exhibited variation in rooting ability. We, therefore, decided to phenotype these clones for their competence to regenerate ARs in more details (Paper III, Figure 1) and to examine the expression of a few genes known to be involved in AR development in *Arabidopsis* and look for a possible correlation with the phenotype.

The 34 SwAsp lines show variability in terms of adventitious rooting competence

To start we selected 34 clones based on their origin in Sweden (Paper III Figure 1A), amplified them and analyzed their rooting competence *in vitro* (Paper III Figure 1B). We determined several parameters such as the timing of rooting as well as the AR pattern types in these clones (Paper III, Figure 2 A-F), the average number of ARs per cuttings, the percentage of rooted cuttings and root length.

Our results showed an important variation in the ability to form ARs among these 34 selected lines (Paper III, Figure 3). The average root number per cutting ranged from 0.3 to 7.5 roots, and the percentage of rooting was between 19.4 % and 100% 15 days after cutting (Paper III, Figure 3A and B). For further analysis of the phenotypic variables related to ARs, we grouped the 34 selected lines into three clusters according to the number of rooted cuttings and the percentage of rooted cuttings from 5 to 15 DAC for each line (Figure 3C). Cluster one, lines (L29, L99, L71, L69, L81, L96, L31, L12 and 82) had a high average number of roots and percentage of rooted cuttings. Cluster two included (lines L37, L10, L62, L56, L46, L116, L19, L73, L100, L42, L34, L91, L23, L7, L114, L55, L77, L1, and L66), had a lower number of ARs compared to cluster one, but most of the microcuttings produced roots. Cluster three (lines L105, L45, L51, L88, and L16) had a low number of ARs per cutting and 19.4% to 75 % of rooted cuttings at 15 DAC.

Since lines with similar rooting traits clustered together (Paper III, Figures 3C and D), we decided to continue with just a few lines that we had classified as good-rooting (99, 81, 71, 29 and 69) (Paper III, Figure 4 and Figure 5), intermediate -rooting (L7, L77, L37, L100 and L62) (Paper III, Figure 6 and Figure 7) and the poor-rooting (L16, L45, L51, L105 and L88) (Paper III, Figure 8 and Figure 9).

Characterization of the rooting performance of selected good-rooting, intermediate-rooting and poor-rooting lines

To better understand the observed differences between the good- intermediate- and poor-rooting lines, we re-analyzed them for their rooting capacity from 5 to 15 DAC (Paper III, Figures 4 to 9).

We observed that among the five good-rooting lines, line 99 reached 100% of rooted cuttings 8 DAC, while the others had already reached this level 5 to 6 DAC (Paper III, Figure 4A).

This was due to a delay in AR regeneration in line 99 since the average number of ARs 5 DAC was the lowest of the lines in this category (Paper III, Figure 5A). We found that among the five intermediate-rooting lines, line 7 and L77 had a

similar profile with very few rooted cuttings 5 DAC (Paper III, Figure 6A and B), and 15 DAC 79 % to 87% of the cuttings developed between 1 to ≥ 7 ARs (Paper III, Figure 7A and B). In the case of line L37, 38% of the cuttings had developed at least 1 AR 5 DAC (Paper III, Figure 6C and 7C) and 95% of the cuttings were rooted 15 DAC but never developed more than 3 ARs per cutting (Paper III, Figure 7C). While line L100, 25 % of the cutting had developed between 1-4 ARs per cutting and rapidly reached 94% to 100% 8 at 15 DAC (Figure 6D) with more than 4 ARs per cutting 15 DAC (Paper III, Figure 7D). Last, line L62 reached 100% of rooted cuttings 7 DAC, but the average number of ARs remained below 3 ARs / cutting 7 to 15 DAC (Paper III, Figure 6E).

Among of the five poor-rooting lines, line L16 was the most affected, with less than 20% of rooted cuttings 15 DAC (Paper III, Figure 8A) and the rooted cuttings never developed more than 2 ARs (Paper III, Figure 9A). Lines L45, L51 and L105 showed a similar rooting profile with no or very few rooted cuttings 5 DAC (Paper III, Figure 8B-D and Figure 9B-D) and 15 DAC, 56.7 % to 75% of the cuttings developed not more 4 to 5 ARs (Paper III, Figure 9B-D). Last, in the case of line L88, 9 % of the cuttings had developed at least 1 AR 5 DAC (Paper III, Figure 8E and 9E) and 57.5% of the cuttings were rooted 15 DAC and developed no more than 4 ARs per cutting (Paper III, Figure 9E).

We then decided to focus on highly contrasting phenotypes considering the average number of ARs per cutting at the beginning and at the end of the rooting process (5 and 15 DAC). Based on (Paper III, Figure 3D), lines L99, L81, L71, L29 and L69 were qualified as good-rooting lines with a high number of ARs 15 DAC despite the initial number of ARs, whereas lines L16, L45, L51, L105 and L37 were considered poor-rooting lines since they formed very low number of ARs 15 DAC. These lines with contrasting phenotype were used for root length measurements and gene expression.

Correlation between the average number and the length of adventitious roots

We observed that all good-rooting lines, which developed the highest average number of ARs per cutting, had a relatively short root system (Paper III, Figure 10 A). In contrast, poor-rooting lines, which presented the lowest average number of ARs per cutting, developed appreciably longer roots (Paper III, Figure 10 B). Therefore, we measured the average length of ARs 15 DAC, and performed a correlation analysis between the number and the average length of ARs. The results showed a significant negative correlation ($R = -0.42$; $p < 2.2 \times 10^{-16}$) between the number and the average length of adventitious roots in rooted cuttings (Paper III, Figure 10 C). This result suggests that poor-rooting lines may attempt to compensate for their poor root system by producing longer roots, therefore increasing the rooting surface.

Expression levels of genes known to be associated with AR formation

In our previous study we showed that *ARF6*, *ARF8*, *ARF17* and *MYC2* played similar roles in *Populus* as in *Arabidopsis* (Paper II) and we wondered whether there could be a correlation between the expression of these genes and the rooting phenotype observed in the good- and poor -rooting lines. Therefore, we used qRT-PCR to analyze the expression level of *ARF6*, *ARF8*, *ARF17* and *MYC2* in these selected lines with contrasting AR phenotypes.

Our results showed that there is no correlation between the expression level of these genes and the phenotypes of the selected good- and poor-rooting lines. Therefore, we suggest that in order to identify potential genes that could be used as early markers for selecting good-rooting clones, transcriptomic analysis of the good- and poor-rooting lines is required.

4. Conclusion and perspectives

Adventitious root development is a complex process and its regulation requires precise interactions of multiple signaling pathways, which mediate endogenous and environmental cues. Defects at any stage of AR development can lead to large economic losses. Several studies have been performed to identify the molecular and genetic networks controlling AR development using a number of species, but the exact mechanistic foundations of this process have remained poorly understood, especially for trees species. In this thesis, we investigated the mechanisms affecting AR formation in trees using *Picea abies* (Norway spruce) de-rooted seedlings and *Populus* spp. stem cuttings.

The first research presented in this thesis succeed in determining the role of the spectral quality of light, which is an important environmental factor known to affect ARI in many species including Norway spruce. We demonstrated that de-rooted Norway spruce seedlings do not develop ARs under white light (WL) possibly because they accumulate cytokinins (CKs) at the base of the cuttings. In contrast to WL, we found that red light (RL) has a positive effect on ARI, most likely, because it represses the jasmonate (JA) biosynthesis and signaling pathways as well as the accumulation of CKs at the base of the cuttings. Indeed, we confirmed that CKs and JA are repressors of ARI in Norway spruce. This mechanism has not been shown previously in any species, highlighting the importance of using different species to elucidate novel molecular pathways. In this study, we also found that blue light (BL) has a negative effect on AR development, which could only be partially countered by exogenous treatments with IBA, but not with NAA or IAA. This is a key observation that requires further investigations. Our research provided important advances towards understanding AR development in Norway spruce. Nevertheless, further research is needed to unravel the missing link that connects the light signaling pathway with its downstream targets (i.e., JA and CKs). More precisely, revealing the molecular players downstream of the light receptors that transduce RL and BL responses during AR development is an important goal. Although it is challenging, generating knockout mutants using the state-of-the-art

CRISPR/Cas9 technology will be illuminating and will certainly help to identify the exact genes involved in this process. Developing protocols to regenerate whole Norway spruce seedlings through somatic embryogenesis is necessary for such functional analysis studies. Moreover, cross-species functional genetic complementation (e.g. with *Arabidopsis*) would provide more insights about the degree of evolutionary conservation of the identified signaling modules. Last but not least, genome-wide transcriptomics may also uncover more candidate genes and molecular networks controlling ARI in Norway spruce.

In the second paper, at the anatomical and molecular level, we compared the development of AR in two genotypes with contrasting phenotypes: easy-to-root (*Populus trichocarpa* × *P. maximowiczii*) clone OP42 and difficult-to-root (*P. tremula* × *P. tremuloides*) clone T89. The comparison of T89 and OP42 under hydroponic growth conditions showed that T89 was unable to produce roots from woody stem cuttings whereas OP42 rapidly produced ARs. It is noteworthy that both genotypes are able to develop ARs under *in vitro* conditions. The comparative histological study of T89 and OP42 under *in vitro* conditions confirmed that in both genotypes ARs initiate in the cambium region. Transcriptomic profiling of cambium tissue of woody stem cuttings of both genotypes under hydroponic conditions identified several transcription factors that may act as regulatory hubs in the cambium and could be involved in adventitious root formation. The role of three transcriptional factors, ARF6 and ARF8, known in *Arabidopsis* to act as positive regulators in AR development, plus ARF17, known to act as a negative regulator in *Arabidopsis*, was confirmed in transgenic hybrid aspen. These data suggest that at least this part of the regulatory interaction is conserved in hybrid aspen. We found that the expression of MYC2 orthologs and the expression of several genes involved in JA signaling increased more in T89 than in OP42, suggesting that JA could be also a negative regulator of ARI in *Populus* as it is in *Arabidopsis*.

Finally, in the third paper, we characterized the rooting phenotype of cuttings from clones of the Swedish Aspen (SwAsp) collection *in vitro*. From this study we reported a remarkable variation in the root system establishment among the

tested clones, as reflected by differences in rooting ability and root length. The comparative study of the expression levels of some genes and the AR number among the tested clones with contrasting AR phenotypes could not discriminate between the good- vs poor-rooting clones in *Populus tremula*. Therefore, it would be interesting to perform genome-wide comparative transcriptomics (RNA sequencing) for some selected clones with contrasting AR phenotypes in order to uncover the transcriptional signatures associated with rooting ability. Comparative transcriptomics might also reveal potential marker gene(s) that can be used for future selection of the best rooting clones from the SwAsp collection. Acquiring fundamental knowledge about the regulation of AR development in tree species to a level similar to that for *Arabidopsis* is certainly the main goal for future research. Exploring the transcriptional and hormonal changes associated with AR development will uncover the secrets behind the adventitious rooting plasticity and recalcitrance in different species. The recent development of single cell RNA sequencing and the rapid progress in mass spectrometry-based techniques will definitely facilitate future research and help to answer these longstanding questions. Alternatively, laser-capture microdissection also allows researchers to precisely target individual or groups of cells for gene expression assays. These technological advances will allow researchers to identify useful marker gene(s) and genetic regulators that control AR formation in woody species. These markers could be used for future selection of the best-rooting clones during breeding programs, especially for economically important trees. In conclusion, although our knowledge of AR development increases daily, it seems that we still have a long way to go if we are to completely understand the molecular foundation for the regulation of this process in different species.

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